DESIGN, SYNTHESIS AND BIOEVALUATION OF STEROIDAL CONJUGATES: A STUDY DIRECTED TOWARDS THE DEVELOPMENT OF NOVEL LEAD MOLECULES

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MARCH 2008

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THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CHEMISTRY

BY

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Dedicated to my Parents….

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March 04, 2008

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "*Design, synthesis and bioevaluation of steroidal conjugates: a study directed towards the development of novel lead molecules*" which is being submitted to the *University of Pune* for the award of *Doctor of Philosophy* in *Chemistry* by *Mr. Deepak Bhalchandra Salunke* was carried out by him under my supervision at the National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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March 04, 2008

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "*Design, synthesis and bioevaluation of steroidal conjugates: a study directed towards the development of novel lead molecules*" submitted by me for the degree of *Doctor of Philosophy* in *Chemistry* to the *University of Pune* is the record of work carried out by me during the period *April, 2004 to February, 2008* and has not been submitted by me for a degree to any other University or Institution. This work was carried out at Organic Chemistry Division, National Chemical Laboratory, Pune, India.

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ACKNOWLEDGEMENTS

I owe the fulfillment of this great endeavor to my research mentors Dr. Braja Gopal Hazra and Dr. Mrs. Vandana S. Pore who introduced me to this fascinating realm of Medicinal Chemistry. Although any eulogy is insufficient, I take this opportunity to express my reverence to both for their erudite guidance, support and excellent work ethics. Their humanitarianism is an attribute that I wish to take forward with me along with the chemistry that I learnt from them.

My thanks are due to Dr. Krishna N. Ganesh, Director Indian Institute of Science Education and Research, Pune (former Head, OCS Division) for his constant support. It is my privilege to thank Dr. Ganesh Pandey, Head, OCD, for his constant encouragement and interest shown during the progress of my work. I got the opportunity to associate myself with Dr. Robert H. Dodd, Research Director, ICSN, France as my research supervisor in France during the Sandwich Thesis Programme. Being an outstanding scientist with a kind and understanding nature, he made the training comfortable, inspiring and enlightening. I wish to place on record my deep sense of gratitude to him. I am indebted to Dr. Josiane Thierry and Dr. Philippe Dauban for their valuable suggestions and useful discussions during this programme. I am also grateful to Dr. M. S. Shashidhar, Dr. N. N. Joshi, Dr. Mrs. V. A. Kumar, Dr. H. V. Thulasiram, Dr. N. P. Argade, Dr. P. L. Joshi, Dr. Mrs. S. Hazra and Dr. D. D. Dhavale for their help and encouragement.

I thank Director NCL for providing infrastructure facilities. DST New Delhi, CSIR New Delhi, Embassy of France in India and LTMT Mumbai are acknowledged for financial assistance. I am also thankful to Mr. Dhananjay Jadhav, former CP Pune City, Mr. Chandrakant Kumbhar, DCP Anti Corruption Bureau Pune, Mr. Rajendra Joshi, PI Crime Branch, Mr. Nitin Israni, Advocate Sudhir Shah and Advocate Pratap Pardeshi for providing partial financial assistance during my stay in France. I also thank all OCS students and staff members for their timely help through out.

I wish to express my gratitude to Dr. Mohan M. Bhadbhade, Dr. Rajesh G. Gonnade, Dr. Mrs. Vedavati G. Puranik and Mr. K. Manoj of the Center for Materials Characterization, NCL for the fruitful discussions and suggestions. Help rendered by the Members of IR, Microanalysis, Mass Spectroscopy, NMR Group and Library Staff Members is also acknowledged. I take this opportunity to thank Dr. Manoj Kumar Bhat, Dr. Debashis Mitra, Dr. Dyavar S. Ravi, Dr. Samit Chattopadhyay, and Mr. Sreenath Kadreppa of National Centre for Cell Science, Pune and Dr. Mukund V. Deshpande, Dr. Pallavi B. Nahar, and Mr. Fazal Shirazi of Biochemical Sciences Division, NCL, Pune for providing the biological data.

I feel great sense of gratitude for Dr. Vijay V. Khanna of Ahmednagar College, Ahmednagar, for his inspirational teaching, ethics and discipline. He has introduced me to this fascinating subject and inspired me to move towards the research.

I enjoyed the cheerful co-operation and accompany of my seniors Pramod Sagar and Anup Thakur who made me feel a member of this family right from the day one in the lab. I am very much indebted to my immediate senior, friend and lab mate Bapu, for his advices, true help, love and care. My special thanks to lab-friends Namdev, Sudhir, Nilkanth, Sachin, Pankaj, Swati, Prabhakar and Khandekar Saab for helping me in various capacities throughout my work and maintaining amazing atmosphere with humor in the lab. Thanks are due to Chetana and Seema for the help during their project tenure in our laboratory. The warm memories of my days in Laboratory No. 201 of NCL, India and days with eq 46 of ICSN, France will haunt me forever.

My warm thanks are due to my langoti yaar Vijay and Dinesh, friends Nitin, George, Abhijeet Fegde, Abhijeet Chaudhari, Soham, Milind, Yogesh Waman, Mehraj, Sandeep, Sachin, Shailesh, Pravin, Yogesh Munot, Sreeji and Sambhaji. I wish to offer my sincere thanks to Dr. Aditya Sanki for teaching me various chemistry related softwares. I would like to extend my thanks to all the NCL and ICSN friends for making my stay at NCL, Pune and ICSN, France very comfortable and memorable one.

If someone can demand that he has made me Doctor, is my Father DADA. I owe my deepest gratitude and affection to him. No word would suffice to express my gratitude and love to AAI, my sister AAKI, brother-in-law BAPU DAGI, brother VISHAL, uncle BALYAMAMA and all family members for their continuous showering of boundless affection on me and supporting me in whatever I chose or did. It is my parent's prayer, constant struggle and relentless hard work to overcome the odds of life, which has inspired me to pursue life with a greater optimism. The warmth and moral value of my parents have stood me in good stead throughout my life and I would always look up to them for strength no matter what I have to go through. This Ph. D. thesis is a result of the extraordinary wills, efforts and sacrifices of my parents. My successes are dedicated to them now and always.

I can't find the right words for the one person whom I like most, my wife Madhuri. She has experienced all my ups and down. Without her patience, love and endless support this thesis wouldn't have been written. Together with our beloved daughter Shourya we are waiting for the next perfect wave. We are curious where it is going to take us.

Finally, my acknowledgement would not be completed without thanking the God Ganesha, for giving me the strength and the determination to overcome the hardship faced in my life.

Deepak

CONTENTS

GENERAL REMARKS

- Independent reference and compound numbering have been employed for Abstract, Part A as well as each Chapter (Chapter B1 to B4) of Part B.
- All the solvents used were purified using the known literature procedures.
- Petroleum ether used in the experiments was of 60-80 °C boiling range.
- Column chromatographic separations were carried out by gradient elution using silica gel (60-120 mesh / 230-400 mesh) or neutral deactivated alumina with light petroleum ether-ethyl acetate mixture, unless otherwise mentioned.
- TLC was performed on E-Merck pre-coated silica gel 60 F_{254} plates and the spots were rendered visible by exposing to UV light, iodine, charring or staining with ninhydrin, *p*-anisaldehyde or phosphomolybdic acid solutions in ethanol.
- Usual work up: organic layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated in *vacuo*.
- Crystallization: Single crystals of the compounds were grown from a hot saturated filtered solution of these compounds in particular solvent. Suitable crystals were obtained by slow evaporation of the solvent at room temperature (RT).
- All the melting points reported are uncorrected and were recorded using an electro-thermal melting point apparatus or with Buchi Melting Point apparatus B-540.
- Ultraviolet (UV) spectra were performed using Perkin-Elemer instrument, Lambda 35 UV/VIS Spectrometer.
- CD spectra were taken on spectropolarimeter, Jasco J-715 at 25 ºC using solutions of the products in methanol exhibiting absorbance values in the range 0.1-0.2 at 220 nm.
- IR spectra were recorded on Shimadzu FTIR instrument, for solid either as nujol mull or in chloroform solution or 1-3 mg in 200 mg of KBr and neat in case of liquid compounds.

- NMR spectra were recorded on Bruker ACF 200 and AV200 (200.13 MHz for ^{1}H NMR and 50.03 MHz for ^{13}C NMR), MSL 300 (300.13 MHz for ^{1}H NMR and 75.03 MHz for ^{13}C NMR), AV 400 (400.13 MHz for ^{1}H NMR and 100.03 MHz for ^{13}C NMR) and DRX 500 (500.13 MHz for ^{1}H NMR and 125.03 MHz for ^{13}C NMR) spectrometers. Chemical shifts (δ) reported are referred to internal reference Tetramethylsilane.
- Mass spectra were recorded on Finnigan-Mat 1020C mass spectrometer and were obtained at an ionization potential of 70 eV or on LC-MS/MS-TOF API QSTAR PULSAR spectrometer, samples introduced by infusion method using Electrosprey Ionization Technique. EI and CI mass spectra were recorded on an AEI MS-50 and AEI MS-9 spectrometer, respectively. High resolution mass spectra were obtained on a Kratos MS-80 spectrometer.
- Micro analytical data were obtained using a Carlo-Erba CHNS-O EA 1108 Elemental Analyzer. Elemental analyses observed for all the newly synthesized compounds were within the limits of accuracy ($\pm$ 0.4%).
- Optical rotations were obtained on Bellingham & Stanley ADP-220 Polarimeter. Specific rotations ($[\alpha]_D$) are reported in deg/dm, and the concentration (c) is given in g/100 mL in the specific solvent.
- All the compounds previously known in the literature were characterized by comparison of their R_f values on TLC, IR and NMR spectra as well as melting point with authentic samples.
- All the new experiments were repeated two or more times.
- Starting materials were obtained from commercial sources or prepared using known procedures.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
Am	Amafalone
Amp B	Amphotericin B
Aq.	Aqueous
AZT	Azidothymidine
BFCA	Bifunctional Chelating Agent
BGTC	Bis(guanidium)-tren-cholesterol
Boc	*tert*-Butoxycarbonyl
Bzl	Benzyl
Cat.	Catalytic
CCDC	Cambridge Crystallographic Data Centre
CD	Circular Dichroism
Cetrimide, CTAB	Cetyltrimethylammonium Bromide
CSA	Cationic Steroid Antibiotics
CSD	Cambridge Structural Database
DCC	Dicyclohexyl Carbodiimide
DCM	Dichloromethane
DECP	Diethyl cyanophosphonate
DEPT	Distortionless Enhancement by Polarization Transfer
DMAP	4-(Dimethylamino)pyridine
DMEM	Dulbecco/Vogt modified Eagle's Minimal Essential Medium
DMF	Dimethylformamide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetraacetic acid

DTPA	Diethylenetriamine Pentaacetic acid
EC	Effective Concentration
ED	Effective Dose
EDCI	1-(3-Dimethylaminopropyl)-3-Ethylcarbodiimide Hydrochloride
ee	Enantiomeric Excess
ELISA	Enzyme-Linked ImmunoSorbent Assay
equiv.	Equivalent(s)
ER	Endoplasmic Reticulum
EtOH	Ethanol
FIC	Fractional Inhibition Concentration
FSC	Forward Scatter
FL	Fluorescence
GFP	Green Fluorescent Protein
Gly	Glycine
gp	Glycoprotein
GR	Glucocorticoid Receptor
h	Hour(s)
HEK293	Human embryonic kidney cells
HGO	Hepatic Glucose Output
HIV	Human immunodeficiency virus
HOBt	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenase
HSV	Herpes Simplex Virus
Hz	Hertz
IC	Inhibitory Concentration
IR	Infra Red

KTP	Kyotorphin
LAH	Lithium Aluminum Hydride
LPS	Lipopolysaccharide
MCF-7	Human Mammary Adenocarcinoma Cells
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
min.	Minute(s)
mL	Millilitre(s)
mmol	Millimole(s)
Mp	Melting Point
MS	Mass Spectrum
MS 4Å	Molecular Sieves (4Å)
MsCl	Mesyl Chloride
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Microwave
NCCLS	National Committee for Clinical Laboratory Standard
NCI	National Cancer Institute
NCIM	National Collection of Industrial Micro-Organisms
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
NSCLC	Non-small-cell Lung Carcinoma
NSI	Non Syncytium Inducing
ORTEP	Orthogonal Thermal Ellipsoid Plots
OSu	*N*-Hydroxysuccinimide Ester
PCC	Pyridinium Chlorochromate
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar

PE	Petroleum Ether
PFC	Pyridinium Fluorochromate
PHMS	Polymethylhydrosiloxane
PMB	Polymyxin B
PTB	Pyridinium Tribromide
p-TSA	*p*-Toluenesulfonic acid
p-TsCl	*p*-Toluenesulfonyl chloride
Py	Pyridine
RNA	Ribonucleic Acid
rt	Room Temperature
RT	Reverse Transcription
SAR	Structure Activity Relationships
SCP	Sterol Carrier Proteins
SI	Syncytium Inducing
S_N1	Unimolecular Nucleophilic Substitution
S_N2	Bimolecular Nucleophilic Substitution
Temp.	Temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMSCl	Trimethylchlorosilane
TMSOI	Trimethyl Sulfoxonium Iodide
TPP	Triphenylphosphine

Research Student	Deepak B. Salunke
Research Guide	Dr. Mrs. Vandana S. Pore
Research Co-guide	Dr. Braja G. Hazra
Title of the Thesis	Design, Synthesis and Bioevaluation of Steroidal Conjugates: A Study Directed Towards the Development of Novel Lead Molecules
Registration Number	EI/68/Ph.D/2005 Dated March 24, 2005
Date of Registration	21.04.2004
Place of Work	Organic Chemistry Division National Chemical Laboratory Pune 411 008

ABSTRACT

The thesis entitled *"**Design, synthesis and bioevaluation of steroidal conjugates: A study directed towards the development of novel lead molecules**"* consist of two parts.

- **Part A:** The developments made over the past few years in the isolation, design and synthesis of steroidal conjugates and their pharmacological applications are reviewed in this part.

- **Part B:** This part comprises present work, which is further subdivided into four chapters. Each chapter describes the design, synthesis and bioevaluation of novel steroid hybrids with the intention to develop innovative lead molecules.

PART A: Steroidal Conjugates and Their Pharmacological Applications.

Nature continues to be the main source of inspiration for synthetic chemists in their quest to make novel conjugates, which can have different physical, biological and medicinal properties. Nature makes these conjugates from mixed biosynthesis and some of these chimeras are found to exhibit unusual biological properties. Among the hybrid natural products, hybrids of steroid frameworks have attracted our attention due to their rigid framework with varying levels of functionalization, broad biological activity profile with significant biological properties [1]. However, the numbers of steroidal natural products are limited, where as millions of hybrids as conjugates of steroids can be prepared. During the past two decades design of such entities has been receiving increasing attention. This new approach seems to be very promising in the development of novel lead molecules. The medicinal applications are based on the fact that the biological activity of the several new hybrids exceeds that of the parent compounds.

This part of thesis highlights the molecular hybrids derived from diverse steroids through integration and/or linkage with other bio-molecules (polyamines, amino acids, and carbohydrates), drugs and other functional molecules, mainly crafted with the object of highlighting their pharmacological applications [2,3].

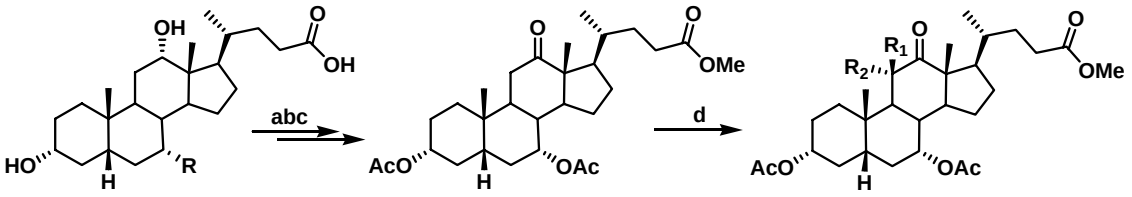
PART B: This part comprises present work.

Chapter 1: ***Synthesis of C-11 Functionalized Novel Bile Acid Derivatives***

Steroids with C-11 functionality are well known for biological activity and are obtained in a number of naturally occurring molecules such as cortisone, hydrocortisone and corticosterone. Stereoselective C-11 functionalization in the steroids is one of the

challenging targets for synthetic organic chemists as it involves severe steric interactions caused due to C-18 and C-19 angular methyl groups. In the course of our studies on synthesis of C-11 functionalized bile acid derivatives cholic acid **1** has been elaborated to its methyl 11α-bromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **4** and methyl 11β-bromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **5** derivatives *via* methyl 3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **3** (Scheme 1).

Scheme 1.



1, R = OH
2, R = H

3

4, R_1 = H, R_2 = Br.
5, R_1 = Br, R_2 = H.
6, R1 = Br, R_2 = Br.

Reagents and conditions: a) CH_3OH, *p*-TSA, 28 °C, 24 h, 96 %; b) Ac_2O, DMAP, Et_3N, CH_2Cl_2, 28 °C, 4-5 h, 84 %; c) CrO_3, H_2SO_4, Acetone, 10 °C, 5 min, 98 %; d) Br_2, Benzene, 28 °C, 96 h, 95 %.

Detailed investigation of the bromination reaction on compound **3** with excess of bromine and longer reaction period led to the isolation of hitherto unknown C-11 dibrominated product namely methyl 11,11-dibromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **6** in 4 % yield. The X-ray crystal structure of this compound showed intermolecular halogen bonding between β-bromine at C-11 and carbonyl oxygen at C-12 (Figure 1). Here we describe the effect of bulky halogen atom (bromine) in the steroid skeleton of cholic acid with different stereo-chemical orientations at C-11 on crystal structure and two-dimensional arrangement of molecules [4].

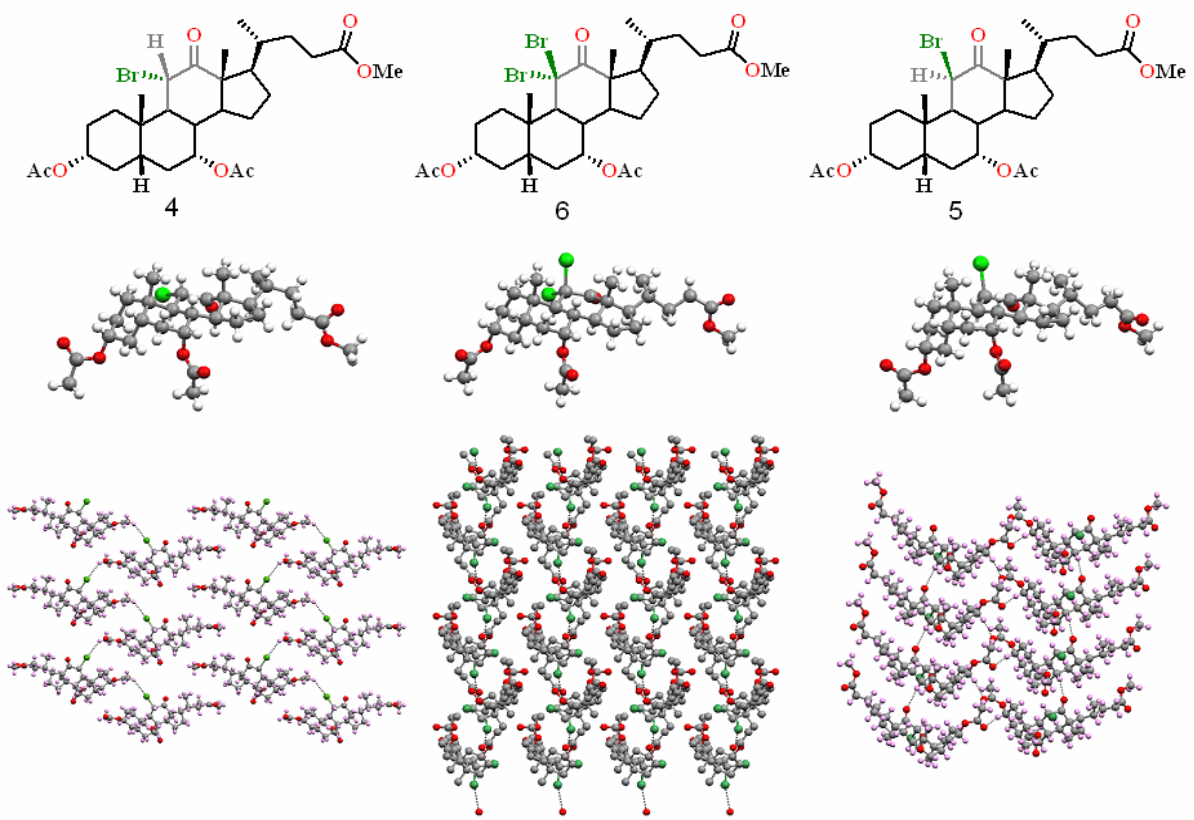
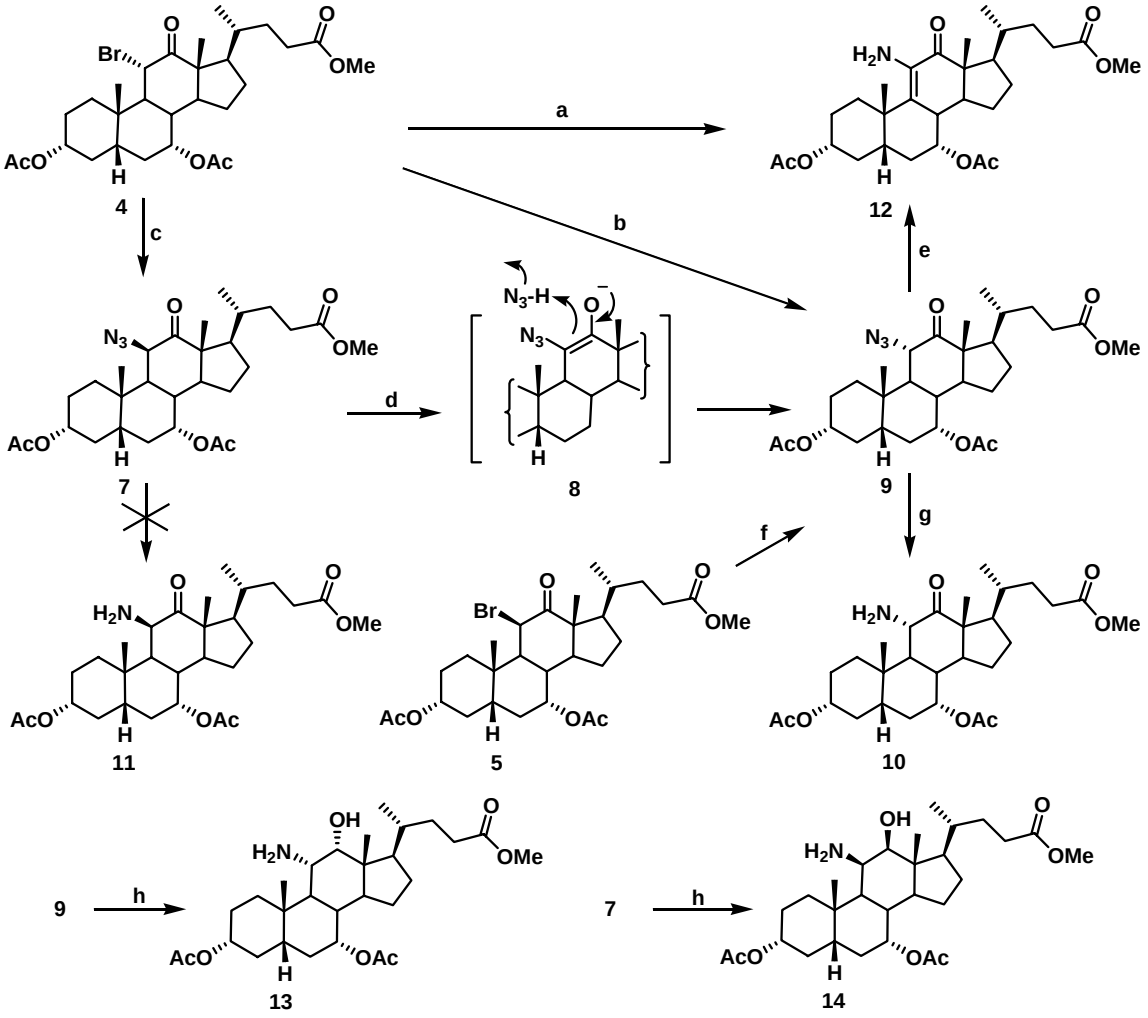


Figure 1: Two-dimensional arrangement of molecules in compounds **4**, **5** and **6** connected by C–Br…O and C–H…O interactions.

Synthesis of C-11 functionalized bile acid analogues namely methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **9**; methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **7**; methyl 11-amino-3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate **12**; methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **10**; methyl 11α-amino-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate **13**; and methyl 11β-amino-3α,7α-diacetoxy-12β-hydroxy-5β-cholan-24-oate **14** have also been achieved in good to excellent yields (Scheme 2) [5]. We have preferred a cholic acid **1** scaffold as it has attracted significant attention due to its availability and orientation of three hydroxyl groups that may be exploited in podant-type receptor, linear dimeric host or facial amphiphiles [6].

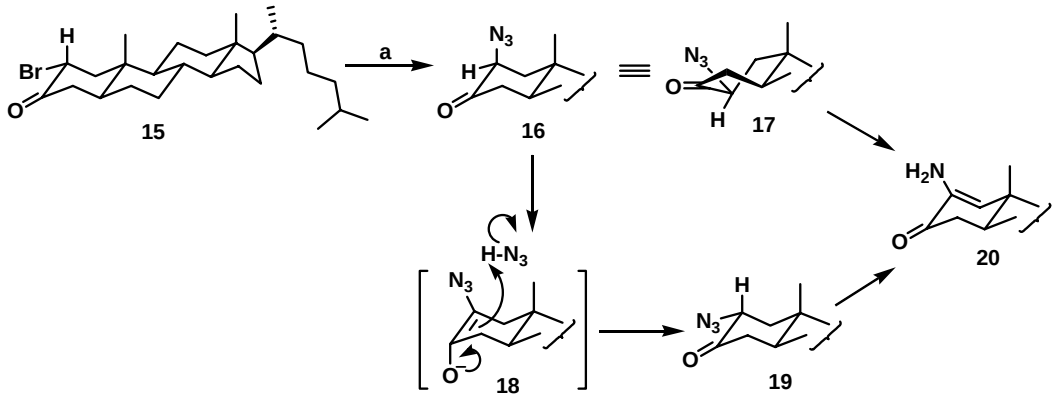
Scheme 2.



Reagents and conditions: a) NaN_3 (12 equiv.), DMF, 100 °C, 48 h, 72 %; b) NaN_3 (5 equiv.), DMF, 60 °C, 16 h, 98 %; c) NaN_3 (1.2 equiv.), DMF, 60 °C, 4 h, 64 %; d) NaN_3 (1.2 equiv.), DMF, 60 °C, 9 h, 98 % or KOAc (1.2 equiv.), DMF, 60 °C, 9 h, 98 %; e) NaN_3 (10 equiv.), DMF, 100 °C, 36 h, 67 %; f) NaN_3 (5 equiv.), DMF, 28 °C, 8 h, 85 %; g) H_2/Pd-C, EtOAc, 40 psi, 28 °C, 5 h, 95 %; h) $NaBH_4$, $CoCl_2$-$6H_2O$, CTAB, CH_2Cl_2, H_2O, CH_3OH, 25 °C, 30 min., 86 % (**13**) and 91 % (**14**).

The similar sets of compounds were synthesized using deoxycholic acid **2** as a starting material. Moreover mechanistic aspects for the decomposition of steroidal azidoketones to its enamines are discussed thoroughly involving both in ring-C as well as ring-A of steroids (Scheme 2 and 3).

Scheme 3.



Reagents and conditions: a) NaN_3 (5 equiv.), DMF, 60 °C, 16 h, 89 %.

Previously such analogous compounds are predicted to be HIV-1 inhibitors by molecular modeling studies [7]. In our study these novel bile acid derivatives induced host cell fusion during the progress of HIV-1 infection and formed multinucleated giant cells [8]. The comprehensive description is includes in this chapter.

Chapter 2: ***Design, Synthesis and Bioevaluation of Novel Steroid-Amino Acid Conjugates***

Endogenous bioactive peptides and steroids play important roles in the normal physiology or disease process of mammalian system [9]. Introduction of the amino acid or peptide to the steroid backbone offers a combination of a hydrophilic functional moiety as well as a hydrophobic carrier in a same molecule and therefore represents as an important class of molecules for drug design and development. Squalamine **21** (Figure 2) is the first sterol-spermidine conjugate that has been isolated from tissues of the dogfish shark, *Squalus acanthias* [10]. This unusual natural product has attracted considerable attention because of its potent antimicrobial activity against a broad spectrum of microbes [11]. A number of peptides have been identified that increases the permeability of the outer membranes of

Gram-negative bacteria and sensitize these organisms to hydrophobic antibiotics that ineffectively transverse the outer membranes. The best studied of these peptides are the polymyxin B (PMB) **23** derivatives. Savage and co-workers [12] have designed a class of cationic steroid antibiotics (CSA). These antibiotics display antibacterial activity comparable or superior to that of squalamine **21** or PMB **23** (Figure 2). Since these natural products are difficult to prepare and purify simple molecules were designed.

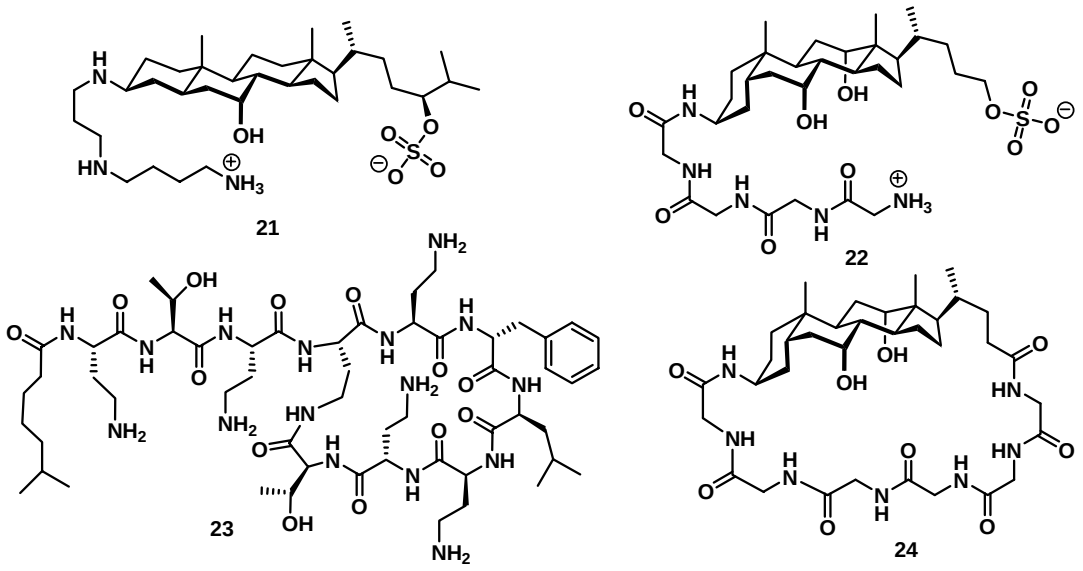
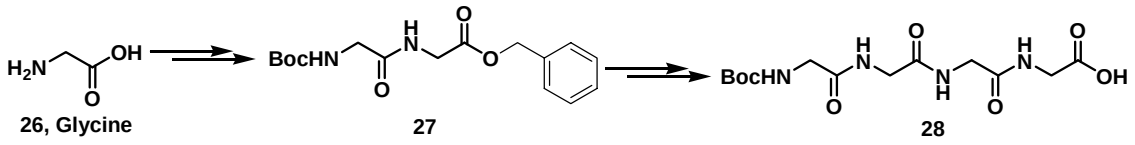


Figure 2: Squalamine **21**, Polymyxin B **23** and bile acid-amino acid conjugates **22** and **24**.

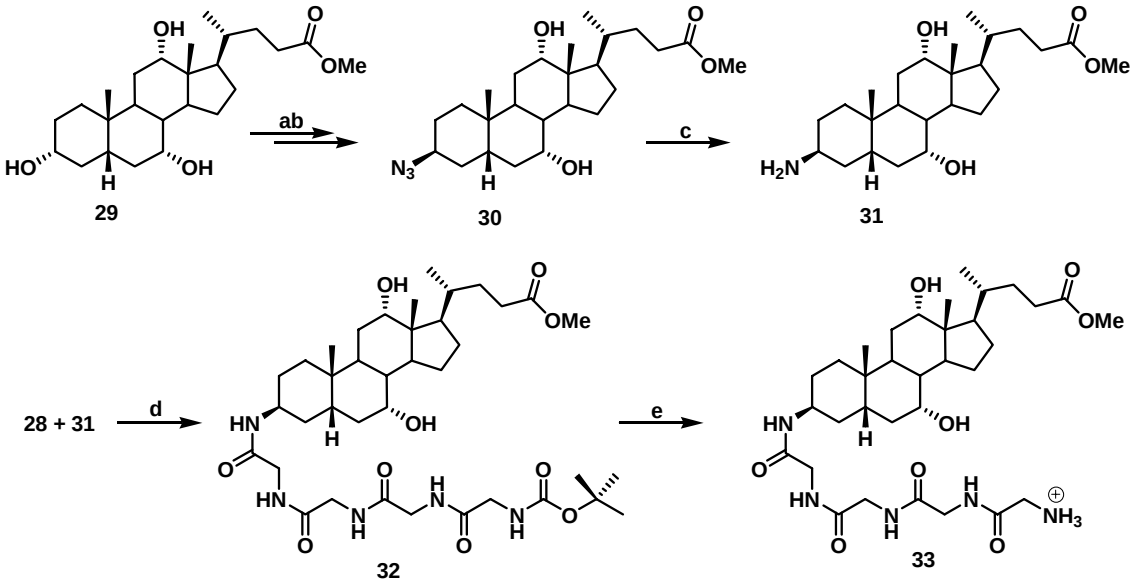
There are two major approaches in designing Squalamine/PMB mimics (i) Modification of steroids with polyamines [13] and (ii) Design of cationic antimicrobial peptides containing cationic and hydrophobic amino acids. This chapter provides a detailed description of the second approach in which we designed to synthesize novel steroid-amino acid conjugates **22** and **24** as squalamine/PMB mimic (Figure 2). A convergent approach is proposed for the synthesis of the desired targets. Suitable polyglycine chain and steroidal amino acid were synthesized as per the procedures reported in the literature. Coupling of the

polyglycine chain **28** with the steroidal amino acid **31** furnished the desired steroid-amino acid conjugate **33** (Scheme 3 and 4).

Scheme 3:



Scheme 4:

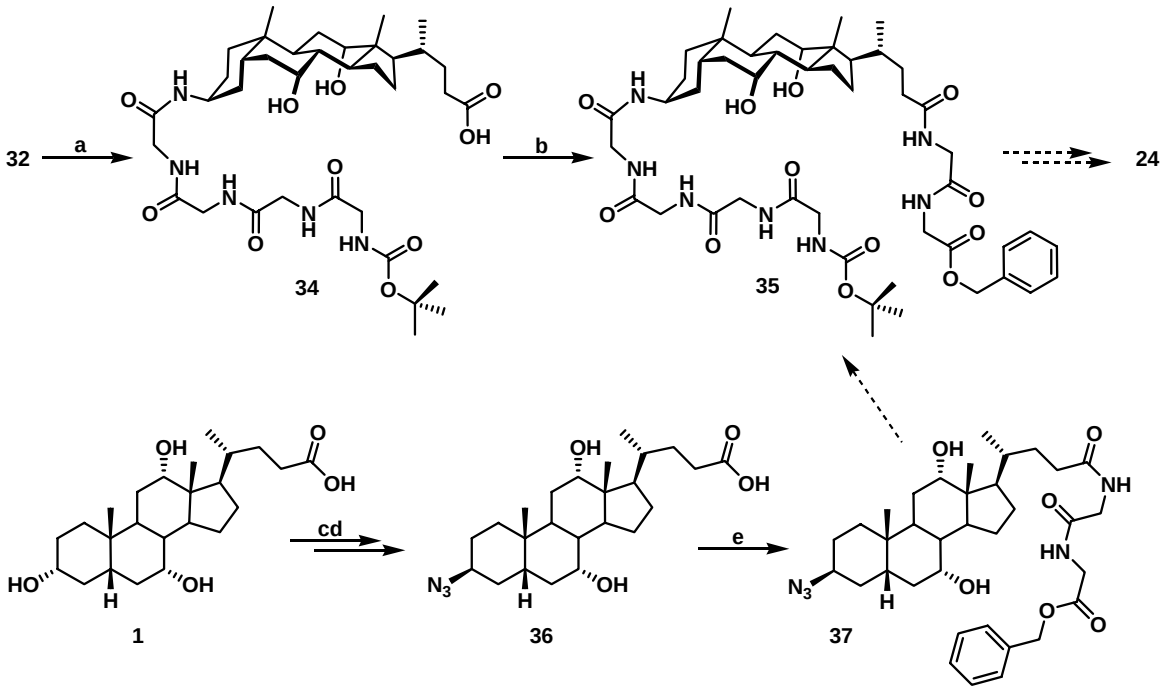


Reagents and conditions: a) MsCl, Et_3N, CH_2Cl_2, 0-25 °C, 15 min, 88 %; b) NaN_3, DMF, 80 °C, 12 h, 74 %; c) H_2, Pd-C, CH_3OH, 25 °C, 4 h, 87%; d) EDCI, HOBT, Et_3N, DMF, 0-25 °C, 12 h, 74 %; e) HCl in THF, 0 °C, 2 h, 95 %.

In conclusion a generic structure wherein fine-tuning of the molecular amphiphilicity is possible, have been designed based on squalamine. All the synthesized compounds were tested against a wide variety of microorganism and their cytotoxicity was evaluated against human embryonic kidney (HEK293) and human mammary adenocarcinoma (MCF-7) cell lines. These compounds interact synergistically with antibiotics such as fluconazole and erythromycin to inhibit growth of fungi and bacteria respectively at 1-24 μg/mL [14].

The first synthetic approach towards the synthesis of steroidal cyclic peptide **24** (Figure 2) is described in Scheme 5. The low overall yield of compound **35** was the major draw back in this approach. Therefore a second approach towards the synthesis of the intermediate steroidal hexapeptide **35** is exploited. Several trials for the chemo-selective reduction of azide functionality in compound **37** were failed.

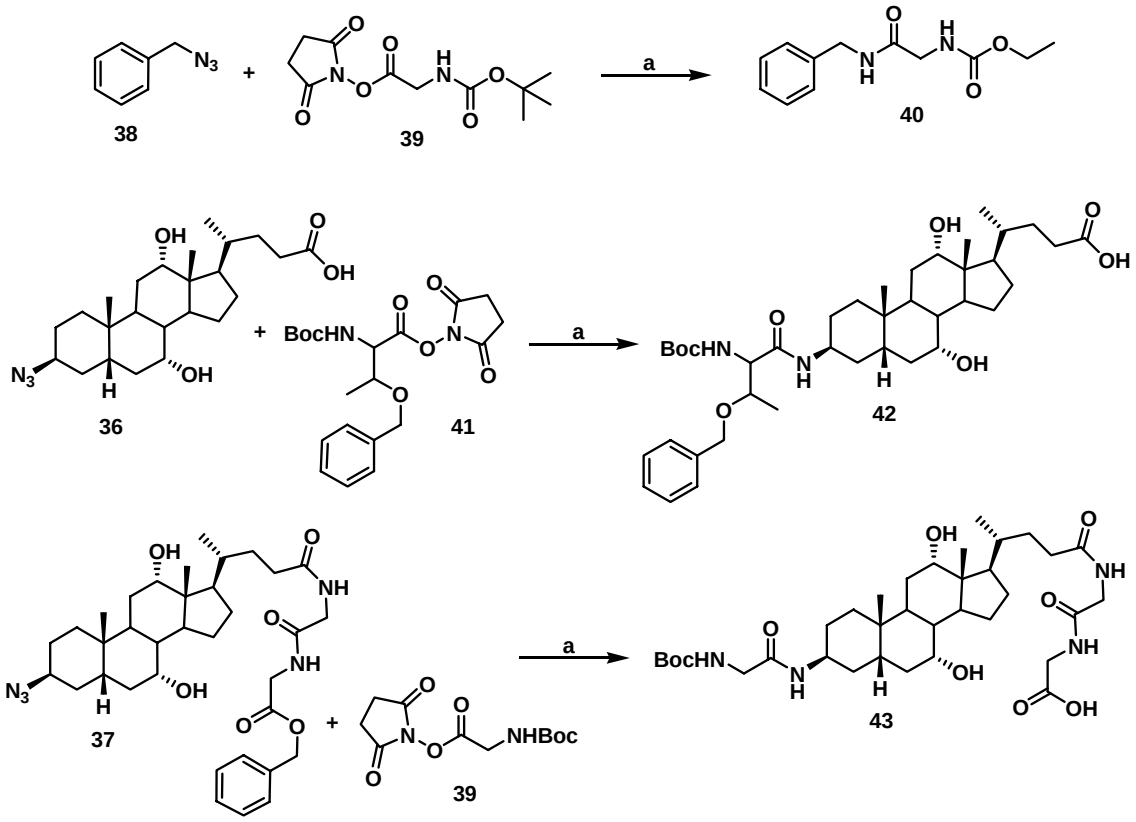
Scheme 5:



Reagents and conditions: a) LiOH, CH_3OH, H_2O, 25 °C, 5h; b) EDCI, HOBT, Et_3N, DMF, 0-25 °C, 12 h; c) MsCl, Pyridine, 0-25 °C, 4 h; d) NaN_3, DMF, 100 °C, 24 h, 86 % in two steps; e) EDCI, HOBT, Et_3N, DMF, 0-25 °C, 12 h, 84 %.

Therefore to increase the overall yield for the intermediate steroidal peptide **35** and to overcome the problem of chemoselective reduction of azide functionality in compound **37** we planned to develop a methodology for the direct one pot conversion of compound **37** to compound **35**. The preliminary results on the progress of this methodology work are illustrated in Scheme 6.

Scheme 6:



Reagents and conditions: a) Pd-$BaSO_4$, H_2 or PHMS, EtOH or THF, 25 ºC, 4-8 h, 32-90 %.

Chapter 3: ***New Steroidal Dimers with Antifungal and Antiproliferative Activity***

During the last four decades, there has been tremendous increase in the frequency of fungal infections. *Candida albicans* is the most dreadful human pathogen for which current drugs include amphotericin B (Amp B) **45** and a variety of azoles. Amp B is reported to be toxic to humans and clinical resistance to azoles is increasing. Therefore, there is need to screen for new antifungal therapeutics, which have high efficacy and low toxicity.

Design of bile acid derived facial antimicrobial. A common feature of bile acid derived antimicrobials is the potential to exhibit facially amphiphilic conformations containing polar and hydrophobic surfaces **44** [15] (Figure 5). Such type of amphiphilicity can be achieved

by polyene macrolide antibiotics like Amp B **45** and also by peptide antimicrobial agents having cationic residues and hydrophobic amino acids such as polymyxin-B **23**. Similar to Amp B, squalamine in the cyclic form **24** functions as an ionophore [16] and several squalamine mimics such as **46** also possesses unusual ionophoric properties.

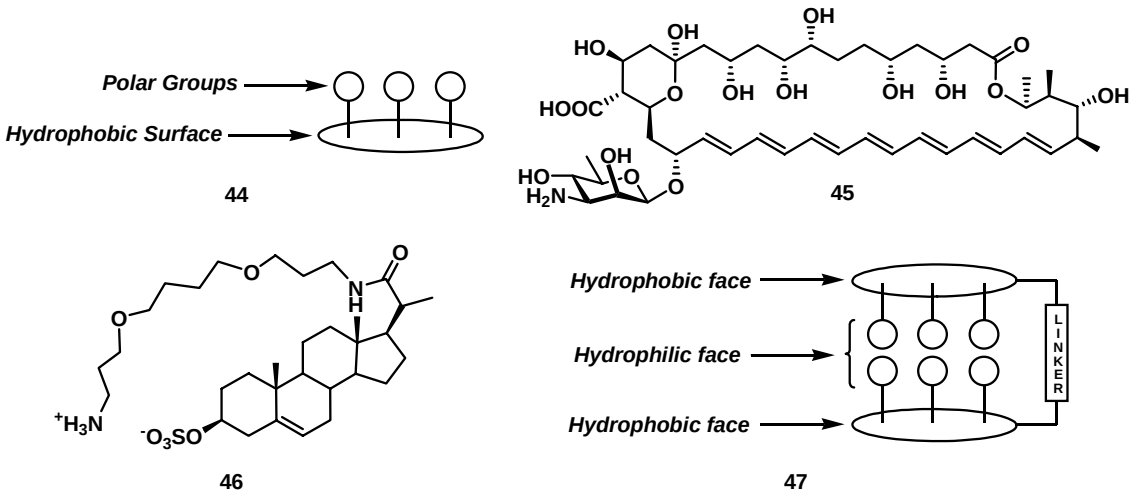
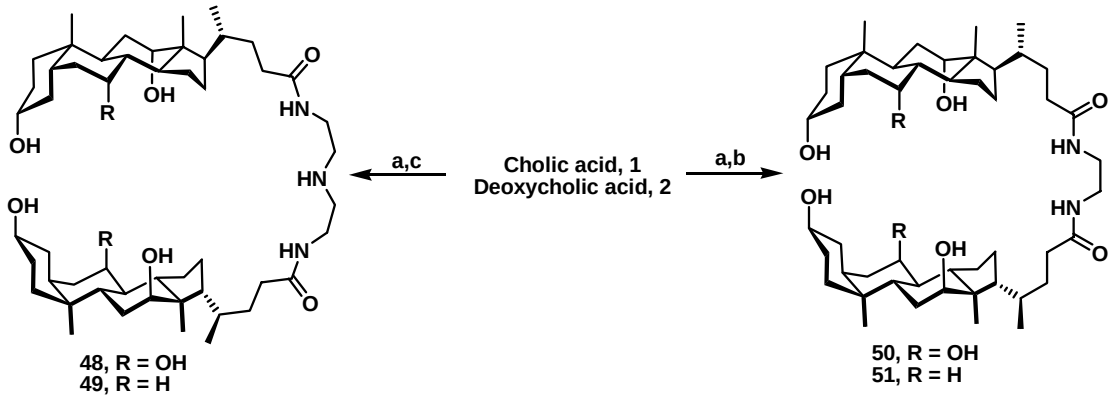


Figure 5. Amphotericin B (Amp B) **45** and bile acid derived facial antimicrobials.

Although the squalamine and polymyxin B mimics are morphologically dissimilar, they display similar activities. Based on these observations we thought of an amphiphilic topology that has not previously received much attention in synthetic systems: partially rigid structure with three discrete faces, one polar face sandwiched within two non-polar faces **47** (Figure 5). In this, steroid skeleton provides hydrophobic surface and hydroxyl groups can play a role of polar head group. Different linkers can be used so that additional hydrophobicity can be achieved. To support our hypothesis novel steroidal dimers **48** to **51** (Scheme 7) were designed, synthesized and their antifungal activity was evaluated [17]. Tested compounds **49** and **51** exhibited antiproliferative activity at micromolar concentrations. (IC_{50} 2-3 μM). Compound **51** exhibited profound effect where as compound **49** inhibited cell growth partially.

Scheme 7:



Reagents and conditions: a) NHS, DCC, THF-CH_3CN, 25 °C, 18 h, 97 %; b) Ethylenediamine, DMF, 25 °C, 1.5 h, 97 %; c) Diethylenetriamine, DMF, 25 °C, 1.5 h, 98 %.

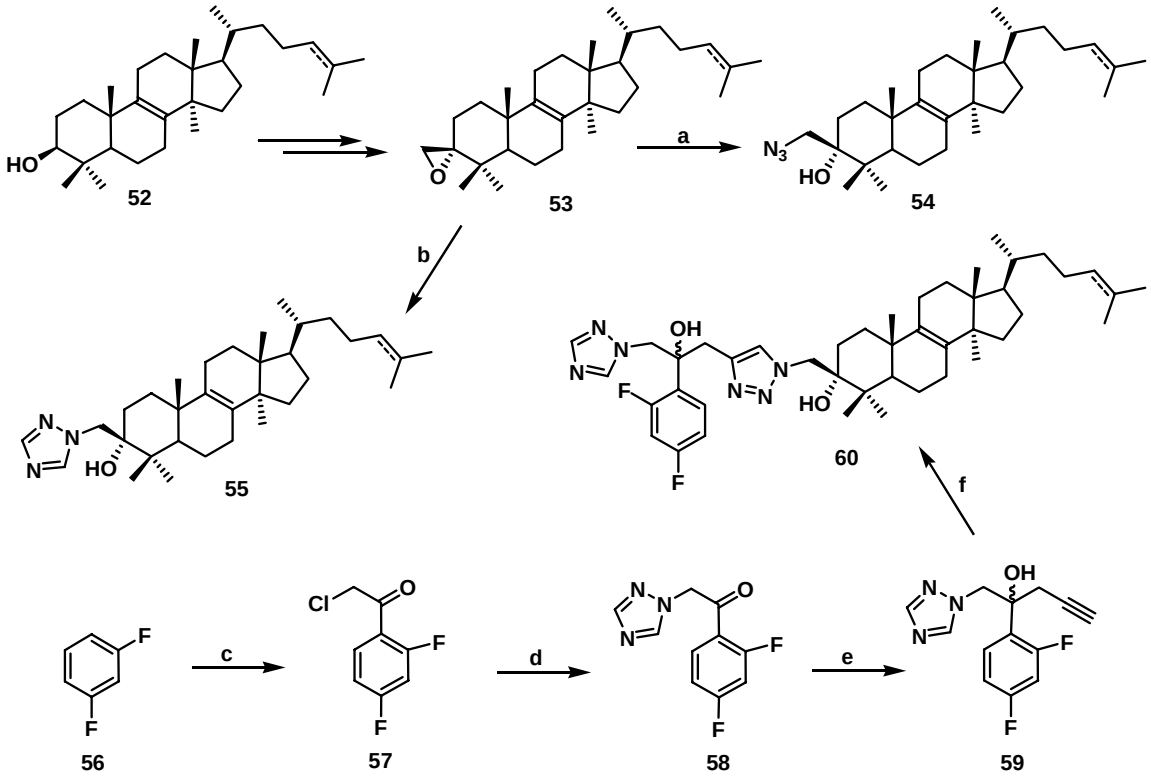
The growth suppressive effect of these compounds appears to be influenced by the length of side chain present. Since compound **48** and **50** had no effect on cell survival, the absence of hydroxyl group at C-7 position plays a critical role in toxicity of these compounds towards human cells. To study the consequence of substitutions at C-3 and C-11 on the physical properties as well as their biological effects, few more dimeric steroids were synthesized.

Chapter 4: ***Synthesis of Steroid Based Potential Inhibitors of Ergosterol Biosynthesis***

The dramatically rising prevalence of multidrug-resistant microbial infections in the past few decades has become a serious health care problem. In order to prevent this serious medical problem, the elaboration of previously known drugs to new class of molecules is highly desirable. These new molecules are expected to have improved antimicrobial activity. Over the past several years there have been quite remarkable advances made in azole antifungal agents [18]. In view of this, we planned to synthesize novel steroid-azole conjugates. In membranes, sterols play a major role both architecturally and functionally.

The most common membrane sterol in animal is cholesterol and the major sterol of most fungi is ergosterol. During the biosynthesis of ergosterol in fungi, a non-catalytic carrier protein (s) (sterol carrier protein) does the job of transfer of lanosterol **52** to the mitochondria and 24-methylene-24,25-dihydrolanosterol back to the endoplasmic reticulum (ER) [19,20]. It is clear from this that the modified sterols having lanosterol or zymosterol like backbone can best fit in to the active site pockets of these sterol carrier proteins thus inhibit the biosynthesis of ergosterol in fungi. Based on the above-mentioned observations we have designed the following new molecules **55** and **60** (Scheme 8). In these compounds we have attached 1,2,4-triazole and modified fluconazole at 3β-position of lanosterol respectively. Compounds **55** and **60** are expected to have improved antifungal activities.

Scheme 8.



Reagents and conditions: a) NaN_3, $LiClO_4$, DMF, 100 ºC, 24 h, 71 %; b) Imidazole, DMF, 100 °C, 36 h, 70 %; c) $CuSO_4 \cdot 5H_2O$, Sodium ascorbate, DMF:H_2O, MW, 5 min, 92 %.

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PART A

Steroidal Conjugates and Their Pharmacological Applications

A.1. Introduction

Nature continues to be the main source of inspiration for synthetic chemists in their quest to make novel conjugates, which can have different physical, biological and medicinal properties. Nature makes these conjugates from mixed biosynthesis and some of these chimeras are found to exhibit unusual biological properties. Among the hybrid natural products, hybrids of steroid frameworks have attracted our attention due to their broad biological activity profile and significant therapeutic effects. Steroids have become ideal synthons for the development of diverse conjugates due to their rigid framework with varying levels of functionalization and ability to penetrate the cell membranes and bind to specific hormonal receptors. Steroids form a group of structurally related compounds that are widely distributed in animals and plants. The medicinal chemistry of steroids covers a large and interesting series of structures and biological activities [1]. The chemistry and biochemistry of this natural product is extensively studied and utilized in the development of various drugs, especially for hormonal imbalance, for the treatment of infections and cancer as well as inflammation. However, the number of steroidal natural products is limited, where as millions of hybrids as conjugates of steroids can be prepared. During the past two decades design of such entities has been receiving increasing attention. This new approach seems to be very promising in the development of lead molecules, which can be used for combating diseases caused by bacteria and fungi that develop resistance due to indiscriminate use of antibiotics. The medicinal applications are based on the fact that the biological activity of the several new hybrids exceeds that of the parent compounds. The advantage of this concept over a combinatorial chemistry approach is the high diversity and the inherent biological properties of the steroid molecules.

A recent survey on hybrid molecules covers a vast literature. Dinan and co-workers [2] carried out an assessment of compounds, representing a number of classes of natural products including ecdysteroid agonist and antagonist. There are two reviews on the synthesis of natural product hybrids mainly designed with the objective of enhancement of therapeutic spectrum [3]. Recently we have reported [4] the use of bile acid-polyamine conjugates as synthetic ionophores and Virtanen has presented a microreview [5] on the use of bile acid based conjugates for pharmacological and supramolecular applications. This part of thesis highlights the molecular hybrids derived from diverse steroids through integration and/or linkage with other biomolecules (polyamines, amino acids, and carbohydrates), drugs and other functional molecules, mainly crafted with the object of highlighting their pharmacological applications.

A.2. Steroid-Polyamine Conjugates

Polyamines are polycationic at physiological pH and play key roles in biological systems [6]. In chromatin, polyamines such as tetra-amine spermine **1** and tri-amine spermidine **2** (Figure 1) help to package DNA into nuclei by neutralization of the poly-anionic phosphate backbone charges. Lipopolyamines condense DNA more efficiently than simple polyamines [7]. Small-molecule lipopolyamines are therefore of considerable interest due to their advantages of low toxicity, low immunogenicity, controllable synthesis and defined molecular structure for pharmaceutical characterization [8]. The sterol-polyamine conjugates as new class of antibiotics have attracted much interest in recent years, due to the dramatically rising prevalence of multidrug-resistant microbial infections such as emergence of penicillin-resistant *Staphylococci*, *Streptococcus pneumoniae* in hospitalized patients [9].

A.2.1. *Squalamine analogues*

Squalamine **3** (Figure 1) is the first sterol-spermidine conjugate that has been isolated from tissues of the dogfish shark, *Squalus acanthias* [10]. This unusual natural product has attracted considerable attention because of its potent antimicrobial activity against a broad spectrum of microorganisms [11]. Squalamine is useful for the treatment of serious diseases such as cancer (lung, ovarian, brain and others), age-related macular degeneration and the control of body weight in man. A minireview has appeared recently which summarizes and highlights the different advances in the understanding of the antimicrobial and antiangiogenic activity of squalamine [12]. Attempts to obtain large amounts of squalmine from the dogfish shark resulted in the discovery, isolation and characterization of family of novel aminosterols [13].

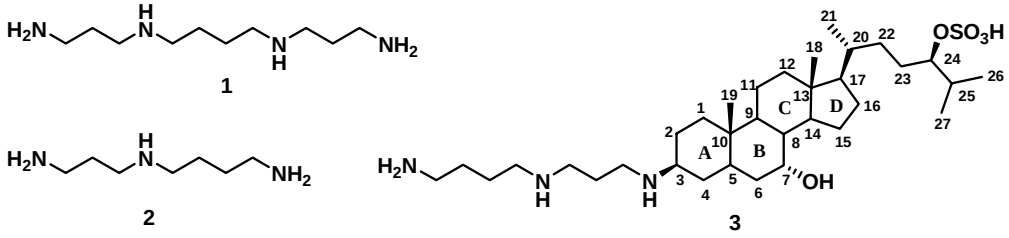


Figure 1.

The fact that insufficient amounts of squalamine were available for mechanistic studies, coupled with clear need for the preparation of analogs prompted several groups to undertake the synthesis of squalamine [14] and its analogues. A short review on synthesis of spermine and spermidine analogs of shark aminosterol squalamine has been reported [15]. Formal synthesis of squalamine has been achieved in twelve steps from desmosterol with 7.4 % overall yield by Takeuchi and co-workers [16] and recently Zhou *et al* have accomplished a concise and stereoselective synthesis of squalamine from chenodeoxycholate [17].

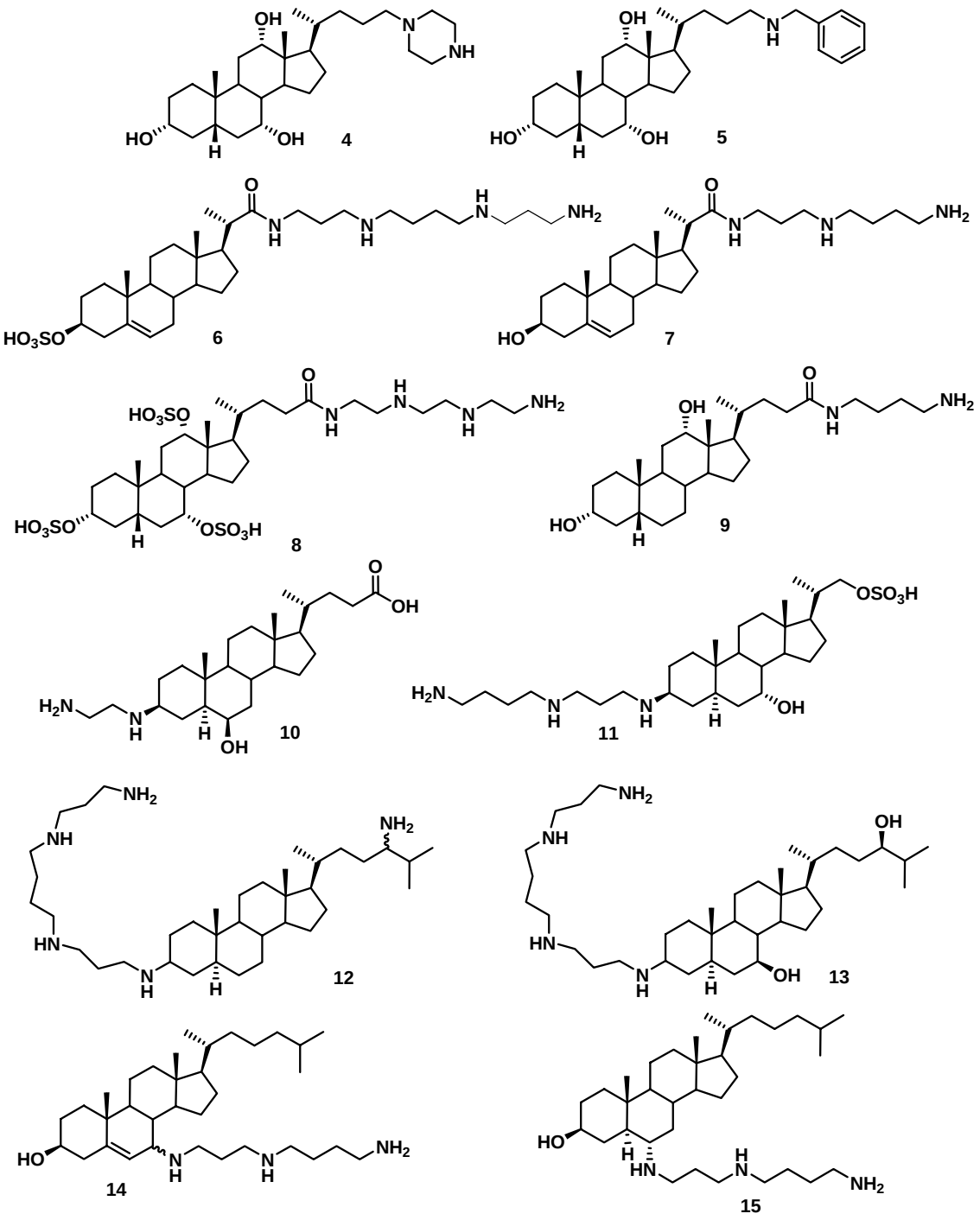


Figure 2.

Before the discovery of squalamine, cholic acid derivatives **4** and **5** (Figure 2) with amine groups incorporated at the C-24 position were described to display only weak antimicrobial activity [18]. Soon after the isolation of squalamine, Regen and co-workers

reported [19] the rapid construction of squalamine mimic **6**. In this molecule they have exchanged the positions of pendant spermidine and sulfate groups on the A and D rings of a closely related sterol. Compound **6** not only mimics the structure of squalamine but also its extraordinary antimicrobial properties. Furthermore, Gilbert *et al* have investigated [20] the role of sulfate groups of squalamine by preparing various simplified analogues of compound **7**. Bradley *et al* have achieved synthesis of same analogue **6** of squalamine by using solid phase synthesis [21]. They have used a new biocompatible pH cleavable linker for this solid phase synthesis. Armstrong and co-workers investigated the antimicrobial properties of several bile acid based squalamine mimics [22]. The mimics like **8** and **9** were prepared by linking putrescine, triethylenetetraamine and spermine in the side chain of different bile acids such as cholic acid, deoxycholic acid, lithocholic acid, ursocholic acid, chenodeoxycholic acid and hyodeoxycholic acid. Jones *et al* described the synthesis and antimicrobial activities of new squalamine analogues, such as 6β-hydroxy-3-aminosterols **10** with *trans* A/B ring junction, from hyodeoxycholic acid [23]. Synthesis of squalamine analogue **11** having short side chain has been reported by Kim and co-workers [24]. Selinsky *et al* synthesized several new analogues *e.g.* **12** and **13** of squalamine and studied their antimicrobial activity [25]. Stereoselective synthesis of squalamine desulfated analogues **14** and **15** in which polyamine chain is attached to B ring of steroid skeleton were reported by Kihel and co-workers (Figure 2) [26]. Some of these analogues showed similar anti-bacterial activity to the parent compound squalamine.

Variations in the structure of the analogues led to changes in the spectrum of activity against a variety of bacteria and yeasts. A number of intuitive assumptions can be drawn from this wide-ranging literature survey.

- Three common elements are required for the characteristic activity (i) long and rigid hydrophobic unit; (ii) a flexible hydrophilic chain which is linked to hydrophobic unit; (iii) a pendant polar head group.
- The precise structure of the polyamine is not important.
- The sulfate groups can be replaced by a carboxylate or hydroxyl or even removed altogether.
- The structure of the rigid hydrophobic unit *i.e.* steroid can also be varied.

A.2.2. *Cationic steroid antibiotics*

Savage and co-workers have designed a class of cationic steroid antibiotics (CSA) as steroid polyamine conjugates, with the intention to mimic the antibacterial activities of polymyxin B (PMB) **16** (Figure 3) [27]. These antibiotics display antibacterial activity comparable or superior to that of PMB against Gram-negative bacteria. They have also developed a means of directly observing cell selectivity with fluorophore-appended antibiotics using intact prokaryotic and eukaryotic cells to observe if the affinity of steroid antibiotics for lipid A correlates with cell selectivity [28].

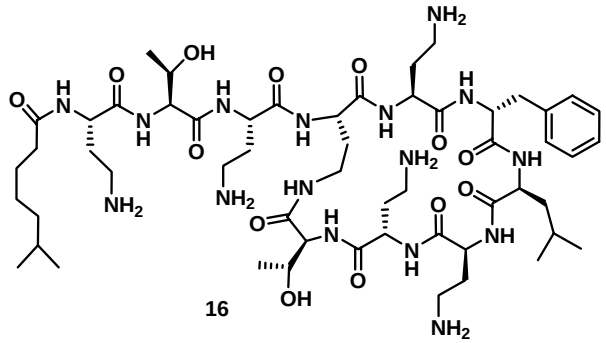


Figure 3.

Acquired resistance to membrane active compounds requires significant changes in the membrane structure to cause alterations in membrane permeability [29]. PMB **16**

contains a lipophilic acyl chain and a heptapeptide ring that is responsible for lipopolysaccharide (LPS) binding. Resistance to PMB involves modification of LPS in the outer membranes of Gram-negative bacteria [30]. Since PMB is difficult to prepare and purify simple molecules capable of associating with LPS and alter the permeability of Gram-negative bacteria were designed [31]. There are two major approaches in designing PMB mimics (i) Modification of steroids with polyamines and (ii) Design of cationic peptides containing cationic and hydrophobic amino acids. This section will provide a detailed description of the first approach. Whereas the second approach will be described briefly in the section entitled steroid-amino acid conjugates (Section A.3).

Initial steroid based mimics of PMB were designed on simple modeling of PMB by using bile acids. Bile acids have attracted significant attention due to availability and the orientation of the hydroxyl groups that may be exploited in podant-type receptors [32], linear dimeric hosts [33] or facial amphiphiles [34]. In addition, bile acids are natural ligands specifically recognized by hepatic cells and are amphiphilic molecules that undergo a biological recycling during enterohepatic circulation [35]. In PMB the amine groups on the macro ring are oriented on one face of the molecule but are segregated from the hydrophobic groups. Based on these observations PMB mimics **17** to **19** were synthesized from cholic acid (Figure 4).

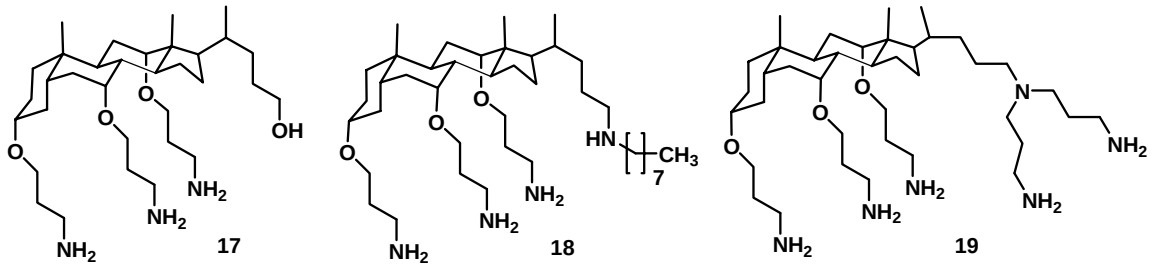


Figure 4.

In these molecules amino groups have been separated from the hydrophobic steroid moiety by ether linkage with stereochemically oriented oxygen atoms, which force the amine groups to occupy one face of the steroid. This allows the cholic acid derivatives **17**, **18** and **19** to exhibit facial amphiphilicity common to cationic peptide antibiotics. Compound **18** shows potent bactericidal activity against Gram-negative and Gram-positive bacteria while compound **17** that has no hydrophobic chain at C-24 does not show bactericidal activity against Gram-negative bacteria. This parallels to that of PMB and its derivatives [36].

A.2.3. *Synthetic ionophores*

Ion transport across membrane is crucial in living cells. Proteins that serve as ion channels or carriers provide this activity. The desire to understand these proteins, along with the need for new antibiotics, has spurred development of ion channel models [37]. Squalamine may not appear to be a facial amphiphile and its relation to cationic peptide antibiotics is questioned. It is proposed [38] that the polyamine group becomes strapped across one face of the steroid when inserted in a membrane, a facial amphiphile is created. Regen and co-workers have described the utilization of bile acid-polyamine conjugates as synthetic ionophores and extremely useful leads in the process of drug discovery. Squalamine mimics **6** (Figure 2) and **20** (Figure 5) functions as ionophores and exhibit membrane selectivity based on surface charge [39]. It functions, as an ionophore by discharging a pH difference across the vesicle membrane. Compound **6** was found to be active for H^+/OH^- transport but not for Na^+ ion transport. Compound **20** showed [40] exactly the opposite properties to that of compound **6**. It is active for Na^+ ion transport but not for H^+/OH^- transport. Recently there are reports [41] on the synthesis of ion conductors **21** to **24**

derived from spermine and cholic acid with varying degrees of facial amphiphilicity (Figure 5). These compounds promote the passive transport of Na^+ across phosphatidylcholine vesicles. However this transport activity decreases substantially as the thickness of the bilayer is increased. A dendritic approach to the construction of a homologous series of pore-forming amphiphiles has been reported [42], based on the use of spermidine, spermine, lysine, and cholic acid.

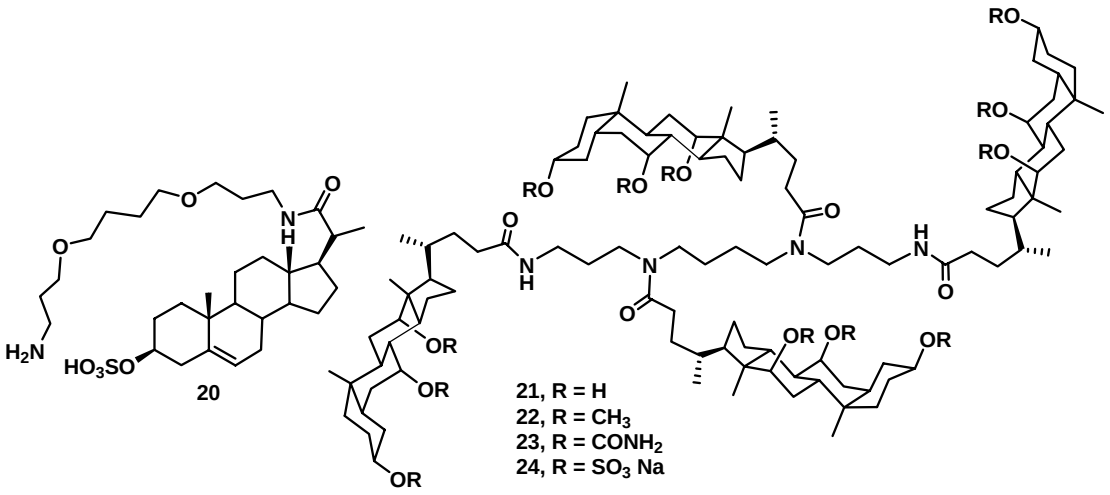


Figure 5.

Amphotericin B (Amp B) **25** (Figure 6) is a widely used antifungal drug for systemic fungal infections [43]. It kills the cells by punching holes in the cell membranes. Drug resistance towards Amp B is extremely rare during its therapeutic use [44]. However, due to its high toxicity and apparent inability to be metabolized, there was a need of biodegradable alternatives with higher membrane selectivity. Regen and coworkers sought the simplest molecules that are capable of forming ion channels and synthesized [45] sterol-oligo conjugate **26** (Figure 6). The compound **26** obtained from 5-androstene-3β,17β-diol, exhibited significant ionophoric activity and is viable as a functional equivalent of Amp B.

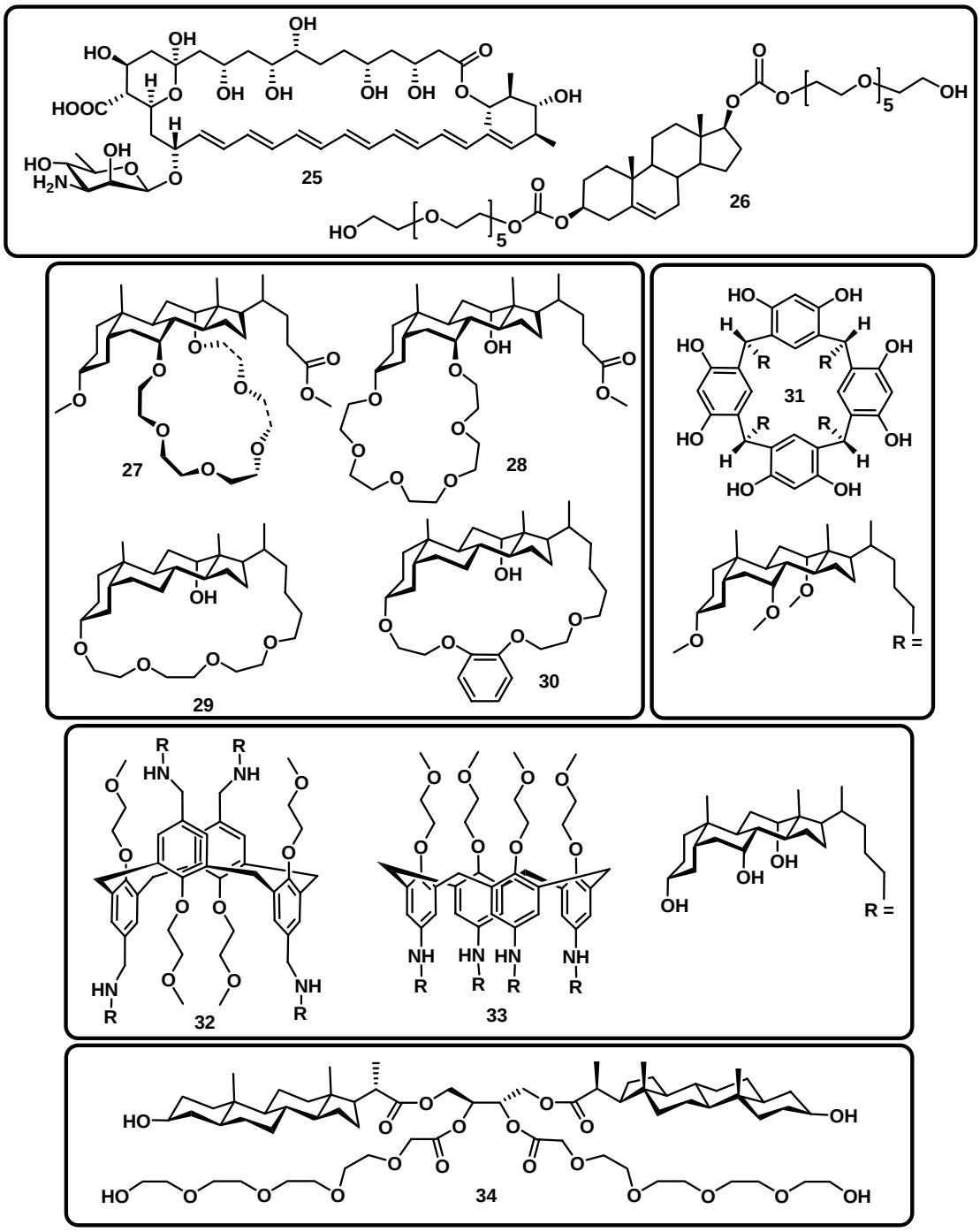


Figure 6.

Davis and co-workers explored the use of steroid nucleus especially cholic acid in the area of anion recognition [46]. The biological and pharmacological implication of synthetic channel forming molecules is currently a focal point of attention [47]. Maitra and

coworkers have synthesized chiral crown ethers **27** and **28** from cholic acid whereas Nair *et al* have synthesized crown ethers **29** and **30** from deoxycholic acid and studied their cation binding properties (Figure 6) [48]. There are two reports [49] on synthesis of artificial ionophores formed by macrocyclic resorcin[4]arene-cholic acid **31**, calix[4]arene-cholic acids **32** and **33** (Figure 6). These compounds seem to act through a unimolecular mechanism. Similar such calix[4]pyrrole-cholic acid derivatives were reported for affecting the enantioselective recognition of organic anions [50]. Kolehmainen and co-workers reported the use of novel porphyrin-cholic acid conjugates as receptors for biologically important anions [51] whereas Kral *et al* used such steroid-porphyrin conjugates for saccharide sensing in protic media [52]. A C_2-symmetrical sterol-polyether conjugate **34** as highly efficient synthetic ionophore has been reported by DeRiccardias and co-workers (Figure 6) [53]. This compound was incorporated into phospholipid vesicles and shown to facilitate Na^+ transport.

A.2.4. *Other steroid-polyamine conjugates*

Steroid-polyamine conjugates are not only exploited for their antimicrobial or ionophoric properties but also for several other different properties. Genzyme's GL-67 **35** (a spermidine bound through a carbamate functional group to a cholesteryl lipid moiety), bis(guanidium)-tren-cholesterol (BGTC) **36** (cationic cholesterol derivatives containing two guanidinium polar headgroups) and its spermidine analogue, are efficient for gene transfection *in vitro* and *in vivo*, transferring a luciferase reporter gene into primary human airway epithelial cells (Figure 7) [54]. Vitamin D_2 (ergocalciferol) polyamine conjugate **37** is a steroid with an opened B-ring, designed to probe the structure-activity effects on

transfection of modifying the geometry within the hydrophobic steroid motif [55]. Sugawara *et al* have synthesized biotinylated lithocholic acid **38** (Figure 7). Compound **38** inhibited mammalian DNA polymerase α and β with dose-dependant manner [56].

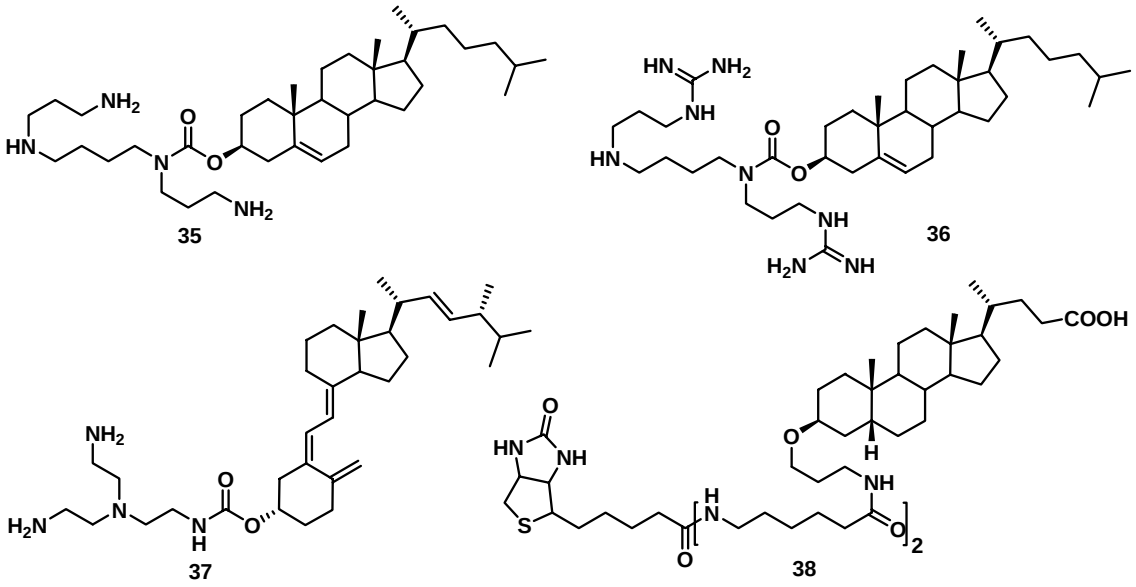


Figure 7.

Ohwada [57] and Blaghrough [58] synthesized several steroid-polyamine conjugates *e.g.* **39** and **40** (Figure 8) consisting of a hydrophobic, structurally rigid steroids (lithocholic acid and cholestane), a flexible hydrophilic polyamines, the nitrogen atom of which can be protonated under physiological conditions, and a linker which connects the hydrophobic and hydrophilic unit. Ohwada studied the hemolytic activity of these conjugates towards the bovine erythrocytes and also found that the gene transfection activity of these steroid-polyamine conjugates is influenced by the polyamine chain length and steroid structure. Blaghrough *et al* demonstrated the use of these novel polyamine conjugates **39** and **40** for efficient DNA condensation and subsequent drug delivery. They have also designed and prepared novel fluorescent molecular probes as tool to throw light on the problematic steps in non-viral gene delivery, which still impede efficient gene therapy.

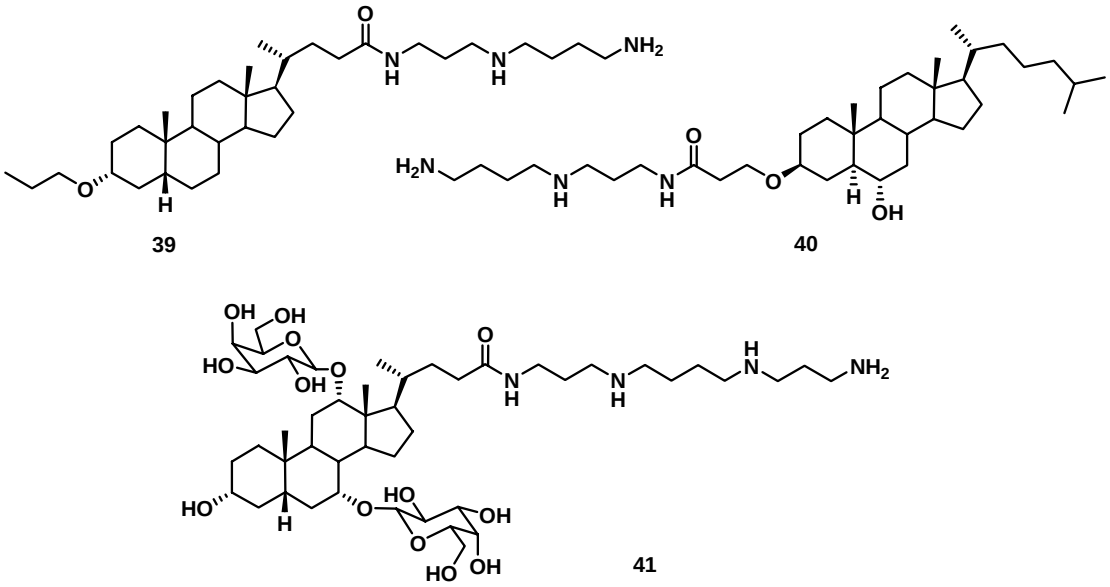


Figure 8.

Walker and co-workers have designed a promising class of compounds such as **41** (Figure 8) for DNA transfection by conjugating various polyamines to bile acid based amphiphiles [59]. Formulations containing these compounds were tested for their ability to facilitate the uptake of a β-galactosidase reporter plasmid into COS-7 cells.

A.3. Steroid-Amino Acid Conjugates

Endogenous bioactive peptides and steroids play important roles in the normal physiology or disease process of mammalian system. Biologically active peptides are recognized to have significant therapeutic potential [60]. Introduction of the amino acid or peptide to the steroid backbone offers a combination of a hydrophilic functional moiety as well as a hydrophobic carrier in a same molecule and therefore represents as an important class of molecules for drug design and development. Steroid-amino acid conjugates include such a class of compounds in which the amino acids are linked with steroids through amide or ester bonds. This class of conjugates covers a spectrum of important molecules in nature,

such as bufetoxin **42**, a 3-arginyl derived steroid product isolated [61] from the Chinese hoptoad (Figure 9). Cholyl glycine **43** and cholyl taurine **44**, which exist in the bile of animals and contains a glycyl or a taurinyl group attached to steroid.

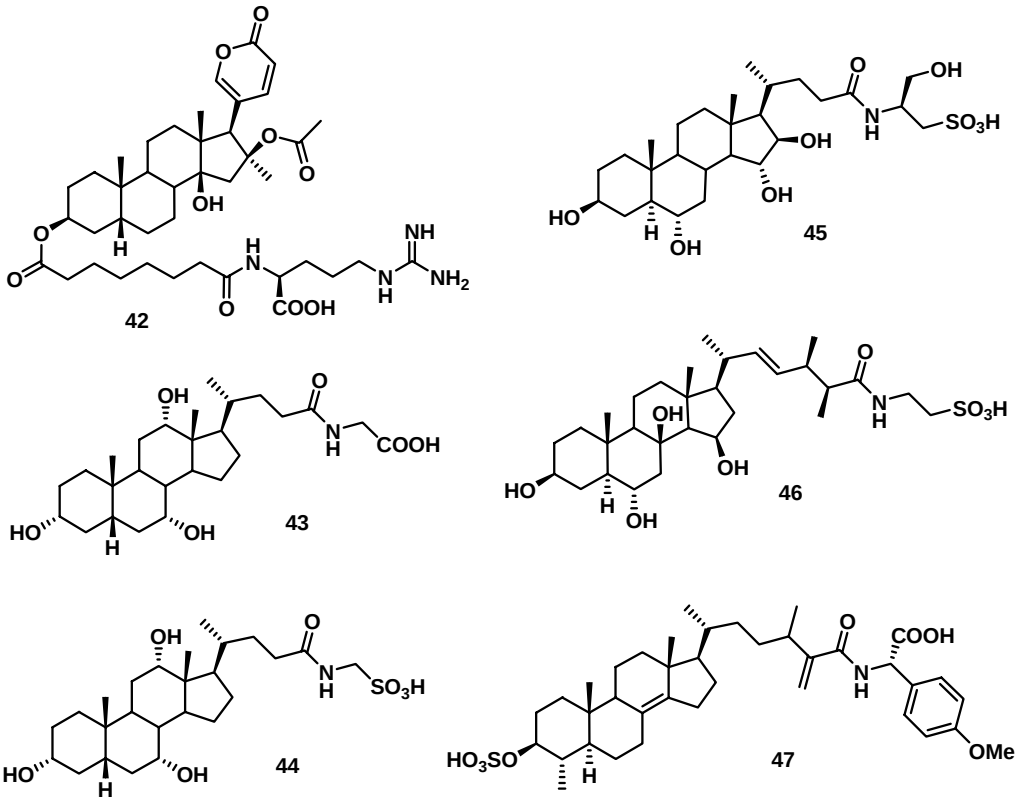


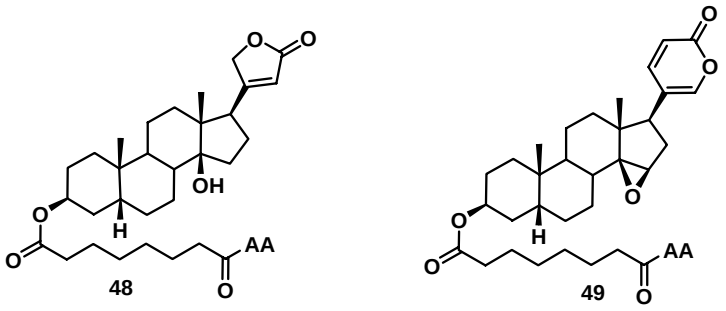
Figure 9.

A starfish steroid, carolisterol C **45** in which a 24-carboxylic acid functionality is linked via an amide bond to D-cysteinolic acid was isolated from the polar extracts of the starfish *Styracaster caroli* by Minale *et al* [62]. In view of the anti-HIV activity reported for polar sulphated sterols [63], carolisterol C was tested in the NCI's primary anti-HIV screen and showed no protection against the cytopathic effects of HIV-1. Triseramide **46** is a new steroid conjugate from the starfish *Astropecten triseriatus* [64] and Myxodermoside A is a novel marine polyhydroxylated steroid from the starfish *Myxoderma platyacanthum* (Figure 9) [65]. Myxodermoside A has been examined for the effects on the development of

fertilized sea urchin eggs and showed a modest activity in comparison with other pentaglycosides [66]. Polymastiamide A **47** (Figure 9), is a tyrosine conjugated steroid analog isolated from the Norwegion marine sponge *Polymastia boletiformis* [67]. It is an antimicrobial metabolite, and involves a linkage of steroid and a nonprotein amino acid. It is the first example of a new type of marine natural product that is formed by combination of steroid and α-amino acid. It exhibited *in vitro* antimicrobial activity against various human and plant pathogens. These types of compounds are obtained in nature having various biological properties and have been found to play a diversity of critical roles in a large number of organisms.

Virtually all-natural bile salts are conjugates of a bile acid with glycine or taurine. In certain instances small amounts of bile salts were found which were conjugates of ornithine, arginine and lysine [68]. Isolation of these conjugated acids from bile is troublesome and uncertain [69], and pure conjugates required for experimentation are best prepared by synthetic conjugation of the components. Bondi and Muller [70] for the first time converted cholic acid through the ester and the acid hydrazide into the azide, which was then coupled with glycine or taurine in an alkaline medium. Bellini *et al* reported [71] number of amino acid conjugates of cholic acid other than with glycine or taurine. A number of them showed antimicrobial activity. To investigate the substrate specificity of bile acid transport across the liver *in vivo*, Ballatore *et al* have synthesized [72] and studied cholic acid conjugates of variety of amino acids. Considering both biological and chemical interest of steroidal polypeptides, Agarwal *et al* reported [73] steroids consisting of a repeating amino acid sequences. Based on the assumption that synthetic steroidal peptides might alter, or otherwise interfere with, established hormone production, Pettit *et al* synthesized [74] 3β-

hydroxy-17β-(L-prolyl-L-prolyl) amino-5α-androstane and 3β-acetoxy-17β-(L-argenyl-L-argenyl-L-prolyl) amino-5α-androstane. In order to examine the physiological activity, Shimada *et al* have synthesized the arginine linked cardiotonic steroids **48** and **49** as bufotoxin analogs from digitogenin and resibufogenin (Figure 10) [75].



AA = Arginine / Arg-Arg / Arg-Proline.

Figure 10.

Novel bile acid-amino acid conjugates *e.g.* **50** (Figure 11) were synthesized in which the amide bond was reversed from its normal configuration [76]. The chemical and physical properties of these reverse amide conjugated bile acid analogs were compared with those of the normal glycine and β-alanine conjugates.

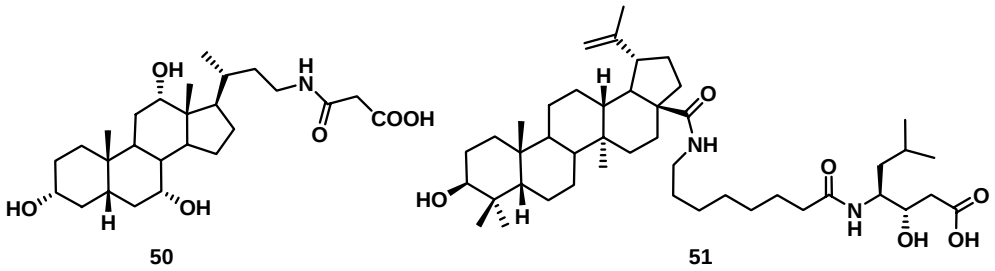


Figure 11.

In recent years a wide variety of such steroid derivatives conjugated with amino acids or peptides have been prepared with various purposes. Mayaux *et al* synthesized [77], N'-{N-[3β-hydroxyl-20(29)-ene-28-oyl]-8-aminooctanoyl}-L-statin **51** (Figure 11), by a five-step procedure starting from betulinic acid. This compound has been postulated to

interfere specifically with virus-cell fusion and inhibited HIV replication at a concentration as low as 0.02 μg/mL.

The metabolism and intestinal absorption of bile salts was studied using cholic acid conjugates *e.g.* **52** (Figure 12) with small peptides of two to six amino acids [78]. To investigate the ability of the human intestinal bile acid transporter to transport cholic acid conjugates with potential HIV-1 protease inhibitory activity, cholic acid was conjugated at the 24 position of the sterol nucleus with various amino acids and amino acid analogues such as **53** (Figure 12) [79]. In this study, one amino acid-cholic acid conjugate showed HIV-1 protease inhibitory activity.

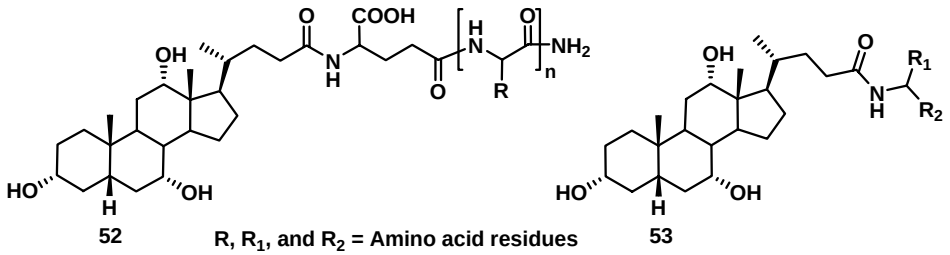


Figure 12.

Kramer *et al* synthesized various peptide conjugates of modified bile acids [80], (i) to investigate whether the hepatic and the intestinal bile acid transport system as well as the intestinal H^+/oligopeptide transporter can be used in drug therapy, (ii) to improve the membrane permeability and intestinal absorption of peptide drugs and (iii) to target a drug to the liver and the bilary system to obtain liver specific drugs. Amafalone **54** (Figure 13) (Am, 3α-amino-2β-hydroxy-5α-androstan-17-one) possessed interesting antiarrhythmic activity in animal models [81] and was developed to the stage of clinical testing, but did not become commercially available because it was deemed not to have sufficient oral bioavailability [82]. In China, Fang and co-workers [83], in an attempt to develop aminosteroid derivatives with more desirable antiarrythmic properties, prepared a number of amide and *N*-alkyl

derivatives of amafalone. These compounds exhibited lower toxicity and retained antiarrhythmic activity. Because of interest in peptides as potential therapeutic agents Mokotoff *et al* reported [84] the synthesis of peptidyl derivatives of the aminosteroid, amafalone. Six analogs were synthesized: the hydrochloride salts of Gly-Am **55**, Ala-gly-Am **56**, D-Ala-Gly-Am **57**, Pro-Am **58**, Pro-Pro-Am **59**, and D-Ala-Pro-Am **60**. Peptidyl aminosteroids **55**, **58**, **59** and **60** when administered to rat intravenously had protective antiarrythmic effects similar to those of amafalone. The oral route observed less marked protection with **58**, in comparison to amafalone, while **59** and **60** were inactive (Figure 13).

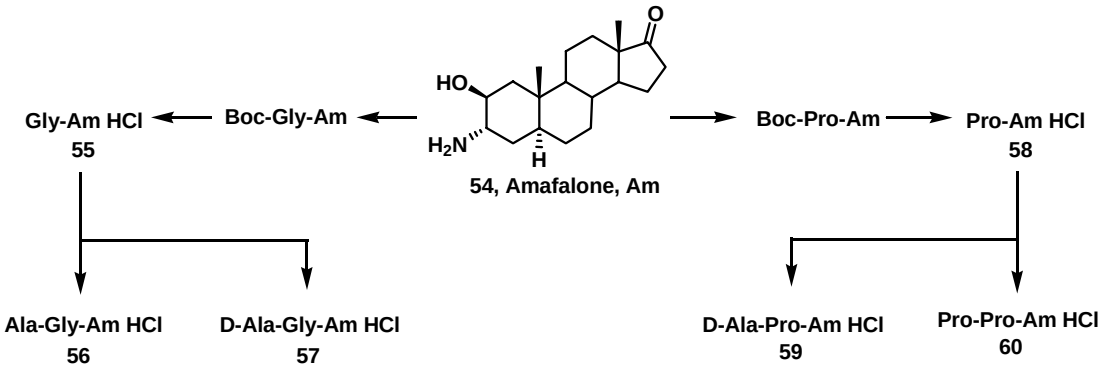


Figure 13.

The phenomenon that the steroids enhanced the effects of the peptides through increasing their receptor numbers was named ‘permissive action’ [85]. Based on this concept, the urotoxins (Glu-Asp-Gly-OH, His-Gly-Glu-OH, His-Gly-Lys-OH, and His-Gly-Lys-$NHNH_2$) were introduced into the convenient sites of hydrocortisone and prednisolone via the amidation or condensation reactions to form the corresponding linkers [86]. The results suggested that the linkers of the steroids and peptides may simulate the ‘permissive action’ and this kind of conjugation of steroids and peptides may provide a special modification for steroids and oligopeptides. Similar such ‘permissive action’ was observed by the same group [87]. For this purpose kyotorphin (KTP, Tyr-ArgOH) was coupled with

hydrocortisone and estrone to furnish the hybrid molecules **61** and **62** (Figure 14). The analgesic activities of the corresponding hybrids were investigated using tail flick test.

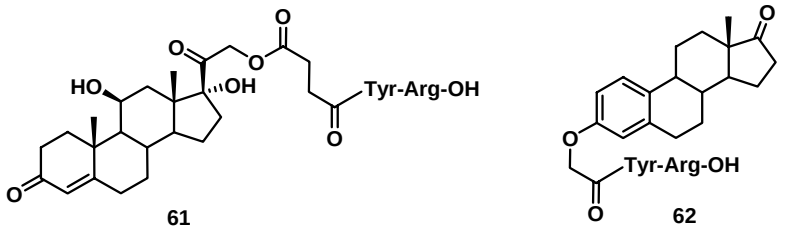


Figure 14.

In order to produce potential inhibitors of type 3, 17β-hydroxysteroid dehydrogenase (17β-HSD), a key steroidogenic enzyme, Poirier *et al* performed solid-phase synthesis of model libraries of 3β-peptido-3α-hydroxy-5α-androstan-17-ones. One of them, the 3β-(*N*-heptanoyl)-L-phenylalanine-L-leucine-aminomethyl)-3α-hydroxy-5α-androstan-17-one inhibited the enzyme with an IC_{50} value of 117 nM, which is twice as potent as the natural substrate Δ^4-dione [88].

A series of bile acid derivatives with variety of amino acids [89], amino alcohols [90] and phenanthroline [91] coupled via an amide bond were reported. These conjugates form small micelles in aqueous solutions and were found to behave as novel organogelators, forming stable, transparent and thermo reversible gels in aromatic solvents.

Thiemann *et al* in their own studies on radiolabelled estradiol derivatives have become interested to find new connective approaches to radiolabelled estrane derivatives and peptide estrane conjugates. For this they have developed a process for the synthesis of estra-1,3,5(10),16-tetraenol [17,16e] pyrimidine **64** from estrone **63** (Figure 15). Compound **64** was then transformed to a steroid-heterocycle-tripeptide conjugate **65** [92]. Complexation of rhenium (Re) to compound **65** was observed in their first exploratory experiments.

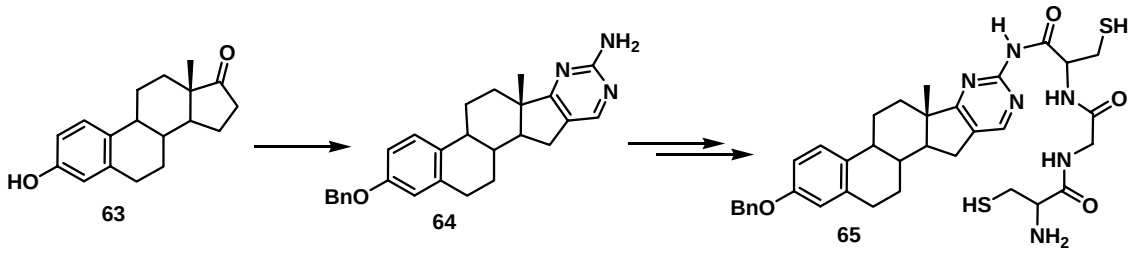


Figure 15.

A number of peptides have been identified that increase the permeability of the outer membranes of Gram-negative bacteria and sensitize these organisms to hydrophobic antibiotics that ineffectively transverse the outer membranes [93]. The best studied of these peptides are the polymyxin B derivatives. Based on these facts Savage *et al* modeled polymyxin B derivatives to determine potential active conformations and determined functionality conserved among antibiotics related to polymyxin B. This conserved functionality was incorporated on to a steroid scaffold yielding compounds **66** and **67** (Figure 16) that sensitize Gram-negative bacteria to hydrophobic antibiotics [94].

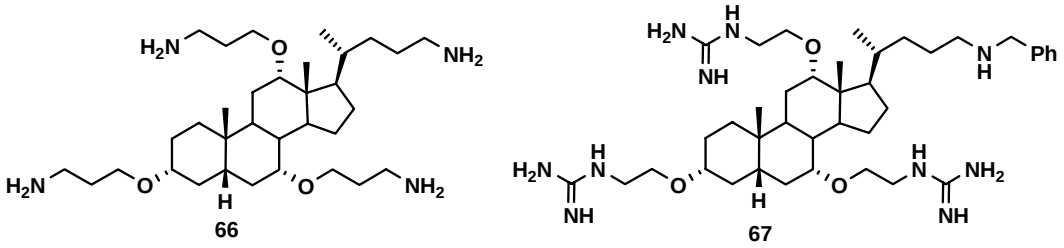


Figure 16.

The same group reported [95] the preparation of cholic acid derivatives such as **68** in which amine bearing groups are attached to the steroid via ester or amide bonds (Figure 17). As a part of this effort, they have developed efficient means of appending cholic acid derivatives with three different amino acids at C-3, C-7 and C-12 positions *e.g.* **69**. A number of the resulting compounds effectively sensitize gram-negative bacteria to erythromycin and novobiocin.

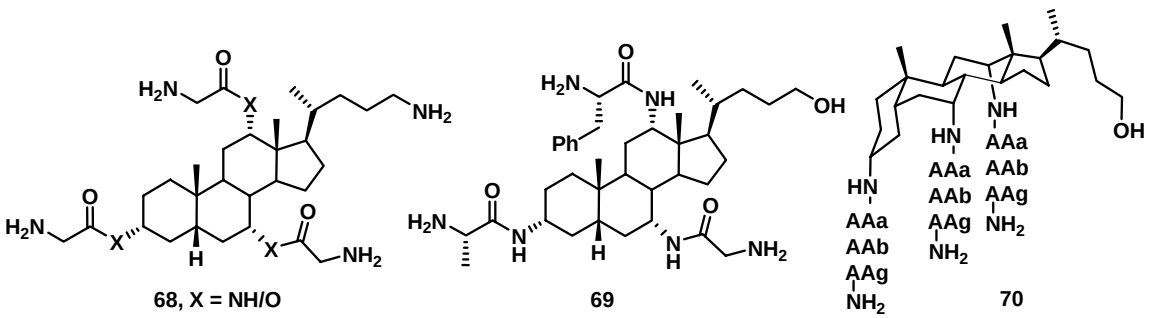
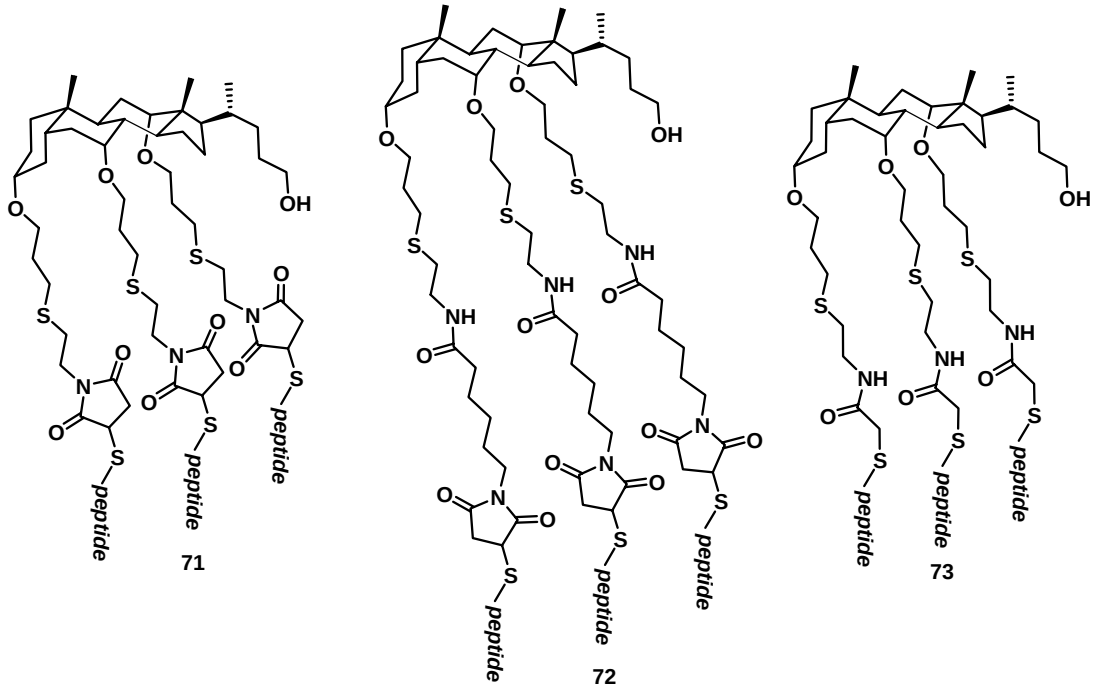


Figure 17.

In continuation with this work new cationic steroid antibiotics (CSA) have been prepared [96] by Savage *et al* by conjugating tripeptides to a triaminoanalogue of cholic acid. These CSA-peptide conjugates such as **70** (Figure 17) were synthesized on a solid phase in an indexed library that was screened for antimicrobial activity against Gram-negative and Gram-positive bacteria. Similar such orthogonally protected triamino scaffold based on the bile acid framework was developed [97] by Davis *et al*. Researchers from Columbia University also described the preparation of a combinatorial library of synthetic receptors based on chenodeoxycholic acid and screening for members that bound a certain pentapeptide such as leuenkephalin [98].

The use of cholic acid as a new template for multivalent peptide assembly has been reported recently [99] by Wang *et al*. The goal of their template-assembled peptide project was to develop mimics of the trimeric gp41 fusion intermediates to serve as vaccines and inhibitors for blocking HIV-1 infection. The desired multivalent peptides were synthesized by a typical chemoselective ligation to afford trivalent peptides **71**, **72** and **73** (Figure 18). The resulting three-α-helix bundles of DP178 are believed to mimic the conformational epitopes of gp41 that are exposed during viral membrane fusion, which should be useful for HIV-1 vaccine development.



peptide, P-37C: Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFC-NH_2
T-helper: CGSSSQYIKANSKFIGITEL-NH_2
P7C: Ac-ELDKWAC-NH_2

Figure 18.

In order to synthesize orally active insulin analogues Byun *et al* modified the recombinant human insulin by covalently attaching deoxycholic acid derivatives. The recombinant insulin conjugates, [N^{B29}-deoxycholyl]insulin **74** and [N^{B29}-bisdeoxycholyl-L-lysil]insulin **75**, were studied for their chemical, structural and biological properties (Figure 19) [100]. Competitive insulin binding assay with HepG2 cells revealed that monosubstituted insulin conjugates retains high binding affinity to the insulin receptor. When the insulin conjugates were intravenously administered (0.33 IU/Kg) to streptozotocin induced diabetic rats, the conjugates showed sustained biological activity for a longer period with the similar lowest blood glucose level compared to native insulin.

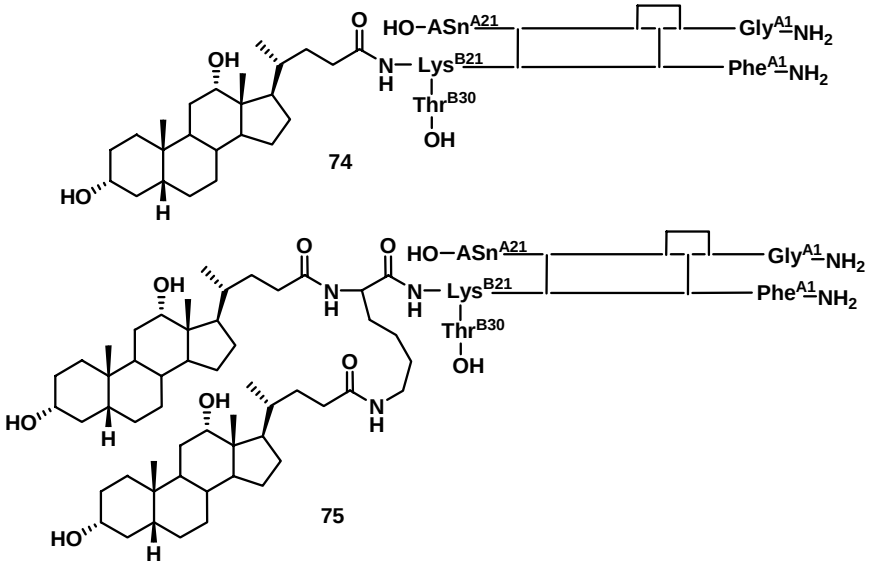
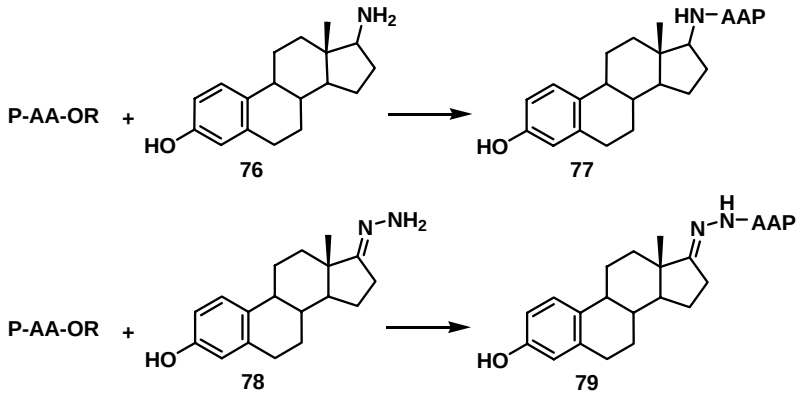


Figure 19.

Ye *et al* synthesized [101] series of *N*-protected amino acid-estradiol conjugates such as **77** and **79** (Figure 20) by coupling of 17β-aminoestra-1,3,5(10)-trien-3-ol **76** or 17β-hydrazonoestra-1,3,5(10)-trien-3-ol **78** with different amino acid *via* the catalysis of subtilisin Carlsberg in organic solvents. *In vitro* biological activity studies revealed that the binding interactions between estradiol conjugates and estrogen receptors can be affected by the properties of conjugated amino acid, but the effects of the change in binding properties did not result in changes in biological activities in both MCF-7 and HeLa cell lines.



AA = Ala, Leu, Phe, Asp(OBzl), Asp, Lys(Z)

Figure 20.

A few reports have been published in literature on the synthesis of cyclic polypeptides such as **80** and **81** as a novel scaffold for the design of combinatorial compound libraries [102-103] (Figure 21).

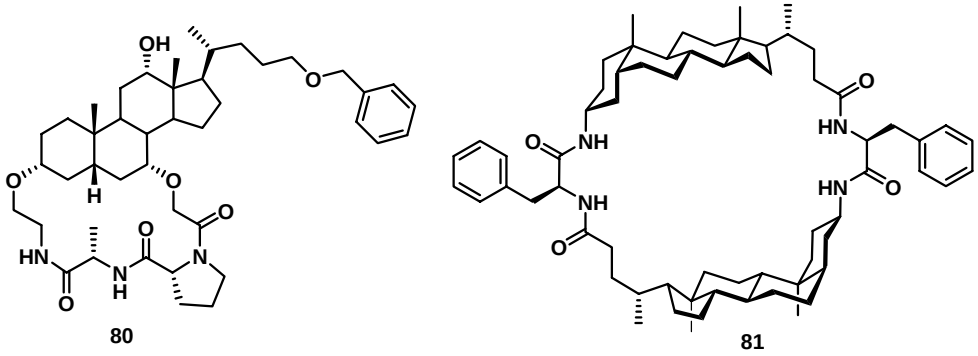


Figure 21.

A.4. Steroid Carbohydrate Conjugates

The recent realization that saccharides and glycoconjugates are involved in a wide range of biological processes has transformed carbohydrate chemistry into a multidisciplinary subject area in which synthetic and structural chemistry are often intertwined with biological studies [104]. Plant based formulations containing glycosides have been used since ancient times as remedial measures against various human and animal ailments [105]. Glycosides are compounds containing carbohydrate and a noncarbohydrate or aglycone residue in the same molecule. Steroidal glycosides are composed of sugar as glycone and sapogenin as aglycone part. Saponins have recently been attracting a surge of interest due to increased understanding of their wide spectrum of biological and pharmacological activities. Steroidal glycosides are divided into two parts: cardioactive group and saponin group.

A.4.1. *Cardiac glycosides*

The cardiac glycosides represent an important class of useful, albeit somewhat dangerous, steroids [106]. Cardiac glycosides are divided into two main types: cardinolides **82** and bufenolides **83** (Figure 22) depending upon the aglycone part.

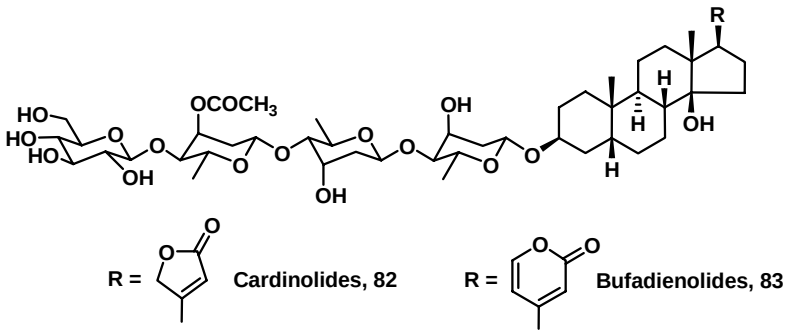


Figure 22.

The most well known use of these compounds is in the preparation of digitalis, which is used to treat congestive heart failure, super ventricular tachycardia, and several other heart conditions. It is a cardiotonic agent that increases the tone of the heart muscle causing more effective emptying of the heart chambers. It has been known and used since at least 1640. Digitalis is prepared from the leaves of *Digitalis purpurea*, a biennial herb known as foxglove [106].

A short review on cardiotonic steroids and their analogues is presented by Feliciano *et al* [107]. The natural, semisynthetic and synthetic derivatives, as well as their mechanism of action and structure-activity relationships (SAR) are described, with a special reference to aminoguanidine derivatives. SAR studies on steroidal cardiac glycosides show that,

- The sugar moiety does not possess biological activity but is important for the partitioning and kinetics of action.
- The "backbone" having U shape of the steroid nucleus is very important. Structures with C/D *trans* fusion are inactive.

- Conversion to A/B *trans* system leads to a marked drop in activity. Thus although not mandatory A/B *cis* fusion is important.
- The 14β-OH group is believed to be dispensable. A skeleton without 14β-OH groups but retaining the C/D *cis* ring fusion was found to retain activity.
- Lactones alone, when not attached to the steroid skeleton, are not active. Thus the activity rests in the steroid skeleton.
- The unsaturated 17-lactone plays an important role in receptor binding. Saturation of the lactone ring dramatically reduced the biological activity.
- The lactone ring is not absolutely required. For example, using α,β-unsaturated nitrile (C=C-CN group) the lactone could be replaced with little or no loss in biological activity.

Schneider and Wolfling presented a review [108] on the recent studies of the synthesis of modified cardenolides, which are expected to have better cardiotonic activities, and with the synthesis of steroids with a variety of heterocycles at C-17 that have been tested against $P450_{17\alpha}$.

4.2. *Steroidal saponins*

A library of biologically active naturally occurring compounds, which are conjugates of a steroid and a carbohydrate, is reported in literature [109]. For this nature has used several steroid skeletons and a huge number of carbohydrates (monosaccherides or polysaccharides). This shows the importance of steroidal saponins as natural product hybrids for the drug design and discovery. As a consequence of amphiphilic nature and surface-active properties of saponins, they act as natural surfactants, or detergents. Several important

biological effects have been ascribed to saponins depending upon their chemical structures, which determine the polarity, hydrophobicity and activity of these compounds. Steroidal saponins have been isolated from a great number of terrestrial plants. In the animal kingdom they are found in most sea cucumbers and starfish, whereas they are found only rarely in alcyonarians, gorgonians, sponges, and as shark-repelling compounds in fish.

Therapeutically, steroidal saponins are mucosal irritants, expectorants, blood cell strengtheners and cholesterol-lowering agents. Many of them also show antifungal [110], antimicrobial [111], anti-HIV [112], anti-inflammatory [113], anticarcinogenic [114] and immunostimulatory properties. The active component in several herbal medicines that have been used as chemotherapeutic agents in Eastern countries was shown to be saponins. Different ginseng species (*e.g.*, *Panax ginseng*, *P. quiquefolius*, *P. japonocus*, *P. pseudoginseng*, *Eleutheroccus senticosus*, *etc.*) are widely employed in Chinese medicine, Eastern Asia regions, and Oriental medicine as tonic and as treatment of cancer, diabetes, and hepatic and cardiovascular diseases [115]. A ginsenolide-Rh1 **84** (Figure 23), a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells [116]. Dioscin **85**, a saponin extracted from the root of *Polygonatum zanlanscianense* exerted significant inhibitory effects on the growth of the human leukemia cell HL-60, inducing differentiation and apoptosis and thus revealing the importance of saponins in related cancer treatment [117]. Dioscin also shows different types of biological activities such as antitumor, antivirus, antifungal, and anti-inflammatory as well as immunostimulant activities. Significant cytotoxic activity against eight cancer cell lines was exhibited by extensumside A **86** (Figure 23), a saponin isolated from *Myriopteron extensum* [118]. The molecules containing two lipophilic arms (fatty acid and the sterol moieties) stretching

outward from central glucosyl unit-acylglucosylsterols have been isolated [119] from Fig Resin (*Ficus carica)* by Mechoulam and coworkers. These novel compounds such as **87** showed *in vitro* inhibitory effects on proliferation of various cancer cell lines.

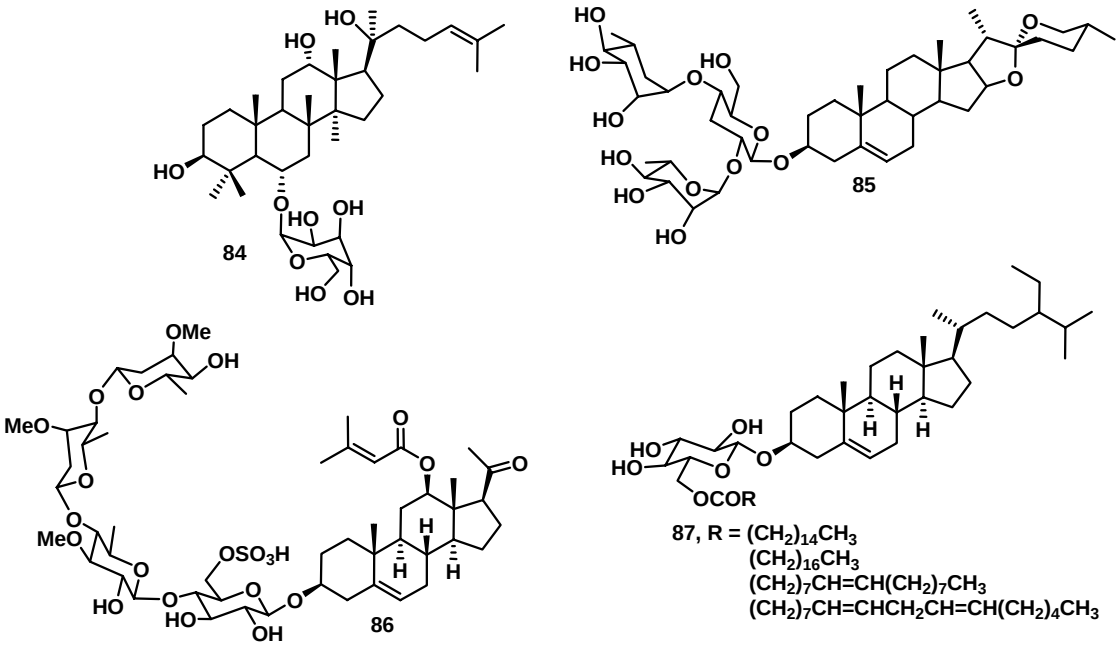


Figure 23.

OSW-1 **88** (Figure 24), a highly potent anticancer natural product, and its four natural analogues have recently been isolated from the bulbs of *Ornithogalum saundersiae*, a perennial grown in South Africa [120]. These are members of the cholestane glycosides. OSW-1 has been found to be much more potent than that of clinically used anticancer agents such as etoposide, adriamycin, and methotrexate. It exhibited exceptionally high cytostatic activities against various human malignant tumor cells which was 10 to 100 fold more than some well-known anticancer agents in clinical use, such as mitomycin-C, cisplatin, camptothecin and even taxol but has significantly lower toxicity to normal human pulmonary cells. The cytotoxicity prophile of OSW-1 is very similar to that of bis steroidal pyrazines-cephalostatins [121].

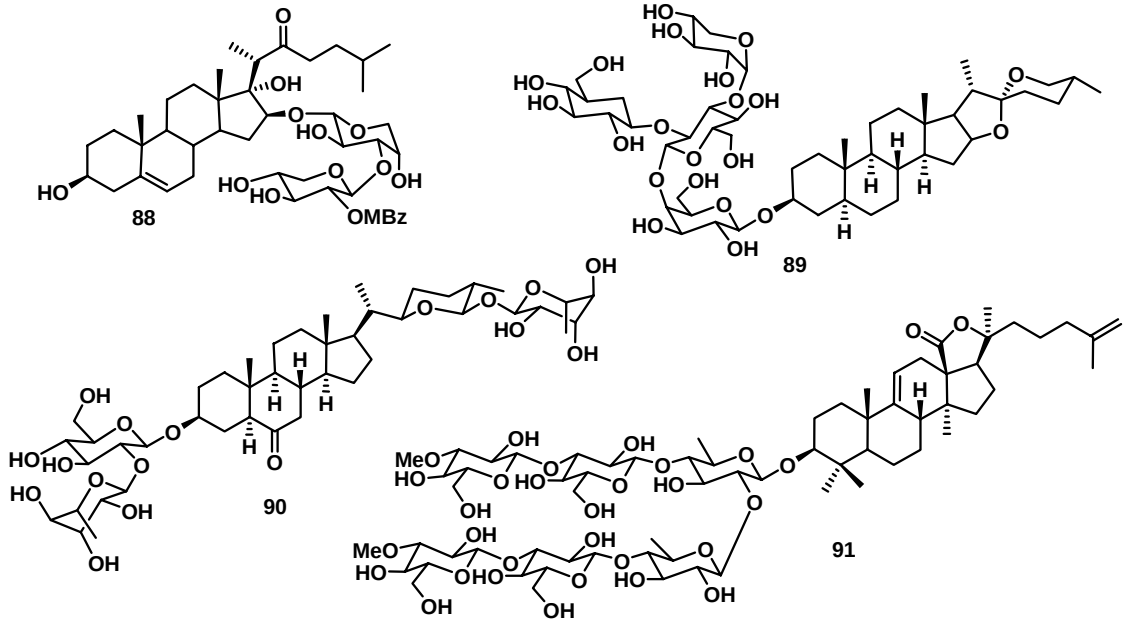


Figure 24.

Isolation of steroidal saponins is a formidable task, which hampers the detailed binding studies of saponins. In 1993, Danishefsky completed [122] the synthesis of complex saponin, desgalactotigonin **89**, Nishizawa reported [123] the total synthesis of osladin **90**, an intensely sweet saponin, and Schmidt prepared [124] holotoxin A **91** (a saponin having six hexose units) without resorting to lengthy protection-deprotection procedures (Figure 24). Yu and Hui have reported chemical syntheses of several steroidal saponins [125]. The extraordinary cytotoxicity of OSW-1 encouraged several research groups to undertake efforts for its synthesis [126]. In recent years there are many reports on the synthesis of OSW-1 [127] and its analogues [128-133].

There are some recent reports on the syntheses of steroid carbohydrate conjugates. Some representative examples are as follows:

Glucosides of nonaminated and glycine and taurine conjugated bile acids were identified in normal urine by Marschall *et al* [134]. These glucosides have also identified as bile acid *N*-acetylglucosaminides **92** (Figure 25). Synthesis of Mosasin-4 **93**, a naturally

occurring steroid saponin with shark repellent activity and its analogue 7β-galactosyl ethyl cholate **94** has been reported [135] by Gargiulo and coworkers.

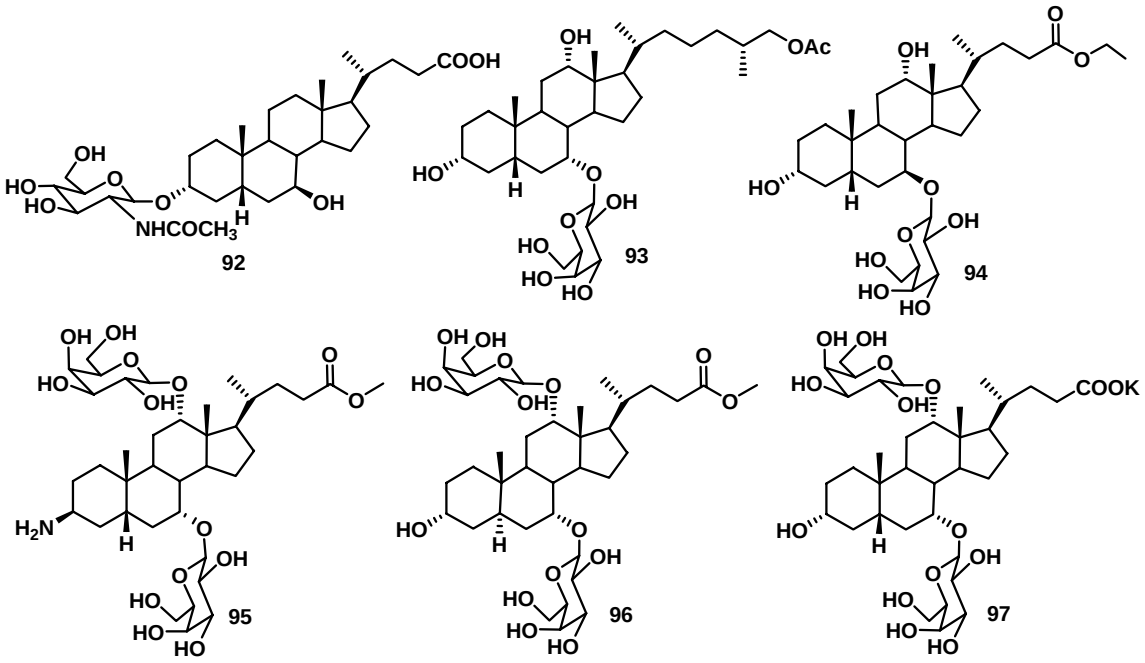


Figure 25.

Sofia and co-workers synthesized methyl 3β-amino-7,12-diglucosyl cholate **95** as drug transport agent [136] where as synthesis of enhanced facial amphiphiles **96** from allocholic acid and **97** from cholic acid by glycosylation of the hindered C-7 and C-12 hydroxyl groups was reported by Kahne *et al* [34a] (Figure 25).

Very recently Kim *et al* reported the synthesis and physicochemical characterization of self-assembled nanoparticles of glycol chitosan-deoxycholic acid conjugate **98** (Figure 26) [137]. In this study they have attempted to prepare deoxycholic acid modified glycol chitosan self-aggregates by covalent attachment of deoxycholic acid to glycol chitosan as a new drug delivery system.

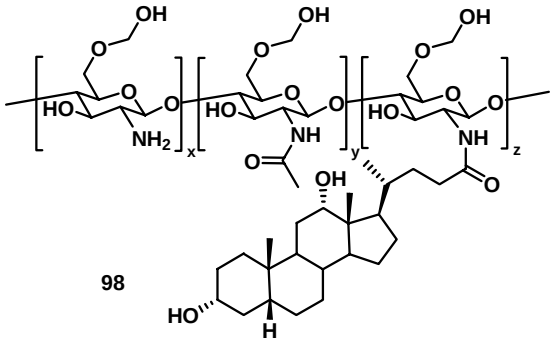


Figure 26.

Menger *et al* [138] have reported the synthesis of bidesmosidic saponins such as **99** (Figure 27) using three different sugars (glucose, galactose and mannose) and 5α-androstane-3β,17β-diol, and evaluated their influence on phospholipid membranes. Their effect on melting behavior of a lipid bilayer depends on the nature of the sugar used. Packing constraints dictate how the lipid bilayer responds to the sugar.

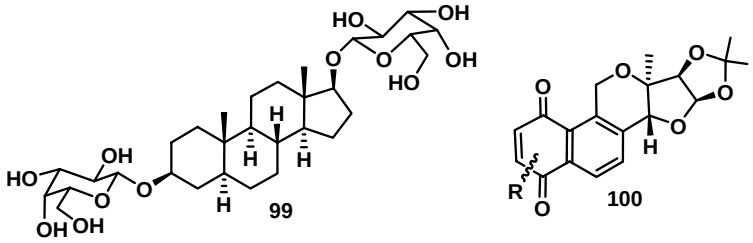


Figure 27.

In an effort to combine the reactivity of the acetal moiety of furanose and the quinone unit with the steroidal backbone, very recently Kaliappan and co-workers have disclosed [139] a versatile strategy to a new class of hybrid molecules *e.g.* **100** (Figure 27) having three (sugar-oxasteroid-quinone) different structural motifs.

A.5. Steroid-Drug Conjugates

In the world of hybrid molecules steroid-drug conjugates are known for long. Nature synthesizes Pachystermine A **101** (Figure 28)**,** a hybrid molecule which is an adduct of a

steroid and a four membered β-lactam ring (pharmacophore of several potent antibacterial agents). Pachystermine A **101** is a naturally occurring β-lactam-steroid found in the buxaceous plant *Pachysandra terminalis* [140].

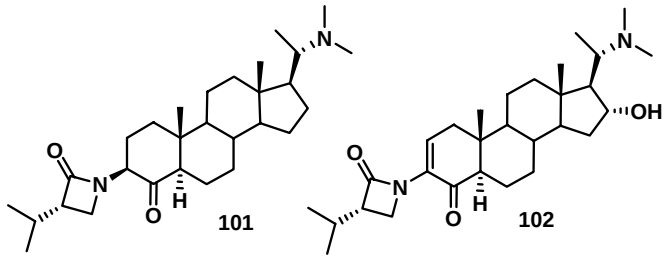


Figure 28.

It is a novel type of 3,20-diamino-5α-pregnane alkaloid carrying a four membered β-lactam ring system. A similar steroidal alkaloid (+)-(20S)-20-(dimethylamino)-3-(3'α-isopropyl)-lactam-5α-pregn-2-en-4-one **102** (Figure 28) was isolated from *Pachysandrs procumbens* [141] using a bioassay guided fractionation based on inhibiton of 3H-tamoxifen binding at the antiestrogen binding site.

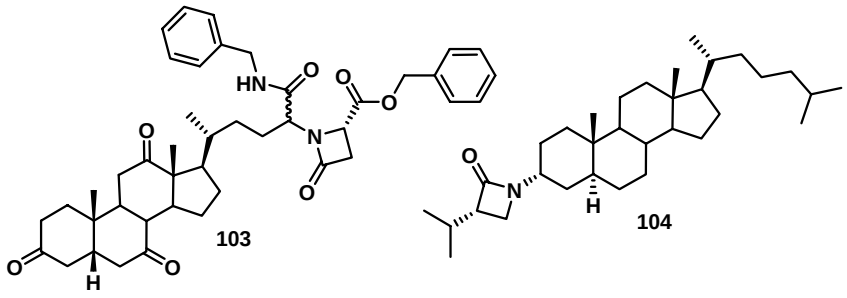


Figure 29.

Ugi and coworkers have shown a simple one-pot synthesis of a β-lactam-steroid adduct **103** (Figure 29) *via* the building block approach using four-component reaction [142]. Bose and co-workers have prepared analogue **104** of steroidal alkaloid Pachystermine A by the cyclization of the β-chloroamide from 3α-aminocholestane [143]. Recently there is

a report from our laboratory on the synthesis of novel 1,2,3-triazole linked β-lactam-bile acid conjugates such as **105** (Figure 30) using 1,3-dipolar cycloaddition reaction [144].

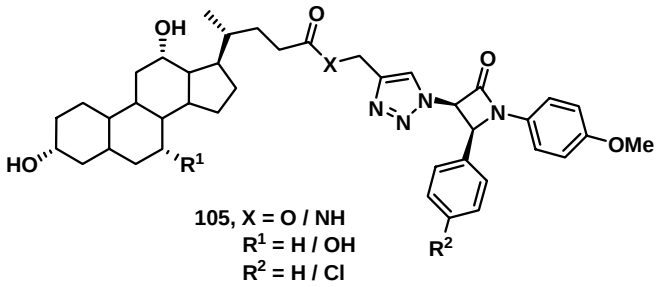


Figure 30.

A.5.1. *Cholesterol based conjugates*

Cholesterol **106** (Figure 31) [145] is one of the most widely distributed natural materials and has a unique chemical structure and is a synthetic building block for artificial lipids with characteristic physical, chemical and biological properties.

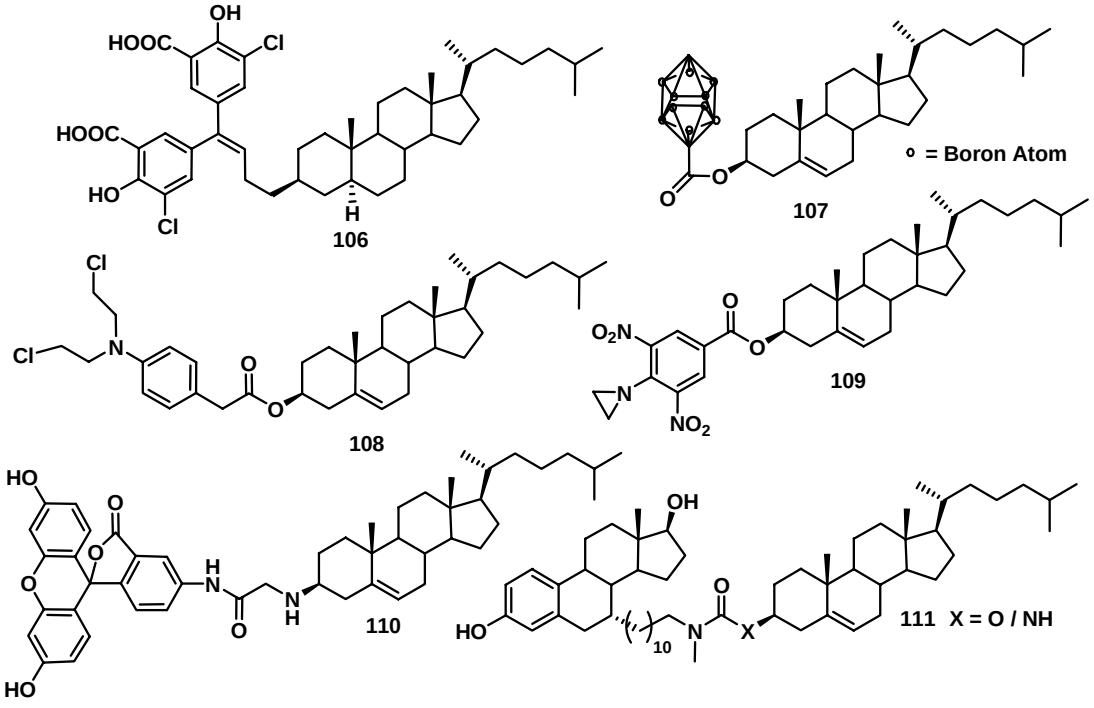


Figure 31.

Cosalane **106** is an anti-HIV agent displaying a wide range of activity against a variety of laboratory, drug-resistant and clinical HIV-1 isolates, as well as HIV-2 [146]. Cosalane was designed conceptually by taking a dichlorinated disalicylmethane fragment of the polymeric anti HIV agent aurintricarboxylic acid and attaching a cholestane moiety to it through a three-carbon linker chain [147]. In this conjugate dichlorodisalicyl methane is acting as a "pharmacophore" and the cholestane fragment is serving as an accessory module to increase potency by directing the molecule to the lipid environment of the cell membrane and the viral envelope. The cholesterol-carborane conjugate **107** (Figure 31) has been designed and synthesized by Lu *et al* [148] to selectively deliver boron to tumor cells by means of reconstituted lipoprotein. They have examined the chemical stability and cytotoxicity of this new compound. Degteva and co-workers [149] have described the preparation of 3β-hydroxy-5-cholestane *p*[*N*,*N*-bis-(2-chloroethyl)amino]phenylacetate, which they called phenesterin **108** and have made detailed studies of phenesterin against a variety of solid tumor systems. Taylor and co-workers have synthesized a series of *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid, steroidal sulfides of *p*-(*N*,*N*-bis-2-chloroethylamino) thiophenol, and a variety of steroidal ethylenimine derivatives such as **109** and tested for antitumor activity in a number of experimental tumor systems [150]. Cellular membranes can be chemically altered to facilitate macromolecular uptake. As an example of this Peterson and coworkers [151] have synthesized fluorescein-cholesterylamine chimers **110** that enable uptake of antifluorescein antibodies and associated protein complexes by mammalian cells. Recently, the same group has synthesized 7α-substituted β-estradiol derivatives bearing side chains terminated with cholesterol and 3β-cholesterylamine **111**. These chimeric compounds exhibited substantial affinity for estrogen receptors.

A.5.2. *Steroid hormone based conjugates*

The lack of selectivity of several antitumor agents as well as their acute toxicity towards rapidly proliferating tissues constitutes the major drawbacks in their use for the treatment of human cancer. In order to circumvent this problem, one approach is to couple these agents to carriers, which have shown selectivity towards the tumors, or the tissues from which the tumors are originated. Lee *et al* have studied binding of conjugates of prednisolone and non-steroidal anti-inflammatory drugs such as ibuprofen **112** and indomethacin **113** to glucocorticoid receptors (Figure 32) [152]. The local and systemic anti-inflammatory activities of the conjugates were evaluated using the cotton pellet granuloma bioassay and their topical activity evaluated by the croton oil-induced ear edema in male Sprague-Dawley rats. The results indicated that these conjugates possess greater local and topical anti-inflammatory activity than prednisolone.

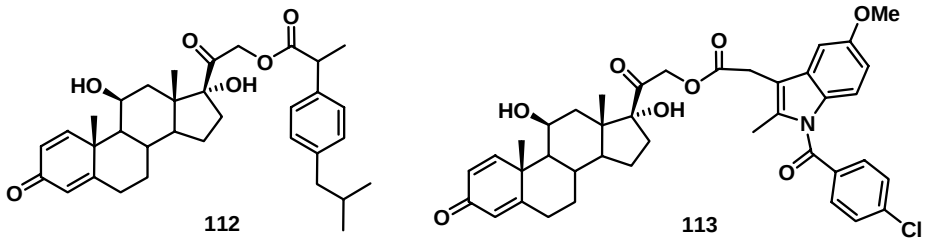
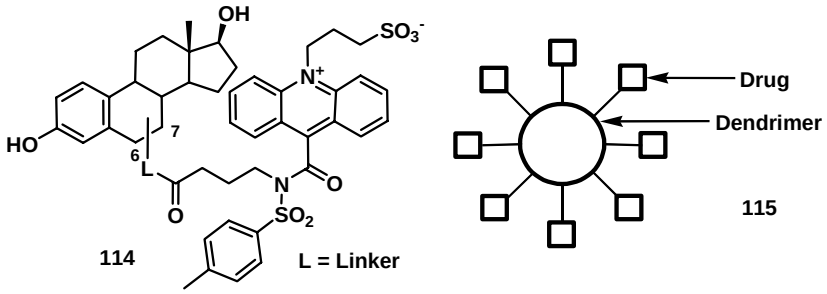


Figure 32.

Adamczyk *et al* synthesized a series of chemiluminescent 17β-estradiol probes *e.g.* **114** (Figure 33) by conjugating hydrophilic acridinium acid with commercially available 17β-estradiol at C-6 and C-7 using several linkers [153]. There solution-binding affinities for an anti-E_2 Fab fragment were measured using a single E_2 analog biosensor surface on a BIACORE surface plasmon resonance instrument. An assay has been developed to determine the concentration of estradiol, which is required while addressing fertility problems.

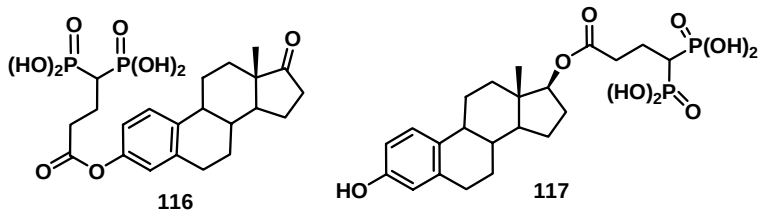


114 115

Figure 33.

Dendrimers have emerged as promising multifunctional nanomaterials for drug delivery due to their well-defined size and tailorability. Their multiple surface functional groups can be easily modified to potentially attach a large number of drug molecules making them ideal vehicles for targeted drug delivery. A schematic representation of dendrimer-drug conjugate **115** is shown in Figure 33. Chen *et al* have synthesized estrone dendrimers containing six estrones attached through polyoxyethylene chains to a benzene core [154]. Maitra *et al* reported the first bile acid-based chiral dendrons [155]. Very recently Rissanen and co-workers have studied thermal behavior of novel steroidal dendrons based on 2,2-bis(hydroxymethyl)propionic acid and bile acids [156]. These conjugates can potentially further conjugated with a targeting moiety to deliver the drugs to specific cells *in vivo*.

Geminal bisphosphonates are stable pyrophosphate analogues, which bind efficiently to bone surface. They have been used to inhibit bone resorption. These molecules are useful in the treatment of osteoporosis and Paget's disease. Page *et al* have carried out novel synthesis of bis(phosphonic acid)-steroid conjugates **116** and **117** (Figure 34) as a new class of potential bone resorption inhibitors [157].



116 117

Figure 34.

The use of radiolabelled derivatives of potent estrogens possessing receptor affinity towards use as diagnostic agents has been extensively and amply demonstrated. Incorporation of radioisotopes for breast cancers to steroidal substrates suitable for use as radiotherapeutic agents poses significant chemical challenges. ^{177}Lu is presently being considered as one of the most promising radionuclide for targeted therapy due to its suitable decay characteristics. Banerjee and co-workers have reported a novel attempt to introduce ^{177}Lu in the estradiol moiety through steroidal-BFCA (Bifunctional Chelating Agent) conjugate **118** (Figure 35). Synthesis of a steroidal conjugate via coupling of 6α-amino-17β-estradiol with a C-functionalized DOTA (1,4,7,10-tetraazacyclododecane 1,4,7,10-tetraacetic acid) derivative and thereafter radiolabeling the conjugate with ^{177}Lu has been described [158]. Biological activity of this conjugate was studied for *in vitro* cell uptake as well as binding studies with anti-estradiol antibodies. Porphyrins occupy a central position in photodynamic therapy of cancer, which relies upon the selective accumulation of a photosensitizer (*e.g.* porphyrins, psolarenns *etc.*) into cancerous tissue followed by irradiation of the diseased tissue [159].

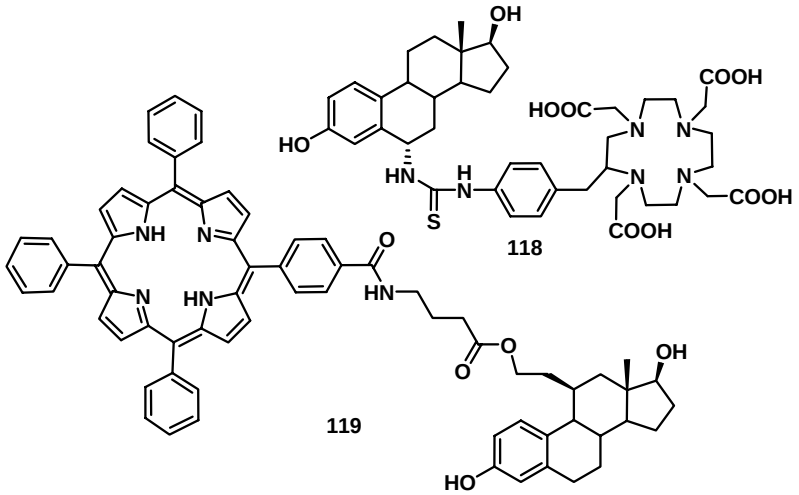


Figure 35.

Upon irradiation, the exited state of the photosensitizer generates singlet oxygen, which damages cellular component and ultimately leads to cell death. Tumor-localizing

property of a porphyrin in estradiol-porphyrin conjugates **119** (Figure 35) could be significantly enhanced by strong interaction between estradiol part and over expressed estrogen receptors in breast tumor cells which may result in the higher uptake of the conjugate by tumor cells.

A.5.3. *Bile acid based conjugates*

Bile acids are essential for digestion and absorption of lipids and lipid soluble vitamins. Intestinal bile acid transporters are solute carrier transporters regulating the enterohepatic circulation of bile salts [35]. Bile acids are pharmacologically interesting as potential carriers of liver-specific drugs, absorption enhancers and as new cholesterol lowering agents. Recent research shows that bile acids are interesting and useful starting materials in the preparation of different polyamines for various pharmacological applications [160]. The treatment of chronic diseases in most cases involves long-term use of drugs. For this a site-specific drug action without adverse side effects and noninvasive, preferably oral administration of drugs is necessary. The physiology of bile transport exclusively involves only liver and small intestine and hence use of bile acid as putative shuttles of pharmaceuticals should be ideal. Current research efforts are focused on specific drug targeting to the liver and on improving the intestinal absorption of poorly absorbed drugs. Very recently use of bile acids and their derivatives in designing prodrugs capable of exploiting the enterohepatic circulation of bile acids is summarized by Sievänen [161]. Large numbers of drug bile acid conjugates have been synthesized [162] by Kramer *et al*, which include chlorambucil-bile acid conjugate **120**, peptide bile acid conjugate **121**, HMG-CoA reductase inhibitor-bile acid conjugates **122** and **123** (Figure 36).

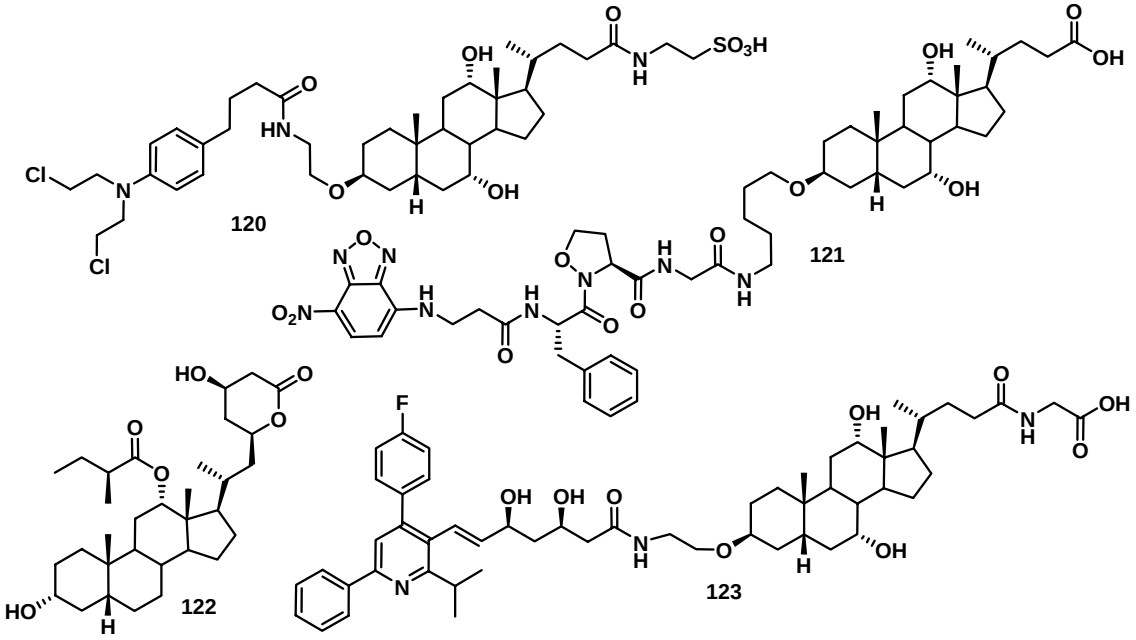


Figure 36.

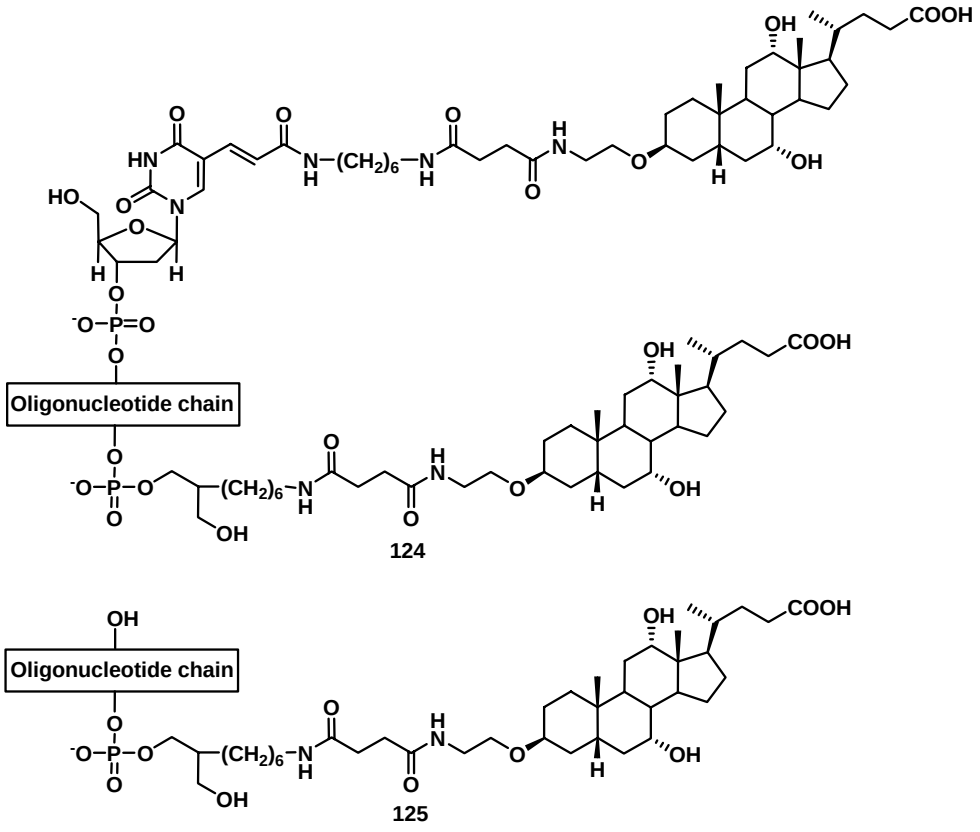
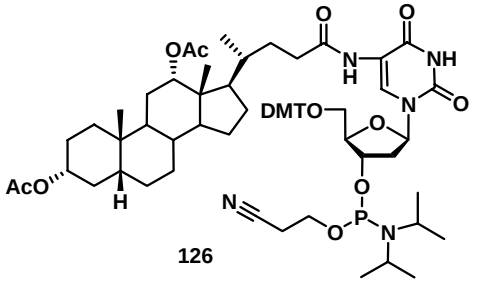


Figure 37.

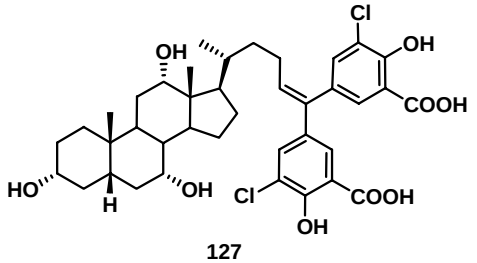
Synthesis of bile acid oligonucleotide conjugates **124** and **125** has been reported (Figure 37) [163]. When used *in vivo* in rats, these conjugates resulted in an increased biliary excretion of bile acid oligodeoxynucleotide conjugates compared to unconjugated oligodeoxynucleotides. Ganesh and co-workers at National Chemical Laboratory [164] synthesized several oligonucleotides carrying the deoxycholic acid moiety by substituting 5-amido-(7-deoxycholic)-2'deoxyuridine **126** (Figure 35) for thymidine at predetermined positions in the oligonucleotide sequences. The enhancement in the stability of derived triplexes was observed due to the incorporation of deoxycholic acid to nucleobase deoxyuridine in the third strand of DNA triplex.



126

Figure 38.

Cosalane **106** (Figure 31) as mentioned earlier is an inhibitor of HIV replication with activity against both HIV-1 and HIV-2. The poor oral availability of cosalane is mainly attributed to its poor permeation across the intestinal epithelium due to its very high lipophilicity and membrane interacting nature.



127

Figure 39.

With the aim to retain the anti HIV activity and to enhance bioavailibility of cosalane, Cushman and his group has synthesized [165] cosalane-bile acid conjugate **127** (Figure 39). Unfortunately this conjugate was found to be less potent than cosalane.

Bile acid conjugates, specifically lithocholic acid conjugates, interact with muscarinic receptors on gastric chief cells. Structural similarities between acetylcholine **128** and lithocholyltaurine **129** (Figure 40) suggested a potential molecular basis for their interaction with the same receptor. Based on this information Cheng *et al* synthesized a hybrid molecule consisting of the steroid nucleus of the lithocholyltaurine and the choline moiety of acetylcholine [166]. The new molecule, lithocholylcholine **130** inhibited binding of a cholinergic radioligand to Chinese hamster ovary cells. The bioactivity data suggest that these hybrid molecules may be used to develop selective musacrinic receptor ligands.

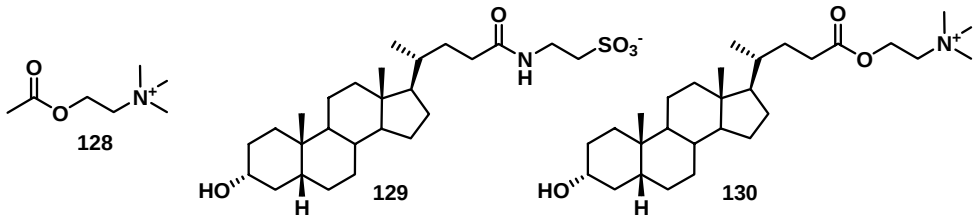


Figure 40.

In 1980, the French pharmaceutical company Roussel-Uclaf announced the discovery [167] of RU-486, now known by the generic name mifepristone **131** (Figure 41). This was the first antiprogestin to be developed. Mifepristone **131**, when used in combination with prostaglandin, effectively and safely terminates early pregnancies. From the SAR [168], two new analogues, **132** and **133**, of mifepristone were designed. We have achieved the syntheses of these two analogues in eleven steps [169] through modified synthetic sequences and improved procedures starting from (+)-estrone. In comparison with

mifepristone **131**, the relative binding affinities of compound **133** for the progesterone receptor was found to be more, whereas that of compound **132** was less (Figure 41).

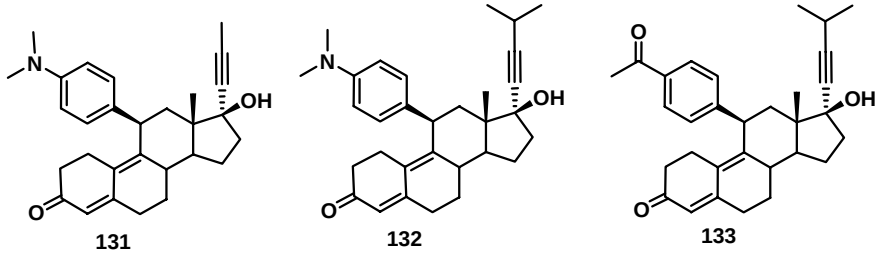


Figure 41.

Glycemic control depends on a precise match between glucose inputs and outputs. Any disturbances in this balance can result in hypo or hyperglycemia. Glucocorticoid receptor (GR) antagonism has been validated as a strategy for regulating hepatic glucose output (HGO). Mifepristone (RU-486) **131** has been used for this validation study.

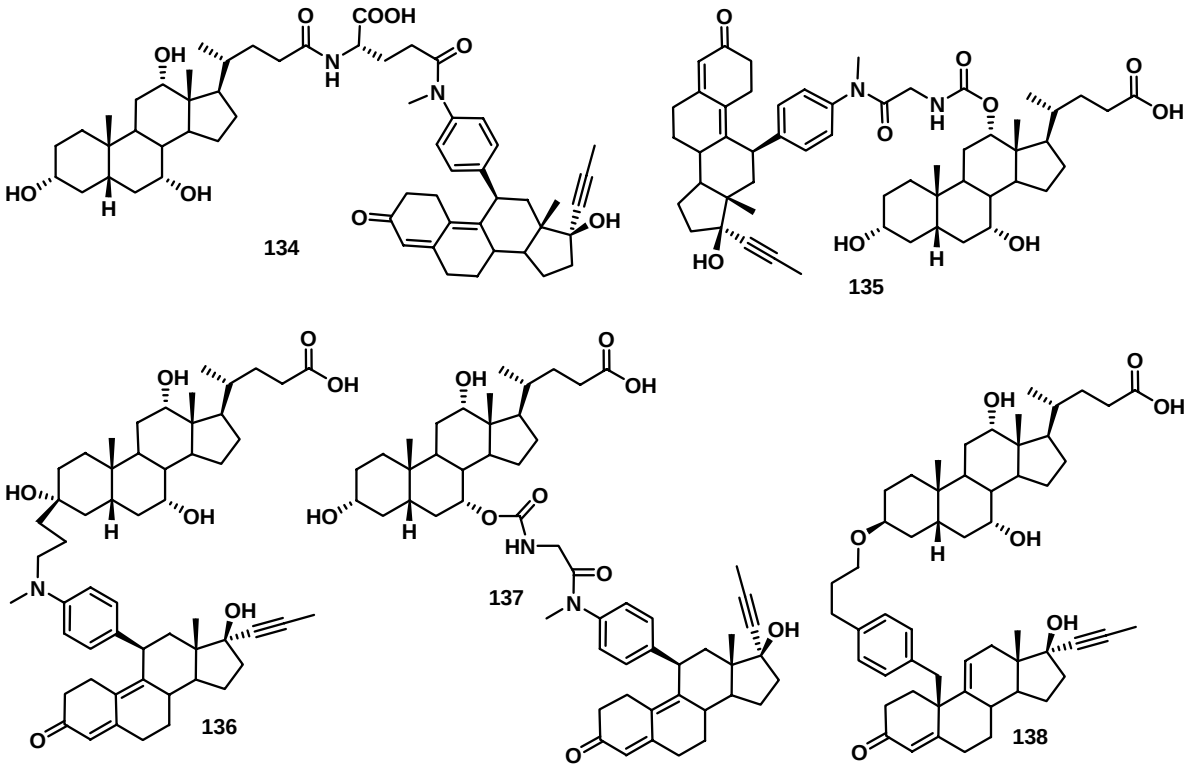


Figure 42.

Long-term systematic GR antagonism is not a viable approach for the treatment of type 2 diabetes. A liver specific derivative of mifepristone would be expected to decrease HGO and improve glucose metabolism without the risk of side effects. With this assumption Geldern and coworkers have synthesized [170] number of bile acid conjugates *e.g.* **134-137** (Figure 42) of mifepristone with **131** at different positions of bile acid using linkers to provide novel drugs for type 2 diabetes. They have also synthesized bile acid-RU-43044 conjugate **138**, which is a selective GR antagonist.

It has been shown that bile acids can be used as carrier units for preparation of MRI contrast agents, which enter hepatocycle by means of active transport mechanism [171]. A series of structurally different gadolinium conjugates incorporating a bile acid moiety have been prepared. Polyaminopolycarboxylic acids such as diethylenetriamine-pentaacetic acid (DTPA) and DOTA have been selected as chelating subunits for the Gd (III) ion. These conjugates *e.g.* **139**, **140** (Figure 43) showed high biliary elimination as well as good tolerabilites.

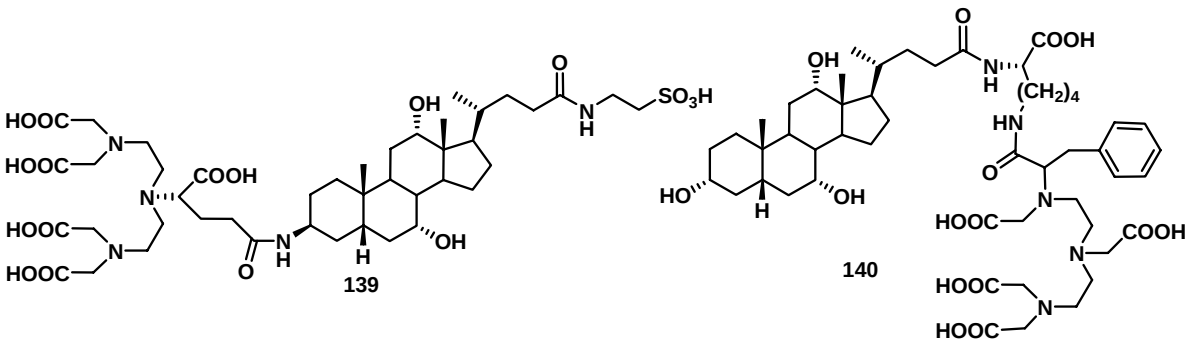


Figure 43.

Aromatic heterocycles are widely used in the pharmaceutical industry, for example as anti inflammatory, antimicrobial and anticancer drugs. Based on this concept there are reports on synthesis of lithocholic acid-pyrrole conjugates [172], corticosterone-imidazole-bis(guanidinium) conjugates [173] and lanosterol-imidazole conjugates [174]. Novel

pyrazole fused bile acids were conjugated with anti-inflammatory drugs such as neproxen and drug surrogate [175]. These annulated pyrazoles and their drug conjugates **141** and **142** (Figure 44) showed good affinity for the human liver bile acid transporter as compared to human ileal bile acid transporter. Potentially such annulated pyrazoles can be used as shuttles for drug targeting.

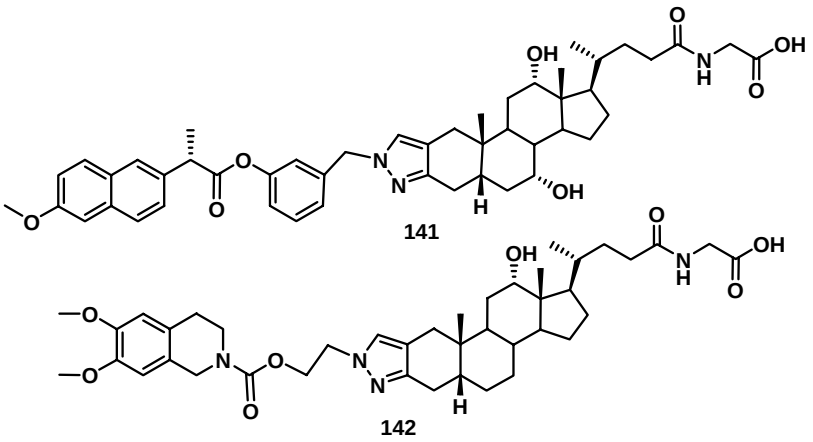


Figure 44.

The 1,2,4,5-tetraoxacyclohexane (tetraoxane) moiety became an increasingly interesting pharmacophore since its antimalarial activity was found to be very similar to that of 1,2,4-trioxanes such as naturally occurring artemisinin.

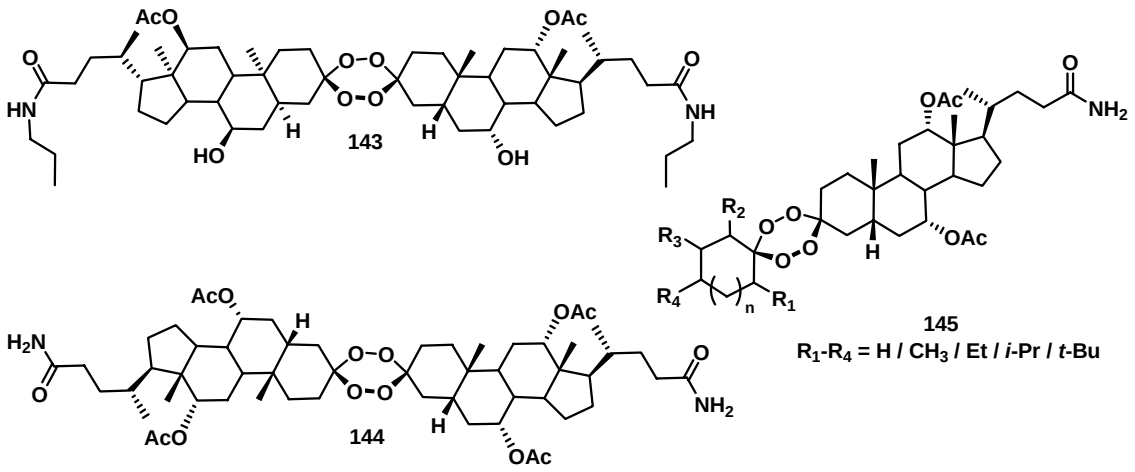


Figure 45.

Solaja and his group has synthesized [176] cholic acid-derived 1,2,4,5-tetraoxanes **143**, **144** and mixed 1,2,4,5-tetraoxanes such as **145** (Figure 45) in order to explore the influence of steroid carrier on its antimalarial, antiproliferative and antimicrobial activities.

A series of sulfonamides incorporating bile acid moieties as potent carbonic anhydrase inhibitors have been reported [177]. Some of the most active derivatives **146** and **147** (Figure 46), incorporating 1,3,4-thiadiazole-2-sulfonamide or benzothiazole-2-sulfonamide functionalities in their molecules, showed excellent affinity for several isozymes of carbonic anhydrase.

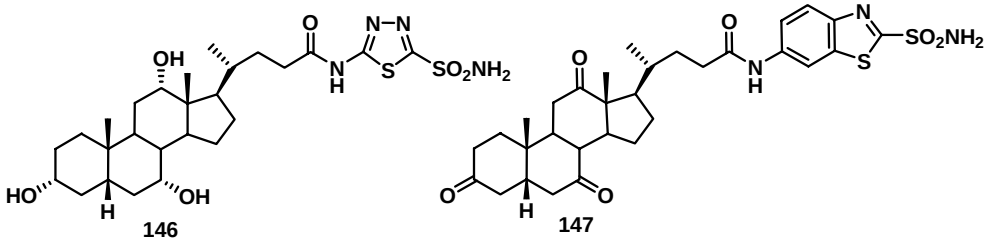


Figure 46.

Recently there are two reports from our laboratory on the synthesis of bile acid conjugates *e.g.* **148** from chiral amino alcohols based on a broad-spectrum antibiotic chloramphenicol and a novel bile acid-fluconazole conjugates *e.g.* **149** (Figure 47) *via* Cu(I) catalyzed intermolecular 1,3-dipolar cycloaddition reaction. These conjugates showed antibacterial as well as antifungal activities [178].

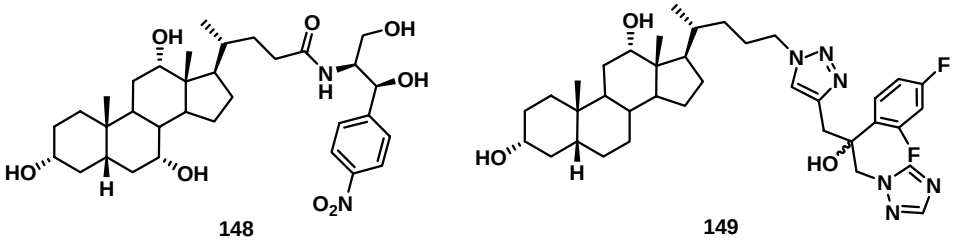


Figure 47.

A.6. Bis(Steroidal) Conjugates

Since the discovery of Japindine **150** (Figure 48), the first example of novel sulphur containing dimeric alkaloid isolated from the root-bark of *Chonemorpha macrophylla* [179], several examples of bis(steroid) derivatives have appeared in the literature.

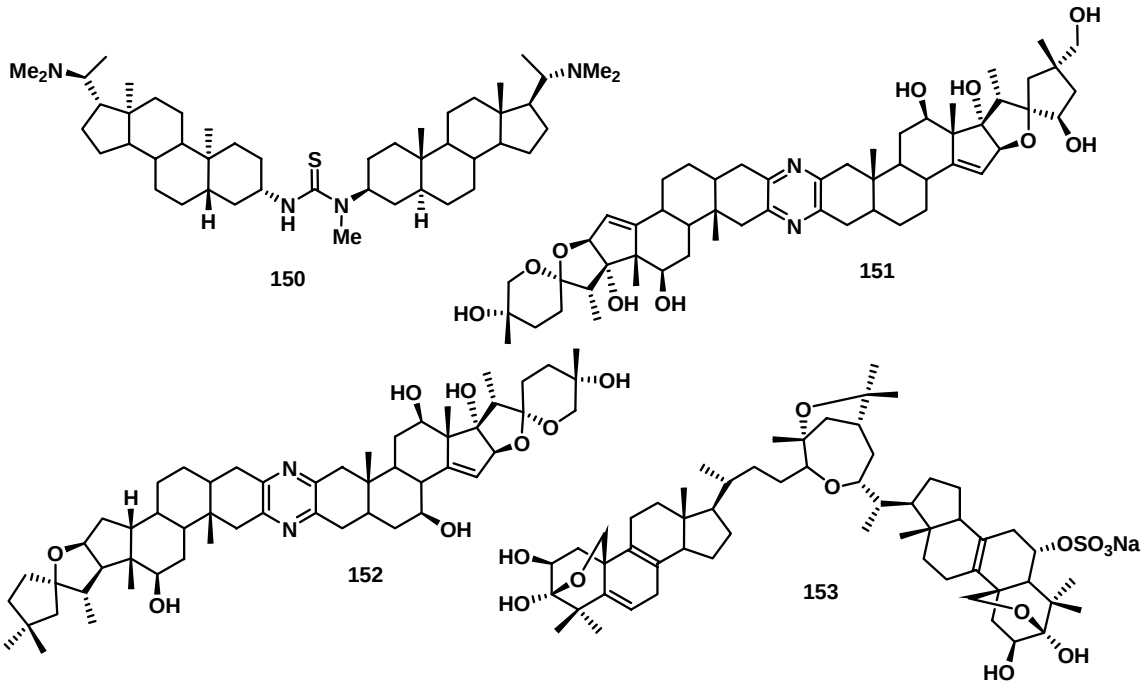


Figure 48.

These dimeric and oligomeric steroids possess interesting micellar, detergent, and liquid crystal properties and many of them led to enhance pharmacological activities [180]. Among these the most pertinant with regard to their extraordinary biological activities are cephalostatins **151** and ritterazines **152** (Figure 48) [181]. Cephalostatins are a group of complex steroidal pyrazine alkaloids that were isolated from the marine worm *Cephalodiscus gilchrist* [182]. They are powerful cytotoxins against the PS cell line (ED_{50} 10^{-7}-10^{-9} μg/mL) and therefore have potential applications as antitumor agents. However, they are rare marine natural products and are available in only small amounts. Heathcock and co-workers [183] for the first time achieved the synthesis of various analogs of

cephalostatins and biological activities of these unsymmetrical bis-steroidal pyrazines was evaluated in the National Cancer Institute's new *in vitro* disease oriented antitumor screen. Recently Haak and co-workers have reported a simple biomimetic route to nonsymmetric pyrazines [184]. Fuchs *et al* [185] reported the first total synthesis of cephalostatin 1. The dimeric steroidal alkaloid ritterazines **152** are closely related to cephalostatin **151**. Seketsu and co-workers have examined the cytotoxicity of ritterazine derivatives [186]. Their studies showed that ritterazine A **152** which contains the highest number of hydroxyl groups is the most potent. The activity decreases with decreasing the number of hydroxyl groups. Isolation of crellastatin A **153** (Figure 48) [187] from Vanuatu marine sponge *Crella sp* is the first example of a dimeric steroid connected through its side chains. Crellastatins exhibit *in vitro* antitumor activity against human bronchopulmonary non-small-cell lung carcinoma cell lines (NSCLC) with IC_{50} values in the range of 2-10 μg/mL.

Adopting the concept of synthesizing dimeric steroids from nature several groups have synthesized various dimeric steroids as models in planning novel receptors. Davis and co-workers reported the first macrocyclic steroid derivatives 'cholaphanes', intended for use in molecular recognition chemistry [188]. Furthermore there are reports on synthesis of several new generation cholaphanes for binding polar molecules such as carbohydrates [189], cations [190] and halide ions [191]. In the intention to develop molecular hosts of designed characteristics by combining the rigid concave surface of cholanic acids with the flexibility and functionality of amino acids, Feigel *et al* reported [102c] the synthesis and characterization of the chola-cyclopeptide **154** (Figure 49) by the cyclodimerization of the pentafluoro phenol ester of bis(phenylalaninyl)-3-amidolithocholic acid.

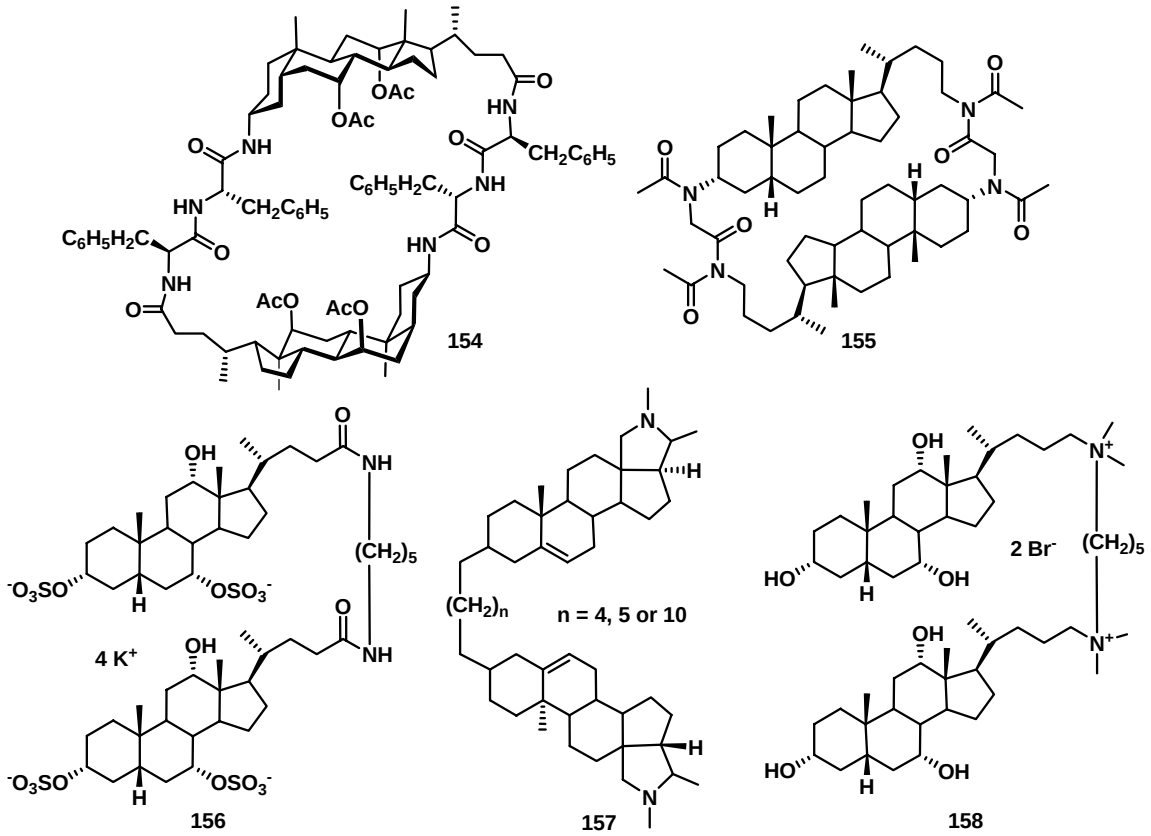


Figure 49.

Similarly Wessjohann and co-workers synthesized a steroid-peptoid hybrid such as **155** by using a multicomponent reaction [102d]. They have presented a straightforward strategy to generate a collection of chimeric peptoid macrocycles, specifically with steroid moieties with size and structural complexity that bears no resemblance to any known natural product. These compounds are potentially useful in chemical genomic approaches, as well as for artificial receptors for molecular recognition studies. Shawakfeh *et al* reported [192] synthesis and selective catalytic oxidation of several dimeric steroids, where as the use of bis-steroids *e.g.* **156**-**158** (Figure 49) as potential enzyme models was reported by McKenna and co-workers [33a]. Synthesis of novel bile acid dimers **159**, **160** and oligomers **161**, **162** (Figure 50) containing 1,2,3-triazole as a linker using click chemistry have been very

recently reported form our laboratory [193]. These new molecules may find applications in molecular recognition, supramolecular chemistry and pharmacology.

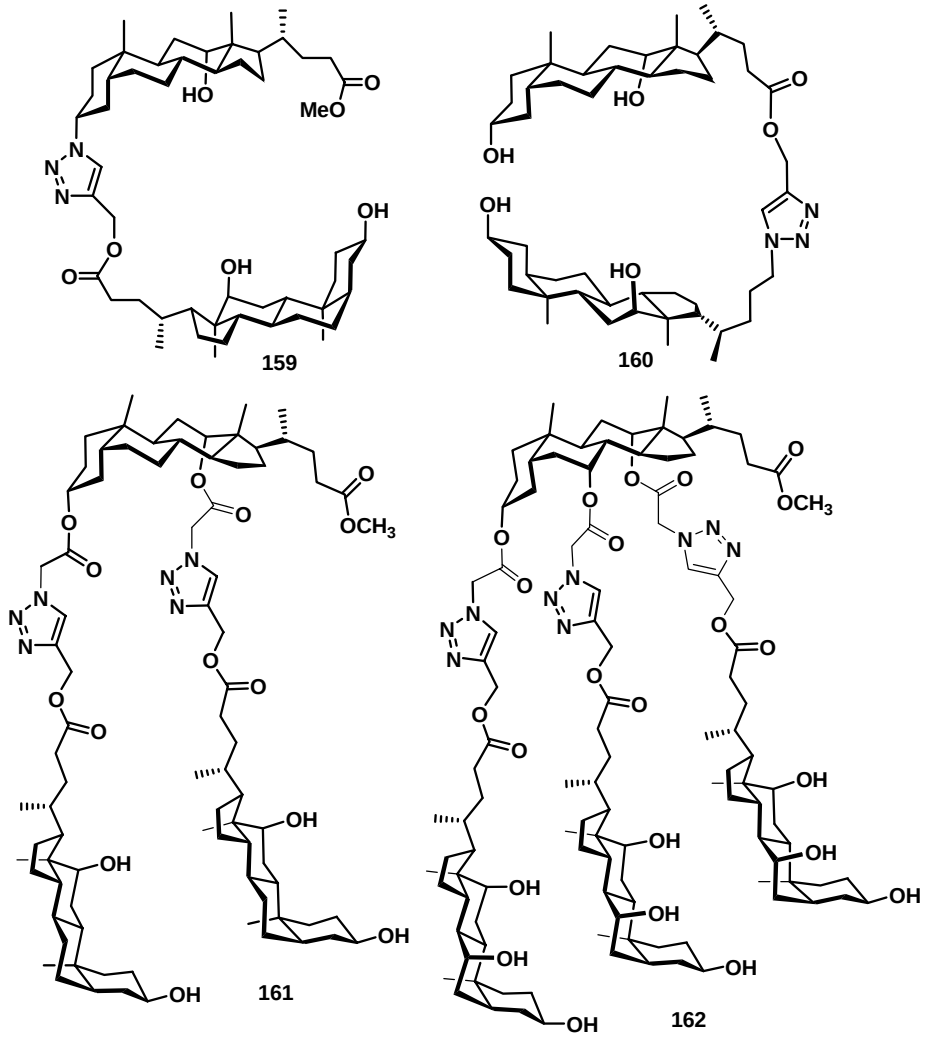


Figure 50.

Efforts to understand the interactions of both drugs and toxins with DNA as well as the desire for new methods of DNA manipulation have spurred the design and synthesis of small molecules that bind in specific ways to DNA. Along this vein, Burrows and co-workers prepared a steroidal dimer **163** (Figure 51), a tetraamine, and examined its binding to DNA. The tetraamine **163** binds to DNA nearly an order of magnitude better than does spermine **1** (Figure 1) [194].

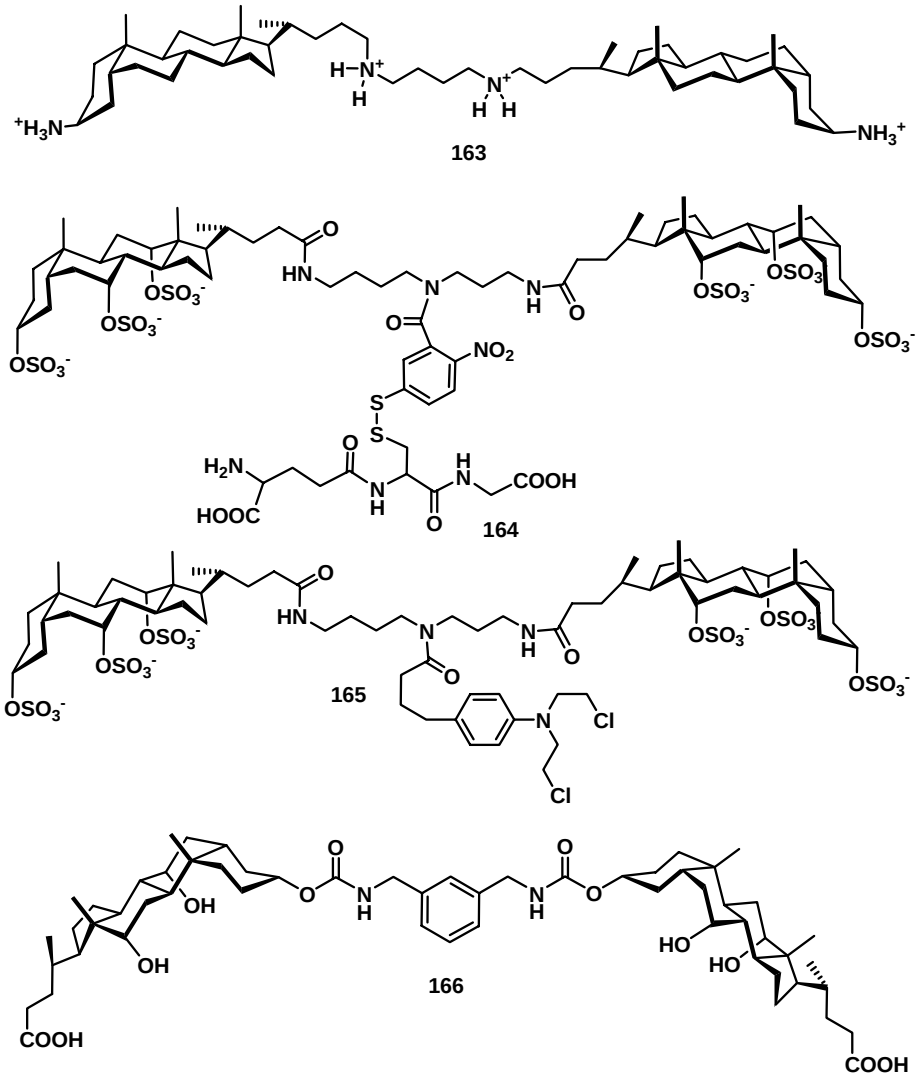


Figure 51.

Based on the facts that, (i) a di-walled molecular umbrella, bearing three sulfate groups on each of two cholyl moieties, is capable of crossing phospholipid bilayers and (ii) anionic polymers such as dextran/dextrin sulfate and cellulose sulfate are known to inhibit cellular binding of HIV and HSV by competing for viral envelope glycoproteins, Regen and co-workers have synthesized [195] persulfated molecular umbrellas such as **164** (Figure 51) as anti-HIV and anti-HSV agents. Based on the similar concepts, chlorambucil, aromatic nitrogen mustard, has been conjugated to putrescine and spermidine based scaffold bearing one, two and four persulfated cholic acid units. This conjugate **165** bearing two sterols

showed improved hydrolytic stability and water solubility relative to chlorambucil. Recently Kobuke and co-workers have synthesized bischolic acid derivatives *e.g.* **166** linked by *m*-xylene dicarbamate at the 3-3'-position and examined their single ion channel properties [196]. The single ion channel currents were measured using the established method for a planar bilayer membrane under 500 mM KCl symmetric conditions [197]. Morzycki and coworkers have synthesized dimeric steroids *e.g.* **167** and **168** (Figure 52) using diosgenin and pregnanoic esters respectively. These dimers were used for the formation of phospholipid membranes. The physicochemical properties of these membranes were examined by various electroanalytical techniques [198].

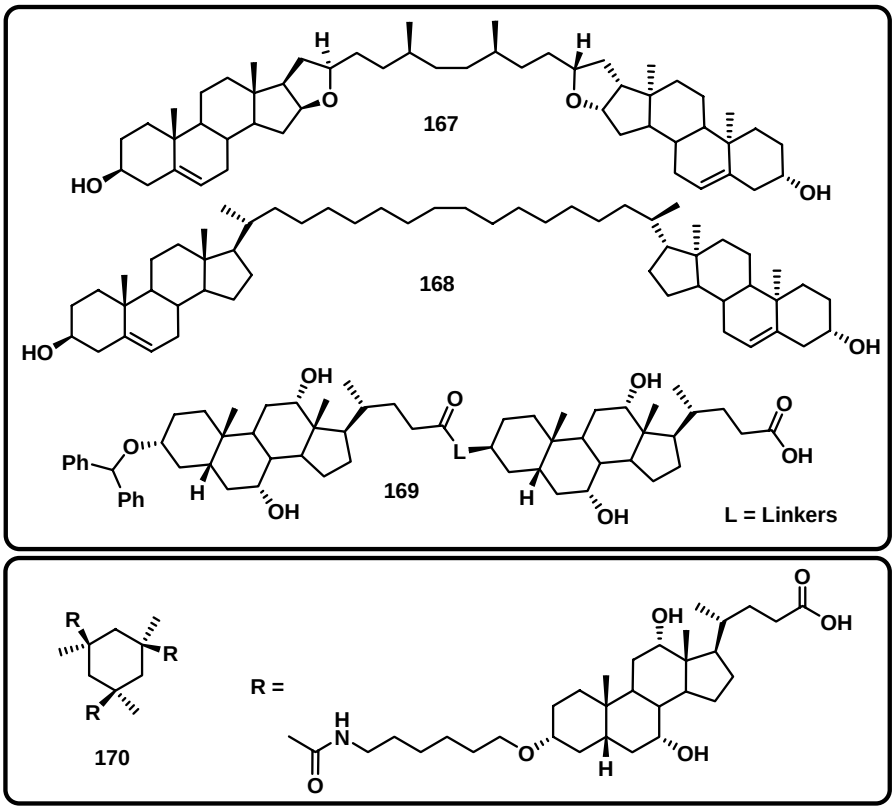


Figure 52.

Wess and co-workers synthesized various bile acid dimers and trimers such as **169** and **170**, as bile acid reabsorption inhibitors for potential use in hypercholesterolemia. The

interaction of these compounds with the specific ileal bile acid transport system was studied by inhibition of Na^+-dependent taurocholate uptake into ileal brush border membrane vesicles. Compounds **169** and **170** showed strong inhibition. These compounds were further characterized pharmacologically by *in situ* ileal perfusion experiments in rats [199].

A.7. Summary

One of the most fascinating challenges in modern medicinal chemistry is the design of strategies capable of providing structurally diverse and complex molecules, which are useful for the study of important biological processes. The possibilities of generating hybrid systems for creating molecular diversity through covalent linkage of different steroid molecules with other biomolecules are almost unlimited. Combinatorial chemistry is the emerging field of science by use of which libraries of such hybrid molecules can be synthesized. In addition, by using the concept of bioisosterism novel drug molecules can be designed for the enhancement of the therapeutic spectrum of existing drug conjugates. The present work describes the design, synthesis and bioevaluation of novel steroid hybrids with the intention to develop innovative lead molecules.

A.8. References

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PART B : CHAPTER - 1

Synthesis of C-11 Functionalized Novel Bile Acid Derivatives

B1.1. Abstract

Stereoselective C-11 functionalization in steroids is one of the challenging targets for synthetic organic chemists as it involves severe steric interactions caused due to C-18 and C-19 angular methyl groups. Synthesis of bromo/azido/amino functionalized bile acid analogues with different stereo-chemical orientation at C-11, have been achieved in good to excellent yields. Mechanistic aspects for the decomposition of steroidal azidoketones to its enamines are also discussed. The crystal structures of these molecules were resolved, to study the effect of substitution in the steroid skeleton of cholic acid with different stereo-chemical orientations at C-11 on the two-dimensional arrangement of molecules and solid-state properties. Previously such analogous compounds are predicted to be Human Immunodeficiency Virus type 1 (HIV-1) inhibitors by molecular modeling studies. In our study these novel cholic acid derivatives induced host cell fusion during the progress of HIV-1 infection and formed multinucleated giant cells. This is the first report of syncytia formation and thereby enhancement of viral replication in HIV-1 infected T cells by bile acid derivatives.

B1.2. Introduction

A C_2-homodimer containing two aspartate residues in the catalytic site has been reported [1] to have therapeutic potential as a specific inhibitor of HIV-1 protease. Among the numerous HIV-1 protease inhibitors the most active inhibitors which are competitive by nature, either contain the partial structure **1** (hydroxyethylamine) or the related carbonyl equivalent **2** which are believed to mimic the hydrolysis transition state leading to tetrahedral *gem* diol **3** (Figure 1), thought to be involved in the aspartate catalysed proteolysis [2].

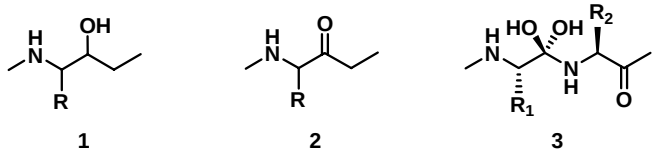


Figure 1. Partial structure to be a specific inhibitor of HIV-1 protease.

Other important features include incorporation of lipophilic residues such as cyclohexyl methyl or benzyl and bulky hydrophobic group or solubility increasing substituent on nitrogen [3]. Typical of this is the symmetrical inhibitor **4** (Figure 2), a cyclohexyl relative **5** [4], the difluoroketone derivative **6** [5] and the pyrrolidine based α-keto amide **7** [6].

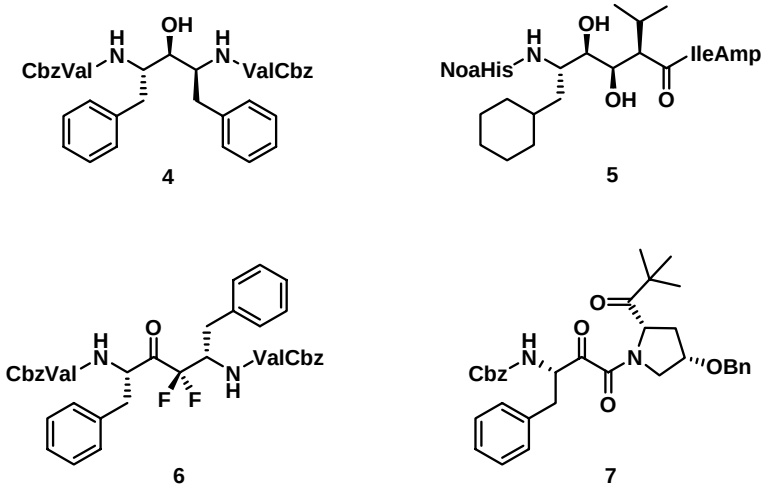


Figure 2. Potent inhibitors of HIV-1 protease.

More recently inhibitors have been based upon sulfonamide-substituted cyclooctylpyranones [7], *trans*-oxabicyclo{3,3,0}octane system [8] and C_2 symmetric cyclic urea and sulfamide derivatives [9]. On the basis of these important features, with the help of X-ray crystallographic data [10] and molecular modeling, Marples and co-workers have designed [11] novel steroidal molecules namely, 11-amino-12-oxo/hydroxy-steroids **8** and **9** based upon bile acids and estra 1,3,5(10)-triens.

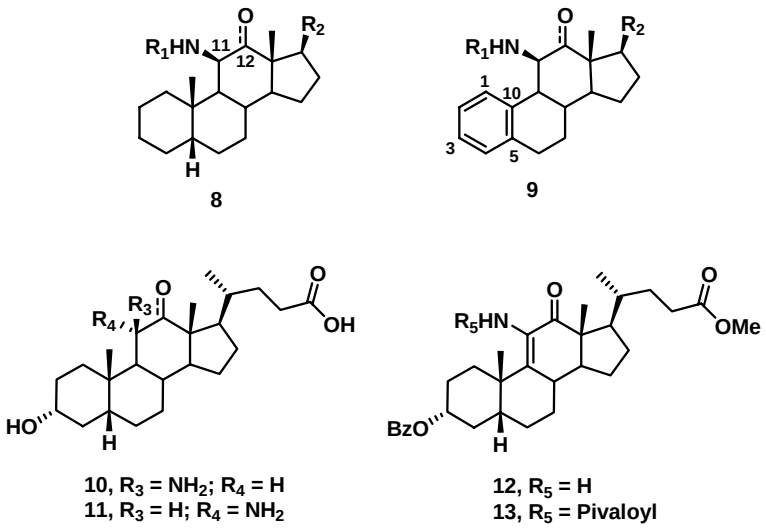


Figure 3. Proposed HIV-1 protease inhibitors.

In the proposed molecules the amino-alcohols and amino-ketones are visualized in steroid skeleton. This group was not able to realize the synthesis of the desired 11-amino-12-oxo compounds **10** or **11** with the specific stereogenic center at C-11 (Figure 3). Instead they synthesized A/B ring cis-steroidal enamine **12**. This steroidal enamine **12** and its *N*-pivaloyl derivative **13** with modest activity against HIV in cell culture were predicted to be protease inhibitors. The failure in the synthesis may be due to the instability of precursor 11-azido ketone that undergoes decomposition to the steroidal enamine. Such type of decomposition was observed in both steroidal [11-13] as well as alicyclic [14,15] α-azidoketones. In the

present study, we have synthesized such analogous compounds and subjected them to biological screening against HIV-1 replication.

B1.2.1. *Rationale for present work*

Stereoselective C-11 functionalization in steroids is one of the challenging targets for synthetic organic chemists as it involves severe steric interactions caused due to C-18 and C-19 angular methyl groups. Introduction of C-11 α-hydroxyl functionality via microbial hydroxylation by Syntex group [16] and *via* long-range chemical functionalization by Breslow [17] are well documented. Steroids with C-11 functionality are well known for biological activity and are obtained in a number of naturally occurring molecules such as cortisone, hydrocortisone and corticosterone [18,19]. Much more potent synthetic corticosteroids such as dexamethasone, triamcinolone and fluticasone also possess C-11 hydroxy functionality [20].

In the previous attempted methods [11] for the synthesis of 11-amino-12-oxo/hydroxy steroids, the required stereochemistry at C-11 position has been lost. To achieve the stereospecificity required in enzymatic reactions there is need for stereoselective synthesis of 11α/β-amino-12-α/β-hydroxy/oxo steroids. Synthesis of C-11 functionalized bile acid analogues namely, methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate, methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate, methyl 11-amino-3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate, methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate, methyl 11α-amino-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate; and methyl 11β-amino-3α,7α-diacetoxy-12β-hydroxy-5β-cholan-24-oate have been achieved in good to excellent yields. In the present study, these novel cholic acid derivatives induced

host cell fusion during the progress of HIV-1 infection and formed multinucleated giant cells. The fold increase in the viral count is also found to be directly proportional to the syncytia formed in T-cells. This is the first report of syncytia formation and thereby enhancement of viral replication in HIV-1 infected T-cells by cholic acid derivatives.

B1.3. Chemistry

Recently, Marples and co-workers reported [11] the synthesis of a steroidal enamine, methyl 11-amino-3α-benzyloxy-12-oxo-5β-chol-9,11-en-24-oate **12** from 11α-bromo compound **14** [11]. Compound **14** on treatment with NaN_3 in DMSO at 100 °C for 48 h afforded enamine **12** instead of the expected β-azido compound **15** (Figure 4). They proposed the formation of enamine **12** through the intermediates **15**, **16** and **17** with the expulsion of nitrogen gas. Thus, the synthesis of 11-aminosteroids with a specific stereogenic center at C-11 was not realized by Marples *et al*. This compound **12** was reported to possess modest activity against HIV-1 protease in cell culture.

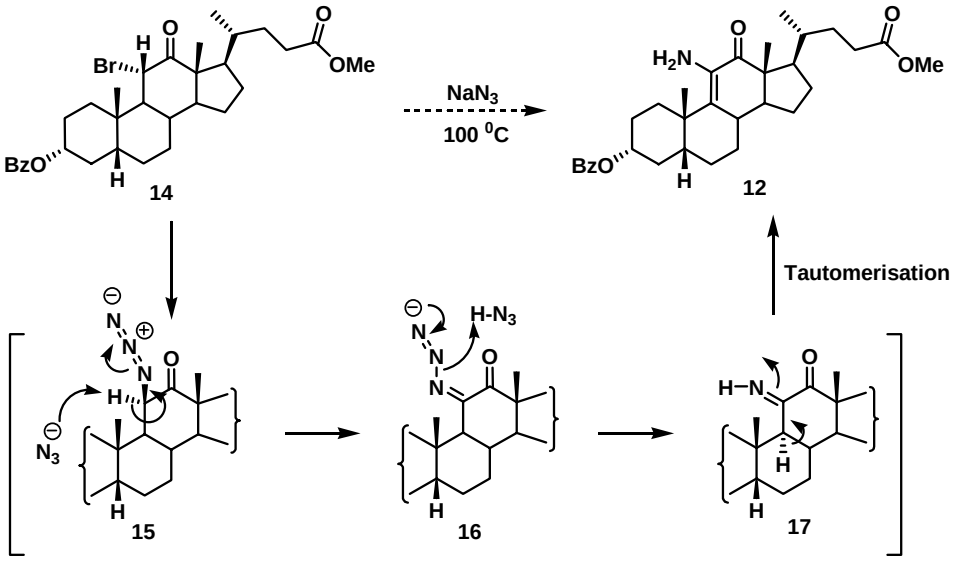
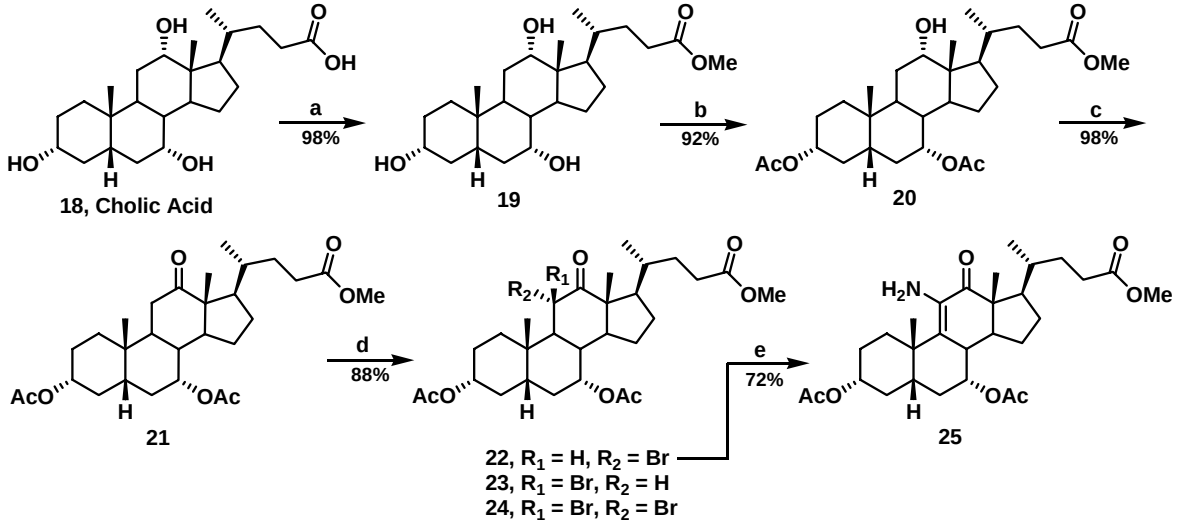


Figure 4. Proposed mechanism for the formation of steroidal enamine **12**.

In the course of our studies on the synthesis of steroidal protease inhibitors, we have accepted this challenge of stereoselective C-11 functionalization in cholic acid. Overnight stirring of cholic acid **18** in dry methanol using catalytic amount of *p*-TSA followed by selective acetylation provided diacetoxy methyl ester **20** in 90 % overall yield in two steps (Scheme 1). Improved yield in oxidation of compound **20** [21,22] was obtained by using $CrO_3/H_2SO_4/H_2O$ in acetone at shorter reaction time (98 % yield, 5 min). Literature procedure used two-phase oxidation [11, 23] in diethyl ether (86 % yield, 90 min).

Scheme 1.



Reagents and conditions: a) CH_3OH, *p*TSA, 28 °C, 24 h; b) Ac_2O, DMAP, Et_3N, CH_2Cl_2, 28 °C, 4-5 h; c) CrO_3, H_2SO_4, Acetone, 10 °C, 5 min; d) Br_2, Benzene, 28 °C, 6 days; e) NaN_3 (12 equiv.), DMF, 100 °C, 48 h.

Bromination of 12-oxo steroids has been widely explored [22,24]. A stereoselective high-yield bromination of 12-keto compound **21** to 11α-bromo compound **22** was demonstrated by Yanuka *et al* [25]. However, there are no reports on the study of crystal structure properties of these compounds. We had interest in the investigation of the effect of bulky bromine atom in steroid skeleton of cholic acid with different stereo-chemical orientations at C-11 on the two-dimensional arrangement of these molecules.

Selective C-11 bromination of 12-oxo compound **21** was achieved by us using bromine in acetic acid *via* $BF_3{\cdot}Et_2O$ catalysis to afford methyl 11α-bromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **22** in 93 % yield. We have also elaborated cholic acid **18** to its 11α-bromo and 11β-bromo derivatives **22** and **23** *via* 12-oxo methyl cholate **21** using bromine in benzene. Detailed investigation of the bromination reaction on compound **21** with excess bromine in benzene and longer reaction period led to the isolation of hitherto unknown C-11 dibrominated product, namely methyl 3α,7α-diacetoxy-11α,11β-dibromo-12-oxo-5β-cholan-24-oate **24** in 4 % yield along with 11α-bromo and 11β-bromo derivatives **22** and **23** with 65 % and 19 % yield respectively. The high resolution mass spectra of compound **24** showed the expected molecular ion peak and in the ^{13}C NMR spectra a quaternary C-11 carbon appeared at δ 74.76 ppm. The absolute structures of 12-oxo compound **21** and bromo ketones **22, 23, 24** have been established by single crystal X-ray analysis (Figure 5).

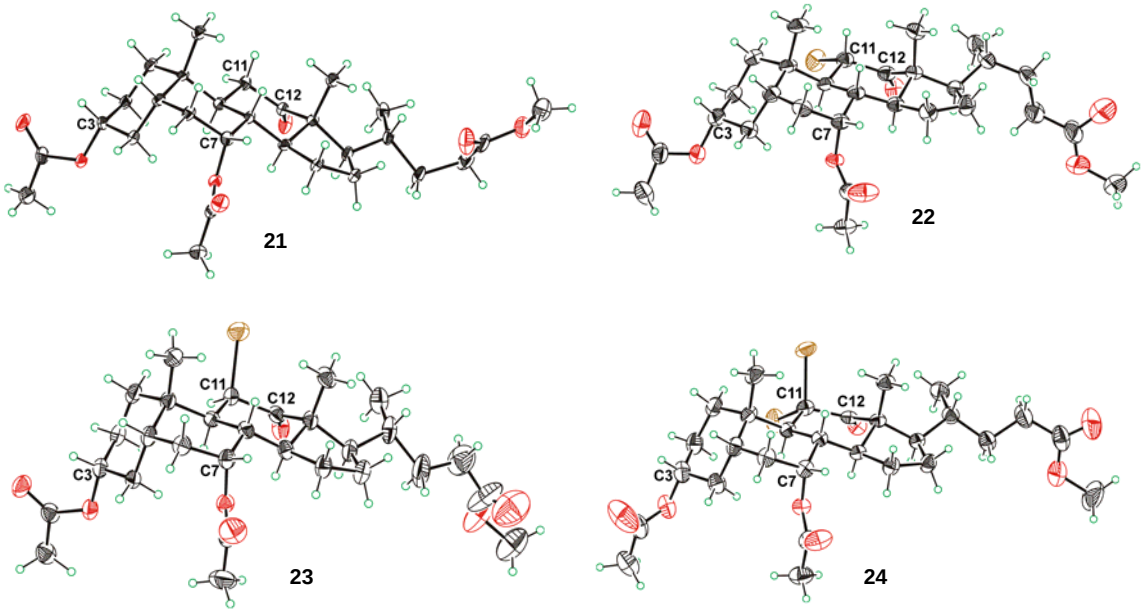
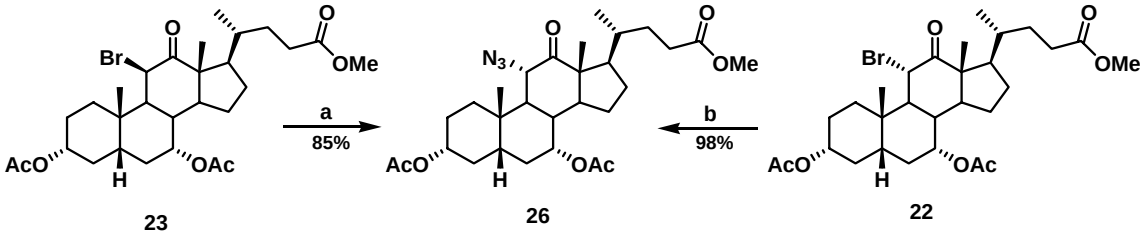


Figure 5. ORTEP [26] views of compounds **21-24**.

Starting from the α-bromo ketone **22** and following the procedure of Marples *et al*, using DMF as solvent in place of DMSO we synthesized steroidal enamine, methyl 11-amino 3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate **25** in 72 % yield (Scheme 1). However, our main aim was to synthesize the hitherto unknown 11-amino steroids.

Substitutions alpha to carbonyl groups are known to follow S_N2 mechanism [27]. Accordingly exposure of 11β-bromo compound **23** with 5 equivalents of NaN_3 in DMF at 28 °C for 8 h furnished the 11α-azido compound **26** in 85 % yield (Scheme 2). Treatment of epimeric α-bromo compound **22** with 5 equivalents of NaN_3 in DMF at 60 °C for 16 h surprisingly resulted in the formation of the same 11α-azido compound **26** in 98 % yield as if the reaction follows the S_N1 type of mechanism. Same α-azido compound **26** was obtained in 92 % yield from the crude bromination reaction mixture.

Scheme 2.



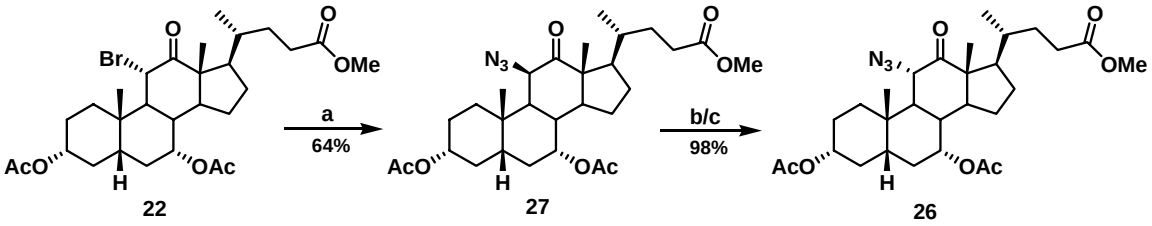
23 26 22

Reagents and conditions: a) NaN_3 (5 equiv.), DMF, 28 °C, 8 h; b) NaN_3 (5 equiv.), DMF, 60 °C, 16 h.

Compound **26** showed characteristic absorbance in IR at 2108 (N_3), 1740 (OAc/COOMe), 1722 (CO) cm^{-1}. The assignment of the stereochemistry at C-11 of compound **26** was supported by the ^1H NMR spectrum in which the C-11 proton appeared as a doublet (δ 4.06 ppm, J = 10.8 Hz) due to trans diaxial coupling between C-11 and C-9 protons (Table 1). Compound **26** obtained from **22** and **23** are identical in all the respect and the absolute configuration at C-11 was confirmed by single crystal X-ray analysis (Figure 6).

In addition, 11α-bromo-12-oxo compound **22** under mild reaction conditions [slight excess of NaN_3 (1.2 equiv.) at 60 °C for 4 h] afforded the unstable 11β-azido-12-oxo compound **27** in 64 % yield (Scheme 3). The assignment of stereochemistry at C-11 of compound **27** was supported by the ^1H NMR spectrum in which the C-11 proton appeared as a doublet (δ 4.14 ppm, J = 5.5 Hz) due to *cis* coupling between the C-11 and C-9 protons (Table 1). The structure of compound **27** was unambiguously confirmed by single crystal X-ray analysis.

Scheme 3.



Reagents and conditions: a) NaN_3 (1.2 equiv.), DMF, 60 °C, 4 h; b) NaN_3 (1.2 equiv.), DMF, 60 °C, 9 h; c) KOAc (1.2 equiv.), DMF, 60 °C, 9 h.

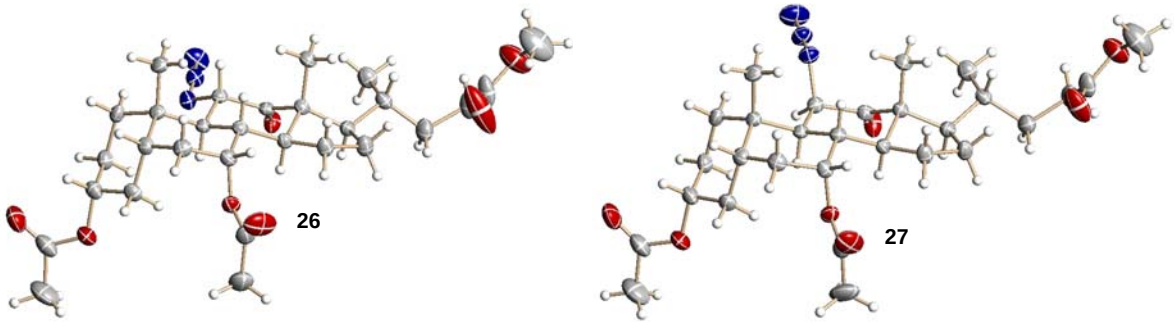


Figure 6. ORTEP views of compound **26** and **27**.

The isolated 11β-azido-12-oxo compound **27**, on treatment with 1.2 equivalents of NaN_3 in DMF at 60 °C for 9 h furnished the relatively stable 11α-azido-12-oxo compound **26** in excellent yield (98 %). A similar transformation of compound **27** to compound **26** was observed with KOAc in DMF (Scheme 3). In these cases NaN_3 and KOAc act as base to promote epimerization to the more stable compound [28]. However, no epimerization was

observed when compound **27** was treated under similar conditions in DMF alone or in the presence of protic acids such as *p*-TSA and camphorsulfonic acid. Thus, compound **26** can be obtained *via* two routes (a) in a single step from 11α-bromo compound **22** following harsh conditions (Scheme 2) or (b) from 11β-azido compound **27** following milder conditions (Scheme 3).

Formation of 11α-azido compound **26** from the 11α-bromo compound **22** suggests that there is formation of unstable intermediate, methyl 11β-azido-3α, 7α-diacetoxy-12-oxo-5β-cholan-24-oate **27** which gets epimerized to stable 11α-azido-12-oxo compound **26**. Such an epimerization of 11β-bromo-12-oxo compound **23** to 11α-bromo-12-oxo compound **22** has been reported [25]. This epimerization is acid catalyzed and proceeds *via* enol formation. In our case the 11β-azide **27** is transformed to 11α-azide **26** via enolate by base catalyzed (NaN_3) enolisation. Since the electrostatic repulsion between the 11β-azide and angular methyl groups in compound **27** seems to be very high, it is probable that there is formation of enolate anion **28** to help relieve the repulsive interactions (Figure 7). This interaction seems to be one of the factors, which can account for the rapid epimerization or enolisation.

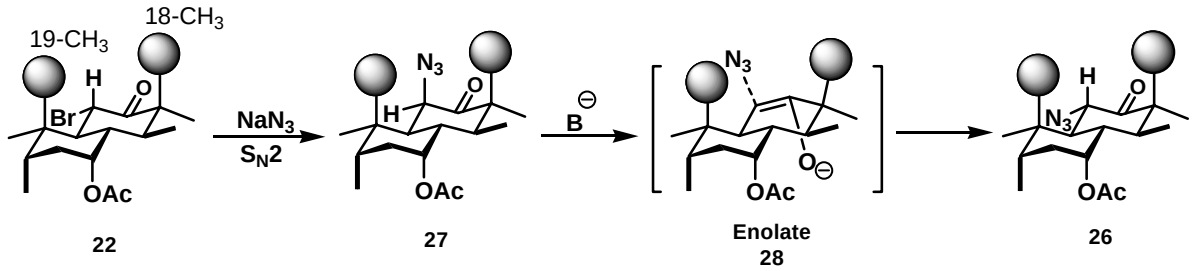
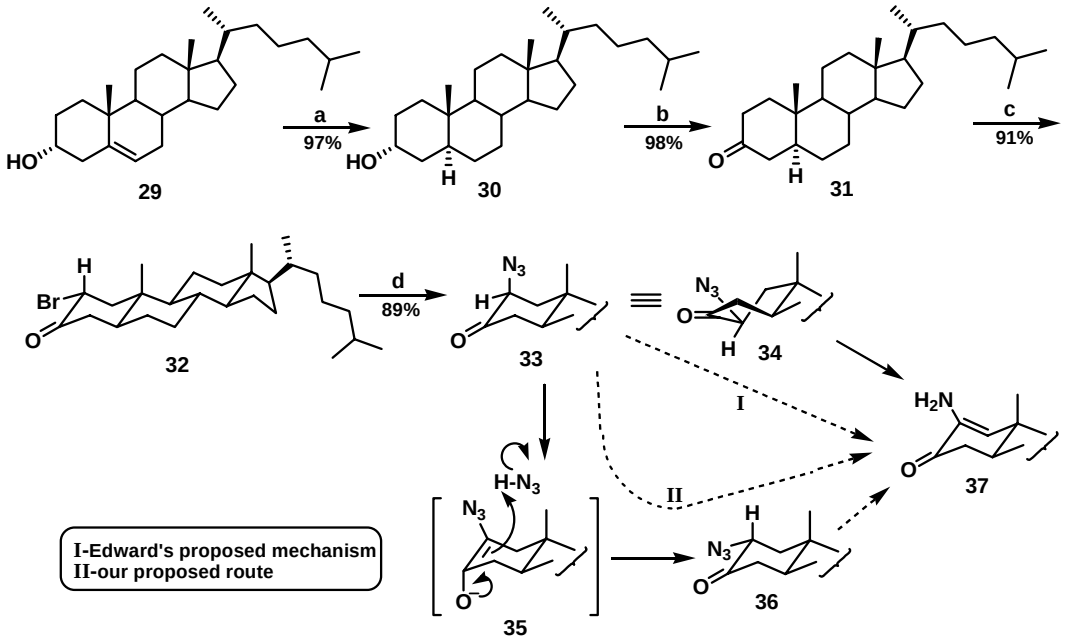


Figure 7. Crowding at beta face of steroid skeleton.

Such precedence of enamine formation (2-aminocholest-1-ene-3-one, **37)** in A ring of steroids (cholesterol, **29**) was reported by Edward *et al* [15]. Attempted preparations of pure

2β-azidocholestan-3-one **33** (Scheme 4) from 2α-bromocholestan-3-one **32** with LiN_3 in methanol or DMF and also with NaN_3 in DMSO were unsuccessful.

Scheme 4.



Reagents and conditions: a) H_2, Pd-C, EtOH, 40 psi, 25 °C, 10 h; b) CrO_3, H_2SO_4, Acetone, 10 °C, 5 min; c) PTB, acetic acid, 50 °C, 15 min; d) NaN_3 (1.2 Equiv.), DMF, 28 °C, 2 h.

Edward [15] proposed a conformational driving force for the accelerated decomposition of the azidoketone **33** to enamino ketone **37**. Since the displacement of the bromine is probably a normal S_N2 reaction, the azide formed from 2α-bromo compound **32** was assumed to be 2β-azidocholestan-3-one **33**. Since C-3 is trigonal in compound **33** it was proposed that the azido ketone **33** adopts ring A conformation closer to the half boat **34** than the chair **33** in order to help relieve the repulsive interactions of the azido group and the C-10-methyl group. This interaction may be one of the factors, which can account for the rapid decomposition of 2β-azidoketone **33** to enamino ketone **37**.

In related work, Heathcock and co-workers [29] prepared the 2α-azidocholestan-3-one **36** from 2α-bromo ketone **32**. When we treated 2α-bromocholestan-3-one **32** [30] (1 mmol) with less amount of NaN_3 (1.2 mmol) in DMF under milder conditions (2 h at 28 °C), we were able to isolate the known 2α-azidocholestan-3-one **36** in 89 % yield (Scheme 4). The signal of C-2 axial proton (δ_H 3.98 ppm) in compound **36** is split to a doublet of doublets by two vicinal couplings (J = 12 Hz and 6 Hz). Thus C-2 proton must adopt an axial orientation. Melting point and other spectral data for compound **36** are in agreement with the data reported [29,31] in the literature. From this it is logical that in the course of reaction starting from 2α-bromo compound **32** there is formation of 2β-azido compound **33**, which on enolisation (the energy difference of about 3.3 kcal/mol is the driving force for such enolisation) [28] produces 2α-azidocholestan-3-one **36** via enolate **35**. This may be converted to steroidal enamine **37** under the reaction conditions used [15, 29]. We propose the formation of 2α-azidocholestan-3-one **36** from 2α-bromocholestan-3-one **32** by base (NaN_3) catalyzed epimerization of unisolated 2β-azidocholestan-3-one **33**.

Decomposition of A-ring substituted azido compounds **33** or **36** to its enamine **37** requires very mild conditions (little excess of NaN_3, low temperature and less time) but decomposition of C-ring substituted azido compounds **26** or **27** to enamine **25** requires drastic conditions. This can be explained on steric grounds. In the progress of reaction α-bromo compound **22** first get converted to β-azido compound **27** in which C-11 equatorial proton is easily accessible for the base (NaN_3) as it is oriented on alpha face of steroid skeleton (Figure 7). But on enolisation C-11 equatorial proton in compound **27** gets transformed to the beta face of the steroid skeleton (compound **26**), which is very crowded due to angular methyl groups. Under these circumstances, it is very difficult for the base

(NaN_3) to pickup the C-11 axial proton and initiates the decomposition process. Comparatively the steric crowding for C-2 equatorial proton in compound **33** or C-2 axial proton in compound **36** is less than that of compounds **26** or **27** (Scheme 4). 11α-Azido-12-keto compound **26** when treated with 10 equivalents of NaN_3 at 100 ºC for 36 h produced the steroidal enamine **25**, this authenticate the predicted route for the formation of enamine **25** from 11α-bromo-12-keto compound **22** (Scheme 5). Compound **25** obtained from **22** and **26** were identical in all the respect.

The observed epimerization in compounds **27** and **33** were supported by molecular modeling calculations. In particular, the calculations have been performed to analyze the energetic of the formation of the more stable products **26** and **36** respectively, obtained by the sodium azide catalyzed epimerization. To get the energy difference between the axial and equatorial azides of compounds **27** and **26** and also **33** and **36** semiemperical molecular orbital calculations (PM3) were performed by using *Gaussian 98* [32]. The initial geometry of all the compounds with different configurations was generated by PCMODEL software.

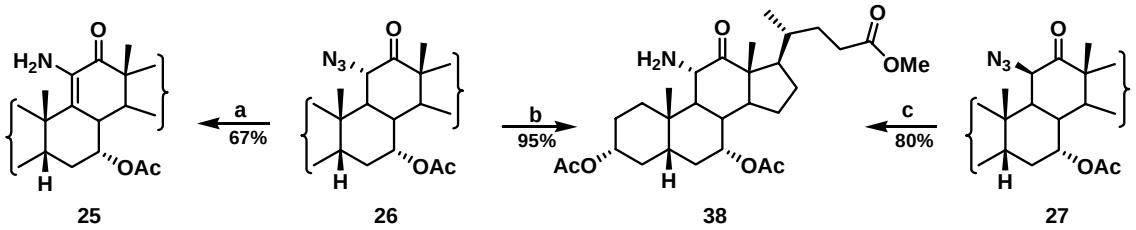
Table 1. The minimized energies for compounds **26**, **27**, **33** and **36**.

Compounds	Minimized Energy (kcal/mol)	Energy Difference (kcal/mol)
27	-254.5142	6.2656
26	-260.7798	
33	-49.1485	3.2938
36	-52.4423	

The values of the energy difference for all the four compounds are given in Table 1. It can be seen that amongst the two-stereochemically different isomers (**27**/**26** or **33**/**36**) the isomer with equatorial azide group (**26** and **36**) are found to be more stable than the isomers with axial azide group (**27** and **33**). This is because of unfavorable severe 1-3 diaxial steric

interaction of C-11 β-azide with two angular methyl groups in compound **27** and C-2 β-azide with C-19 methyl group in compound **36**. These electrostatic interactions seem to be one of the factors, which can account for the rapid epimerization.

Scheme 5.



Reagents and conditions: a) NaN_3 (10 equiv.), DMF, 100 ºC, 36 h; b) H_2/Pd-C, ethylacetate, 40 psi, 28 ºC, 5 h; c) TPP, THF, H_2O, 28 ºC, 48 h.

11α-Azido-12-keto compound **26** on catalytic hydrogenation using 10 % Pd/C was readily converted into the desired 11α-amino-12-keto compound **38** in 97 % yield (Scheme 5). Strong absorption at 3529 cm^{-1} in the IR spectrum of compound **38** attributed to the NH_2 group while in the ^1H NMR spectrum, the C-11 proton appeared as a doublet (J = 9.3 Hz) at δ_H 3.76 ppm due to *trans* diaxial coupling with the C-9 proton. The structure of 11α-amino-12-keto compound **38** was confirmed by single crystal X-ray analysis (Figure 8).

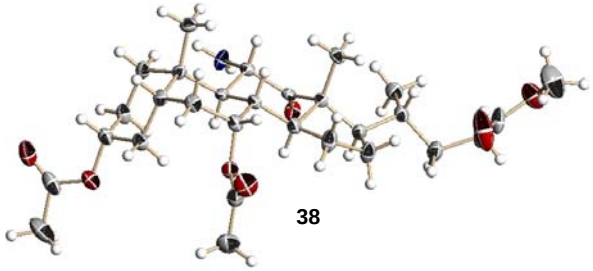


Figure 8. ORTEP view of compound **38**.

Treatment of 11β-azido-12-keto compound **27** with excess of Pd-C and high pressure (40 to 80 psi) in ethanol or ethyl acetate resulted in the recovery of the starting compound.

Attempted reduction of 11β-azide functionality in compound **27** using LAH in THF or dioxane produced complex reaction mixtures. However, treatment of compound **27** with triphenylphosphine in THF followed by aqueous workup (Staudinger Reaction, [33]) furnished 11α-amino-12-keto compound **38** in 80 % yield.

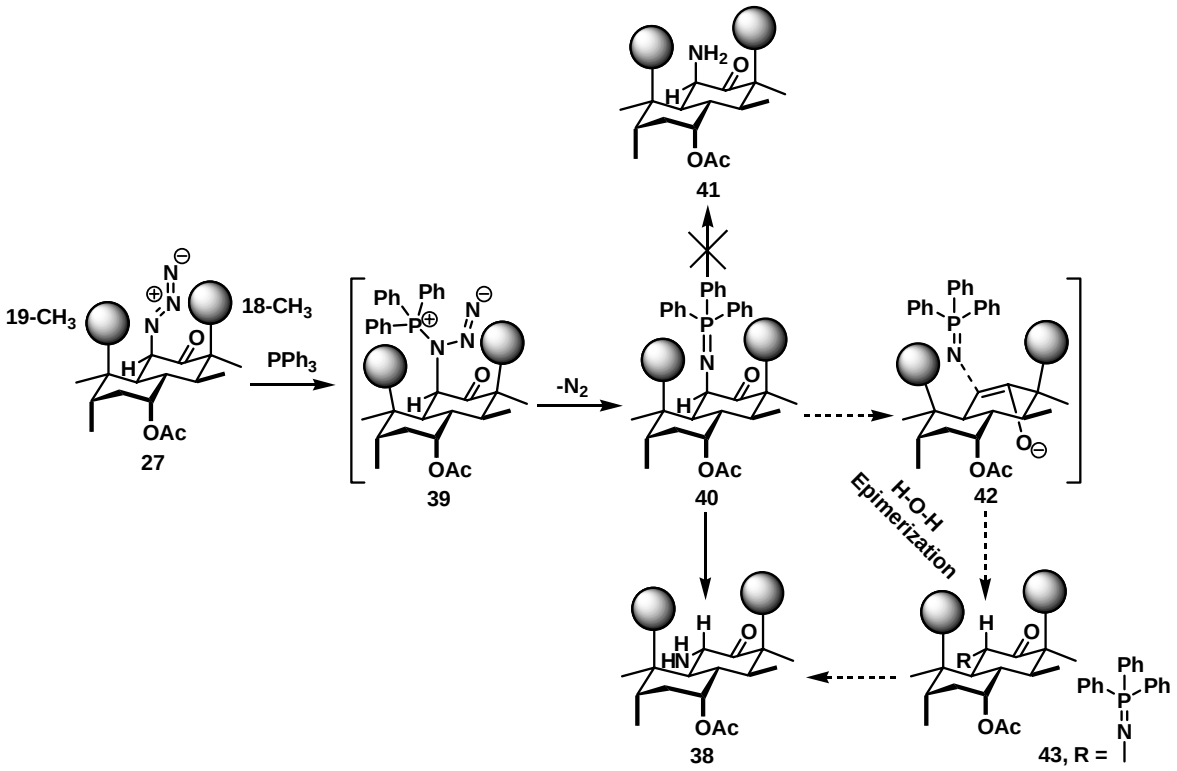
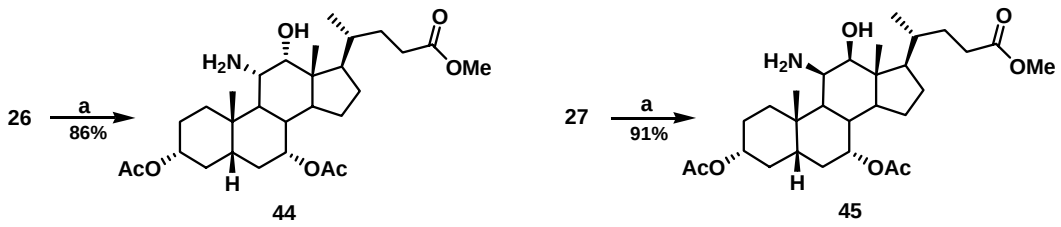


Figure 9. Mechanism for the formation of 11α-amino compound **38** from **27**.

Triphenylphosphine reacts with the β-azide functionality of compound **27** to generate a phosphazide **39** which looses N_2 to form an iminoposphorane **40**. Because of the severe steric crowding at beta face of iminophosphorane **40**, it might epimerized to the stable iminophosphorane **43** during the aqueous workup. This leads to the formation of 11α-amino-12-keto compound **38** and triphenylphosphine oxide (Figure 9). Thus synthesis of desired 11β-amino compound **41** is not realized. Reduction of 11α-amino-12-oxo compound **38** with $NaBH_4$ produced mixture of 12-hydroxy epimers and we failed to isolate the desired

11α-amino-12α-hydroxy compound **44**. However, the desired 11α-amino-12α-hydroxy compound **44** has been synthesized in a single step from the 11α-azido-12-oxo compound **26** using $NaBH_4/CoCl_2{\cdot}6H_2O$ [34] in CH_2Cl_2-H_2O-CH_3OH and cetyltrimethylammonium bromide (CTAB) as a phase transfer catalyst (Scheme 6). This is a unique reaction in which both the azide and the carbonyl functionalities are reduced to its aminoalcohol in a single step. Use of this reagent has not been exploited earlier in steroid chemistry.

Scheme 6.



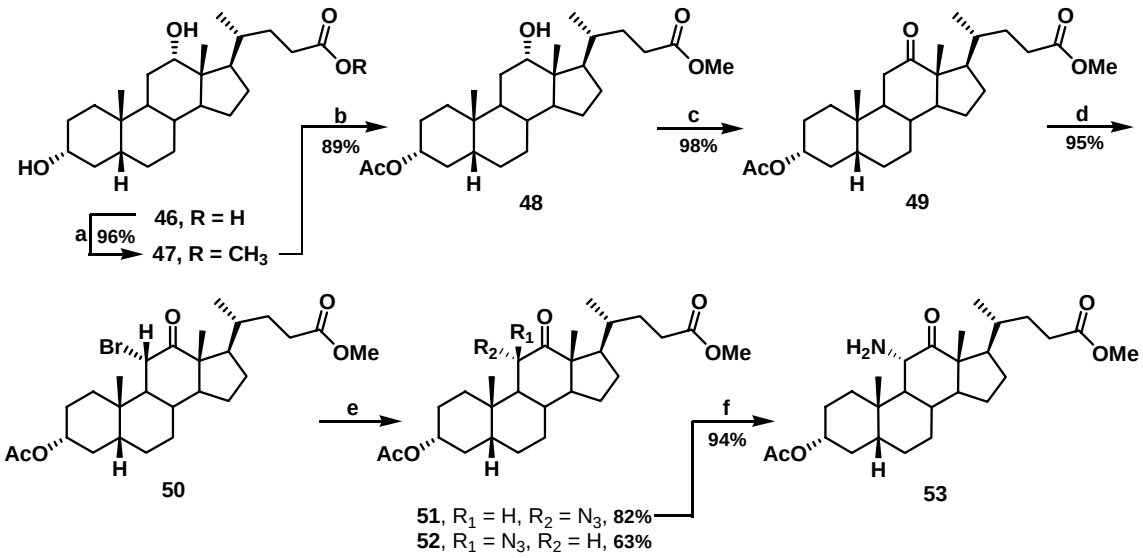
Reagent and condition: a) $NaBH_4$, $CoCl_2$, CTAB, CH_2Cl_2, H_2O, CH_3OH, 25°C, 30 min.

It is also worth mentioning here that 11α-azido-12-oxo compound **26** stereospecifically furnished 11α-amino-12α-hydroxy compound **44** in 86 % yield. In a similar fashion treatment of 11β-azido-12-oxo compound **27** with $NaBH_4/CoCl_2$-$6H_2O$ in CH_2Cl_2-H_2O-CH_3OH and CTAB as a phase transfer catalyst afforded stereospecifically 11β-amino-12β-hydroxy compound **45** in excellent yield.

To understand the effect of C-7 acetate functionality during the observed conversion of 11α-bromo compound **22** to 11α-amino compound **38**, the similar reaction sequences were carried out on deoxy cholic acid **46** (Scheme 7). In this series we could isolate the hitherto unknown C-11 azido compounds **51**, **52** and 11α-amino-12-oxo steroid **53** as observed in cholic acid series, which were not realized by Marples and co-workers, during

their work on deoxycholic acid. Comparable yields were obtained after employing the similar reaction conditions as mentioned for that of cholic acid.

Scheme 7.



Reagents and Conditions: a) CH_3OH, *p*TSA, 28 °C, 24 h; b) Ac_2O, DMAP, Et_3N, CH_2Cl_2, 28 °C, 4-5 h; c) CrO_3, H_2SO_4, Acetone, 10 °C, 5 min; d) Br_2, Acetic acid, $BF_3{\cdot}Et_2O$, 28 °C, 5 days, diazomethane, 28 °C, 2 h; e) NaN_3, DMF, 60 °C, 4-16 h; f) H_2/Pd-C, ethyl acetate, 40 psi, 28 ºC, 5 h.

In conclusion we have demonstrated that 11α-bromo compound **22** on treatment with NaN_3 is converted into 11β-azido compound **27**. In the presence of base, 11β-azido compound **27** epimerized to the thermodynamically more stable 11α-azido compound **26**. Compound **26** on further treatment with NaN_3 under drastic conditions is transformed into the steroidal enamine **25**. The stereoselective synthesis of 11α-amino-12-oxo steroid **38**, 11α-amino-12α-hydroxy steroid **44** and 11β-amino-12β-hydroxy compound **45** has been achieved. Mechanistic aspects for the decomposition of steroidal azidoketones to its enamines in A as well as C ring of steroids are also discussed.

B1.3.1. ***Spectroscopic discussion***

Some interesting observations can be made on the ^{1}H, ^{13}C NMR and IR spectra of the bromo ketones (**22**, **23**, and **24**), azido ketones (**26**, **27**) and amino ketone (**38**) with respect to the starting 12-oxo compound **21** (Table 1). Partial ^{1}H NMR spectra of these compounds showing consequence of C-11 substitution on C-18, C-19 and C-21 methyl groups are pictured in figure 10. Deshielding effect of ~0.18 δ ppm was observed on C-19 methyl group in 11α-bromo compound **22** in comparison with 12-oxo compound **21**, while deshielding effect of ~0.33 to 0.35 δ ppm on both C-18 and C-19 methyl's was observed in 11β-bromo compound **23** in comparison with **21**. Similarly, deshielding effect of ~0.14 δ ppm was observed on C-19 methyl group in 11-dibromo compound **24** in comparison with 11β-bromo compound **23**, while deshielding effect of about 0.31 to 0.32 δ ppm on both C-18 and C-19 methyl's was observed in 11-dibromo compound **24** in comparison with 11α-bromo compound **22**. In case of azido ketones, deshielding effect of ~0.14 δ ppm was observed on C-19 methyl group in 11α-azido compound **26** in comparison with 12-oxo compound **21**, while deshielding effect of ~0.24 to 0.25 δ ppm on both C-18 and C-19 methyl groups was observed in 11β-azido compound **27** in comparison with **21**. Analogous effect was observed in case of 11α-amino-12-oxo **38** in which deshielding effect of ~0.18 δ ppm was observed on C-19 methyl group, where as no effect was observed on C-18 methyl group. Thus, C-11α-bromine (~0.14-0.18 δ ppm), C-11α-azide (~0.14 δ ppm) and C-11α-amine (~0.18 δ ppm) functionalities contributes for the deshielding of only C-18 methyl protons, where as C-11β-bromine (~0.31-0.35 δ ppm) and C-11β-azide (~0.24-0.25 δ ppm) functionalities contributes for the deshielding of both C-18 and C-19 methyl protons. The C-11α-bromine, azide and amine functionalities also contributes for the deshielding of C-1

equatorial (C-1_e) proton through space this can be observed in compounds **22** [22], **24**, **26** and **38** in which there is a presence of C-11α-functionality (Table 2). These observations demonstrate a well-defined effect of C-11 substitution through space on the chemical shifts of C-18 and C-19 angular methyl and C-1_e protons.

Table 2. Some representative ^{1}H, ^{13}C NMR and IR spectroscopy chemical shifts.

Comp	^{1}H NMR, δ ppm and coupling constants *J* in Hz						C-12 carbonyl	
	Methyl group			Proton (H) at			^{13}C NMR δ ppm	IR ν_{max} cm^{-1}
	C-18	C-19	C-21	C-9	C-11	C-1_e		
21	1.03	1.03	0.85 d, *J* = 5.8	—	—	—	213.3	1701
22	1.03	1.21	0.86 d, *J* = 5.8	2.79 dd, *J* = 10.7	5.01 d, *J* = 10.7	2.90 dt, *J* = 15.4 & 3.0	202.3	1718
23	1.36	1.38	0.93 d, *J* = 6.6	2.64 dd, *J* = 11.7 & 5.9	4.42 d, *J* = 5.9	—	203.4	1697
24	1.36	1.52	1.00 d, *J* = 5.9	3.35 d, *J* = 11.4	—	3.02 dt, *J* = 15.2 & 3.1	194.3	1716
26	1.02	1.15	0.88 d, *J* = 6.4	—	4.06 d, *J* = 10.8	2.48 dt, *J* = 14.6 & 3.0	207.0	1687
27	1.25	1.26	0.92 d, *J* = 6.3	—	4.14 d, *J* = 5.5	—	206.7	1715
38	1.01	1.19	0.85 d, *J* = 6.5	—	3.76 d, *J* = 9.3	2.72 dt, *J* = 14.1 & 3.0	214.1	1713

Carbonyl resonance of C-12 in compound **21** appeared at 213.3 ppm (Table 2). As expected [35], marked upfield shift (shielding) of about 10 ppm was observed for carbonyl resonances in 11-bromo-12-oxo compounds **22** (202.3) and **23** (203.4). This clearly suggests that irrespective of the stereochemistry at C-11 position the bromo substitution accounts for deshielding of about 10 ppm, accordingly carbonyl resonance of C-12 in C-11-*gem*-dibromo

compound **24** appeared at 194.3 ppm. In this compound two bromine atoms showed an additive effect of about 19 ppm on C-12 carbonyl resonance. Similar type of upfield shift of about 6-7 ppm was observed for 11-azido-12-keto compounds **26** (207.0) and **27** (206.7).

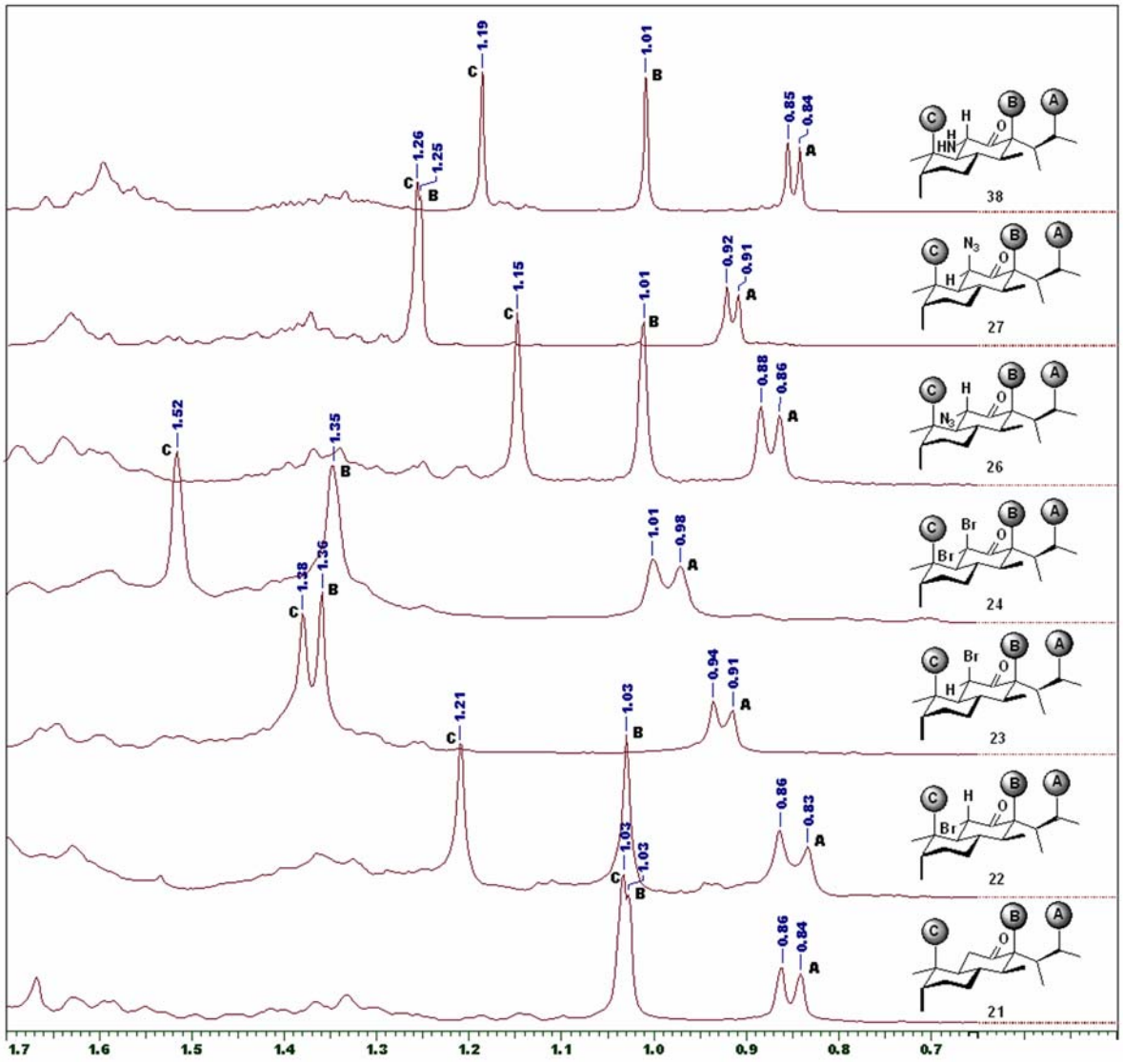


Figure 10. Partial ^{1}H NMR spectra of **21**, **22**, **23**, **24**, **26**, **27** and **38**.

An equatorial α-bromo substituent produced a marked shift in the infrared carbonyl stretching frequency (about 17 cm^{-1} in case of 11α-bromo-12-oxo compound **22** and 15 cm^{-1} in case of C-11-*gem*-dibromo-12-oxo compound **24**) relative to that of the parent ketone but that an axial halogen has a negligible effect this can be observed in case of compounds **23**

and **24** (Table 2). In contrast an equatorial α-azido substituent produced a marked up field shift of about 13 cm^{-1} in the infrared carbonyl stretching frequency relative to that of the parent ketone **21** but that an axial azide produced a marked down field shift of 14 cm^{-1}.

The circular dichroism (CD) curves for the three bromoketones **22**, **23** and **24** are compared with that of the parent 12-oxo compound **21 (**Figure 11). The axial nature of the bromine atom in compounds **23** and **24** is clearly evident from its strong negative Cotton effects, indicating a very high degree of asymmetry in these compounds. The similarity of the circular dichroism absorption (positive sign) of the 11α-bromo ketone **22** and the parent compound **21** is characteristic of the equatorial bromine substituent. These findings are consistent with the octant rule [36].

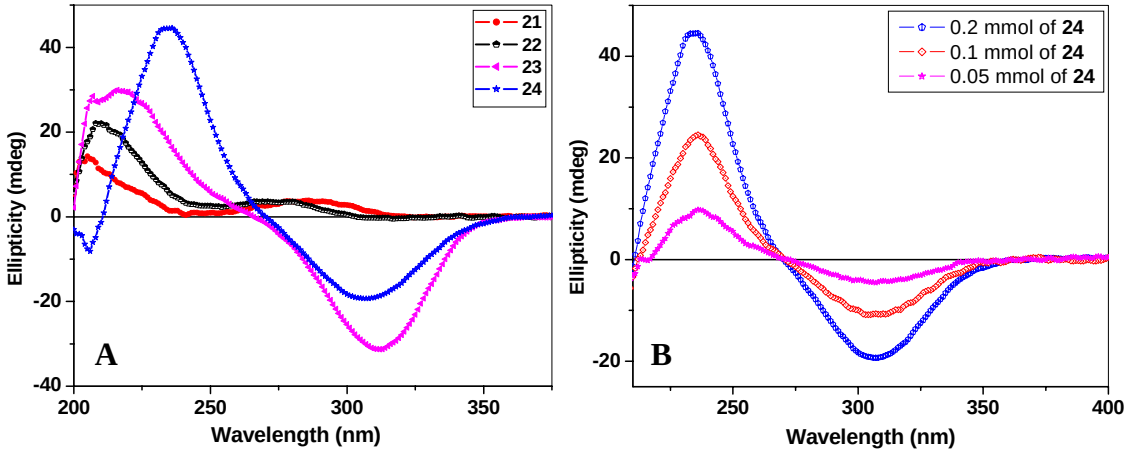


Figure 11. (A) CD curves for **21-24**; (B) CD curves for **24** at variable concentrations.

B1.3.2. *Crystallographic discussion*

Single crystals of compounds **21**, **22**, **23**, **24**, **26**, **27** and **38** were grown from a hot saturated filtered solution of these compounds in ethyl acetate. Suitable crystals were obtained by slow evaporation of the solvent at room temperature (RT). Compound **21** was crystallized as colorless long needles, **26**, **27** and **38** were crystallized as colorless thin

needles, where as crystals of compounds **22**, **23**, and **24** were thin plates and best amongst them were selected using Leica polarizing microscope. X-ray intensity data of all the compounds were collected on a Bruker SMART APEX CCD diffractometer. All the data were corrected for Lorentzian, polarization, and absorption effects using Bruker's SAINT and SADABS programs. The crystal structures were solved by direct method using SHELXS-97 and the refinement was performed by full matrix least squares of F^2 using SHELXL-97 [37]. Hydrogen atoms were included in the refinement as per the riding model. Molecular graphics were from Mercury (http://www.ccdc.cam.ac.uk/prods/mercury). A summary of the crystal data and experimental details are listed in Table 3, 4 and 5.

The effects of sequential replacement of methylene H atoms at the C-11 position of **21** by the bromine atom on molecular organization in crystals were studied. The effect of classical H-bonding interaction on the molecular packing of bile acid skeleton was recently reported by Zhao [38] and Tato [39] *et al*. In order to study the interplay of weak intermolecular interactions on the molecular aggregation of C11 functionalized bile acid skeleton in the absence of conventional H-bonding, the C3 and C7 hydroxyl groups as well as C24 carboxylic acid was masked with acetate and methyl ester functionalities, respectively. As expected, the molecular packing in all the four structures was dominated by C-H…O interactions (Table 5). In **21**, molecules make trifurcated C-H…O interactions with unit-translated molecules along b-axis forming a molecular chain involving carbonyl oxygen O5 and the methyl hydrogen at C18, C19 and methine hydrogen at C8 atoms (Figure 12A). These chains are zipped by 2_1-screw axis *via* C3B-H3B…O2 interactions bringing the head groups in close proximity. These layers make cohesions through C29-H29B…O4 and C29-H29A…O1 interactions along the a-axis and *via* C15-H15A…O4 contacts along the c-axis

(Table 5, Figure 12B). The molecular organization does not exhibit typical 'head-to-tail' organization generally observed in steroids [38].

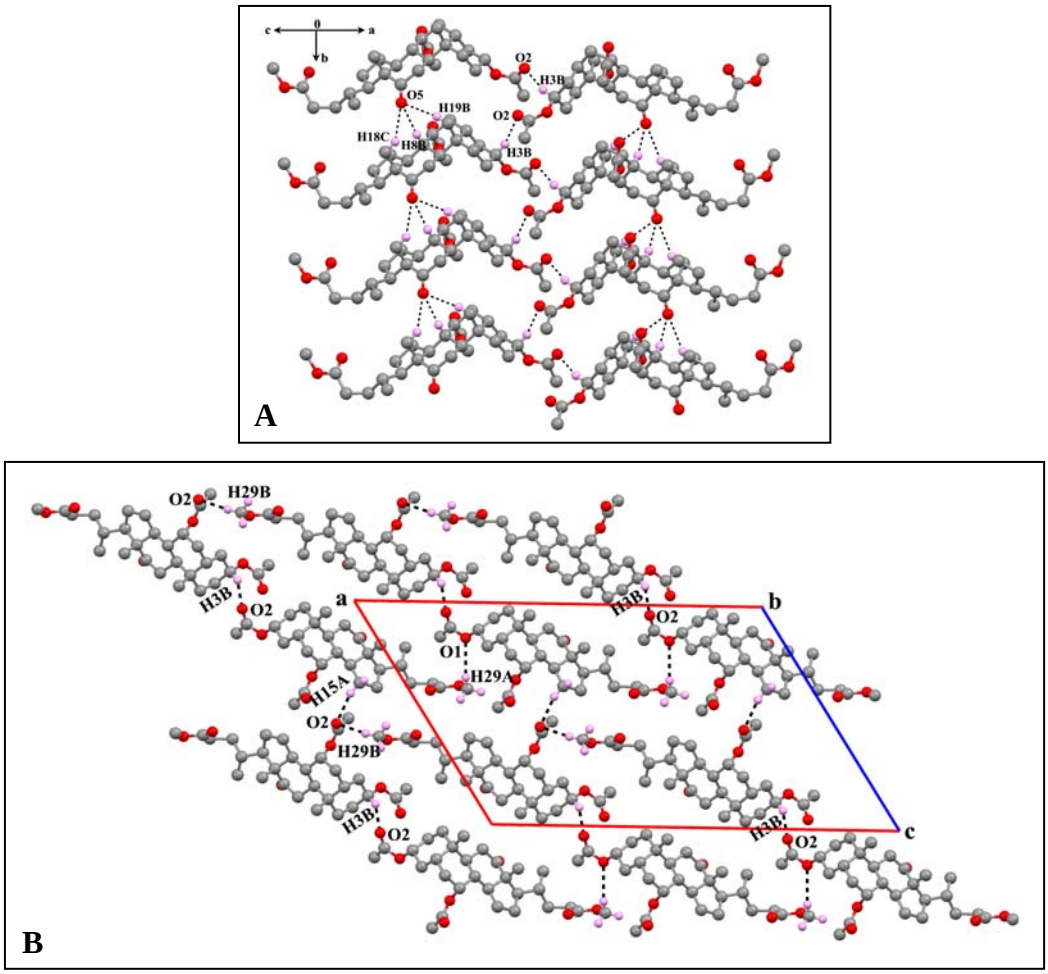


Figure 12. Association of molecules in **21** showing (A) formation of molecular chain *via* trifurcated C-H…O interactions and (B) bridging of the layers viewed down b-axis.

In **22**, bromine at the C-11 alpha position breaks the trifurcated C-H…O linked molecular assembly seen in **21**. Molecules along a-axis are linked by C28-H28B…O5 contacts across crystallographic 2_1-screw axis forming helical assembly (Figure 13A). In addition, four other C-H…O interactions (C2-H2A…O7, C29-H29B…O2, C21-H21A…O1 and C26-H26C…O6, Table 5) are made between the 2_1 related molecules along the helix that brings the C3-acatate group and the side chain in close proximity. These helices are

stitched together by C5-H5…O6, C19-H19A…O6 and C26-H26A…Br1 interactions (Figure 13B). The helices along the a-axis are discretely packed by creating well-guided tunnel.

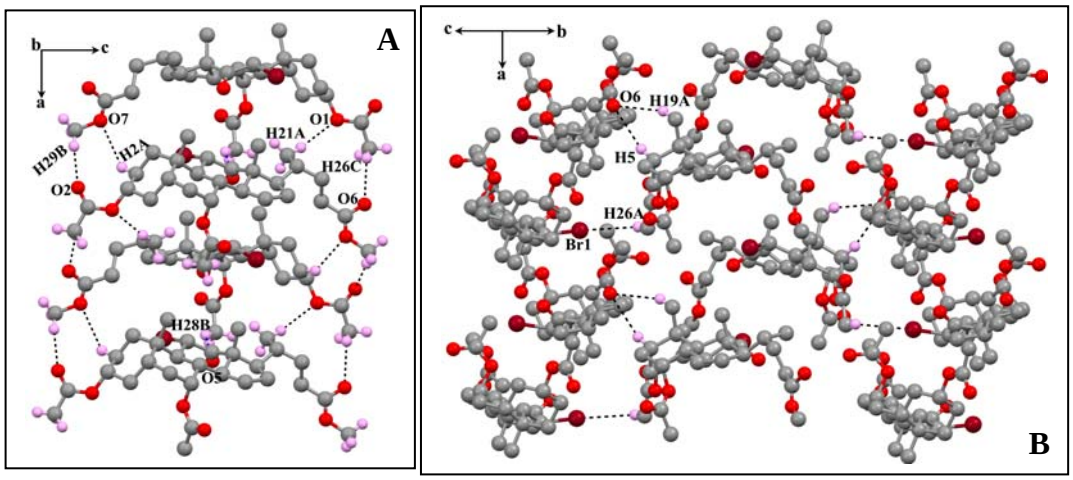


Figure 13. View of molecular packing in **22** showing (A) helical assembly along the a-axis and (B) interlinking of the helices via C-H…O interactions.

In **23** with Br atom at C-11-β position, the organization of molecule was very similar to **21** along b-axis (Figure 14A). Molecules made trifurcated C-H…O assembly with longer C18-H18C…O5, C19-H19B…O5 interactions but similar C8-H8B…O5 interaction as compared to **21** (Figure 12). These molecular chains are glued through C3-H3B…O2 interactions along crystallographic two-fold screw axis by bringing the heads together. In addition, one more C-H…O interaction (C26-H26C…O2) was involved in bridging these chains as compare to **21** giving more stability to the association. These layers are zipped by 2_1-screw axis *via* bifurcated C-H…O interactions (C28-H28A…O6 and C15-H15A…O6) between acetate group at C7 and side chain at C17. Molecular packing viewed down the b-axis shows the dimeric assemblies of layers, which are connected *via* C23-H23A…O4 contact (Figure 14B). It is interesting to note that Br atom in this molecule does not make any short contact.

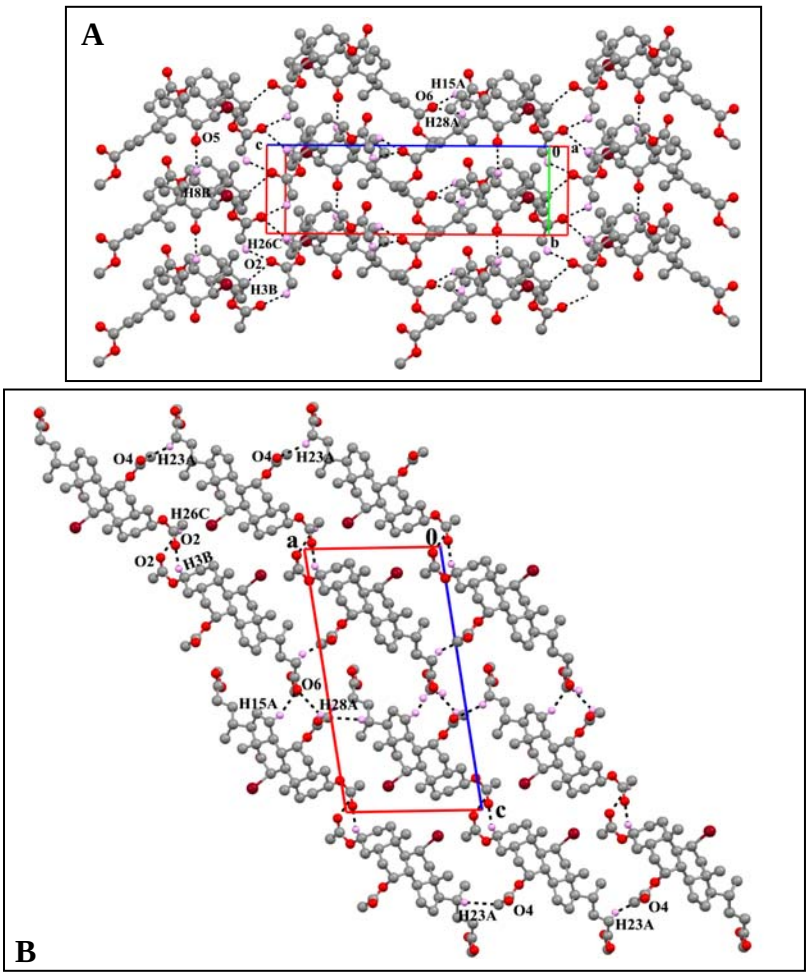


Figure 14. Molecular packing in **23** (A) viewed down a-axis and (B) viewed down b-axis showing association of the molecules via C-H…O interactions.

In **24**, replacement of both the H-atoms at C-11 by Br atoms causes an interesting effect on molecular organization. Molecules in **24** are helically assembled around the crystallographic 2_1-screw axis *via* halogen bonding (Br…O) and C-H…O contacts (Figure 15A). Beta bromine atom at C-11 interacts with the carbonyl oxygen (O5) of the 2_1-screw axis molecule *via* C11-Br2…O5 contact. The same carbonyl oxygen (O5) accepts H atom from C29-H29A of C7 acetate group of the next 2_1-screw axis molecule to make C-H…O interaction (Table 5). In continuing this pattern, each successive molecule gets a twist of 180° to generate helicity along the b-axis, which coincides with the crystallographic two-

fold screw axis. The Br1…O5 distance (3.256 Å) is much less than the sum of their van der Waals radii (3.35 Å) although the angle of approach deviates from linearity (∠C11-Br1…O5 = 139.33°). The same is the case with C29-H29A…O5 contact (H29A…O5 = 2.455 Å & ∠C29-H29A…O5 = 133.93°). These successive helices are held together along the a-axis through C27-H27C…O4 & C15-H15A…Br1 (Figure 15B).

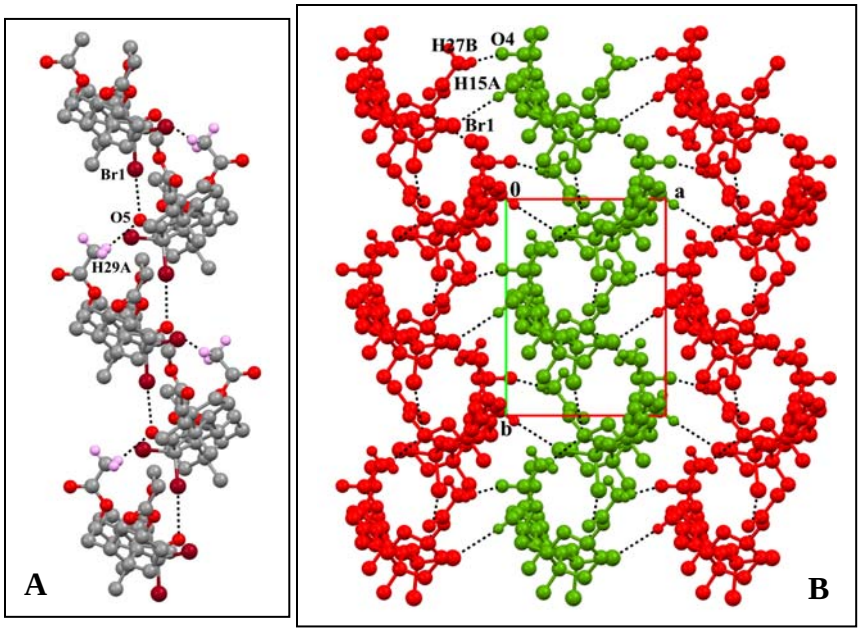


Figure 15. (A) Helical self-assembly in **24** through C-Br…O and C-H…O interactions along the 2_1-axis (B) interlinking of helices along a-axis *via* C-H…O and C-H…Br contacts.

The molecular assembly *via* 'Halogen bonding', a non covalent interaction between the halogen atoms (as acceptors of electron density) and lone-pair possessing atoms (mostly O and N) was vastly studied since last decade due to its application in the field of crystal engineering, molecular recognition, solid state synthesis [40]. To the best of our knowledge, structure of **24** is the first example of halogen bonding recognized in steroid structures. Since we observed halogen bonding contact for the first time in steroids, we carried out a CSD search to examine its occurrence and preferred geometries in other steroid structures [41]. The constrains applied were R < 0.10, distances ≤ sum of van der Waals radii and

angles in the range 130-180º. All searches were carried out with error-free coordinates and restricted entries of disordered, ionic, polymeric and powdered structures. The CSD search included halogens (X = F, Cl, Br, I) and differently hybridized oxygen atoms (carbonyl and ether). Out of 104 structures containing halogen atoms and CO groups in steroid structures, 7 structures showed C-X…O interaction. In all the seven structures, only carbonyl oxygen was involved in the short contact with Cl, Br and I, but no hits were found for F. This weak interaction was considered to have a role in binding of halo-steroids to the carbonyl of the peptide chain of the receptor protein that offers optimum desired binding affinity, as observed in the crystal structure of an inhibitor-protein complex between the inhibitor 4,5,6,7-tetra-bromobenzotriazole and phosphor-CDK2-cyclin A [40].

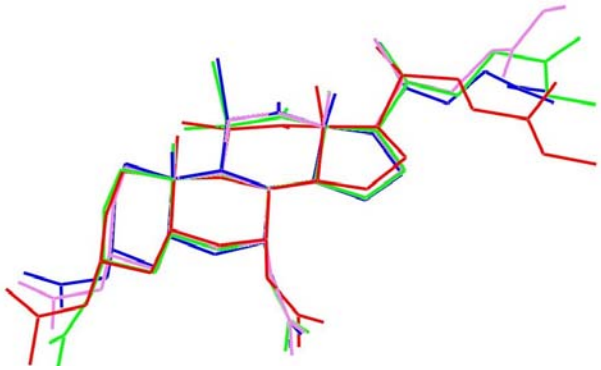


Figure 16. The overlap of molecules **21** (pink), **22** (red), **23** (blue) and **24** (green).

The best fit of the four steroids **21**, **22**, **23** and **24** (Figure 16) reveals the large conformational differences, maximum seen in the side chains at C-3 and C-17. It was note worthy to see some amount of flexibility upon bulky substitution at C-11 in the steroid skeleton itself, which is normally taken as a rigid framework.

Table 3. Crystal data for compounds **21**, **22**, **23** and **24**.

Crystal data	**21**	**22**	**23**	**24**
Molecular Formula	$C_{29}H_{44}O_7$	$C_{29}H_{43}BrO_7$	$C_{29}H_{43}BrO_7$	$C_{29}H_{42}Br_2O_7$
Molecular Mass	504.64	583.54	583.54	662.45
Crystal Size, mm	0.45x0.05x0.03	0.58x0.36x0.17	0.99x0.24x0.04	0.87x0.45x0.12
Temp. (K)	297(2)	297(2)	297(2)	297(2)
Crystal system	Monoclinic	Orthorhombic	Monoclinic	Monoclinic
Space group	C2	$P2_12_12_1$	$P2_1$	$P2_1$
a [Å]	27.845(15)	10.059(2)	10.575(11)	8.825(5)
b [Å]	6.284(4)	11.392(3)	6.541(7)	11.778(6)
c [Å]	18.143(10)	25.629(6)	20.83(2	15.053(8)
α [°]	90	90	90	90
β [°]	122.219(8)	90	97.966(18)	101.755(8)
γ [°]	90	90	90	90
V [$Å^3$]	2686(3)	2936.9(12)	1427(3)	1531.8(13)
Z	4	4	2	2
F(000)	1096	1232	616	684
d calc [g cm^{-3}]	1.248	1.320	1.358	1.436
μ [mm^{-1}]	0.088	1.442	1.484	2.688
Absorption correction	multi-scan	multi-scan	multi-scan	multi-scan
T_{min}	0.9616	0.4884	0.3212	0.2038
T_{max}	0.9972	0.7916	0.9430	0.7351
Reflns. Collected	12738	14791	10137	10530
Unique reflns.	4690	5145	4796	5087
Observed reflns.	2969	4059	3274	4290
Index range	$-32 \Rightarrow h \Rightarrow 32$ $-7 \Rightarrow k \Rightarrow 7$ $-21 \Rightarrow l \Rightarrow 21$	$-5 \Rightarrow h \Rightarrow 11$ $-12 \Rightarrow k \Rightarrow 13$ $-30 \Rightarrow l \Rightarrow 30$	$-12 \Rightarrow h \Rightarrow 12$ $-7 \Rightarrow k \Rightarrow 7$ $-24 \Rightarrow l \Rightarrow 24$	$-10 \Rightarrow h \Rightarrow 10$ $-13 \Rightarrow k \Rightarrow 13$ $-17 \Rightarrow l \Rightarrow 17$
R_1 [$I>2\sigma(I)$]	0.0768	0.0540	0.0630	0.0383
WR_2	0.1703	0.1429	0.1623	0.0898
Goodness-of-fit on F^2	1.003	1.017	0.994	0.990
$\Delta \rho_{max}$, $\Delta \rho_{min}$ (e $Å^{-3}$)	-0.218, 0.276	-0.369, 0.690	-0.346, 0.531	-0.235, 0.363
CCDC number	676282	676283	676284	676285

Table 4. Crystal data for compounds **26**, **27** and **38**.

Crystal data	**26**	**27**	**38**
Molecular Formula	$C_{29}H_{43}N_3O_7$	$C_{29}H_{43}N_3O_7$	$C_{29}H_{45}NO_7$
Molecular mass	545.66	545.66	519.66
Crystal size, mm	0.83x0.08x0.07	0.77x0.24x0.08	0.86x0.15x0.08
Temp. (K)	293(2)	293(2)	293(2)
Crystal system	Monoclinic	Monoclinic	Monoclinic
Space group	$P2_1$	$P2_1$	$P2_1$
a [Å]	6.469(3)	6.470(2)	6.314(4)
b [Å]	16.108(8)	16.307(6)	16.180(9)
c [Å]	14.206(7)	14.081(5)	14.035(9)
α [°]	90	90	90
β [°]	99.118(9)	98.487(6)	98.733(17)
γ [°]	90	90	90
V [$Å^3$]	1461.5(13)	1469.2(8)	1417.2(14)
Z	2	2	2
F(000)	588	588	564
d calc [g cm^{-3}]	1.240	1.233	1.218
μ [mm^{-1}]	0.088	0.088	0.086
Absorption correction	multi-scan	multi-scan	multi-scan
T_{min}	0.9298	0.9352	0.9301
T_{max}	0.9936	0.9931	0.9929
Reflns. Collected	13935	7374	11909
Unique reflns.	5125	4698	4992
Observed reflns.	3779	4206	4166
Index range	$-7 \Rightarrow h \Rightarrow 7$ $-19 \Rightarrow k \Rightarrow 19$ $-16 \Rightarrow l \Rightarrow 16$	$-6 \Rightarrow h \Rightarrow 7$ $-19 \Rightarrow k \Rightarrow 16$ $-14 \Rightarrow l \Rightarrow 16$	$-7 \Rightarrow h \Rightarrow 7$ $-19 \Rightarrow k \Rightarrow 19$ $-16 \Rightarrow l \Rightarrow 16$
R_1 [$I>2\sigma(I)$]	0.0494	0.0455	0.0542
WR_2	0.1077	0.1072	0.1190
Goodness-of-fit on F^2	1.004	1.038	1.099
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e $Å^{-3}$)	-0.213, 0.243	-0.171, 0.254	-0.167, 0.239
CCDC number	253328	253329	253330

Table 5. Bonds lengths (Å) and dihedral angles (˚) for intermolecular interactions.

Compound	D–H…A	d(D–H)	d(H…A)	d(D…A)	<(D–H…A)
21	C(3)-H(3B)…O(2)[a]	0.98	2.61	3.522(7)	155.1
	C(28)-H(28B)…O(4)[b]	0.96	2.92	3.309(7)	105.5
	C(15)-H(15A)…O(4)[b]	0.97	2.73	3.558(6)	143.7
	C(29)-H(29B)…O(4)[c]	0.96	2.55	3.412(8)	149.1
	C(29)-H(29A)…O(1)[d]	0.96	2.82	3.732(8)	159.0
	C(18)-H(18C)…O(5)[e]	0.96	2.63	3.398(6)	137.5
	C(19)-H(19B)…O(5)[e]	0.96	2.52	3.439(6)	159.2
	C(8)-H(8B)…O(5)[e]	0.98	2.46	3.415(6)	165.1
22	C(26)-H(26A)…Br(1)[f]	0.96	3.15	3.856(6)	132.0
	C(28)-H(28B)…O(5)[g]	0.96	2.74	3.477(6)	134.4
	C(29)-H(29B)…O(2)[g]	0.96	2.81	3.748(12)	165.2
	C(26)-H(26C)…O(6)[g]	0.96	2.66	3.483(15)	144.5
	C(19)-H(19A)…O(6)[h]	0.96	2.77	3.628(14)	149.0
	C(5)-H(5)…O(6)[h]	0.98	2.79	3.734(12)	160.8
	C(2)-H(2A)…O(7)[i]	0.97	2.82	3.601(8)	138.5
	C(21)-H(21A)…O(1)[i]	0.96	2.70	3.651(7)	172.0
	C(8)-H(8B)…O(4)[j]	0.98	2.57	3.358(6)	137.6
	C(6)-H(6A)…O(4)[j]	0.97	2.78	3.518(7)	133.1
23	C(26)-H(26C)…O(2)[k]	0.96	2.68	3.503(10)	143.9
	C(28)-H(28C)…O(7)[l]	0.96	2.76	3.687(16)	161.3
	C(28)-H(28A)…O(6)[m]	0.96	2.71	3.22(2)	114.1
	C(15)-H(15A)…O(6)[m]	0.97	2.56	3.393(19)	144.1
	C(29)-H(29A)…O(4)[n]	0.96	2.74	3.270(17)	115.1
	C(23)-H(23A)…O(4)[o]	0.97	2.60	3.476(17)	151.0
	C(3)-H(3B)…O(2)[p]	0.98	2.44	3.366(9)	158.5
	C(19)-H(19B)…O(5)[q]	0.96	2.74	3.698(10)	172.5
	C(8)-H(8B)…O(5)[q]	0.98	2.47	3.382(9)	154.4
24	C(4)-H(4B)…O(6)[r]	0.97	2.82	3.704(9)	152.6
	C(6)-H(6A)…O(6)[r]	0.97	2.73	3.630(9)	155.2
	C(14)-H(14A)…Br(2)[s]	0.98	3.11	4.032(4)	157.7
	C(28)-H(28A)…O(5)[s]	0.96	2.46	3.198(6)	134.0
	C(15)-H(15B)…Br(1)[t]	0.97	2.99	3.920(5)	161.4
	C(20)-H(20B)…O(4)[k]	0.98	2.80	3.647(6)	144.9
	C(23)-H(23A)…O(2)[u]	0.97	2.58	3.330(10)	134.1
	C(26)-H(26B)…O(4)[v]	0.96	2.51	3.461(8)	171.2
	C(26)-H(26C)…O(6)[w]	0.96	2.73	3.376(9)	125.3

Symmetry codes: [a] -x+1/2, y-1/2, -z; [b] -x+1/2, y+1/2, -z+1; [c] x-1/2, y+1/2, z; [d] x-1/2, y-1/2, z; [e] x, y-1, z ; [f] -x+1, y+1/2, -z+3/2; [g] x-1/2, -y+1/2, -z+2; [h] -x+3/2, -y+1, z-1/2; [i] x+1/2, -y+1/2, -z+2; [j] x+1/2, -y+3/2, -z+2 ; [k] -x + 2, y+1/2, -z; [l] x+1, y-1, z; [m] -x+1, y-1/2, -z+1; [n] -x+1, y+3/2, -z+1; [o] x-1, y+1, z; [p] -x+2, y-1/2, -z; [q] x, y-1, z [r] x, y, z+1; [s] -x+1, y-1/2, -z; [t] x+1, y, z; [u] x, y, z-1; [v] x-1, y, z; [w] x-1, y, z+1.

B1.4. Bioevaluation

Based on the previous report by Marples and coworkers [11], in the present study the compounds synthesized from cholic acid series were subjected to biological screening against HIV-1 replication. To our surprise, compounds **25**, **26**, **27**, **38**, **44** and **45** induced host cell fusion during the progress of HIV-1 infection and produced multinucleated giant cells. They were found to enhance HIV-1 replication and increase the number of syncytia formed by HIV-1 infected T cells. The fold increase in the viral count was also found to be directly proportional to the syncytia formed in T-cells. This is the first report of syncytia formation and thereby enhancement of viral replication in HIV-1 infected T cells by bile acid derivative. Biological screening of the compounds synthesized from deoxychoilc acid series is in active progress.

HIV-1 induces host cell fusion during the progress of infection and form multinucleated giant cells (syncytia). Depending on the induction of syncytia, HIV-1 viral isolates are classified in to Syncytium Inducing (SI) and Non Syncytium Inducing (NSI) strains. Most of the T cell infecting HIV-1 viruses and dendritic cells are SI [42], where as Monocyte infecting HIV-1 viruses are NSI. HIV-1 induced syncytia is the primary cause of T lymphocyte cell death [43] and are characterized by high rate of cell-cell fusion events between infected and uninfected cells eventually leading to cell death. Syncytia are observed in *'in vitro'* cell culture and *'in vivo'* in lymph nodes (including the tonsils) and brain region in HIV-1 infected patients [44]. HIV-1 infected cells express gp120 envelope protein on their surface, which interact with CD4 receptor and co-receptors (CXCR4 and CCR5) on surface of uninfected bystander cells. This interaction leads to the fusion of both of these cells to form syncytium [42].

B1.4.1. ***Enhancement of syncytia formation and viral replication***

CEM-GFP cells were infected with HIV-1_{NL4-3} virus at 0.1 MOI and cells were cultured in 24 well plates. Cholic acid analogues were tested for their effect on viral replication at similar, non-cytotoxic concentration of 1 μg/mL. We have tested the HIV-1 released in to the culture supernatants of HIV-1 infected CEM-GFP cells on day 5-post infection by p24 antigen ELISA, which is a sensitive method to quantitate HIV-1. Cholic acid analogues **25**, **26**, **27**, **38**, **44** and **45** have enhanced HIV-1 replication during the HIV-1 infection, where as cholic acid **18** and compound **20** did not increase viral replication (Figure 17C). About 1.5 to 2.5 fold increase in p24 levels was observed with syncytia inducing compounds. Visualization of progress of infection using GFP fluorescence under fluorescence microscope also indicated an increase in syncytia formation and large sized syncytia when compare to the DMSO treated controlled-infected cells. This property of increase in syncytia is proportional with increase in virus production in the respective treatments of cholic acid derivatives **25**, **26**, **27**, **38**, **44** and **45**. Highest density of syncytia was observed with compound **45** (Figure 17A and 17B), which also enhanced maximum viral replication.

For quantification of HIV-1 mediated syncytia, HIV-$1_{NL4.3}$ infected CEM-GFP cells were fixed on Day 5 post infection and DNA is stained with Propidium Iodide and the number of syncytia formed was quantitated in density plots using forward scatter (FSC) versus DNA content (FL-2), obtained by flow cytometry as described previously [45] (Figure 17B). In mock-infected controls, single nucleus-containing cells (R1 region) represent Gap-1 (G-1) phase cells and two nuclei containing cell populations (R2 region) represent cells in DNA Synthesis (S), Gap-2 (G-2) or Mitosis (M) phases of the cell cycle

were clearly separated. In HIV-1 infected cells, multinucleated syncytia having increased size and greater than 2N DNA content fall in R3 region (Figure 17B).

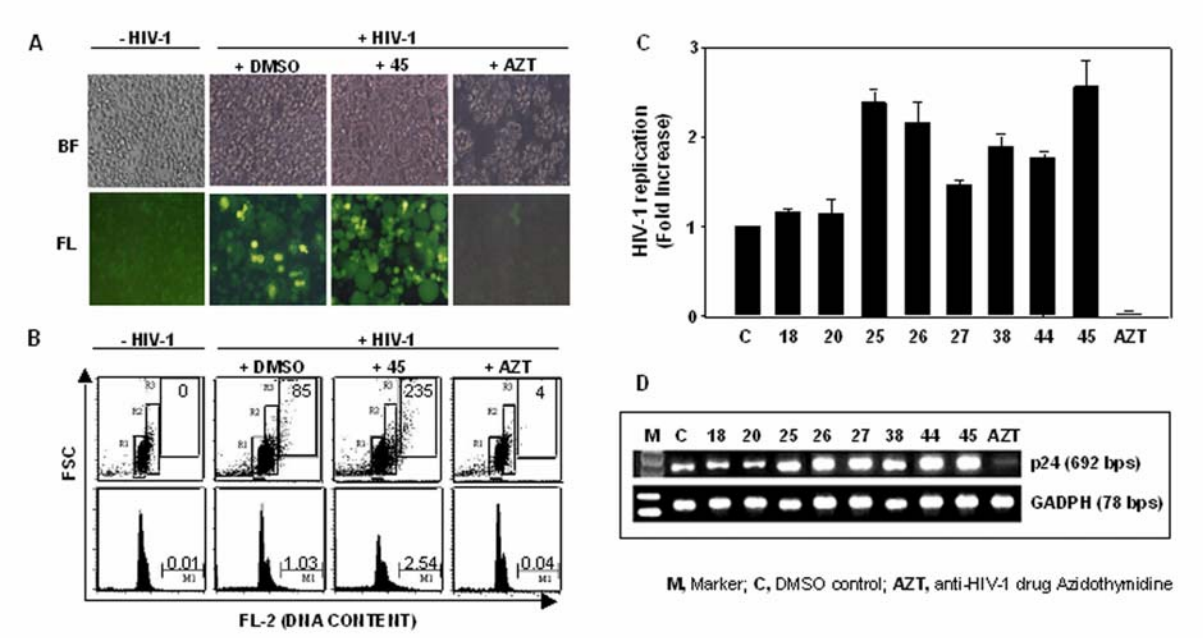


Figure 17A. Cholic acid derivative (45) induces syncytia formation in HIV-1 infected CEM-GFP cells as visualized by Fluorescence microscopy. Bright Field (BF) and Fluorescence (FL) microscopic images of un-infected (-HIV-1), infected (+HIV-1) CEM-GFP cells in control (+DMSO), compound **45** (+ **45**) and anti-HIV-1 drug AZT (+AZT) treatments.

Figure 17B. Quantification of HIV-1 induced syncytia. Syncytia are quantified in uninfected (-HIV-1), infected (+HIV-1) control (+DMSO), compound **45** (+ **45**) and anti-HIV-1 drug AZT (+ AZT) treated CEM-GFP cells by Flow cytometry as described in Experimental Section.

Figure 17C. Effect of cholic acid derivatives on HIV-1 replication in HIV-1_{NL4-3} infected CEM-GFP cells. HIV-1 released in to the culture supernatants of DMSO treated (**C**), and cholic acid derivatives **18**, **20**, **25-27**, **38**, **44** and **45** treated (**18**, **20**, **25**, **26**, **27**, **38**, **44** and **45**) and anti-HIV-1 drug Azidothymidine (**AZT**). **C**, 1.00±0.00; **18**, 1.14±0.04; **20**, 1.14±0.16; **25**, 2.39±0.09; **26**, 2.16±0.22; **27**, 1.46±0.04; **38**, 1.89±0.1; **44**, 1.76±0.05; **45**, 2.56±0.28; **AZT**, 0.02±0.06.

Figure 17D. RT-PCR analysis of viral p24 expression in Cholic acid derivative treated HIV-1 infected CEM-GFP cells. p24 transcripts were amplified by RT-PCR as described in experimental section. Lanes: **M**, Marker; **C**, DMSO control; **18**, **20**, **25**, **26**, **27**, **38**, **44** and **45**, Cholic acid analogues; **AZT**, anti-HIV-1 drug Azidothymidine treatments. GAPDH is used as internal control.

When we compared the syncytia formed in cholic acid analogues treated HIV-1 infected CEM-GFP cells with DMSO treated cells, we observed further increase in number of syncytia in case of compounds **25**, **26**, **27**, **38**, **44** and **45** treated HIV-1 infected CEM-GFP cells, where as in case of compound **18** and **20** treated infected cells, very little or no increase in the number of syncytia were observed (Table 6). In Figure 17A and 17B, compound **45** treated HIV-1 infected cells were compared with DMSO treated and anti-HIV-1 drug AZT treated cells as it had shown more number of syncytia as well as large sized syncytia among all the compounds tested. Finally, increase in viral gene expression was monitored by reverse transcription-polymerase chain reaction (RT-PCR), using RNA isolated from infected cells treated with various compounds. The RT-PCR data (Figure 17D) clearly correlates with syncytia formation (Table 6) and p24 ELISA results (Figure 17C) showing increased p24 expression in compounds **25**, **26**, **27**, **38**, **44** and **45** treatments but very little or no increase in compounds **18** and **20** treatments.

Table 6. Enhancement of syncytia formation due to treatment with cholic acid derivatives.

Treatment	No. of syncytia
DMSO	85
18	69
20	92
25	214
26	136
27	108
38	170
44	173
45	235
Inophyllum B	2
AZT	4

Our primary object was to understand the relationship between molecular structure and biological activity. From Figure 17 and Table 6 it is clear that the fold increase in the viral count is directly proportional to the syncytia formed in T-cells. The 11β-amino-12β-

hydroxy compound **45** is inducing the maximum viral replication in T-cells followed by 11-imino-12-oxo compound **25**, 11α-azido-12-oxo compound **26**, 11α-amino-12-oxo compound **38**, 11α-amino-12α-hydroxy compound **44** and 11β-azido-12-oxo compound **27** whereas cholic acid **18** and diacetoxy methyl cholate **20** are almost acting as control samples. Looking at the current set of data and comparing the structural features of compounds **18**, **20** and **25-27**, **38**, **44**, **45** with respect to the biological activity, it is very much clear that C-11 fuctionalization by amine or azide functionality is responsible for the observed enhancement of viral replication with induction of syncytium formation.

B1.5. Summary

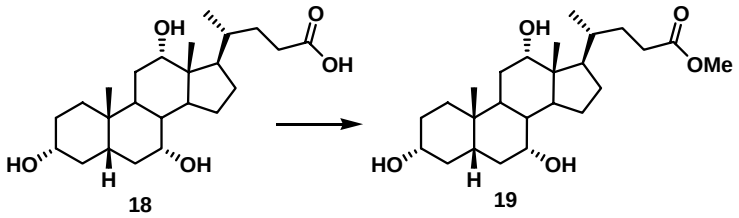
Synthesis of difficultly accessible C-11 functionalized bile acid analogues namely, methyl 11-amino-3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate **25**; methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **38**; methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **26**; methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **27**; methyl 11α-amino-3α,7α-diaetoxy-12α-hydroxy-5β-cholan-24-oate **44**; and methyl 11β-amino-3α,7α-diaetoxy-12β-hydroxy-5β-cholan-24-oate **45** have been achieved in good to excellent yields. Mechanistic aspects for the decomposition of steroidal azidoketones to its enamines are studied and established for the first time. We have demonstrated that 11α-bromo compound **22** on treatment with NaN_3 is converted into 11β-azido compound **27**. In the presence of base, compound **27** epimerized to the thermodynamically more stable 11α-azido compound **26**. Compound **26** on further treatment with NaN_3 under drastic conditions is transformed into the steroidal enamine **25**. The crystal structures of these molecules were resolved to study the effect of bromo/azido/amino functionalization in the steroid skeleton of

cholic acid with different stereo-chemical orientations at C-11 on the two-dimensional arrangement of molecules and solid-state properties.

Surprisingly these compounds enhanced HIV-1 replication with induction of syncytia formation. The fold increase in the viral load was also found to be proportional to the increase in syncytia formation. As syncytium is formed by cell-cell fusion, it will be interesting to study the molecular basis of this induction. The syncytia inducing property of cholic acid analogues may also be useful for screening the efficacy of compounds which inhibit syncytia induction and also for screening the efficacy of the existing and novel anti-retroviral drugs by artificial enhancement of HIV-1 replication and syncytia formation.

B1.6. Experimental Section

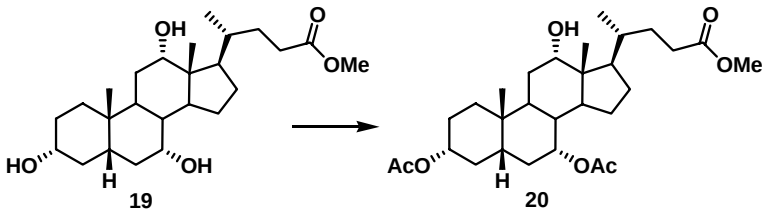
B1.6.1. *Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (19):*



To a solution of cholic acid (0.3 g, 0.74 mmol) in dry methanol (10 mL) was added *p*-TSA (0.03 g, 0.17 mmol). The mixture was allowed to stand at 28 °C for 24 hrs. Methanol was evaporated and the residue was extracted with CH_2Cl_2 (3x50 mL). The organic extract was washed with cold H_2O (2x10 mL), 10 % $NaHCO_3$ (2x10 mL), brine (2x10 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (5 %, $CH_3OH/CHCl_3$) afforded compound **19** (0.3 g, 98 %) as a white foamy solid. Mp. 157-158 °C; $[\alpha]_D^{28}$ + 31.33 (*c* 1.0, $CHCl_3$); IR ν_{max} (Nujol) 3670, 1739 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.65 (s, 3H), 0.86

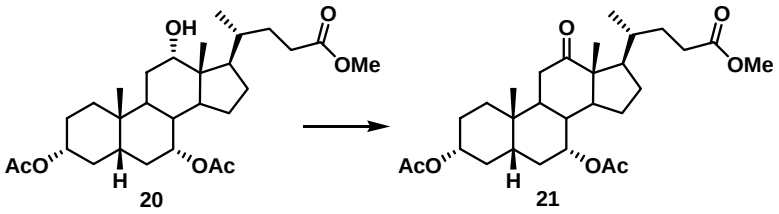
(s, 3H), 0.95 (d, *J* = 6 Hz, 3H), 3.43 (m, 1H), 3.64 (s, 3H), 3.82 (bs, 1H), 3.94 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 17.7, 22.8, 23.6, 26.6, 27.9, 28.5, 31.2, 31.4, 31.5, 35.0, 35.2, 35.7, 35.8, 39.8, 39.9, 41.9, 42.0, 46.8, 47.3, 51.8, 68.8, 72.2, 73.4, 175.2; Anal. Calcd. for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 70.93; H, 10.36.

B1.6.2. ***Methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate (20):***



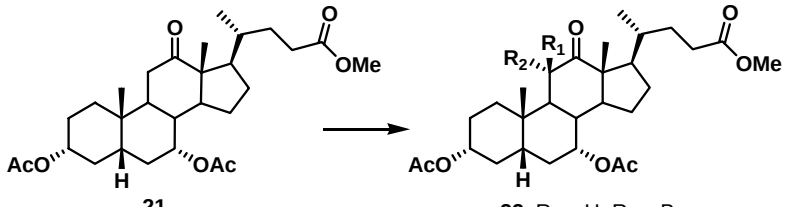
To a solution of methyl ester **19** (0.211 g, 0.5 mmol), DMAP (0.07 g, 0.06 mmol) and acetic anhydride (0.11 g, 1.05 mmol) in dry CH_2Cl_2 (10 mL) was added Et_3N (0.22 g, 2.13 mmol) at 0 °C. The reaction mixture was allowed to stir at 0-28 °C for 4-5 hrs. The residue was extracted with CH_2Cl_2 (3x50 mL). The organic extract was washed with cold H_2O (2x10 mL), 5 % cold HCl (2x10 mL), 10 % $NaHCO_3$ (2x10 mL), brine (2x10 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (25 %, EtOAc/PE) afforded compound **20** (0.232 g, 92 %) as a white crystalline solid. Mp. 185-188 °C; $[\alpha]_D^{28}$ + 20.78 (*c* 0.56, $CHCl_3$); IR ν_{max} (Nujol) 3678, 1746, 1735 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.66 (s, 3H), 0.90 (s, 3H), 0.95 (d, *J* = 6.0 Hz, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 3.64 (s, 3H), 3.98 (bs, 1H), 4.56 (m, 1H), 4.87 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.4, 17.2, 21.3, 21.4, 22.8, 22.9, 26.6, 27.2, 27.8, 28.4, 31.0, 31.2, 34.5, 34.7, 34.8, 35.0, 38.0, 40.9, 42.0, 46.5, 47.6, 51.3, 70.8, 72.5, 74.0, 170.5, 174.5; Anal. Calcd. for $C_{29}H_{46}O_7$: C, 68.74; H, 9.15. Found: C, 68.53; H, 9.33.

B1.6.3. ***Methyl 3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (21):***



Compound **20** (0.506 g, 1.0 mmol) in acetone (20 mL) was stirred with Jones Reagent (1 mL) at 5-10 °C for 5 min. Methanol (5 mL) was added after 5 min, the solvent was evaporated and the crude solid material was dissolved in EtOAc/H_2O (5:1) mixture (100 mL). The organic layer was washed with cold H_2O (2x10 mL), 10 % $NaHCO_3$ (2x10 mL), brine (2x10 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (20 %, EtOAc/PE) afforded compound **21** (0.49 g, 98 %) as a white crystalline solid. Mp. 175-176 °C (EtOAc); IR ν_{max} (Nujol) 1737, 1730, 1701 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.86 (d, *J* = 5.8 Hz, 3H), 1.03 (s, 3H), 1.04 (s 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.52 (t, *J* = 12 Hz, 1H), 3.66 (s, 3H), 4.57 (m, 1H), 4.98 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.4, 18.4, 21.0, 22.0, 23.6, 26.5, 27.7, 30.2, 31.1, 31.2, 34.3, 34.4, 35.4, 37.7, 37.8, 40.5, 46.4, 51.2, 53.0, 57.0, 70.4, 73.4, 169.4, 170.3, 174.2, 213.3; MS (LCMS) *m/z*: 527.3 $[M+Na]^+$; HRESIMS *m/z* 527.3005 $[M+Na]^+$ ($C_{29}H_{44}NaO_7$; calcd. 527.2985). Anal. Calcd. for $C_{29}H_{44}O_7$: C, 69.02; H, 8.79. Found: C, 69.34; H, 8.57.

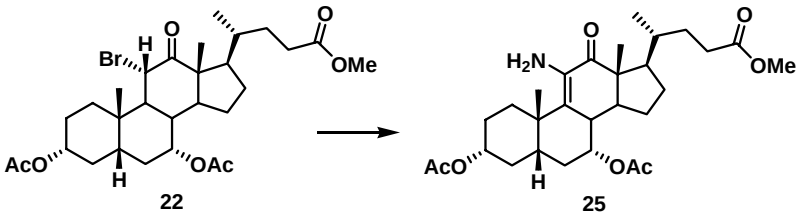
B1.6.4. ***Methyl 11α-bromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (22), Methyl 11β-bromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (23), Methyl 11,11-dibromo-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (24):***



To a solution of **21** (1.008 g, 2 mmol) in benzene (10 mL), a bromine solution (1 mL, 2M in benzene) was slowly added with stirring, at 30 °C in the dark. After 6 days, TLC analysis showed the total consumption of the starting material. The solvent was evaporated and the crude solid material was dissolved in EtOAc (150 mL). The organic layer was washed with 10 % $Na_2S_2O_5$ (2x10 mL), cold H_2O (2x10 mL), brine (2x10 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. The residue was chromatographed on flash silica gel (10 %, EtOAc/PE) to yield the dibromo compound **24** (0.05 g, 4 %). Mp. 181-182 °C (EtOAc); $[\alpha]_D^{25}$ + 40.00 (*c* 1.1, $CHCl_3$); IR (KBr) ν_{max} 2962, 1743, 1731, 1716 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.02 (d, *J* = 6.3 Hz, 3H), 1.37 (s, 3H), 1.54 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 3.04 (bd, *J* = 15.3 Hz, 3Hz), 3.38 (d, *J* = 11.4 Hz, 1H), 3.67 (s, 3H), 4.67 (m, 1H), 4.97 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 15.6, 18.4, 21.4, 21.4, 24.0, 26.6, 27.0, 29.7, 30.4, 31.1, 31.3, 34.5, 35.5, 36.2, 39.6, 40.3, 46.3, 49.0, 49.5, 51.5, 54.1, 54.7, 71.4, 73.6, 74.8, 169.9, 170.7, 174.5, 194.4; MS (LCMS) *m/z*: 685.1 $[M+Na]^+$; HRESIMS *m/z* 685.1152 $[M+Na]^+$ ($C_{29}H_{42}Br_2NaO_7$; calcd. 685.1174). Anal. Calcd. for $C_{29}H_{42}Br_2O_7$: C, 52.58; H, 6.39. Found: C, 52.60; H, 6.04. On further elution with the same solvent system furnished β-bromo compound **23** (0.22 g, 19 %). Mp. 183-184 °C

(EtOAc); $[\alpha]_D^{25}$ + 19.20 (*c* 1.25, $CHCl_3$); IR (KBr) ν_{max} 2937, 1739, 1730, 1697 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.93 (d, *J* = 6.6 Hz, 3H), 1.36 (s, 3H), 1.38 (s, 3H), 2.03 (s, 6H), 2.64 (dd, *J* = 11.7 & 5.9 Hz, 1H), 3.67 (s, 3H), 4.42 (d, *J* = 5.9 Hz, 1H), 4.60 (m, 1H), 5.03 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.5, 18.1, 21.1, 21.2, 23.7, 24.6, 26.9, 27.3, 30.4, 30.6, 31.1, 33.7, 34.6, 35.6, 35.8, 36.9, 39.9, 43.8, 47.4, 51.3, 52.0, 52.2, 56.3, 70.6, 73.1, 169.6, 170.3, 174.3, 203.4; MS (LCMS) *m/z* 605.2 $[M+Na]^+$. Followed by α-bromo compound **22** (0.76 g, 65 %). Mp. 202-203 °C (EtOAc); $[\alpha]_D^{25}$ + 43.3 (*c* 1.20, $CHCl_3$); IR (KBr) ν_{max} 2962, 1743, 1731, 1712 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.85 (d, *J* = 5.8 Hz, 3H), 1.04 (s, 3H), 1.22 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 2.79 (dd, *J* = 10.7 Hz, 1Hz), 2.90 (dt, *J* = 15.4 Hz & 3.0 Hz, 1H), 3.66 (s, 3H), 4.64 (m, 1H), 4.97 (bs, 1H), 5.01 (d, *J* = 10.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.7, 17.9, 20.8, 22.3, 23.7, 26.6, 27.5, 29.8, 30.6, 31.3, 34.5, 35.0, 36.5, 37.5, 39.5, 41.7, 46.1, 47.6, 50.8, 55.9, 57.6, 70.3, 72.9, 169.4, 170.0, 173.8, 202.3; MS (LCMS) *m/z* 605.2 $[M+Na]^+$. {A stereoselective high-yield bromination of compound **21** to compound **23** was also carried out by the procedure as reported [25] by Yanuka *et al*}.

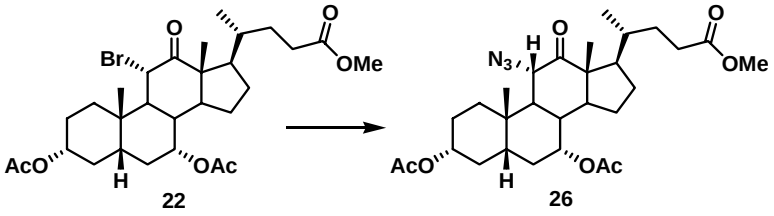
B1.6.5. ***Methyl 11-amino-3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate (25):***



To a solution of compound **22** (0.15 g, 0.257 mmol) in dry DMF (5 mL) was added solid sodium azide (0.2 g, 3.1 mmol). The reaction mixture was stirred at 100 °C for 48 h and it was poured into crushed ice. It was extracted with EtOAc (3x50 mL), washed with cold

water (2x25 mL), brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (1 % CH_3OH/CH_2Cl_2) produced compound **25** as a yellowish solid (0.096 g, 72 %). Compound **25** was also synthesized from methyl 11α-azido-3α,7α-diacetoxy-12-keto-5β-cholan-24-oate **26**. To a solution of compound **26** (0.1 g, 0.183 mmol) in dry DMF (5 mL) was added solid sodium azide (0.12 g, 1.83 mmol). The reaction mixture was then stirred at 100 °C for 36 h. This was further worked up and purified as described above to yield compound **25** (0.064 g, 67 % yield). Mp. 65-68 °C; $[\alpha]_D^{25}$ + 20.00 (*c* 2.5, $CHCl_3$); IR ν_{max} (Nujol) 3500, 3365, 2923, 1730, 1685, 1604 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.97 (s, 3H), 1.02 (d, *J* = 6.1 Hz, 3H), 1.31 (s, 3H), 2.05 (s, 6H), 3.66 (s, 3H), 3.93 (m, 2H), 4.72 (m, 1H), 5.02 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.3, 19.2, 21.3, 21.3, 23.7, 25.5, 26.6, 27.4, 30.0, 30.5, 31.3, 32.0, 35.2, 35.6, 40.5, 40.7, 42.6, 45.9, 47.8, 51.3, 51.6, 70.6, 73.0, 126.6, 135.3, 170.3, 170.4, 174.5, 202.4; MS (LCMS) *m/z* 519.0 $[M+H]^+$, 541.0 $[M+Na]^+$. Anal. Calcd. for $C_{29}H_{45}NO_7$: C, 67.27; H, 8.39; N, 2.71, Found: C, 66.96; H, 8.56; N, 2.74.

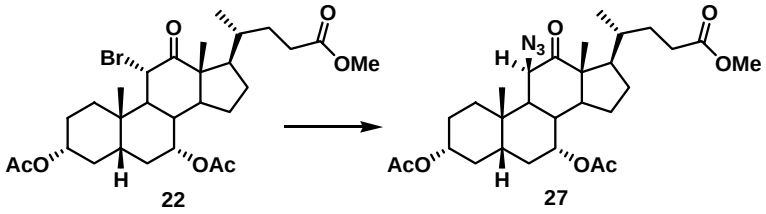
B1.6.6. ***Methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (26):***



To a solution of compound **22** (0.1 g, 0.172 mmol) in dry DMF (5 mL) was added solid sodium azide (0.056 g, 0.86 mmol). The reaction mixture was stirred at 60 °C for 16 h and allowed to cool to room temperature. It was then poured into H_2O (50 mL) and extracted with Et_2O (3x50 mL). The organic extract was washed with cold water (2x25 mL) followed

by brine (20 mL) and it was dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (10 % EtOAc/PE) afforded compound **26** (0.092 g, 98 %) as a white crystalline solid. Compound **26** was also synthesized from methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **27**. To a solution of compound **27** (0.05 g, 0.092 mmol) in dry DMF (2 mL) was added solid sodium azide (0.007 g, 0.11 mmol) or potassium acetate (0.011 g, 0.11 mmol). The reaction mixture was then stirred at 60 °C for 9 h for the complete conversion of β-azide to α-azide (TLC). This was worked up and purified as described above to yield compound **26** in quantitative yield. Mp. 213 °C (CH_2Cl_2/PE); $[\alpha]_D^{28}$ + 61.68 (*c* 1.07, $CHCl_3$); IR ν_{max} (Nujol) 2922, 2108, 1740, 1722 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (d, *J* = 6.4 Hz, 3H), 1.02 (s, 3H), 1.15 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.48 (dt, *J* = 14.6 Hz & 3.0 Hz, 1H), 3.66 (s, 3H), 4.06 (d, *J* = 10.8 Hz, 1H), 4.58 (m, 1H), 4.96 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.7, 18.3, 21.2, 21.2, 22.7, 23.9, 27.0, 27.4, 30.1, 30.9, 31.4, 35.2, 35.2, 37.0, 37.2, 37.9, 41.6, 42.9, 47.0, 51.3, 51.7, 56.1, 64.4, 70.4, 73.4, 169.8, 170.6, 174.1, 207.0; Anal. Calcd. for $C_{29}H_{43}N_3O_7$ C, 63.81; H, 7.96; N, 7.70 Found: C, 63.68; H, 7.91; N, 7.63.

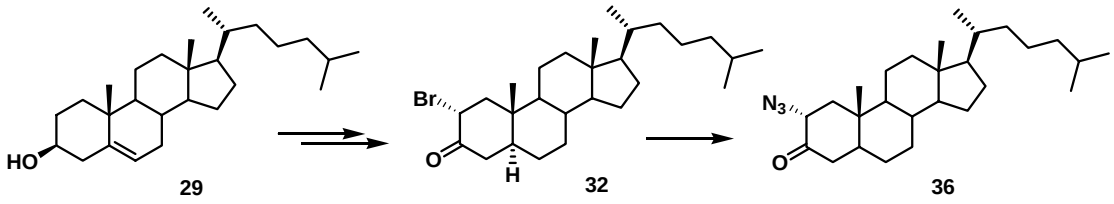
B1.6.7. ***Methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (27):***



To a solution of compound **22** (0.18 g, 0.3 mmol) in dry DMF (10 mL) was added solid sodium azide (0.023 g, 0.36 mmol). The reaction mixture was then stirred at 60 °C for 4 h. It was then diluted with H_2O (50 mL) and extracted with Et_2O (3x50 mL). The organic layer

was washed with cold water (2x20 mL), brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to give 0.17 g of crude product. Purification by column chromatography on silica gel (10 % EtOAc/PE), furnished compound **27** (0.105 g, 64 %) as white solid. Mp. 181 °C (CH_2Cl_2/PE); $[\alpha]_D^{27}$ + 98.61 (*c* 1.44, $CHCl_3$); IR ν_{max} (Nujol) 2910, 2108, 1738, 1715 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.92 (d, *J* = 6.3 Hz, 3H), 1.25 (s, 3H), 1.26 (s, 3H), 2.03 (s, 6H), 3.67 (s, 3H), 4.14 (d, *J* = 5.5 Hz, 1H), 4.60 (m, 1H), 5.01 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.3, 18.7, 21.3, 21.3, 23.6, 25.2, 27.0, 27.1, 30.4, 30.8, 31.2, 34.3, 34.6, 35.4, 35.5, 36.0, 39.3, 43.1, 47.2, 51.4, 52.7, 56.4, 69.0, 70.7, 73.3, 169.8, 170.4, 174.4, 206.7; MS (LCMS) *m/z* 546.02 $[M+H]^+$; Anal calcd. for $C_{29}H_{43}N_3O_7$ C, 63.81; H, 7.96; N, 7.70 Found: C, 63.83; H, 8.04; N, 7.24.

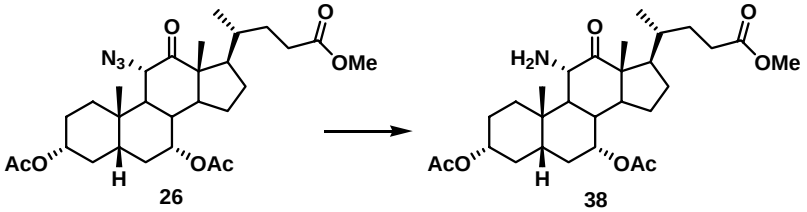
B1.6.8. ***2α-Bromocholestan-3-one (32) and 2α-Azidocholestan-3-one (36):***



2α-Bromocholestan-3-one **32** was synthesized from cholesterol **29** using the literature procedures [29] in three steps with an overall 87 % yield. To a solution of **32** (0.465 g, 1.0 mmol) in dry DMF (20 mL) was added solid sodium azide (0.078 g, 1.2 mmol). The reaction mixture was then stirred at 28 °C for 2 h. It was then diluted with H_2O (50 mL) and extracted with Et_2O (3x50 mL). The organic layer was washed with cold water (2x20 mL), brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to give 0.43 g of crude product. Purification by column chromatography on silica gel (60-120 Mesh, 10 %

EtOAc/PE), furnished compound **36** (0.38 g, 89 %) as white solid. Spectroscopic data was consistent with that reported in the literature [29].

B1.6.9. ***Methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (38):***

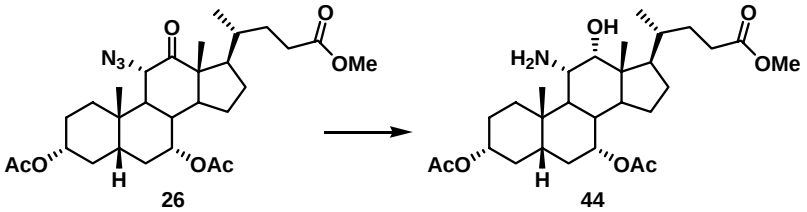


Method A: Methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **26** (0.25 g, 0.46 mmol) in EtOAc (15 mL) was hydrogenated at 28 °C and 40 psi pressure using 10 % Pd/C (0.025 g) for 5 h. After filtration of the catalyst and evaporation of the solvent, afforded compound **38** (0.224 g, 95 %) as white solid.

Method B: Compound **38** was also synthesized from compound **26** using Staudinger type of reaction. A solution of **26** (0.11 g, 0.2 mmol) was stirred with triphenylphosphine (0.08 g, 0.3 mmol) in dry THF (5 mL) for 24 h. Water (0.1 mL) was added and the solvent was removed after additional 24 h. After dilution with EtOAc (100 mL), the organic layer was separated, washed with water (2x20 mL), brine (20 mL), dried and solvent was evaporated under reduced pressure. Chromatography of the residue on alumina (10 % EtOAc/PE) afforded compound **38** (0.08 g, 80 %) as white solid. Mp. 154 °C (EtOAc/PE); $[\alpha]_D^{28}$ + 61.84 (*c* 1.52, $CHCl_3$); IR ν_{max} (Nujol) 3389, 2954, 1731, 1714 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.85 (d, *J* = 6.5 Hz, 3H), 1.01 (s, 3H), 1.19 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.27 (m, 1H), 2.40 (m, 1H), 2.72 (dt, *J* = 14.1 Hz & 3.0 Hz, 1H), 3.66 (s, 3H), 3.76 (d, *J* = 10.0 Hz, 1H), 4.64 (m, 1H), 4.94 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.0, 18.5, 21.4, 21.4, 23.1, 24.3, 27.4, 28.0, 30.4, 31.2, 31.8, 35.4, 35.4, 37.6, 37.8, 38.5, 42.2, 46.0, 47.3, 51.4,

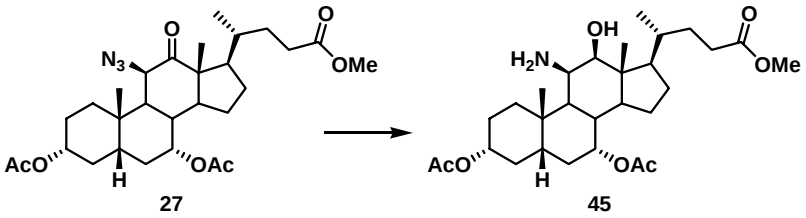
52.3, 56.0, 57.0, 71.0, 74.0, 170.1, 170.6, 174.5, 214.1; MS (LCMS) *m/z* 520.03 $[M+H]^+$; Anal calcd. for $C_{29}H_{45}NO_7$ C, 67.01; H, 8.74; N, 2.69 Found: C, 66.77; H, 9.14; N, 2.72.

B1.6.10. ***Methyl 11α-amino-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate (44):***



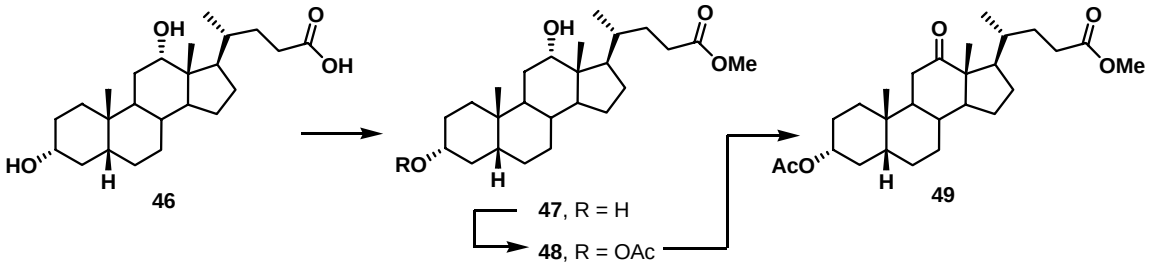
Azido compound **26** (0.1 g, 0.18 mmol) was dissolved in a mixture of CH_3OH (2 mL) and CH_2Cl_2 (1 mL). To this $CoCl_2 \cdot 6H_2O$ (0.004 g, 0.018 mmol) in water (1 mL) and cetyltrimethylammonium bromide (CTAB, 0.01 g, 10 %) was added and reaction mixture was stirred for 15 min. at room temperature. Finally solid $NaBH_4$ (0.02 g, 0.54 mmol) was added slowly in fractions for the period of 5 min. The whole reaction mixture was stirred for 30 min. at room temperature. Water (10 mL) was added and the reaction mixture was extracted with EtOAc (3x25 mL), washed with water (2x25 mL) and brine (1x20 mL). The organic extract was dried over Na_2SO_4 and evaporated in vacuum. The residue was chromatographed on the column of neutral deactivated alumina (CH_2Cl_2-CH_3OH, 20:1) to afford 0.082 g (86 %) of compound **44** as colorless foam; IR ν_{max} (Nujol) 3433, 3361, 1735 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.67 (s, 3H), 0.99 (d, *J* = 6 Hz, 3H), 1.08 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 3.44 (bs, 1H), 3.63 (m, 1H), 3.67 (s, 3H), 4.64 (m, 1H), 4.86 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 11.4, 17.0, 21.2, 21.4, 23.0, 23.3, 27.0, 27.4, 29.1, 29.5, 30.8, 31.4, 34.9, 35.5, 37.7, 38.5, 40.5, 42.7, 45.7, 47.4, 50.6, 51.3, 53.0, 70.9, 73.8, 77.3, 170.3, 170.4, 174.5; MS *m/z* 522.32 $[M+H]^+$; Anal. Calcd for $C_{29}H_{47}NO_7$: C, 66.77; H, 9.08; N, 2.69. Found: C, 66.39; H, 9.38; N, 3.10.

B1.6.11. ***Methyl 11β-amino-3α,7α-diacetoxy-12β-hydroxy-5β-cholan-24-oate (45):***



Compound **45** was synthesized using the similar procedure as described for compound **44**. Crude amino alcohol was subjected to column chromatographic purification to afford pure compound **45** as a foamy solid (87 mg, 91 %); IR ν_{max} (Nujol) 3421, 3357, 1733 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.73 (s, 3H), 1.05 (d, *J* = 6 Hz, 3H), 1.21 (s, 3H), 2.04 (s, 3H), 2.06 (s, 3H), 3.30 (m, 1H), 3.67 (s, 3H), 3.75 (bs, 1H), 4.62 (m, 1H); 4.99 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 10.5, 20.6, 21.4, 21.6, 22.9, 23.4, 27.0, 29.4, 29.7, 30.7, 32.2, 32.4, 33.1, 34.5, 34.8, 35.1, 37.4, 43.9, 47.3, 48.7, 51.6, 53.5, 58.0, 70.9, 73.6, 77.2, 170.1, 170.6, 175.0; MS *m/z* 522.32 $[M+H]^+$; Anal. Calcd for $C_{29}H_{47}NO_7$: C, 66.77; H, 9.08; N, 2.69. Found: C, 66.55; H, 9.06; N, 2.76.

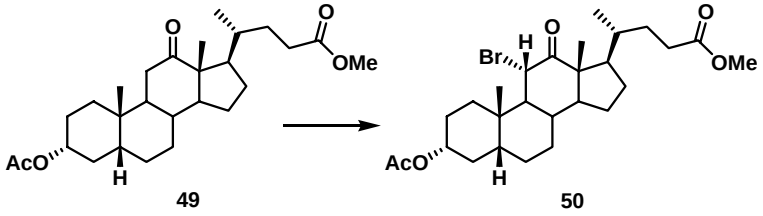
B1.6.12. ***Methyl 3α,12α-dihydroxy-5β-cholan-24-oate (47), Methyl 3α-acetoxy-12α-hydroxy-5β-cholan-24-oate (48) and Methyl 3α-acetoxy-12-oxo-5β-cholan-24-oate (49):***



Compound **49** was synthesized from deoxycholic acid **46** using the similar procedures as reported earlier for cholic acid series in three steps with an overall 84 % yield. Compound **47**, IR ν_{max} (Nujol) 3678, 1744 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.65 (s, 3H), 0.88 (s,

3H), 0.95 (d, *J* = 6 Hz, 3H), 2.28 (m, 2H), 3.55 (m, 1H), 3.64 (s, 3H), 3.96 (bs, 1H), 3.94 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ12.6, 17.2, 23.0, 24.1, 26.1, 27.2, 27.3, 28.6, 29.9, 30.8, 30.9, 33.1, 33.2, 35.2, 35.2, 35.7, 36.4, 42.1, 46.1, 47.3, 48.1, 51.3, 71.6, 73.0, 174.6. Compound **48**, IR ν_{max} (Nujol) 3669, 1742 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.68 (s, 3H), 0.92 (s, 3H), 0.98 (d, *J* = 6 Hz, 3H), 2.02 (s, 3H), 2.31 (m, 2H), 3.67 (s, 3H), 3.99 (bs, 1H), 4.71 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ12.6, 17.2, 21.2, 23.0, 23.5, 26.0, 26.8, 27.0, 27.3, 28.6, 30.9, 31.0, 32.0, 33.6, 33.9, 34.7, 35.0, 36.0, 41.9, 46.5, 47.3, 48.2, 51.2, 72.9, 74.2, 170.4, 174.4. Compound **49**, Mp. 151 °C (CH_3OH); IR ν_{max} (Nujol) 1742, 1735, 1710 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.82 (d, *J* = 6 Hz, 3H), 1.00 (s, 6H), 2.00 (s, 3H), 2.51 (t, *J* = 12 Hz, 1H), 3.65 (s, 3H), 4.68 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.4, 18.2, 21.0, 22.4, 24.0, 25.7, 26.0, 26.6, 27.2, 30.2, 31.0, 31.8, 34.6, 35.0, 35.3, 37.8, 41.1, 43.7, 46.2, 51.1, 57.2, 58.3, 73.3, 170.1, 174.2, 214.1; MS *m/z* 446 $[M]^+$.

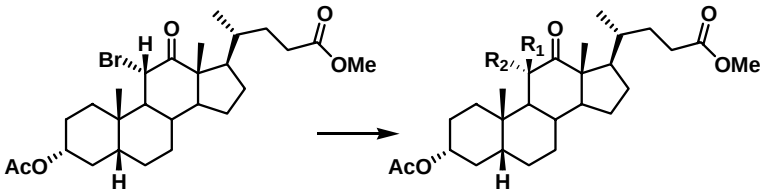
B1.6.13. ***Methyl 3α-acetoxy-11α-bromo-12-oxo-5β-cholan-24-oate (50):***



A stereoselective high-yield bromination of compound **49** to compound **50** was carried out by using the procedure as reported [25] by Yanuka *et al*. Mp. 158 °C; IR ν_{max} (Nujol) 1728, 1703 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (d, *J* = 6.8 Hz, 3H), 1.03 (s, 3H), 1.19 (s, 3H), 2.02 (s, 3H), 2.17-2.43 (m, 4H), 2.99 (m, 1H), 3.66 (s, 3H), 4.77 (m, 1H), 4.98 (d, *J* = 11 Hz, 1H); ^{13}C NMR (175 MHz, $CDCl_3$) δ 11.4, 18.5, 21.3, 23.1, 24.9, 26.6, 27.3, 27.6, 27.7, 30.4, 31.2, 33.1, 35.4, 37.2, 38.0, 38.1, 43.8, 48.2, 51.4, 51.6, 56.2, 57.1, 58.4, 73.8,

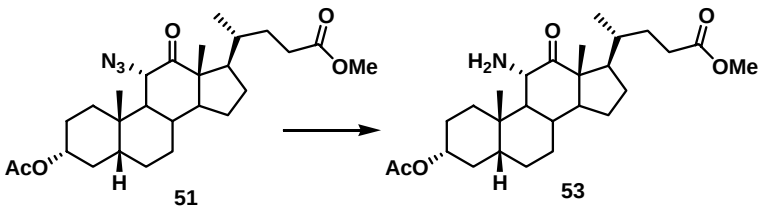
170.6, 174.5, 203.6; LCMS *m/z* 547.2 $[M+Na]^+$; Anal. Calcd for $C_{27}H_{41}BrO_5$: C, 61.71; H, 7.86. Found: C, 61.55; H, 7.93.

B1.6.14. ***Methyl 3α-acetoxy-11α-azido-12-oxo-5β-cholan-24-oate (51) and Methyl 3α-acetoxy-11β-azido-12-oxo-5β-cholan-24-oate (52):***



11-azido-12-oxo compounds **51** and **52** were synthesized from compound **50** using the similar procedures as reported for cholic acid analogues **26** and **27**, respectively. Compound **51**; IR ν_{max} (Nujol) 2104, 1726 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (d, *J* = 6.5 Hz, 3H), 1.02 (s, 3H), 1.14 (s, 3H), 2.02 (s, 3H), 2.14 (m, 1H), 2.27 (m, 1H), 2.40 (m, 1H), 2.51 (m, 1H), 3.67 (s, 3H), 3.99 (d, *J* = 11.0 Hz, 1H), 4.72 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.1, 18.4, 21.3, 23.2, 24.6, 26.0, 27.3, 27.4, 27.4, 30.3, 31.1, 32.8, 35.3, 35.8, 36.9, 37.4, 43.0, 47.1, 48.6, 51.4, 56.7, 56.7, 64.8, 73.7, 170.6, 174.4, 207.9; LCMS *m/z* 510.3 $[M+Na]^+$; Anal. Calcd for $C_{27}H_{41}N_3O_5$: C, 66.50; H, 8.47; N, 8.62 Found: C, 66.45; H, 8.33; N, 8.47. Compound **52**; IR ν_{max} (Nujol) 2089, 1730, 1703 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.91 (d, *J* = 5.8 Hz, 3H), 1.23 (s, 3H), 1.24 (s, 3H), 2.02 (s, 3H), 2.29 (m, 1H), 2.39 (m, 1H), 3.67 (s, 3H), 4.06 (d, *J* = 5.3 Hz, 1H), 4.72 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.4, 18.8, 21.3, 24.1, 25.6, 26.0, 26.6, 26.7, 27.1, 30.4, 31.3, 31.9, 32.3, 34.5, 35.5, 35.7, 43.8, 45.3, 47.4, 51.5, 57.0, 59.1, 69.3, 73.4, 170.5, 174.6, 207.7; LCMS *m/z* 510.3 $[M+Na]^+$; Anal. Calcd for $C_{27}H_{41}N_3O_5$: C, 66.50; H, 8.47; N, 8.62 Found: C, 66.41; H, 8.27; N, 8.53.

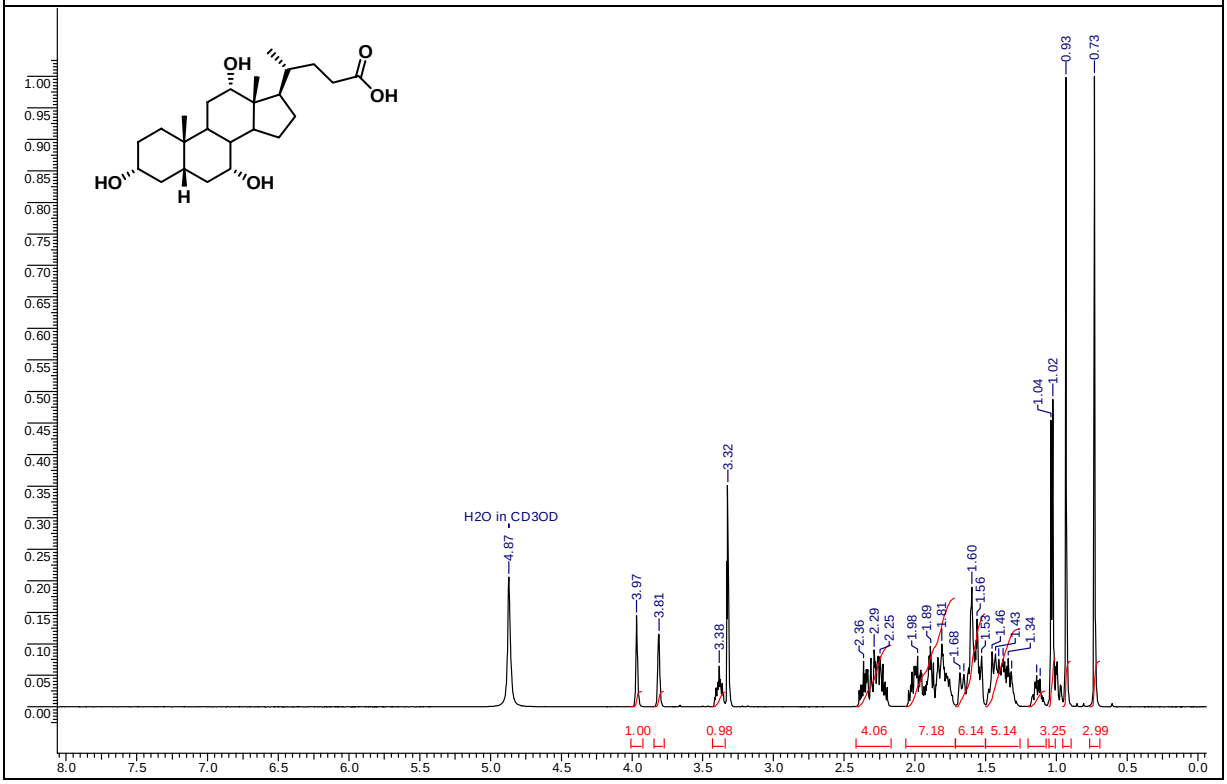
B1.6.15. ***Methyl 3α-acetoxy-11α-amino-12-oxo-5β-cholan-24-oate (53):***



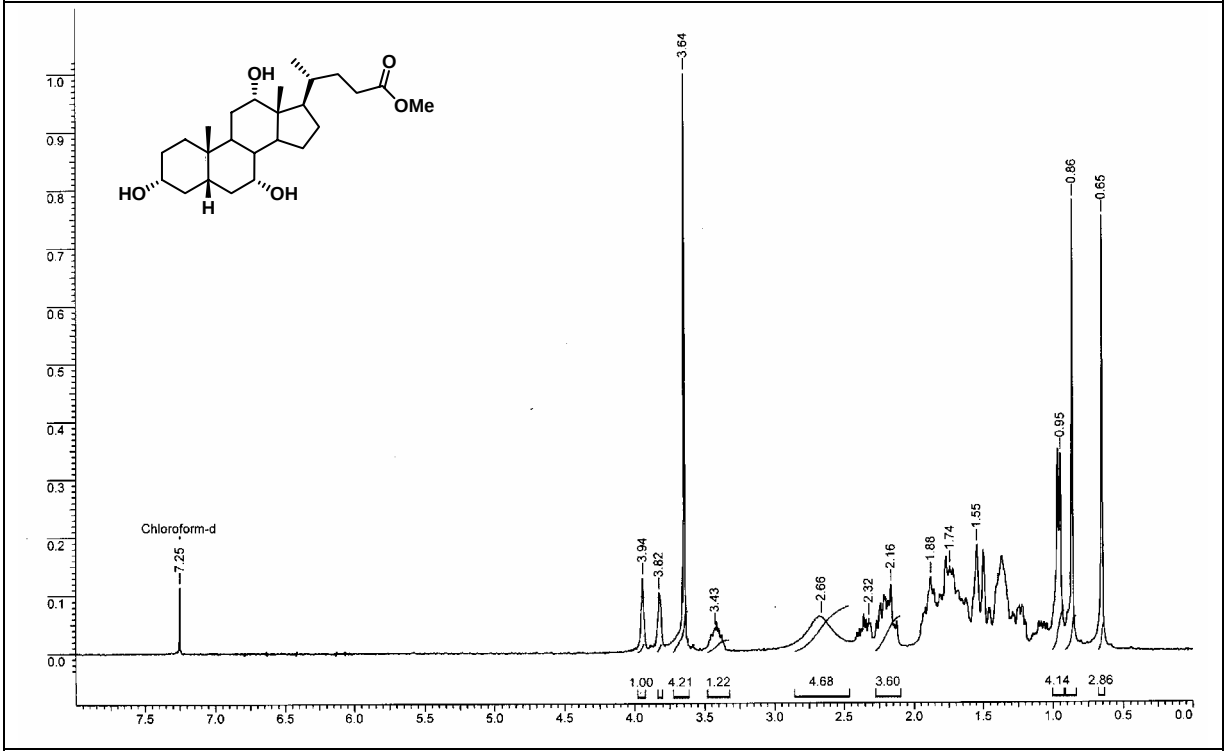
Compound **53** was synthesized from **51** using the similar procedure as reported for cholic acid analogue **38**. IR ν_{max} (Nujol) 3358, 2966, 1738, 1713 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.85 (d, *J* = 6.8 Hz, 3H), 1.01 (s, 3H), 1.18 (s, 3H), 2.01 (s, 3H), 2.10 (m, 1H), 2.26 (m, 1H), 2.40 (m, 1H), 2.82 (dt, *J* = 14.0 Hz & 3.0 Hz, 1H), 3.67 (s, 3H), 3.75 (d, *J* = 12.0 Hz, 1H), 4.76 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.1, 18.4, 21.2, 23.4, 24.8, 26.3, 27.3, 27.6, 27.6, 30.4, 31.1, 32.8, 35.4, 36.2, 37.3, 37.6, 43.4, 43.5, 47.3, 51.2, 56.3, 57.1, 57.4, 74.1, 170.4, 170.3, 214.5; MS (LCMS) *m/z* 461.3 $[M+H]^+$, $[M+Na]^+$; Anal calcd. for $C_{27}H_{43}NO_5$ C, 70.25; H, 9.39; N, 3.03 Found: C, 70.17; H, 9.52; N, 2.86.

B1.7. Selected Spectra

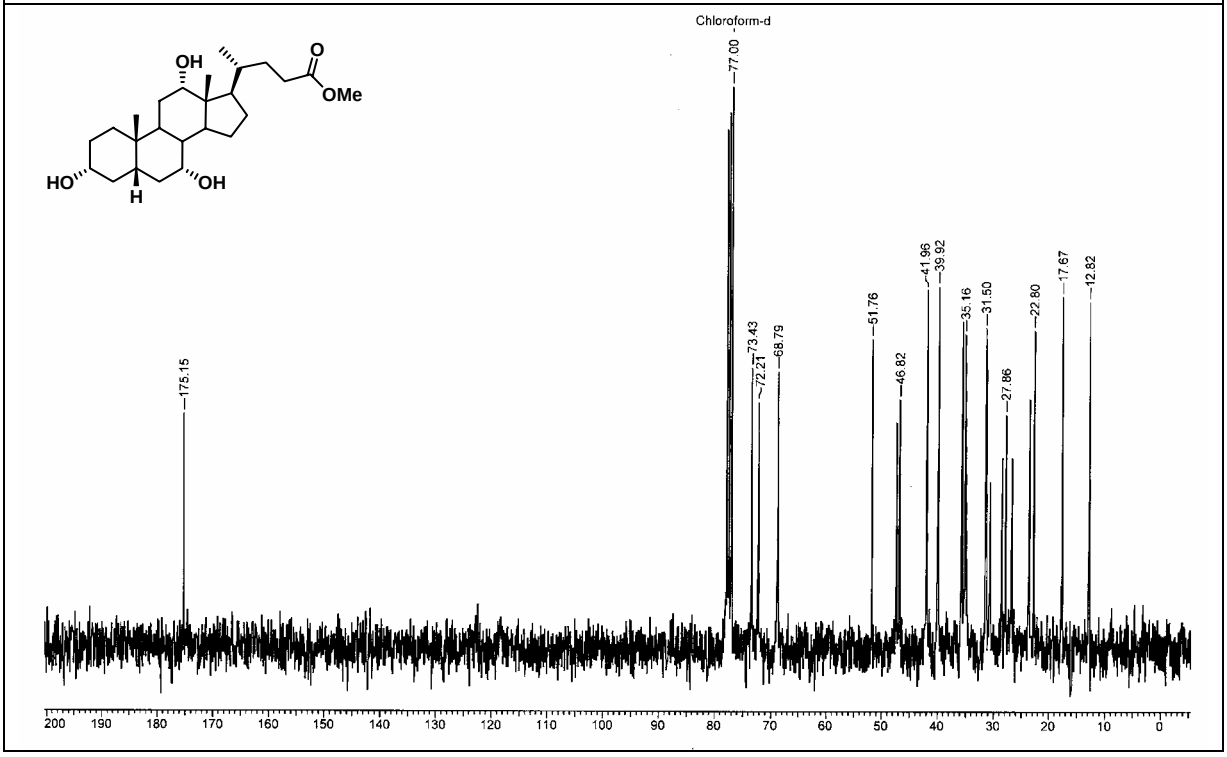
18: ^{1}H NMR, 500 MHz, CD_3OD



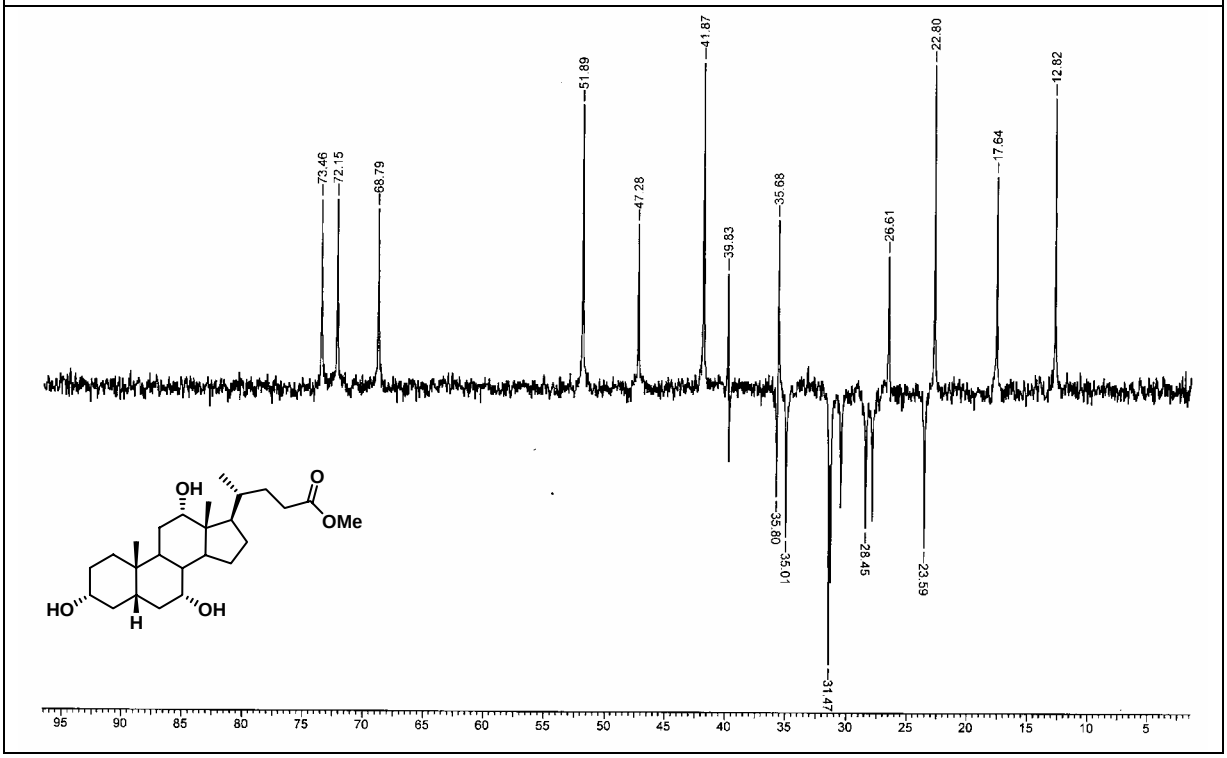
19: ^{1}H NMR, 300 MHz, $CDCl_3$



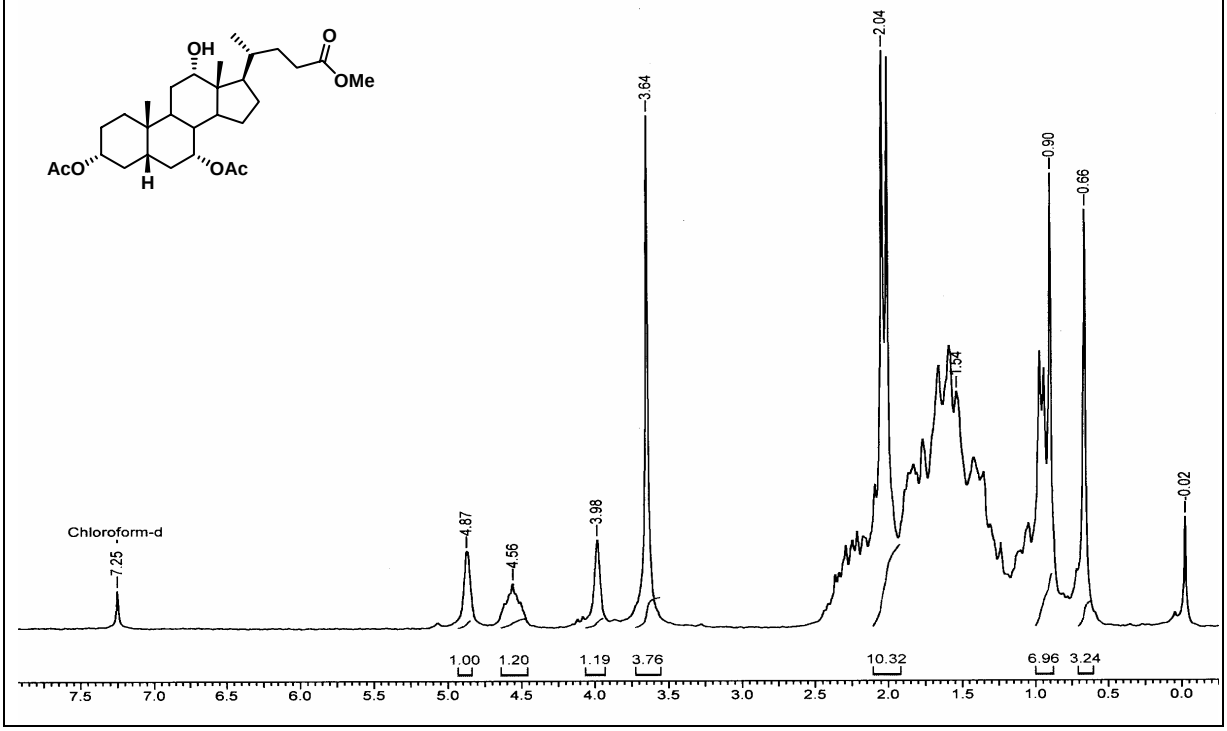
19: ^{13}C NMR, 75 MHz, $CDCl_3$



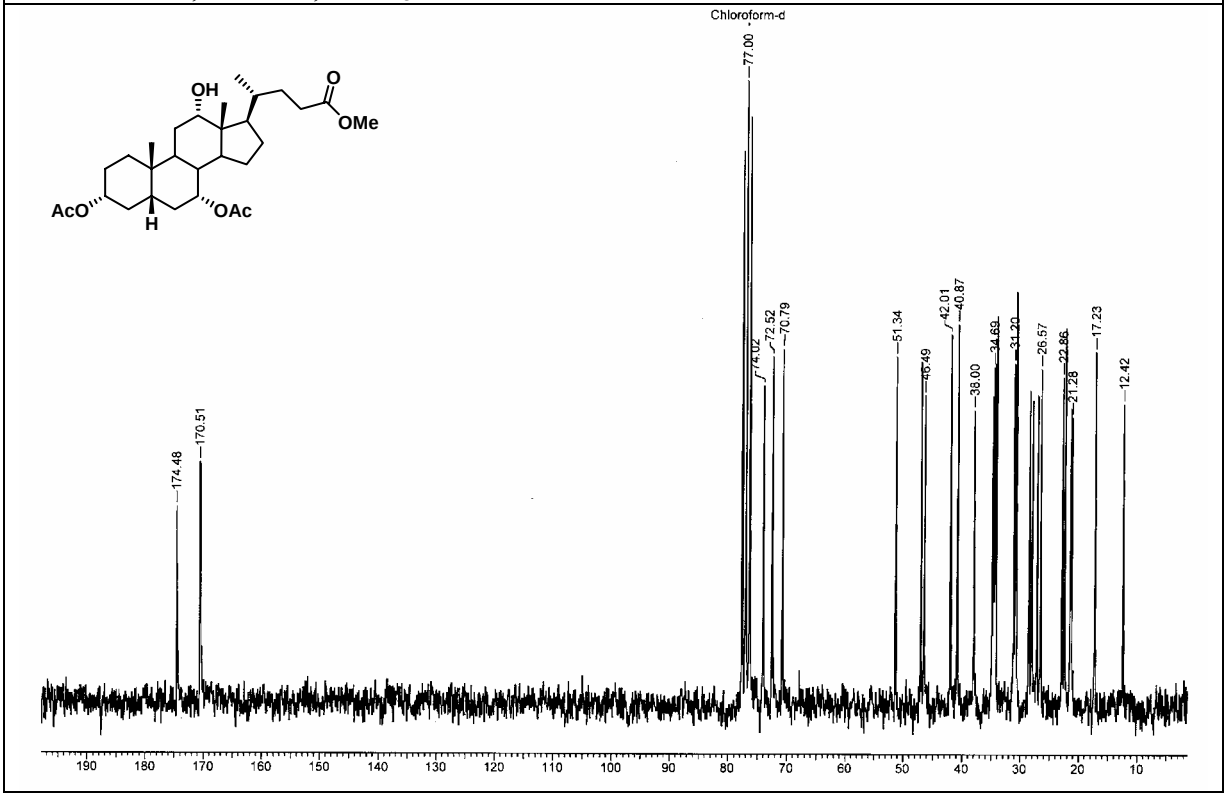
19: DEPT, 75 MHz, $CDCl_3$



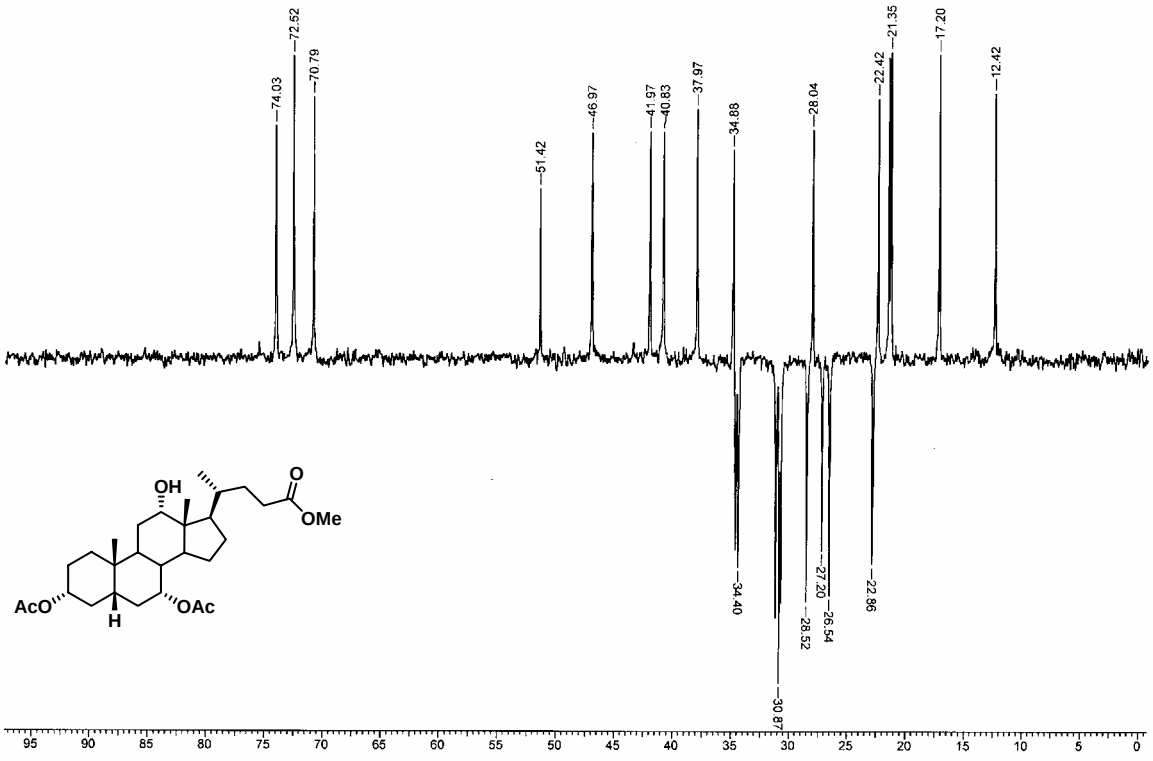
20: ^{1}H NMR, 200 MHz, $CDCl_3$



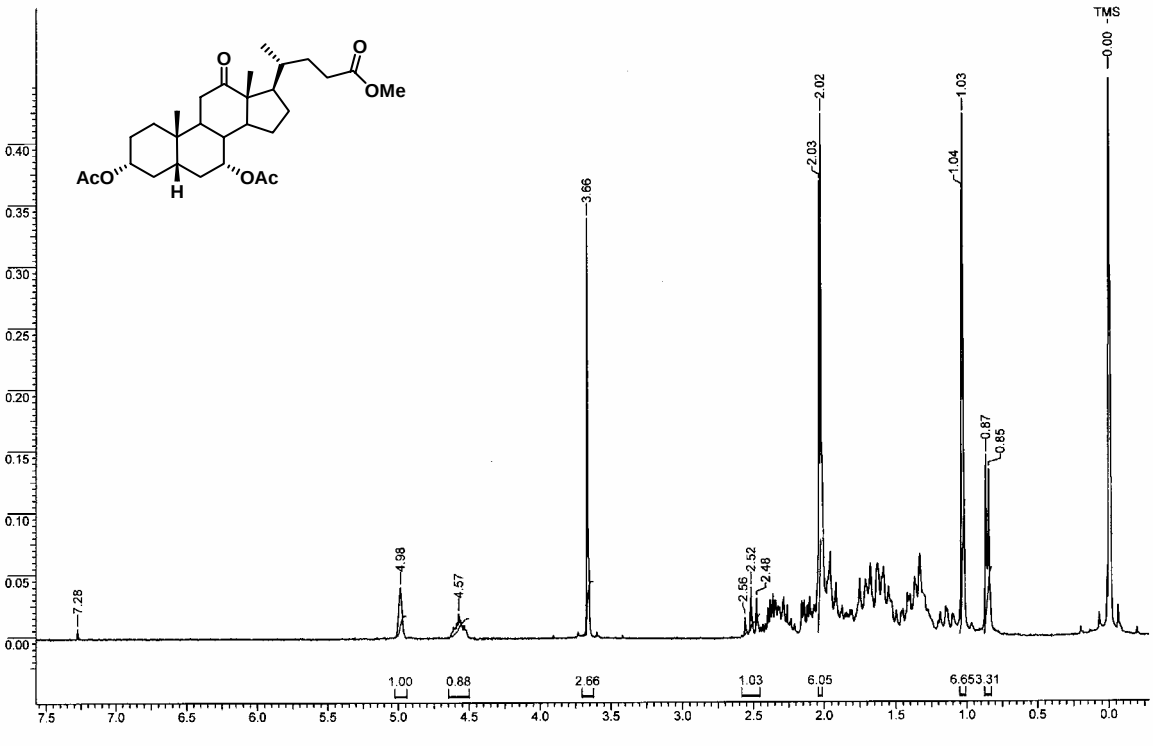
20: ^{13}C NMR, 50 MHz, $CDCl_3$



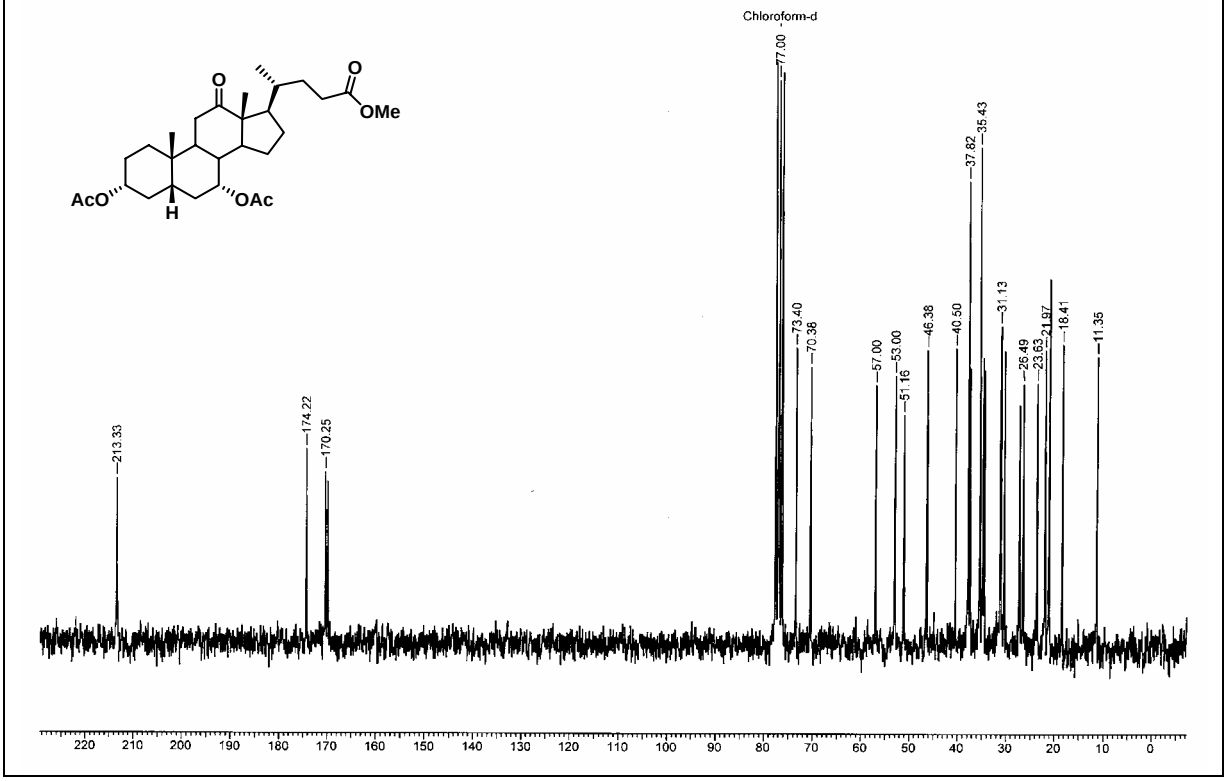
20: DEPT, 50 MHz, $CDCl_3$



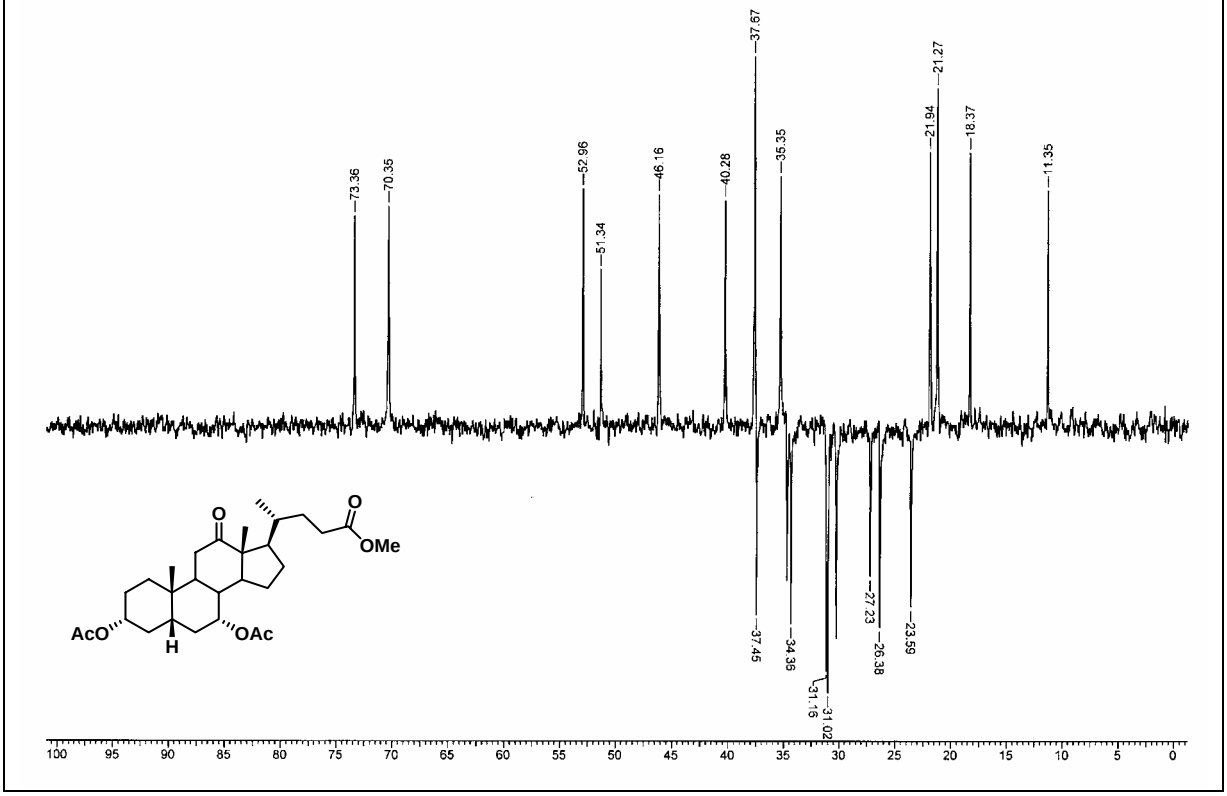
21: ^1H NMR, 300 MHz, $CDCl_3$



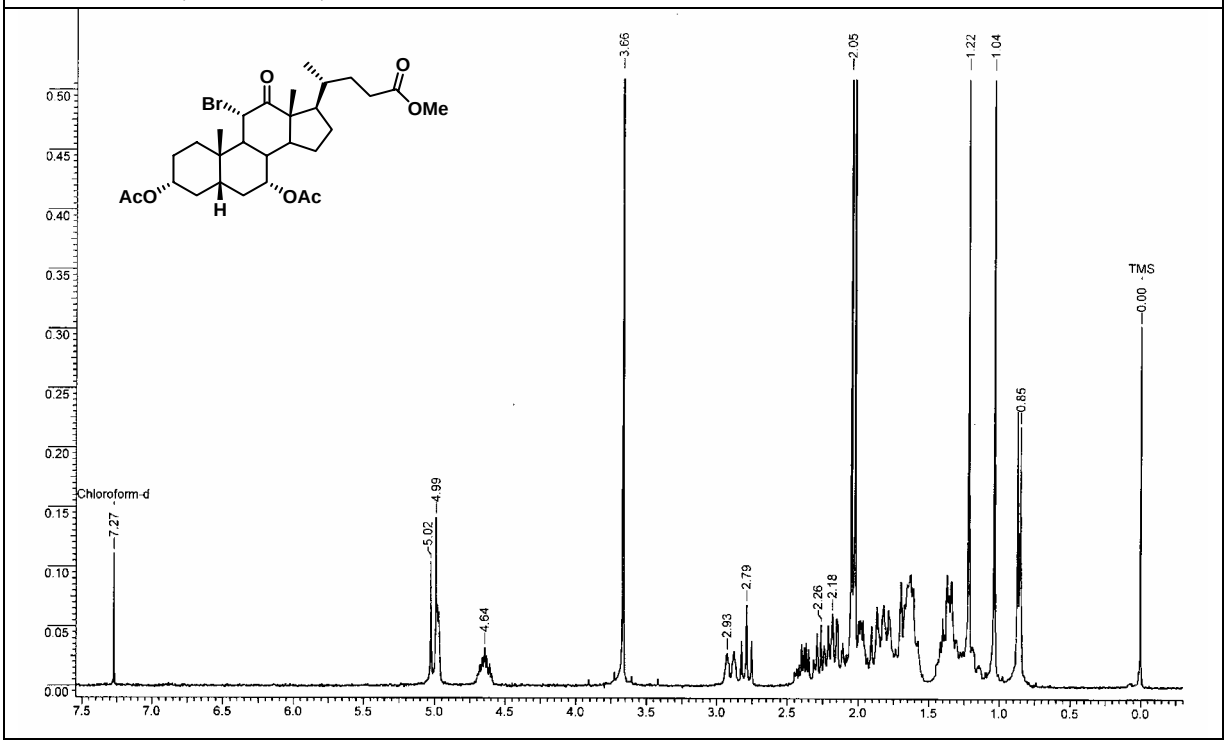
21: ^{13}C NMR, 75 MHz, $CDCl_3$



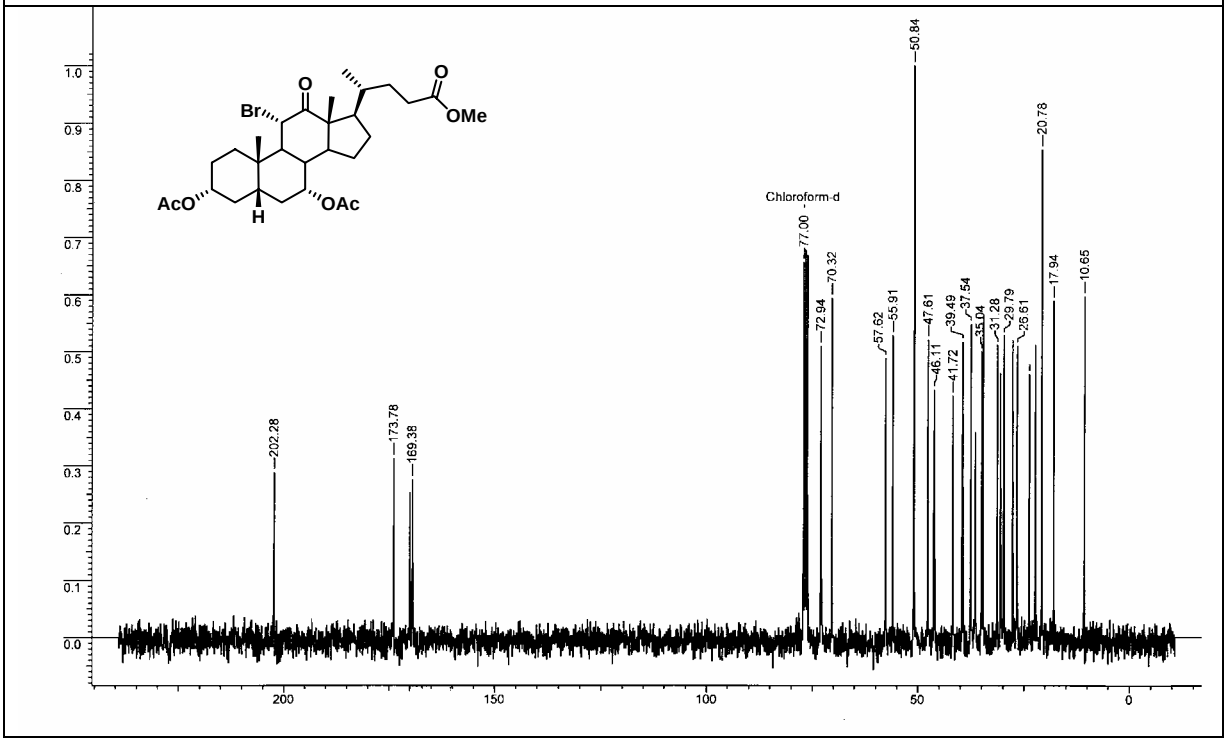
21: DEPT, 75 MHz, $CDCl_3$



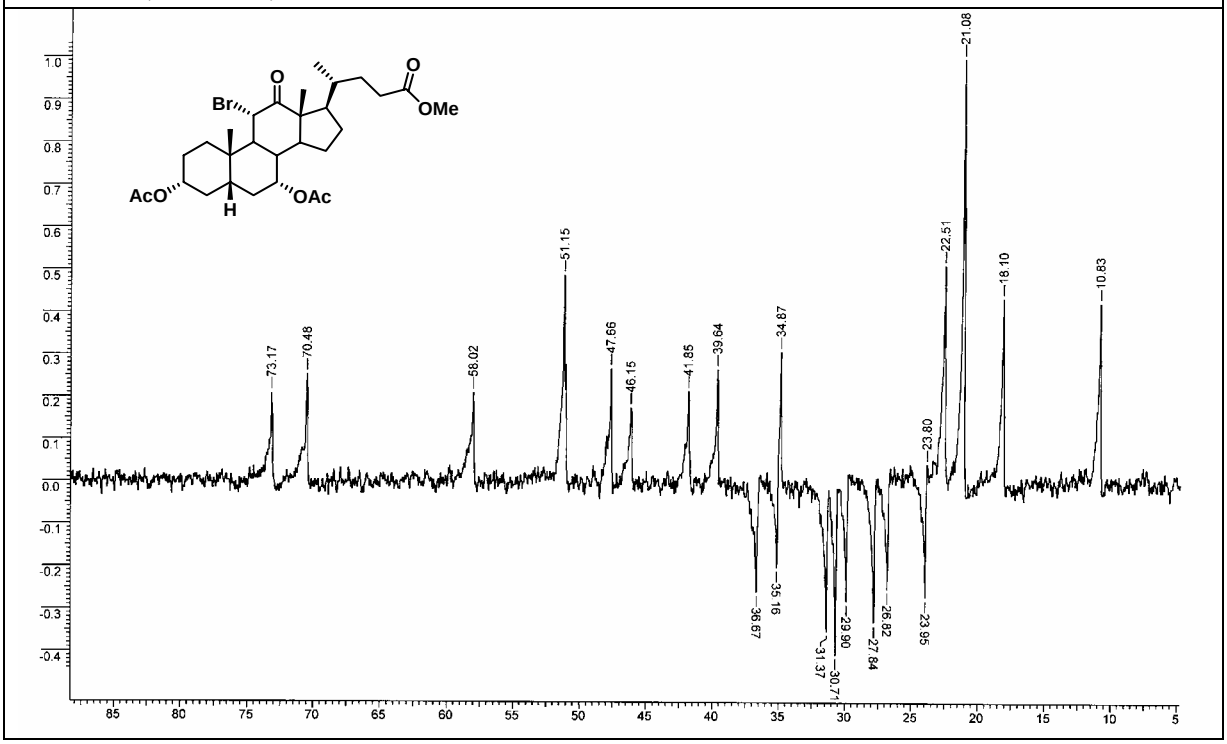
22: ^{1}H NMR, 300 MHz, $CDCl_3$



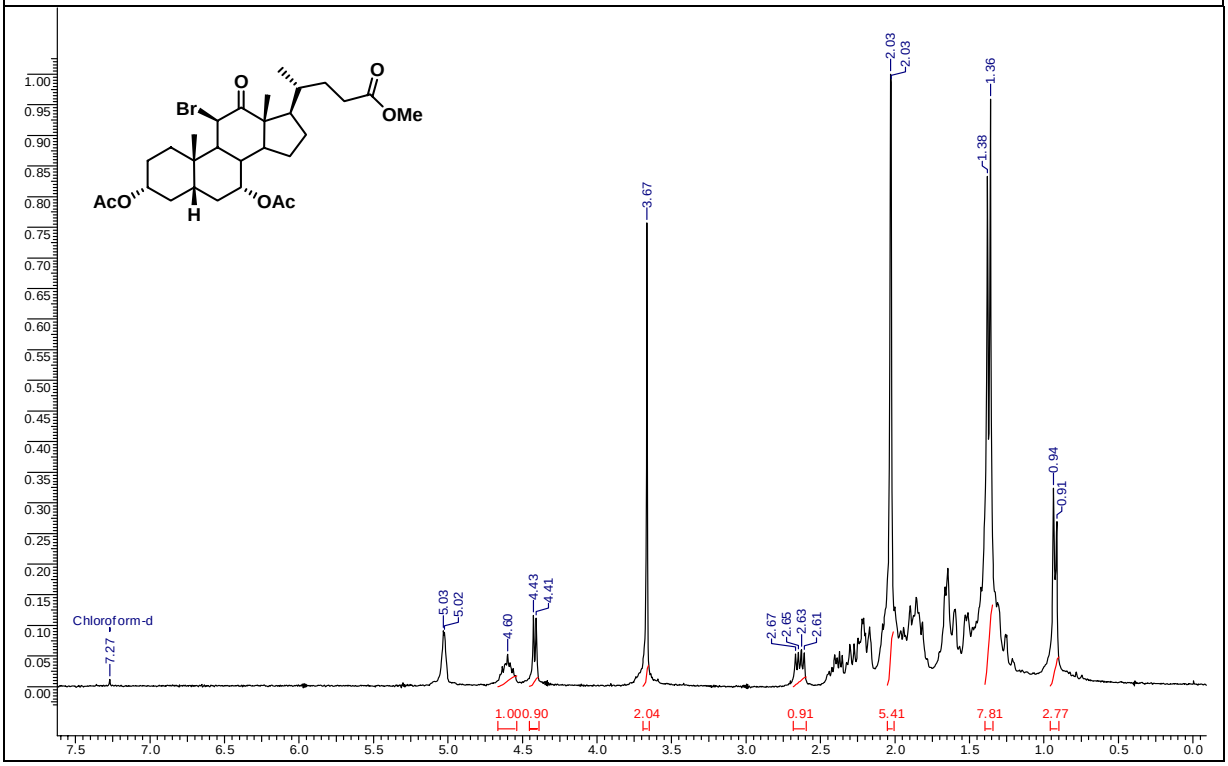
22: ^{13}C NMR, 75 MHz, $CDCl_3$



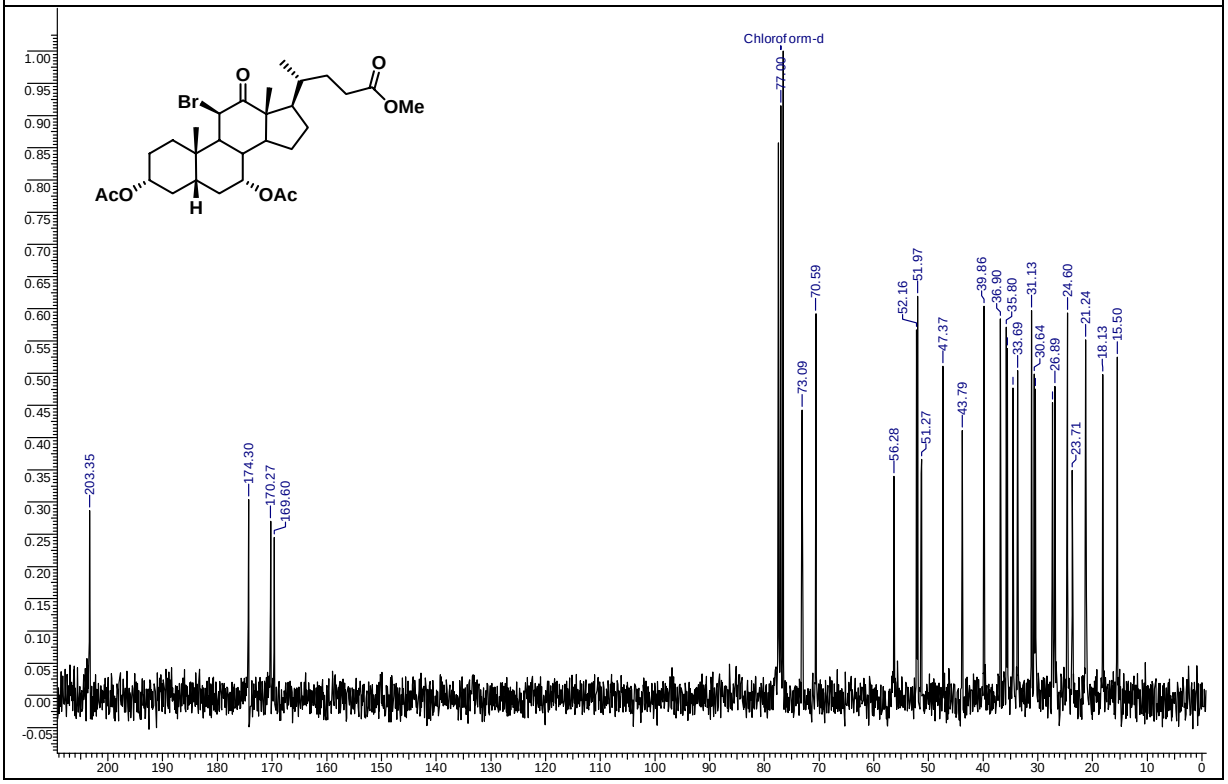
22: DEPT, 75 MHz, $CDCl_3$



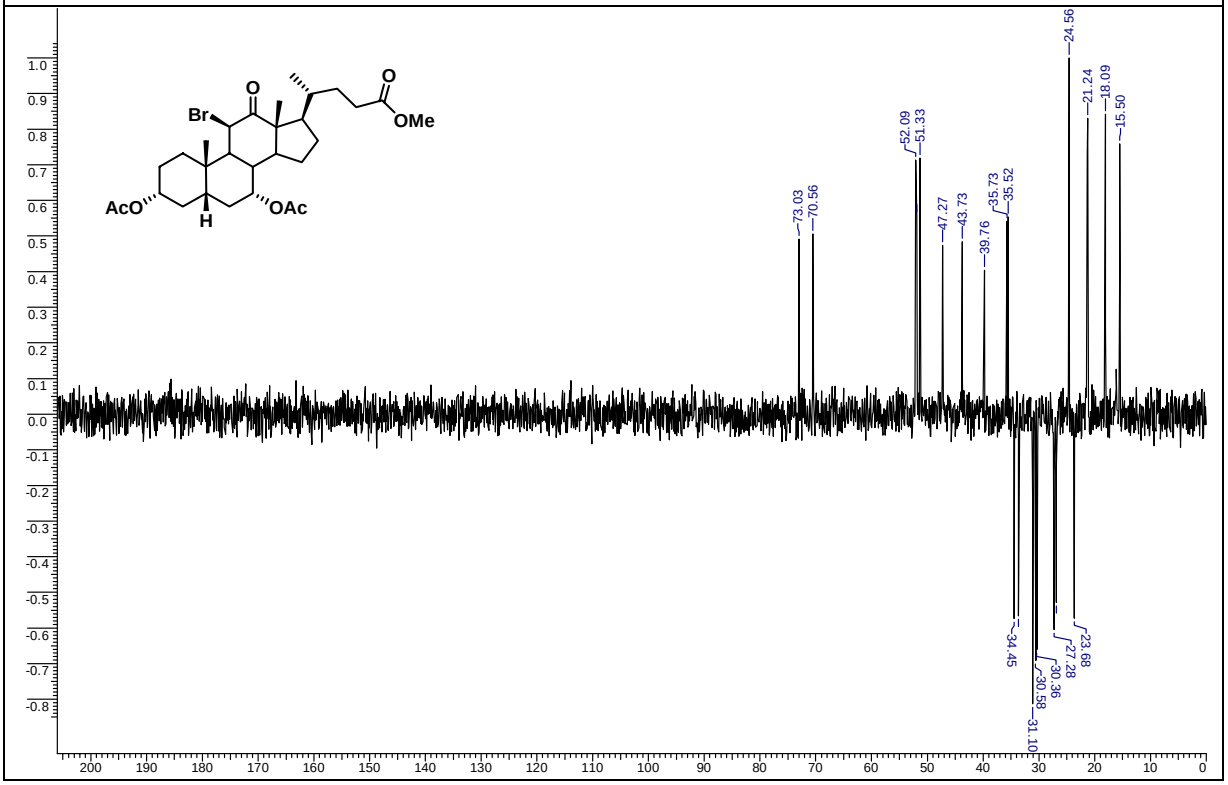
23: ^1H NMR, 300 MHz, $CDCl_3$



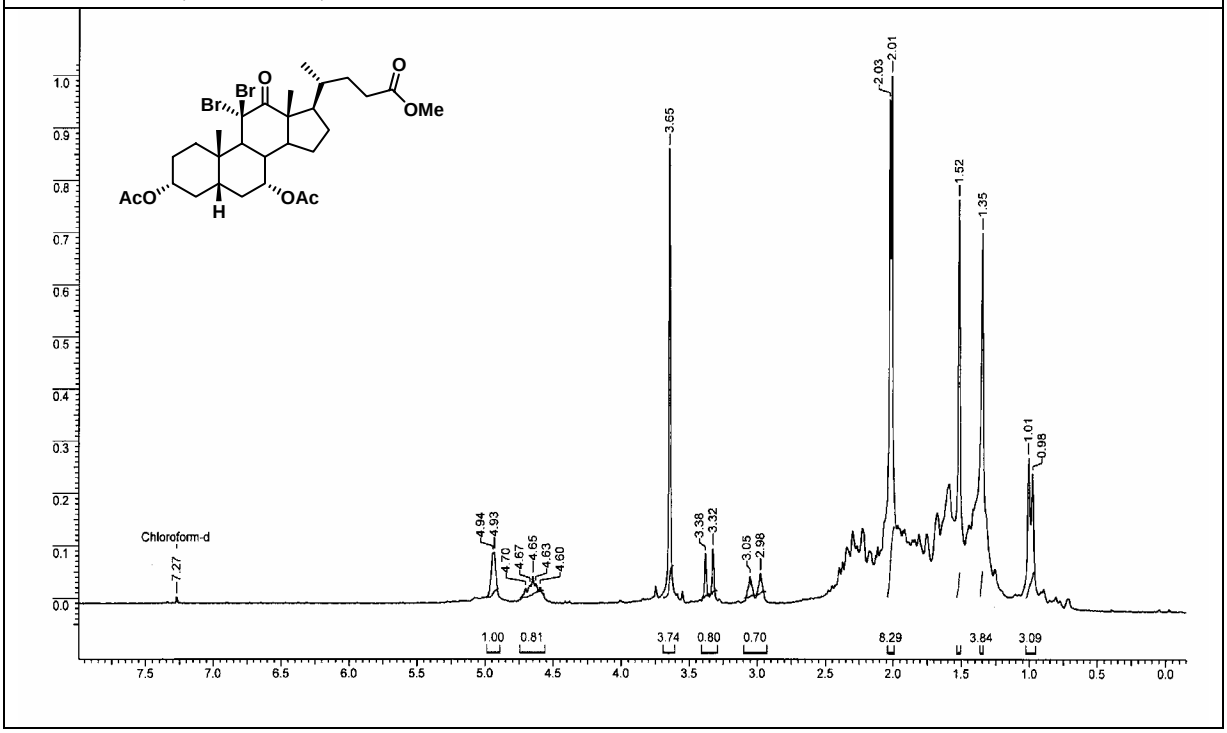
23: ^{13}C NMR, 75 MHz, $CDCl_3$



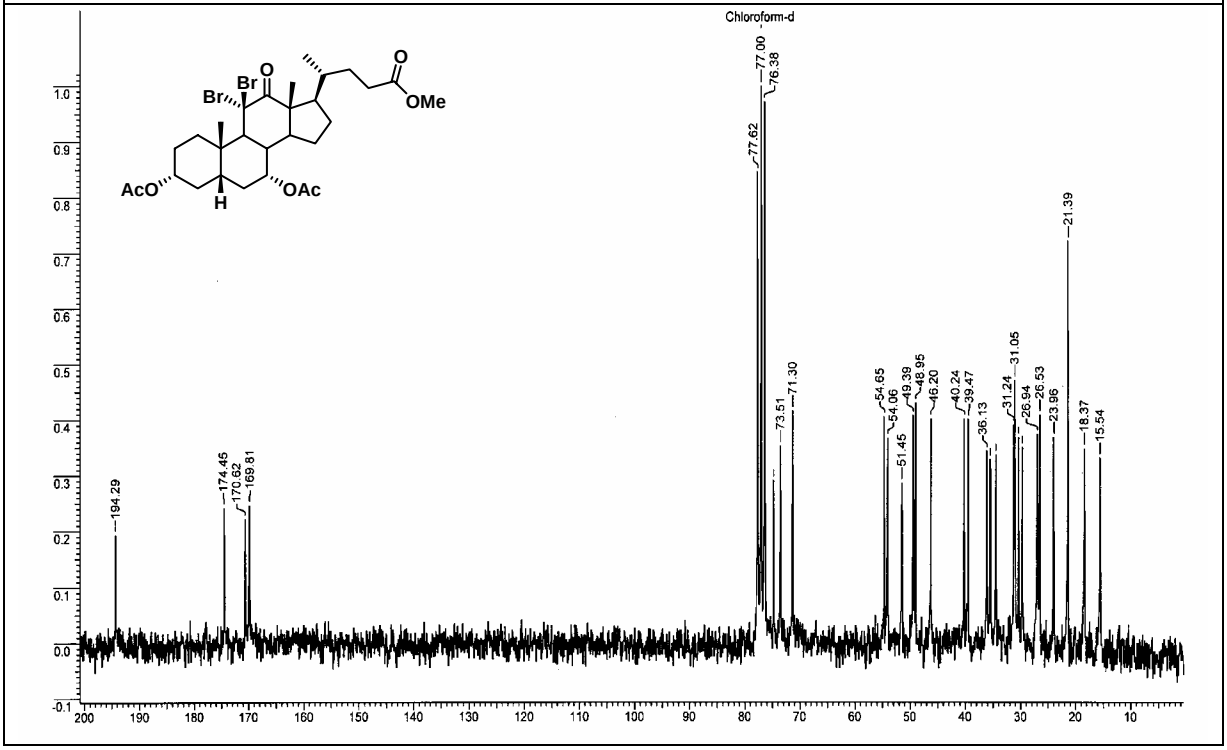
23: DEPT, 75 MHz, $CDCl_3$



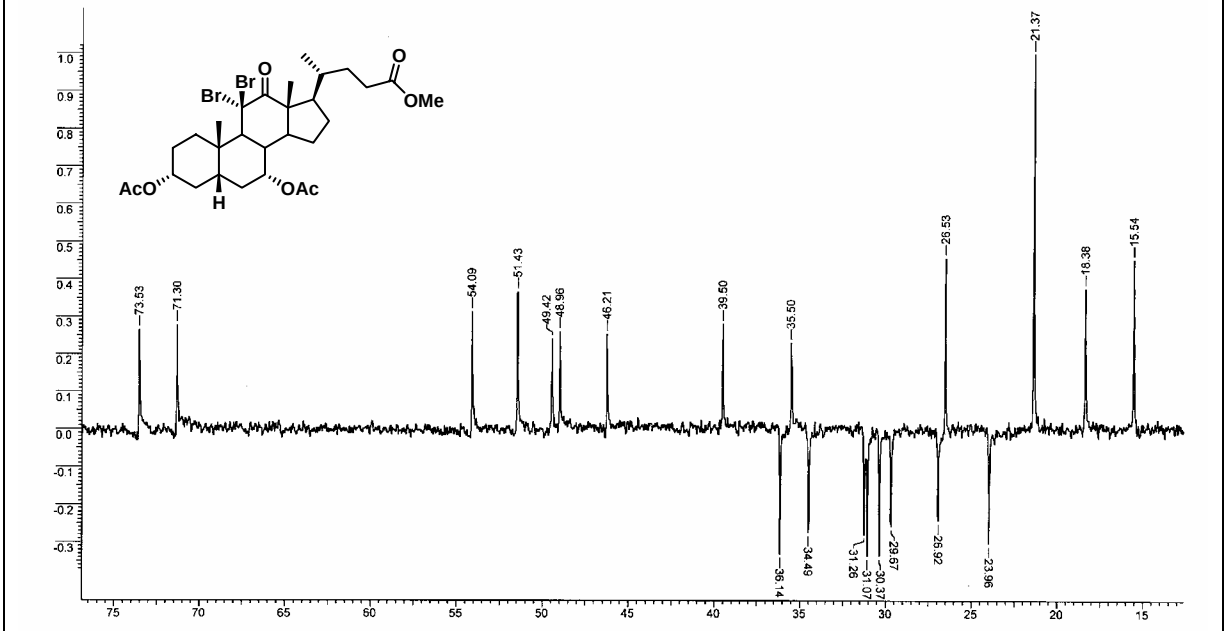
24: ^{1}H NMR, 200 MHz, $CDCl_3$



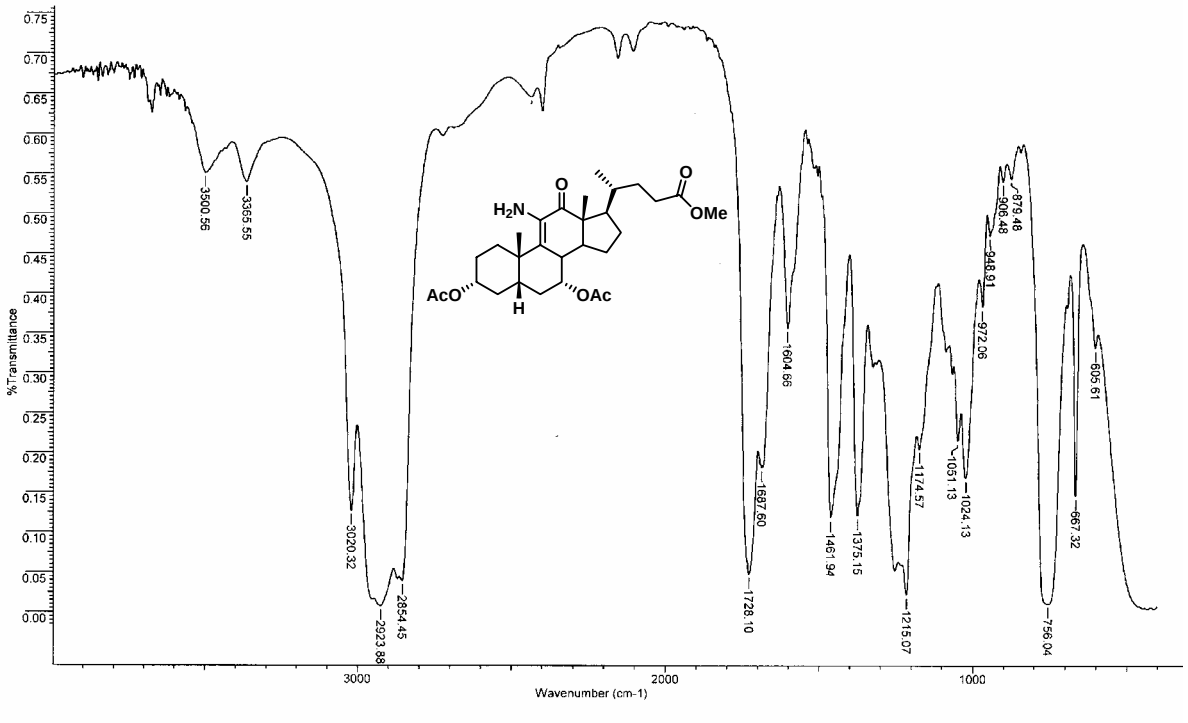
24: ^{13}C NMR, 50 MHz, $CDCl_3$



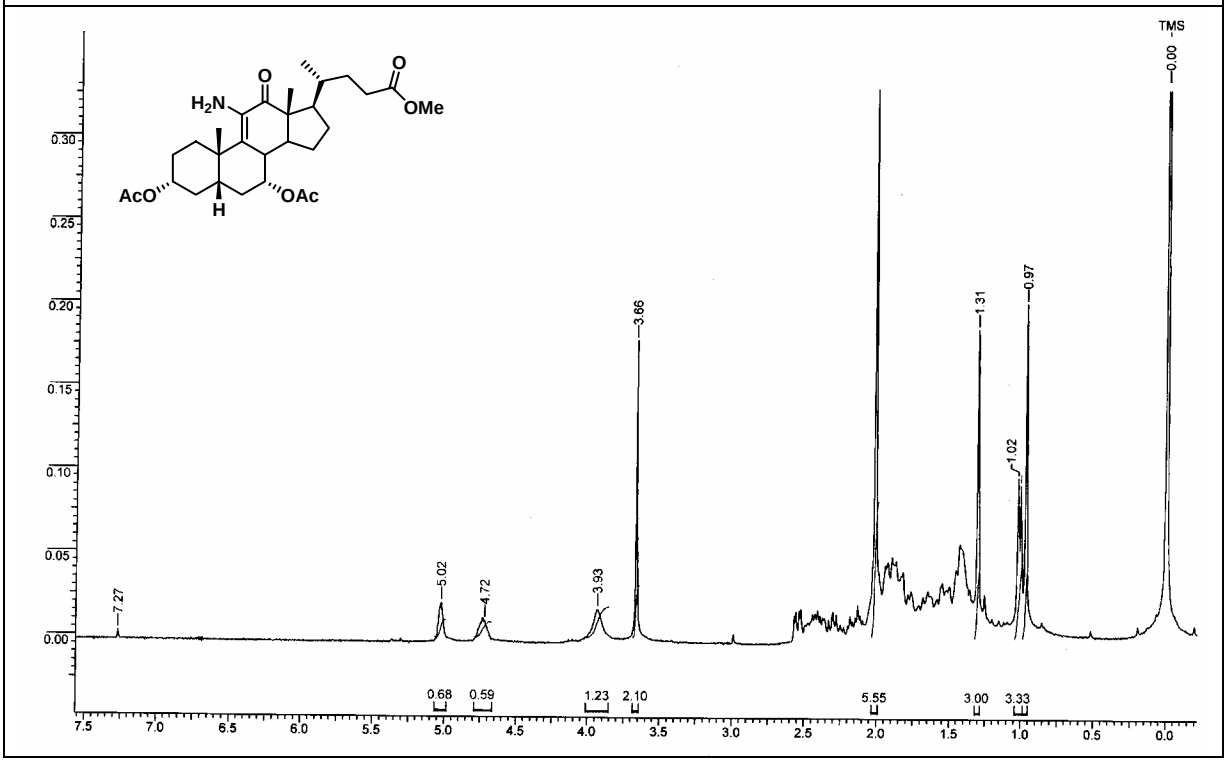
24: DEPT, 50 MHz, $CDCl_3$



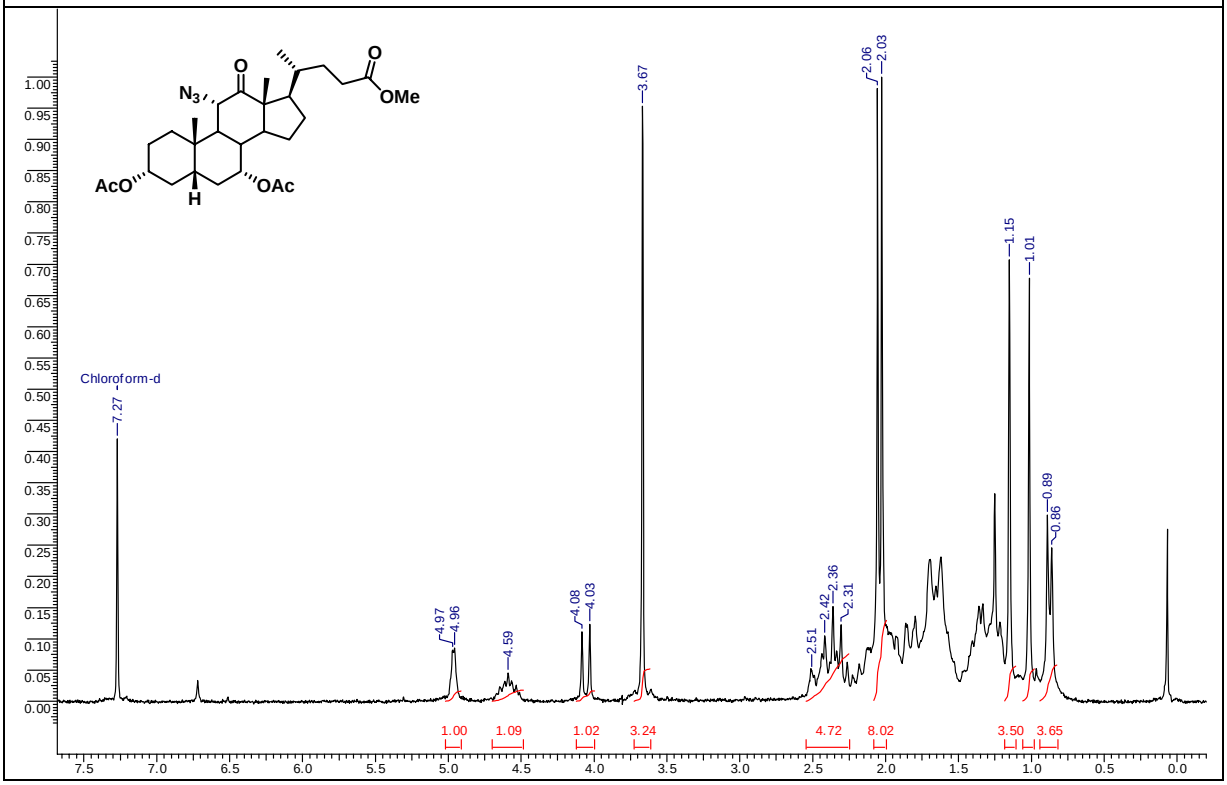
25: IR, Nujol.



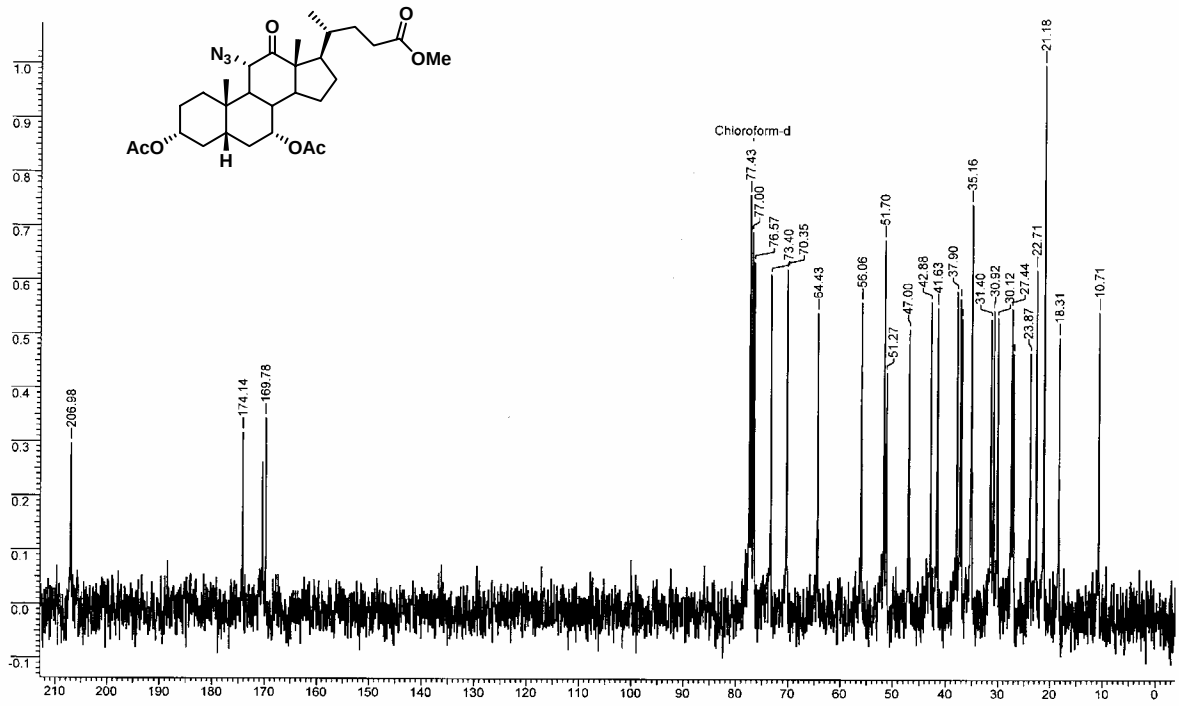
25: ^{1}H NMR, 300 MHz, $CDCl_3$



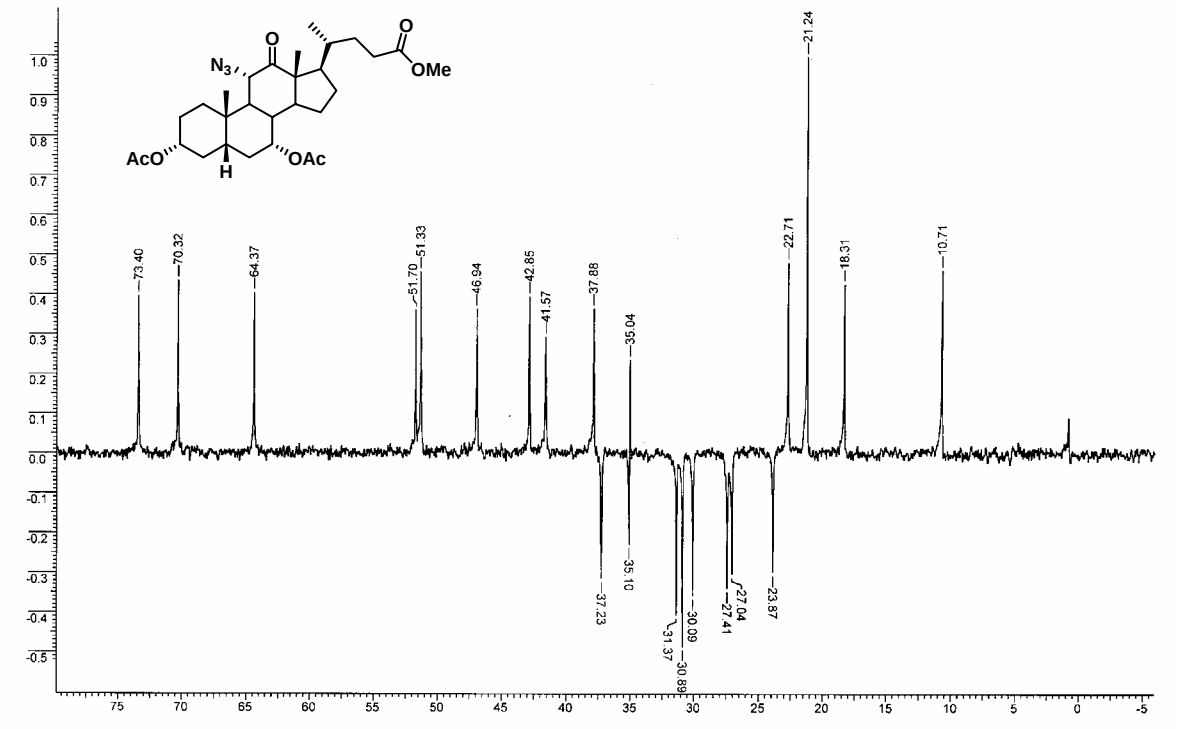
26: ^{1}H NMR, 300 MHz, $CDCl_3$



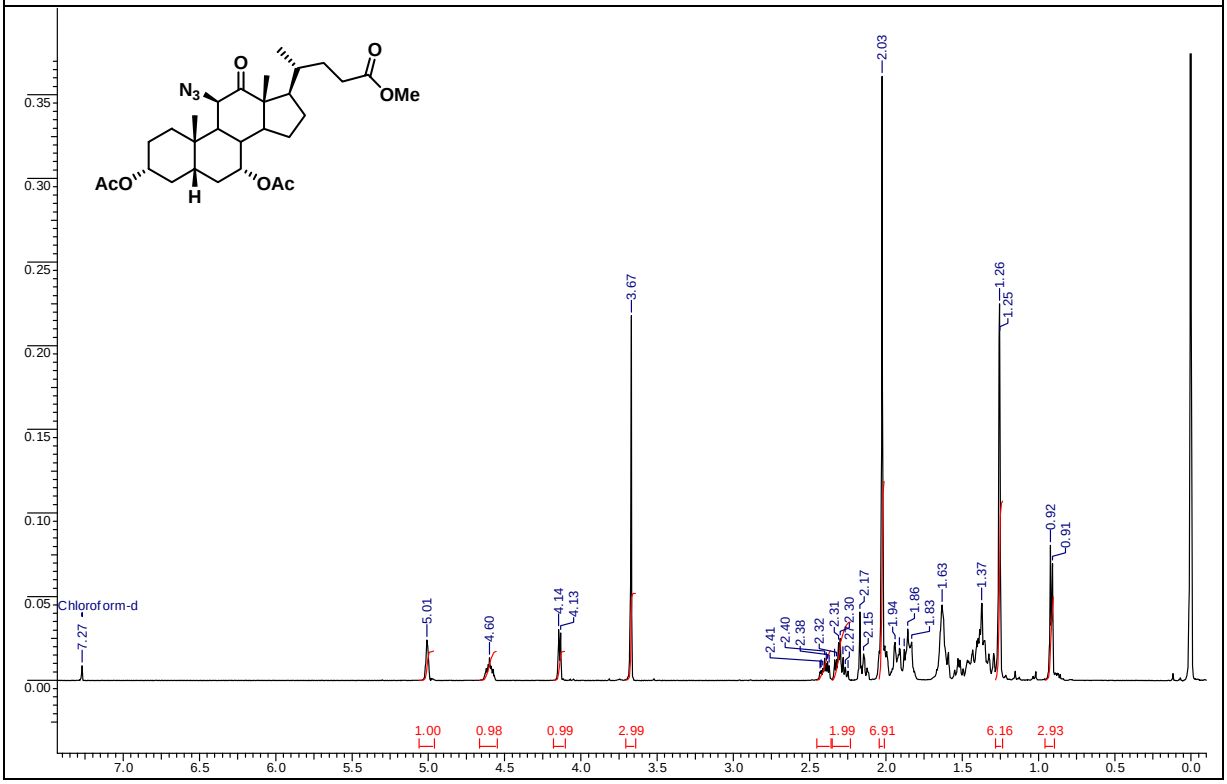
26: ^{13}C NMR, 75 MHz, $CDCl_3$



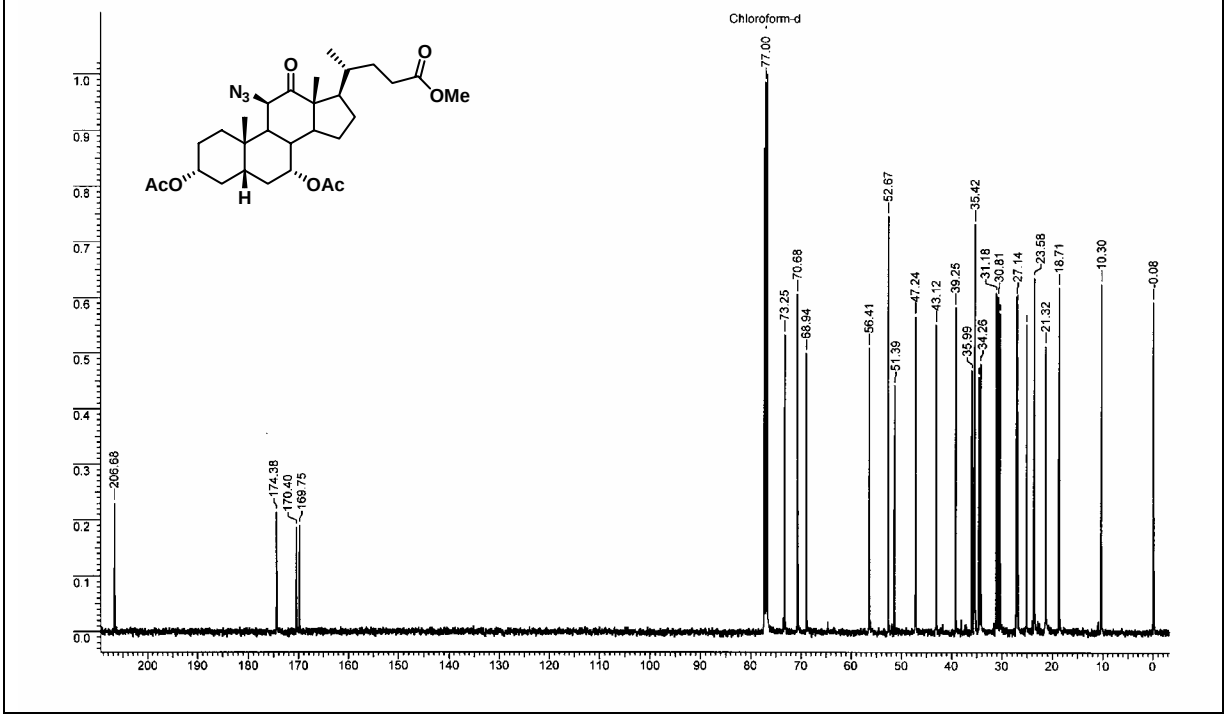
26: DEPT, 75 MHz, $CDCl_3$



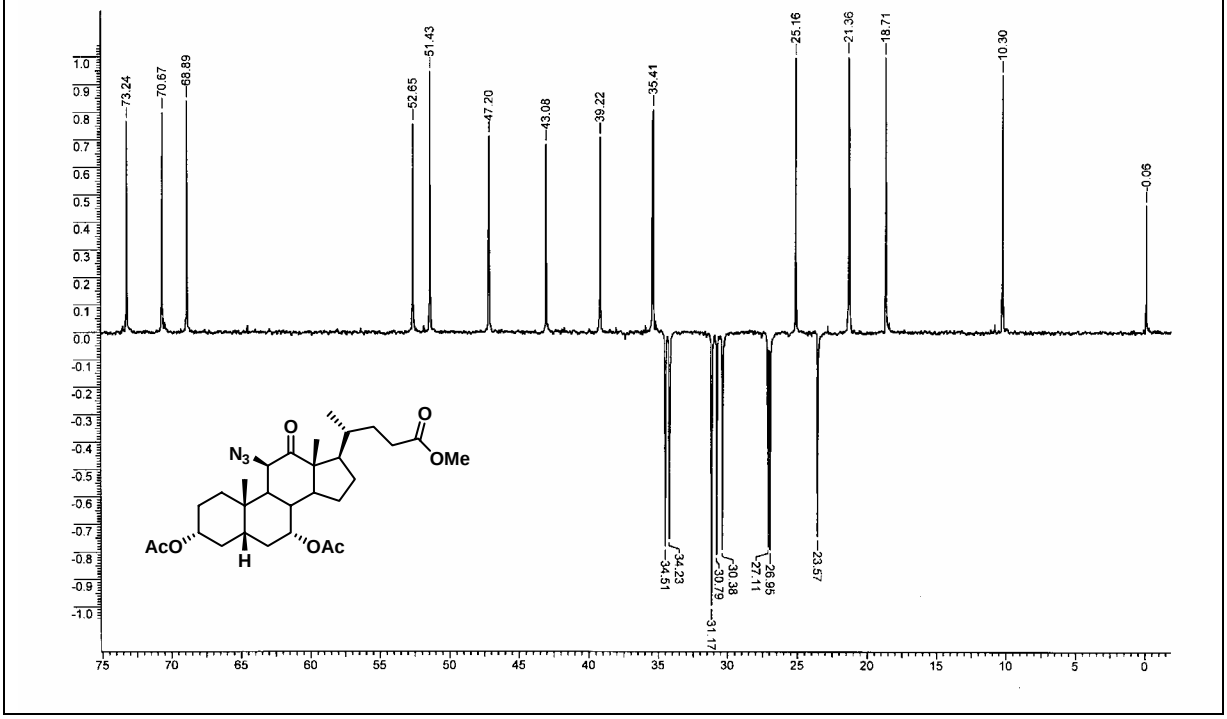
27: ^{1}H NMR, 500 MHz, $CDCl_3$



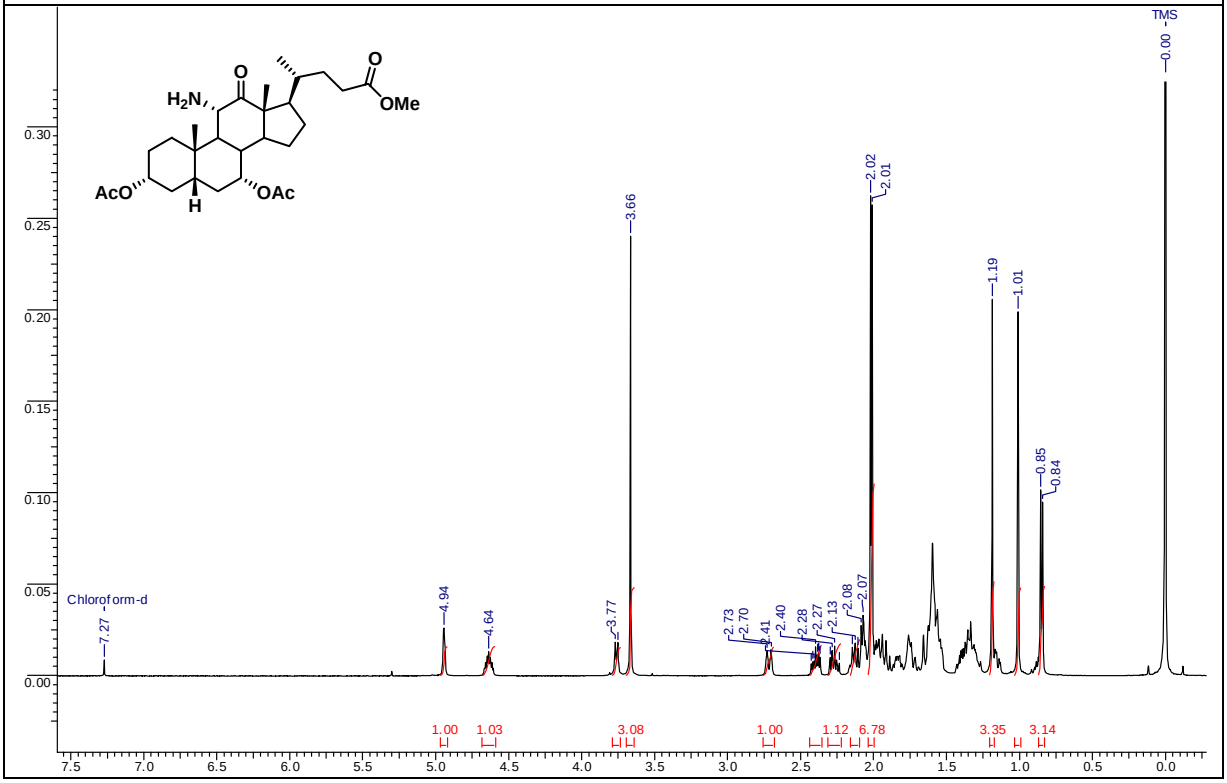
27: ^{13}C NMR, 125 MHz, $CDCl_3$



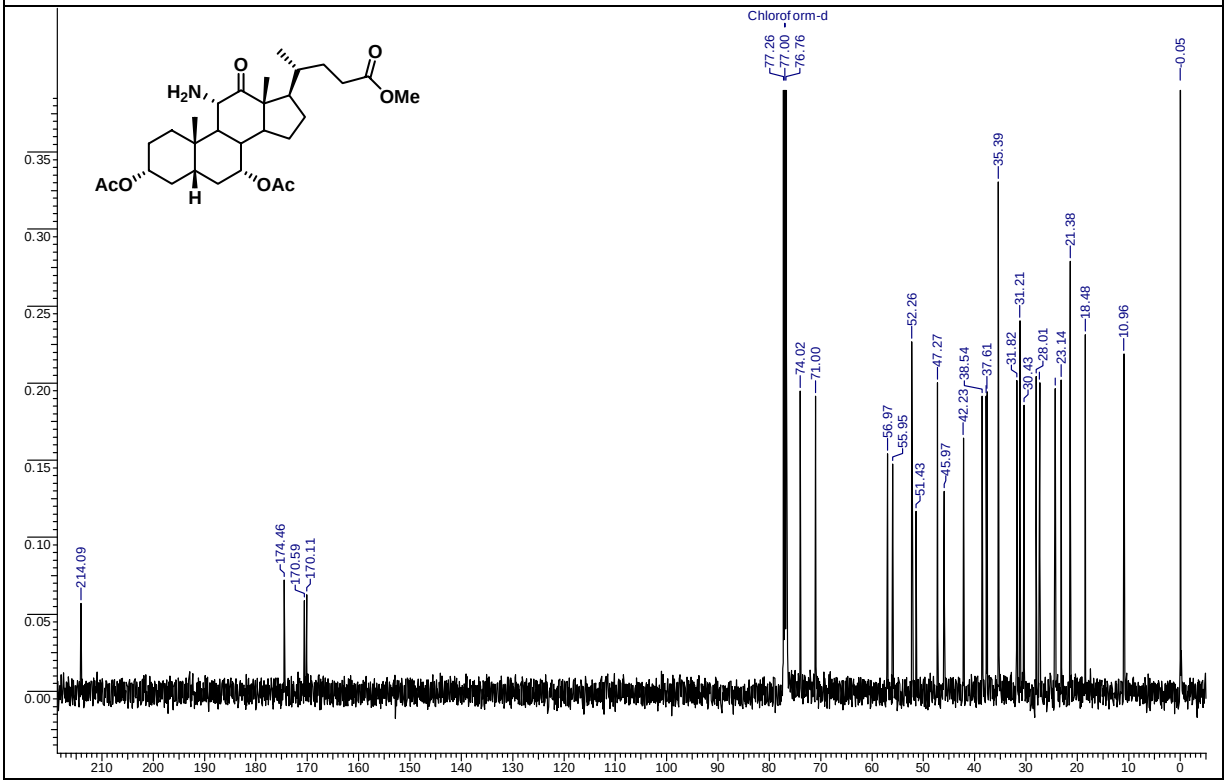
27: DEPT, 125 MHz, $CDCl_3$



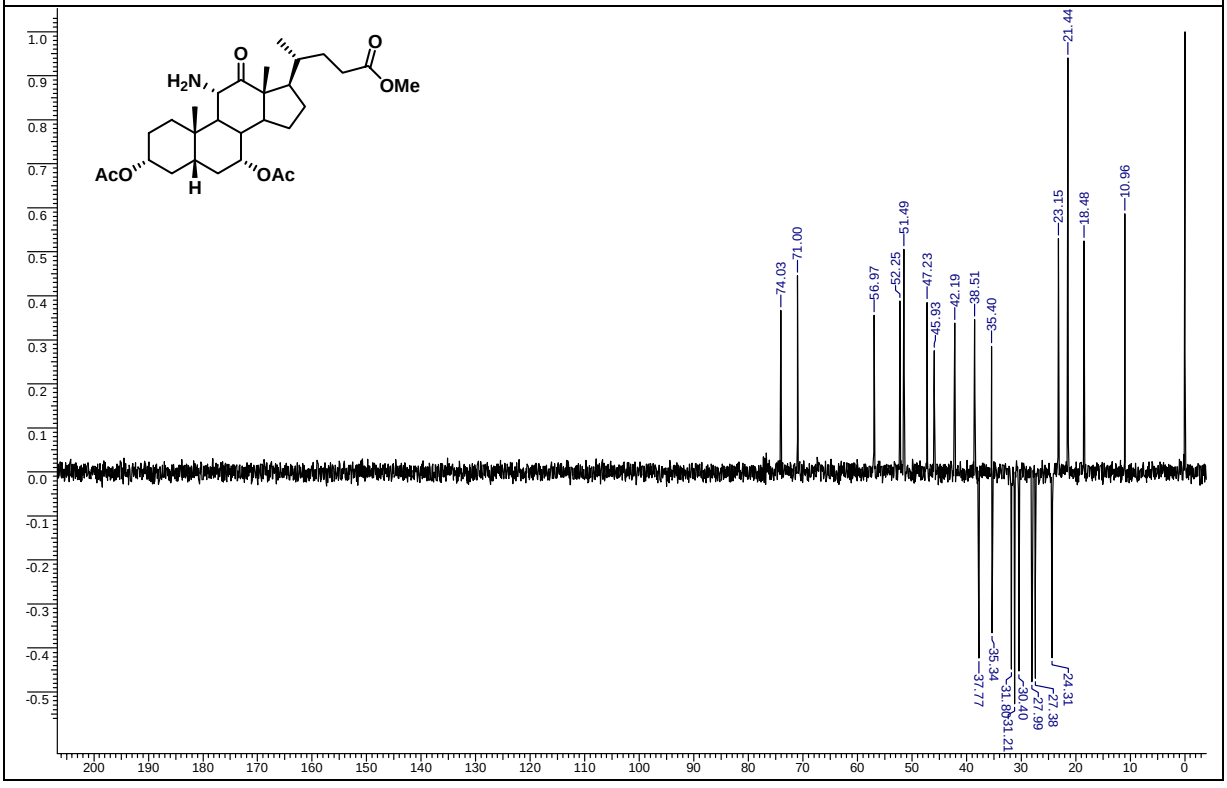
38: ^1H NMR, 500 MHz, $CDCl_3$



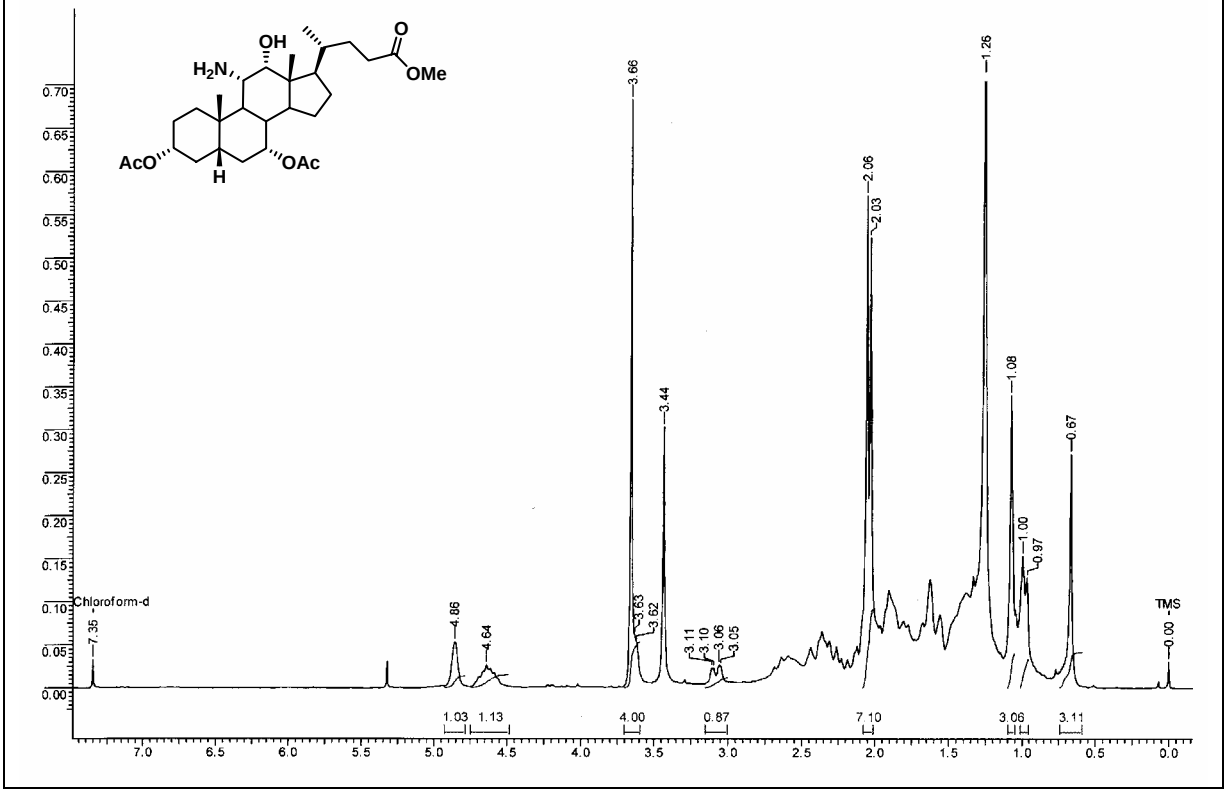
38: ^{13}C NMR, 125 MHz, $CDCl_3$



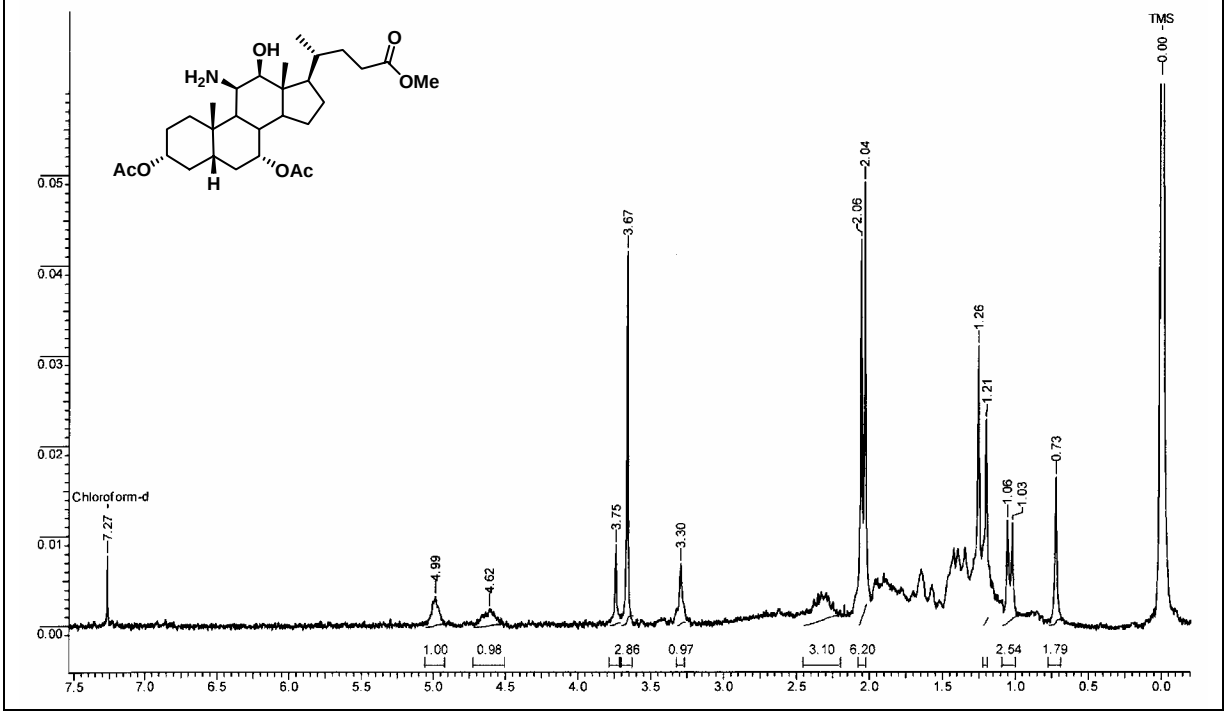
38: DEPT, 125 MHz, $CDCl_3$



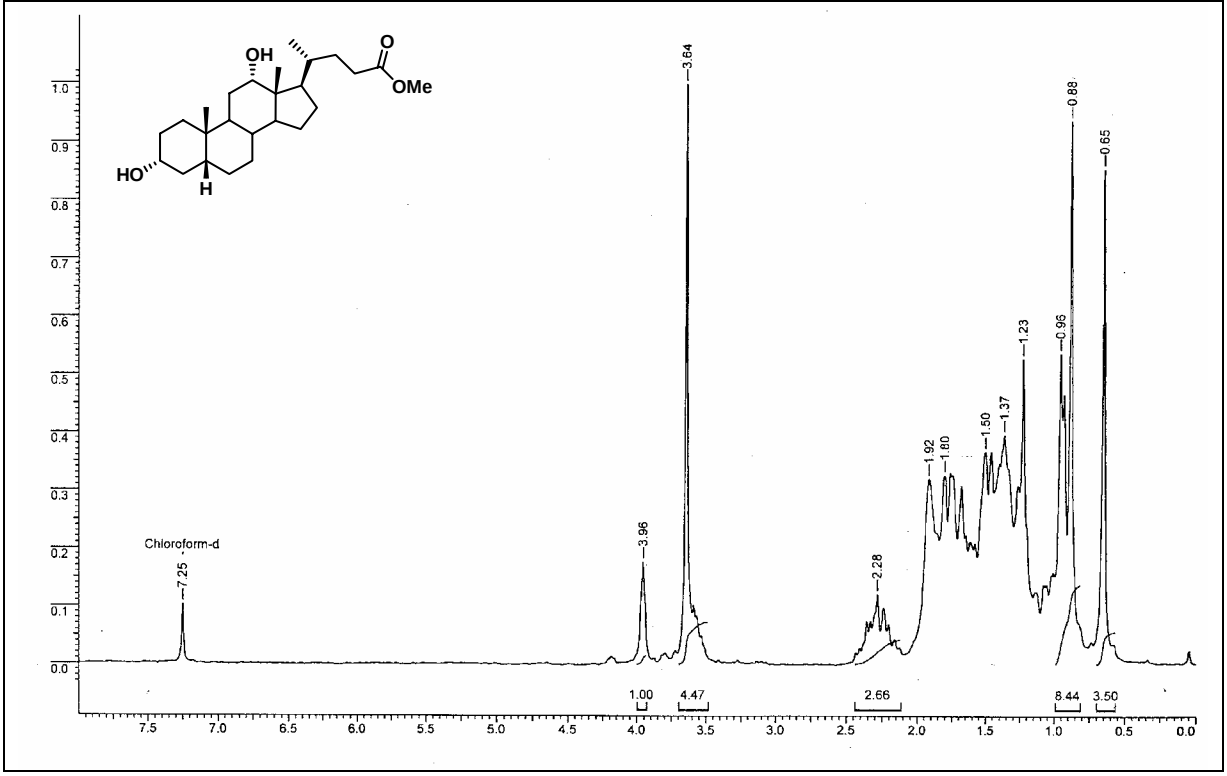
44: ^{1}H NMR, 200 MHz, $CDCl_3$



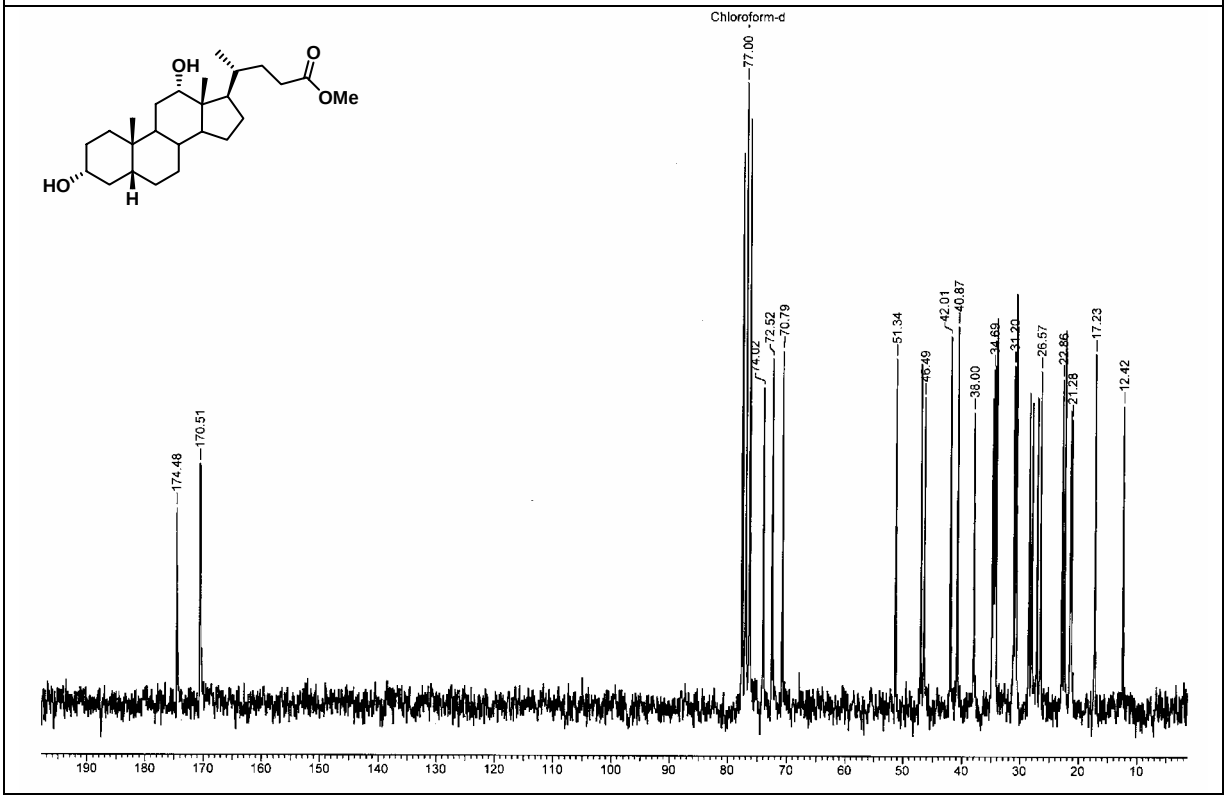
45: ^{1}H NMR, 200 MHz, $CDCl_3$



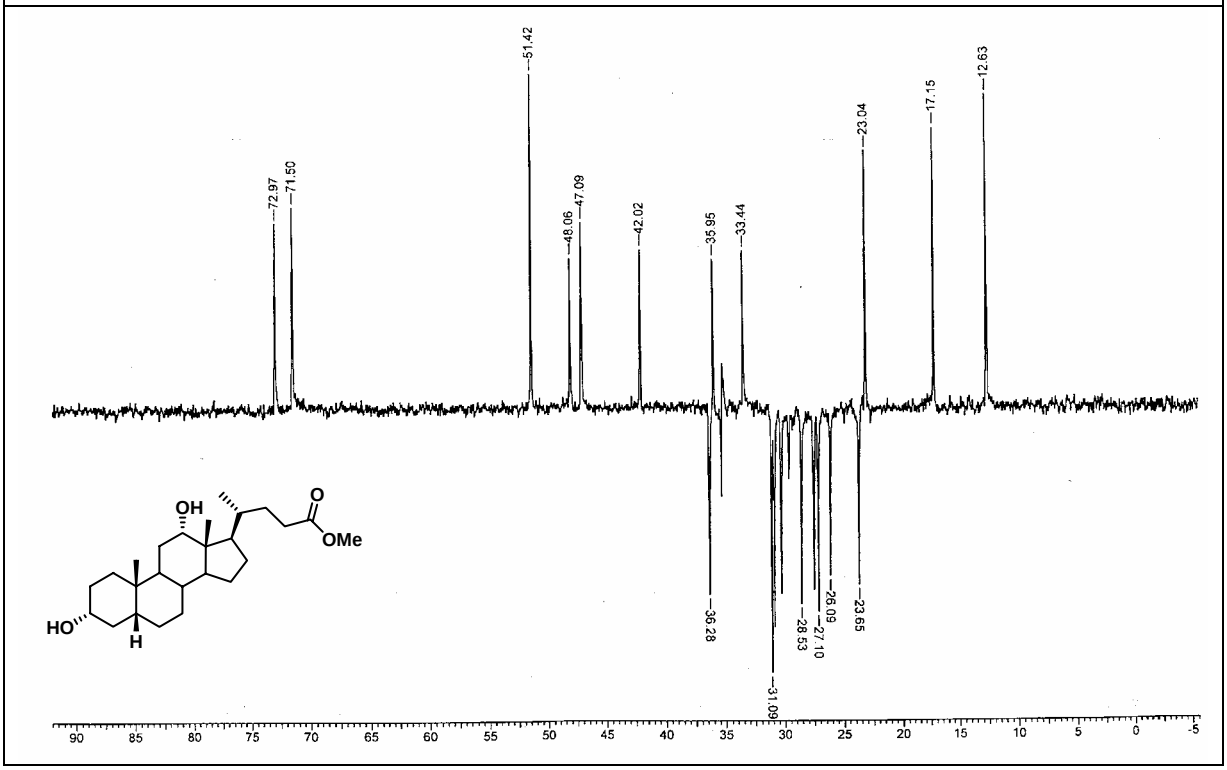
47: ^{1}H NMR, 200 MHz, $CDCl_3$



47: ^{13}C NMR, 50 MHz, $CDCl_3$



47: DEPT, 50 MHz, $CDCl_3$



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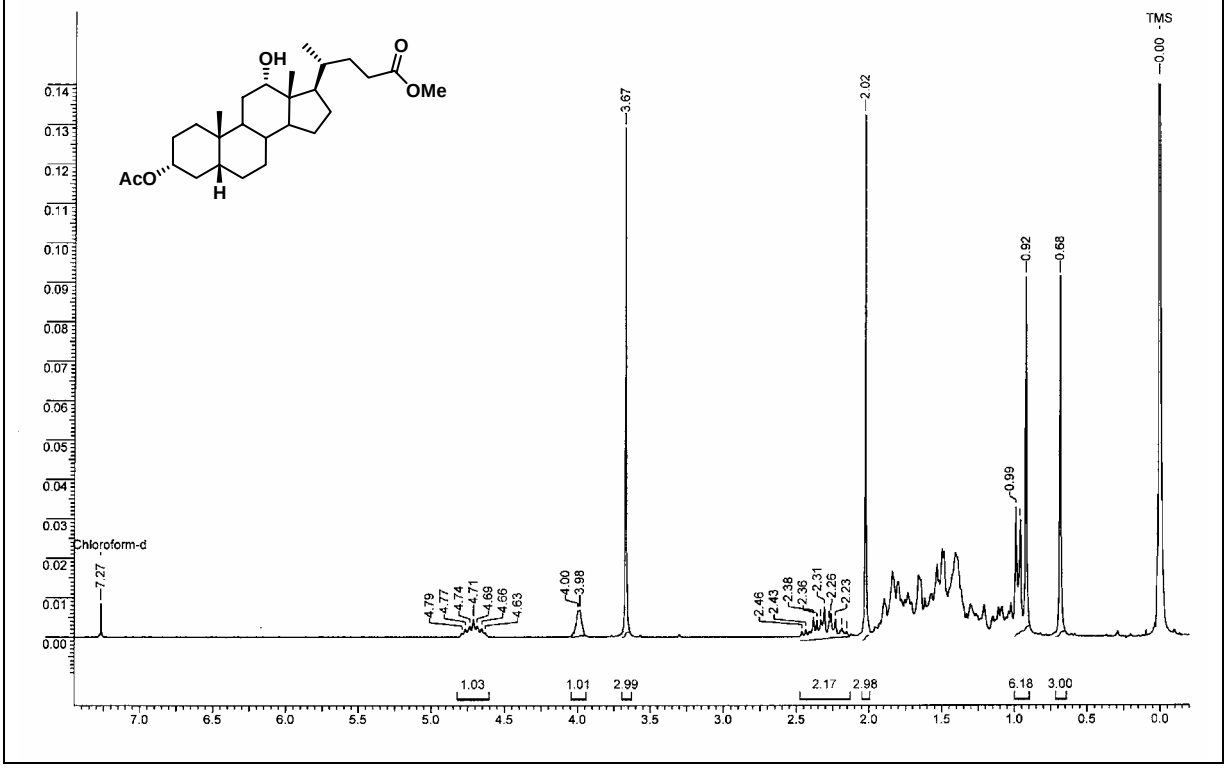

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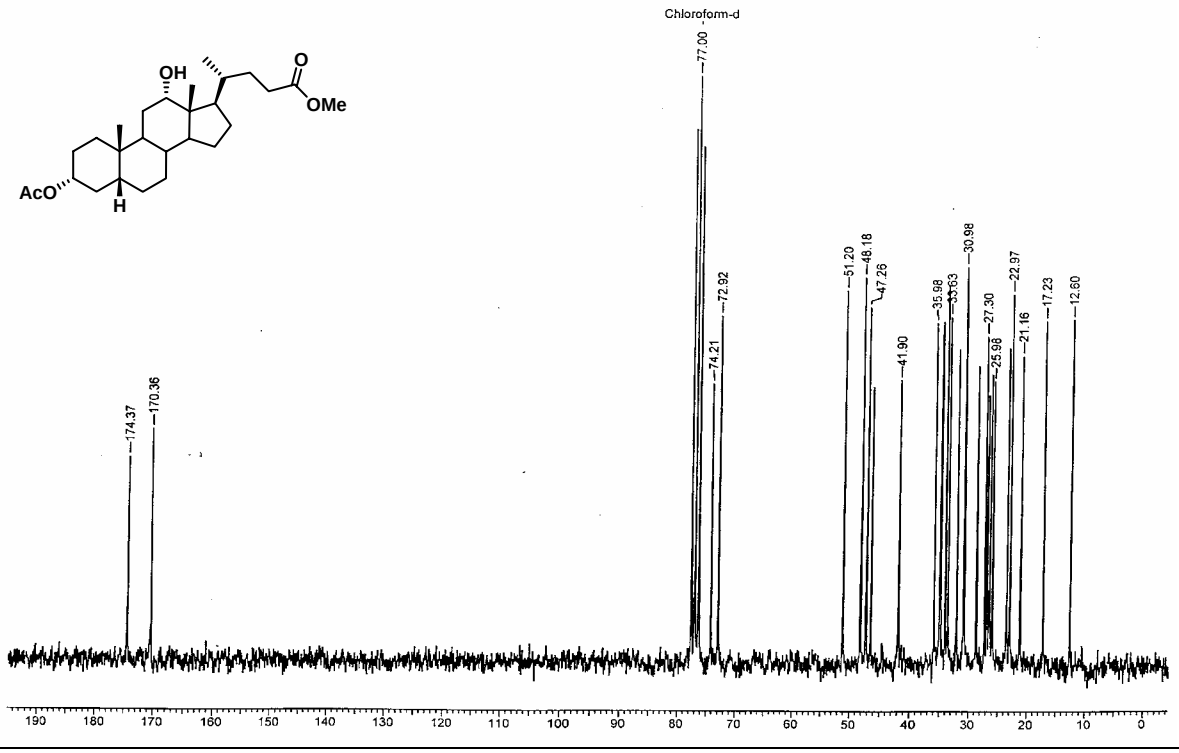
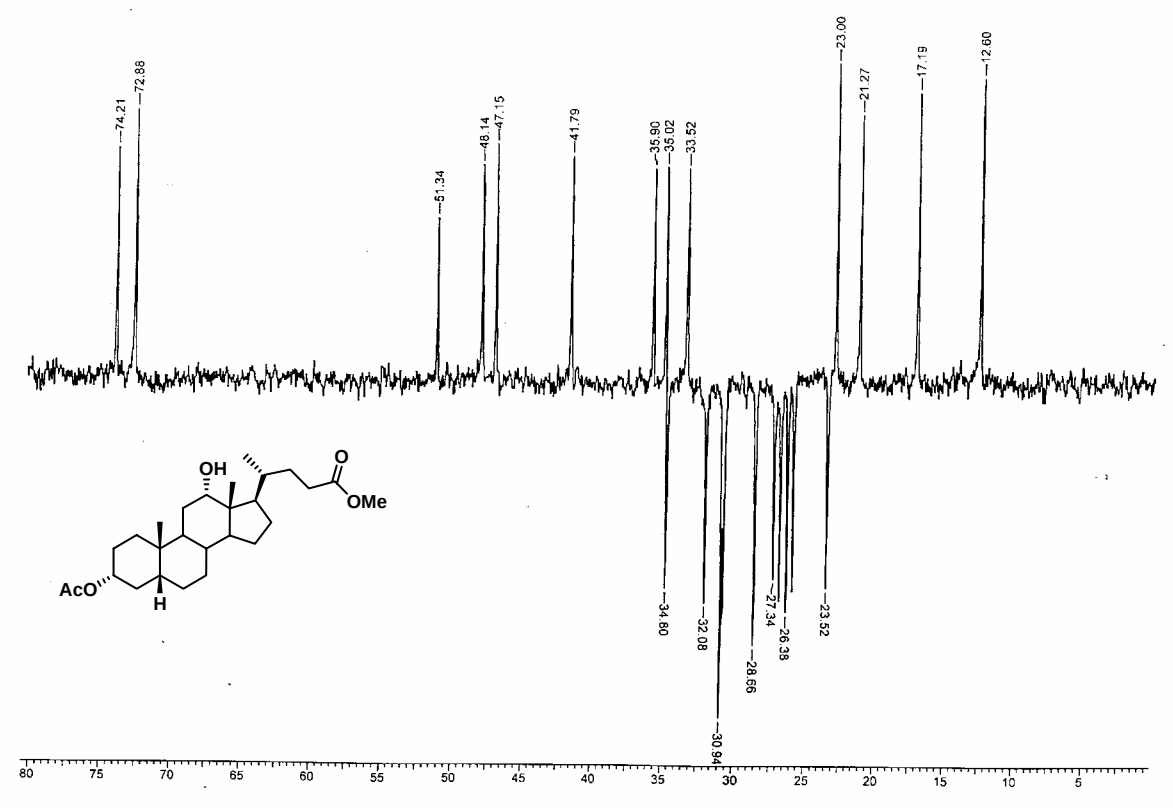
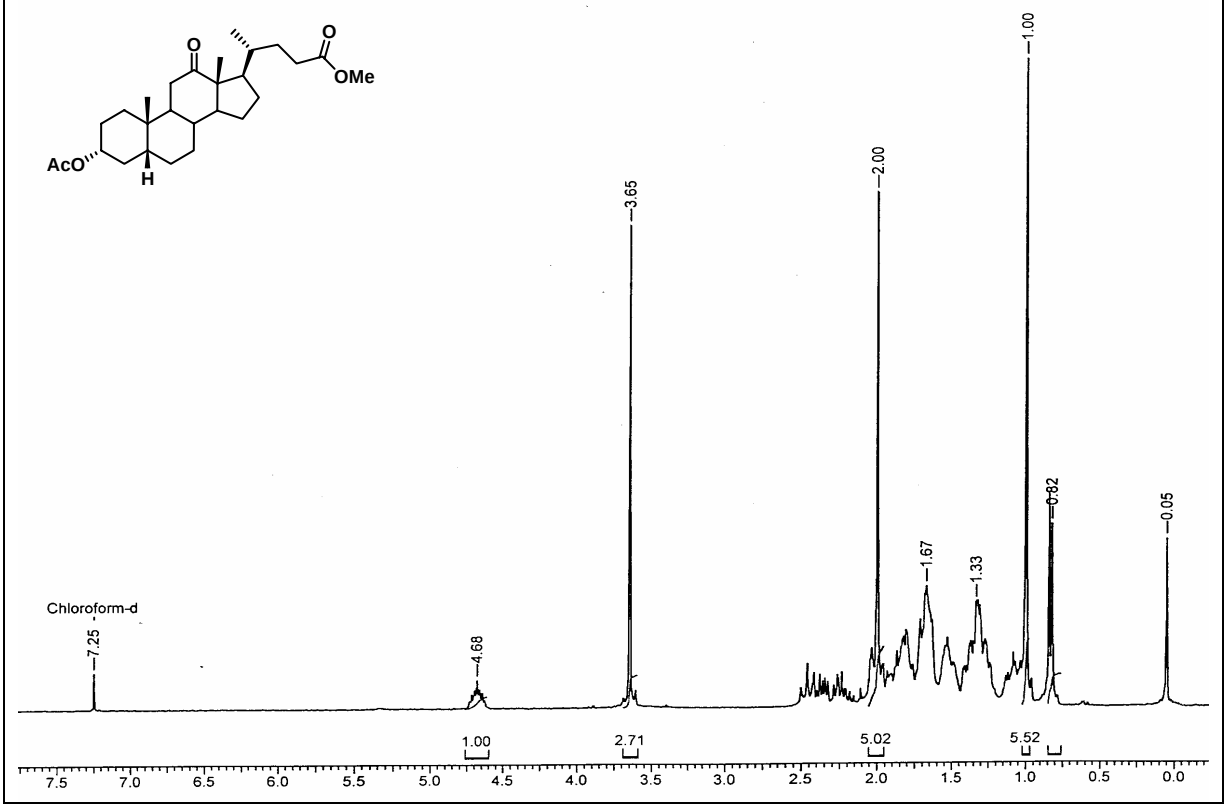


48: DEPT, 50 MHz, $CDCl_3$



49: ^{1}H NMR, 300 MHz, $CDCl_3$



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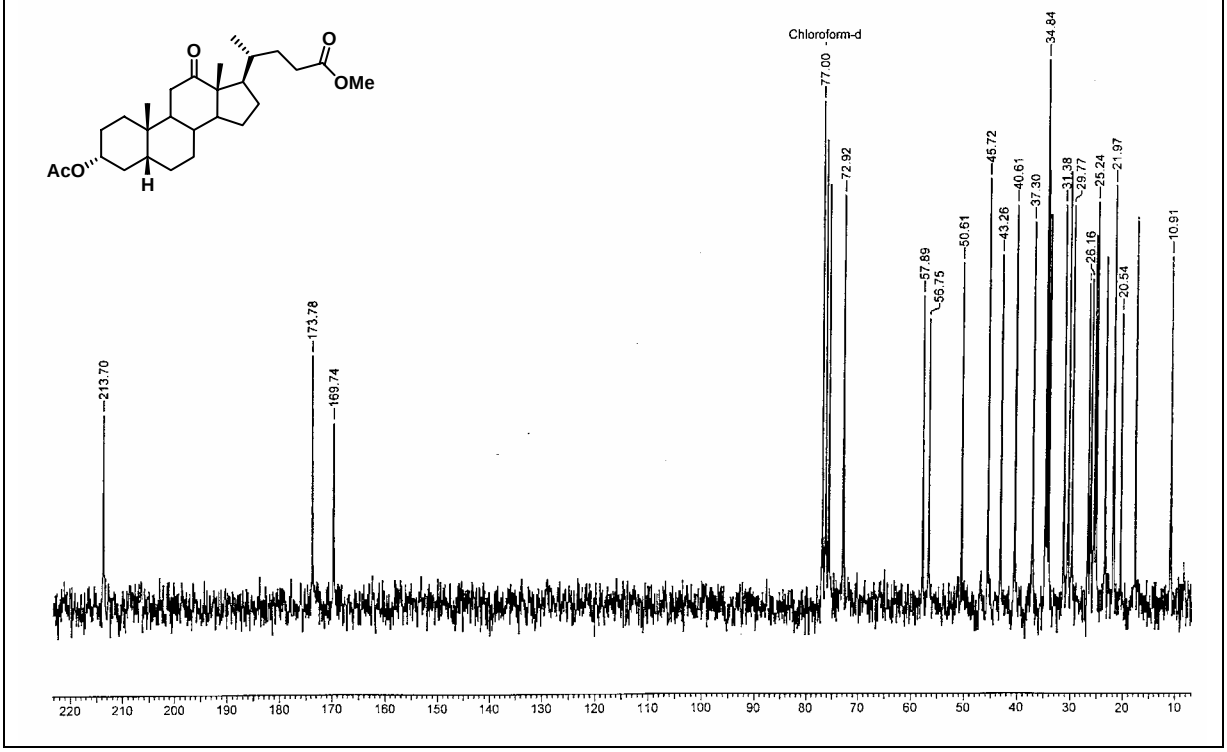
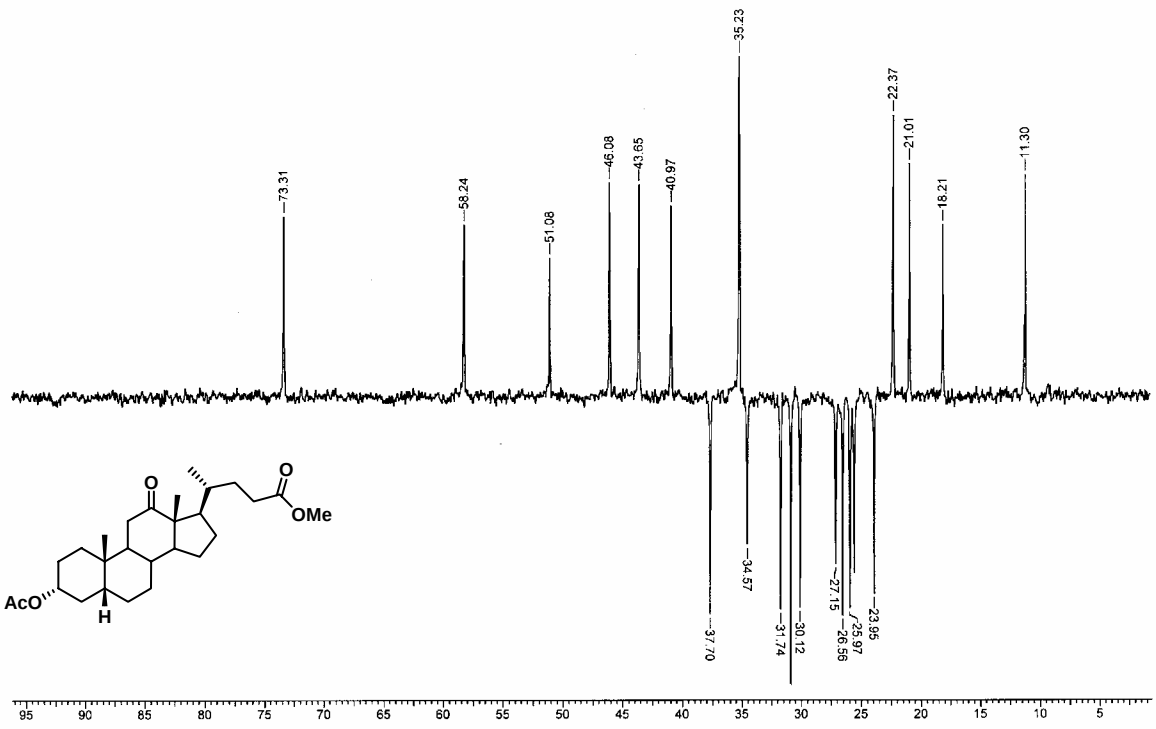


49: DEPT, 75 MHz, $CDCl_3$



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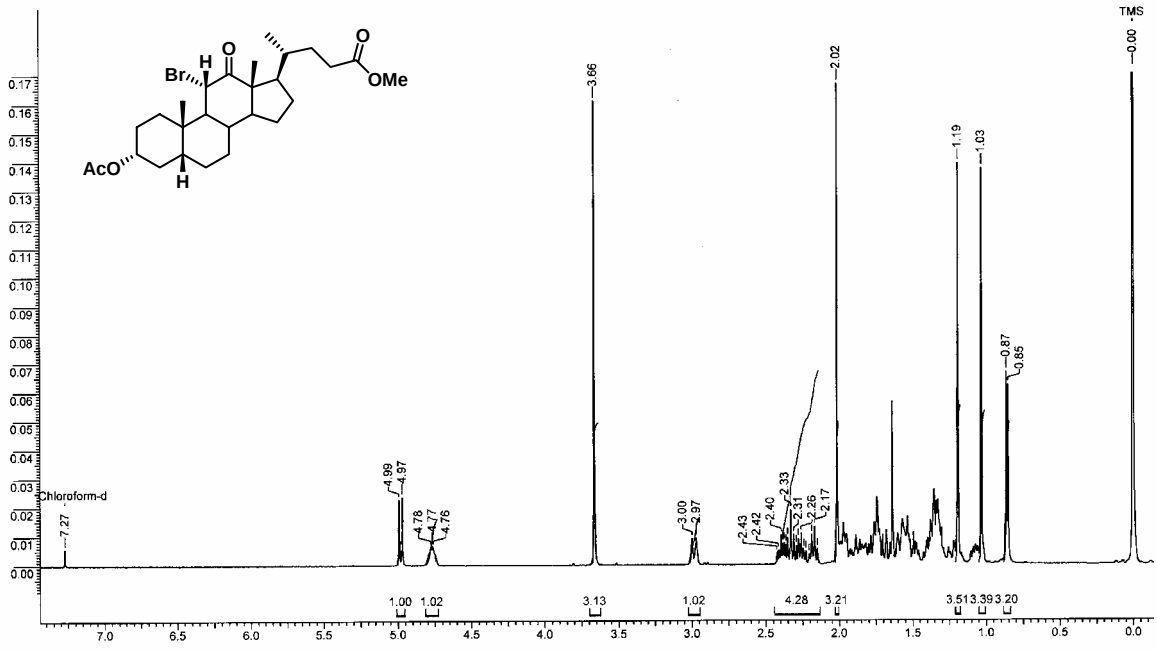

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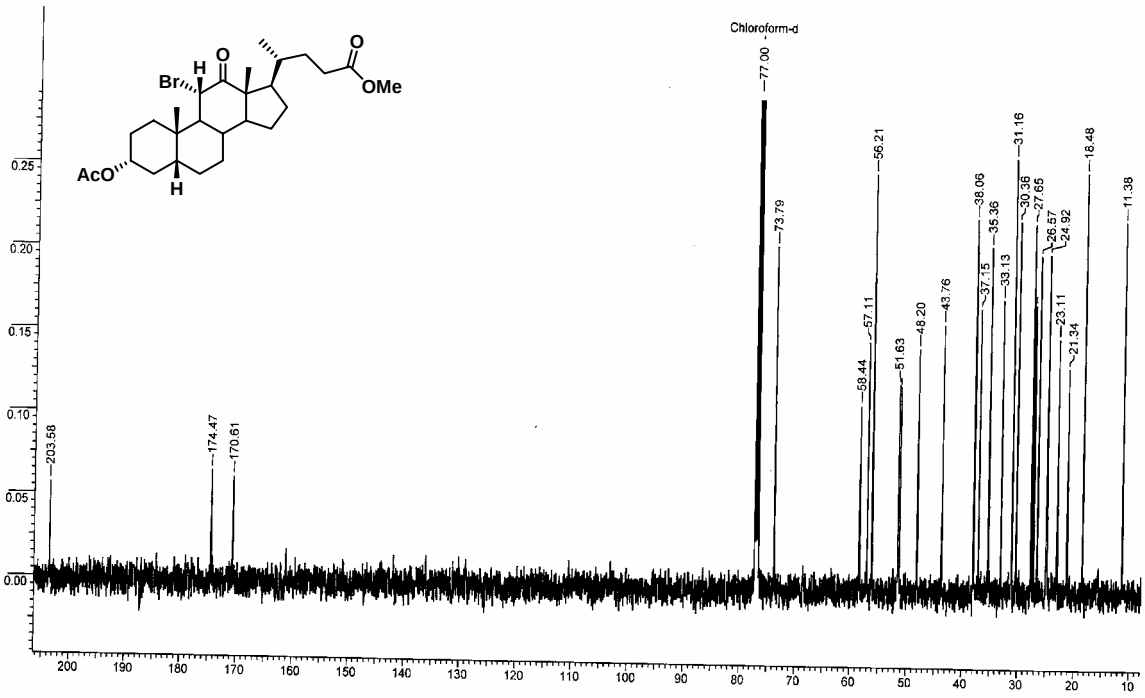

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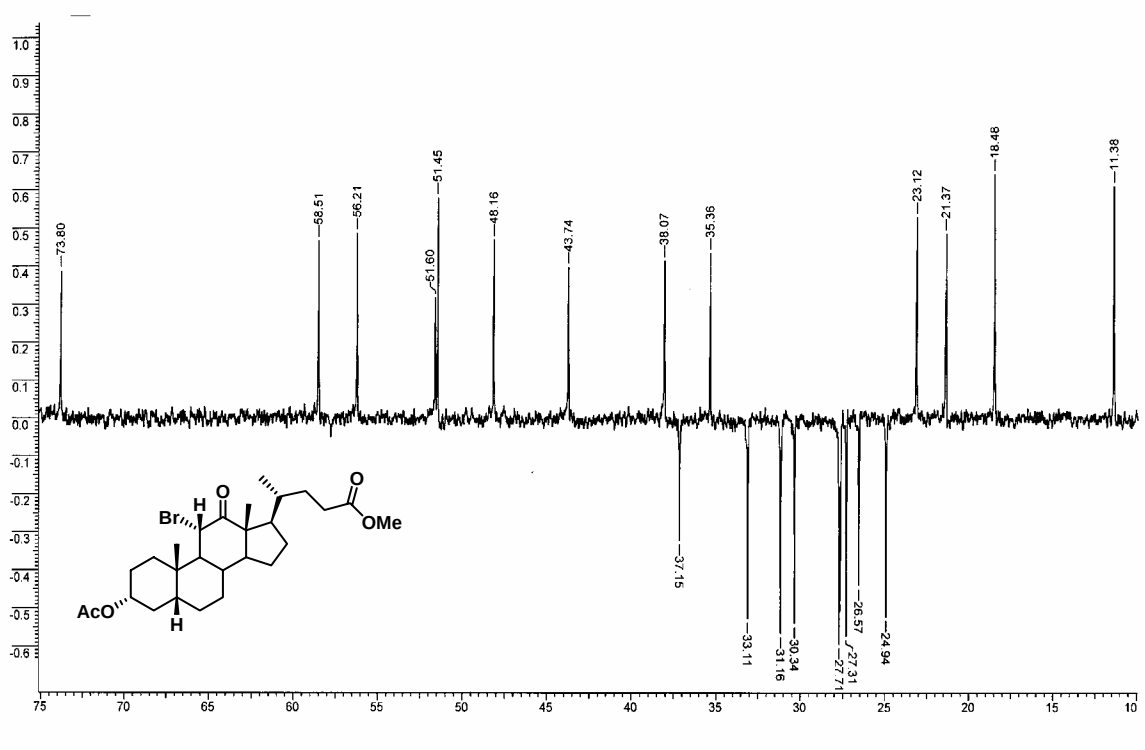

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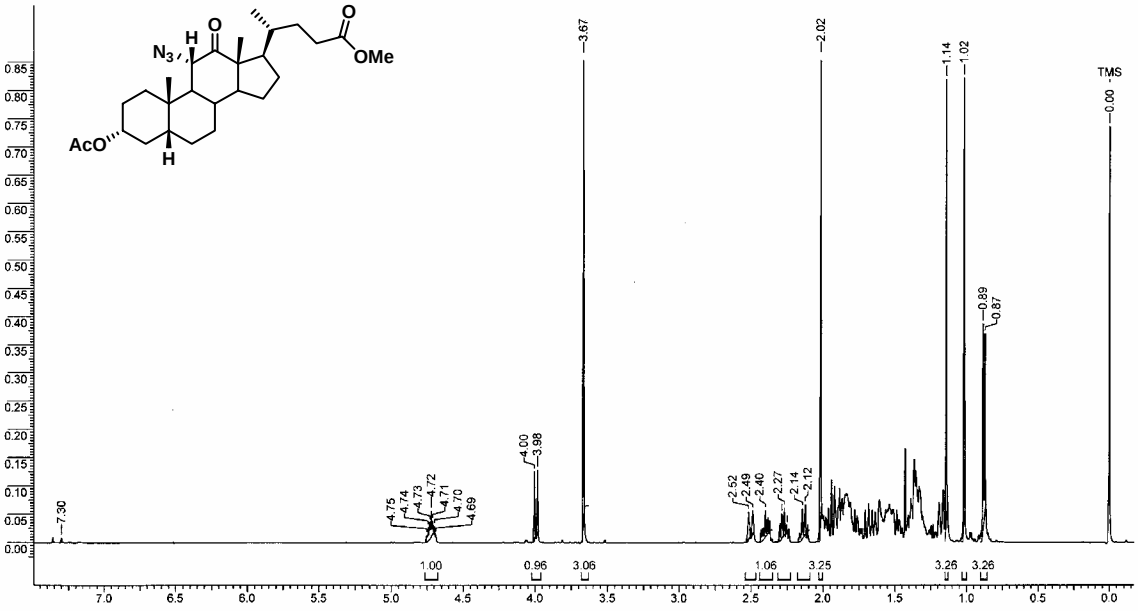

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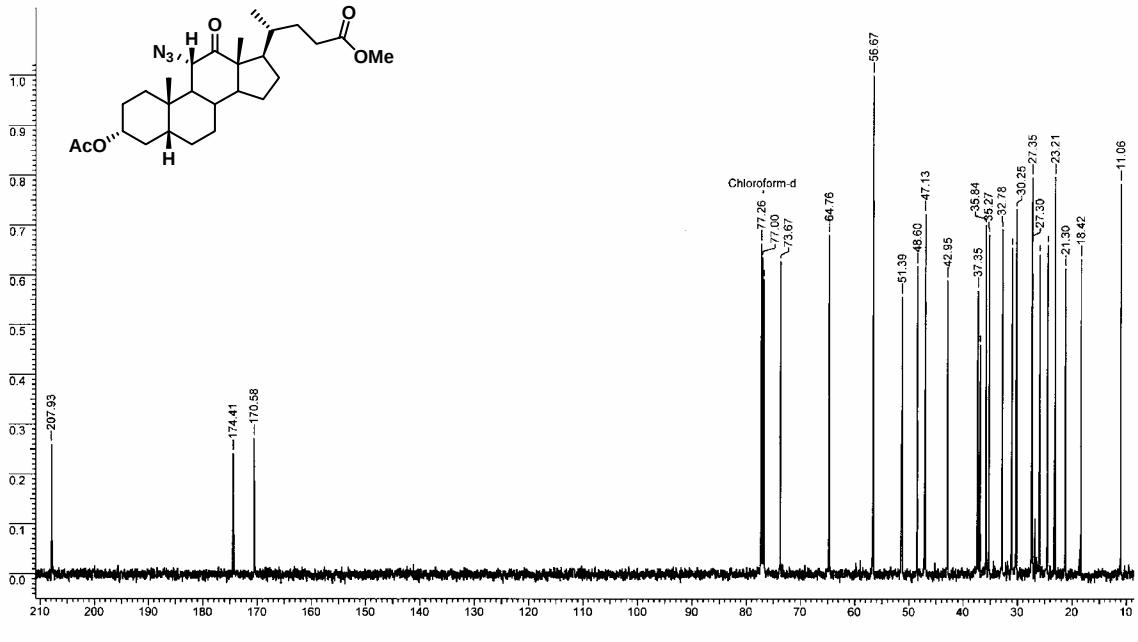

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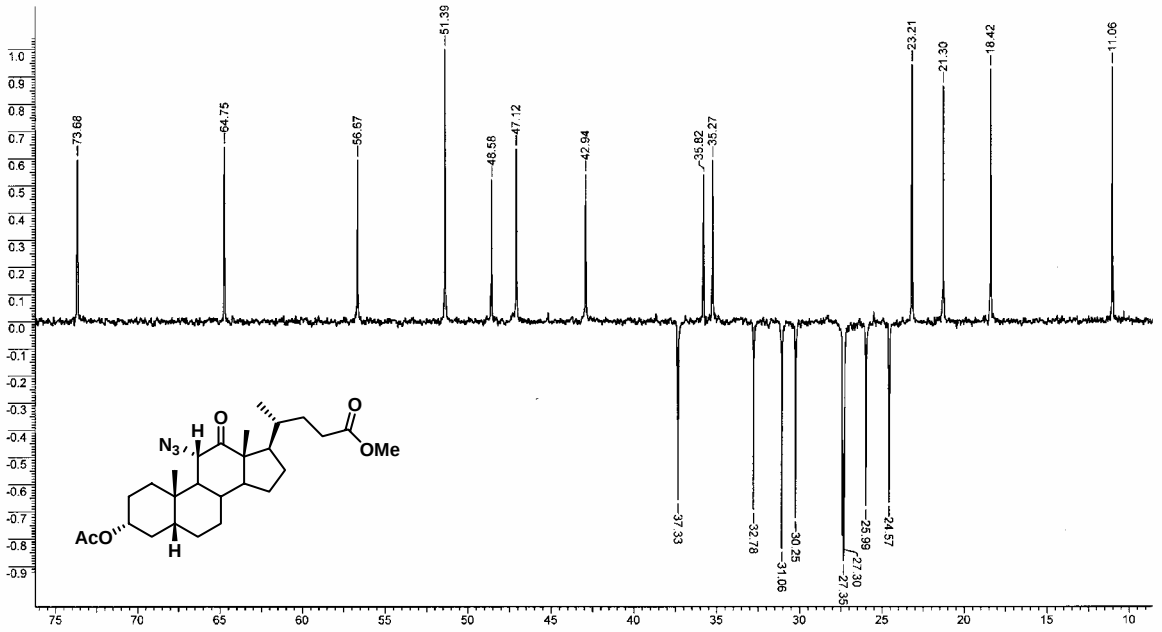

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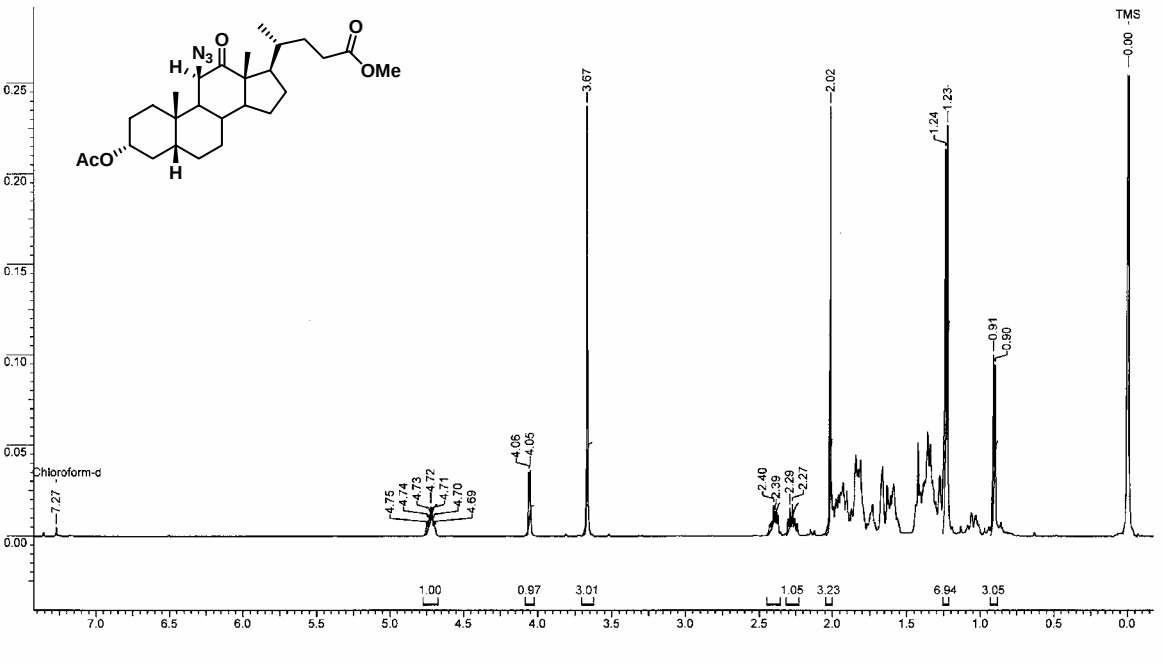

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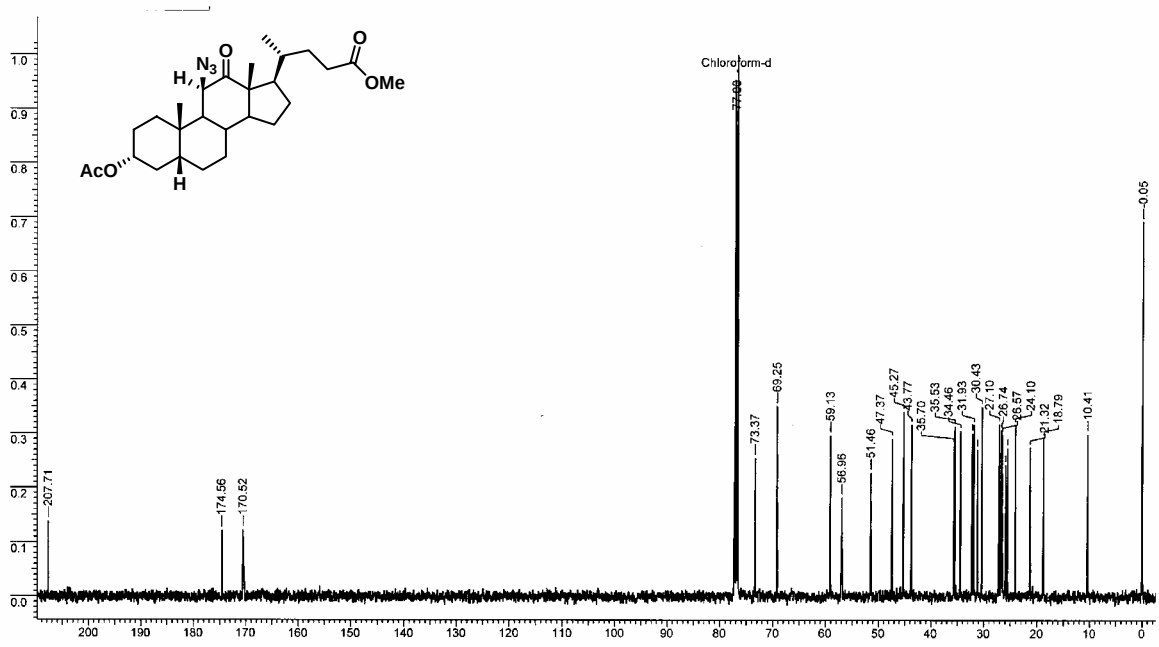

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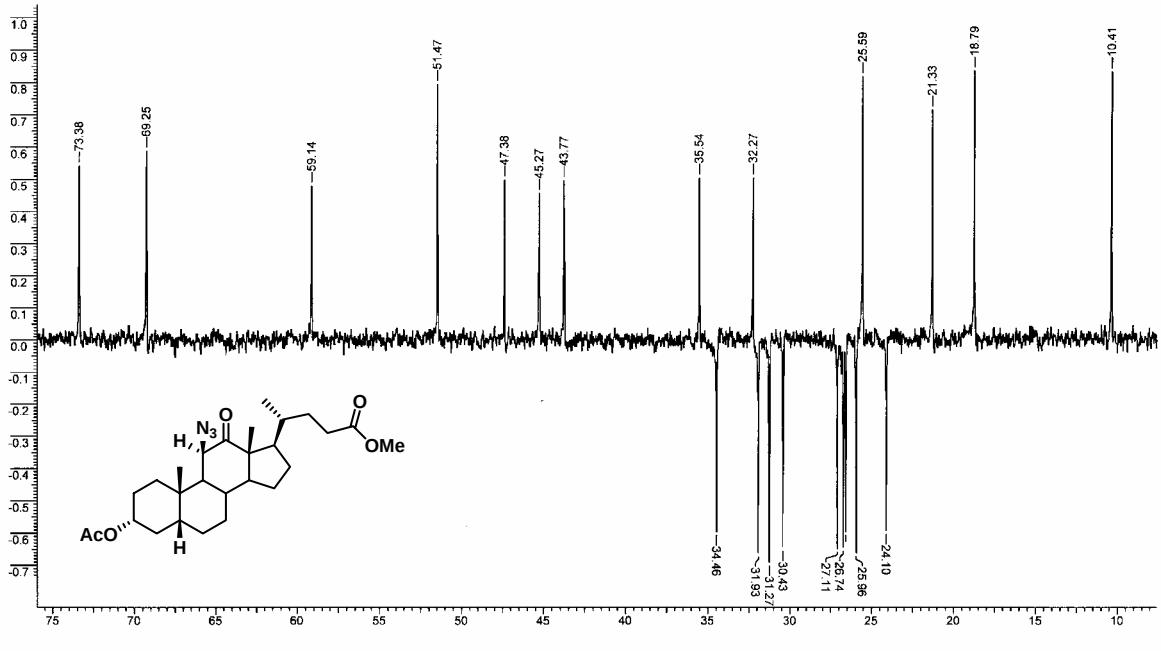

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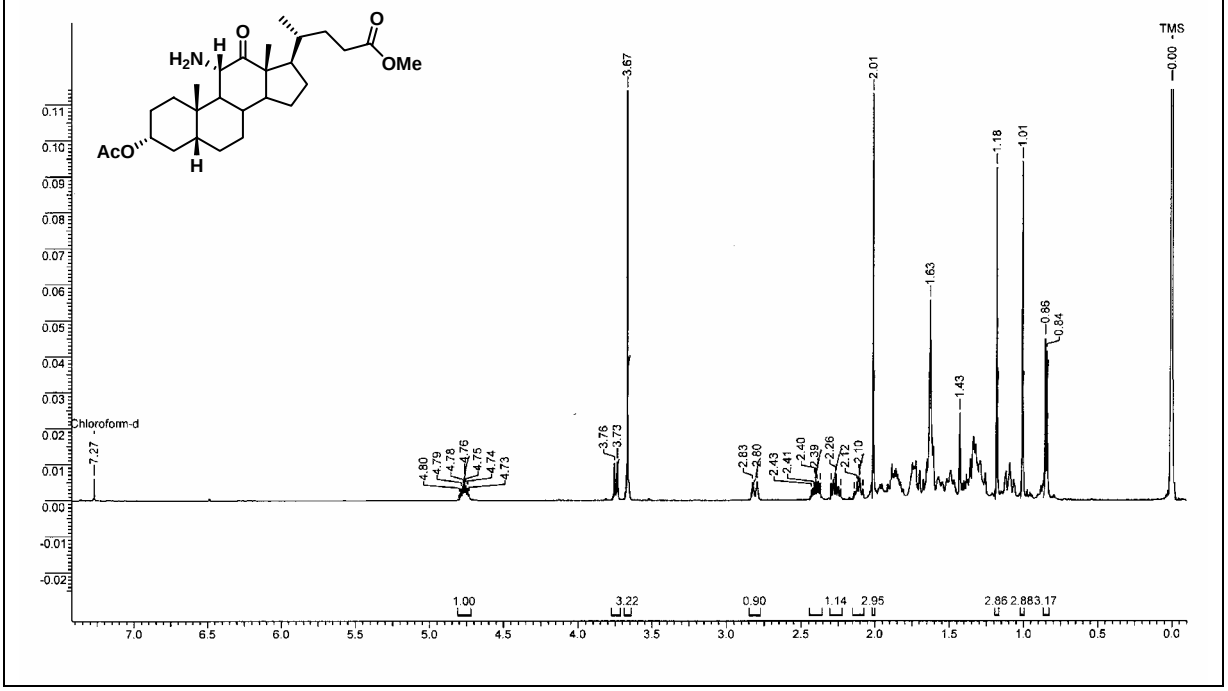

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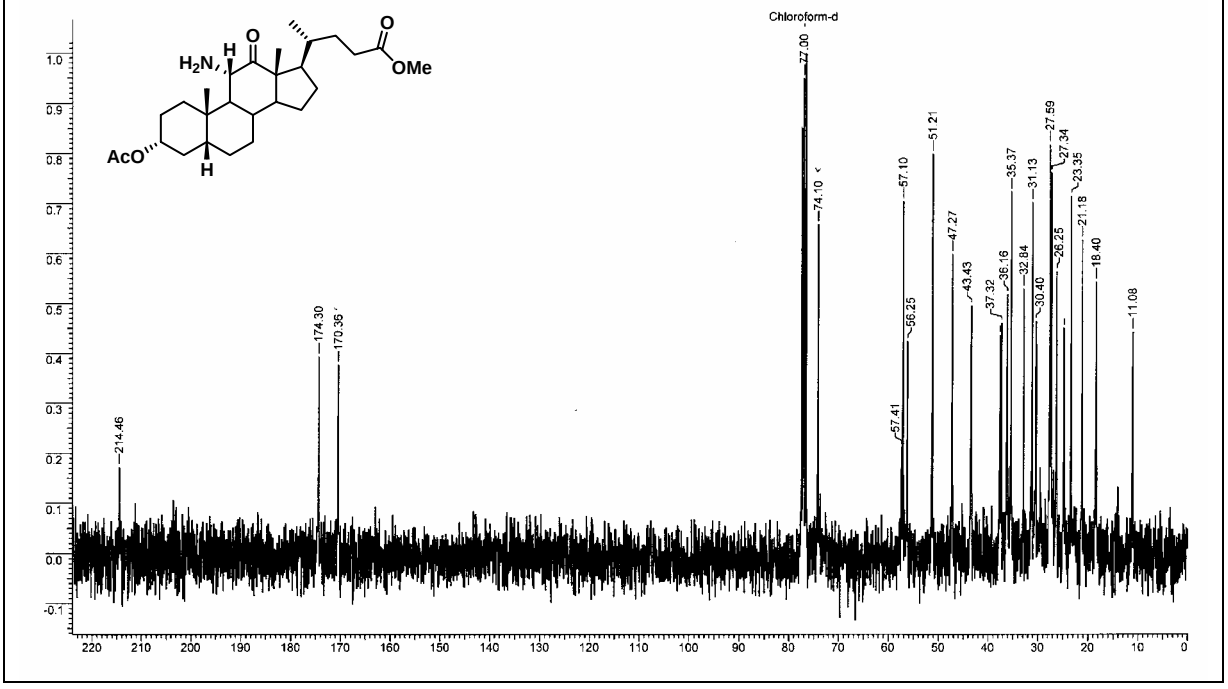

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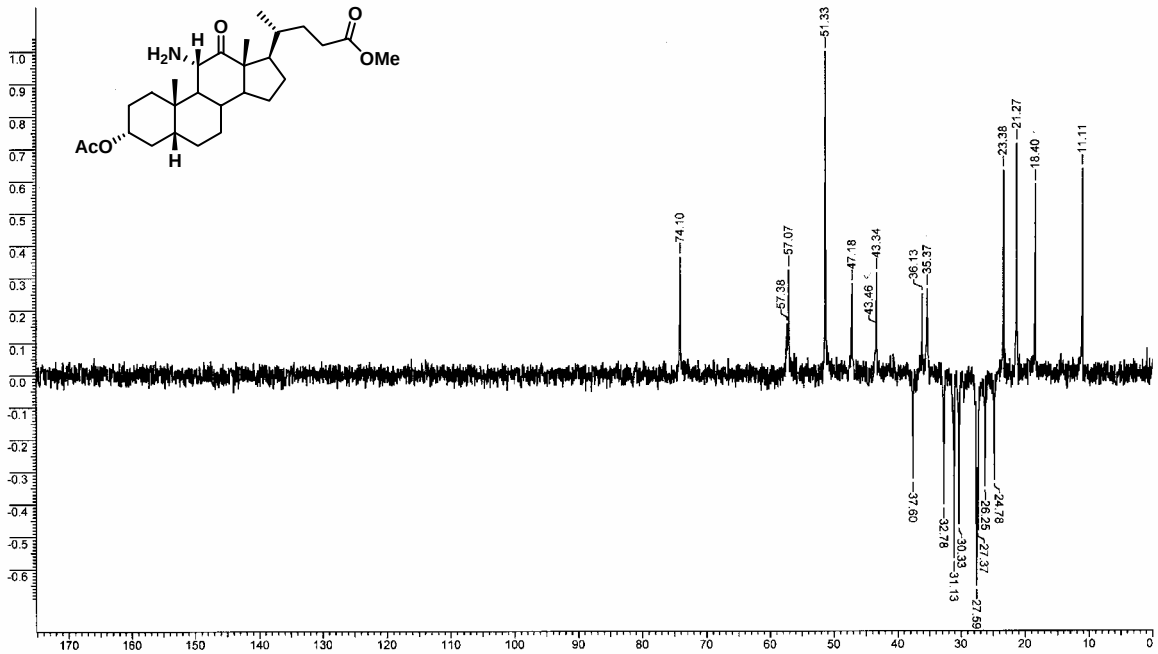

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PART B : CHAPTER - 2

Design, Synthesis and Bioevaluation of Novel Steroid-Amino Acid Conjugates

B2.1. Abstract

A generic structure wherein fine-tuning of the molecular amphiphilicity is possible, has been designed based on squalamine and polymyxin B (PMB). Tetrapeptide derived from glycine was attached at the C-3β position of the modified cholic acid to realize novel linear tetrapeptide-linked cholic acid derivatives. These linear tetrapeptide-linked cholic acid derivatives were planned to further elaborate to steroidal cyclopeptides, during this process a novel methodology for the palladium catalyzed simple and chemoselective one-pot reduction of alkyl azides followed by in situ amidation with the commercially available activated esters was developed. All the synthesized compounds were tested against a wide variety of microorganisms (Gram negative bacteria, Gram positive bacteria and fungi) and their cytotoxicity was evaluated against human embryonic kidney (HEK293) and human mammary adenocarcinoma (MCF-7) cell lines. While relatively inactive by themselves, these compounds interact synergistically with antibiotics such as fluconazole and erythromycin to inhibit growth of fungi and bacteria respectively.

B2.2. Introduction

The dramatically rising prevalence of multidrug-resistant microbial infections over the past few decades has become a serious health problem. In order to circumvent this increasingly serious situation, there is an urgent need to develop new antimicrobial therapeutics having high efficacy and low toxicity in resistant strains of infectious organisms. The class of membrane-disrupting drugs is ideal as antimicrobial agents because microbes are unlikely to develop resistance to them [1,2]. As the outer membrane or cell wall of microbes provides a protective barrier against many types of antibiotics [3], amphipathic molecules that can act synergistically with various hydrophobic antibiotics as outer membrane permeabilizers may represent a new class of antibiotic agents.

B2.2.1. *Design of novel amphipathic molecules*

A literature survey of antimicrobial steroids reveals that several amino cholesterol derivatives exhibit potent antimicrobial activity against Gram-positive, Gram-negative bacteria and yeast [4]. The *in vitro* antibacterial properties of bile acids against certain Gram-positive microorganisms are well known [5]. Consequently, the preparation of various bile acid-based aminosterols was reported with a view to examine their activity as anti-microbial agents [6]. Recent approach to combat against microorganism is to introduce a polycationic chain on to a steroid scaffold. One such chimeric natural product, namely squalamine [7] **1**, has attracted considerable attention because of its potent antimicrobial activity against a broad spectrum of microorganisms [8,9] (Figure 1). Soon after the isolation of squalamine, Regen reported [10] the construction of squalamine mimic **2**.

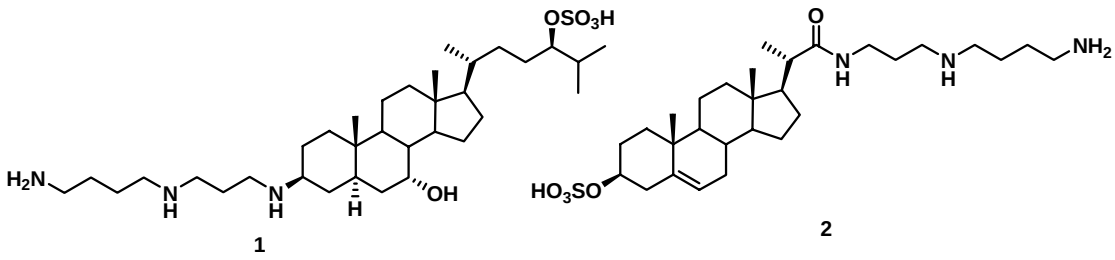


Figure 1. Structure of squalamine (**1**) and potent steroid-polyamine conjugate (**2**).

These results stimulated the synthesis of a wide range of squalamine analogues by several research groups [11]. Some of these analogues showed anti-bacterial activity similar to that of parent compound squalamine. Interestingly, variations in structure of analogues led to the changes in their spectrum of activity against a variety of bacteria and yeasts [12].

The structure and the biological significance of naturally occurring and synthetic polyamine and polypeptide steroid conjugates are well known [13]. Natural steroid-amino acid conjugates such as bufetoxin **3** and polymastiamide A **4** (Figure 2), exhibit *in vitro* antimicrobial activity [14]. The information about the steroid-amino acid conjugates having pharmacological applications was briefly summarized in the part A of this thesis (Section A.3, Page 14). Several peptides have been identified that increase the permeability of outer membranes of Gram-negative bacteria and sensitize these organisms to hydrophobic antibiotics [15]. These amphipathic peptides exhibit antibiotic, fungicidal, hemolytic, virucidal, and tumoricidal activities [16]. Although the exact mechanism by which they kill microbes is not clearly understood, it has been shown that peptide-lipid interactions leading to membrane permeation play a role in their activity [16]. The best studied of these peptides are PMB **5** derivatives. Savage and co-workers [17] designed a class of cationic steroid antibiotics (CSA) **6** as PMB mimics which display antibacterial activities comparable or superior to that of squalamine **1** or PMB **5** (Figure 2).

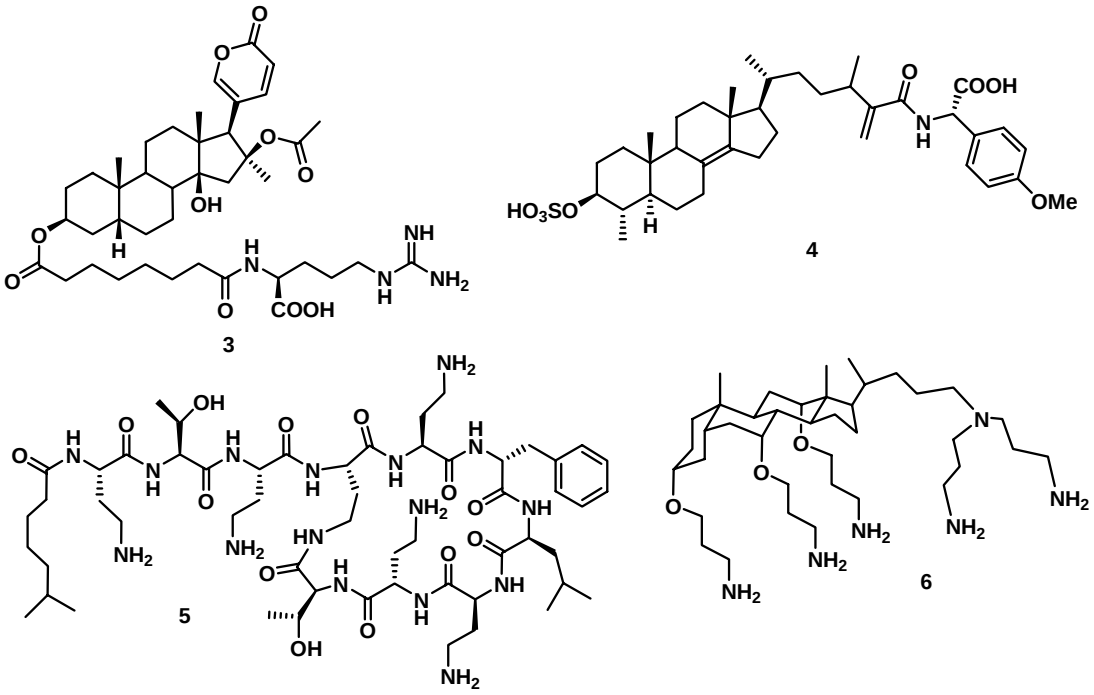


Figure 2. Steroid-amino acid conjugates **3** and **4**, PMB **5** and CSA **6**.

A number of intuitive assumptions can be drawn from this wide-ranging literature survey.

- A common feature of steroid derived antimicrobials is their potential to exhibit facially amphiphilic conformations containing polar and hydrophobic surfaces [18].
- Three common elements are required to obtain squalamine-type activities (i) a long and rigid hydrophobic unit; (ii) a flexible hydrophilic chain which is linked to the hydrophobic unit (the length of the polyamine chain is not important); (iii) a pendant polar head group (the precise structure of this group is also unimportant).
- The uncontrolled incorporation of polycationic or polyanionic structures on a steroid scaffold may result in cytotoxic molecules.
- The overall amphiphilicity of the molecule plays a key role in determining the level of antimicrobial activity.

With this in view, design of a generic structure with fine-tuning of the molecular amphiphilicity will lead to novel molecules. These molecules are expected to selectively permeabilize the outer membrane of the microbes. We have hypothesized that the cholic acid-polypeptide conjugate **8** could represent such a generic structure amenable to fine-tuning (Figure 3).

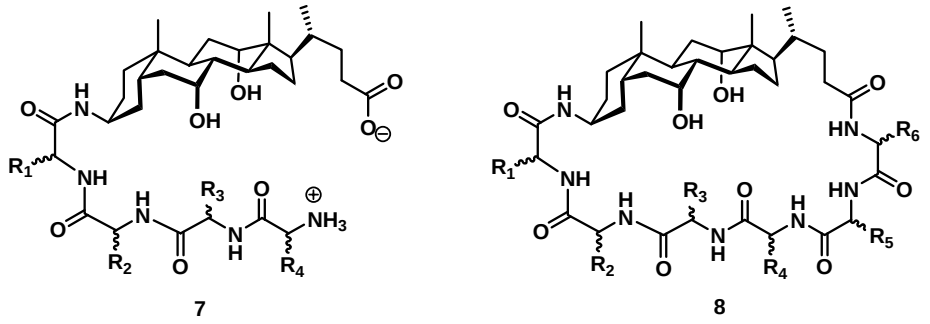


Figure 3. Fine-tunable generic structures **7** and **8**.

Cholic acid has been found to be very useful for the preparation of new pharmaceuticals and more recently for the preparation of peptide and protein drugs because of its natural amphiphatic nature [19]. However, it differs from conventional head-to-tail amphiphiles because the polar and non-polar domains are separated along the longitudinal axis of the molecule. This gives rise to distinct polar and non-polar faces [20].

Introduction of an amino acid or peptide on cholic acid backbone offers a combination of a hydrophilic functional moiety as well as a hydrophobic carrier in the same molecule. Synthetic variations of the amino acid residues can produce library of compounds with variable amphiphilicity. There are very few reports of steroidal polypeptides consisting of a repeating amino acid sequence in which each amino acid unit is attached to a sterol molecule [21]. It was considered that such compounds would be of both biological as well as chemical interest [14].

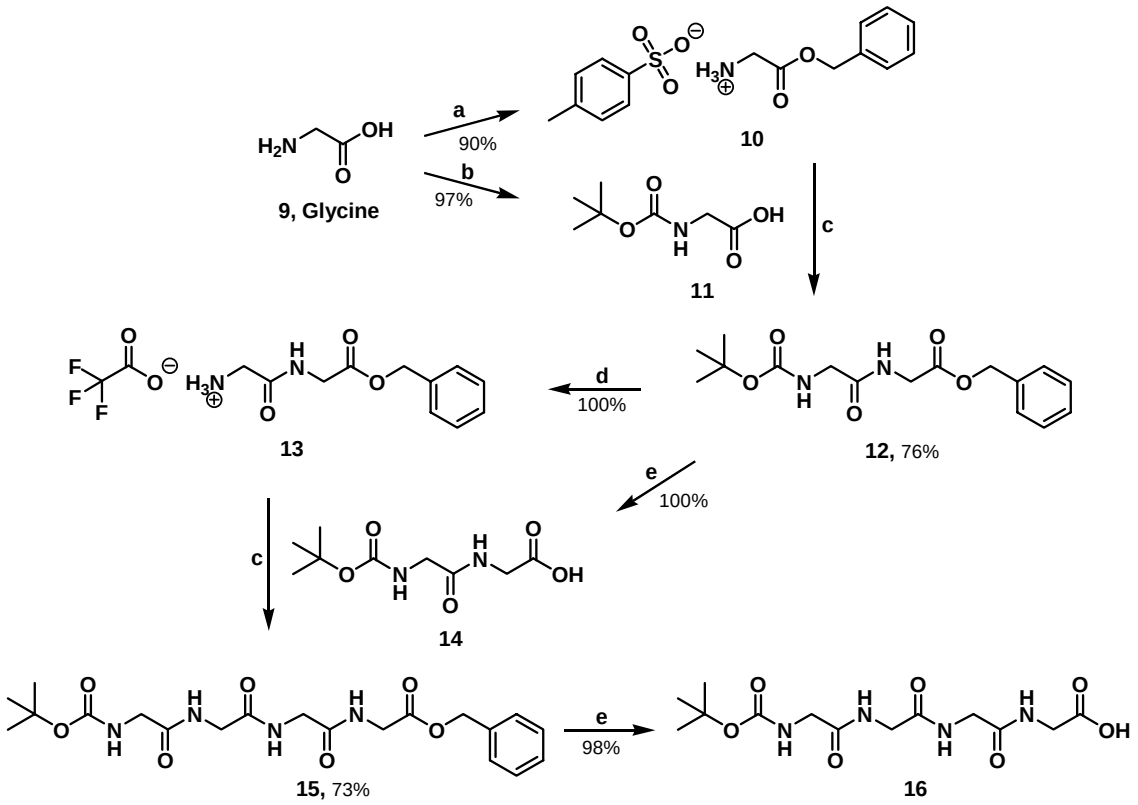
To support this hypothesis, novel cholic acid-tetrapeptide conjugates of glycine **23** and **24** were synthesized and were found to display remarkable synergistic activity with respect to fluconazole and erythromycin as documented herein for the first time.

B2.3. Chemistry

The history of steroidal peptide synthesis begins with the Bondi and Muller preparations of glycocholic and taurocholic acids from cholic acid azide [22]. Later, an alternate synthetic route based on the triformyl derivative of cholic acid chloride was employed [23]. Efficient synthesis of bile conjugates with the aid of dicyclohexyl carbodiimide [24] or through a mixed anhydride technique [25] is also reported. We undertook the synthesis and evaluation of a new family of cholic acid-polypeptide conjugates by exploiting a convergent approach, using classical solution phase synthesis.

The linear Boc-$(Gly)_4$-OBzl **15** was synthesized starting from the suitably protected monomers **10** and **11** (Scheme 1). The stepwise elongation was performed in dichloromethane (DCM) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) activation to furnish Boc-$(Gly)_2$-OBzl **12** in 76 % yield. For the preparation of tetrapeptide **16** the removal of the *tert*-butoxycarbonyl (Boc) group from peptide **12** was performed in trifluoroacetic acid (TFA/DCM) to get compound **13** and removal of the benzyl (Bzl) group by hydrogenolysis in MeOH in the presence of Pd/C (10 %) as catalyst to get compound **14**. Compound **15** was obtained by fragment condensation of **13** with **14** in DMF using EDCI activation in the presence of 1-hydroxybenzotriazole (HOBt) as catalyst. This tetrapeptide was purified by chromatography to furnish compound **15** as white solid in 73 % yield.

Scheme 1: Synthesis of polypeptide chain.



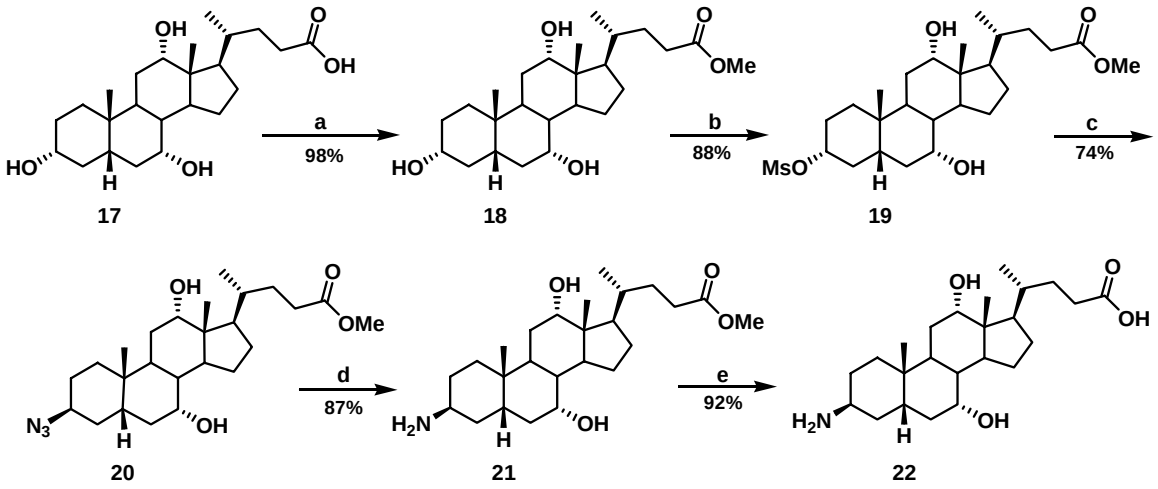
Reagents and conditions: a) Benzyl alcohol, *p*-TSA, toluene, reflux, 4 h; b) Boc anhydride, NaOH (1N), dioxane:water, 0-25 °C, 30 min; c) EDCI, HOBt, Et_3N, CH_2Cl_2, 0-25 °C, 6 h; d) TFA, CH_2Cl_2, 0-25 °C, 2 h; e) H_2, Pd-C, CH_3OH, 25 °C, 1 h.

The Bzl groups of Boc-$(Gly)_4$-OBzl **15** was removed by a similar catalytic hydrogenation reaction to furnish compound **16** in 98 % yield. Synthesis of Boc-$(Gly)_4$-OBzl **15** was previously reported using diethyl cyanophosphonate (DECP) as a coupling agent [26]. Following the modified procedure (using EDCI and HOBt) we have drastically reduced the reaction time (7-8 h) compared to the earlier reported method, which required three days for the reaction to complete [27].

Synthesis of the cholic acid intermediates, which are the building blocks for the realization of the desired conjugates, is depicted in Scheme 2. From a purely synthetic perspective the easiest way to functionalize cholic acid is to take advantage of its C-24

carboxylic acid moiety and controlled reactivity of C-3, C-7 and C-12 hydroxyl groups. Overnight stirring of cholic acid **17** in dry methanol (Chapter B1, Scheme 1) using a catalytic amount of *p*-TSA to get the methyl ester **18** followed by selective mesylation provided methyl 3α-mesyl-7α,12α-dihydroxy-5β-cholane-24-oate **19** in two steps, nucleophilic S_N2 type of displacement of 3α-mesyl functionality with sodium azide furnished methyl 3β-azido-7α,12α-dihydroxy-5β-cholane-24-oate **20** and hydrogenation of azido functionality using Pd-C furnished methyl 3β-amino-7α,12α-dihydroxy-5β-cholane-24-oate **21** as a white solid with an overall yield of 77 % in four steps and using only one final chromatographic purification [28]. Hydrolysis of C-24 methyl ester functionality of compound **21** using LiOH (2M solution in H_2O) in CH_3OH afforded a steroidal amino acid **22** in 92 % yield.

Scheme 2. Synthesis of steroid backbone.

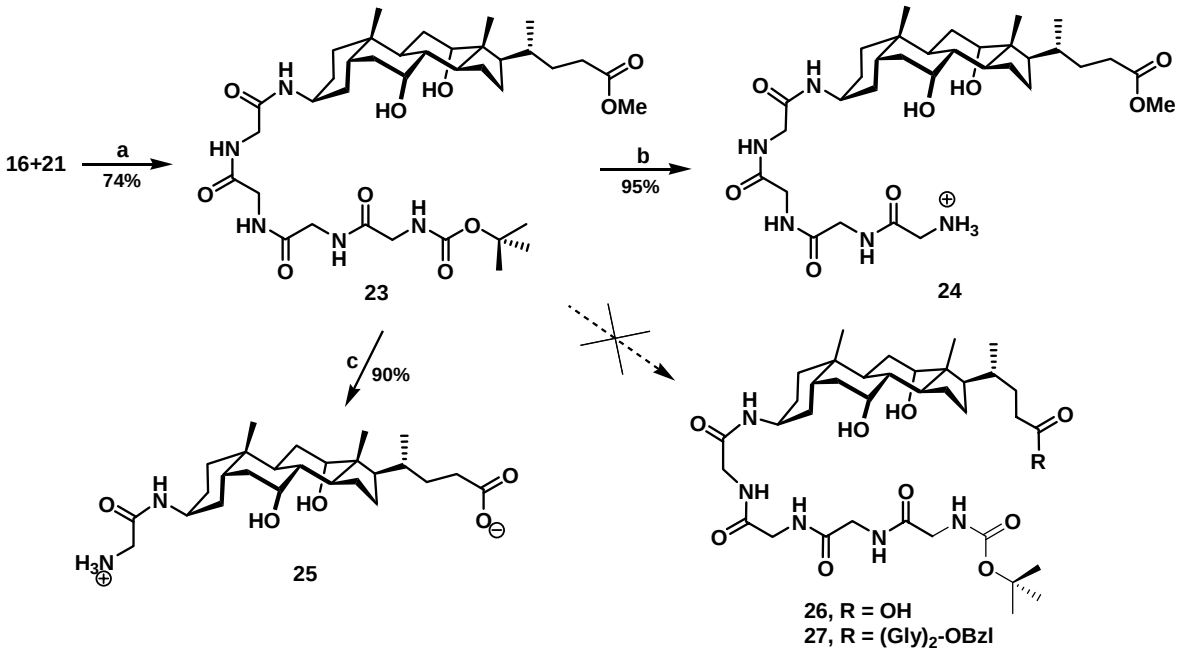


Reagents and conditions: a) CH_3OH, *p*TSA, 28 °C, 24h; b) MsCl, Et_3N, CH_2Cl_2, 0-25 °C, 6h; c) NaN_3, DMF, 60 °C, 12h; d) H_2, Pd-C, 40 psi, 25 °C, 4h; e) LiOH, MeOH, 0 °C, 12h.

Synthesis of cholic acid-tetrapeptide conjugates is depicted in Scheme 3. Coupling of 3β-amino cholic acid intermediate **21** (Scheme 2) with Boc-$(Gly)_4$-OH **16** (Scheme 1) under

mild condition using EDCI/HOBt and Et_3N in DMF provided compound **23** in 74 % yield. Subsequent cleavage of the Boc protecting group in compound **23** was accomplished with 2M HCl:Et_2O to afford free amino compound **24** in excellent yield.

Scheme 3.

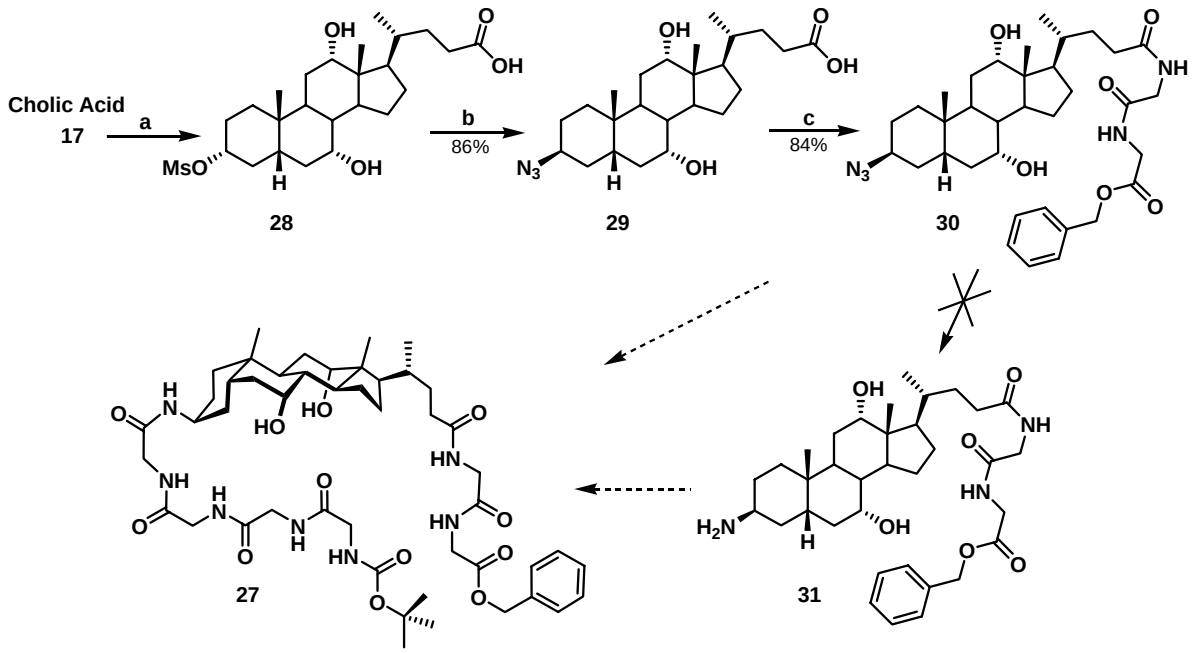


Reagents and conditions: a) EDCI, HOBt, Et_3N, DMF, 0-25 °C, 6h; b) 2M HCl:Et_2O, 0-25 °C, 1.5h; c) LiOH/KOH, CH_3OH, H_2O, 0-25 °C, 2-8h.

Several efforts for the chemoselective hydrolysis of the methyl ester functionality in compound **23** failed, indeed a zwitter ionic compound **25** was obtained in good yield. In this step the amide hydrolysis was observed during the acidic (5 % citric acid/dil. HCl/acetic acid) work up. The mass spectra of the reaction mixture before acidic work showed a required molecular ion peak for the hydrolysis product **26** {MS (LCMS) *m/z* 758.4 $[M+Na]^+$; HRESIMS *m/z* 758.4285 $[M+Na]^+$ ($C_{37}H_{61}N_5O_{10}Na$; calcd. 758.4316)}. The further reaction of this product with compound **13** (Scheme 1) produced a trace amount of desired steroidal heptapeptide **27** which was confirmed by mass spectral analysis {MS (LCMS) *m/z* 962.3 $[M+Na]^+$}. Consequently, synthesis of steroidal heptapeptide **27**, which

is an important intermediate for the realization of steroidal cyclic peptide, was not accomplished by this route. A second probable and more efficient approach for the synthesis of cyclic steroidal peptide is depicted in Scheme 4.

Scheme 4.



Reagents and conditions: a) MsCl, Py, 25 °C, 1 h; b) NaN_3, DMF, 100 °C, 24 h, 86 % in two steps; c) **13**, EDCI, HOBt, Et_3N, DMF, 0-25 °C, 6 h.

In this approach our important object was to reduce the number of steps by employing novel methodology for chemoselective reduction of 3β-azido functionality. The 3α-hydroxyl group in cholic acid **17** was selectively mesylated using MsCl in pyridine. The crude mesylate **28** was further subjected for substitution reaction by NaN_3. Little harsh reaction condition (100°C) and longer reaction time (24 h) were required to substitute the 3α-mesyl functionality of compound **28** as compared to that of **19** (Scheme 2). Azido cholic acid-digly conjugate **30** was obtained by coupling azido acid **29** with digly **13** (Scheme 1) using EDCI/HOBt and Et_3N in DMF. Compound **30** was obtained with an overall 72 % yield starting from choilc acid **17** within three steps and a single column chromatographic

purification. The next goal was to achieve the one-pot amidation of the 3β-azido functionality in compound **30** with Boc-$(Gly)_4$-OH **16**. A systematic literature survey for the amidation of carboxylic acid with azide was carried out to find out the rational behind the projected methodology.

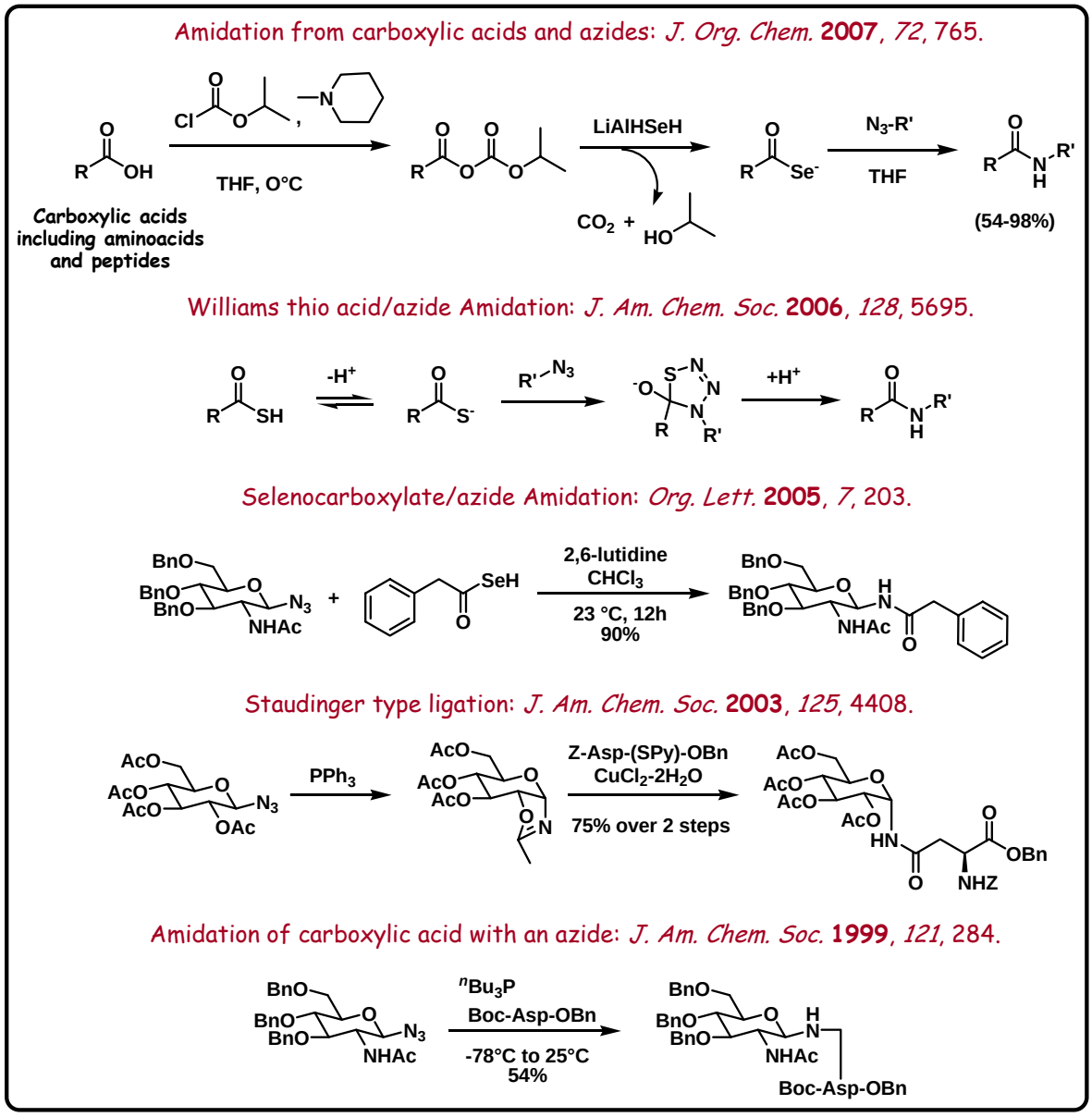


Figure 4. One-pot amidation of carboxylic acids with azides.

A chemoselective facile one-pot procedure for the coupling of carboxylic acid and azide *via* selenocarboxylate and selenatriazoline was developed and successfully applied to the coupling of amino acids and peptides with azides by Hu *et al* [29] (Figure 4). By treatment with Woollins' reagent in toluene, carboxylic acids were converted to selenocarboxylic acids by Knapp and co-workers [30]. The latter reacts in situ with azides to provide amides. A one-pot procedure for the conversion of carboxylic acids to *N*-acyl sulfonamides, *via* thio acid/azide amidation, was presented recently by Williams *et al* [31]. *N*-Acyl sulfonamide synthesis on solid support, peptide thio acid/sulfonazide coupling, and *N*-alkyl amide synthesis *via* selective cleavage of sulfonyl from an *N*-alkyl-*N*-acyl sulfonamide were also reported by him. A new chemical ligation method in which a phosphinobenzenethiol was used to link a thioester and azide was demonstrated by Raines and co-workers [32]. Transformation of azido-group to *N*-(*t*-butoxycarbonyl)amino group under mild conditions *via* Staudinger reaction by Afonso [33] and a one-pot synthesis of glycosyl amides from glycosyl azides using a modified Staudinger reaction was demonstrated independently by Inazu [34] and Boullanger [35] research groups. Stereoselective synthesis of α- and β-glycosylamide derivatives from glycopyranosyl azides *via* isoxazoline intermediates was also reported [36].

Palladium catalyzed single-step conversion of azides [37], benzyl carbamates [38], *N*-benzyl, *N*-trityl or *N*-diphenylmethyl amines [39] to *t*-butyl carbamates using hydrogen [40] or triethylsilane [41] or polymethylhydrosiloxane (PHMS) [38,39] was reported by several researchers (Figure 5). Based on this literature survey we planned, palladium catalyzed simple and chemoselective one-pot reduction of alkyl azides followed by in situ amidation with the commercially available activated esters.

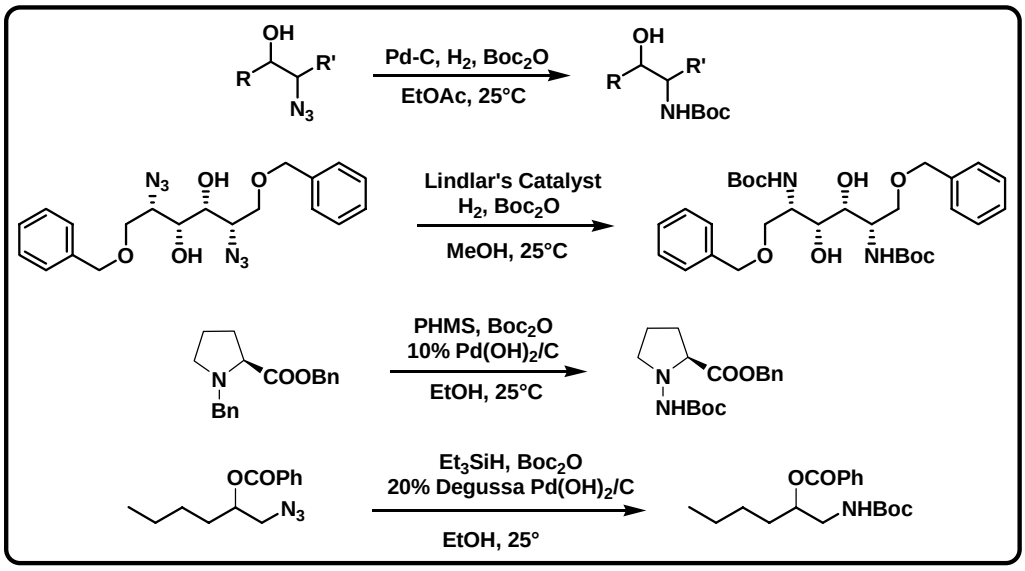
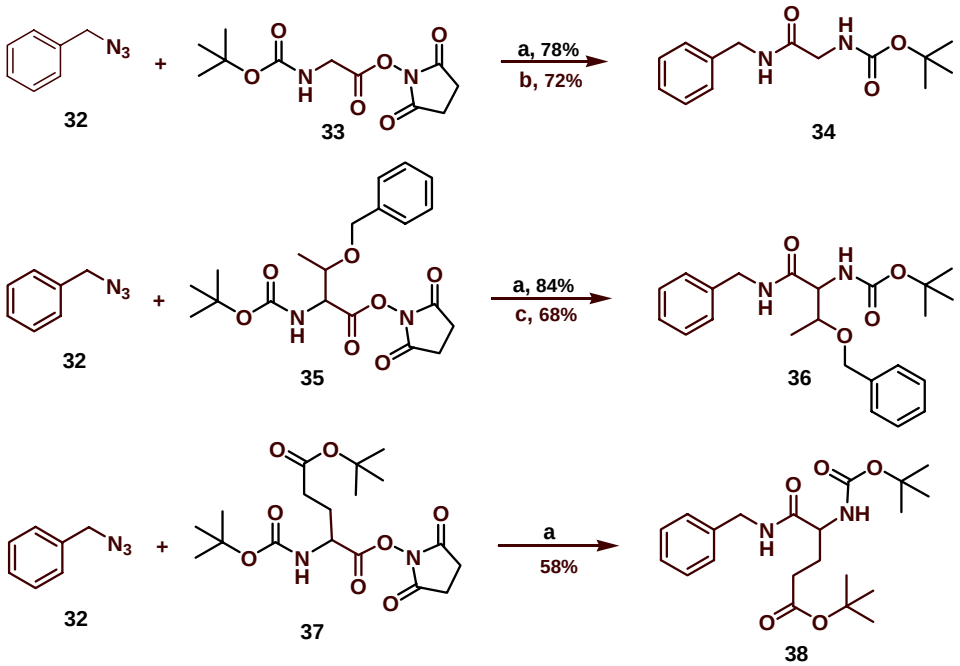


Figure 5: Reductive transformation of azides to *N*-Boc amines.

Scheme 5.

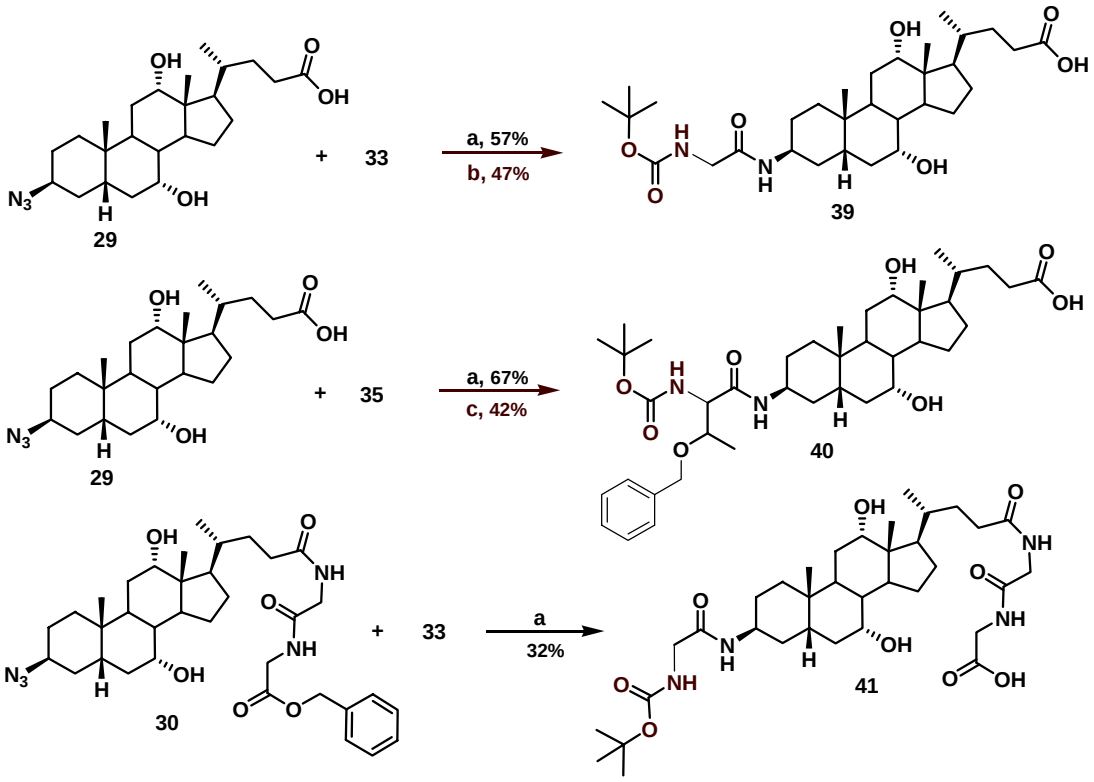


Reagents and conditions: a) Pd-$BaSO_4$, H_2, THF, 25 °C, 18 h; b) Pd-$BaSO_4$, H_2, CH_3OH, 25 °C, 1 h; c) Pd-$BaSO_4$, PHMS, EtOH, 25 °C, 20 min.

The first successful result for the amidation of benzyl azide **32** with Boc-Gly-OSu **33** was obtained using Pd-$BaSO_4$ in THF to furnish compound **34** (Scheme 5). THF was

preferred over CH_3OH to prevent the possible transesterification of the activated esters. Chemoselectivity was also achieved for the reduction of azides over benzyl ethers (compound **36**). This methodology was successfully utilized for the amidation of steroidal azides **29** and **30** (Scheme 6). Unfortunately we could not achieve the required chemoselectivity for the reduction of azides over benzyl esters, as the benzyl ester in compound **30** was hydrogenolyzed under the present reaction conditions.

Scheme 6.



Reagents and conditions: a) Pd-$BaSO_4$, H_2, THF, 25 °C, 18 h; b) Pd-$BaSO_4$, H_2, EtOH, 25 °C, 30 min; c) Pd-$BaSO_4$, H_2, CH_3OH, 25 °C, 30 min.

The longer reaction time may be one of the reasons for the observed hydrogenolysis of the benzyl ester functionality. To reduce the reaction time we planned to use methanol/ethanol as a solvent. For this the stability of the *N*-hydroxy succinimide ester in compound **33** was verified by simply stirring the activated ester in CH_3OH/EtOH. The ester

33 was found to be stable in CH_3OH/EtOH for the period of about 12 hrs. We observed methanolysis of compound **33** at elevated temperatures as well as when the reaction time was more than 12 hrs. This suggests that CH_3OH/EtOH can be utilized for the reaction provided the reaction completes within 10-12 hrs. The first trial experiment for the amidation of benzyl azide **32** with Boc-Gly-OSu **33** in MeOH using H_2 balloon produced the desired amide **34**. Moreover PHMS was also used effectively as a hydrogen source for the amidation of benzyl azide with compound **33** in EtOH. The amidation of compound **29** with Boc-Gly-OSu **33** in EtOH or Boc-Ser(OBz)-OSu **35** in CH_3OH produced the desired amides **39** and **40** respectively. Unfortunately, the amidation of compound **30** with Boc-Gly-OSu **33** in CH_3OH/EtOH was unsuccessful and produced complex reaction mixtures.

Lindlar catalyst mediated one-pot chemoselectie conversion of azides to *t*-butyl carbamates in the presence of benzyl esters was demonstrated previously by Baskaran and co-workers [40]. Total synthesis of desired steroidal cyclopeptide **8** using this methodology and continuation of this work with the use of Lindlar catalyst is underway in our laboratory.

B2.4. Bioevaluation

B2.4.1. *Antimicrobial activity*

Cholic acid **17**, 3β-amino steroids **21** and **22** and cholic acid-tetrapeptide conjugates **23** and **24** were examined for antimicrobial activity against a wide variety of microorganisms (fungal strains *viz.*, *Candida albicans*, *Cryptococcous neoformans*, *Benjaminiella poitrasii*, *Yarrowia lipolytica, Fusarium oxysporum* and bacterial strains *Escherichia coli* and *Staphylococcus aureus*) to find out MIC (minimum inhibitory concentration) and IC_{50} (half maximal inhibitory concentration). The MIC and the IC_{50} are

presented in Table 1. To characterize synergism of the synthesized compounds with fluconazole (an antifungal agent) and erythromycin (a hydrophobic antibacterial agent), we determined the concentrations of the cholic acid derivatives necessary to lower the MIC values of these antibiotics to 1 μg/mL {a concentration at which many clinically useful antibiotics are active}. This measurement entailed incubating a known population of yeast suspension (*C. albicans*) for 48 h in YPG and bacterial cells (*E. coli*) for 24 h in a nutrient broth with fluconazole and erythromycin, respectively, with incrementally varied concentrations of the synthesized compounds as described [15a] by Savage *et al*.

Unexpectedly, none of the tested compounds demonstrated appreciable activity when tested for their ability to act as antifungals or antibacterials alone (Table 1). No significant inhibitory effects by any of these compounds were observed against a pathogenic fungus *C. neoformans* and a saprophyte *Y. lipolytica* (columns B and D, Tables 1). Cholic acid **17** and 3β-amino cholic acid derivatives **21** and **22** did not show any remarkable antifungal or antibacterial effects. By comparison, the tetrapeptide-linked cholic acid derivatives showed good to moderate activity against the fungal strains of *C. albicans*, *B. poitrasii*, *F. oxysporum* and the bacterial strain of *S. aureus* (columns A, C, E and G; entries 4 and 5) whereas these compounds are less active towards *E. coli* (column F) than *S. aureus* (columns G). The antifungal activity of most of the compounds showing some activity was found to be similar to that of fluconazole (32 μg/mL). The difference in the toxicity of the synthesized compounds against a wide variety of microorganisms can be attributed to the differences in their cell wall/cell membrane compositions which affect the passage of these compounds through cell wall/cell membrane.

Squalamine offers termini of opposite charges with a hydrophobic core, whereas the target compounds **23** and **24** offers non-conventional head-to-tail amphiphilic conformations with distinct polar and non-polar faces. These kind of amphiphiles can permeabilize the outer membranes of microbes.

Table 1: Antimicrobial activity: Determination of MIC and IC_{50}

Entry	Compound Number	Antimicrobial Activity $\frac{MIC}{IC_{50}}$ (μg/mL)						
		Fungal Strains					*Bacterial Strains*	
		A	**B**	**C**	**D**	**E**	**F**	**G**
1	**17**	>128 128	>128 >128	128 96	>128 >128	>128 64	>128 64	>128 64
2	**21**	**64** **32**	>128 100	>128 64	>128 >128	>128 72	>128 96	>128 64
3	**22**	**64** **32**	>128 >128	>128 96	>128 >128	128 64	>128 64	>128 128
4	**23**	**16** **4**	>128 >128	**64** **32**	>128 128	**64** **32**	**96** **32**	**32** **16**
5	**24**	**32** **8**	>128 >128	**64** **48**	>128 >128	128 56	>128 64	96 32
6	**Amp**[a]	2 0.5	16 8	16 8	16 8	16 8	NT	NT
7	**Flu**[b]	32 4	32 16	32 16	64 32	8 4	NT	NT
8	**Tet**[c]	NT	NT	NT	NT	NT	8 1	16 4
9	**Ery**[d]	NT	NT	NT	NT	NT	64 16	32 16

A, *C. albicans*; **B**, *C. neoformans*; **C**, *B. poitrasii*; **D**, *Y. lipolytica*; **E**, *F. oxysporum*; **F**, *E. coli*; **G**, *S. aureus*; NT, Not Tested. [a]Amphotericin B, [b]Fluconazole, [c]Tetracycline, [d]Erythromycin.

With this idea in mind compounds **17** and **21-24** were also tested for their ability to permeabilize the outer membrane of Gram negative bacteria such as *E. coli* causing sensitization to hydrophobic antibiotics that inefficiently cross the outer membrane. We also demonstrated such permeabilization by cholic acid derivatives with *C. albicans* a pathogenic fungus. The MICs of compounds **21** to **24** with respect to *C. albicans* was measured to be inbetween 16-64 μg/mL whereas the concentration of these compounds required to lower

the MIC of fluconazole from 32 μg/mL to 1 μg/mL was within the range of 4-8 μg/mL in the same strain (Table 2). Similerly, The MICs of compounds **21** to **24** with respect to *E. coli* was measured to be >128 μg/mL whereas the concentration of these compounds required to lower the MIC of erythromycin from 64 μg/mL to 1 μg/mL was within the range of 12-24 μg/mL in the same strain. A profound effect of the charge on the termini of the conjugate molecules can be noticed if we compare the synergism of compound **21** with that of compounds **22** or **23** with that of compounds **24**.

Table 2: MIC and FIC data for **B1.18** and **B2.15-18** with *C. albicans* and *E. coli*.

Compound Number	a (μg/ml)	a' (μg/ml)	a"	b (μg/ml)	b' (μg/ml)	b"
17	>128	64	<0.53	>128	64	<0.52
21	64	8	0.156	>128	16	<0.14
22	64	4	0.094	>128	12	<0.11
23	16	8	0.53	96	24	<0.265
24	32	6	0.22	>128	12	<0.11

a: MIC of the synthesized compounds against *C. albicans*.
a': Concentration of the synthesized compounds required to lower the MIC of fluconazole from 32 μg/mL to 1 μg/mL (a concentration at which many clinically useful antibiotics are active).
a": FIC values with fluconazole.
b: MIC of the synthesized compounds against *E. coli*.
b': Concentration of the synthesized compounds required to lower the MIC of erythromycin from 64 μg/mL to 1 μg/mL.
b": FIC values with erythromycin.

To quantify the synergistic behaviour of our compounds with fluconazole and erythromycin, fractional inhibition concentration (FIC) values were calculated [42] (Table 2). An FIC value is a standard measure of the ability of two antibiotics to inhibit microbial growth synergistically and is defined as FIC = $[A]/MIC_A + [B]/MIC_B$ where [A] and [B] are the concentrations of compounds A and B that in combination inhibit bacterial growth and MIC_A and MIC_B are the MICs of compounds A and B, respectively. Synergism between antibiotics is indicated by FIC values of less than 0.5 [42]. In the present case all the

synthesised compounds display FIC values of less than 0.5 with both fluconazole and erythromycin (Table 2). Many of the FICs shown in Table 2 are comparable to those reported for PMB derivatives [15b]. In conclusion, compounds **21**, **22** and **24** are effective permeabilizers of the outer membranes of fungi and bacteria.

B2.4.2. *Antiproliferative activity*

Bile acids are known to be extremely toxic at high doses, presumably damaging cell membranes and mitochondrial membranes [43]. At low doses, they are known to stimulate cell-signalling effects involving various cell signalling pathways [44]. They are also known to promote proliferation and metastasis of cells of cancer origin and inhibit the proliferation of cells of non-cancer origin [45]. Hence we tested the cytotoxicity of the synthesized compounds for two different cell lines, one of cancer origin MCF-7 and the other of non-cancer origin HEK293. The cytotoxicity of the synthesized compounds against HEK293 and MCF-7 cells was evaluated using the MTT assay [46]. Percentage of cell death at 10 μM concentaration and 50 % inhibitory concentrations (IC_{50}) are presented in Table 3.

Table 3: Antiproliferative activity.

Entry	Compound Number	Cytotoxicity			
		% cell death[¶]		IC_{50} (μM)	
		HEK293	MCF-7	HEK293	MCF-7
1	**17**	20	0	50	>1000
2	**21**	26	0	75	20
3	**22**	19	0	400	800
4	**23**	17	0	300	600
5	**24**	13	68	500	80

[¶] % of cell death at 10 μM concentration.

Compound **21** displayed significant cytotoxic activity at 75 and 20 μM, respectively, in both the cell lines tested. Incorporation of peptide residue on this compound drastically reduced

their cytotoxicities. At 10 μM concentration almost all the tested compounds are non toxic to the MCF-7 cell lines, except compound **24**. The IC_{50} values are in the range of 80-800 μM for HEK293 as well as MCF-7 cell lines. Moreover, it was found that all the compounds except compound **24** enhanced the proliferation of MCF-7 cells and not HEK293 cells. This confirms the observation that cholic acid and its derivatives promote the proliferation of cells from cancerous origin and not normal cells. This study thus confirms that the synthesized cholic acid-peptide conjugates are not toxic to human cells up to 75 μM concentrations.

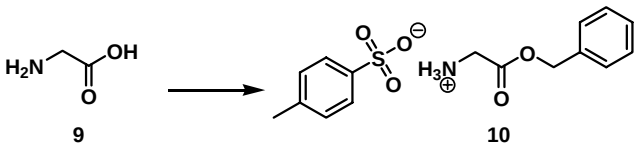
B2.5. Summary

Molecular amphiphilicity is one of the important factor which accounts for the easy transport of the molecules through the membranes, as a result a generic structure wherein fine-tuning of the molecular amphiphilicity is possible, have been designed utilizing amphiphilic nature of cholic acid. To realize a designed generic structure, tetra-peptide derived from glycine was linked at C3β-position of modified cholic acid. For the first time a palladium catalyzed simple and chemoselective one-pot reduction of alkyl azides followed by in situ amidation with the commercially available activated esters is demonstrated. The synergism of the most active compounds with fluconazole and erythromycin greatly improves the activity of these antibiotics against *C. albicans* and *E. coli* respectively. Because the synthesized modified steroids act synergistically with unrelated hydrophobic antibiotics, these compounds most likely act as outer membrane permeabilizers. In summary, we have demonstrated that the molecules having optimum amphiphilicity can produce potent sensitizers of Gram-negative bacteria and fungi. Extension of this work with

direct incorporation of cationic antimicrobial peptides on cholic acid scaffold and variation of amino acid residues in the proposed generic structures **7** and **8** is in progress.

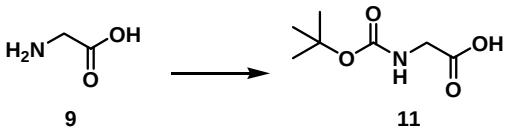
B2.6. Experimental Section

B2.6.1. *H-Gly-OBzl·pTSA (10):*



Glycine **9** (18.8 g, 2.50 mol) and *p*-toluenesulfonic acid (*p*-TSA) (monohydrate, 48.5 g, 2.25 mol) were added to a mixture of freshly distilled benzyl alcohol (100 mL) and toluene (50 mL) in a 500 mL round-bottom flask. The mixture was heated to reflux and the water formed in the reaction was trapped in a Dean-Stark receiver. When no more water appeared in the distillate the mixture was allowed to cool to room temperature, diluted with ether (500 mL) and cooled in an ice-water bath for two hours. The crystalline *p*-toluenesulfonate of glycine benzyl ester was collected by filtration, washed with ether, air dried and used without further purification [47].

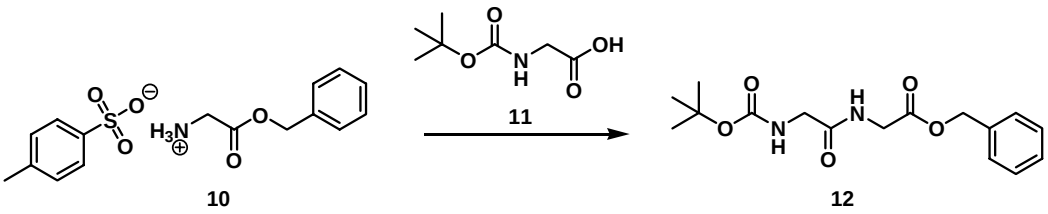
B2.6.2. *Boc-Gly-OH (11):*



A solution of glycine **9** (0.75 g, 10 mmol) in a mixture of dioxane (20 mL), water (10 mL) and 1N NaOH (10 mL) was stirred and cooled in an ice-water bath. Di-*tert*-butyl dicarbonate (Boc anhydride) (2.4 g, 11 mmol) was added and stirring was continued at room temperature for 30 min. The solution was concentrated under vacuum to about 10 to 15 mL,

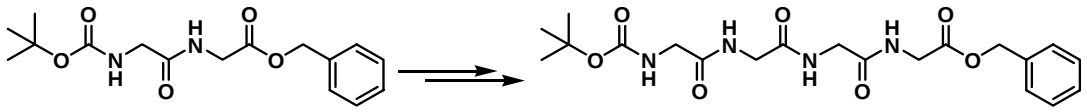
cooled in an ice-water bath, covered with a layer of ethyl acetate (30 mL) and acidified with a dilute solution of $KHSO_4$ to pH 2-3. The usual work up afforded crude product which was used without further purification [47].

B2.6.3. ***Boc-Gly-Gly-OBzl (12):***



H-Gly-OBzl·*p*TSA **10** (3.37 g, 10 mmol), Boc-Gly-OH **11** (1.75 g, 10 mmol) and 1-hydroxybenzotriazole (HOBt) (0.68 g, 5 mmol) were dissolved in dry CH_2Cl_2 (80 mL) and the reaction mixture was cooled to 0 °C under argon atmosphere. EDCI (2.1 g, 11 mmol) and Et_3N (2.02 g, 20 mmol) were added. The reaction mixture was allowed to warm to room temperature and was stirred for 6 h. The solvent was evaporated and the residue was dissolved in ethyl acetate (200 mL). The organic phase was washed successively with H_2O, 5 % citric acid, H_2O, sat. $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was recrystallized from MeOH·Et_2O to give dipeptide **12** as colorless crystals (2.44 g, 76 %). Mp. 78-79 ºC (lit. colorless oil [26]); IR ν_{max} (neat) 3321, 2982, 2935, 1752, 1716, 1682, 1526 cm^{-1}; ^1H NMR ($CDC1_3$, 400 MHz) δ 7.28-7.33 (m, 5H), 6.89 (bs, 1H), 5.36 (bs, 1H), 5.13 (s, 2H), 4.04 (d, 2H, *J* = 5.4 Hz), 3.81 (d, 2H, *J* = 5.3 Hz), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.9, 169.6, 156.0, 135.1,128.6, 128.5, 128.3, 80.2, 67.2, 44.1, 41.2, 28.3 (3C); FABMS (NBA-NaI) *m/z* 323 $[M+H]^+$.

B2.6.4. ***Boc-(Gly)$_4$-OBzl (15):***

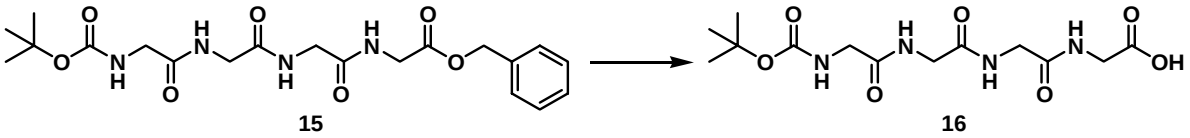


12 **15**

Boc-Gly-Gly-OBzl **12** (1.6 g, 5 mmol) was added to a mixture of TFA and CH_2Cl_2 (1:1, 5 mL) at 0 °C and the reaction mixture was stirred for 1 h at 0 °C and additionally for 1 h at room temperature. The solvent was evaporated and the H-Gly-Gly-OBzl·TFA **13** obtained was used without further purification. 10 % Pd/C (0.18 g) was added to the solution of compound **12** (1.6 g, 5 mmol) in CH_3OH and the reaction mixture was hydrogenated for 1 h at 40 psi pressure. Pd/C was removed by filtration and the solvent was evaporated. This residue of compound **14** was dissolved in dry DMF (50 mL) under an argon atmosphere and the solution was cooled to -10 ºC. HOBt (337 mg, 2.5 mmol) and EDCI (1.09 g, 5.5mmol) were added and stirring was continued for 15 min. The H-Gly-Gly-OBzl·TFA **13** obtained above and triethylamine (1.01 g, 10 mmol) were added and the reaction mixture was allowed to warm to room temperature and was stirred for 6 hrs. The solvent was evaporated and the residue was dissolved in ethyl acetate (200 mL). The organic phase was washed successively with H_2O, 5 % citric acid, H_2O, saturated $NaHCO_3$ and brine, dried with anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by flash chromatography using neutral alumina (CH_2Cl_2/CH_3OH, 19:1) and further recrystallized using MeOH/iPr$_2$O to afford Boc-(Gly)$_4$-OBzl **15** (1.58 g, 73 %) as a white powder. Mp. 173-174 ºC. (lit. [26] 174-175 ºC); IR ν_{max} (KBr) 3360, 3306, 2980, 2934, 1740, 1690, 1650, 1533 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.33 (t, 1H, J = 5.9 Hz), 8.21 (t, 1H, J = 5.7 Hz), 8.06 (t, 1H, J = 5.7 Hz), 7.33-7.42 (m, 5H), 7.03 (t, 1H, J = 5.9 Hz), 5.19 (s, 2H), 3.92 (d, 2H, J = 5.9 Hz), 3.76 (d, 4H, J = 5.7 Hz), 3.60 (d, 2H, J = 5.9 Hz), 1.40 (s, 9H); ^{13}C

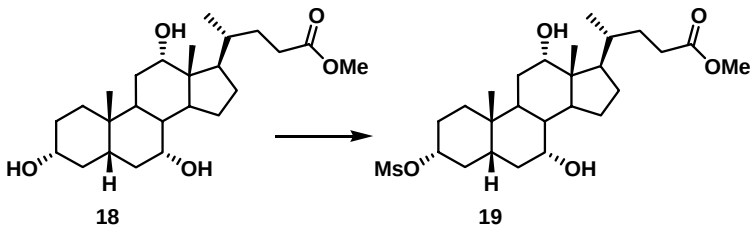
NMR (DMSO-d_6, 100 MHz) δ 169.8, 169.7, 169.4, 169.2, 155.9, 136.0, 128.5 (2C), 128.1, 128.0 (2C), 78.2, 65.9, 43.3, 42.1, 41.8, 40.7, 28.2 (3C); FABHRMS (NBA) *m/z* 437.2044 (M+H, $C_{20}H_{28}N_4O_7$ requires 437.2036).

B2.6.5. ***Boc-(Gly)$_4$-OH (16):***



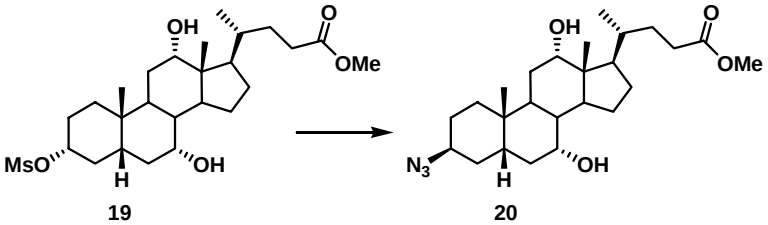
A solution of Boc-(Gly)$_4$-OBzl **15** (872 mg, 2 mmol) in anhydrous CH_3OH (40 mL) was treated with 10 % Pd-C (87 mg, 10 % wt. equiv.) and hydrogenated for 1 h at 40 psi pressure at 25 °C. The reaction mixture was filtered through celite (CH_3OH wash), concentrated under vacuum, and dried thoroughly under vacuum to afford **16** (680 mg, 98 %) as a white solid, which was further recrystallized from 10% CH_3OH/*i*Pr_2O as a white powder. Mp 126-127 ºC dec. (lit. [26] 126-128); IR (KBr) ν_{max} 3308, 3089, 2982, 2934, 1650, 1552 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.19 (t, 1H, *J* = 5.8 Hz), 8.12 (t, 1H, *J* = 5.7 Hz), 8.06 (t, 1H, *J* = 5.5 Hz), 7.03 (t, 1H, *J* = 6.0 Hz), 3.73-3.85 (m, 6H), 3.59 (d, 2H, *J* = 6.0 Hz), 1.40 (s, 9H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 171.2, 169.8, 169.2, 169.1, 155.9, 78.2, 43.3, 42.1, 41.8, 40.8, 28.3 (3C); FABHRMS (NBA) *m/z* 347.1580 (M+H, $C_{13}H_{22}N_4O_7$ requires 347.1567).

B2.6.6. ***Methyl 3α-mesyl-7α,12α-dihydroxy-5β-cholane-24-oate (19):***



To a solution of **18** (1.0 g 2.4 mmol) in dry CH_2Cl_2 (20 mL) was added Et_3N (0.36 mL, 2.6 mmol) at 0 °C. Methane sulfonyl chloride (MsCl) (0.2 mL, 2.6 mmol) was added drop wise in 10 min at 0 °C, and ice was added to the reaction mixture immediately after addition was complete. The reaction mixture was extracted with CH_2Cl_2. Organic layer was washed with $NaHCO_3$, water, and brine. Solvent was evaporated under reduced pressure to obtain crude product as a white solid which was generally used in the next step without further purification, yield 1.03 g, 87 %; Mp. 83-85 ºC; $[\alpha]_D^{25}$ +29.98 ($CHCl_3$, *c* 0.9); IR ν_{max} (Nujol) 3460, 1728 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.69 (s, 3H), 0.91 (s, 3H), 0.99 (d, 3H, *J* = 6.06 Hz), 2.99 (s, 3H), 3.67 (s, 3H), 3.88 (bs, 1H), 4.00 (bs, 1H), 4.51 (m, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 12.4, 17.2, 22.1, 23.0, 26.3, 27.4, 27.8, 28.0, 30.7, 30.9, 34.1, 34.4, 34.7, 35.1, 35.9, 38.7, 39.3, 41.3, 41.6, 46.4, 47.0, 51.4, 68.0, 72.8, 82.9, 174.7; Anal. calcd. for $C_{26}H_{44}O_7S$: C, 62.37; H, 8.86; S, 6.40; Found: C, 62.23; H, 8.92; S, 6.23; MS (LCMS *m/z* 501.07 $[M+H]^+$, 523.17 $[M+Na]^+$.

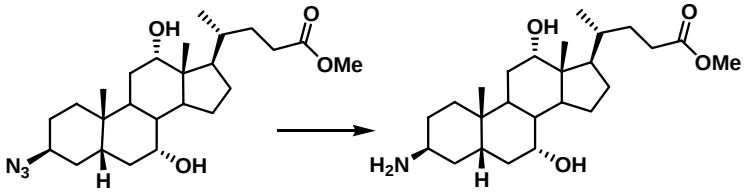
B2.6.7. ***Methyl 3β-azido-7α,12α-dihydroxy-5β-cholane-24-oate (20):***



To a solution of **19** (0.5 g, 1.0 mmol) in dry DMF (10 mL) solid sodium azide (0.325 g, 5.0 mmol) was added and stirring was continued at 60 °C for 12 h and allowed to cool to room temperature. It was then poured in to ice cold water (30 mL) and extracted with EtOAc (3×50 mL). The organic extract was washed with cold water (3×50 mL), brine (25 mL) and was dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude

product **20**. Purification by column chromatography on silica gel (10 % EtOAc/PE) produced compound **20** as a white solid, yield (0.402 g) 90 %; mp = 169-170 ºC (lit. [28c] 157 ºC); $[\alpha]_D^{28}$ + 22.45 (MeOH, *c* 1.16), (lit. [28c] + 23.7); IR ν_{max} (Nujol) 1728, 2098, 3439 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.70 (s, 3H), 0.93 (s, 3H), 0.97 (d, 3H, *J* = 6.06 Hz), 3.86-3.89 (bs, 2H), 3.99 (bs, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 12.4, 17.2, 22.7, 23.2, 24.5, 26.0, 27.4, 28.3, 30.4, 30.8, 31.0, 33.0, 34.2, 35.1, 35.2, 36.7, 39.3, 41.7, 46.5, 47.2, 51.5, 58.7, 68.4, 70.0, 174.7; Anal calcd for $C_{25}H_{41}N_3O_4$: C, 67.08; H, 9.23; N, 9.39; found: C, 67.21; H, 9.18; N, 9.31; MS (LCMS) *m/z* 448.24 $[M+H]^+$, 470.22 $[M+Na]^+$.

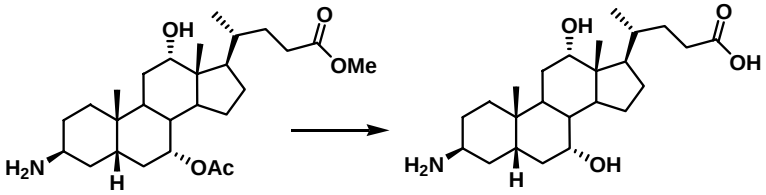
B2.6.8. ***Methyl 3β-amino-7α,12α-dihydroxy-5β-cholane-24-oate (21):***



Compound **20** (0.25 g, 0.54 mmol) in MeOH (15 mL) was hydrogenated at 28 °C and 40 psi pressure using 10 % Pd/C (25 mg) for 4 h. After filtration of the catalyst and evaporation of the solvent, afforded compound **21** (0.225 g, 87 %) as a white solid, mp 225–230 °C dec.; $[\alpha]_D^{25}$ + 27.12 (MeOH, c 1.08); IR ν_{max} (Nujol) 1732, 3439 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.68 (s, 3H), 0.94 (s, 3H), 0.99 (d, 3H, *J* = 6.0 Hz), 3.29 (bs, 1H), 3.66 (s, 3H), 3.84 (bs, 1H), 3.97 (bs, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 12.4, 17.2, 22.8, 23.3, 25.7, 27.4, 27.5, 28.4, 29.8, 30.8, 31.1, 34.7, 35.3, 35.4, 35.9, 39.3, 41.5, 46.3, 46.4 (2C), 47.1, 51.4, 68.2, 73.0, 174.8; Anal calcd for $C_{25}H_{43}NO_4$: C, 71.22; H, 10.28; N, 3.32; found: C, 71.41; H, 9.98; N, 3.01.

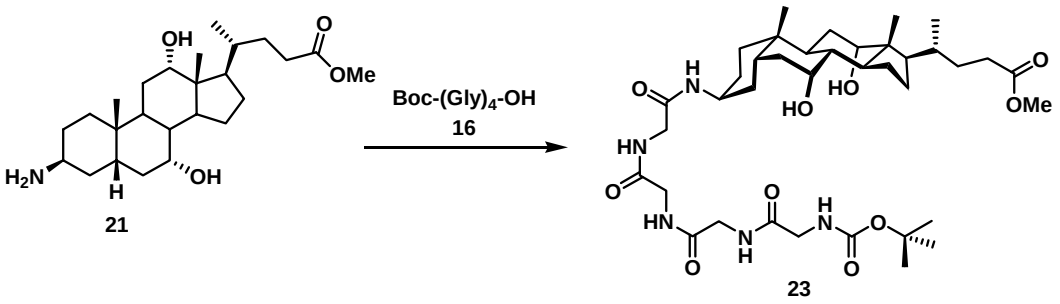
{***Modified high yield protocol:*** Overnight stirring of cholic acid **17** in dry methanol using a catalytic amount of *p*TSA followed by selective mesylation, nucleophilic displacement with sodium azide and hydrogenation of azido functionality using Pd-C furnished 3β-amino methyl cholate **21** as a white solid with an overall yield of 77 % in four steps and using only one final chromatographic purification.}

B2.6.9. ***3β-Amino-7α,12α-dihydroxy-5β-cholane-24-oic acid (22):***



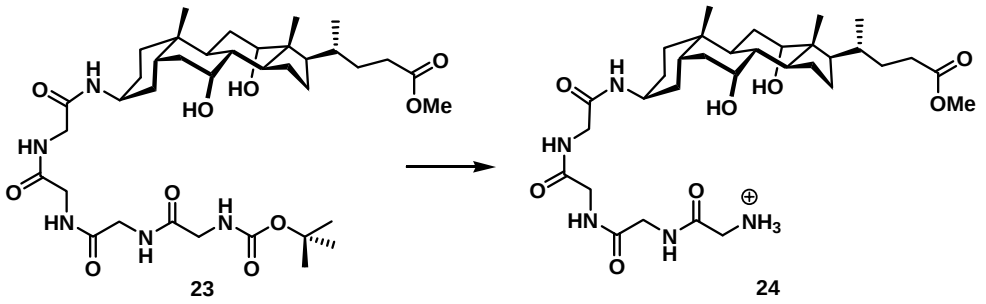
This compound was prepared according to adopted literature procedures [48]. LiOH (2M, 5 mL) was added to the solution of compound **21** (0.135 g, 0.321 mmol) in methanol (10 mL). The mixture was stirred at room temperature for 21 hrs. HCl (2N) was added until pH = 7–8. Solvent was removed in *vacuo*. Residue was purified by column chromatography using MeOH/Et_3N (50/1) as eluent to give white solid (0.121 g, 0.298 mmol, 93 % yield). Mp 238–242 °C, dec. (lit. [49] 240-245 ºC, dec); IR ν_{max} (Nujol) 1724, 3377-3462 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.67 (s, 3H), 0.95 (bs, 6H), 3.54 (bs, 1H), 3.83 (bs, 1H), 3.98 (bs, 1H). MS (LCMS) *m/z* 408.3 $[M+H]^+$; HRESIMS *m/z* 408.3121 $[M+H]^+$ ($C_{24}H_{42}NO_4$; calcd. 408.3114); Anal Calcd. for $C_{24}H_{42}NO_4 \cdot H_2O$: C, 67.73; H, 10.18; N, 3.29; found: C, 67.41; H, 9.98; N, 3.22.

B2.6.10. ***Steroid-amino acid conjugate (23):***



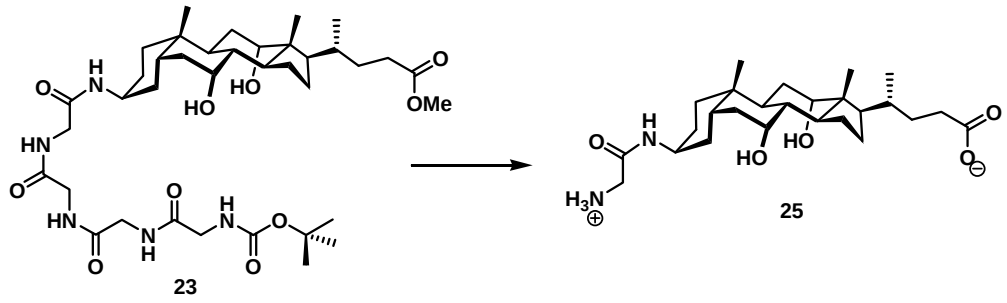
Compound **21** (1.0 g, 2.37 mmol) and Boc-$(Gly)_4$-OH **16** (0.905 g, 2.61 mmol) were dissolved in dry DMF (25 mL) under an argon atmosphere and the solution was cooled to 0 ºC. HOBt (0.175 g, 1.3 mmol) and EDCI (0.55 g, 2.87 mmol) were added and stirring was continued for 30 min. The reaction mixture was allowed to warm to room temperature and was stirred for 6 h. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (300 mL). The organic phase was washed successively with H_2O, 5 % citric acid, H_2O, sat. $NaHCO_3$ and brine, dried with anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH, 23:2) to afford compound **23** (1.3 g, 74 %) as white powder. Mp. 133-135 ºC; $[\alpha]_D^{25}$ + 17.55 (*c* 1.60, MeOH); IR ν_{max} (Nujol) 1655, 1666, 1738, 3292, 3305 cm^{-1}; ^1H NMR (~10 % CD_3OD in $CDCl_3$, 400 MHz) δ 0.69 (s, 3H), 0.95 (s, 3H), 0.99 (d, 3H, *J* = 5.91 Hz), 1.44 (s, 9H), 3.67 (s, 3H), 3.79 (bs, 2H), 3.81-3.94 (m, 7H), 3.95 (bs, 1H), 4.01 (bs, 1H); ^{13}C NMR (~10 % CD_3OD in $CDCl_3$, 100 MHz) δ 12.8, 14.3, 17.4, 23.0, 23.6, 24.7, 26.7, 27.9, 28.5, 28.9, 31.1, 31.4, 31.5, 33.6, 34.6, 35.6, 35.8, 37.2, 39.9, 42.1, 43.1, 43.2, 43.3, 44.4, 46.5, 46.8, 47.3, 51.9, 61.1, 68.6, 73.4, 80.7, 157.7, 169.5, 171.0, 171.3, 172.6, 176; MS (LCMS) *m/z* 772.4 $[M+Na]^+$; HRESIMS *m/z* 772.4440 $[M+Na]^+$ ($C_{38}H_{63}N_5O_{10}Na$; calcd. 772.4473); Anal Calcd. for: $C_{38}H_{63}N_5O_{10}$: C, 60.86; H, 8.47; N, 9.34; found: C, 61.01; H, 8.34; N, 9.49.

B2.6.11. ***Steroid-amino acid conjugate (24):***



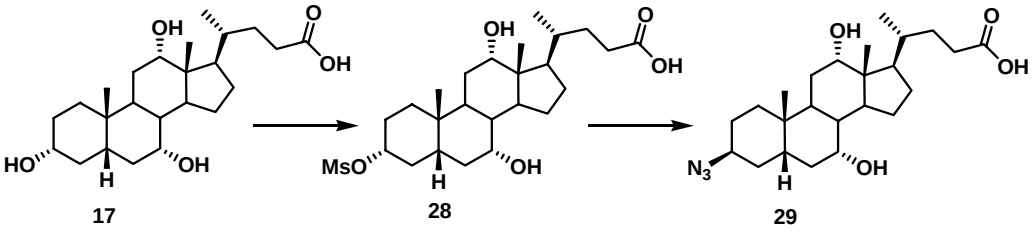
A solution of compound **23** (75 mg, 0.1 mmol) in 2M HCl:Et_2O (5 mL) was stirred at 0 ºC for 30 min and 25 ºC for 1.5 h. The volatiles were removed under vacuum, and the residue was dried thoroughly to afford compounds **24** (65 mg, 95 % yield) as a white solid. $[\alpha]_D^{25}$ +18.99 (*c* 1.58, MeOH); IR ν_{max} (Nujol) 1666, 1681, 1714, 1737, 3303, 3544 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 0.71 (s, 3H), 0.97 (s, 3H), 1.0 (bs, 3H), 3.31 (bs, 1H), 3.35 (bs, 1H), 3.64 (s, 3H), 3.75-3.84 (m, 3H), 3.86-3.93 (m, 4H), 3.94-4.04 (m, 4H); ^{13}C NMR (CD_3OD, 125 MHz) δ 13.0, 17.6, 23.4, 24.2, 25.5, 27.4, 28.7, 29.7, 30.7, 31.9, 32.2, 34.7, 35.4, 36.2, 36.7, 38.2, 40.9, 41.6, 43, 43.4, 43.6, 43.7, 47.4, 47.5, 48, 52, 69.1, 74, 168.5, 170.6, 171.9, 172, 176.5; MS (LCMS) *m/z* 650.2 $[M+H]^+$, 672.3 $[M+Na]^+$; HRESIMS *m/z* 650.4138 $[M+H]^+$ ($C_{33}H_{56}N_5O_8$; calcd. 650.4129); Anal Calcd. for: $C_{33}H_{56}N_5O_8 \cdot HCl \cdot H_2O$: C, 56.28; H, 8.30; N, 9.94; found: C, 56.42; H, 8.63; N, 10.21.

B2.6.12. ***Steroid-amino acid conjugate (25):***



LiOH/KOH (2M in H_2O, 1 mL) was added to a solution of compound **23** (0.201 g, 0.25 mmol) in methanol (5 mL). The mixture was stirred at room temperature for 2-8 h and the solvent was removed in *vacuo*. The white residue was dissolved in cold water. 5 % citric acid/dil. HCl/acetic acid was added until pH = 7-8. The residue was filtered, washed with water and diethyl ether and dried thoroughly under vacuum to afford compounds **25** (0.112 g, 90 % yield) as a sticky solid. IR ν_{max} (KBr) 3633, 3348 broad, 1724, 1684 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 0.72 (s, 3H), 0.99 (s, 3H), 1.02 (d, J = 6.3 Hz, 3H), 2.23 (m, 2H), 2.37 (m, 1H), 2.59 (dt, J = 3.3 and 14 Hz, 1H), 3.74 (bs, 2H), 3.83 (m, 1H), 3.97 (m, 1H), 4.08 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 13.0, 17.6, 23.3, 24.0, 25.3, 27.0, 28.4, 29.4, 31.5, 31.9, 32.0, 34.2, 35.1, 36.0, 36.4, 37.8, 40.5, 41.4, 42.7, 47.3, 47.3, 47.8, 68.9, 73.8, 166.2, 178.1; MS (LCMS) *m/z* 465.4 $[M+H]^+$, 487.3 $[M+Na]^+$.

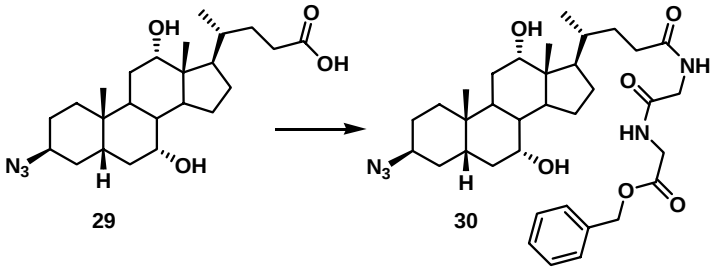
B2.6.13. ***3α-Mesyl-7α,12α-dihydroxy-5β-cholane-24-oic acid (28) and 3β-azido-7α,12α-dihydroxy-5β-cholane-24-oic acid (29):***



To a solution of cholic acid **17** (0.408, g 1.0 mmol) in dry pyridine (10 mL) was added MsCl (0.085 mL, 1.1 mmol). The reaction mixture was stirred at 25 °C for 1 h and 5 % citric acid was added to the reaction mixture. Pyridine was evaporated and the reaction mixture was extracted with EtOAc (3x50mL). Organic layer was washed with cold water, and brine. Solvent was evaporated under reduced pressure to obtain crude **28** as a white solid which was used in the next step without further purification. ^1H NMR (CD_3OD, 300 MHz) δ 0.72

(s, 3H), 0.93 (s, 3H), 1.0 (d, *J* = 6 Hz, 3H), 3.01 (s, 3H), 3.89 (bs, 1H), 4.03 (bs, 1H), 4.55 (m, 1H); ^{13}C NMR (CD_3OD, 75 MHz) δ 12.5, 17.3, 22.2, 23.1, 26.5, 27.5, 27.9, 28.1, 30.7, 30.9, 34.1, 34.5, 34.8, 35.3, 36.1, 38.8, 39.4, 41.4, 41.7, 46.5, 47.0, 68.2, 73.0, 82.9, 179.1; MS (LCMS) *m/z* 486.3 $(M+H)^+$, 509.3 $[M+Na]^+$. To a solution of **28** (0.5 g, 1.0 mmol) in dry DMF (10 mL) solid sodium azide (0.325 g, 5.0 mmol) was added and stirring was continued at 100 °C for 24 h and allowed to cool to room temperature. It was then poured in to ice cold water (30 mL) and extracted with EtOAc (3×50 mL). The organic extract was washed with cold water (2×10 mL), brine (20 mL) and was dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude **29**. Purification by column chromatography on silica gel (10 % EtOAc/PE) produced compound **29** as a white solid. (0.372 g, 86 % yield); IR ν_{max} (Neat) 1709, 2095, 3394 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 0.74 (s, 3H), 0.96 (s, 3H), 1.02 (d, *J* = 6Hz, 3H), 2.01 (m, 2H), 2.24 (m, 2H), 2.36 (m, 1H), 2.64 (m, 1H), 3.81 (bs, 1H), 3.91 (bs, 1H), 3.97 (bs, 3H); MS (LCMS) *m/z* 433.4 $[M+H]^+$, 456.4 $[M+Na]^+$.

B2.6.14. ***Steroid-amino acid conjugate (30):***



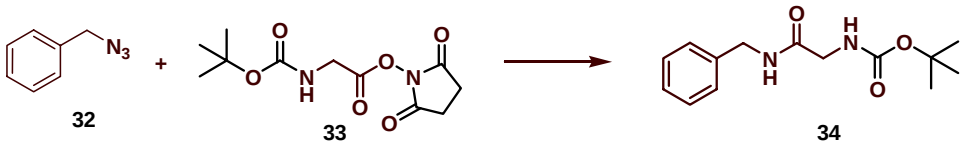
Compound **29** (0.22 g, 0.5 mmol) and **13** (0.26 g, 0.6 mmol) were dissolved in dry DMF (10 mL) under an argon atmosphere and the solution was cooled to -10 ºC. HOBt (0.034 g, 0.25 mmol) and EDCI (0.115 g, 0.6 mmol) were added and stirring was continued for 30 min. The reaction mixture was allowed to warm to room temperature and was stirred for 12 h.

The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (300 mL). The organic phase was washed successively with cold H_2O (2x5 mL), 5 % citric acid (2x5mL), sat. $NaHCO_3$ (10 mL) and brine (10 mL), dried with anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH, 19:1) to afford compound **30** as white powder. (0.321 g, 84 % yield); IR ν_{max} (Neat) 3396, 1693, 1698, 1729, 2092, 1532 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 0.71 (s, 3H), 0.95 (s, 3H), 0.98 (d, *J* = 6Hz, 3H), 2.54 (m, 1H), 2.89 (m, 2H), 3.99 (m, 3H), 4.10 (m, 2H), 5.20 (s, 2H), 6.40 (m, 1H), 6.68 (m, 1H), 7.37 (m, 5H); MS (LCMS) *m/z* 637.4 $[M+H]^+$, 660.4 $[M+Na]^+$.

B2.6.15. ***General Procedure:*** *Palladium catalyzed one-pot reduction of alkyl azides followed by in situ amidation with the commercially available activated esters.*

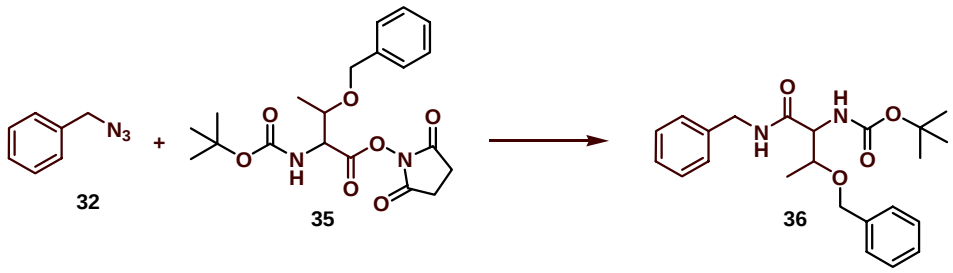
NHS ester (1 mmol) and 10 % $Pd/BaSO_4$ (10 %) were added successively to a stirred solution of alkyl azide (1 mmol), in THF or EtOH or MeOH (10 mL). The reaction flask was evacuated and flushed with hydrogen gas. The resultant mixture was stirred under hydrogen atmosphere (balloon) at 25 ˚C for 20 min to 18 h. after completion of the reaction, the catalyst was filtered through a pad of celite, the filter cake was washed with the appropriate solvent (10 mL) and the filtrate was concentrated under reduced pressure. This crude product was dissolve in EtOAc (100 mL) washed with with 10 % citric acid (2x10 mL), 20% $NaHCO_3$ (2x10 mL), cold water (2×10 mL), brine (10 mL) and was dried over Na_2SO_4. The residue was purified over silica gel (100-200 mesh) using appropriate solvent system.

B2.6.16. ***Compound (34):***



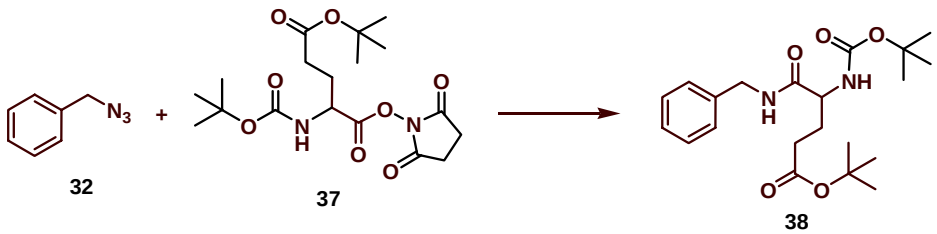
IR ν_{max} (Nujol) 3322, 1703, 1658, 1530 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.41 (s, 9H), 3.82 (d, *J* = 8H, 2H), 4.45 (d, *J* = 8Hz, 2H), 5.30 (m, 1H), 6.67 (m, 1H), 7.24-7.37 (m, 5H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 28.1, 43.1, 44.1, 80.0, 127.3, 127.5x2, 128.5x2, 137.9, 156.1, 169.5; MS (LCMS) *m/z* 264.2 $[M+H]^+$, 287.2 $[M+Na]^+$.

B2.6.17. ***Compound (36):***



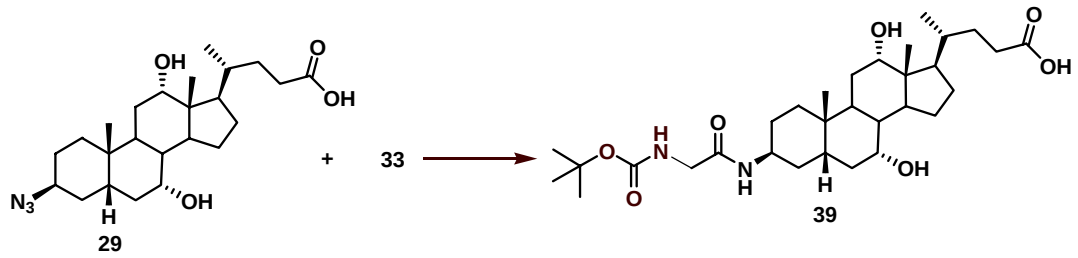
IR ν_{max} (Nujol) 3320, 1706, 1667, 1547, 1223 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.10 (d, *J* = 6.0 Hz, 3H), 1.45 (s, 9H), 4.10-4.60 (m, 6H), 5.50 (m, 1H), 6.82 (m, 1H), 7.20-7.38 (m, 10H); ^{13}C NMR (CD_3OD, 75 MHz) δ 15.8, 28.1, 43.2, 47.8, 71.5, 74.8, 79.9, 127.5, 127.7, 127.8, 128.4, 128.7, 138.0, 156.1, 170.1; MS (LCMS) *m/z* 421.2 $[M+Na]^+$.

B2.6.18. ***Compound (38):***



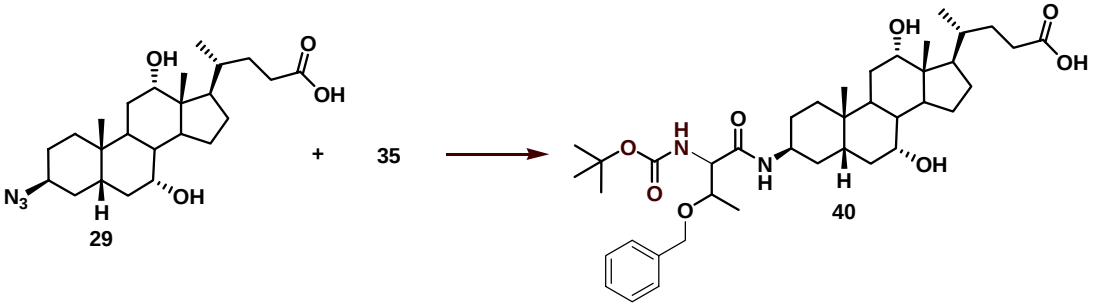
IR ν_{max} (Nujol) 3347, 1739, 1652, 1537 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.38 (s, 9H), 1.86 (m, 1H), 2.01 (m, 1H), 2.24 (m, 1H), 2.31 (m, 1H), 4.09 (bs, 1H), 4.36 (bs, 2H), 5.33 (bs, 1H), 6.71 (bs, 1H), 7.18-7.25 (m, 5H); MS (LCMS) *m/z* 637.4 $[M+H]^+$, 660.4 $[M+Na]^+$.

B2.6.19. ***Steroid-amino acid conjugate (39):***



IR ν_{max} (Neat) 1650, 1697, 1705, 3351 (broad) cm^{-1}; ^1H NMR ($CDCl_3$+CD_3OD, 400 MHz) δ 0.71 (s, 3H), 0.97 (s, 3H), 1.02 (d, *J* = 6.0 Hz, 3H), 1.47 (s, 9H), 2.22 (m, 2H), 2.35 (m, 1H), 2.57 (m, 1H), 3.69 (bs, 2H), 3.83 (bs, 1H), 3.96 (bs, 1H), 4.05 (bs, 1H); ^{13}C NMR ($CDCl_3$+CD_3OD, 100 MHz) δ 12.9, 17.5, 23.4, 23.8, 25.0, 26.6, 28.2, 28.6x3, 29.1, 31.5, 31.8, 34.0, 34.8, 35.8, 36.1, 37.9, 40.2, 42.3, 44.6, 46.3, 47.0, 47.5, 50.0, 68.6, 73.5, 80.6, 157.8, 170.8, 178.0; MS (LCMS) *m/z* 587.4 $[M+Na]^+$, HRESIMS *m/z* 587.3701 $[M+Na]^+$ ($C_{31}H_{52}N_2NaO_7$; calcd. 587.3672).

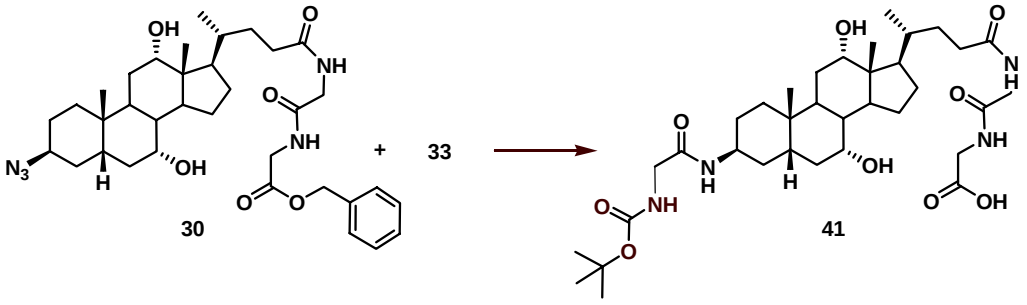
B2.6.20. ***Steroid-amino acid conjugate (40):***



IR ν_{max} (Neat) 3380 (broad), 1712, 1660, 1546 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.66 (s, 3H), 0.69 (s, 3H), 0.97, (s, 3H), 1.15 (d, *J* = 6 Hz, 3H), 1.46 (s, 9H), 3.81 (bs, 1H), 3.95 (bs,

1H), 4.11 (bs, 2H), 4.31 (bs, 1H), 4.60 (s, 2H), 5.70 (bs, 1H), 6.90 (bs, 1H), 7.32 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.4, 14.9, 17.2, 22.7, 23.2, 24.6, 25.8, 27.5, 28.3x3, 29.6, 30.7, 30.9, 31.0, 33.5, 34.2, 35.0, 35.3, 37.2, 39.3, 41.7, 45.5, 46.5, 46.9, 56.9, 60.4, 68.3, 71.8, 73.0, 75.1, 79.9, 127.8x3, 128.4, 137.8, 155.8, 168.6, 178.3; MS (LCMS) *m/z* 699.4 $[M+H]^+$, 721.4 $[M+Na]^+$.

B2.6.21. ***Steroid-amino acid conjugate (41):***



IR ν_{max} (Neat) 3360 (broad), 1708, 1656 cm^{-1}; ^{1}H NMR (CD_3OD, 300 MHz) δ 0.75 (s, 3H), 1.00 (s, 3H), 1.07, (d, *J* = 6 Hz, 3H), 1.48 (s, 9H), 3.71-4.05 (m, 9H); MS (LCMS) *m/z* 678.4 $[M+H]^+$, 701.4 $[M+Na]^+$.

B2.6.24. ***Antimicrobial activity: materials and methods***

Candida albicans, *Cryptococcus neoformans* (human pathogen), *Benjaminiella poitrasii* and *Yarrowia lipolytica* (non pathogen) strains were maintained on YPG (yeast extract, 0.3 %, peptone, 0.5 %, and glucose, 1 %) agar slants. *Fusarium oxysporum* (plant pathogen) strain was maintained on PDA (potato, 20 % dextrose, 2 %) agar slants at 28 ^oC. *Escherichia coli* (NCIM no. 2574) and *Staphylococcus aureus* (NCIM no. 2122) were maintained on NA (beef extract, 0.3 %, peptone, 0.5 %, sodium chloride, 0.5 %) slants. Strains of *C. albicans, C. neoformans, Y. lipolytica* were inoculated in YPG broth at 28 ^oC and *B. poitrasii* at 37 ^oC for 24 h respectively. *F. oxysporum* was inoculated in potato dextrose at 28 ^oC for 48 h

whereas bacterial strains *E. coli* and *S. aureus* were inoculated in NA broth for 24 h. Compounds **17**, **21-24** were solubilized in DMSO, and stock solutions of 1.28 mg/mL were prepared. Amphotericin B, Fluconazole, Tetracycline and Erythromycin were also dissolved in DMSO and were used as a positive control.

B2.6.25. *MIC and IC_{50} determination*

In *vitro* antifungal and antibacterial activity of the newly synthesized compounds were studied against the fungal strains viz., *C. albicans*, *C. neoformans*, *B. poitrasii*, *Y. lipolytica, F. oxysporum* strains and bacterial strains *E. coli* (NCIM No.2574), and *S. aureus* (NCIM No.2122) respectively to determine MIC (Minimum Inhibitory Concentration) and IC_{50} (50 %, Inhibition of Growth) values. Experiments were performed in triplicate under similar experimental conditions. MIC and IC_{50} of the synthesized compounds were determined according to standard broth microdilution technique as per NCCLS guidelines [50]. Testing was performed in U bottom 96 well tissue culture plates in YPG, PDA for fungal strains and NA for bacterial strains. The concentration range of tested compounds and standard was 128-0.25 μg/ml. The plates were incubated at 28 °C for all the microorganisms except for *B. poitrasii* (37 °C), absorbance at 600 nm was recorded to assess the inhibition of cell growth after 24 h for *B. poitrasii* and *Y. lipolytica*, 48 h for *C. albicans* and *F. oxysporum*, 72 h for *C. neoformans* and 24 h for bacterial cultures. MIC was determined as 90 % inhibition of growth with respect to the growth control and IC_{50} was the concentration at which 50 % growth inhibition was observed.

B2.6.26. *Antiproliferative activity. materials and methods*

Human embryonic kidney (HEK293) and human mammary adenocarcinoma (MCF-7) cell lines were grown in a monolayer in nutrient media DMEM supplemented with fetal bovine

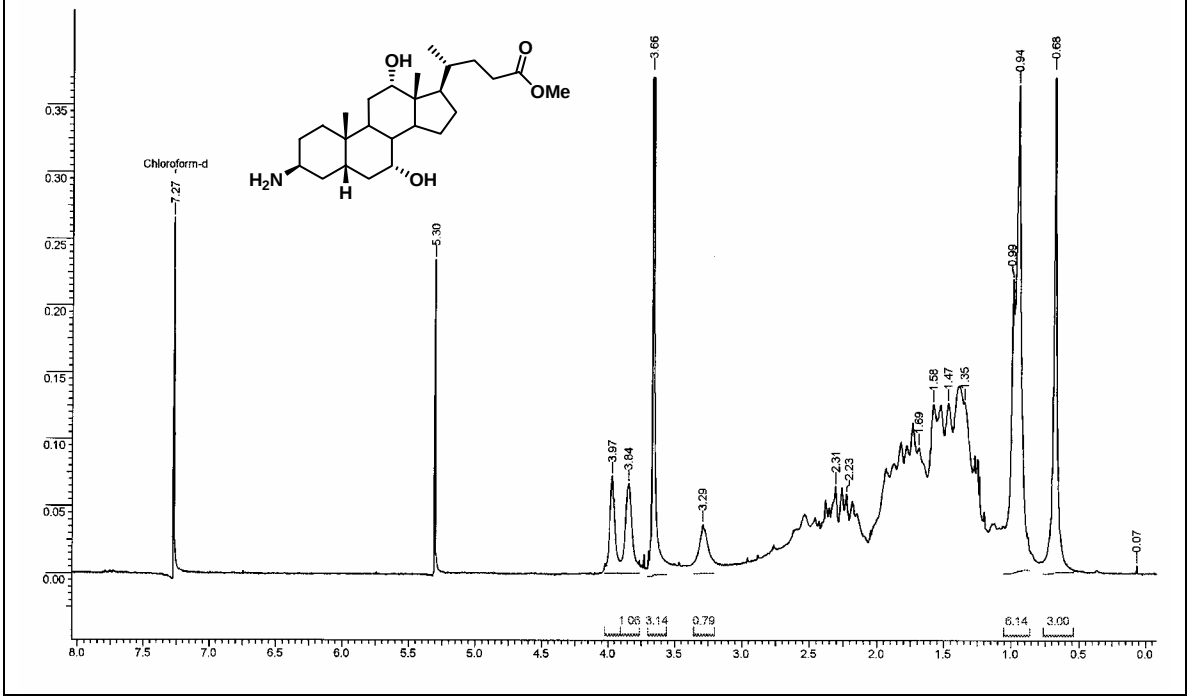
serum (10 %), penicillin (100 U/mL), and streptomycin (100 μg/mL) (all from Invitrogen Life Technologies, MD). The cells were grown at 37 °C in presence of 5 % CO_2.

B2.6.27. ***MTT cell proliferation assay***

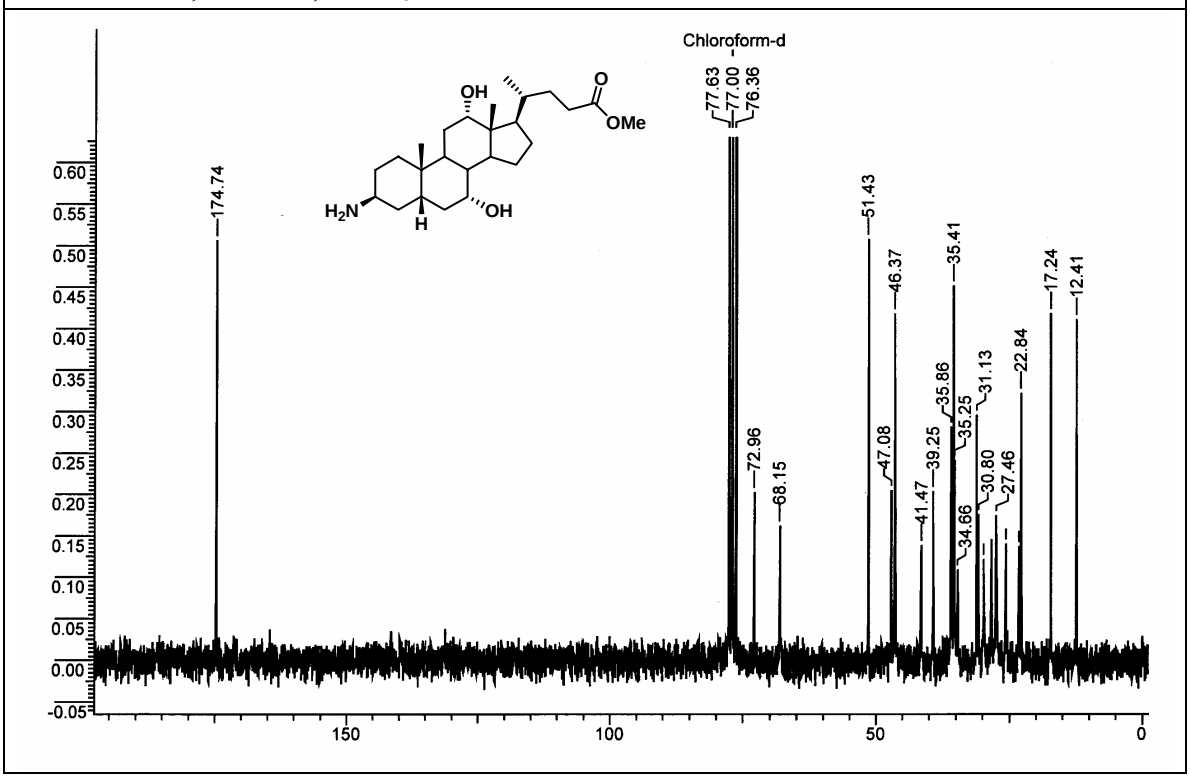
HEK293 and MCF-7 cells were plated at a density of 10^4 cells per well in 96-well tissue culture plates. Cells were allowed to adhere for 24 h at 37 °C. Stock solutions of all the compounds were prepared in DMSO at a concentration of 10 mM and diluted to the required concentration. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved (5 mg/mL) in DMEM (without phenol red) and filtered through a 0.22 μm filter before use. The cells were treated with various concentrations (0, 1, 10, 100 and 1000 μM) of compounds dissolved in DMSO for an additional 48 h, in triplicate. In the control wells, nutrient medium with a corresponding concentration of DMSO only was added to the cells. Thereafter, the drug containing media was replaced with 50 μL media containing 1 mg/mL MTT and incubated for 4 h at 37 °C. Medium was then aspirated off from the formazan crystals, which were then solubilized in 100 μL of acidified isopropanol. The optical density was read on a microplate reader at 570 nm using 630 nm as a reference filter against a blank prepared from cell free wells. Absorbance given by cells treated with the carrier DMSO alone was taken as 100 % cell growth. All assays were performed in triplicate.

B2.7. Selected Spectra

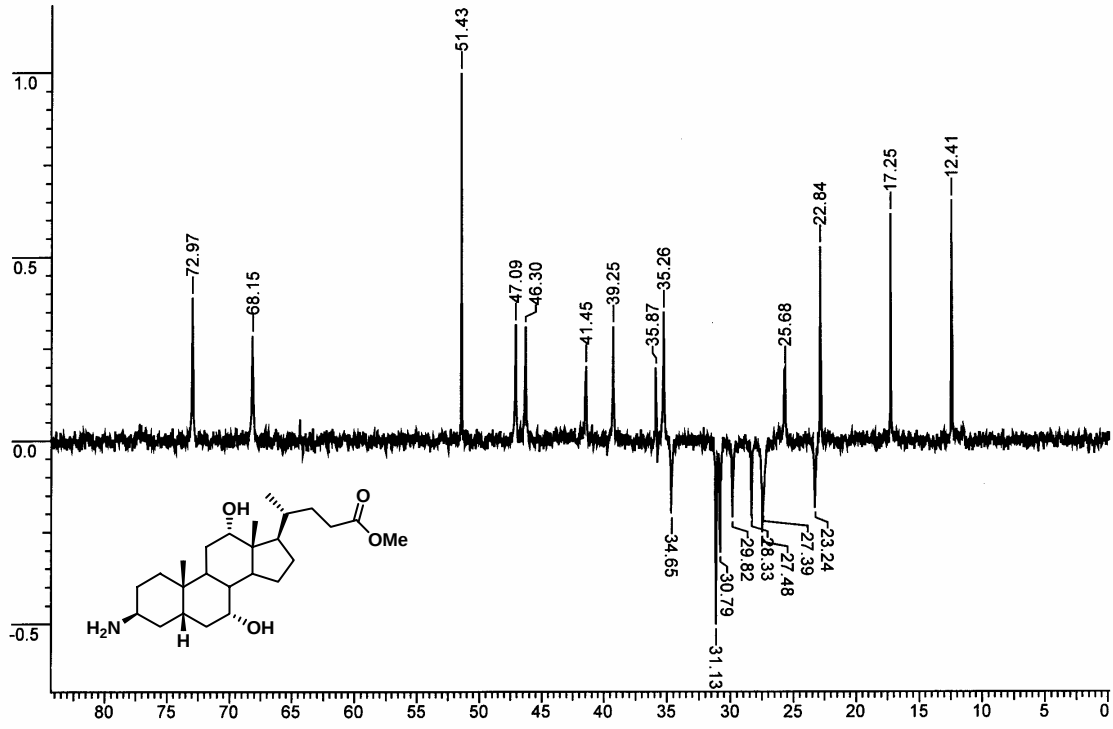
21: ^{1}H NMR, 200 MHz, $CDCl_3$



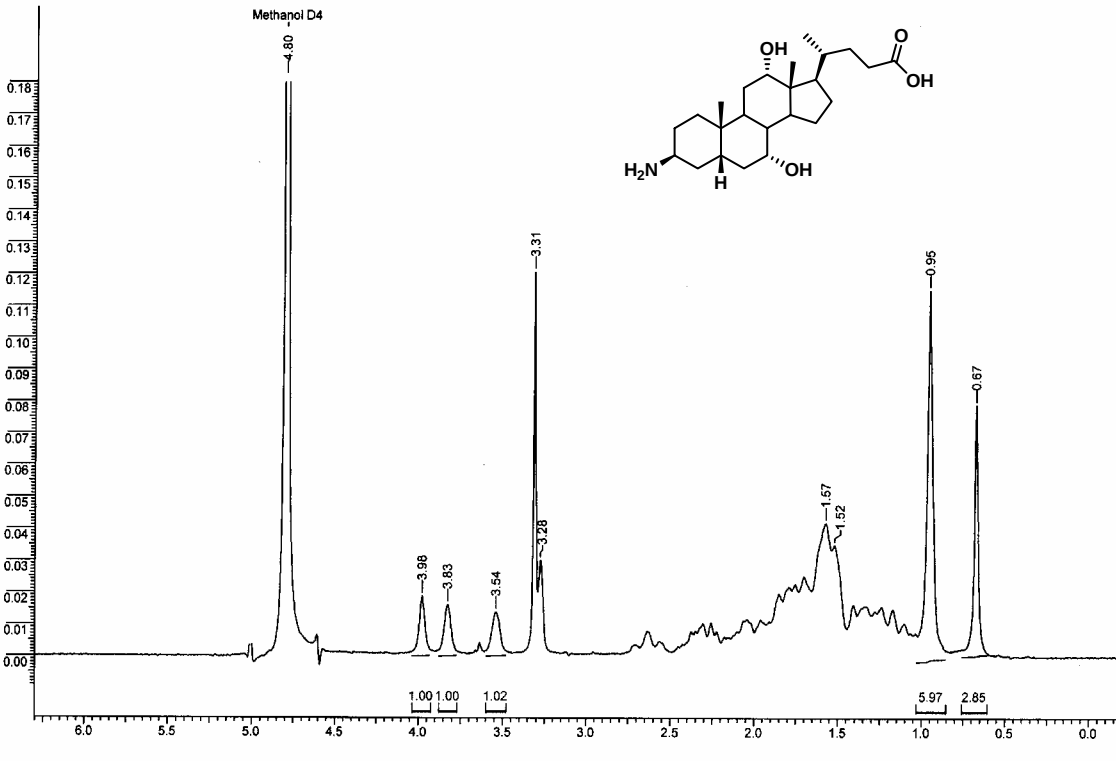
21: ^{13}C NMR, 50 MHz, $CDCl_3$



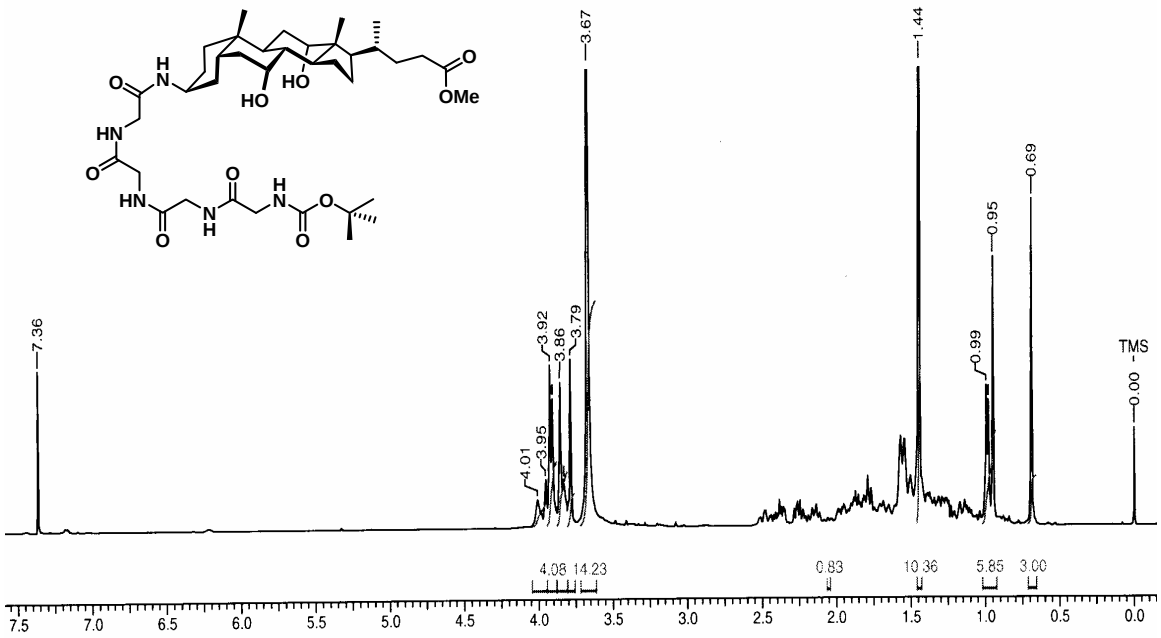
21: DEPT, 50 MHz, $CDCl_3$



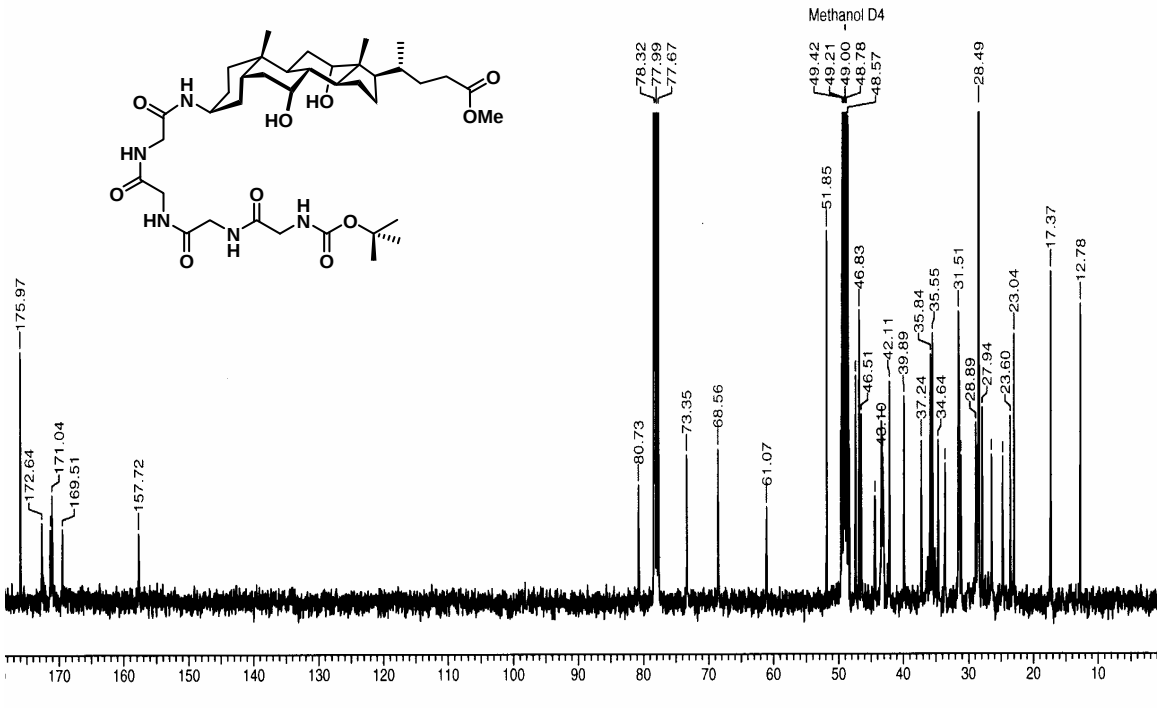
22: ^1H NMR, 200 MHz, CD_3OD



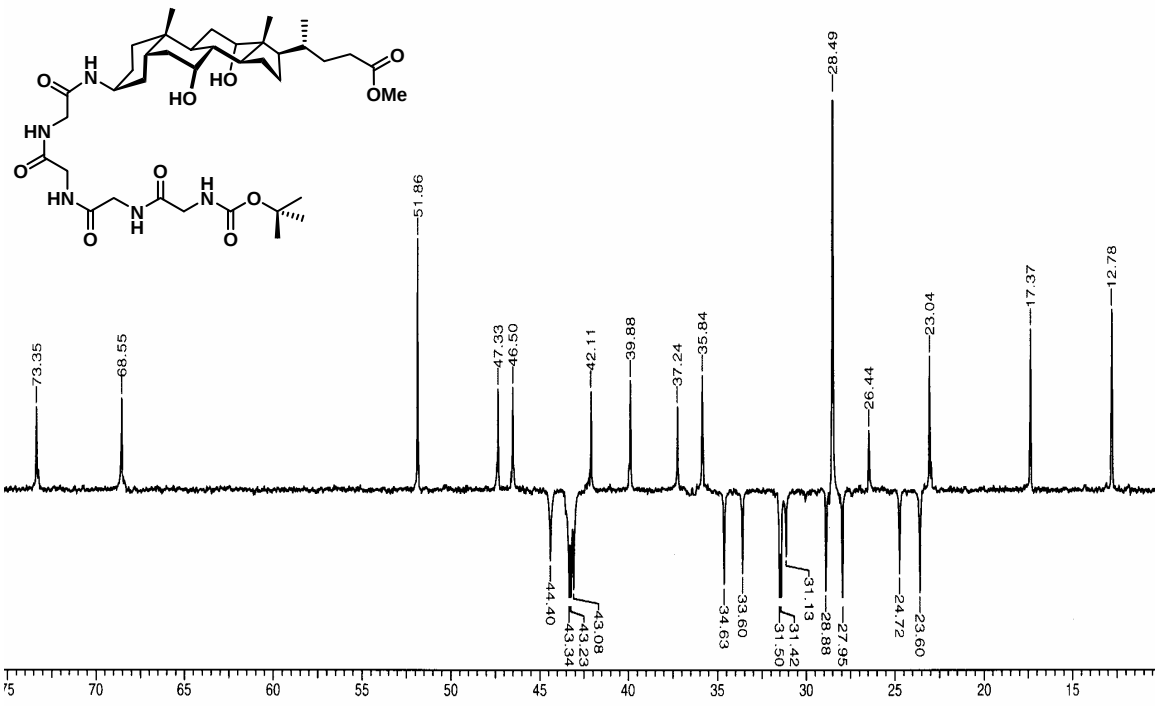
23: ^{1}H NMR, 400 MHz, $CDCl_3$+CD_3OD



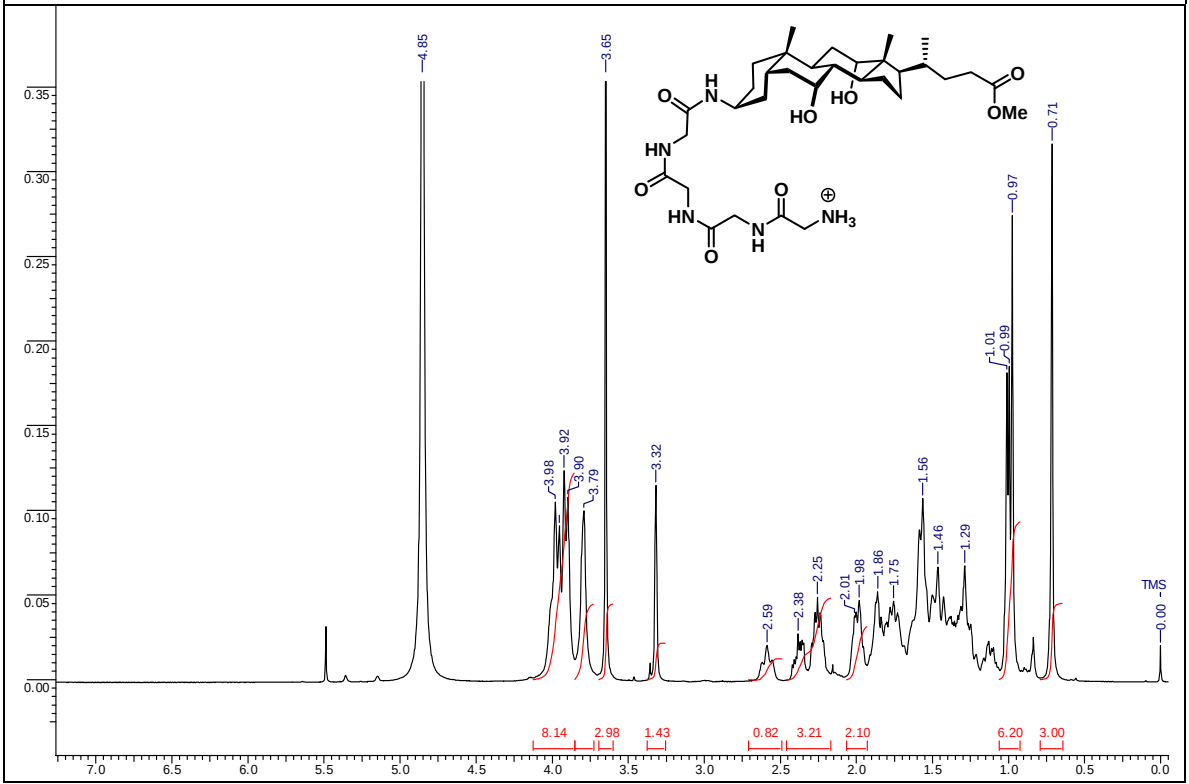
23: ^{13}C NMR, 100 MHz, $CDCl_3$+CD_3OD



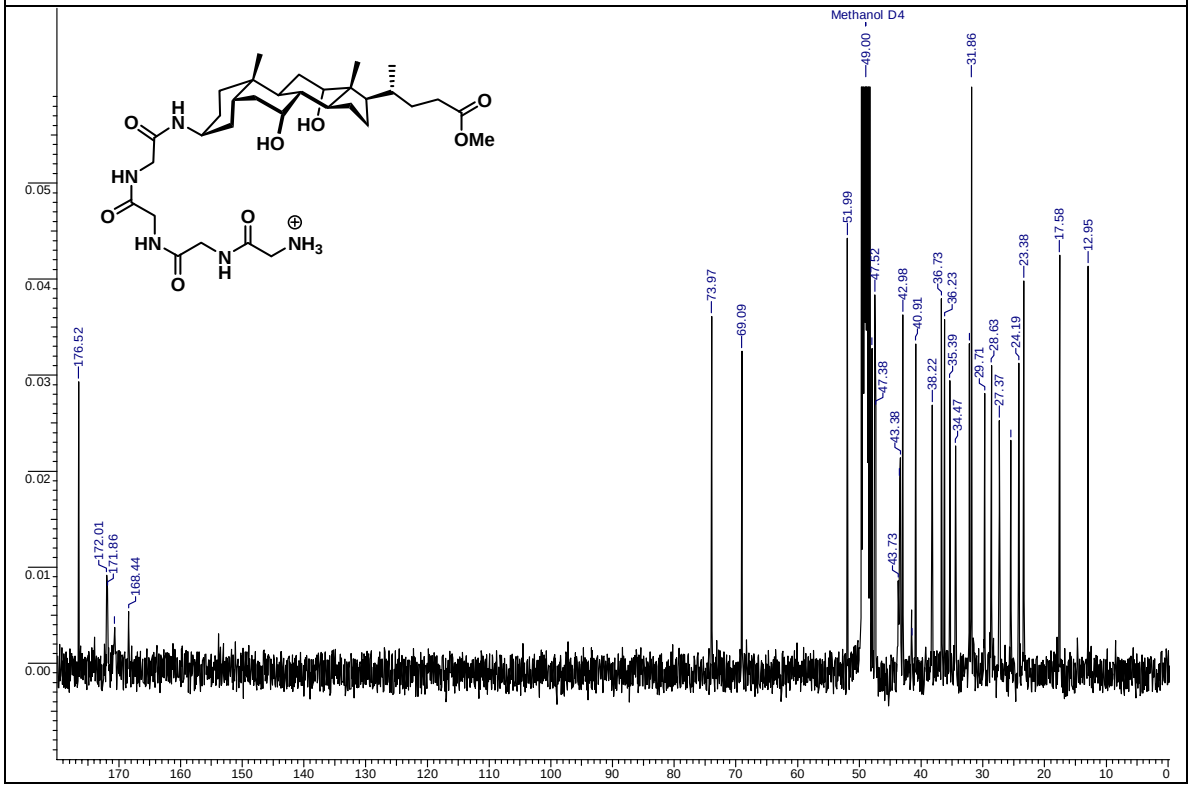
23: DEPT, 100 MHz, $CDCl_3$+CD_3OD



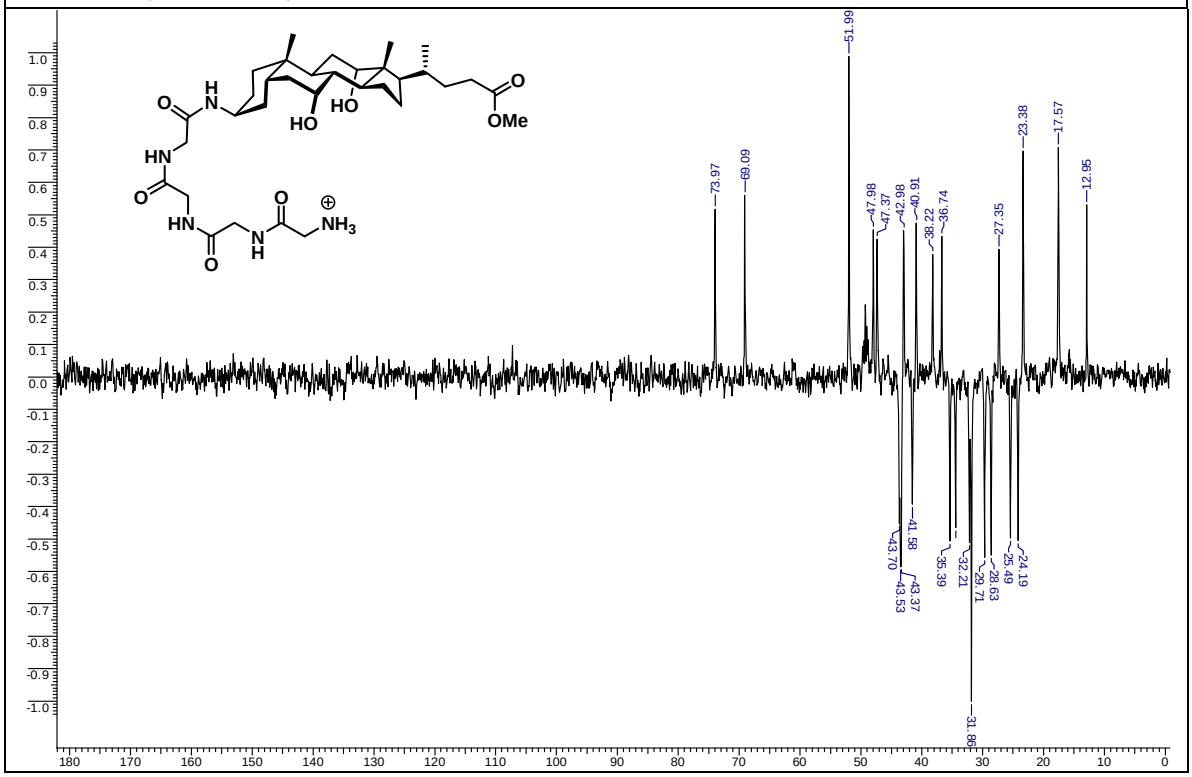
24: ^1H NMR, 400 MHz, CD_3OD



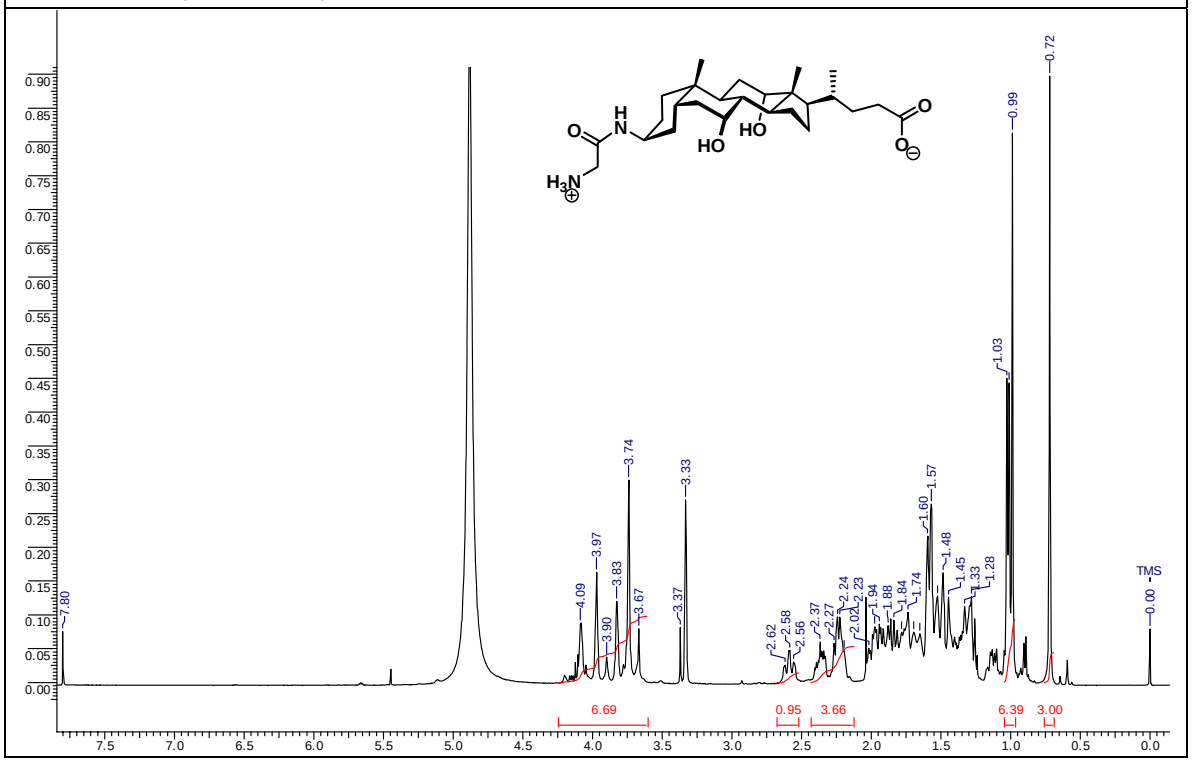
24: ^{13}C NMR, 100 MHz, CD_3OD



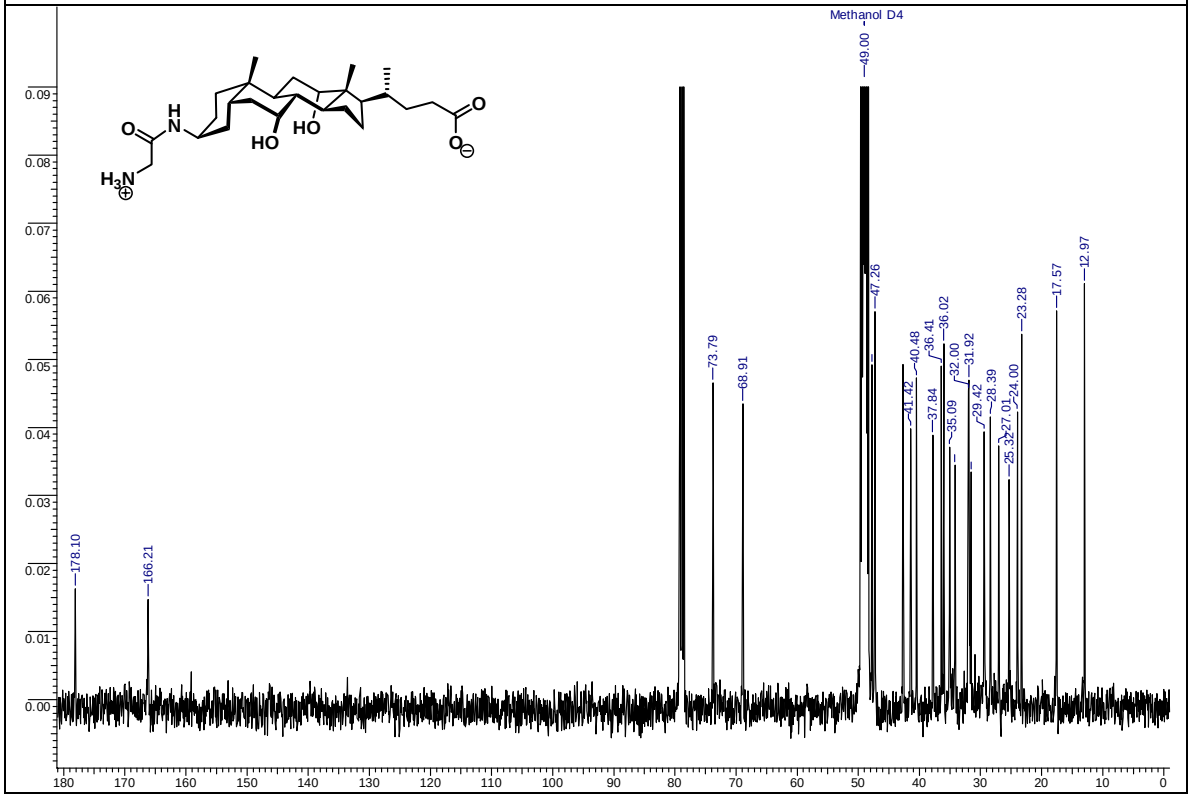
24: DEPT, 100 MHz, CD_3OD



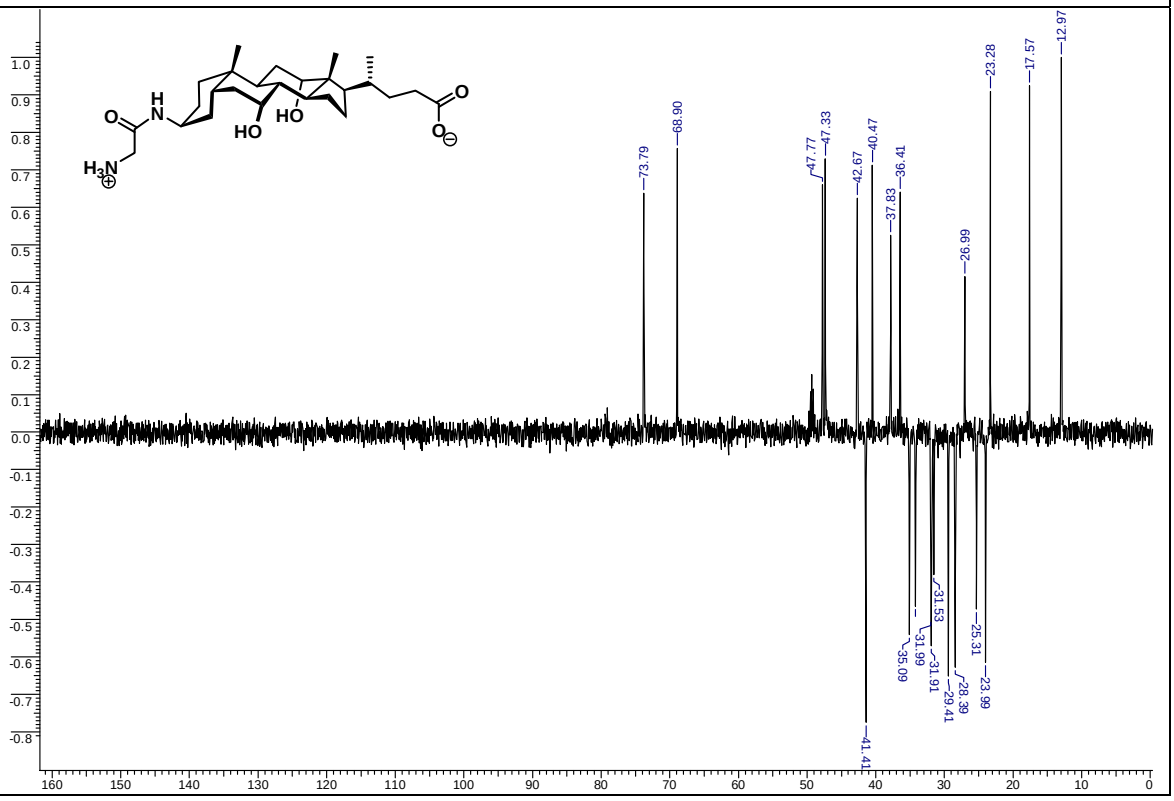
25: ^{1}H NMR, 400 MHz, $CDCl_3$+CD_3OD



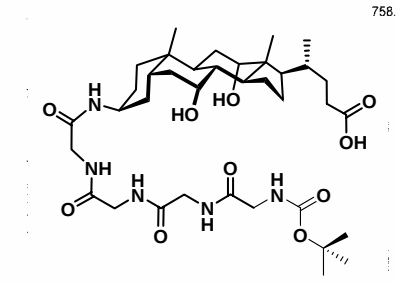
25: ^{13}C NMR, 100 MHz, $CDCl_3$+CD_3OD



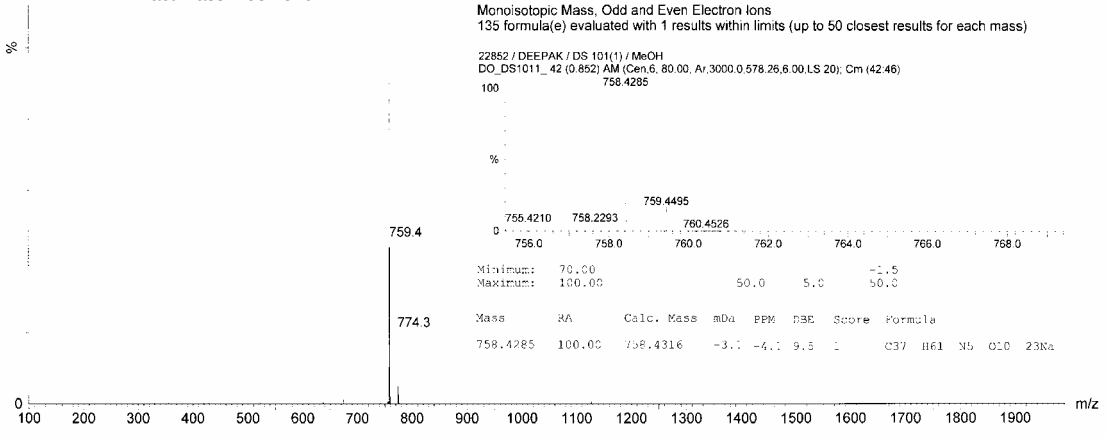
25: DEPT, 100 MHz, $CDCl_3$+CD_3OD



26: LCMS & ESIHRMS



Chemical Formula: $C_{37}H_{61}N_5NaO_{10}$
Exact Mass: 758.4316



Elemental Composition Report

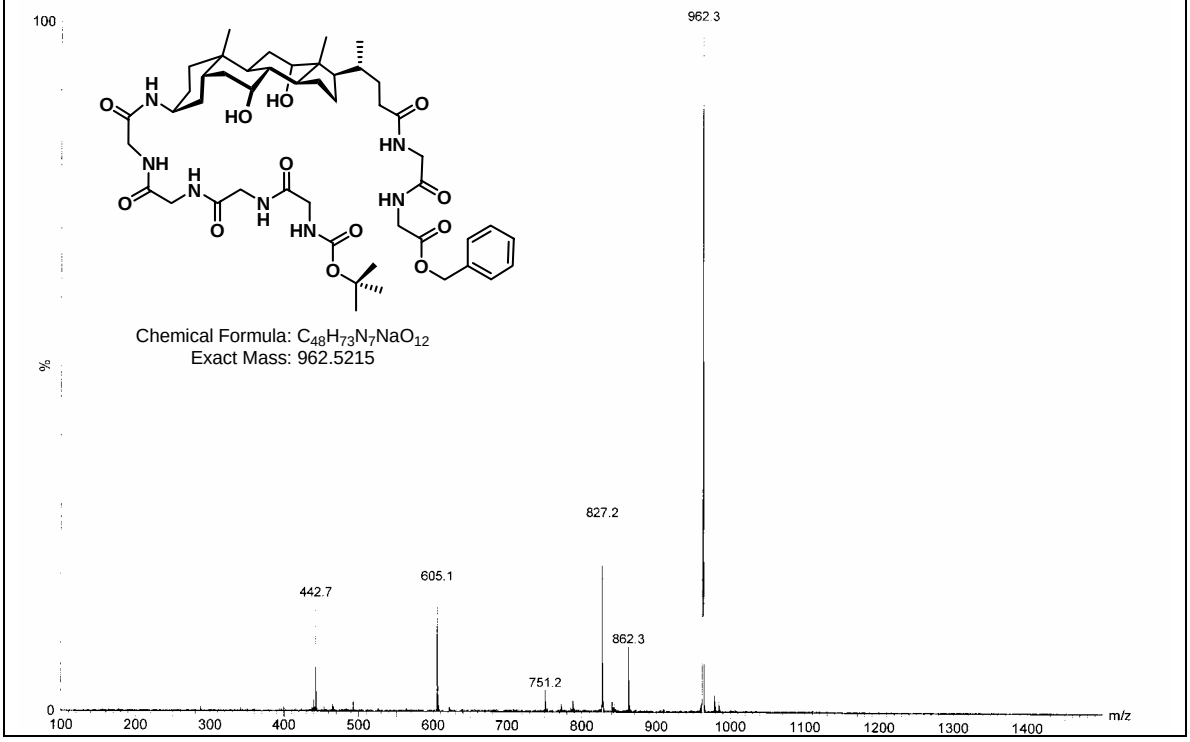
Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 50.0 mDa / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
135 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

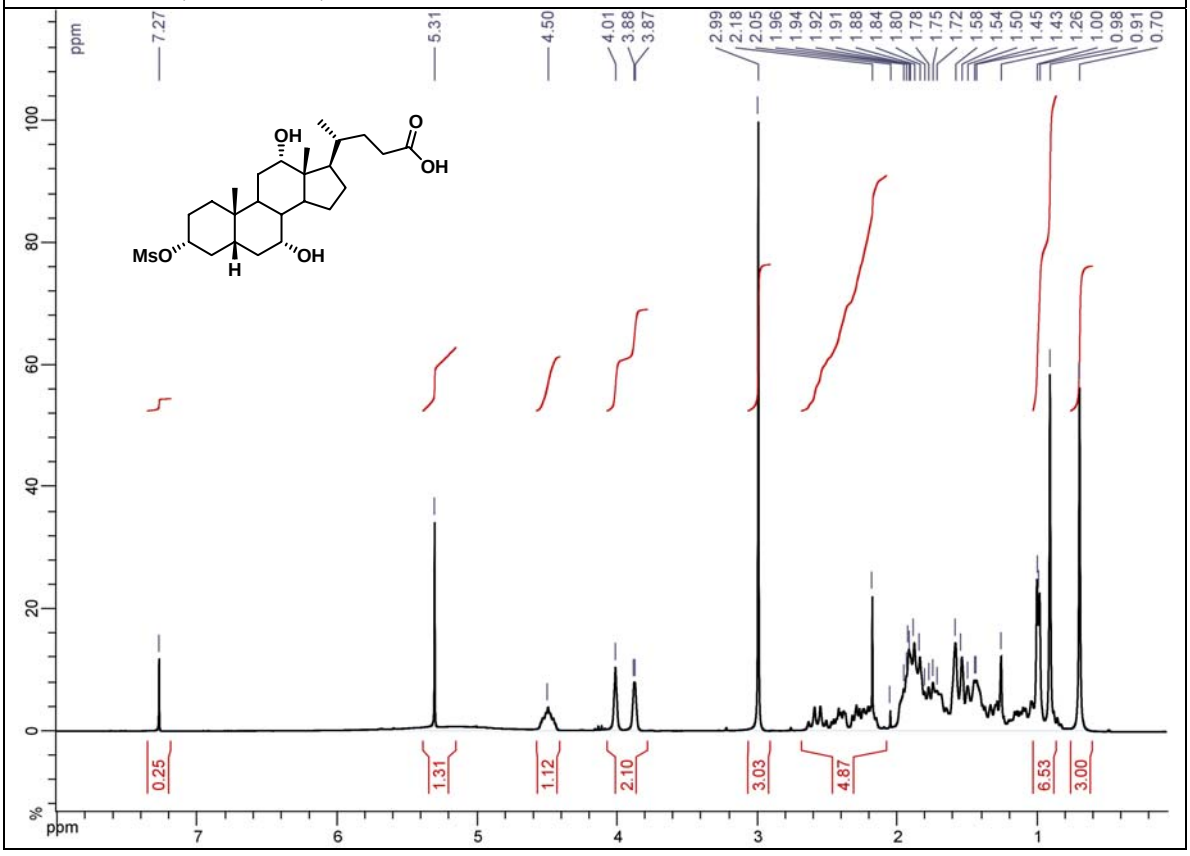
22852 / DEEPAK / DS 101(1) / MeOH
DO_DS1011_ 42 (0.852) AM (Cen,6, 80.00, Ar,3000.0,578.26,6.00,LS 20); Cm (42:46)

Minimum:	70.00					-1.5		
Maximum:	100.00				50.0	5.0	50.0	
Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula	
758.4285	100.00	758.4316	-3.1	-4.1	9.5	1	C37 H61 N5 O10 23Na	

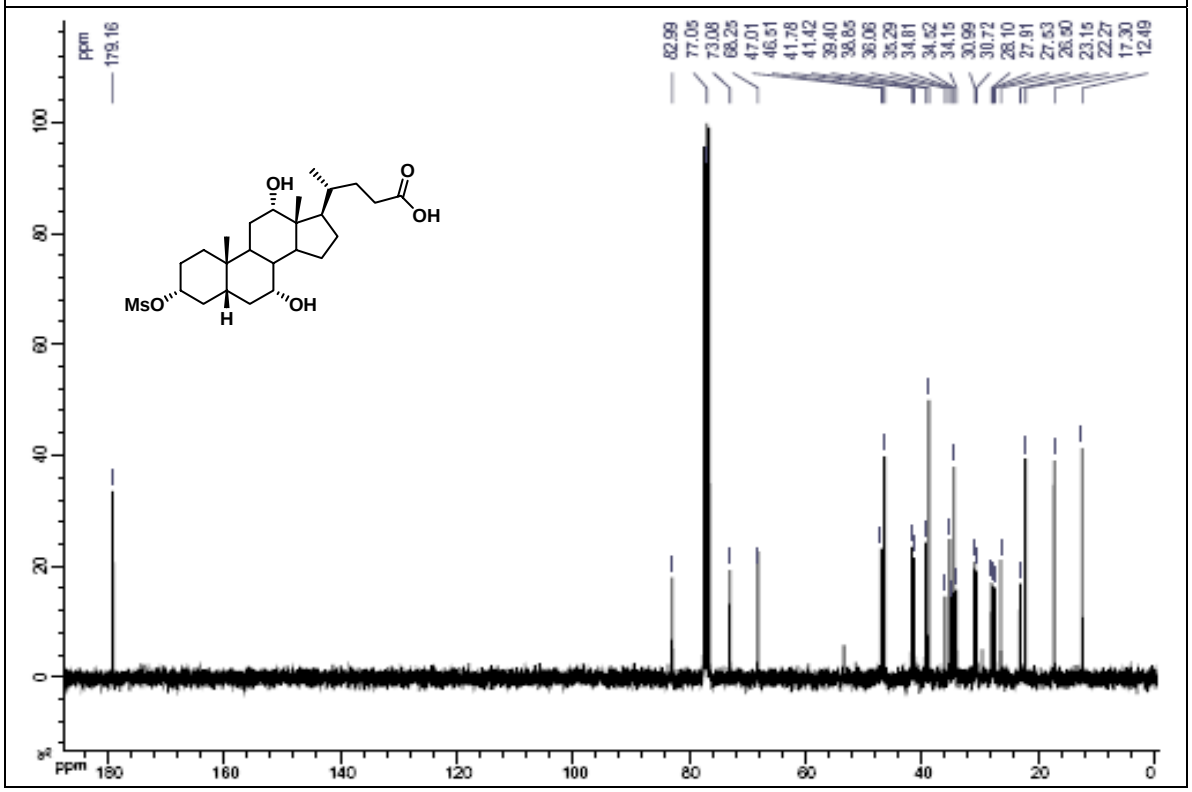
27: LCMS



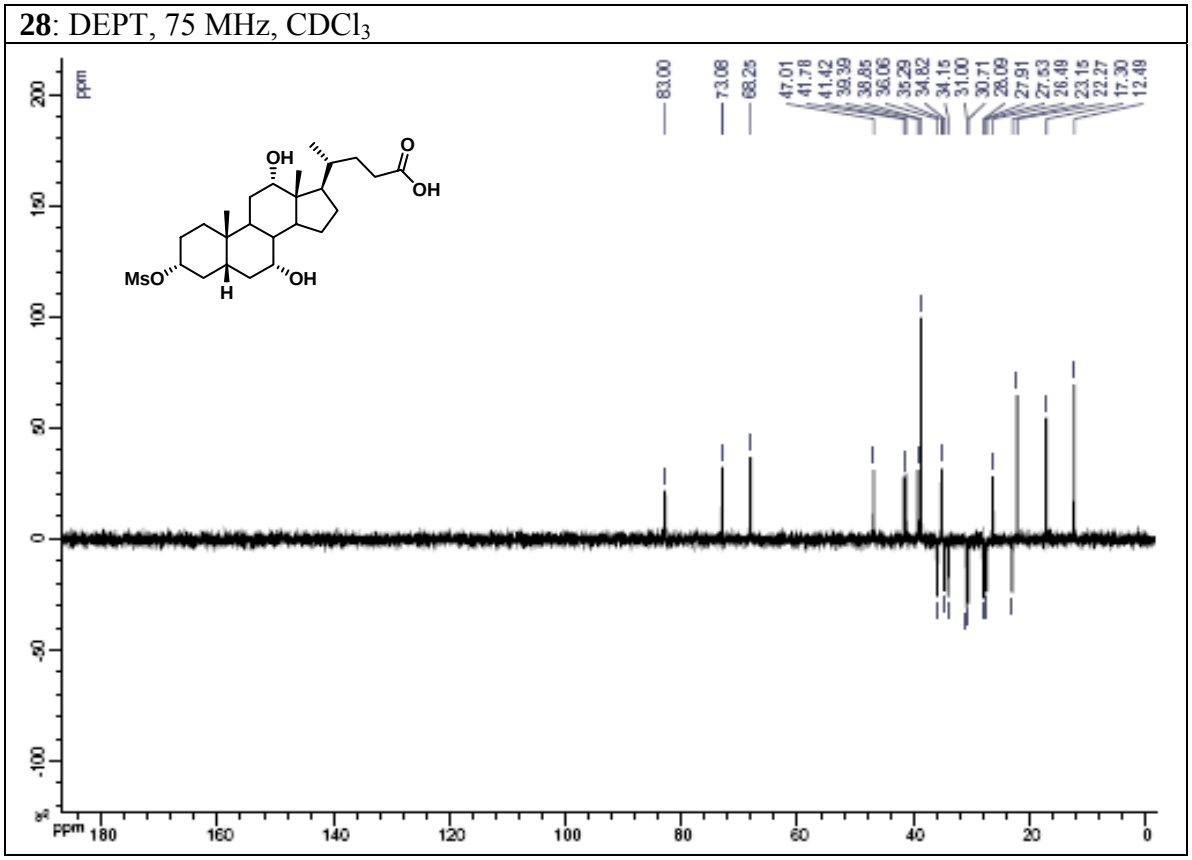
28: ^{1}H NMR, 300 MHz, $CDCl_3$



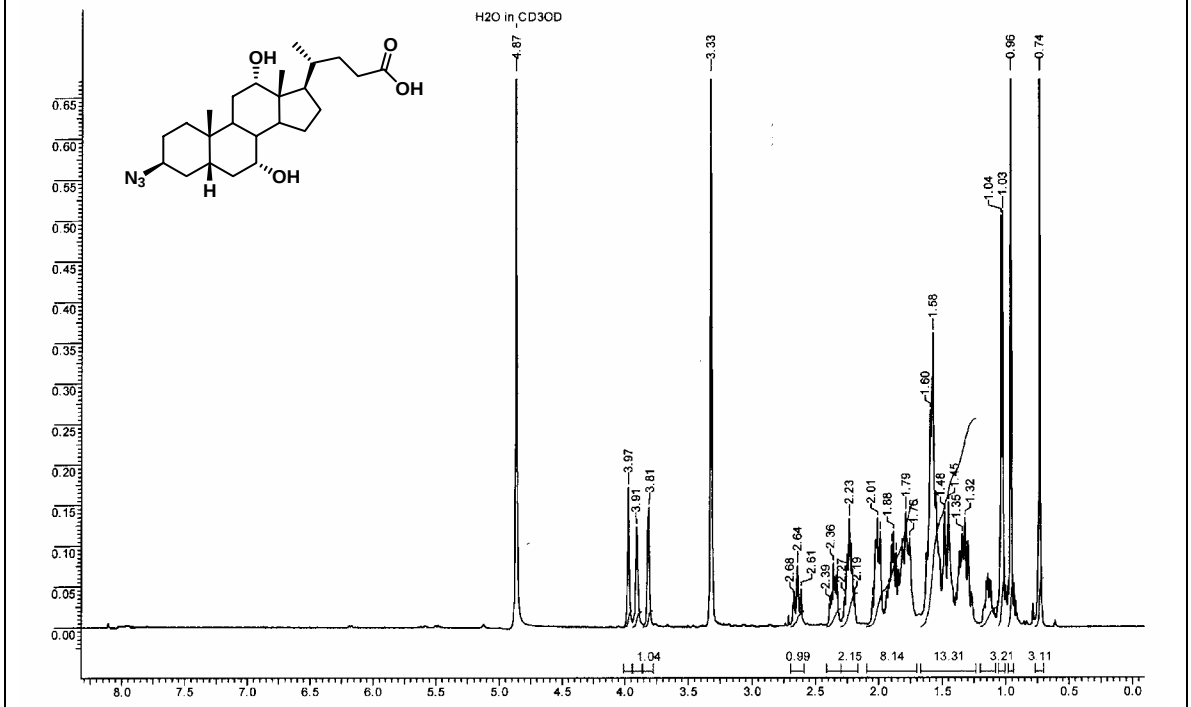
28: ^{1}H NMR, 75 MHz, $CDCl_3$



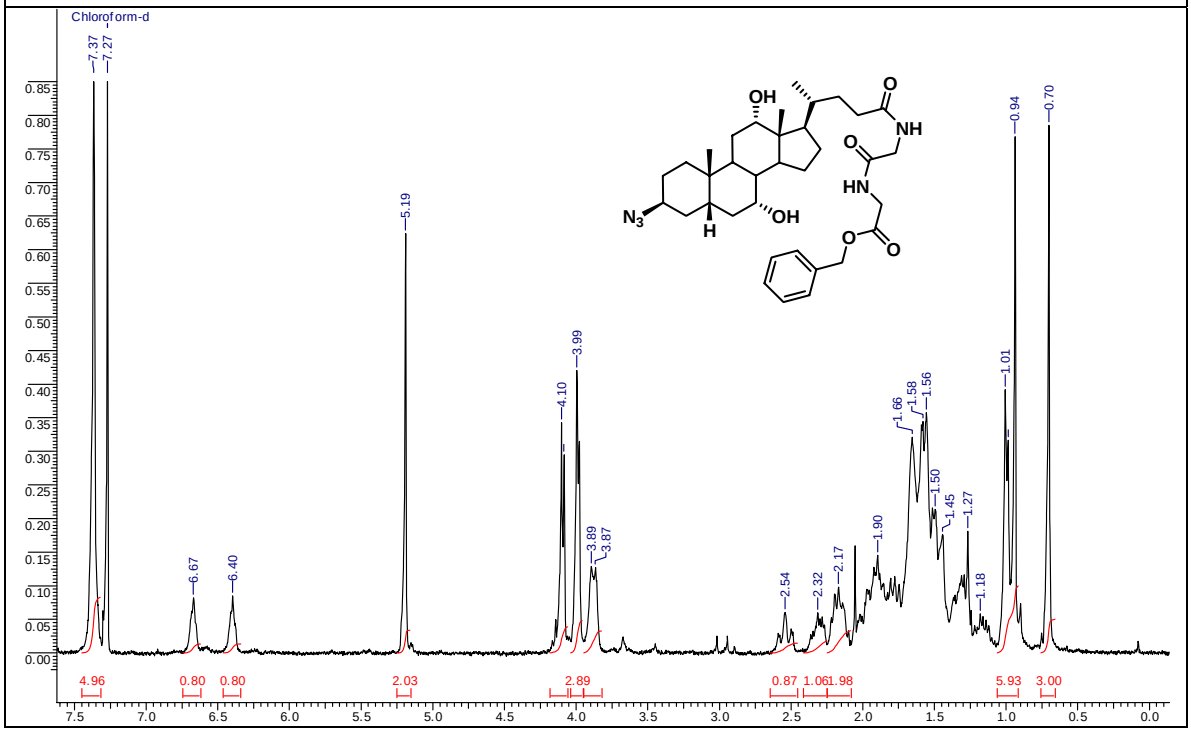
28: DEPT, 75 MHz, $CDCl_3$



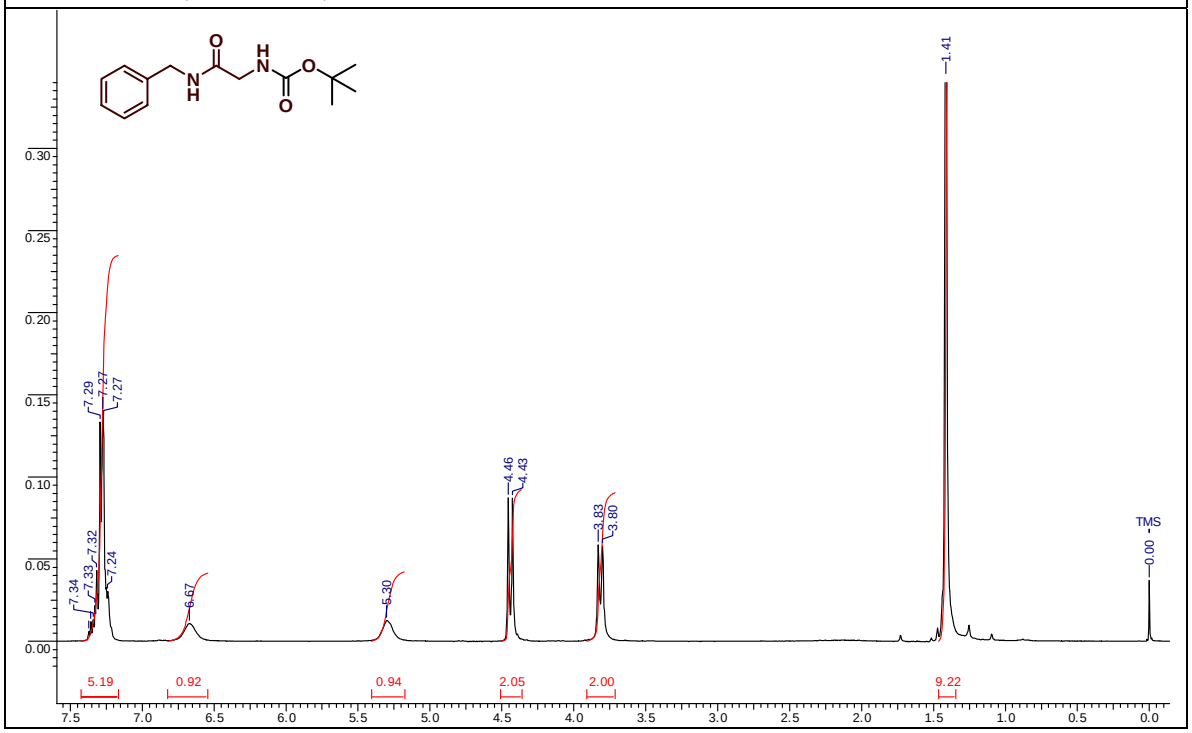
29: ^{1}H NMR, 500 MHz, CD_3OD



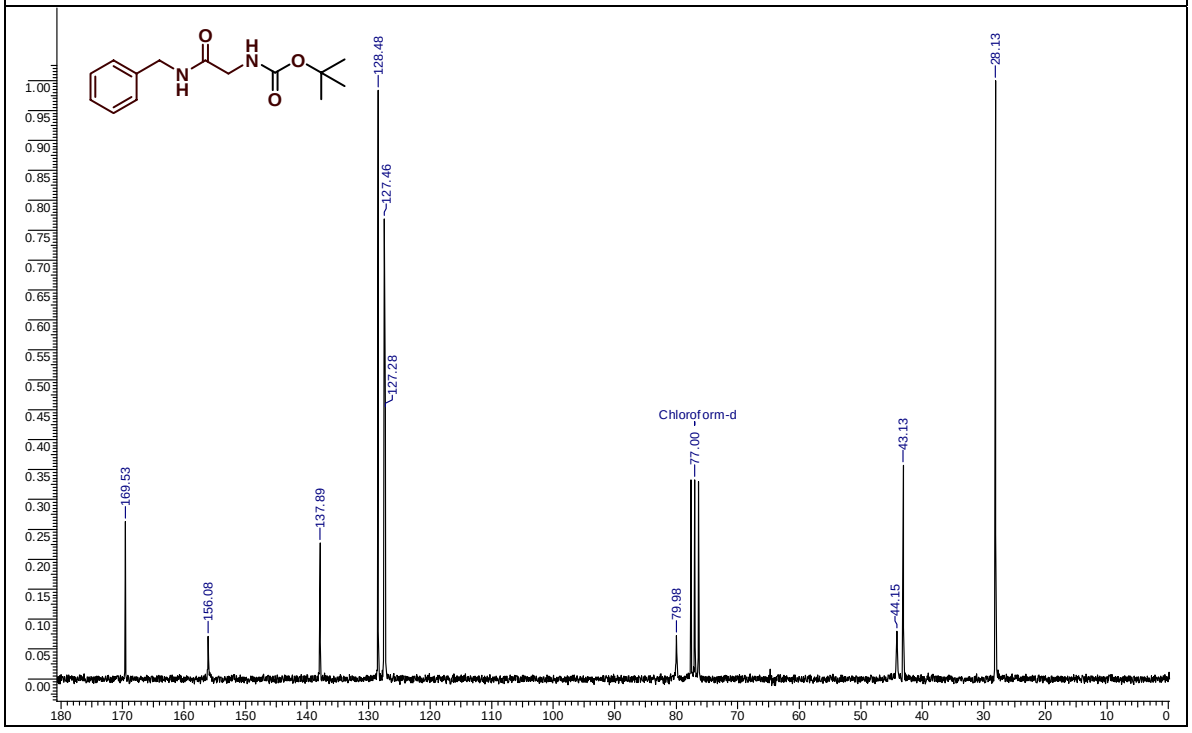
30: ^{1}H NMR, 300 MHz, $CDCl_3$



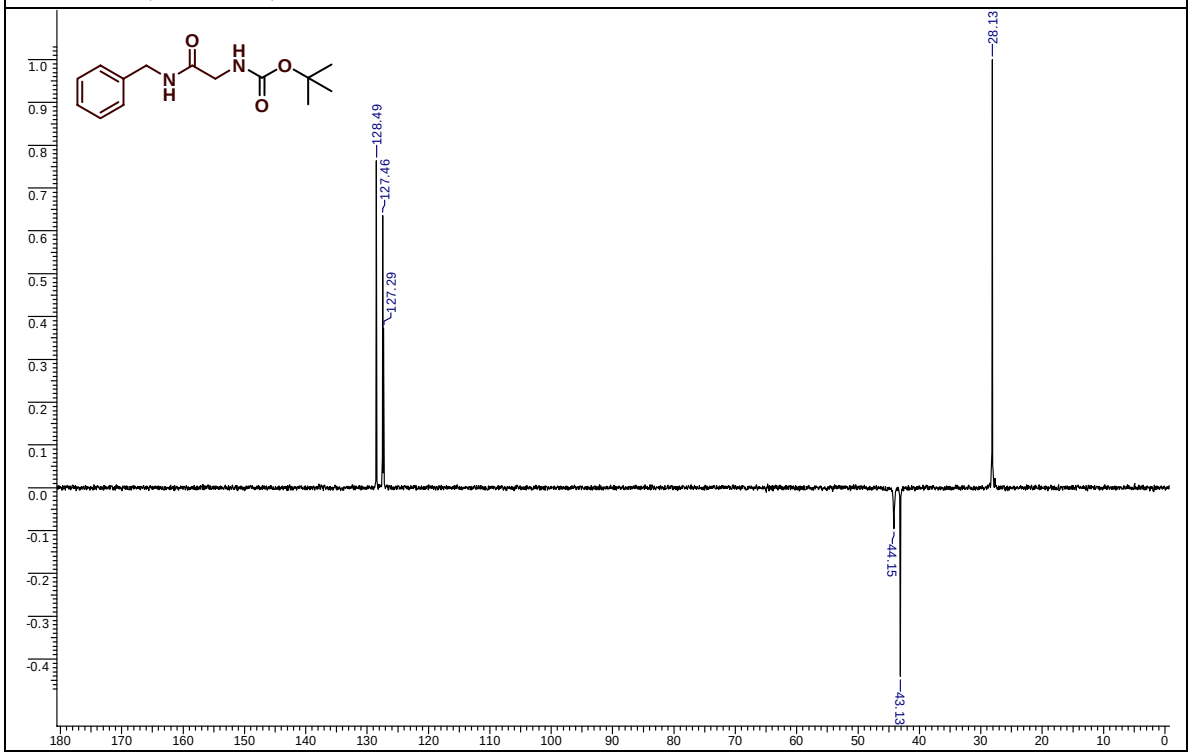
34: ^{1}H NMR, 200 MHz, $CDCl_3$



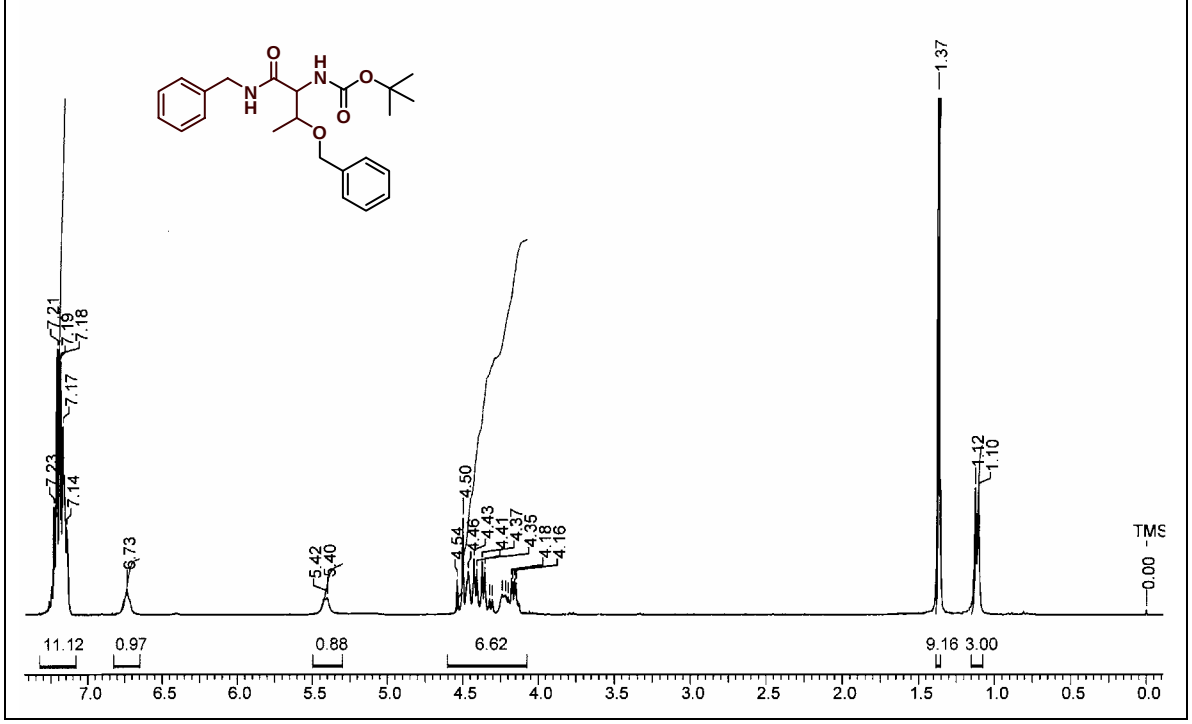
34: ^{13}C NMR, 50 MHz, $CDCl_3$



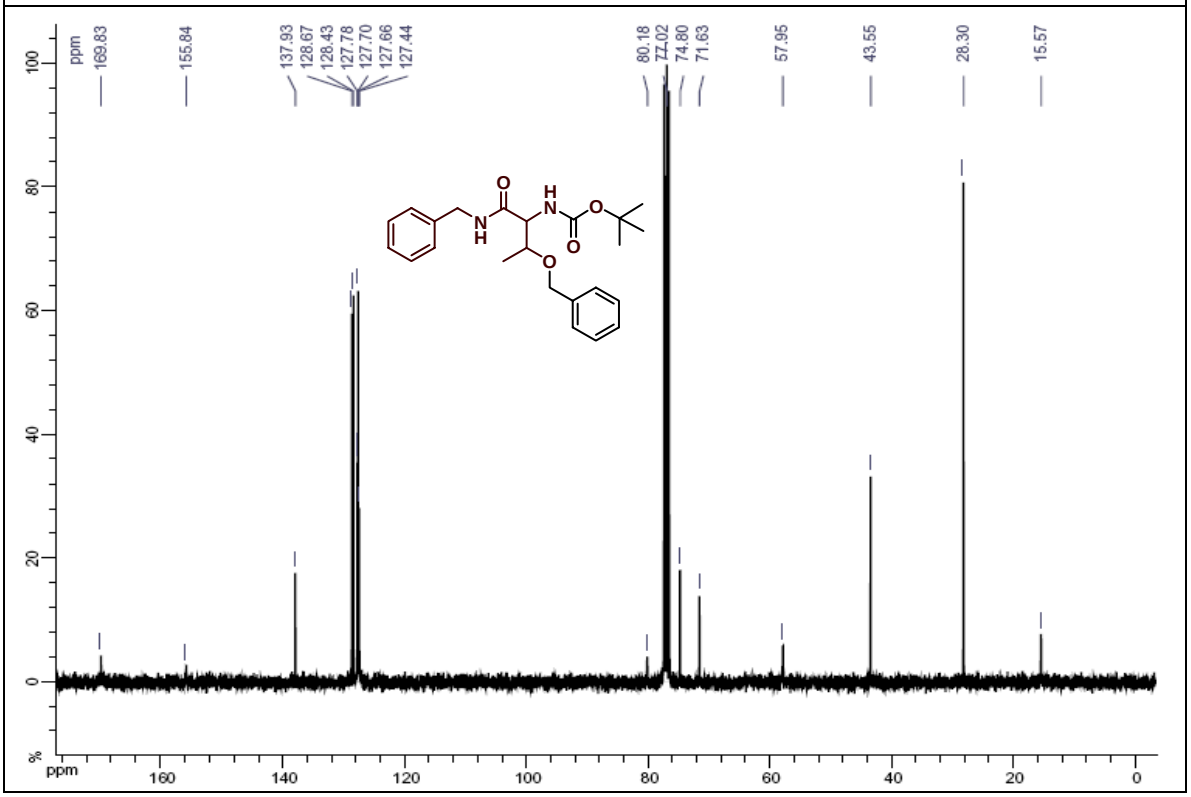
34: DEPT, 50 MHz, $CDCl_3$



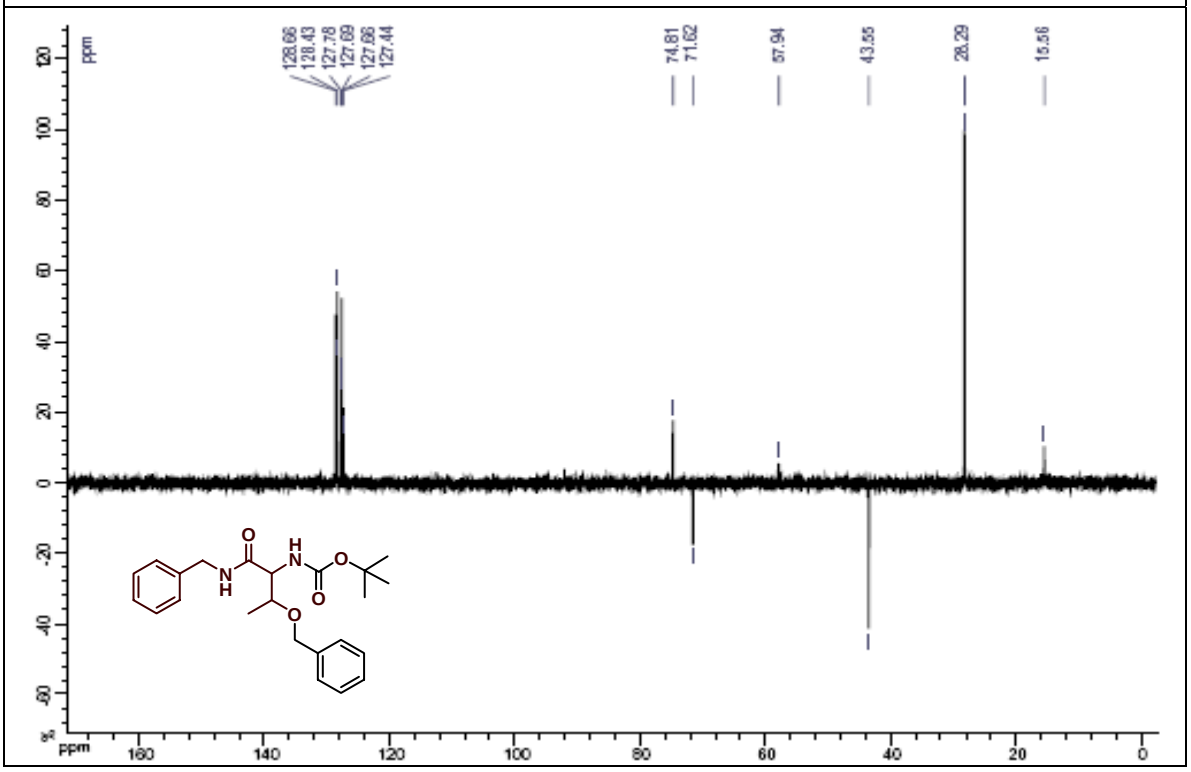
36: ^1H NMR, 300 MHz, $CDCl_3$



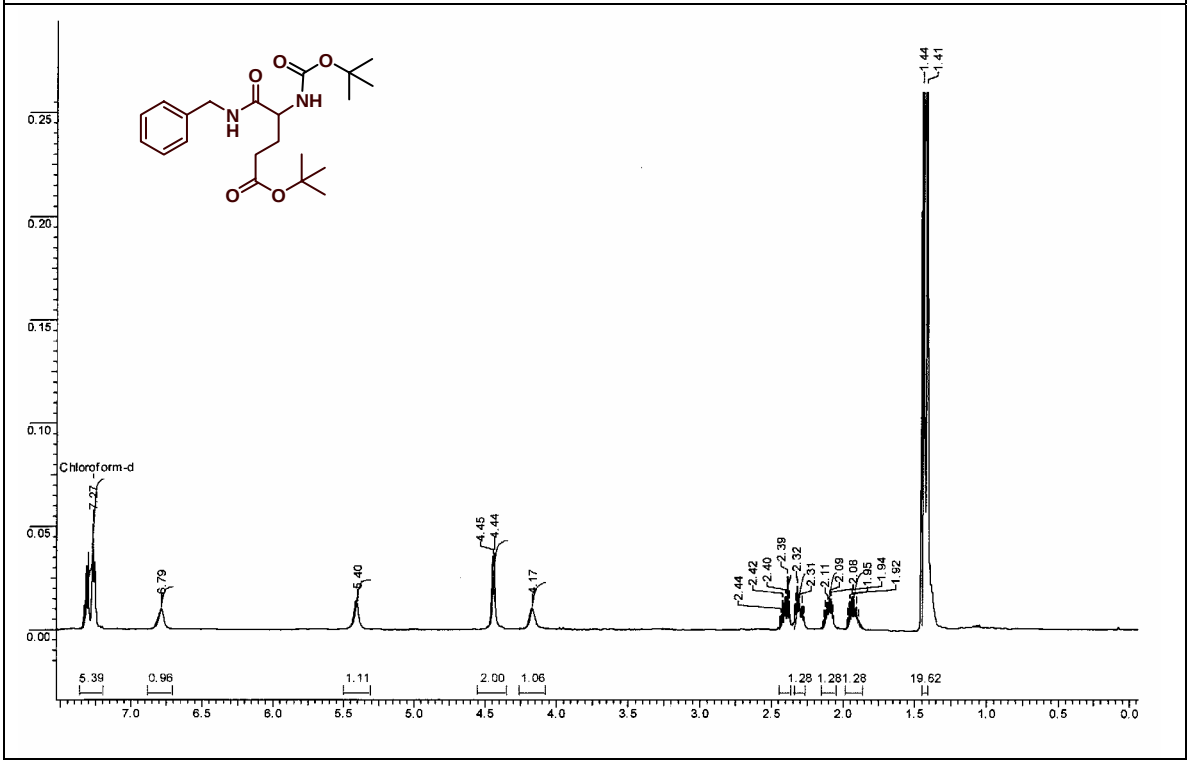
36: ^{13}C NMR, 75 MHz, $CDCl_3$



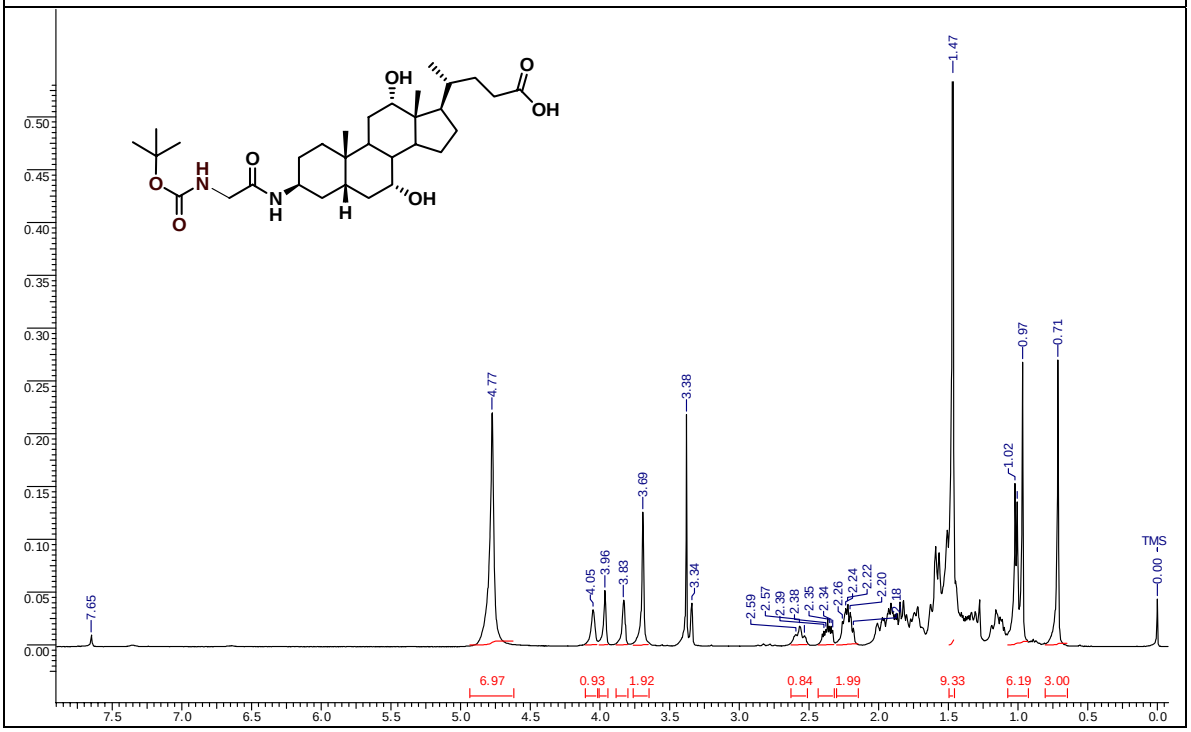
36: DEPT, 75 MHz, $CDCl_3$



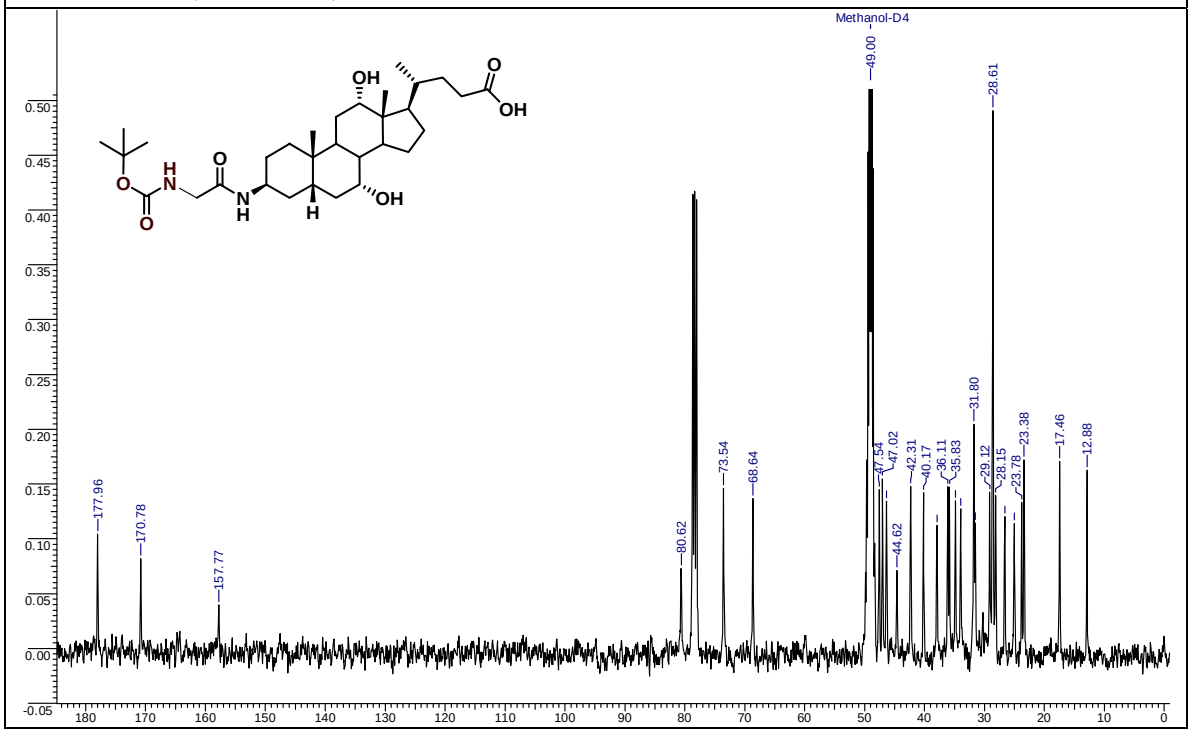
38: ^{1}H NMR, 300 MHz, $CDCl_3$



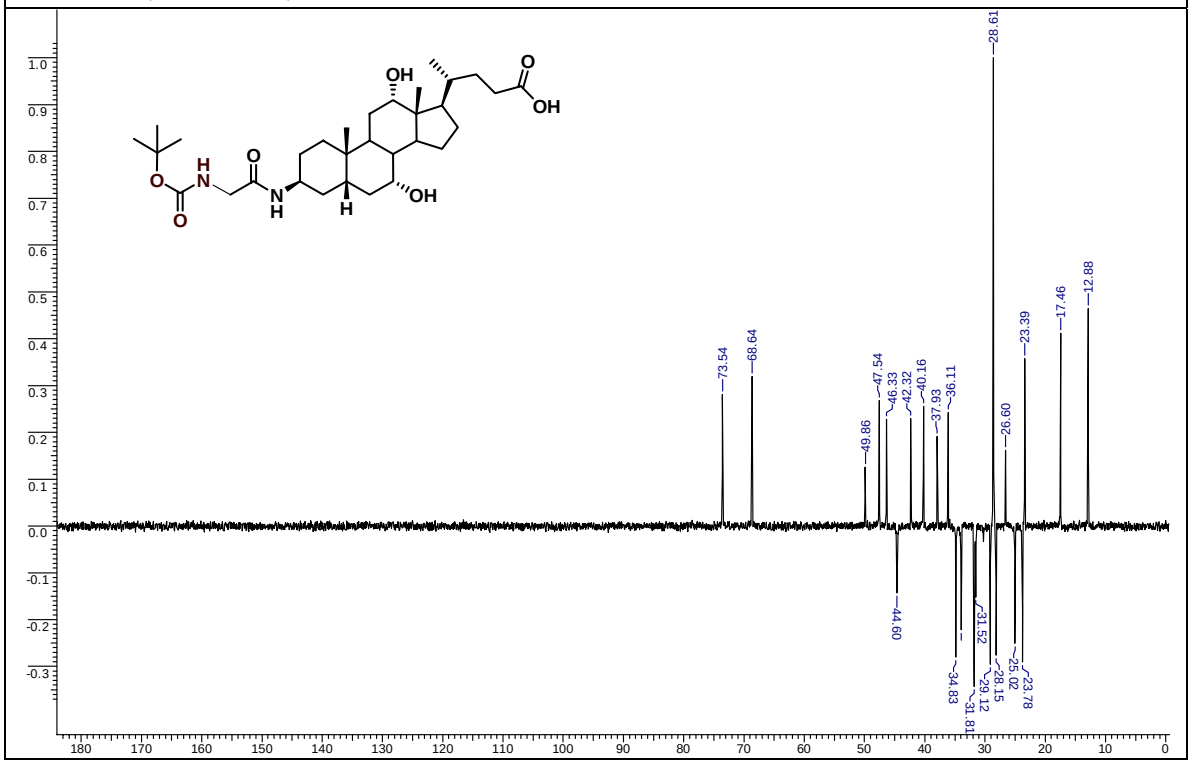
39: ^{1}H NMR, 400 MHz, $CDCl_3$+CD_3OD



39: ^{13}C NMR, 100 MHz, $CDCl_3$



39: DEPT, 100 MHz, $CDCl_3$



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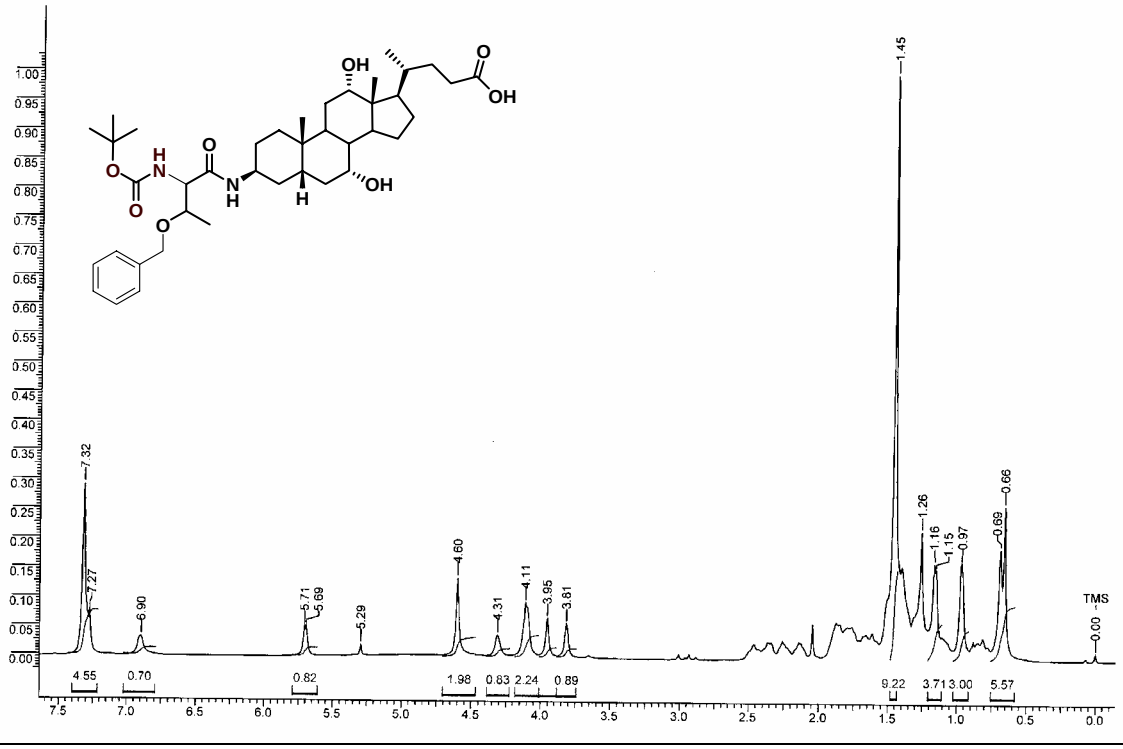

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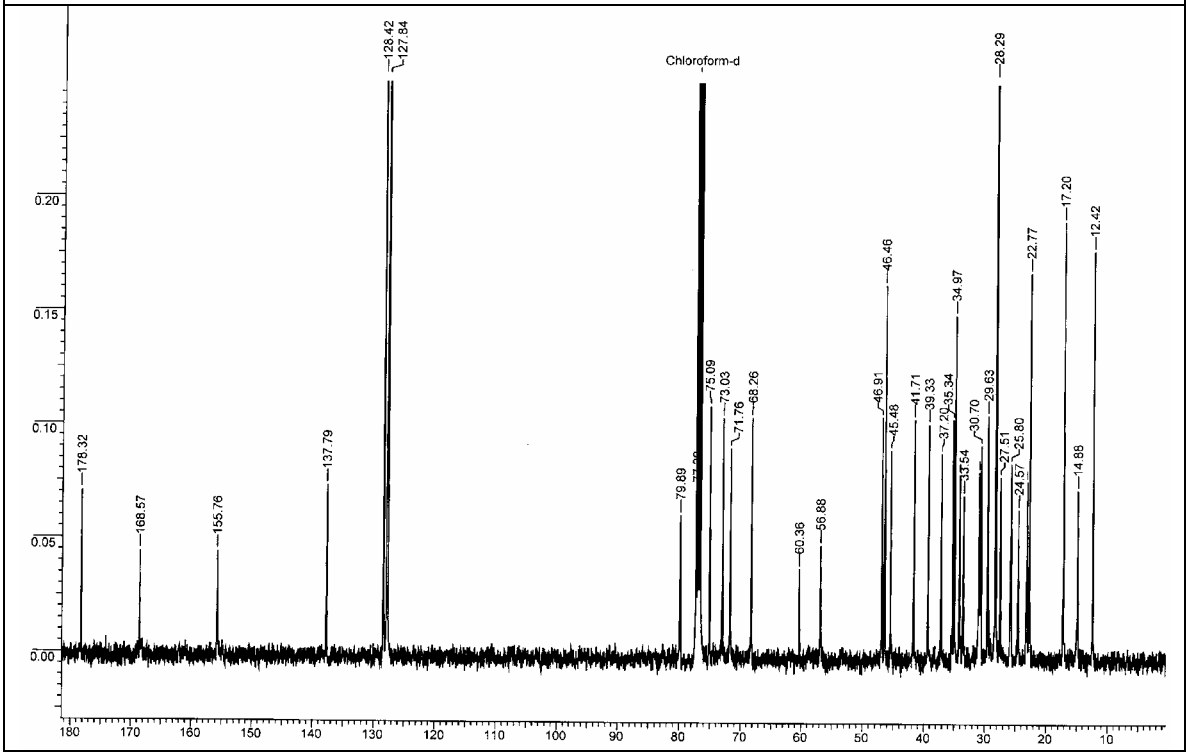
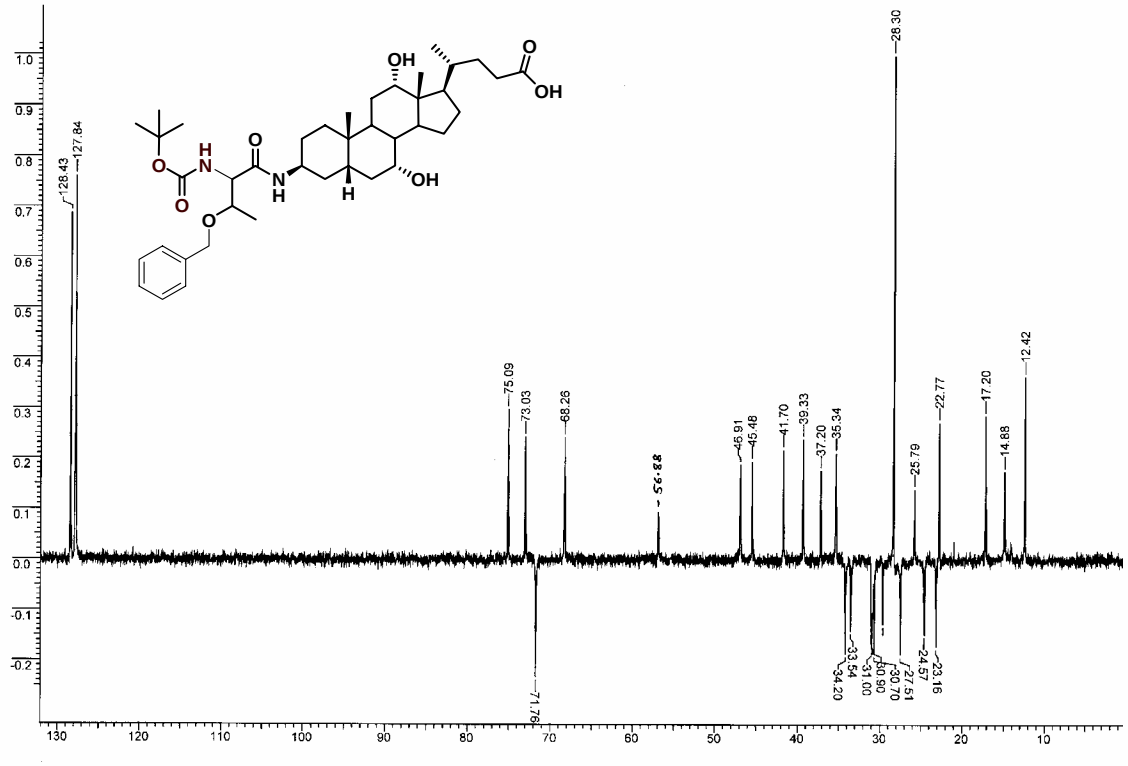
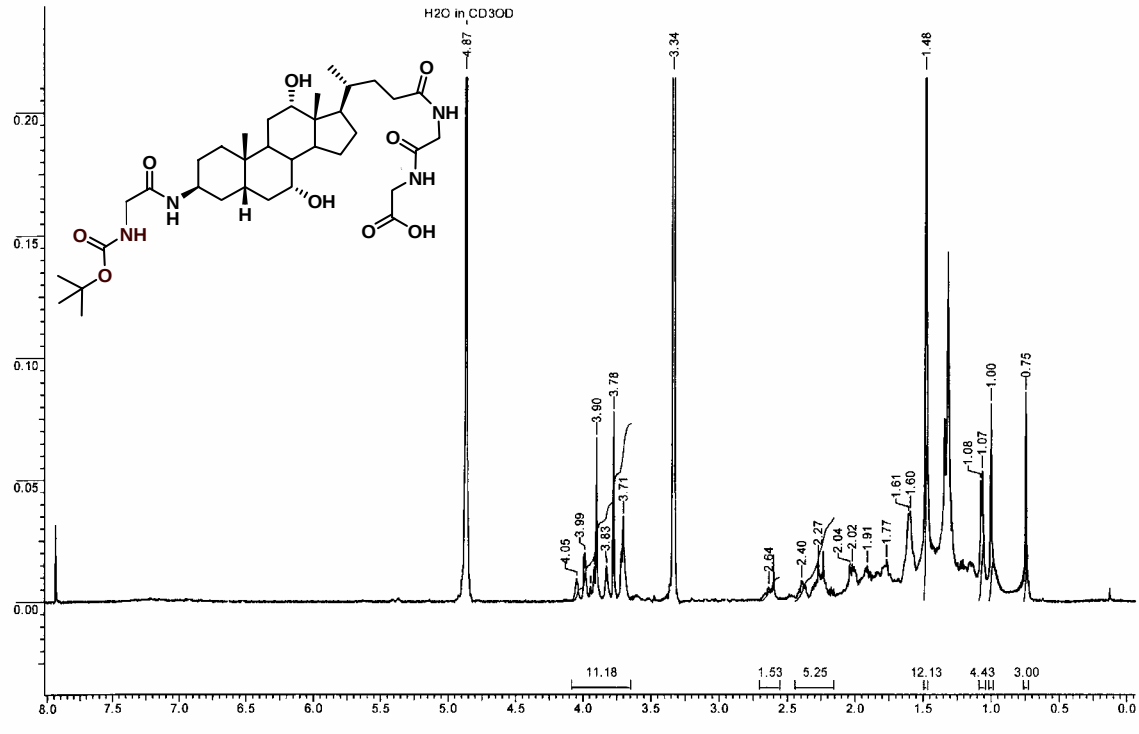


40: DEPT, 100 MHz, $CDCl_3$



41: ^1H NMR, 500 MHz, CD_3OD



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PART B : CHAPTER - 3

New Steroidal Dimers with Antifungal and Antiproliferative Activity

B3.1. Abstarct

Bile acid derived novel amphiphilic topology was designed and synthesized using cholic acid, deoxycholic acid, ethylenediamine and diethylenetriamine in the form of steroidal dimers. These dimers were tested for antifungal as well as antiproliferative activity *in vitro*. Cholic acid dimer with diethylenetriamine as a linker was found to be active against *C. albicans*, *Y. lipolytica* and *B. poitrassi* at nanomolar concentration and did not show any effect on cell proliferation. On the other hand deoxycholic acid dimer with ethylenediamine as a linker totally inhibited the growth of human oral cancer (HEp-2) and human breast cancer (MCF-7) cells.

B3.2. Introduction

During the last four decades, there has been tremendous increase in the frequency of fungal infections, especially disseminated systemic mycoses in immunocompromised hosts. These mycoses are mainly the result of opportunistic infections by organisms that are normally harmless, but can be pathogenic under certain conditions [1]. Species of *Aspergillus*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma* and *Sporothrix* are important causative agents, of which, *Candida albicans* is the most dreadful human pathogen. Current drugs against *C. albicans* include amphotericin B (Amp B) and a variety of azoles. But, Amp B is reported to be toxic to humans, and clinical resistance to azoles is increasing [2]. Therefore, there is need to screen for new antifungal therapeutics, which have high efficacy and low toxicity. Ideally new antimicrobial agents would include compounds to which microbes would unlikely develop resistance. Development of novel membrane active compounds may yield useful antimicrobials to which bacteria or fungi are unable to become resistant [3].

Accordingly, bile acid derived novel amphiphilic topology was designed and synthesized using cholic acid, deoxycholic acid, ethylenediamine and diethylenetriamine in the form of steroidal dimers. The information about the dimeric steroids having pharmacological applications was briefly summarized and presented as Bis(Steroidal) Conjugates in the part A of this thesis (Section A.6, Page 47). In the present case the synthesized dimeric steroids were tested for antifungal as well as antiproliferative activity *in vitro*. Cholic acid dimer with diethylenetriamine as a linker was found to be active against *C. albicans*, *Y. lipolytica* and *B. poitrassi* at nanomolar concentration and did not show any effect on cell proliferation. On the other hand deoxycholic acid dimer with ethylenediamine

as a linker totally inhibited the growth of human oral cancer (HEp2) and human breast cancer (MCF7) cells.

B3.2.1. *Design of Bile Acid Derived Facial Antimicrobial*

A common feature of bile acid derived antimicrobials is the potential to exhibit facially amphiphilic conformations. Facial amphiphiles are compounds containing polar and hydrophobic surfaces (Figure 1).

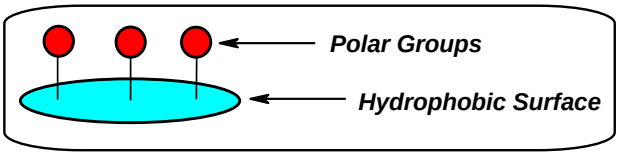


Figure 1. Schematic representation of facial amphiphile.

Such type of amphiphilicity can be achieved by polyene macrolide antibiotics [4] such as AmpB **1** (Figure 2). Number of membrane active, peptide antimicrobial agents [5] generally have both cationic residues along with hydrophobic amino acids, such as Polymyxin-B (PMB) **3**. In compounds with known structures [6,7] generally the cationic residues are clustered on one face of the molecule, while the hydrophobic side chains are oriented on an opposite face. These molecules therefore can be considered as facial amphiphiles [8]. Similar to polyene antibiotic Amp B, squalamine may be depicted in the cyclic form **2**. In this form it functions as an ionophore in common with large class of ionophoric antibiotics [4]. This salt-bridged cyclic form consists of an upper lipophilic sterol part and the lower part is the polyaminochain [9]. There is also a report in which squalamine mimic **4** possesses unusual inophoric properties [10]. At 0.5 % of compound **4**, a critical micelle concentration is reached on the membrane surface leading to cooperative insertion of an aggregate-active form allowing passage to anions such as chloride ions through membrane. This may lead to

destruction of membrane. Although the squalamine and PMB mimics are morphologically dissimilar, they display similar activities [11].

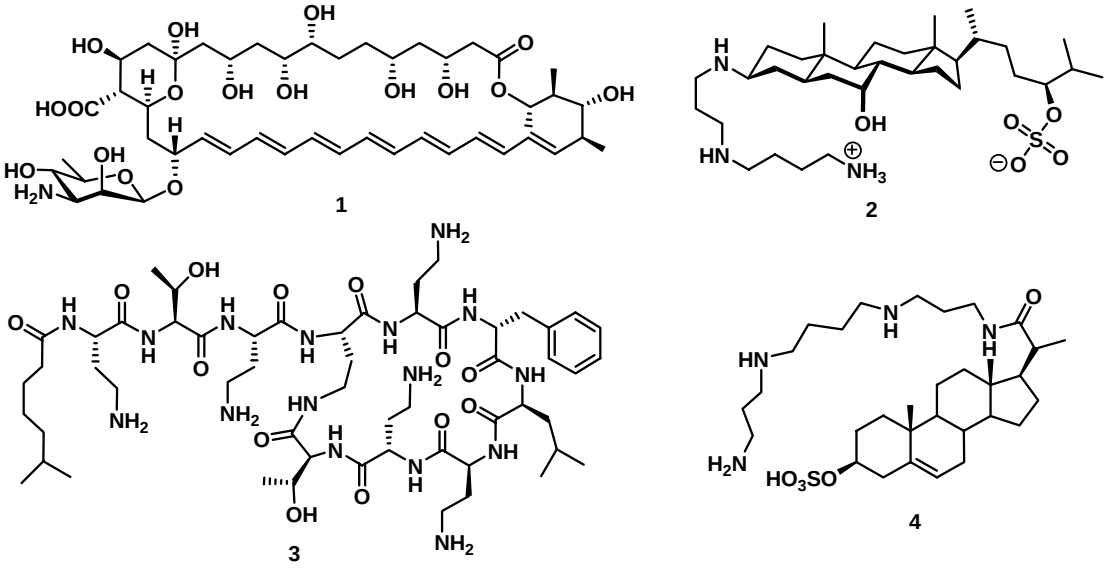


Figure 2. Amphotericin B **1**, squalamine in salt bridged cyclic form **2**, polymyxin B **3** and squalamine mimic **4**.

Based on these observations we thought of an amphiphile topology (Figure 3) that has not previously received much attention in synthetic systems: partially rigid structure with three discrete faces, one polar face sandwiched within two non polar faces.

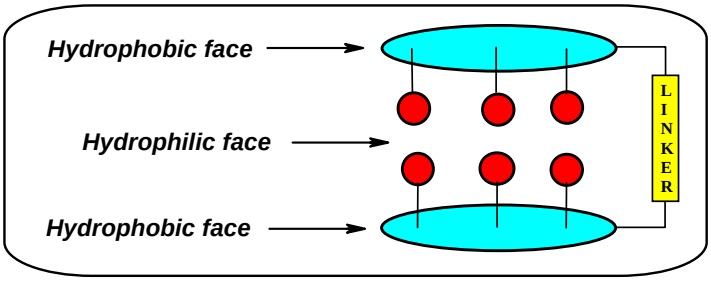


Figure 3. Schematic representation of novel facial amphiphile.

In this topology, steroid skeleton provides hydrophobic surface and hydroxyl groups can play a job of polar head group. Different linkers can be used so that additional hydrophobicity can be achieved. To support our hypothesis novel steroidal dimers **5** to **8**

were synthesized (Figure 4) and their antifungal activity was evaluated. Recently clinical researchers in London reported [12,13] exciting antitumor activity by the antifungal drug Trichostatin A. Therefore we examined antiproliferative activity of these newly synthesized steroidal dimers **5** to **8**.

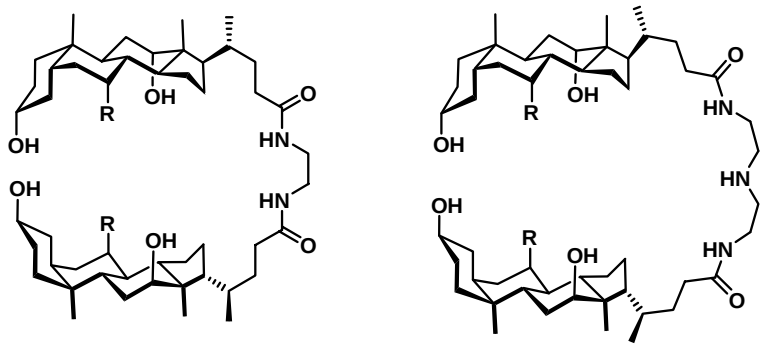


Figure 4. Proposed target molecules designed from cholic acid and deoxycholic acid.

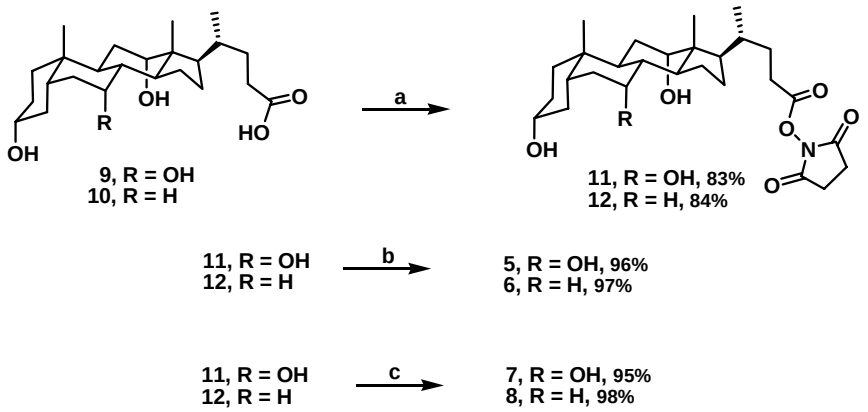
B3.3. Chemistry

The steroid nucleus is one of the largest rigid units readily available with multiple chiral centers and biological importance of this structural entity is well documented [14, 15]. Dimeric steroids were first observed as synthetic by-products [16] and then discovered in nature [17]. Dimerization of steroid skeleton leads to unique characteristics and applications gradually began to emerge in different areas. Many dimeric and oligomeric steroids exhibit micellular, detergent and liquid crystal behavior [18, 19]. Steroidal dimers have been used as catalysts for some types of reactions [20] and may lead to new pharmacologically active steroids [21]. Cephalostatins is a group of dimeric steroids that are among the most potent natural cytotoxins. This exceptional activity of cephalostatins has led to interest in the synthesis of steroidal dimers as potential antitumor agents [22]. Oligomeric steroids with or

without spacer groups can be used as chiral building blocks to construct artificial receptors and as architectural components in biomimetic/molecular recognition chemistry [15].

Cholic acid steroidal skeleton plays pivotal role in formation of dimeric and oligomeric forms. In dimeric form cholic acid exists in a rigid conformation with the steroid hydroxyl groups intramolecularly hydrogen bonded [23]. It is ideal building block for artificial enzymes because i) formation of a cavity due to rigidity of A/B *cis* junction, ii) the two faces of the molecule are dramatically different, α face is hydrophyllic due to the three hydroxyl groups while β face is hydrophobic due to steroid skeleton and two angular methyl groups, iii) all the three chemically different hydroxyl groups and side chain carboxylate can be readily derivatised, iv) cholic acid is a chiral molecule with many asymmetric centers and is inexpensive [14]. This chapter highlights an efficient synthesis, characterization and bioevaluation of four new dimeric steroids **5** to **8** having different spacer groups (Scheme 1).

Scheme 1.



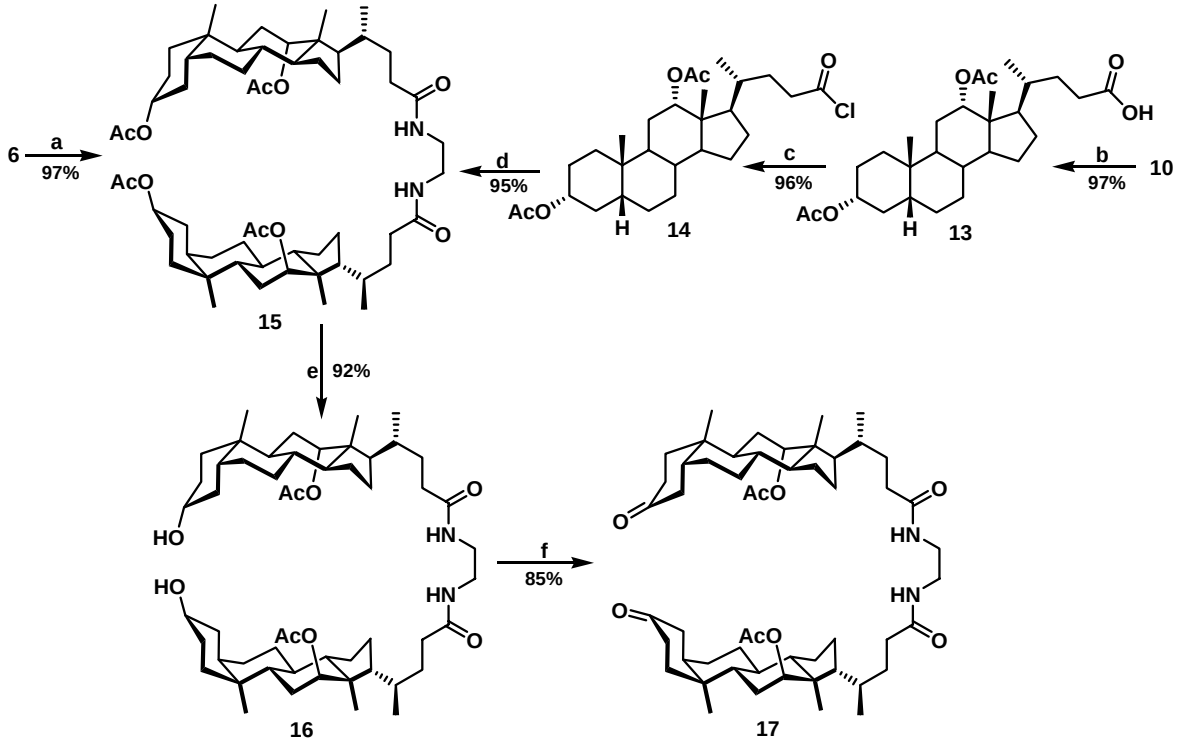
Reagents and conditions: a) *N*-hydroxy succinimide, DCC, THF-CH_3CN, 25 ˚C, 18 h; b) Ethylenediamine, DMF, 25 ˚C, 1.5 h; c) Diethylenetriamine, DMF, 25 ˚C, 1.5 h.

Cholic acid **9** and deoxycholic acid **10** were activated as *N*-succinimidyl esters **11** and **12** respectively (Scheme 1) [24]. These esters were reacted with ethylenediamine in DMF at 25 °C for 1.5 h to give the dimeric compounds **5** and **6**. Similarly esters **11** and **12**

were reacted with diethylenetriamine in DMF at 25 °C for 1.5 h to afford the dimers **7** and **8** in very high yields (95-98 %). There are two reports [25] in which synthesis of compound **5** and **6** have been carried out in four steps as an intermediate for the total synthesis of cholaphanes. Eur. Pat. Application EP 489423 describes a process for the preparation of bile acid derivatives and their use in medicine. In this patent application steroidal dimers have been synthesized as inhibitors of bile acid resorption than the compounds described in the present chapter. The dimeric compounds **5** and **6** with ethylenediamine as a linker showed 25 singlets in ^{13}C FT NMR spectra though it has total 50 carbon atoms. The respective monomer unit has 26 C-atoms. This reduction in number of C-atoms is due to symmetry of the molecule. Similar observations found in case of compounds **7** and **8** in which ^{13}C FT NMR spectra showed 26 singlets though it has total 52 carbon atoms. The respective monomer unit has 28 C-atoms. Mass spectroscopy and C, H, N analysis finally confirmed the formation of dimeric compounds.

To study the consequence of substitutions at C-3 and C-12 on the physical properties as well as their biological effects, few more dimeric steroids were synthesized from compound **6** as shown in Scheme 2. In dimeric form compound **6** was expected to addopt a rigid conformation with the steroid hydroxyl groups intramolecularly hydrogen bonded. To break this expected hydrogen bonded network all the hydroxyl groups in compound **6** were acetylated to afford tetra-acetate compound **15**. This can be achieved by two easy routes as depticted in Scheme 2. The 3,3'-acetate groups of compound were then selectively hydrolyzed to furnish compound **16**, which still can addopt a conformation with the steroid hydroxyl groups intramolecularly hydrogen bonded. The 3,3'-hydroxyl groups of compound **16** were then oxidized to 3,3'-oxo functionalities to afford compound **17**.

Scheme 2.



Reagents and conditions: a) Ac_2O, Pyridine, 25 ˚C, 18 h; b) Ac_2O, Pyridine, 25 ˚C, 18 h; c) $SOCl_2$, 25 ˚C, 1.5 h; d) Ethylenediamine, DMF, 25 ˚C, 1.5 h; e) Na_2CO_3, CH_3OH, H_2O, 12 h, f) CrO_3, H_2SO_4, H_2O, 10 ˚C, 5 min.

B3.4. Bioevaluation

Compounds **5**, **6**, **7** and **8** were examined for antifungal activity against five different isolates of *Candida albicans*, a human pathogen and two non-pathogenic dimorphic fungi *Benjaminiella poitrasii* and *Yarrowia lipolytica* as model systems. The minimum inhibitory concentrations (*MIC*) were determined for the respective compounds by the Antibiotic Disc Diffusion Method against *C. albicans*, *B. poitrasii* and *Y. lipolytica*. Cycloheximide, a protein synthesis inhibitor was used as a standard inhibitor for comparison. The *MIC* for **7** was 11.32 nM for all five *C. albicans* isolates, *B. poitrassi* and *Y. lipolytica*, the *MIC* values for the compound **8** ranged between 10-25 nM and compounds **5** and **6** did not show any zone of inhibition even upto 500 nM concentration. However, in case of cycloheximide, the

MIC value was much higher and ranged between 250-500 nM for isolates of *C. albicans*. In a microtitre dilution broth assay, the *MIC* of cycloheximide against a clinical isolate of *C. albicans* was reported to be greater than 900 nM [26]. *MIC* of cycloheximide was 71 nM in *B. poitrasii* and 106 nM in *Y. lipolytica*. The difference in the toxicity of cycloheximide towards *C. albicans*, a pathogenic fungus and *B. poitrasii* and *Y. lipolytica*, non-pathogenic fungi can be attributed to the differences in their cell wall composition that affects the passage of the compound through the fungal cell wall. DMSO did not show any zone of inhibition. The results suggest that the compounds **7** and **8** have potential as antifungal agents when compared to the standard inhibitor, cycloheximide, when tested against both the pathogenic as well as non-pathogenic fungi.

Table 1. *In Vitro* antifungal activity of compounds **7, 8** and cycloheximide.

Fungi	**7**		**8**		Cycloheximide	
	MIC nM	Inhibition Zone, cm	*MIC* nM	Inhibition Zone, cm	*MIC* nM	Inhibition Zone, cm
C. albicans, Isolate no.1	11.32	0.8	11.75	0.9	355.8	1.0
C. albicans, Isolate no.2	11.32	0.8	11.75	0.7	538.8	0.9
C. albicans, Isolate no.3	11.32	0.8	11.75	1.0	266.9	0.9
C. albicans, Isolate no.4	11.32	0.8	11.75	0.8	533.8	1.0
C. albicans, Isolate no.5	11.32	0.8	17.63	0.9	533.8	0.8
B. poitrassi	11.32	0.7	17.63	0.6	71.2	1.45
Y. lipolytica	11.32	0.9	23.50	0.8	106.8	0.9

The antiproliferative activity of all the compounds was tested against human cancer cells (Human oral cancer cells, HEp-2 and human breast cancer cells, MCF-7). To assess the anti-proliferation activity of these compounds HEp-2 and MCF-7 cells were grown *in vitro*. The cells were treated with varying concentrations of compounds individually for 72

hrs. Compounds **7** and **5** up to a concentration of 10 μM had no effect on the survival of HEp-2 and MCF-7 cells. However, the compounds **6** and **8** elicited a significant reduction in proliferation of both HEp-2 and MCF-7 cells. The compound **6** at 10 μM concentration totally inhibited the growth of MCF-7 and HEp-2 cells. IC_{50} values for compound **6** on HEp-2 and MCF-7 cells were between 2-3 μM. Whereas IC_{50} values for compound **8** appear to be greater than 10 μM. The compound **8** at 10 μM concentration inhibited the growth of HEp-2 by 20 % whereas it inhibited MCF-7 growth 40 %.

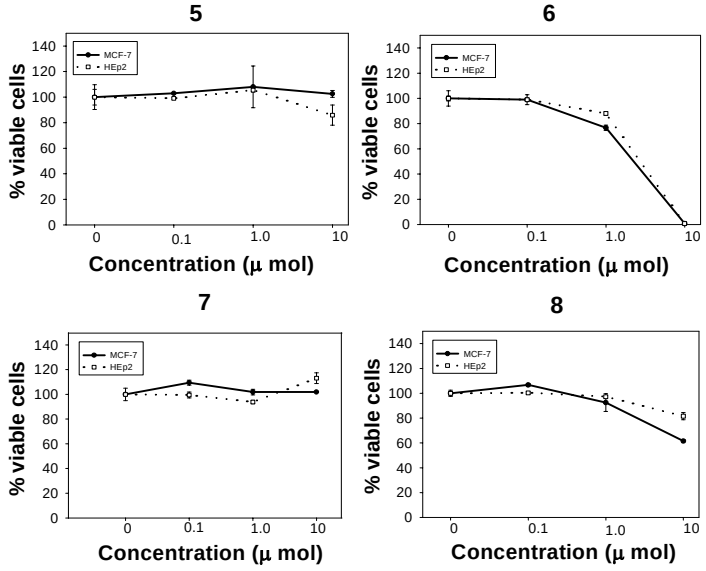


Figure 5. Graphical representation for antiproliferative activities.

It was found that compound **7** is active against *C. albicans* (Isolate 1-5), *B. poitrassi* and *Y. lipolytica.* Compound **8** also at little higher concentration showed similar antifungal activity. Where as compound **5** and **6** were found to be inactive up to 500 nM concentratioin. Compound **5** and **7** up to a concentration of 10 μM had no effect on the survival of Hep-2 and MCF-7 cells. Compound **8** at 10 μM concentration inhibited the growth of Hep-2 cells by 20 % where as it inhibited MCF-7 growth by 40 %. Compound **6** at 10 μM concentration totally inhibited the growth of Hep-2 and MCF-7 cells.

Table 2. Comparison of antifungal activity and cytotoxicity of **6** and **7**.

Compound No.	Antifungal Activity *MIC* in nM	Antiproliferative Activity IC_{50} in μM
6	> 500	2.2
7	11.32	Not active

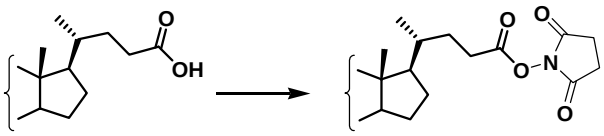
The results suggest that compound **7** has a potential as antifungal agent when tested against both pathogenic and non-pathogenic fungi. The compounds **15**, **16** and **17** are under biological evaluation to study their antiproliferative activity against human cancer cell lines.

B3.5. Summary

Two series of steroidal dimers with different linkers were synthesized, characterized and their biological activities were evaluated. The antifungal activity of compound **7** was very good in comparison with cycloheximide, so considering its low toxicity on cancer cell lines tested; it represents a good lead for further research in this area. Tested compounds **6** and **8** exhibited antiproliferative activity at micromolar concentrations (IC_{50} 2-3 μM). Compound **6** exhibited profound effect where as compound **8** inhibited cell growth partially. The growth suppressive effect of compound **6** and **8** appears to be influenced by the length of side chain present. Since compound **5** and **7** had no effect on cell survival, the absence of hydroxyl group at C-7 position plays a critical role in toxicity of these compounds towards human cells. To study the consequence of substitutions at C-3 and C-12 on the physical properties as well as their biological effects, few more dimeric steroids were synthesized from compound **6** which are under biological evaluation. Thus, an extensive SAR studies should be carried out so that one can reach to the appropriate explanation for the presence or absence of particular functionality in the dimers.

B3.6. Experimental Section

B3.6.1. *Typical procedure for the synthesis of N-succinimidyl ester (11) of cholic acid (9) and the N-succinimidyl ester (12) of deoxycholic acid (10):*

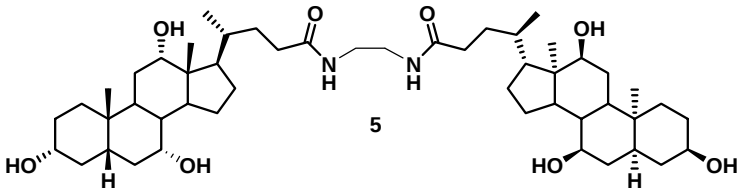


Cholic acid **9** (0.2g, 0.489 mmol) was dissolved in a mixture of dry THF (4 mL) and dry acetonitrile (1 mL) and *N*-hydroxy succinimide (0.067 g, 0.589 mmol) was added. To the resulting homogeneous solution dicyclohexylcarbodiimide (0.1g, 0.489 mmol) in dry THF (2 mL) at 10-15 ˚C was added dropwise. The mixture was stirred at 25 °C for 18 h and the precipitated *N*,*N*-dicyclohexyl urea was removed by filtration. THF was removed under reduced pressure and the residue was extracted with ethyl acetate (3x20 mL) and the extract was washed successively with aqueous $NaHCO_3$, water and then with brine. The extract was dried over Na_2SO_4 and ethyl acetate was removed under reduced pressure to get crude solid compound. Column chromatographic purification afforded a pure material **11** (0.206 g, 83 %). This was then crystallized from ethyl acetate-hexane (50 %). Mp. 118 ˚C (colorless powder, lit. [24a] Mp. 119-120 ˚C); IR ν_{max} (Nujol) 3328, 1731, 1631, 1209 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 3.98 (m, 1H), 3.83 (m, 1H), 3.42 (m, 1H), 2.82 (bs, 4H), 2.61 (m, 2H), 1.02 (d, *J* = 4.2 Hz, 3H), 0.87 (s, 3H), 0.68 (s, 3H); Anal Calcd. for $C_{28}H_{43}NO_7$ C, 66.51; H, 8.57; N, 2.77 Found: C, 66.24; H, 8.69; N, 2.39.

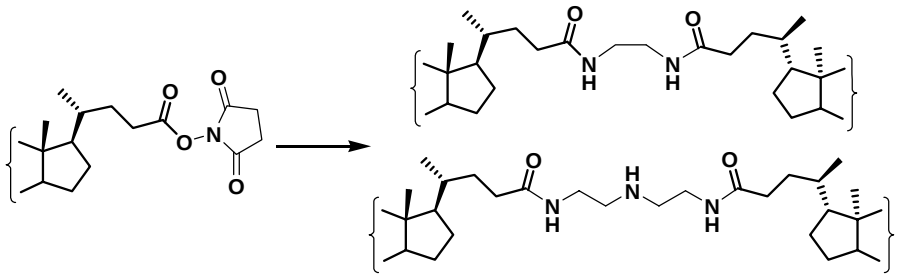
In the similar manner deoxycholic acid **10** (3.92 g, 10 mmol), was converted to its *N*-hydroxy succinimide ester **12** (4.13 g, 84 %) which was crystallized from ethanol. Mp. 195-197 ˚C, lit. [24b] Mp. 194-196 ˚C; IR ν_{max} (Nujol) 3330, 1730, 1632, 1211 cm^{-1}; ^1H NMR

(200 MHz, $CDCl_3$) δ 3.98 (m, 1H), 3.60 (m, 1H), 2.82 (s, 4H), 2.63 (m, 2H), 1.01 (d, *J* = 4.2 Hz, 3H), 0.89 (s, 3H), 0.68 (s, 3H); Anal. Calcd. for $C_{28}H_{43}NO_6$: C, 68.68; H, 8.85; N, 2.86 Found: C, 68.23; H, 8.92; N, 2.54.

B3.6.2. *N^1, N^2- Ethylenediaminebis[cholic acid amide] (5):*



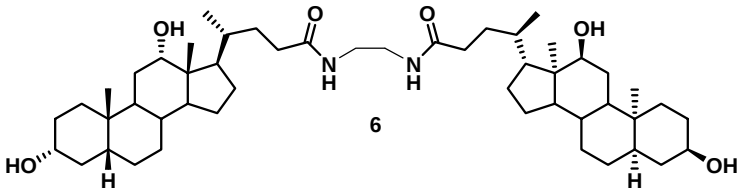
Typical procedure for the synthesis of dimers (5-8) from *N*-succinimidyl ester (11) of cholic acid (9), *N*-succinimidyl ester (12) of deoxycholic acid (10) and polyamines such as ethylenediamine and diethylenetriamine:



N-succinimidyl ester **11** of cholic acid **9** (1.01g, 2 mmol) was dissolved in 1.5 mL of dimethylformamide. To it ethylenediamine (0.073 mL, 1.1 mmol) was added and reaction mixture was stirred for 1.5 h at 25 ˚C. Reaction was quenched by the addition of crushed ice. Solid crude product was filtered and dried under vacuum. Column chromatographic purification of the crude product (neutral deactivated alumina, eluent: chloroform/methanol (19:1)) afforded N^1,N^2- Ethylenediaminebis[cholic acid amide] **5** (0.81g, 96 %). It was further crystallized from methanol/chloroform. Mp. 205 ˚C (colourless powder); IR ν_{max} (Nujol) 3344, 2921, 1656, 1631 cm^{-1}; ^1H NMR (500 MHz, CD_3OD+$CDCl_3$) δ 4.01 (bs, 2H),

3.84 (s, 2H), 3.40 (m, 2H), 3.33 (bs, 4H), 1.01 (d, *J* = 5 Hz, 6H), 0.89 (s, 6H), 0.69 (s, 6H). ^{13}C NMR (125 MHz, CD_3OD+$CDCl_3$) δ 176.8, 73.4, 72.0, 68.7, 46.8, 46.7, 42.0, 41.7, 39.7, 39.5, 36.0, 35.6, 35.1, 34.9, 33.2, 33.1, 32.2, 30.2, 28.3, 28.0, 26.7, 23.5, 22.7, 17.5, 12.7; MS (LCMS) *m/z* 859.02 $[M+NH_4]^+$, 842.02 $[M+H]^+$; $[\alpha]_D^{25}$ + 8.36 (*c* 1.53, CH_3OH); Anal. Calcd. for $C_{50}H_{84}N_2O_8$: C, 71.39; H, 10.06; N, 3.33 Found: C, 71.09; H, 10.30; N, 3.51.

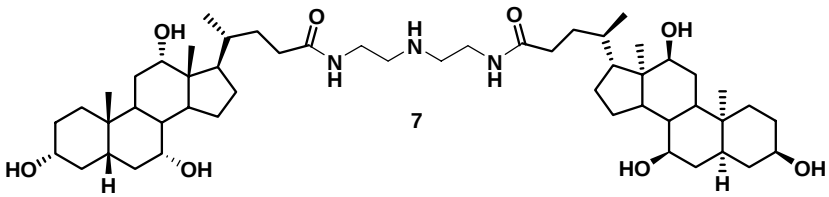
B3.6.3. *N^1, N^2- Ethylenediaminebis[deoxycholic acid amide] (6):*



6

Yield 97 %; Mp. 170 ˚C; IR ν_{max} (Nujol) 3303, 2921, 1653, 1637 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD + $CDCl_3$) δ 3.97 (bs, 2H), 3.56 (m, 2H), 3.31 (m, 4H), 0.99 (d, *J* = 5 Hz, 6H), 0.90 (s, 6H), 0.68 (s, 6H). ^{13}C NMR (125 MHz, CD_3OD+$CDCl_3$) δ 175.7, 72.9, 71.3, 47.9, 46.6, 46.3, 42.0, 39.2, 35.9, 35.8, 35.4, 35.2, 34.0, 33.4, 33.0, 31.5, 29.8, 28.3, 27.4, 27.0, 26.1, 23.6, 22.9, 17.0, 12.5; MS (LCMS) *m/z* 827.02 $[M+NH_4]^+$, 810.02 $[M+H]^+$; $[\alpha]_D^{25}$ + 46.61 (*c* 0.47, CH_3OH); Anal. Calcd. for $C_{50}H_{84}N_2O_6$: C, 74.21; H, 10.46; N, 3.46 Found: C, 73.77; H, 10.72; N, 3.26.

B3.6.4. *N^1, N^3- Diethylenetriaminebis[cholic acid amide] (7):*

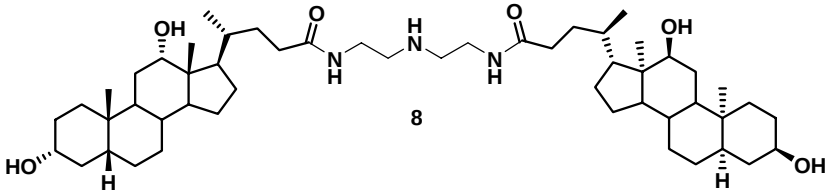


7

Yield 95 %; Mp. 168 ˚C; IR ν_{max} (Nujol) 3298, 2921, 1670 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD+$CDCl_3$) δ 3.95 (s, 2H), 3.83 (bs, 2H), 3.37 (m, 2H), 3.32 (m, 4H), 2.27 (m, 4H),

1.01 (d, *J* = 5 Hz, 6H), 0.90 (s, 6H), 0.69 (s, 6H); ^{13}C NMR (125 MHz, CD_3OD+$CDCl_3$) δ 175.4, 72.8, 71.4, 68.0, 48.0, 46.2, 46.2, 41.2, 41.3, 39.3, 39.1, 38.5, 35.2, 35.1, 34.5, 34.4, 32.5, 31.4, 29..8, 27.9, 27.3, 26.2, 23.0, 22.2, 17.0, 12.1; MS (LCMS) *m/z* 885.01 $[M+H]^+$; $[\alpha]_D^{25}$ + 8.264 (*c* 0.605, CH_3OH); Anal. Calcd. for $C_{52}H_{89}N_3O_8$: C, 70.63; H, 10.14; N, 4.75 Found: C, 70.23; H, 10.47; N, 4.45.

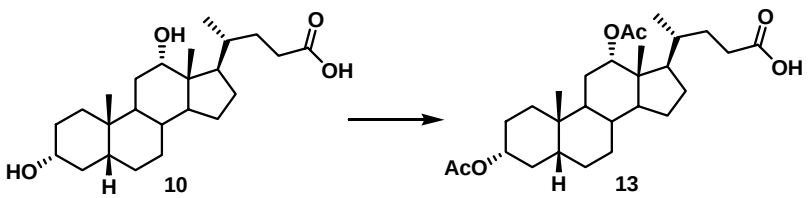
B3.6.5. ***N^1, N^3- Diethylenetriaminebis[deoxycholic acid amide] (8):***



8

Yield 98 %; Mp. 130 ˚C; IR ν_{max} (Nujol) 3298, 2921, 1650 cm^{-1}; $[\alpha]_D^{25}$ + 39.23 (*c* 1.56, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD+$CDCl_3$) δ 3.97 (s, 2H), 3.57 (m, 2H), 3.33 (m, 4H), 2.70 (m, 4H), 0.99 (d, *J* = 5 Hz, 6H), 0.91 (s, 6H), 0.67 (s, 6H); ^{13}C NMR (125 MHz, CD_3OD+$CDCl_3$) δ 174.8, 73.0, 71.4, 48.4, 48.1, 46.6, 46.4, 42.0, 38.9, 38.8, 36.2, 36.0, 35.3, 34.1, 33.6, 32.9, 31.5, 30.2, 28.5, 27.5, 27.1, 26.2, 23.7, 23.1, 17.2, 12.6; MS (LCMS) *m/z* 853.05 $[M+H]^+$; Anal. Calcd. for $C_{52}H_{89}N_3O_6$: C, 73.28; H, 10.53; N, 4.93 Found: C, 72.87; H, 10.92; N, 5.19.

B3.6.6. ***3α,12α-Diacetoxy-5β-cholan-24-oic acid (13):***

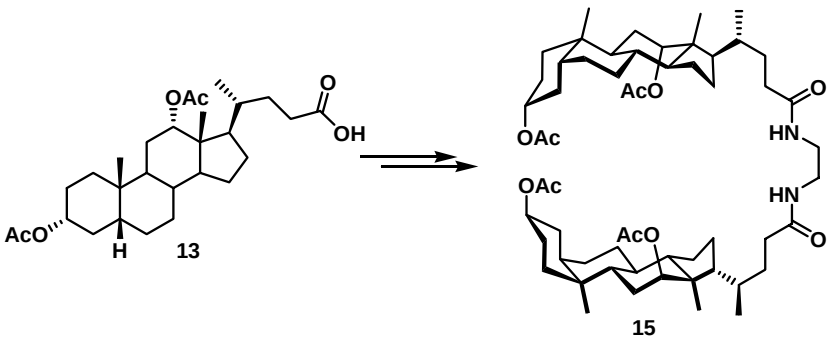


10 13

Deoxycholic acid (0.5 g, 1.3 mmol) and *N*,*N*-dimethyl amino pyridine (DMAP) (0.015 g, 0.13 mmol) was dissolved in dry pyridine (7 mL) at 25 °C. To this solution acetic anhydride

(0.53 g ~ 0.5 mL, 5.2 mmol) was added drop wise and the reaction mixture was heated at 60 °C for 3 hrs. Crushed ice (10 g) was added and the reaction mixture was extracted using EtOAc (3x30 mL). The organic layer was washed with dil. HCl (3x10 mL), H_2O (2x10 mL) and brine (2x10 mL). The extract was dried over Na_2SO_4 and ethyl acetate was removed under reduced pressure to get crude solid material. Column chromatographic purification using silica gel (60-120 mesh, 10 % CH_3OH/CH_2Cl_2) afforded a pure white solid **13** (0.59 g, 97%). IR ν_{max} (Nujol) 3450, 3171, 1741, 1731, 1712 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.73 (s, 3H), 0.83 (d, *J* = 6.0 Hz, 3H), 0.91 (s, 3H), 2.04 (s, 3H), 2.11 (s, 3H), 2.32 (m, 2H), 4.71 (m, 1H), 5.09 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.2, 17.3, 21.2, 21.3, 22.9, 23.2, 25.4, 25.7, 26.4, 26.7, 27.1, 30.4, 30.8, 32.0, 33.8, 34.2, 34.5 (2C), 35.5, 41.6, 44.8, 47.4, 49.2, 74.1, 75.8, 170.6, 170.6, 179.7; MS (LCMS) *m/z* 476.3 $[M+H]^+$, 499.3 $[M+Na]^+$; Anal. Calcd. for $C_{28}H_{44}O_6$ C, 70.56; H, 9.30 Found: C, 70.39; H, 9.53.

B3.6.7. ***3,3',12,12'-Tetraacetoxy dimer (15):***

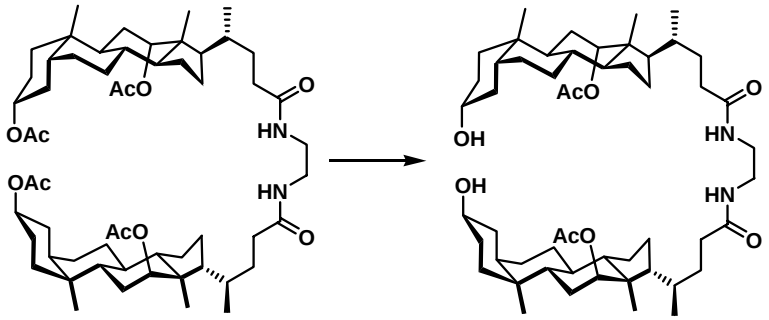


Method A: Compound **13** (0.59 g, 1.2 mmol) was dissolved in $SOCl_2$ (2 mL) and the reaction mixture was stirred at 25 °C for 2 hrs. The excess $SOCl_2$ was removed using vacuum pump by co-evaporation with dry benzene. The crude product **14** (0.69 g, 100 %) obtained was further used without any purification. IR ν_{max} (Nujol) 1798, 1736, 1730 cm^{-1}. This crude product **14** (0.69 g, 1.2 mmol) was further dissolved in dry CH_2Cl_2 (6 mL) and

ethylenediamine (0.036 g ~ 0.04 mL, 0.6 mmol) was added and the reaction mixture was stirred at 25 °C for 1 hr. The reaction mixture was further diluted with CH_2Cl_2 (100 mL) and the organic layer was washed with dil. HCl (2x10 mL), H_2O (2x10 mL) and brine (2x10 mL). The extract was dried over Na_2SO_4 and CH_2Cl_2 was removed under reduced pressure to get crude solid material. Column chromatographic purification using silica gel (60-120 mesh, 5 % MeOH/CH_2Cl_2) afforded pure white crystalline solid **15** (0.55 g, 91 %).

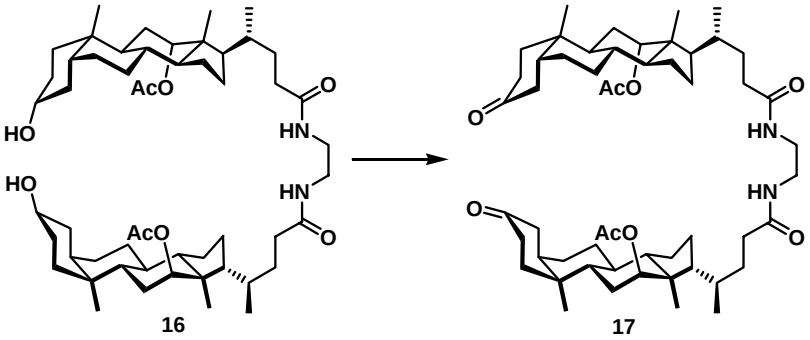
Method B: Compound **6** (0.162 g, 0.2 mmol) and DMAP (2.5 mg, 0.02 mmol) were dissolved in dry pyridine (2 mL) to it Ac_2O (0.23 mL, 2.4 mmol) was added and the reaction mixture was stirred at 30 °C for 3 hrs. Crushed ice (5 g) was added and the reaction mixture was diluted with EtOAc (50 mL). The organic layer was washed with dil. HCl (2x10 mL), 10 % $NaHCO_3$ (2x5 mL), H_2O (2x10 mL) and brine (2x10 mL). The extract was dried over Na_2SO_4 and ethyl acetate was removed under reduced pressure to get crude solid material. Column chromatographic purification using silica gel (60-120 mesh, 5 % CH_3OH/CH_2Cl_2) afforded pure white crystalline solid **15** (0.189 g, 97 %). IR ν_{max} (Nujol) 3322, 1743, 1738, 1683 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.72 (s, 6H), 0.80 (d, J = 6.0 Hz, 6H), 0.90 (s, 6H), 2.03 (s, 6H), 2.10 (s, 6H), 3.37 (bs, 4H), 4.70 (m, 2H), 5.07 (bs, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.3, 17.5, 21.3, 21.3, 22.9, 23.3, 25.5, 25.7, 26.4, 26.7, 27.2, 31.5, 32.1, 33.4, 33.9, 34.3, 34.6, 34.7, 35.5, 40.0, 41.6, 44.9, 47.6, 49.3, 74.1, 75.8, 170.5, 170.5, 174.9; MS (LCMS) *m/z* 976.7 $[M+H]^+$; Anal. Calcd. for $C_{58}H_{92}N_2O_{10}$ C, 71.28; H, 9.49; N, 2.87 Found: C, 70.99; H, 9.23; N, 2.52.

B3.6.8. ***12,12'-Diacetoxy dimer (16):***



To the solution of compound **15** (0.488 g, 0.5 mmol) in CH_3OH (10 mL) was added Na_2CO_3 (0.212 g, 2 mmol) in H_2O (1 mL). The resulting reaction mixture was stirred at 28 °C for 12 hrs. CH_3OH was evaporated and the reaction mixture was extracted in EtOAc washed with H_2O (2x10 mL), brine (2x10 mL) dried over Na_2SO_4. Ethyl acetate was removed under reduced pressure to get crude solid material. Column chromatographic purification using silica gel (60-120 mesh, 10 % CH_3OH/CH_2Cl_2) afforded pure white solid **16** (0.410 g, 92 %). IR ν_{max} (Nujol) 3442, 1739, 1687 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.71 (s, 6H), 0.80 (d, *J* = 6.0 Hz, 6H), 0.89 (s, 6H), 2.03 (s, 6H), 3.36 (bs, 4H), 3.61 (m, 2H), 5.07 (bs, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.3, 17.5, 21.3, 23.0, 23.4, 25.5, 25.9, 26.9, 27.2, 30.2, 31.5, 33.4, 33.9, 34.3, 34.7, 34.9, 35.6, 36.1, 33.9, 41.9, 44.9, 47.6, 49.3, 71.5, 75.9, 170.6, 174.9; MS (LCMS) *m/z* 892.7 $[M+H]^+$; Anal. Calcd. for $C_{54}H_{88}N_2O_8$: C, 72.61; H, 9.93; N, 3.14 Found: C, 72.49; H, 9.71; N, 2.97.

B3.6.9. ***3,3'Dioxo-12,12'-diacetoxy dimer (17):***



Compound **16** (0.223 g, 0.25 mmol) was dissolved in acetone (10 mL) to this Jones reagent (0.25 mL) was added drop wise at 5 °C. CH_3OH (5 mL) was added to quench the excess of Jones reagent. All the solvent was evaporated and the crude product was dissolved in EtOAc (100 mL), washed with 10 % $NaHCO_3$ (2x10 mL), H_2O (2x10 mL), brine (2x10 mL). The extract was dried over Na_2SO_4 and ethyl acetate was removed under reduced pressure to get crude solid material. Column chromatographic purification using silica gel (60-120 mesh, 7 % CH_3OH/CH_2Cl_2) afforded pure white crystalline solid **17** (0.189 g, 85 %). IR ν_{max} (Nujol) 3305, 1733, 1716, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 6H), 0.81 (d, *J* = 6.0 Hz, 6H), 1.00 (s, 6H), 2.07 (s, 6H), 2.69 (m, 2H), 3.36 (bs, 4H), 5.12 (bs, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.4, 17.6, 21.2, 22.3, 23.4, 25.3, 25.9, 26.4, 27.2, 31.5, 33.5, 34.2, 34.6, 34.7, 35.3, 36.5, 36.8, 40.2, 42.2, 43.9, 45.0, 47.7, 49.3, 75.7, 170.3, 174.7, 212.9; MS (LCMS) *m/z* 888.6 $[M+H]^+$; Anal. Calcd. for $C_{54}H_{84}N_2O_8$: C, 72.94; H, 9.52; N, 3.15 Found: C, 72.83; H, 9.63; N, 2.87.

B3.6.10. ***Antifungal Activity. Materials and Methods:***

Organisms: *Candida albicans* (5 isolates), *Benjaminiella poitrasii* and *Yarrowia lipolytica* maintained on Difco Yeast extract peptone glucose agar (YPG-yeast extract, 0.3 %, peptone, 0.5 %, glucose, 1 % and agar 2 %) slants at 28 ˚C for 7 days.

Inoculum Development: All the three fungal cultures were inoculated in YPG broth and incubated at 28 ˚C in case of *C. albicans* and *Y. lipolytica* and 37 ˚C in case of *B. poitrasii* for 24 hours.

Inhibitor Solutions: The dimeric compounds **5-8** were solubilized in dimethyl sulphoxide (DMSO) and stock solution of mg/ml was prepared. Cycloheximide, which was used as the standard inhibitor was also dissolved in DMSO and then the varying concentrations were added on to the filter paper discs.

B3.6.11. ***Antibiotic Inhibition Assay Using Disc Method:***

100 μL of the yeast cell suspensions for all five *C. albicans* isolates, *B. poitrasii* and *Y. lipolytica* were spread plated on sterile YPG agar plates separately. On each plate, five sterile whatmann filter paper discs were placed four at the corners and one in the center. To the central disc, DMSO was added as the control and to other four discs, different concentrations of the compounds were added. The plates were then incubated at 28 ˚C for 24 hours. Upon incubation, the plates were observed for the zone of inhibition around the disc. The minimum concentration that gave the zone of inhibition was determined as the minimum inhibitory concentration (*MIC*) of the compound for that fungal culture. All the experiments were done in triplicates. Similar procedure was followed to determine the *MIC* of cycloheximide against *C. albicans*, *B. poitrasii* and *Y. lipolytica*.

B3.6.12. ***Antiproliferative Activity. Materials and Methods:***

Human oral squamous cell carcinoma cell line (HEp-2) and human mammary adenocarcinoma cells (MCF-7) were obtained from National Centre for Cell Science, Pune, India. Cells were maintained as a monolayer in nutrient media MEM supplemented with heat inactivated fetal bovine serum (HyClone, Utah, USA) (10%), penicillin (100 U/mL)

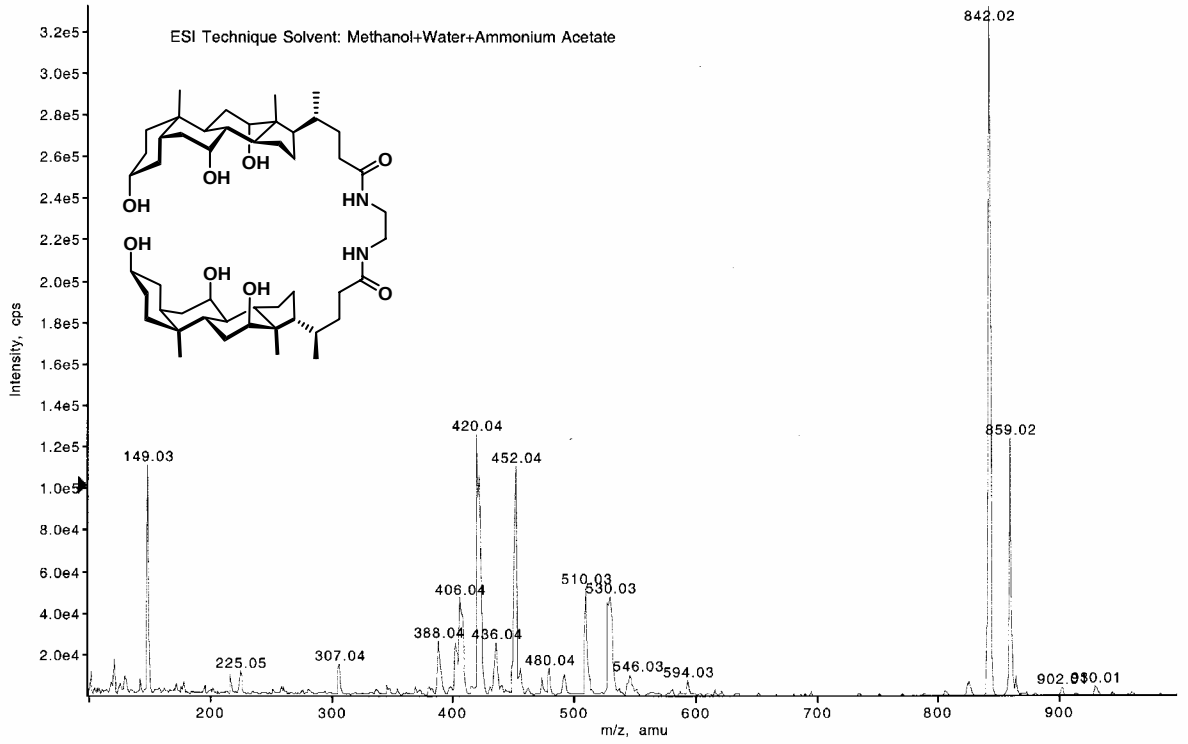
and streptomycin (100 µg/mL) (Invitrogen Life Technologies, MD, USA). The cells were grown at 37 ˚C in 5 % CO_2 and humidified air atmosphere. Stock solutions of compounds **5** to **8** were prepared in DMSO at a concentration of 10-11.5 mM and afterwards diluted to the required concentrations. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemicals, St. Louis, USA) was dissolved (1 mg/mL) in MEM (without phenol red) and filtered through a Millipore filter, 0.22 µm, before use.

B3.6.13. ***MTT Cell Proliferation Assay:***

HEp-2 and MCF-7 cells were plated at a density of 15,000 cells per well in 96 wells tissue culture plates. Cells were allowed to adhere for 24 h at 37 ˚C and then treated with various concentrations (0, 0.1,1.0, and 10 µM) of compounds dissolved in DMSO for additional 72 h, in triplicates. In the control wells nutrient medium with correspondent concentration of DMSO only was added to the cells. Thereafter cell proliferation was assessed by replacing treatment medium with 50 µL media containing 1 mg/mL MTT and incubated for 4 h at 37 ˚C. Medium was then aspirated off and formazan crystals were solubilized in 50 µL of *iso*-propanol. The optical density was read on a microplate reader at 570 nm using 630 nm as a reference filter against a blank prepared from cell free wells. Absorbance given by cells treated with the carrier DMSO alone was taken as 100 % cell growth. All assays were performed in triplicates.

B3.7. Selected Spectra

5: ESI Mass



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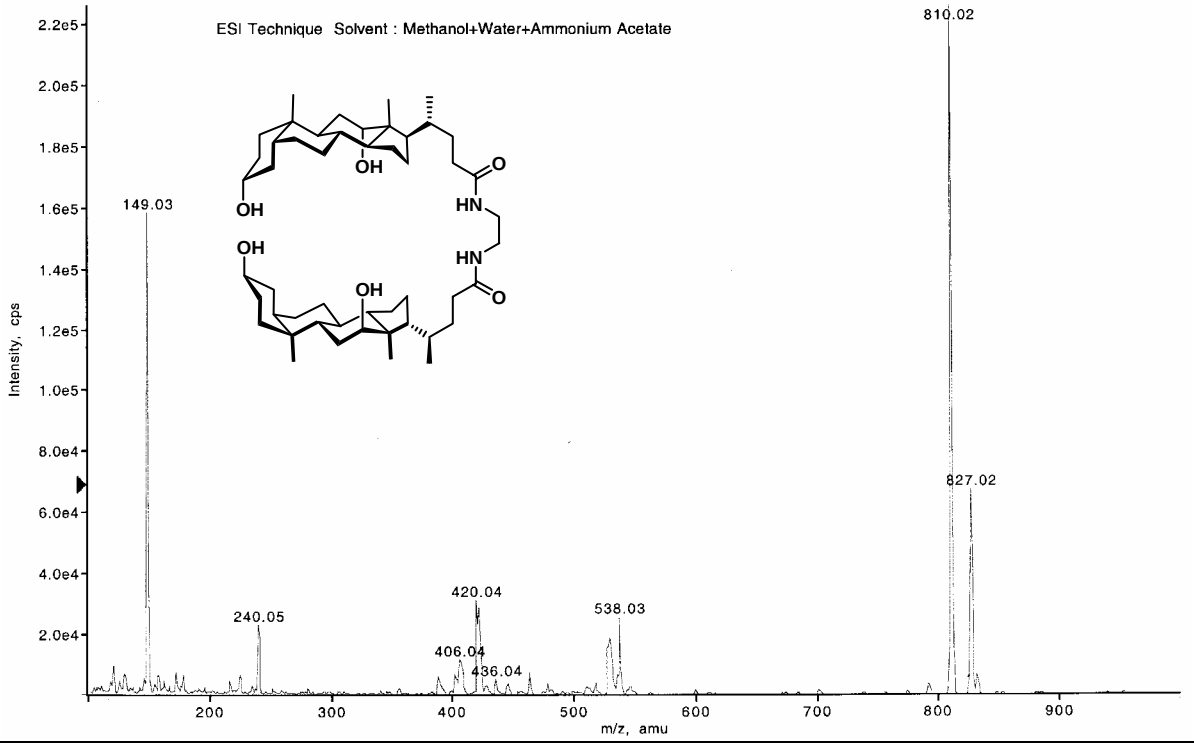
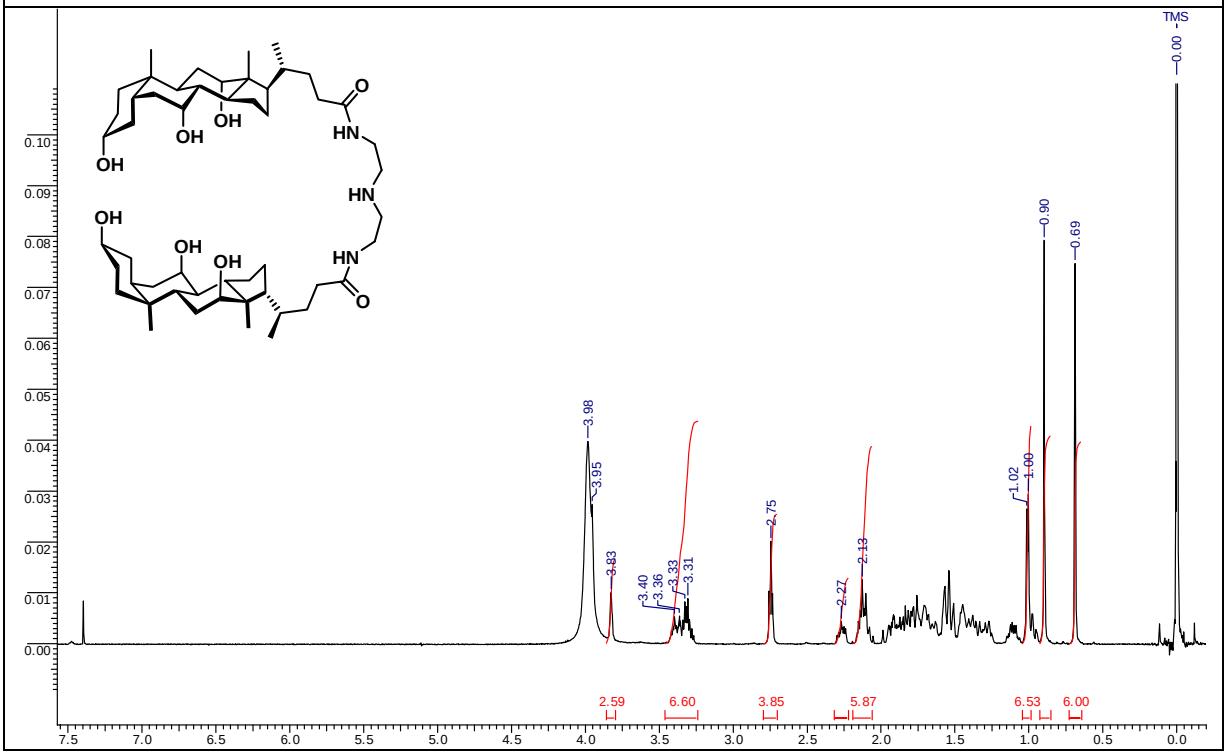
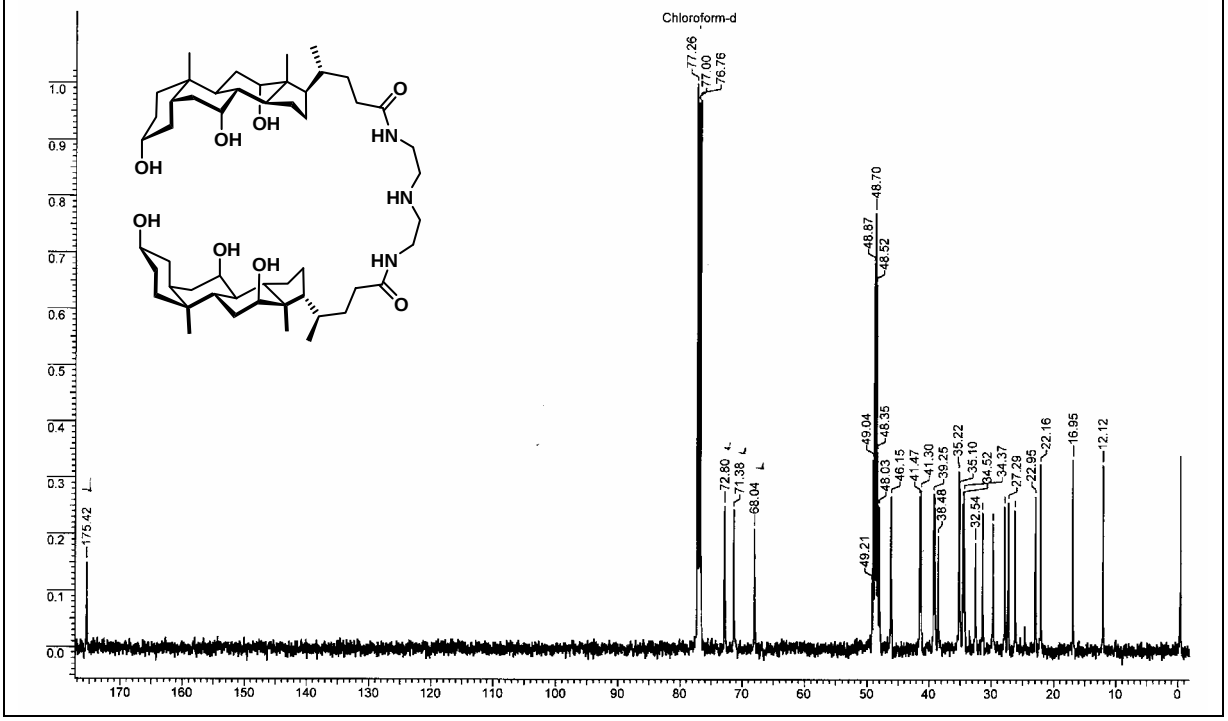


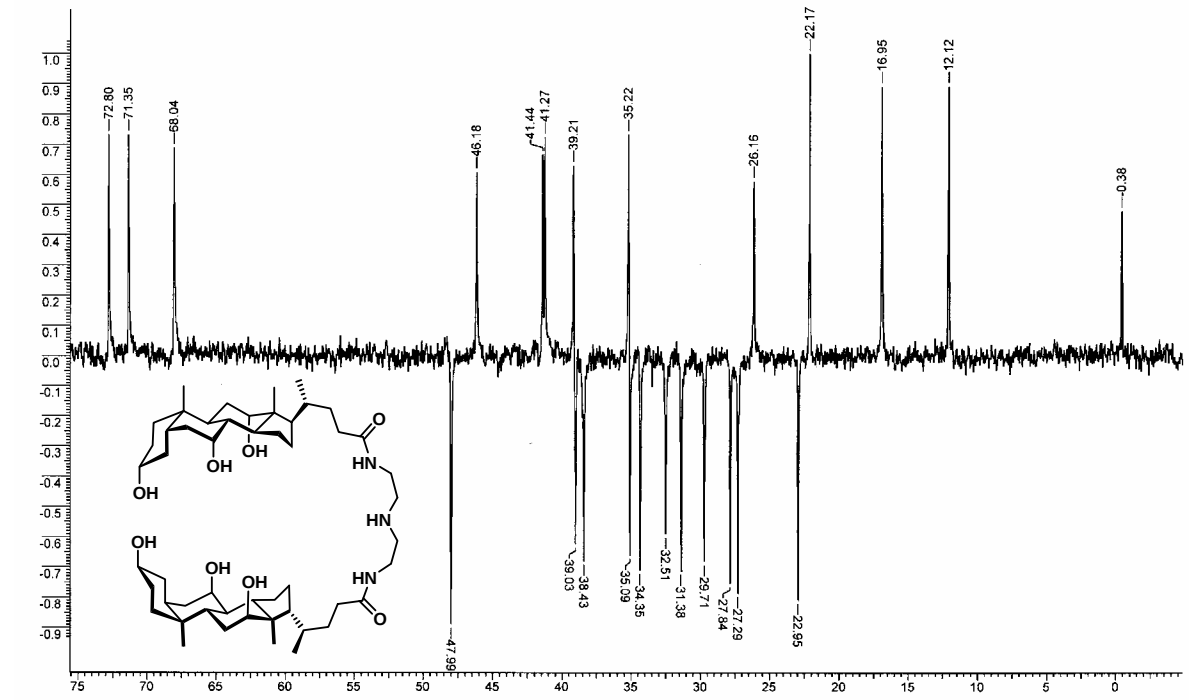
7: ^{1}H NMR, 500 MHz, $CDCl_3$+CD_3OD



7: ^{13}C NMR, 125 MHz, $CDCl_3$+CD_3OD



7: DEPT, 125 MHz, $CDCl_3$+CD_3OD



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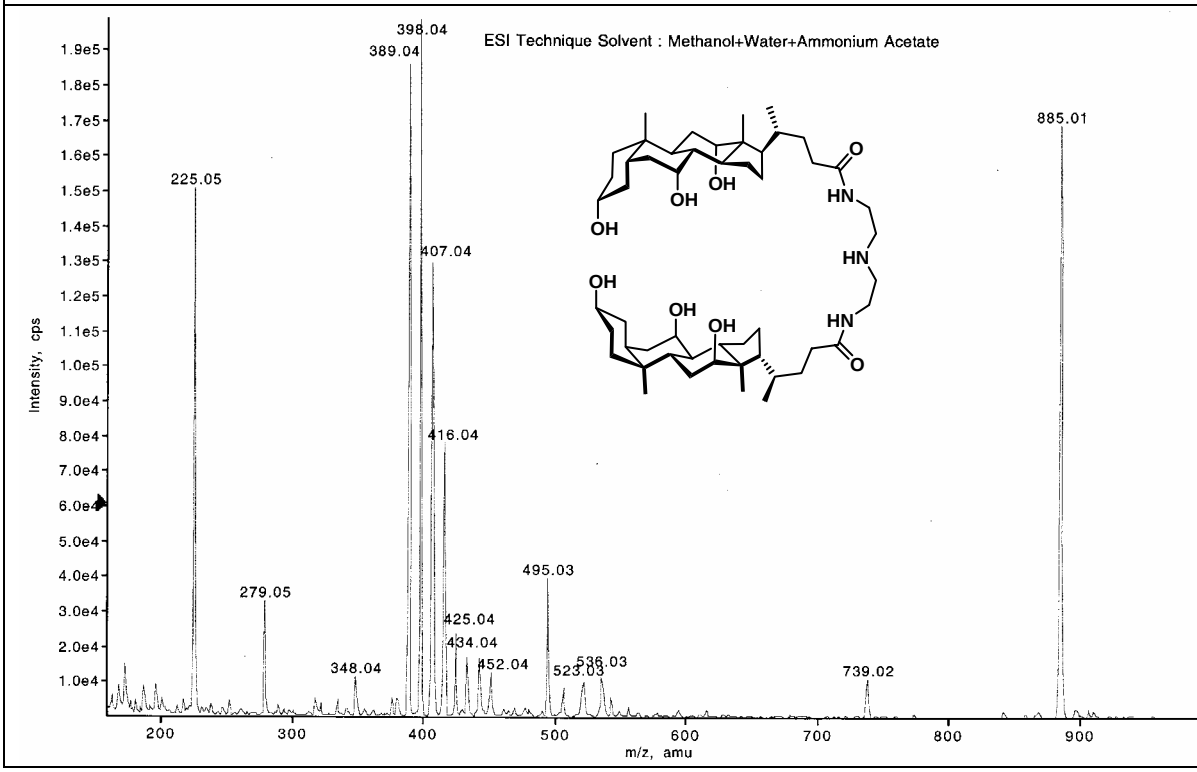
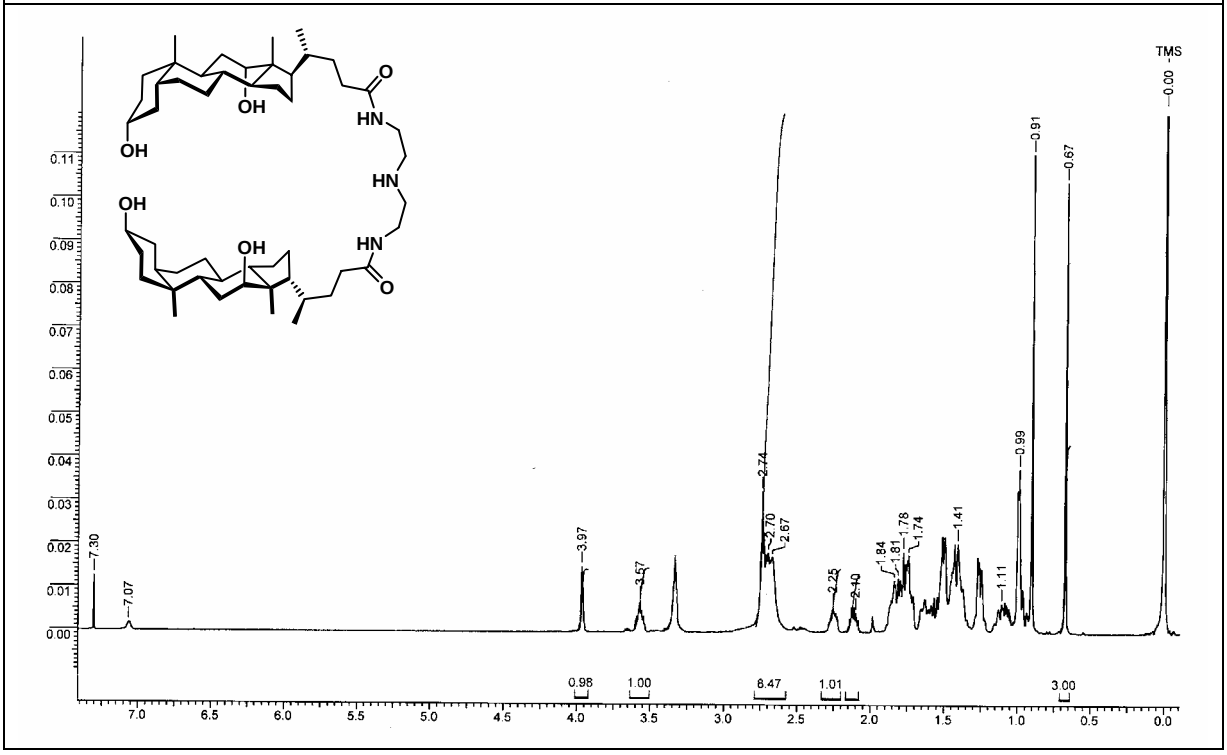
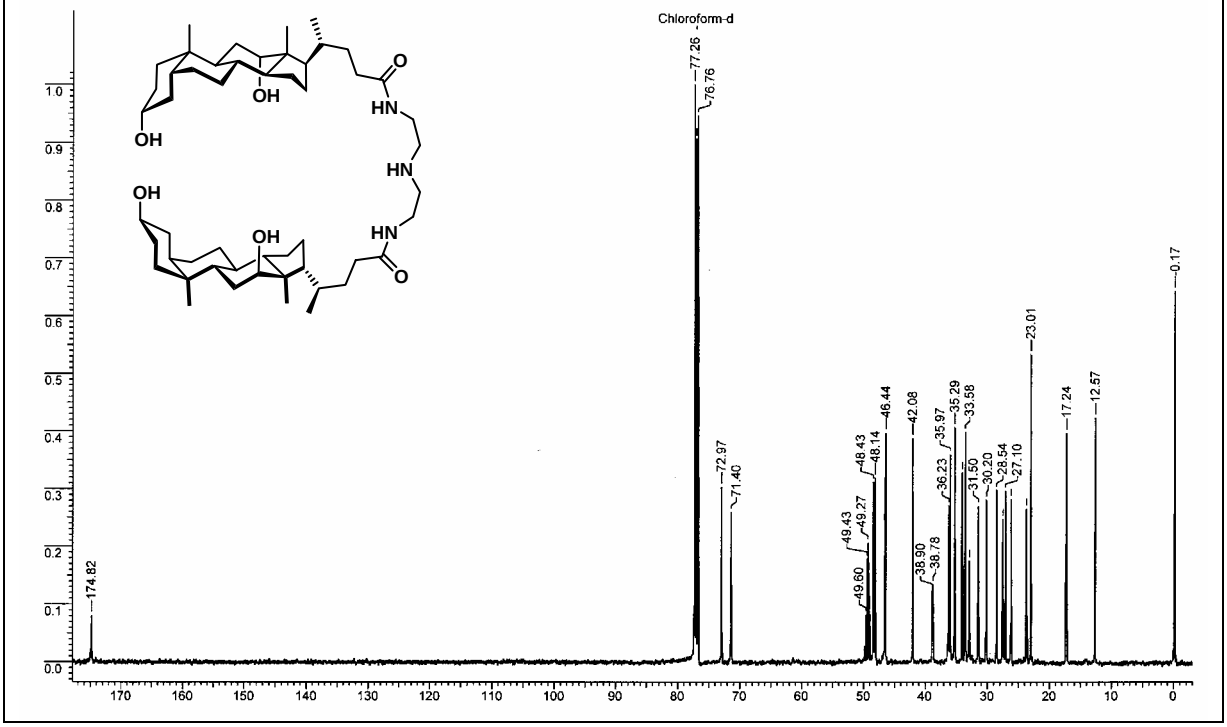


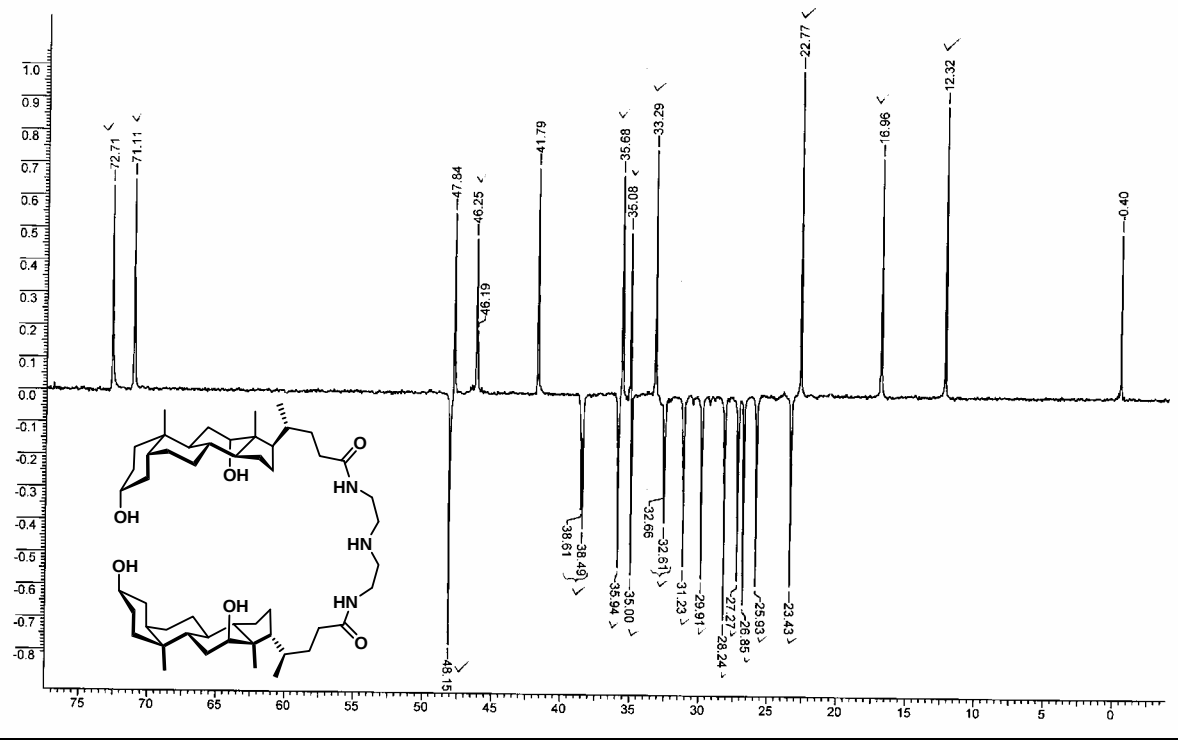
8: ^{1}H NMR, 500 MHz, $CDCl_3$+CD_3OD



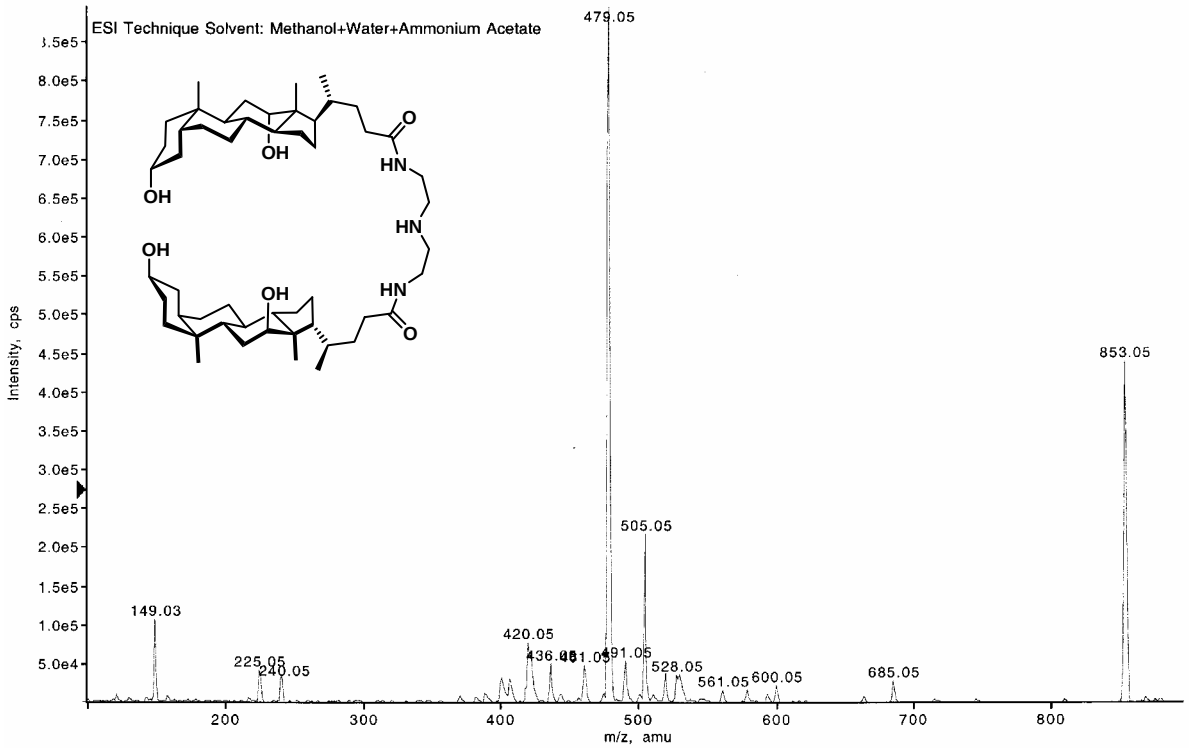
8: ^{13}C NMR, 125 MHz, $CDCl_3$+CD_3OD



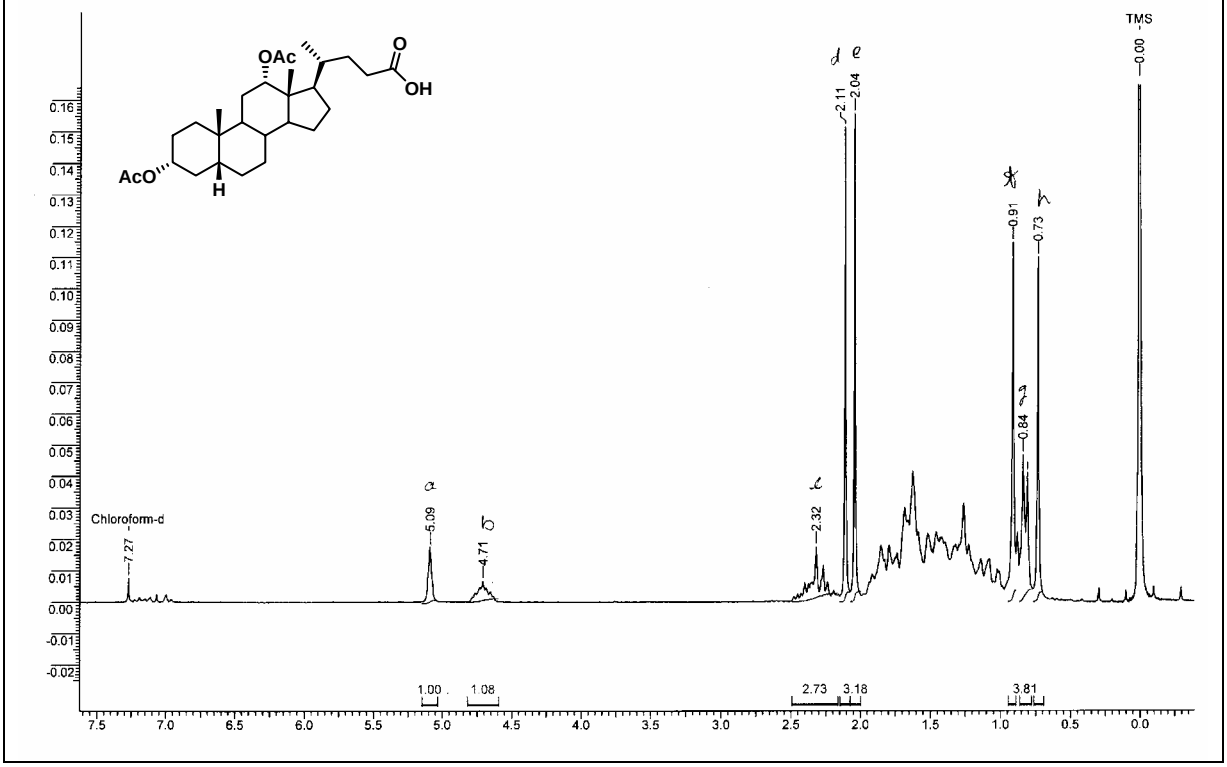
8: DEPT, 125 MHz, $CDCl_3$+CD_3OD



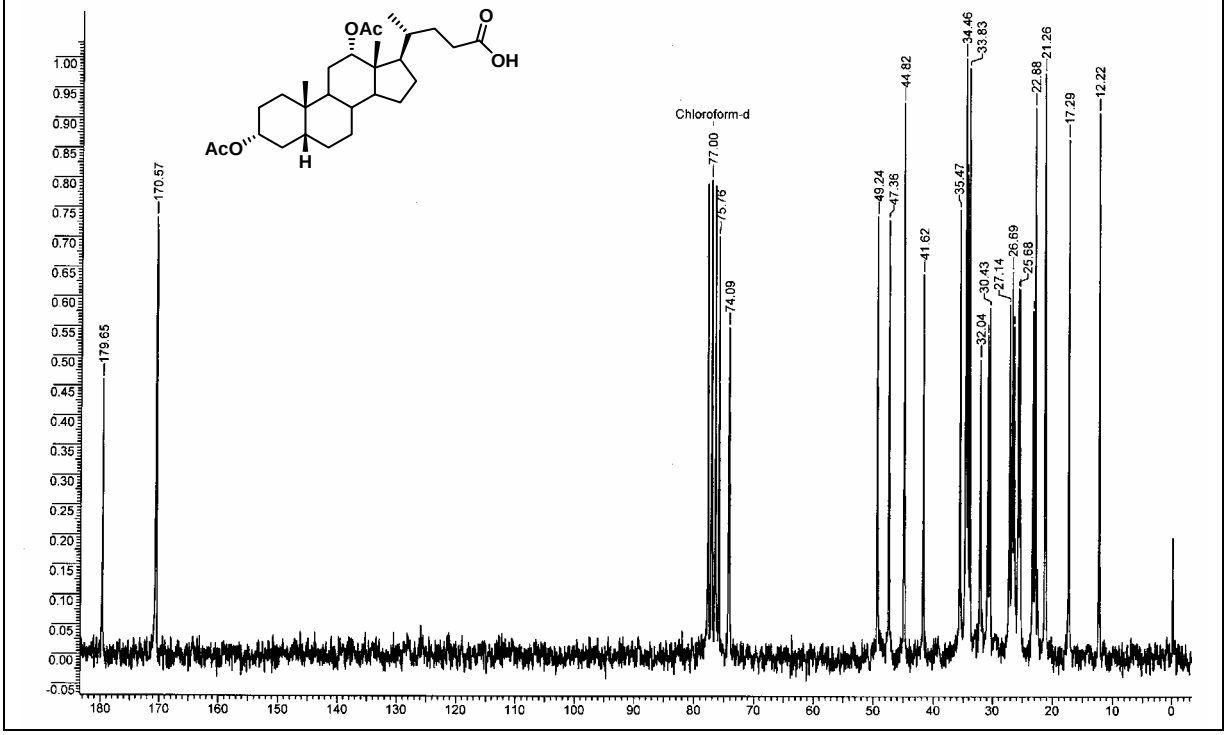
8: ESI Mass



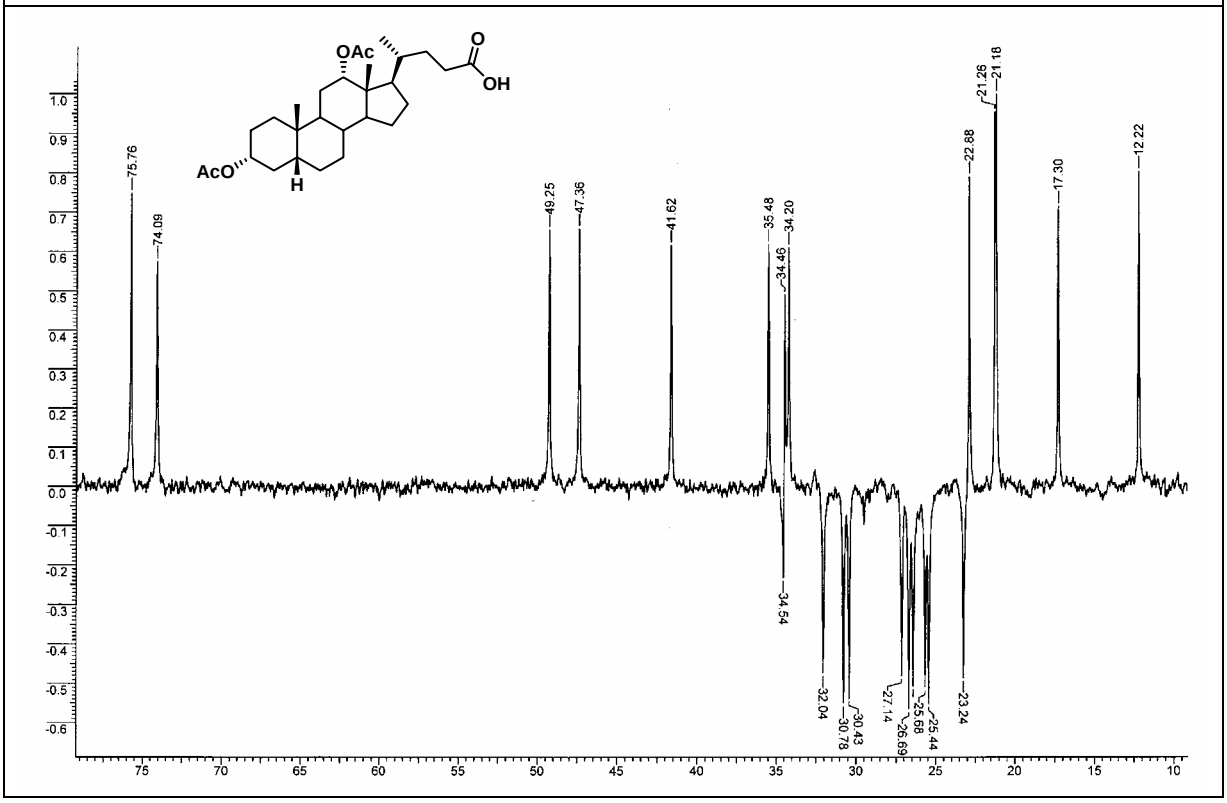
13: ^{1}H NMR, 200 MHz, $CDCl_3$



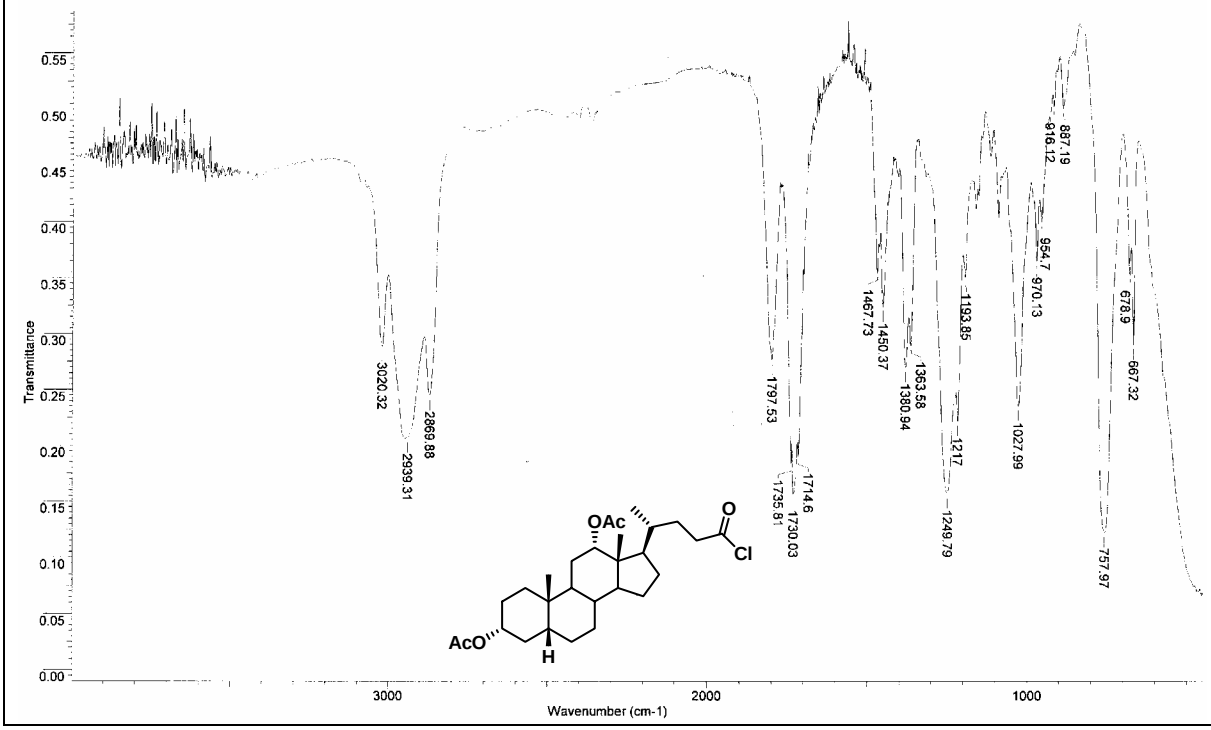
13: ^{13}C NMR, 50 MHz, $CDCl_3$



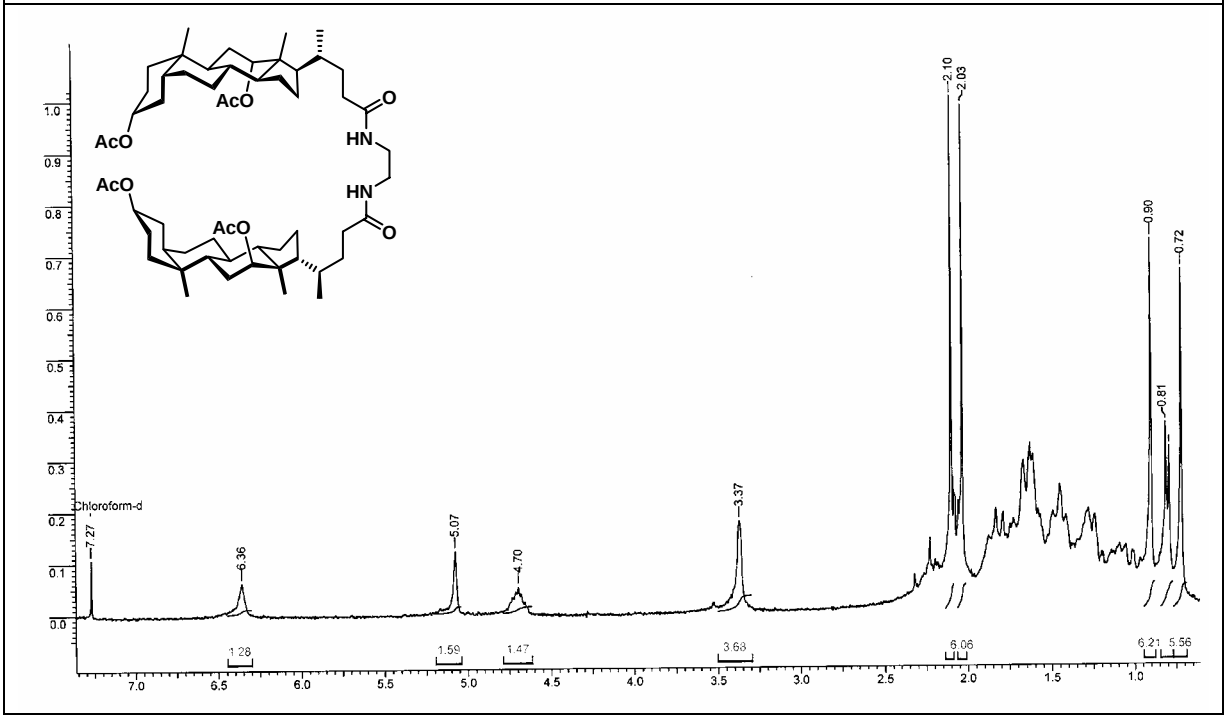
13: DEPT, 50 MHz, $CDCl_3$



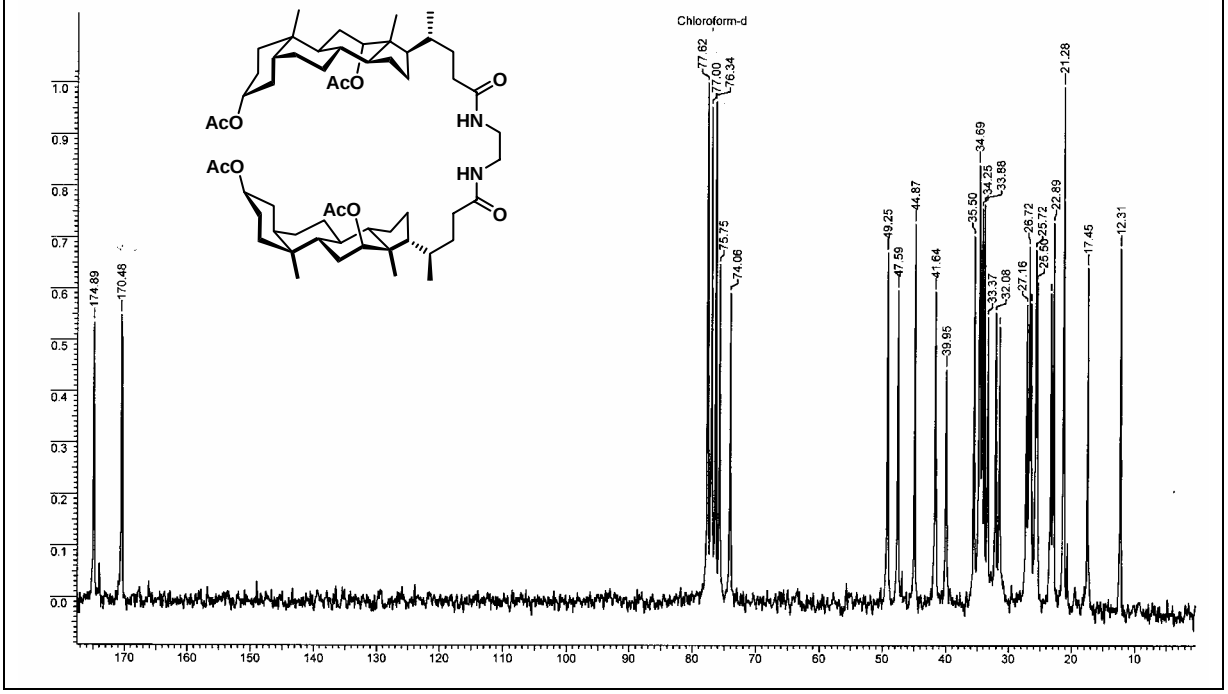
14: IR



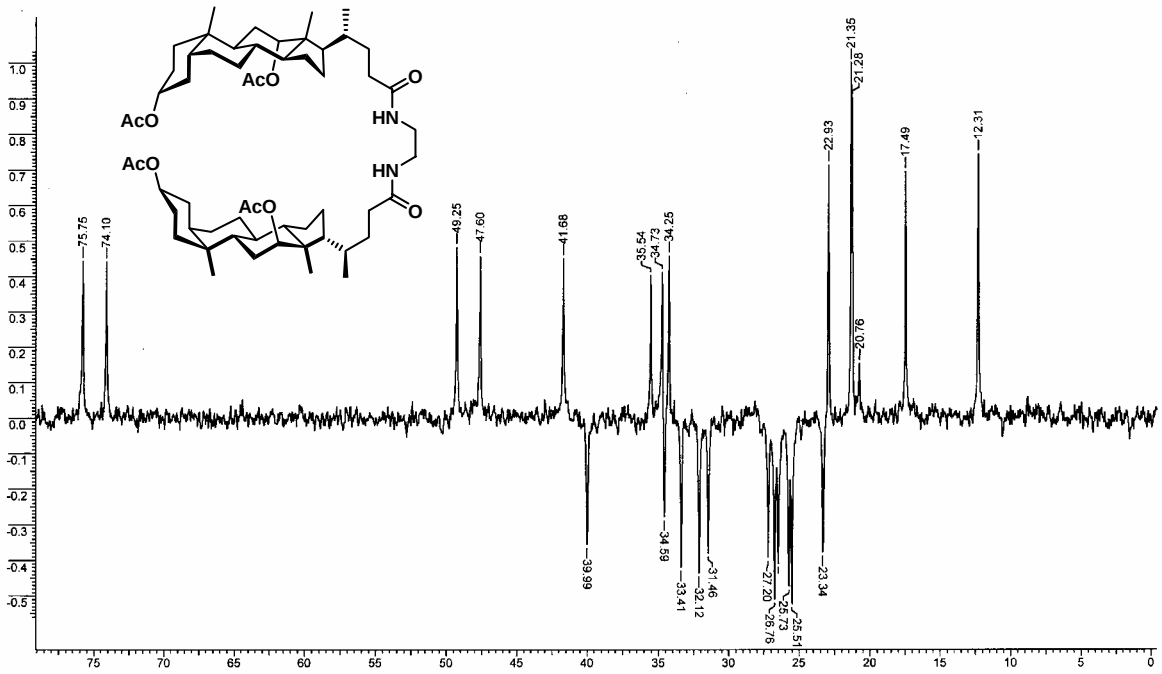
15: ^{1}H NMR, 300 MHz, $CDCl_3$



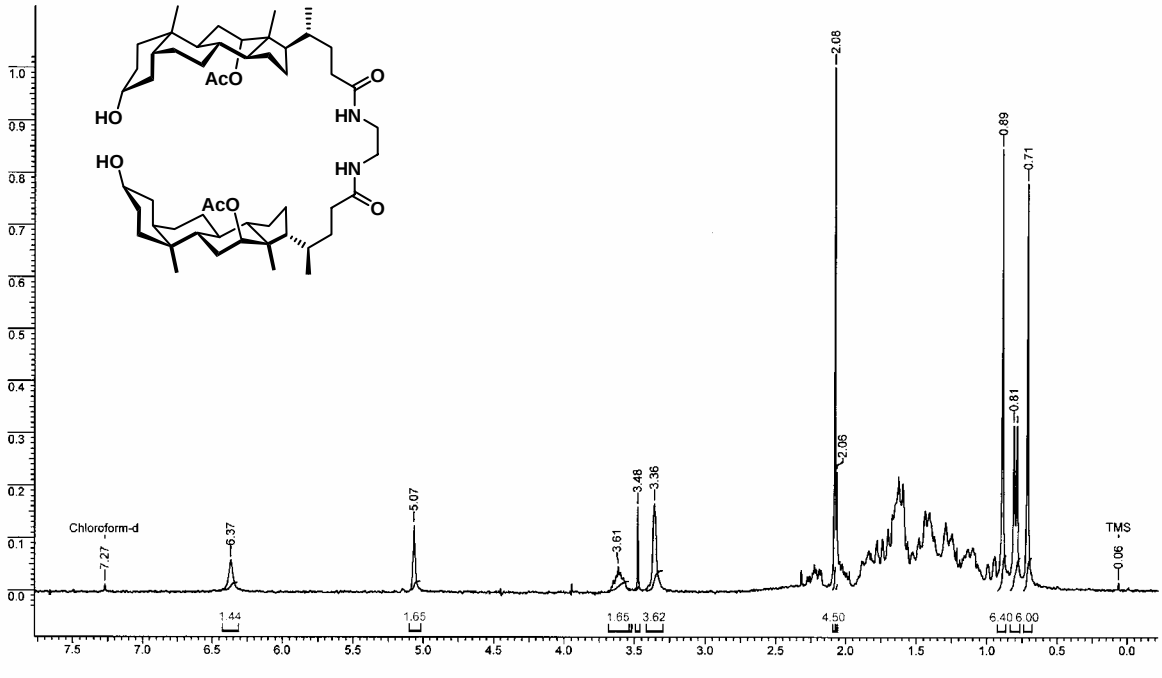
15: ^{13}C NMR, 50 MHz, $CDCl_3$



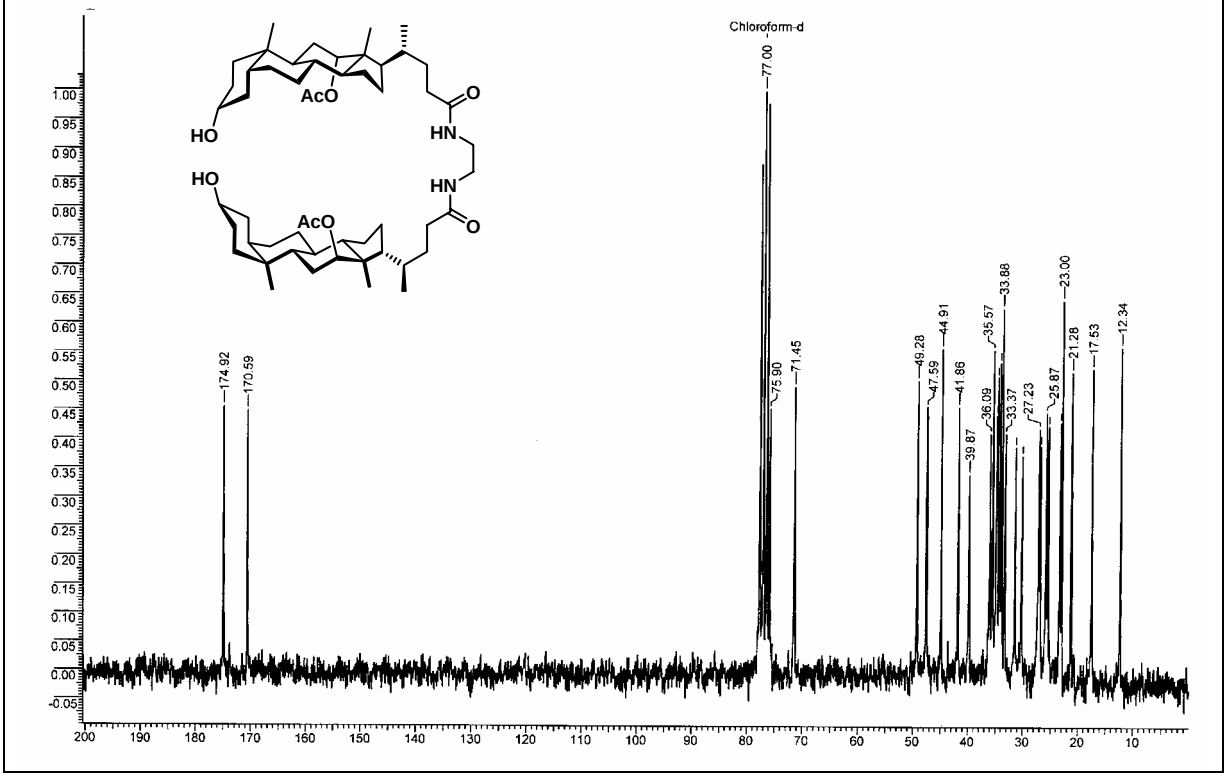
15: DEPT, 50 MHz, $CDCl_3$



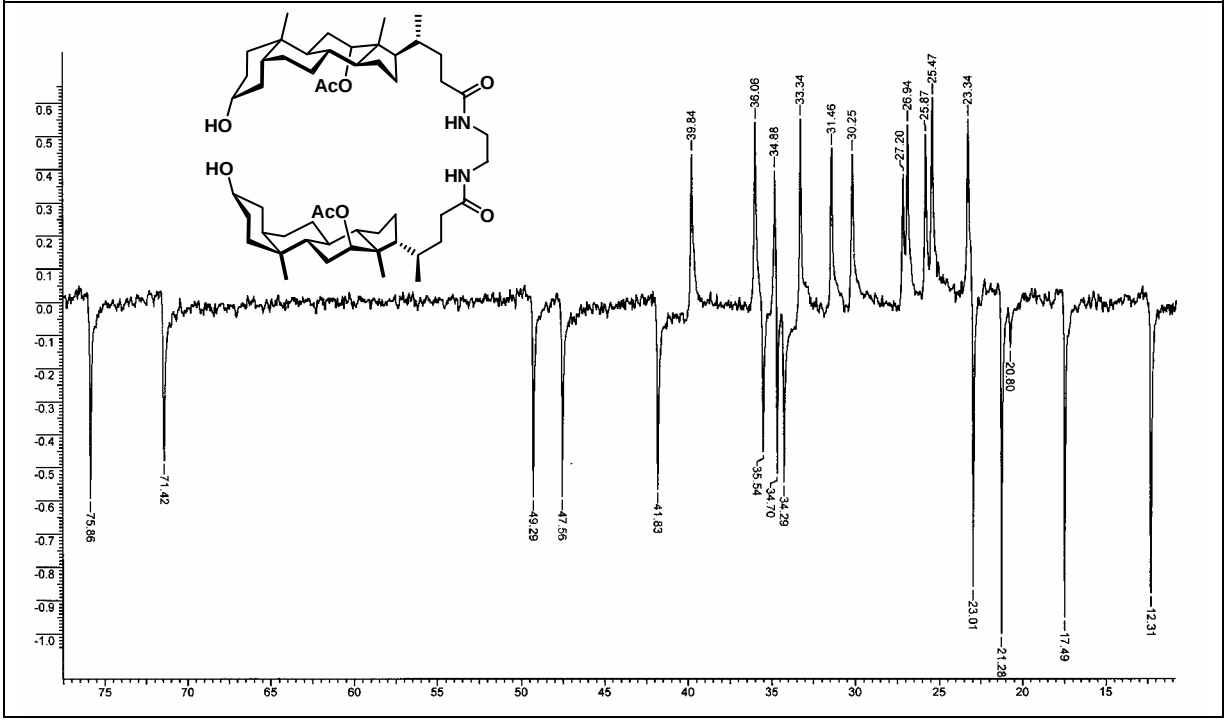
16: ^1H NMR, 300 MHz, $CDCl_3$



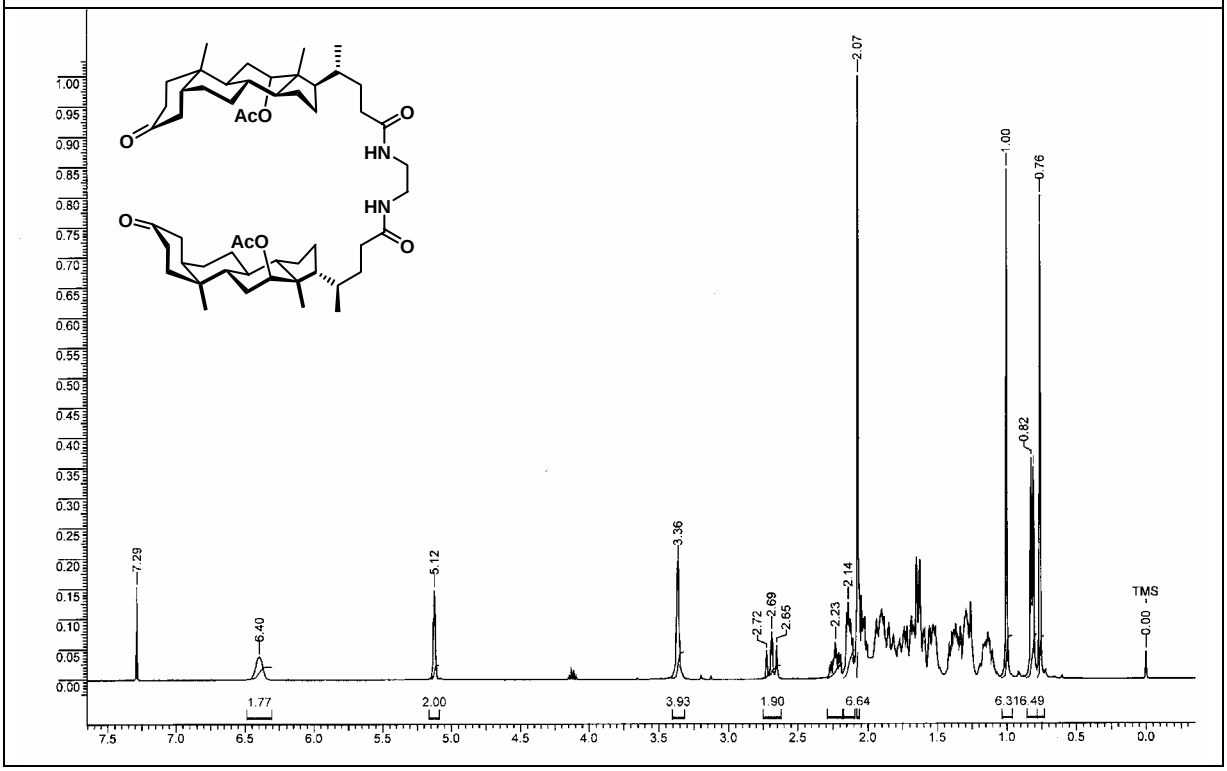
16: ^{13}C NMR, 50 MHz, $CDCl_3$



16: DEPT, 50 MHz, $CDCl_3$



17: ^{1}H NMR, 400 MHz, $CDCl_3$



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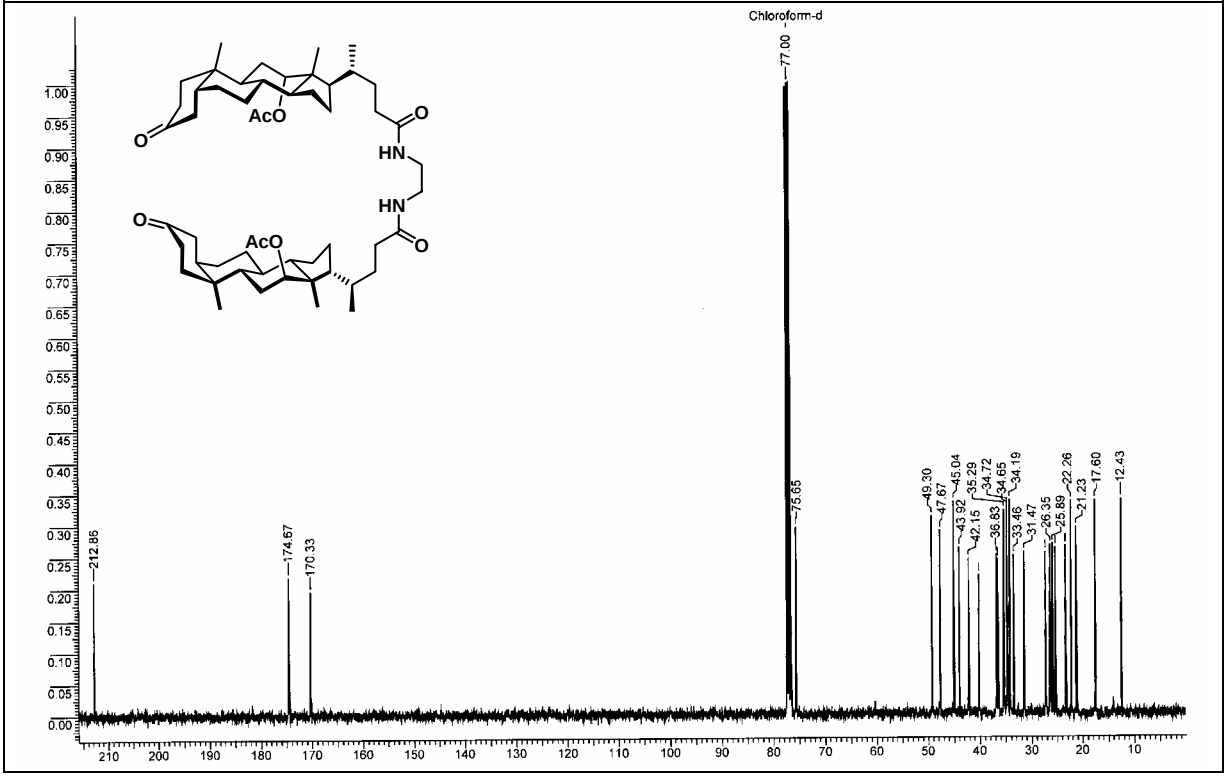
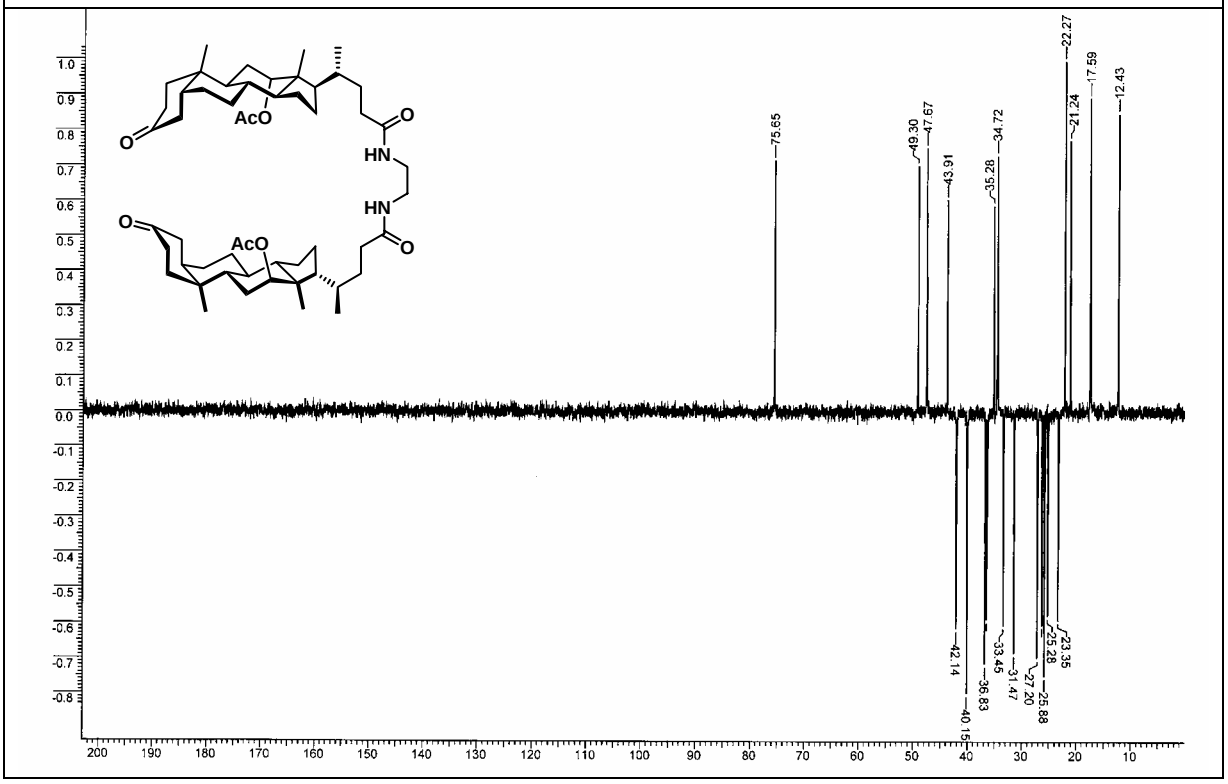


17: DEPT, 100 MHz, $CDCl_3$



B3.8. References

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PART B : CHAPTER - 4

Synthesis of Steroid Based Potential Inhibitors of Ergosterol Biosynthesis

B4.1. Abstract

Fungi are eukaryotic organisms, whose cellular functions resemble those of plants and animals. Hence, selectivity plays a crucial role in the quest for safe and effective chemotherapeutic drugs. For the first time sterol carrier proteins (SCP) are suggested to be one of the key targets for the development of safe and effective antifungal therepeutics. With this hypothesis novel lanosterol/fluconazole conjugates were designed and their regioselective synthesis was achieved in very high yield *via* Cu(I) catalyzed intermolecular 1,3-dipolar cycloaddition. As a control lanosterol/imidazole conjugate was also synthesized. These molecules are under biological screening for their antifungal activity and cytotoxicity.

B4.2. Introduction

In recent years fungal infections have emerged as a major cause of disease and mortality, in part as a consequence of the increase in acquired immunodeficiency syndrome (AIDS), the greater use of immunosuppressive drugs in transplantation and chemotherapeutic agents in cancer, long term use of corticosteroids, and even the indiscriminate use of antibiotics [1-3]. Fungi are eukaryotic organisms, whose cellular functions resemble those of plants and animals. Hence, selectivity plays a crucial role in the quest for safe and effective chemotherapeutic drugs.

The biomedical literature abounds with reports of macromolecules essential for fungal survival, growth, virulence or cellular morphogenesis that have been proposed as potential targets for novel antifungal agents. The arrival of whole-genome sequence data for pathogenic fungi, such as *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, as well as for non-pathogens, such as *Saccharomyces cerevisiae*, has paved the way for discovery of genes encoding candidate antifungal targets on a previously unprecedented scale. However, the antifungal discovery process based on screening compounds against molecular targets, which dates back before the genomics era, has so far not resulted in a single new agent emerging into the clinic or even the development pipeline. (The same can be said for most areas of drug discovery research). It takes time to generate practically useful results from fundamental biomedical research, and it is probably still too early to expect new antifungal agents to be discovered this way [4].

The targets of all antifungal agents used in the clinic (and of some agents that entered or approached clinical development but have not been marketed) are summarized in Figure 1 [4]. This figure shows that, in terms of numbers of classes of agents that can be used to treat

lifethreatening mycoses, the targets are heavily focused, directly or indirectly, on the cell envelope, particularly on the fungal membrane sterol, ergosterol, and its biosynthesis.

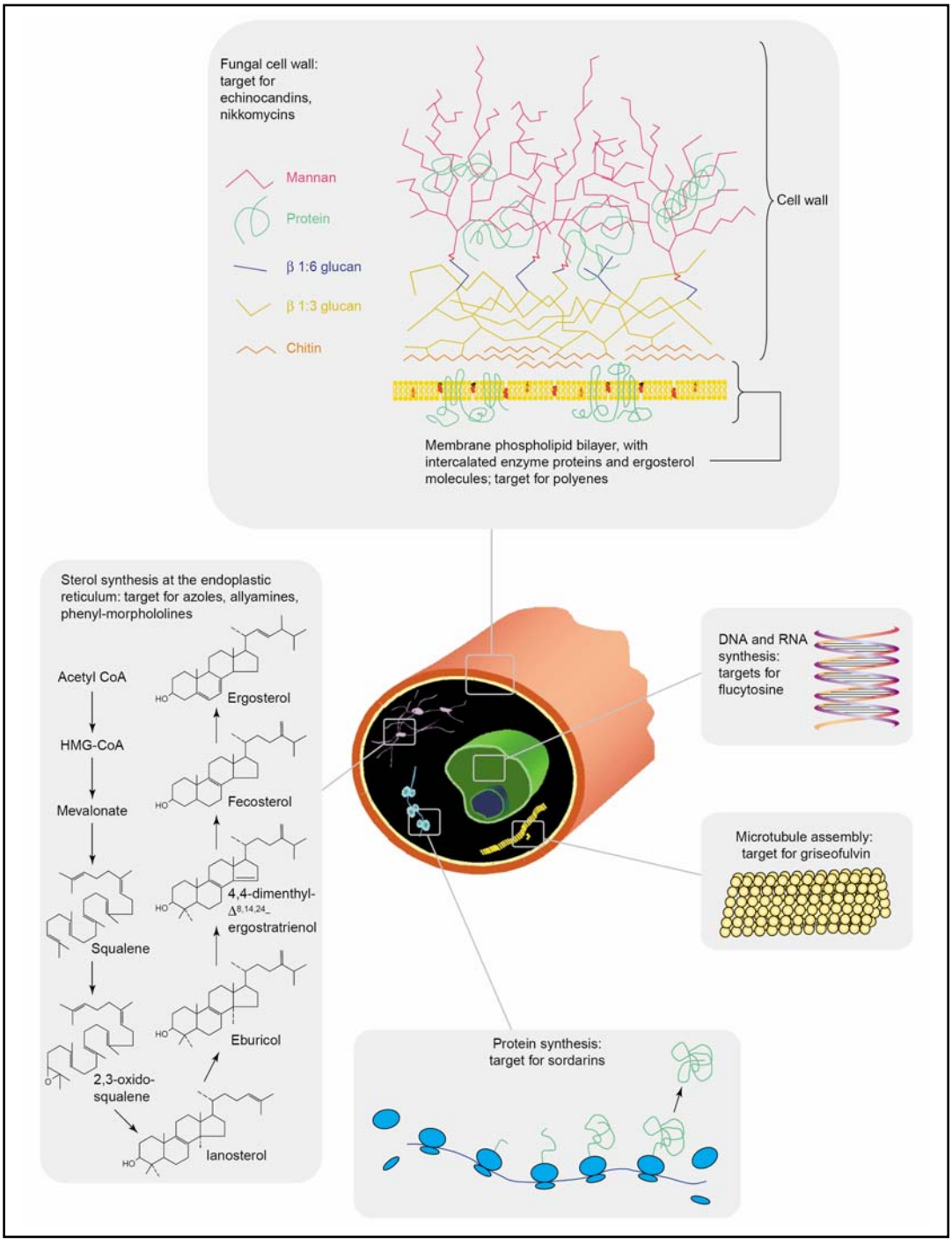


Figure 1. Generalised cartoon showing target areas for antifungal agents. The cross-section through a fungal hypha shows the intracellular sites of action of antifungal agents. The callouts show details for each site. The steps illustrated for ergosterol synthesis are the major steps found in all fungi.

B4.2.1. ***Antifungal antibiotics currently in use***

Antifungals play an important role in human medicine, agriculture and veterinary medicine. They can be broadly classified into five major types [4].

- ✓ The polyene antibiotics
- ✓ Allylamines & thiocarbamates
- ✓ The azole derivatives
- ✓ Morpholines
- ✓ Miscellaneous class

The first three groups bind to ergosterol **18** (Figure 6), the main fungal sterol present in the plasma membrane. They are ineffective against *Pneumocystis carinii*, which has cholesterol instead of ergosterol in its membrane. Morpholines are known to inhibit sterol synthesis. Griseofulvin **10** and 5-fluorocytocine **11** (Figure 4) have been classified under miscellaneous class of compounds. Griseofulvin inhibits nuclear division and is also a membrane tubule inhibitor while nucleoside analogues such as 5-fluorocytosine target DNA synthesis. There are also cell wall antagonist like echinocandins *e.g.* cilofungin or nikkomycins.

The polyene antifungals comprises a large family of drugs but only two (amphotericin B **1** and nystatin **2**) are used for human disease. These drugs bind to sterol within the fungal membrane disrupting its integrity. This makes the membrane leaky, causing loss of small molecules from the fungal cell leading to lysis and finally death of fungal cell. Amphotericin B is a very successful and widely used antifungal drug but its use has been curtailed due to its toxicity. It causes unpleasant side effects including chills, fever and lowering of blood pressure. It may also cause kidney damage. But still amphotericin B remains the drug of choice for life threatening fungal infections and may often be

administered in lower doses together with other antifungal agents, reducing the risk of therapeutic complications.

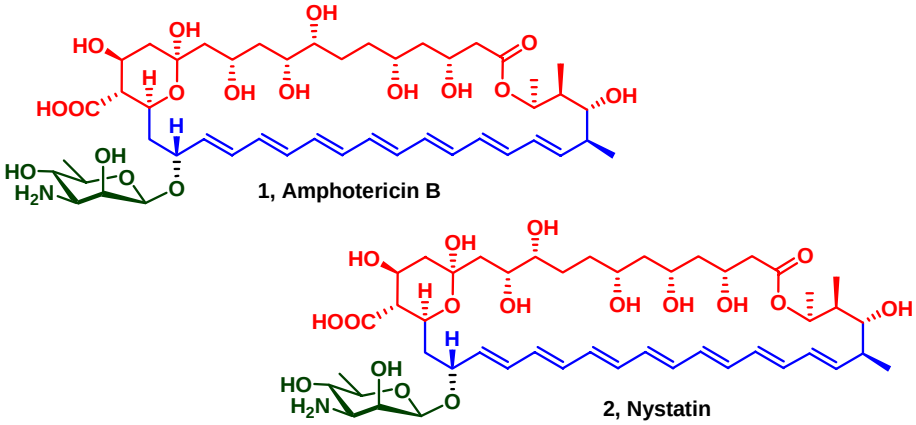


Figure 2. Polyene antifungals amphotericin B **1** and nystatin **2**.

Azole derivatives such as imidazoles and triazoles are emerging groups of antifungal agents. The principal mode of action of this series of compounds is the inhibition of cytochrome P-450 dependent C-14 demethylation of lanosterol **16** (Figure 6), which is the key step in the synthesis of ergosterol in the fungal cell membrane. The first group to be developed is imidazoles, which contains large number of compounds primarily aimed at topical use.

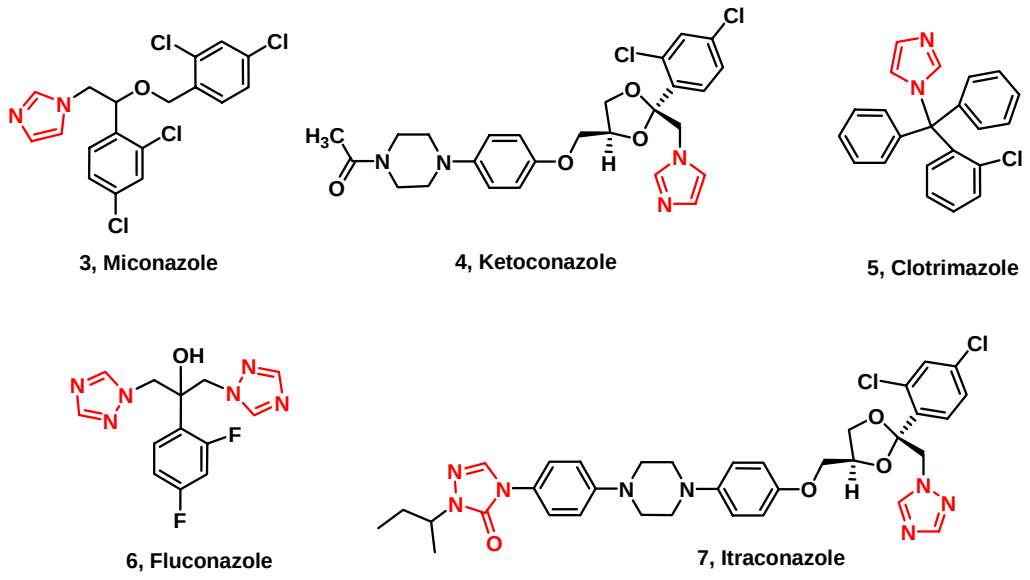


Figure 3. Azole antifungals imidazoles and triazoles.

They include miconazole **3**, ketoconazole **4**, clotrimazole **5** *etc* (Figure 3). These compounds have broad spectrum of antifungal activity. Some of them are also active against Gram-positive bacteria. One of the potential disadvantages of these compounds is interference with human cytochrome P-450. The newer triazole series includes fluconazole **6**, itraconazole **7**, terconazole and very recent ones voriconazole, posaconazole etc. They act by the same mechanism as the imidazoles but show less affinity for the mammalian cytochrome P-450.

There are two allylamine antifungals in current use namely naftifine **8** and terbinafine **9** (Figure 4). Allylamines act by inhibiting squalene epoxidase, which is an important enzyme in the membrane synthesis of fungi. Naftifine is active against dermatophytes and some yeast. Terbinafine is active against wider range of fungi. Its *tert*-butyl side chain is essential for the specific activity.

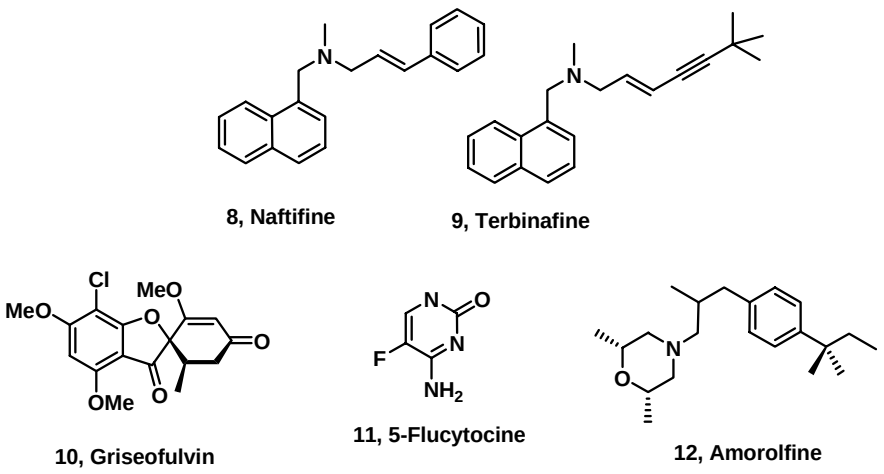


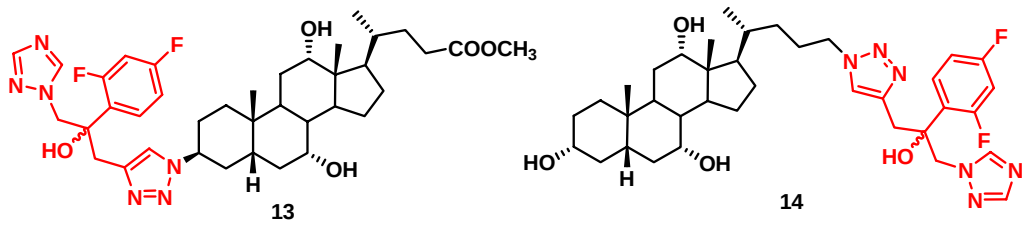
Figure 4. Other antifungal agents.

Other antifungal agents include griseofulvin **10**, which is a natural antibiotic and acts by inhibition of microtubule formation, thus inhibiting fungal mitosis. It is used in the treatment of ringworm, dermatophytis and fungal infections of nails. Flucytosine interferes with the formation of fungal RNA/DNA. It can be used alone or with amphotericin B. It derives

selective toxicity from inability of human cells to convert it into 5-fluorouracil. Its major drawback is the ease with which resistance develops.

Morpholine antifungal amorolfine **12**, is known to inhibit sterol synthesis. There are also cell wall antagonist like echinocandins *e.g.* cilofungin or nikkomycins. The echinocandins are fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid sidechain responsible for antifungal activity. Antifungal activity in the prototypes, echinocandin B and aculeacin A, was discovered by random screening in the 1970s. The target for the echinocandins is the complex of proteins responsible for synthesis of cell wall β-1,3 glucan polysaccharides. The sordarin antifungal class, although not developed for clinical use, merits mention among the new mechanisms of action. Sordarins inhibit protein synthesis by blocking the function of fungal translation Elongation Factor 2 (EF2).

The field of antifungal has become static and there are only a few new groups of antifungals under development. Due to emergence of new fungal pathogens, nephrotoxicity to polyene antifungals and development of resistance to emerging azole antifungals, there is always need for new antifungal compounds with novel modes of action for treating or preventing fungal infections. In order to seek new triazole antifungal agents, recently fluconazole/bile acid conjugates **13** and **14** were designed in our group and their regioselective synthesis was achieved in very high yield *via* Cu(I) catalyzed intermolecular 1,3-dipolar cycloaddition [5].



13 14

Figure 5. Novel bile acid-fluconazole conjugates.

These new molecules showed good antifungal activity against *Candida* species. In these conjugates bile acid part was thought to be useful to permeabilize the fungal cell membranes as well as transporter for the active component of the fluconazole because of its amphiphilic nature. Hence, the conjugates were predicted to be more active than fluconazole. Unfortunately, these molecules were found to be less active than fluconazole. In this chapter our aim is to select a suitable steroid unit which will be responsible for the perfect transport of the whole conjugate at the appropriate site of action.

B4.2.2. *Rational for the design of lanosterol-azole conjugates*

The medicinal chemistry of steroids covers a large and interesting series of structures and biological activities [6]. The chemistry and biochemistry of this natural product is extensively studied and utilized in the development of various drugs. A vast literature highlighting the medicinal properties of natural, unnatural and molecular hybrids derived from diverse steroids through integration and/or linkage with other biomolecules (polyamines, amino acids, and carbohydrates), drugs and other functional molecules is summarized in the part A of this thesis.

Literature survey of antimicrobial steroids reveals that several amino cholesterol derivatives exhibit potent antimicrobial activity against Gram-positive, Gram-negative bacteria and yeast [7]. A series of oxygenated cholesterol derivatives were reported to exhibit high activities against *C. albicans* and Amphotericin B and miconazole resistant strains of *C. albicans* at a concentration of 1.5 μg/mL [8]. The oxygenated cholesterol derivatives are endowed with apoptotic properties, potentially useful for immunosuppression

and anticancer therapy [9]. For understanding the potential of these sterol units as antifungal agents we should study the ergosterol biosynthesis.

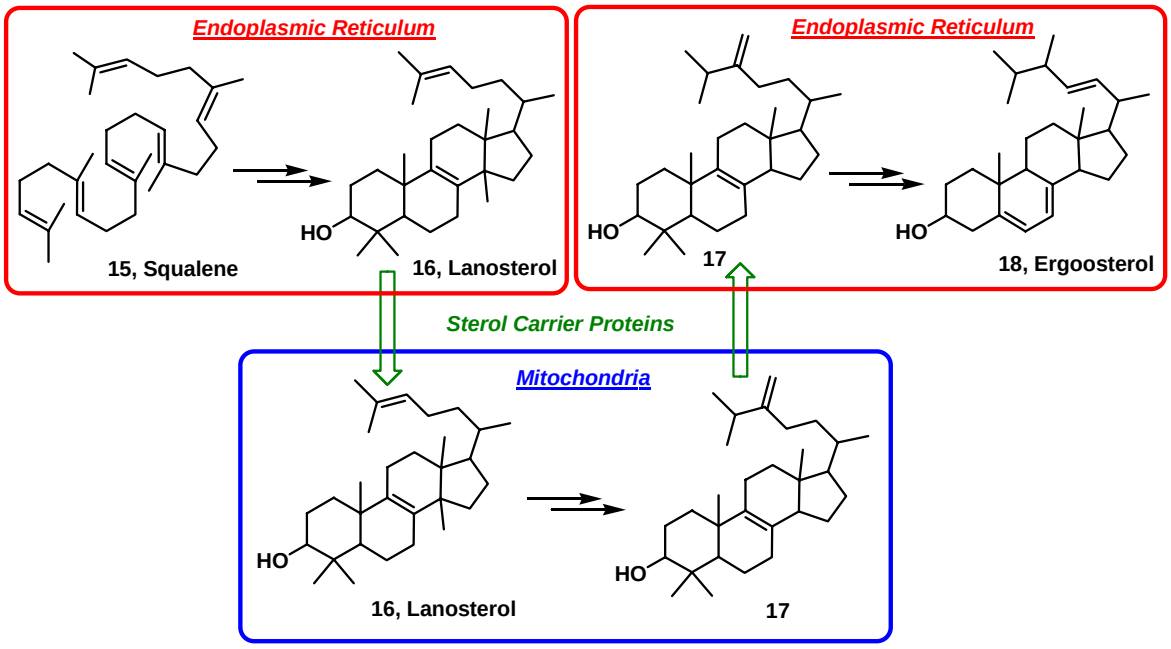


Figure 6. Transfer of sterols within ER to mitochondria during ergosterol biosynthesis.

Lewis Thomas (1974) pointed out that, "It takes a membrane to make sense out of disorder." In these membranes, sterols play a major role both architecturally and functionally. The most common membrane sterol in animals is cholesterol [10] and the major sterol of most fungi is ergosterol [11]. The major steps involved in the ergosterol biosynthesis are illustrated as one of the callout in Figure 1. It is known that the viscosity of the lipid core of the plasmamembrane is a function of the concentration of sterol. A decrease in the ratio of cholesterol-phospholipids or ergosterol-phospholipids indicates an increase in membrane fluidity [10]. It has been shown that in sterol deficient cells an increased fluidity of the membrane alters several of its functions [12]. In fungi, biosynthesis of ergosterol takes place in Endoplasmic Reticulum (ER) during which the intermediate Lanosterol **16** gets transported to Mitochondria. Lanosterol then gets transformed to 24-methylene-24,25-

dihydrolanosterol **17** in Mitochondria; fungi then need to transport **17** back to ER for further biosynthetic transformations. (Figure 5) [10].

A non-catalytic carrier protein (s) (sterol carrier protein) does this job of transfer of sterols within ER to mitochondria [13]. It is clear from this discussion that only the sterols having lanosterol like backbone can best fit in to the active site pocket of these sterol carrier proteins and the molecules derived from these sterols may be more active as antifungal agents than those derived from cholesterol. Based on the above-mentioned observations we have designed novel lanosterol-azole conjugates **19** and **20** (Figure 7) which can mimic the substrate lanosterol. These compounds can play a duel role. They can act as an azole derivative and lanosterol backbone can target whole drug at the appropriate site of action.

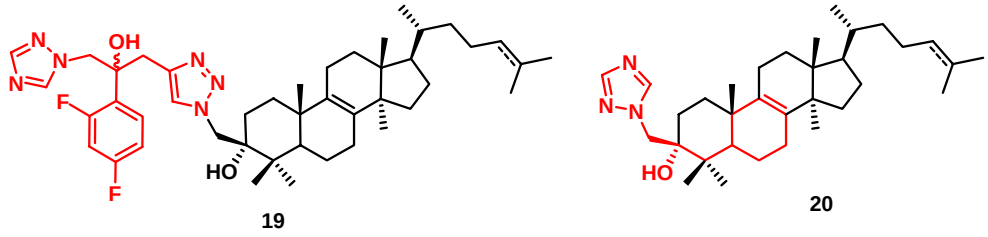


Figure 7. Novel lanosterol-azole conjugates.

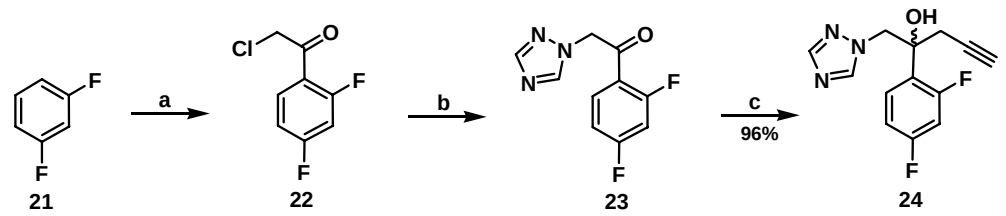
B4.3. Chemistry

Bioconjugation has recently emerged as a fast growing technology that affects almost every discipline of life science. It aims at the ligation of two or more molecules to form new complexes with the combined properties of its individual components [14]. In continuation of our work on bile acids, we designed new bioconjugates **19** and **20** (Figure 7) of lanosterol and pharmacophore of fluconazole, linked together with 1,2,3-triazole, which may be viewed as an isoster of one of the 1,2,4-triazole component of fluconazole. 1,2,3-Triazole moieties are attractive connecting units, since they are stable to metabolic

degradation and capable of hydrogen bonding, which can be favorable in binding of biomolecular targets and for solubility [15]. 1,2,3-Triazole moiety does not occur in nature, although the synthetic molecules containing 1,2,3-triazole unit shows diverse biological activities including antibacterial, herbicidal, fungicidal, antiallergic, and anti-HIV [16]. 1,3-Dipolar cycloaddition of terminal acetylene and organic azides has been a method of choice for the synthesis of 1,2,3-triazoles [17].

In our approach to synthesize these new molecules, we considered performing Huisgen (click) reaction [18] to connect fluconazole part containing terminal alkyne **24** (Scheme 1) and 24,25-dihydrolanosterol containing terminal azide **27** (Scheme 2), in the presence of Cu(I) catalyst to form lanosterol-fluconazole conjugate **19**. Accordingly, we synthesized 2-(2,4-difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)pent-4-yn-2-ol **24** by propargylation of the corresponding ketone **23** [19] using propargyl bromide and zinc dust.

Scheme 1.



Reagents and conditions: (a) $AlCl_3$, 1,2-dichloroethane, chloroacetyl chloride, 25 °C, 7 h; (b) 1,2,4-triazole, $NaHCO_3$, toluene, reflux, 4 h (55 % in two steps); (c) Zn, propargyl bromide, DMF/THF, 25 °C, 5 h.

The racemic compound **24** constitutes an alkyne component. In the ^{1}H NMR spectrum of compound **24**, the acetylenic proton was identified as triplet at δ 2.06 ppm and doublet of doublet at δ 2.87 ppm, due to β methylene. In the ^{13}C NMR spectrum it showed intricacies of various ^{19}F-^{13}C coupling as in fluconazole molecule [20]. The presence of acetylenic group was also evident from IR spectrum wherein the absorption due to

acetylenic group was observed at 3307 cm^{-1}. Halogen bonding and other weak interactions driven self-assembly of 11-bromo-12-oxo-5β-cholan derivatives was systematically investigated and described in chapter 1 of this thesis. With inspiration from this the crystal structures of difluoro compounds **23** and **24** were resolved, to study the two-dimensional arrangement of these molecules. Unfortunately, we have not observed any halogen bonding (C–F…O or C–F…N) in the molecular organization of compounds **23** and **24**.

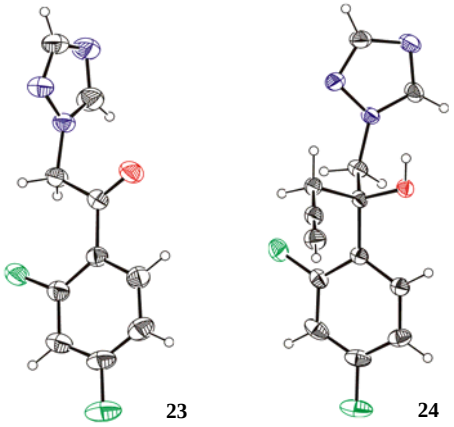
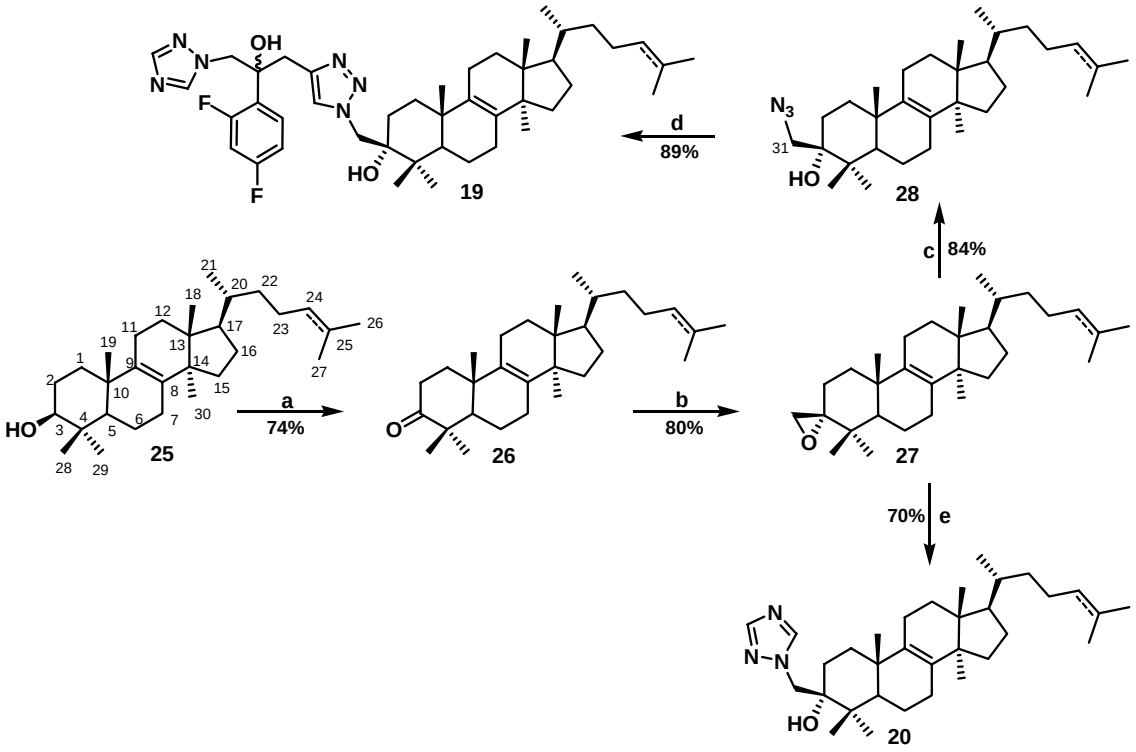


Figure 8. ORTEP views of compounds **23** and **24**.

The synthesis of azido lanosterol unit **27** was attempted from the commercially available (60/40) mixture of lanosterol and dihydrolanosterol. In the first step C-3 hydroxyl functionality of compound **25** was oxidized to lanostanone **26** using CrO_3/H_2SO_4 (50 %). Improved yields for the oxidation of **25** were obtained using pyridinium fluorochromate (PFC, 74 %). Lanostanone **26** was then stereoselectively converted to spiro-3(R)-oxirane **27** by reaction with trimethyl sulfoxonium iodide (TMSOI). The stereochemistry of carbonyl addition to cyclohexanones varies depending on the ylide; the oxosulfonium ylide reacts by equatorial addition of methylene and the sulfonium ylide shows a preference for axial addition [21].

Scheme 2.



Reagents and conditions: (a) CrO_3, H_2SO_4, H_2O, 10 °C, 10 min. or PFC, CH_2Cl_2, 25 °C, 2.5 h; (b) TMSOI, NaH, DMSO/THF, 100 °C, 5h; (c) NaN_3, $LiClO_4$, DMF, 60 °C, 4h; (d) **24**, $CuSO_4$-$5H_2O$ (5 mol %), sodium ascorbate (40 mol %), DMF/H_2O (9:1), microwave, 5 min; (e) Triazole, DMF, 100 °C, 4h.

The regioselective epoxide ring opening of oxirane **27** using NaN_3 and catalytic amount of $LiClO_4$ furnished the required β-azido alcohol **28** in 84 % yield. The absolute structure of β-azido alcohol **28** has been established by single crystal X-ray analysis (Figure 10), which indirectly confirms the equatorial addition of methylene by oxosulfonium ylide. IR of this compound showed absorption due to azido group at 2102 cm^{-1} and in ^1H NMR spectrum, resonance corresponding to C-31-methylene group was observed as two separate doublets at δ 3.21 ppm (C-31H_a) and δ 3.63 ppm (C-31-H_b)(H_aH_b geminal coupling J = 12 Hz).

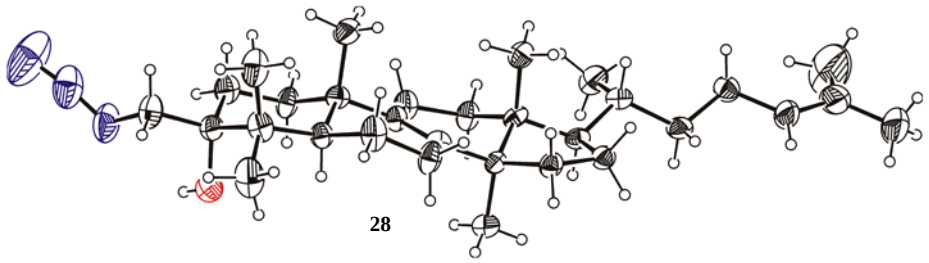


Figure 8. ORTEP view of compounds **28**.

Among the various reaction conditions, microwave assisted Cu(I) catalyzed reaction was found to be suitable for the desired cycloaddition reaction between alkyne **24** and azide **28** [22]. Under microwave irradiation compound **24** was reacted with azide 2**8** in DMF/H_2O using catalytic amount of Cu(I) to give dihydrolanosterol-fluconazole conjugate **19** as a diastereomeric mixture in 89 % yield (Scheme 2). In the ^{1}H NMR spectrum of compound **19**, resonances corresponding to C-31-methylene protons were identified as two separate peaks at δ 3.12 and 3.52 ppm. The expected six aromatic peaks were observed at 6.66-6.77 (m, 2H), 7.30-7.40 (m, 2H), 7.83 (s, 1H), 8.16-8.25 (m, 1H). In addition, mass spectrum showed molecular ion peak at 745.5 (M+H). To investigate the exact effect of fluconazole moiety in conjugate **19**, as a control lanosterol-triazole conjugate **20** was also synthesized by the regioselective opening of epoxide ring in compound **27** using 1,2,4-triazole (Scheme 2). Synthesis of this compound was confirmed by ^{1}H NMR spectroscopy in which C-31 methylene protons were appeared as two separate doublets at δ 4.19 ppm (C-31H_a) and δ 4.38 ppm (C-31-H_b)(H_aH_b geminal coupling J = 12 Hz). The two aromatic protons of 1,2,4-triazole functionality were appeared at 7.94 and 8.19 δ ppm. Synthesis of the similar compounds starting from dihydrolanosterol is in progress and we have achieved the synthesis of pure spiro epoxide **27**. The ^{1}H and ^{13}C NMR spectra of C-24,25-dihydro analogues of compound **25**, **26** and **27** are enclosed.

B4.4. Bioevaluation

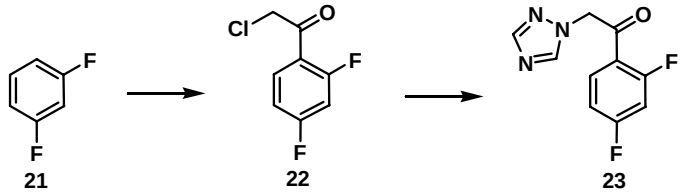
The synthsized lanosterol-azole conjugates **19**, **20** and the other intermediates are under preliminary biological evaluation to study their antifungal activity against the available strains of *Candida albicans*, *Candida parapsilosis*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes* and *Aspergillus fumigatus*. Isolation and structural investigation of sterol carrier proteins (SCP) in fungi and study its *in vitro* inhibition are the further goals of this project.

B4.5. Summary

In conclusion, for the first time sterol carrier proteins (SCP) are suggested to be one of the key targets for the development of safe and effective antifungal therepeutics. With this hypothesis novel lanosterol-fluconazole conjugates were designed and their regioselective synthesis was achieved in very high yield *via* Cu(I) catalyzed intermolecular 1,3-dipolar cycloaddition. The synthesized molecules and intermediates are under biological screning for their antifungal activity and cytotoxicity.

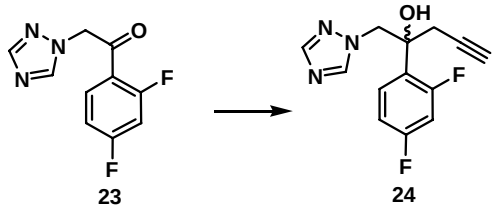
B4.6. Experimantal Section

B4.6.1. *1-(2,4-Diflurophenyl)-2-(1H-1,2,4-triazole-1-yl)ethanone (23):*



The ketone **23** was synthesized from dofluoro benzene **21** using literature procedures [5,19]. White solid; yield 95 %; Mp. 115 ºC (lit. [19d] Mp. 103-105 ºC); IR ν_{max} 1703, 1614, 1593 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.59 (d, *J* = 3.54 Hz, 2H), 6.63-7.10 (m, 2H), 7.99-8.11 (m, 2H), 8.21 (bs, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 187.6 (d, $^{3}J_{CF}$ = 5.53 Hz), 166.6 (dd, $^{1}J_{CF}$ = 259.9 Hz, $^{3}J_{CF}$ = 12.58 Hz), 163.0 (dd, $^{1}J_{CF}$ = 256.38 Hz, $^{3}J_{CF}$ = 13.08 Hz), 151.7, 144.8, 132.9 (dd, $^{3}J_{CF}$ = 4.52 Hz, 10.81 Hz), 118.8 (dd, $^{2}J_{CF}$ = 14.09 Hz, $^{4}J_{CF}$ = 3.52 Hz), 112.9 (dd, $^{2}J_{CF}$ = 21.63 Hz, $^{4}J_{CF}$ = 3.02 Hz), 104.8 (dd, $^{2}J_{CF}$ = 25.66 Hz), 58.2 (dd, $^{4}J_{CF}$ = 14.09 Hz); Anal. calcd. for $C_{10}H_7F_2N_3O$: C, 53.82; H, 3.16; F, 17.03; N, 18.83; Found: C, 54.10; H, 3.02; F, 16.87; N, 18.71; MS (LCMS) *m/z* 224.57 $[M+H]^+$, 246.57 $[M+Na]^+$.

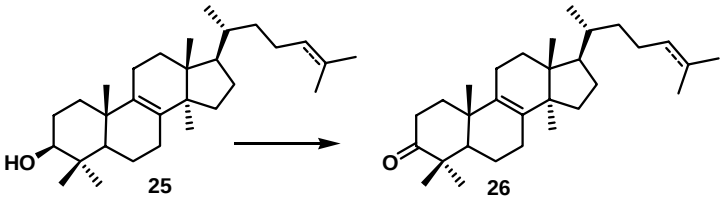
B4.6.2. *2-(2,4-diflurophenyl)-1-(1H-1,2,4-triazole-1-yl)pent-4-yn-2-ol (24):*



The ketone **23** (0.500 g, 2.24 mmol) and propargyl bromide (4 mL, 6.73 mmol) were dissolved in a mixed solvent DMF-THF (10 mL, 1:1). To this well stirred solution, activated zinc dust (washed with 2 % HCl, water and dried in vacuum) (0.439 g, 6.73 mmol) was slowly added at room temperature. After 5 min exothermic reaction brought itself to reflux,

which was allowed to cool to 25 ºC (room temp). The whole reaction mixture was then stirred for 5 h at 25 ºC. Ice-cold HCl solution (5mL, 5 %) was added to the reaction mixture and it was extracted with EtOAc, washed with water and brine. Solvent was evaporated under reduced pressure to afford crude product, which was purified by column chromatography on silica gel (5 % MeOH/DCM) to furnish compound **24** (0.551 g, 96 %) as white solid. Mp. 145-146 ºC; IR ν_{max} 3272, 3137 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 2.06 (t, *J* = 2.65 Hz, 1H), 2.87 (dd, *J* = 17.06, 5.13 Hz, 2H), 4.13 (bs, 1H, OH), 4.77 (dd, *J* = 14.14, 4.80 Hz, 2H), 6.73-6.87 (m, 2H), 7.50-7.59 (m, 1H), 7.87 (s, 1H), 8.20 (s, 1H); ^{13}C NMR ($CDCl_3$+CD_3OD, 50 MHz) δ 162.6 (dd, $^1J_{CF}$ = 249.59 Hz, $^3J_{CF}$ = 12.08 Hz), 158.6 (dd, $^1J_{CF}$ = 246.58 Hz, $^3J_{CF}$ = 12.08 Hz), 150.4, 144.2, 129.6 (dd, $^3J_{CF}$ = 6.04 Hz, 9.56 Hz), 124.2 (dd, $^2J_{CF}$ = 13.08 Hz, $^4J_{CF}$ = 3.52 Hz), 111.1 (dd, $^2J_{CF}$ = 20.63 Hz, $^4J_{CF}$ = 3.52 Hz), 103.9 (dd, $^2J_{CF}$ = 27.42 Hz), 78.1, 73.4 (d, $^3J_{CF}$ = 4.53 Hz), 71.9, 56.3 (dd, $^4J_{CF}$ = 5.03 Hz), 29.1 (dd, $^4J_{CF}$ = 5.03 Hz); Anal. calcd. for $C_{13}H_{11}F_2N_3O$: C, 29.31; H, 4.21; F, 14.43 N, 15.96; Found: C, 29.45; H, 4.13; F, 15.31; N, 15.87; MS (LCMS) *m/z* 264.06 $[M+H]^+$, 286.05 $[M+Na]^+$.

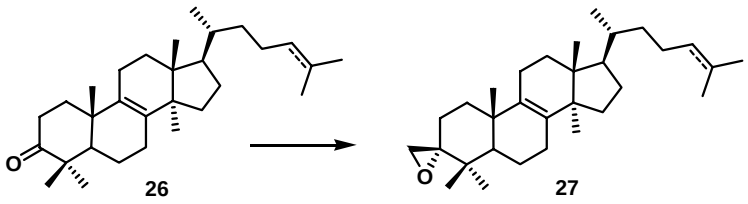
B4.6.3. ***Lanostanone (26):***



Compound **25** (0.426 g, 1.0 mmol) in acetone (20 mL) was stirred with Jones Reagent (1 mL) at 5-10 °C for 5 min. Methanol (5 mL) was added after 5 min, the solvent was evaporated and the crude solid material was dissolved in EtOAc/H_2O (5:1) mixture (100 mL). The organic layer was washed with cold H_2O (2x10 mL), 10 % $NaHCO_3$ (2x10 mL),

brine (2x10 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (5 %, EtOAc/PE) afforded compound **26** (0.212 g, 50 %) as a white crystalline solid. Improved yields for the oxidation of **25** were obtained using pyridinium fluorochromate (PFC). Compound **25** (5.0g, 11.7 mmol) was dissolved in dry DCM (80 mL), to it PFC (4.6 g, 23.5 mmol) was added and the resulted suspension was stirred at 25 °C for 2.5 hrs. The reaction mixture was filtered through celite and further diluted with 200 mL of DCM. The organic layer was washed with cold H_2O (2x15 mL), 10% $NaHCO_3$ (2x10 mL), brine (2x15 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to afford crude product. Purification by column chromatography on silica gel (2 %, EtOAc/PE) afforded compound **26** (3.68 g, 74%) as a white crystalline solid. IR ν_{max} (Nujol) 1711 cm^{-1}. With the similar reaction conditions 24,25-dihydrolanostanone was synthesized from 24,25-dihydrolanosterol in 78 % yield. The spectroscopic data is consistent with that reported in the literature (^1H and ^{13}C NMR spectra are attached).

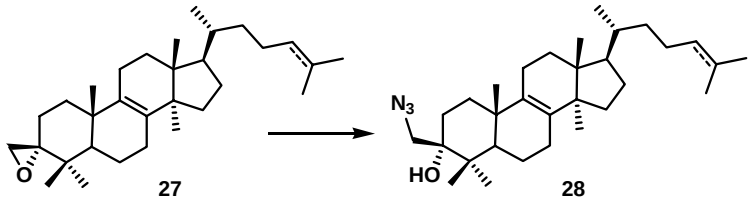
B4.6.4. ***Compound (27):***



TMSOI (0.33 g, 1.5 mmol) and NaH (0.036 g, 1.5 mmol) were dissolved in DMSO (6 mL) and heated at 50 °C for 1h. To this hot reaction mixture lanostanone **26** (0.424 g, 1.0 mmol) in THF (4 mL) was added drop wise for the period of 10 min. The resulted reaction mixture was heated at 70 °C for 7 hrs. Crushed ice (10 g) was added and the compound was extracted with Et_2O (3x50 mL). The organic layer was washed with cold water (2x20 mL),

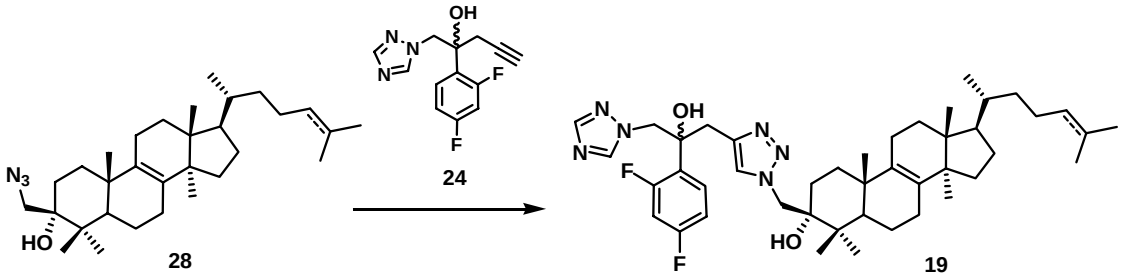
brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to give 0.39 g of crude product. Purification by column chromatography on silica gel (1 % EtOAc/Hexane), furnished compound **27** (0.35 g, 80 %) as gummy solid. Likewise the 24,25-dihydroanalogue of **27** was synthesized from 24,25-dihydrolanostanone.^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.71 (d, *J* = 4.36 Hz, 3H), 0.84-0.90 (m, 15H), 0.99 (s, 3H), 1.08 (s, 3H), 2.42 (d, *J* = 4.41 Hz, 1H), 2.82 (d, *J* = 4.41 Hz, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 15.7, 18.6, 18.7, 19.1, 21.0, 21.7, 22.5, 22.7, 22.8, 24.1, 24.2, 26.3, 27.9, 28.0, 28.2, 29.7, 30.8, 31.0, 33.8, 36.4, 36.5, 36.7, 37.3, 39.5, 44.4, 49.0, 49.8, 50.1, 50.4, 63.3, 134.3, 134.4; MS (LCMS) *m/z* 439.39 $[M+H]^+$.

B4.6.5. ***Compound (28):***



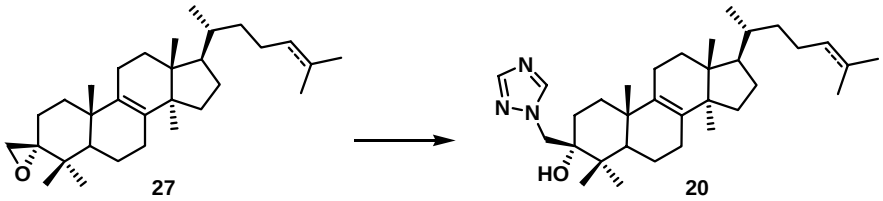
The oxirane 27 (0.219 g, 0.5 mmol) was reacted with NaN_3 (0.390 g, 6 mmol) in the presence of catalytic amount of $LiClO_4$ (0.020 g, 10%) in DMF (10 mL) at 100 °C for the period of 24 hrs. Crushed ice (10 g) was added and the compound was extracted with Et_2O (3x50 mL). The organic layer was washed with cold water (2x20 mL), brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to give 0.24 g of crude product. Purification by column chromatography on silica gel (0.5 % EtOAc/PE), furnished compound **28** (0.17 g, 71 %) as white solid. IR ν_{max} (Nujol) 3576, 2103 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.69 (s, 3H), 0.83 (s, 3H), 0.88 (s, 3H), 0.92 (d, *J* = 6.75 Hz, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.60 (s, 3H), 1.68 (s, 3H), 3.21 (d, *J* = 12.0 Hz, 1H), 3.62 (d, *J* = 12.0 Hz, 1H), 5.10 (t, *J* = 7.0 Hz, 0.7H); MS (LCMS) *m/z* 504.39 $[M+Na]^+$.

B4.6.6. ***Compound (19)*****:**



The alkyne **24** (0.132 g, 0.5 mmol) and the azide **28** (0.289 g, 0.6 mmol) were dissolved in DMF/H_2O 4:1 (5 mL). To this solution, $CuSO_4$-$5H_2O$ (0.05 equiv) and sodium ascorbate (0.40 equiv) were added. The reaction mixture was placed in a domestic microwave reactor and irradiated for 5 min at 415 W. The reaction mixture was cooled, ice was added, and it was then extracted with Et_2O. The extract was washed with water and brine. Solvent was evaporated under reduced pressure and crude product was purified by column chromatography on silica gel using EtOAc to obtain lanosterol-fluconazole conjugate **19** (0.343 g, 92 %) linked with 1,4-disubstituted 1,2,3-triazole. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.68 (s, 3H), 0.86 (s, 3H), 0.87 (s, 3H), 0.89 (s, 3H), 0.94 (s, 9H), 1.60 (s, 3H), 1.68 (s, 3H), 3.11 (m, 1H), 3.52 (m, 1H), 4.26-4.38 (m, 2H), 4.60-4.70 (m, 2H), 5.10 (t, *J* = 6.5 Hz, 0.6H), 6.64-6.67 (m, 2H), 7.30-7.41 (m, 2H), 7.83 (s, 1H), 8.20 (d, *J* = 16 Hz, 1H); MS (LCMS) *m/z* 767.48 $[M+Na]^+$.

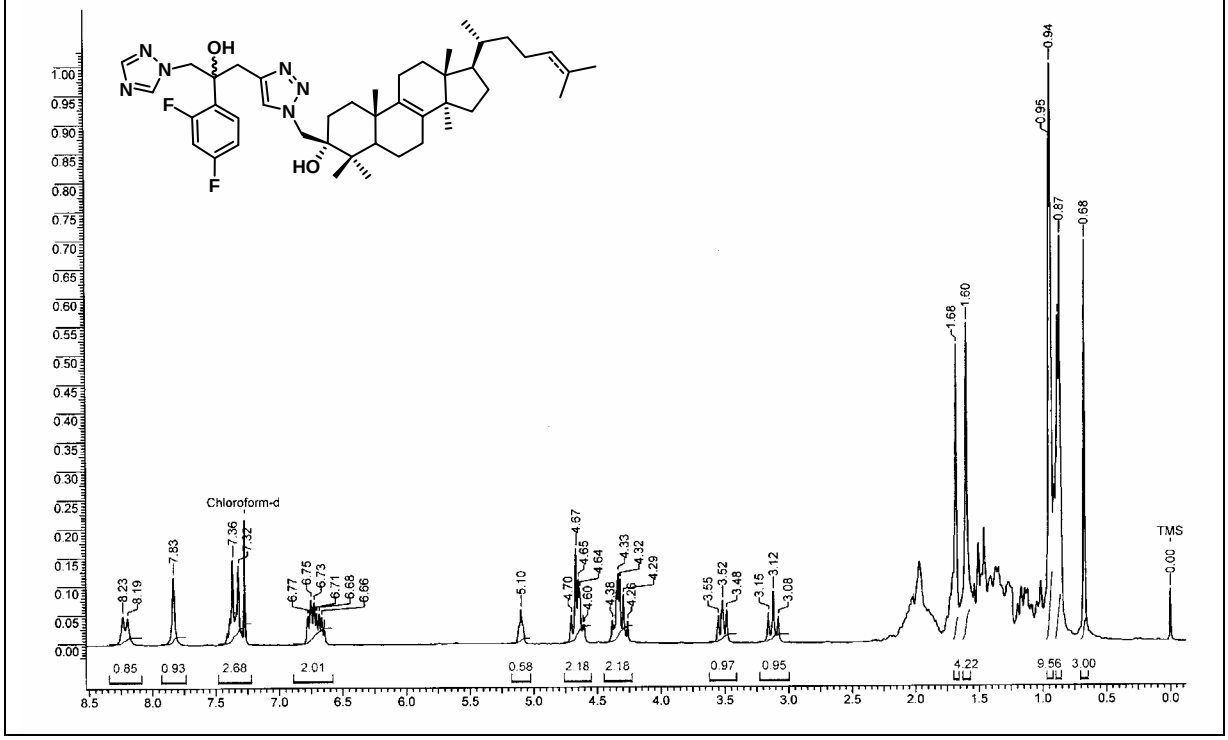
B4.6.7. ***Compound (20):***



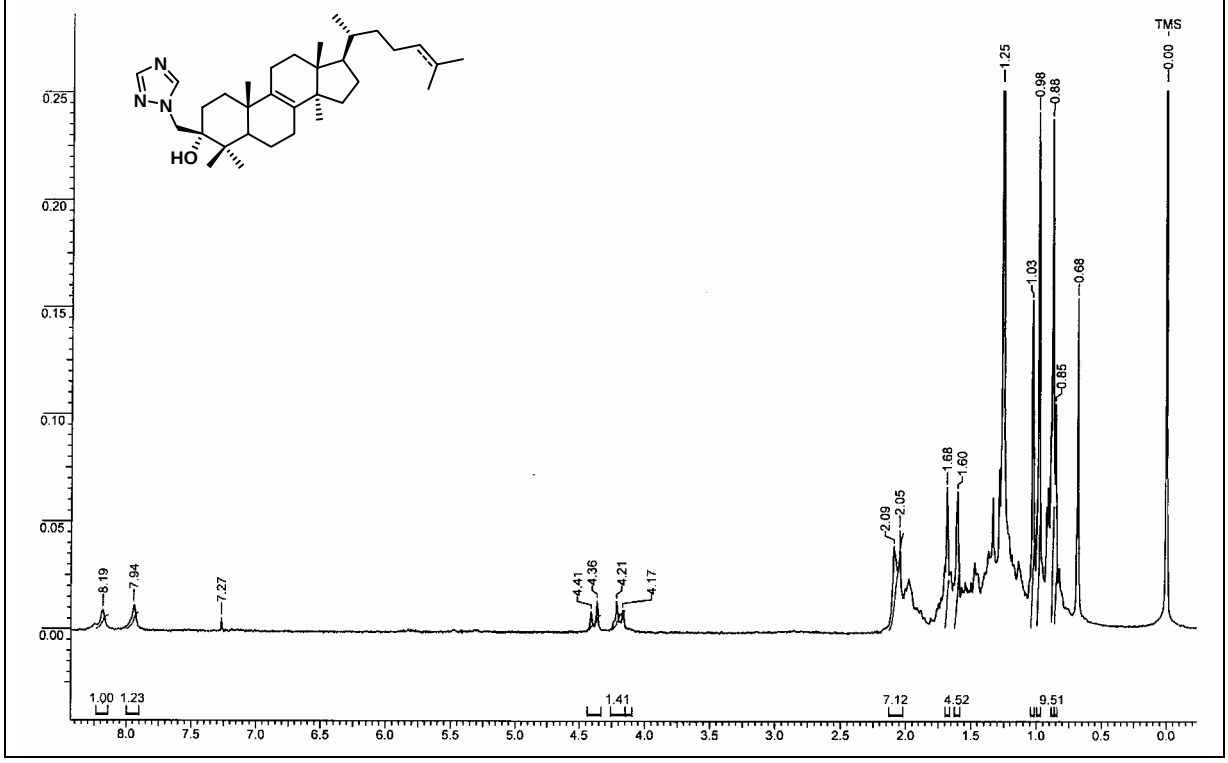
The oxirane **27** (0.27g, 0.62 mmol) and 1,2,4-triazole (0.086g, 1.24 mmol) were dissolved in DMF (10 mL) and were heated at 100 °C for 36 hrs. Crushed ice (10 g) was added and the compound was extracted with Et_2O (3x50 mL). The organic layer was washed with cold water (2x20 mL), brine (20 mL) and dried over Na_2SO_4. Solvent was evaporated under reduced pressure to furnish 0.30 g of crude product. Purification by column chromatography on silica gel (1.5 % EtOAc/PE), afforded compound **20** (0.22 g, 70 %) as white solid. IR ν_{max} (Nujol) 3391, 1618, 1597 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.68 (s, 3H), 0.85, 0.88, 0.98, 1.03, 1.60, 1.68 (multiple singlets, 21-H), 4.20 (d, *J* = 14.2 Hz, 1H), 4.38 (d, *J* = 14.2 Hz, 1H), 5.10 (m, 0.5H), 7.94 (bs, 1H), 8.19 (bs, 1H); MS (LCMS) *m/z* 530.41 $[M+Na]^+$.

B4.7. Selected Spectra

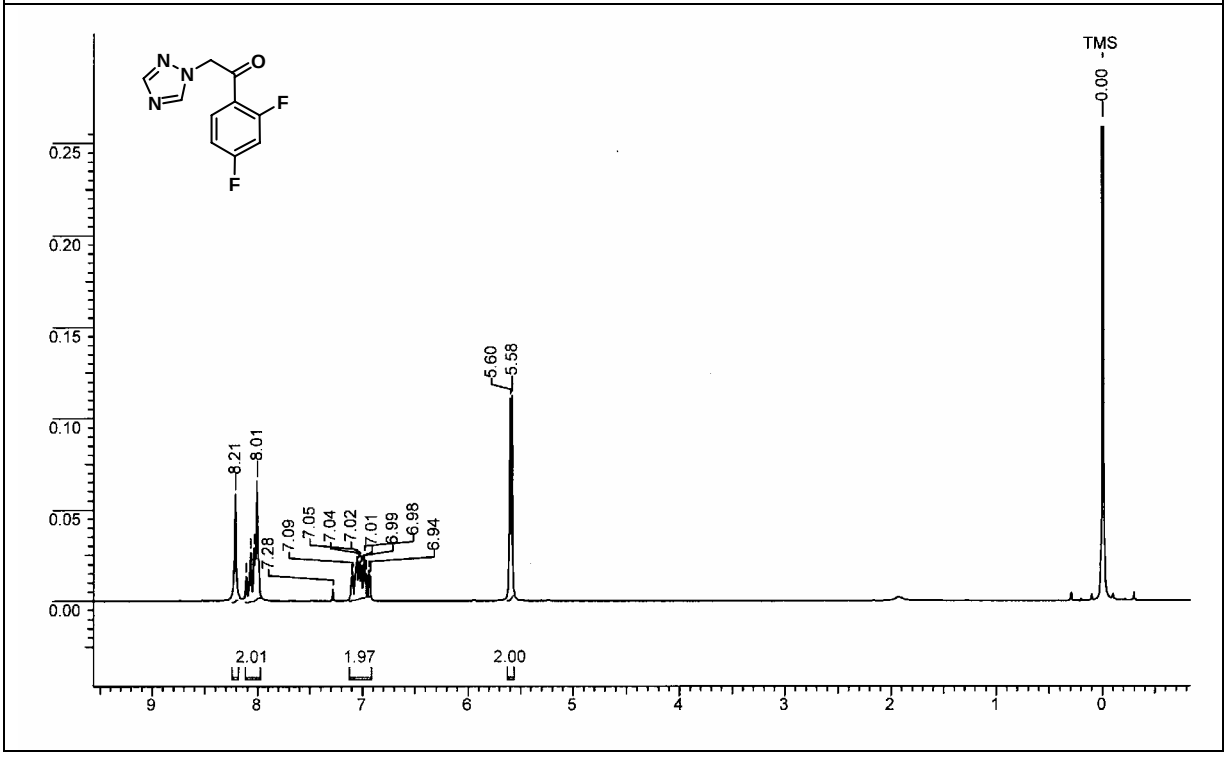
19: ^{1}H NMR, 400 MHz, $CDCl_3$



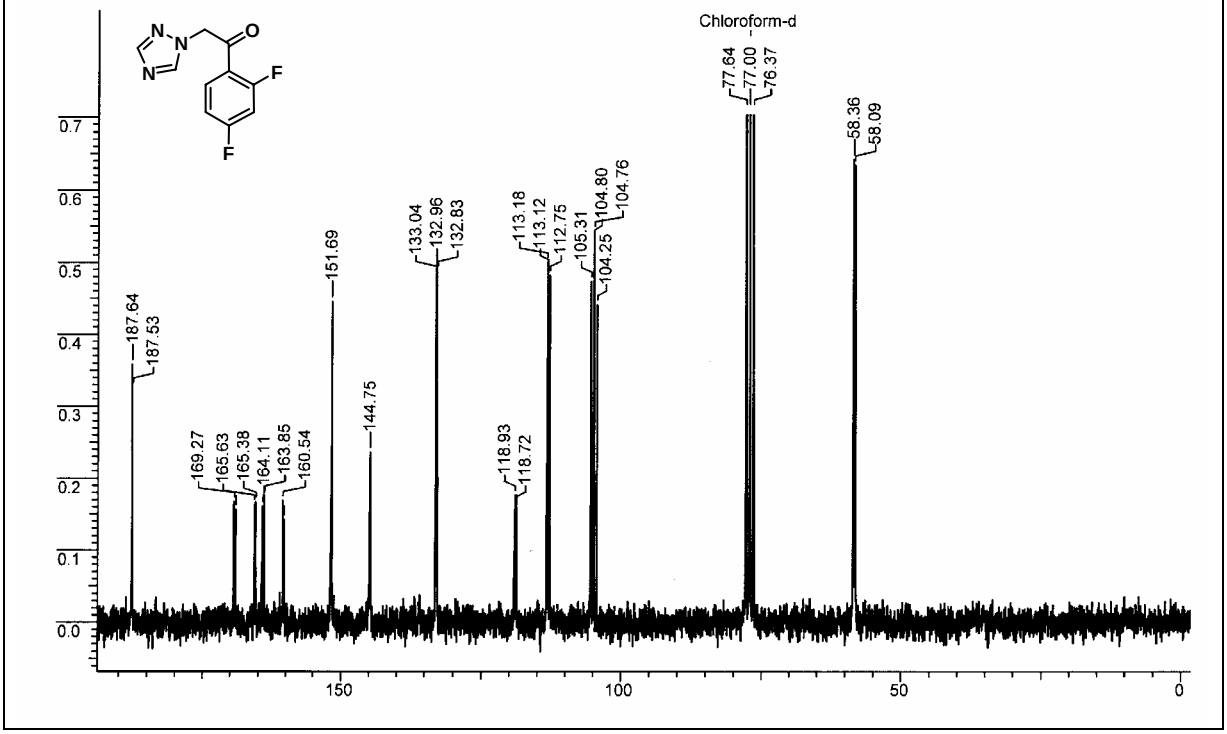
20: ^{1}H NMR, 200 MHz, $CDCl_3$



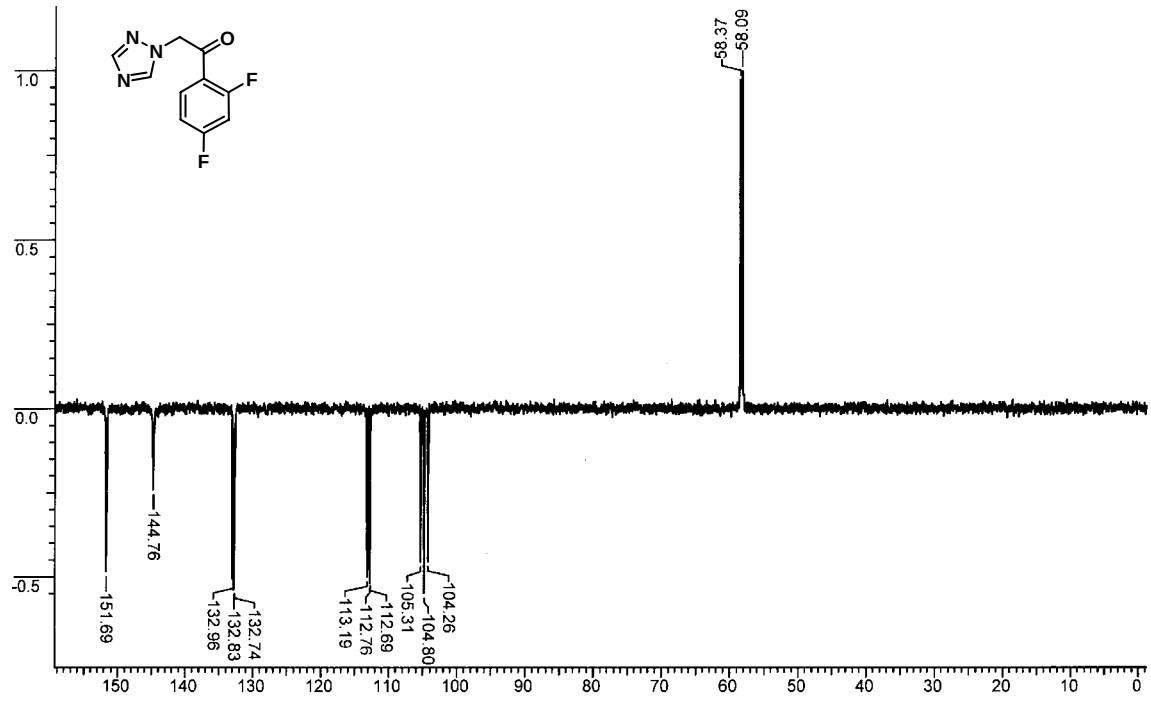
23: ^{1}H NMR, 200 MHz, $CDCl_3$



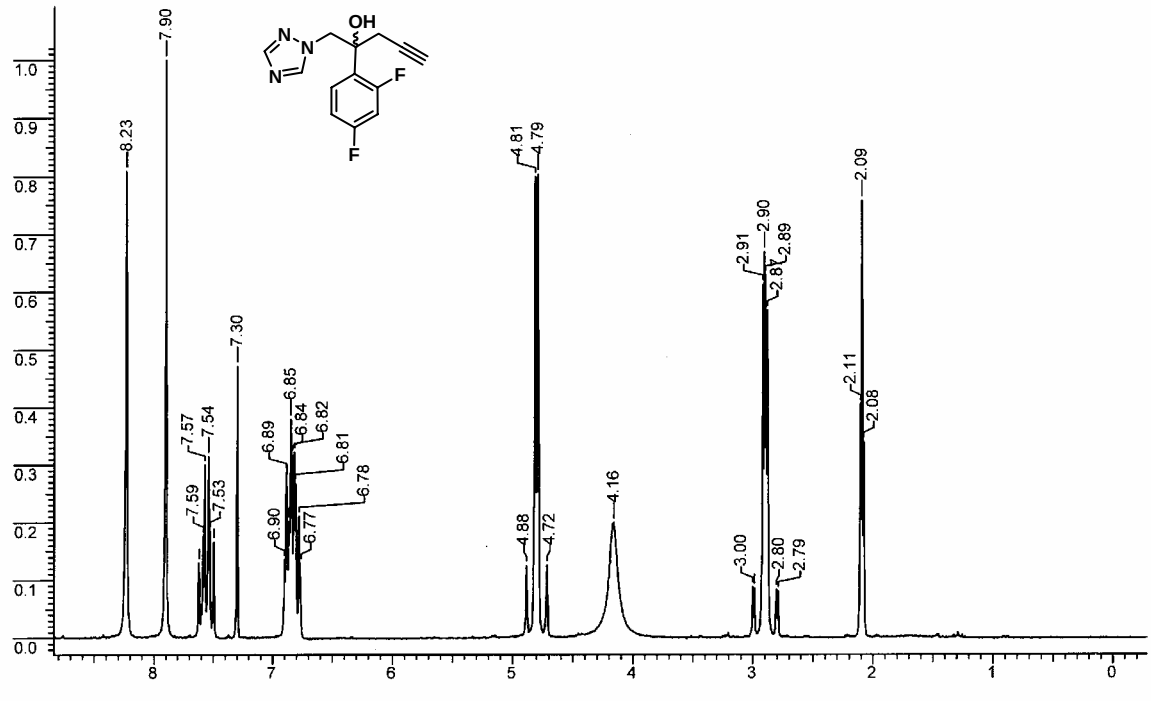
23: ^{13}C NMR, 50 MHz, $CDCl_3$



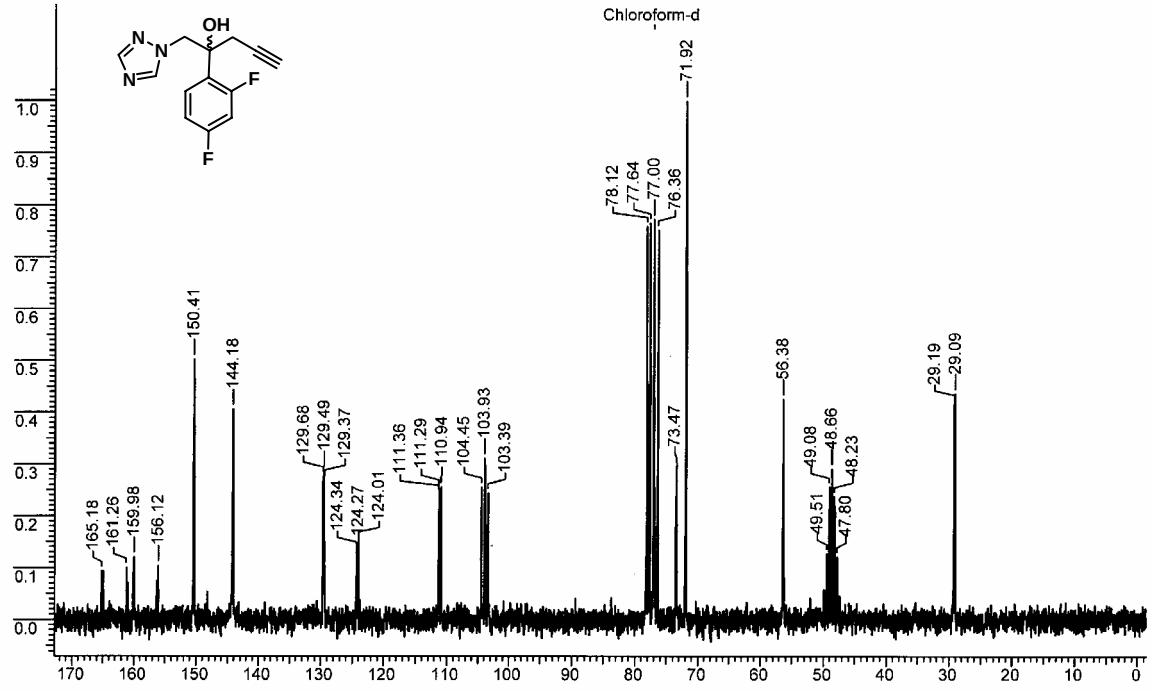
23: DEPT, 50 MHz, $CDCl_3$



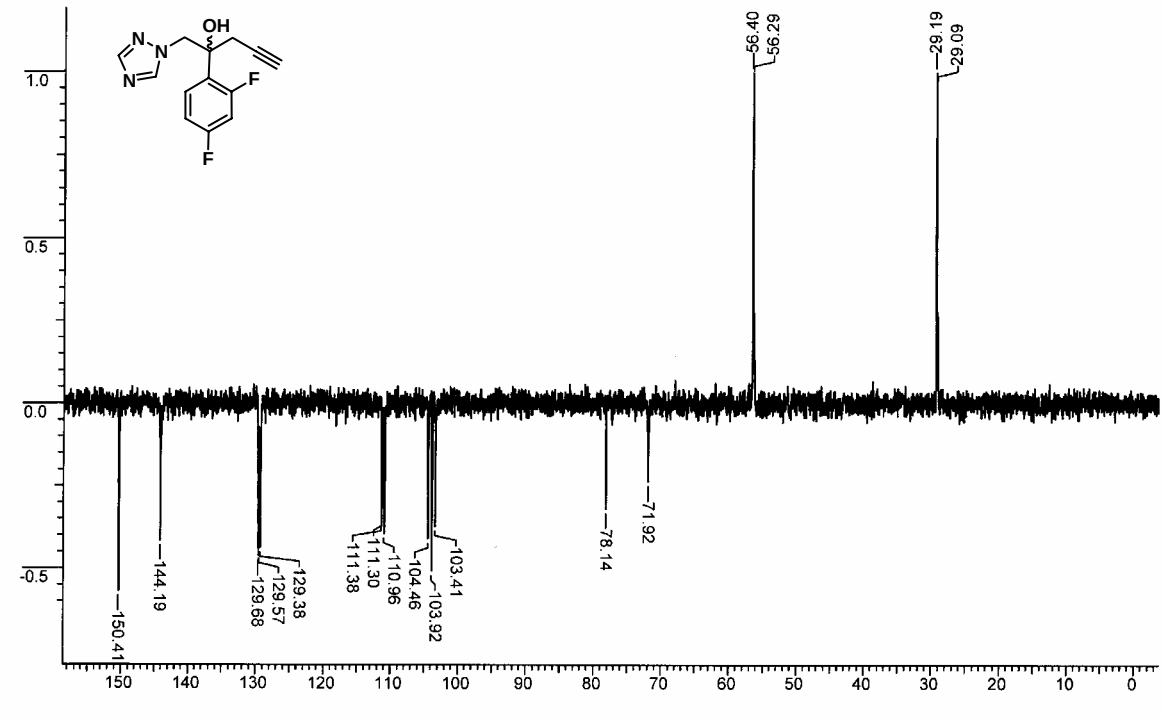
24: ^1H NMR, 300 MHz, $CDCl_3$



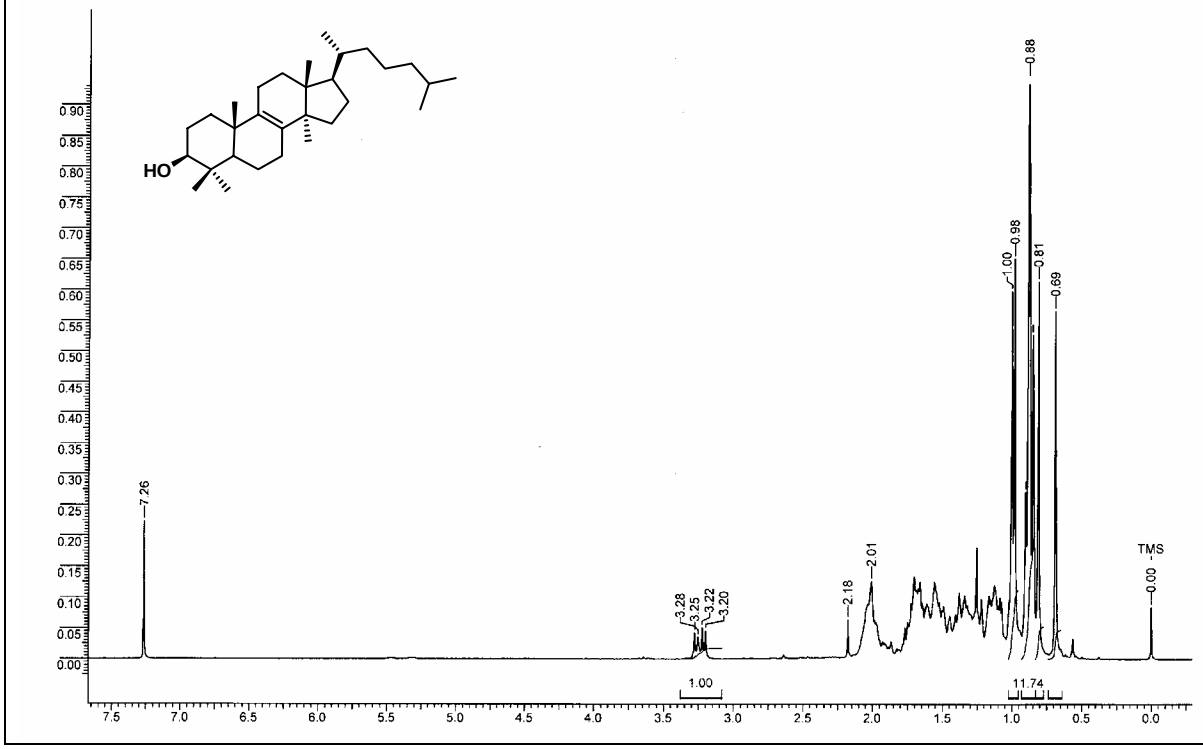
24: ^{13}C NMR, 50 MHz, $CDCl_3$



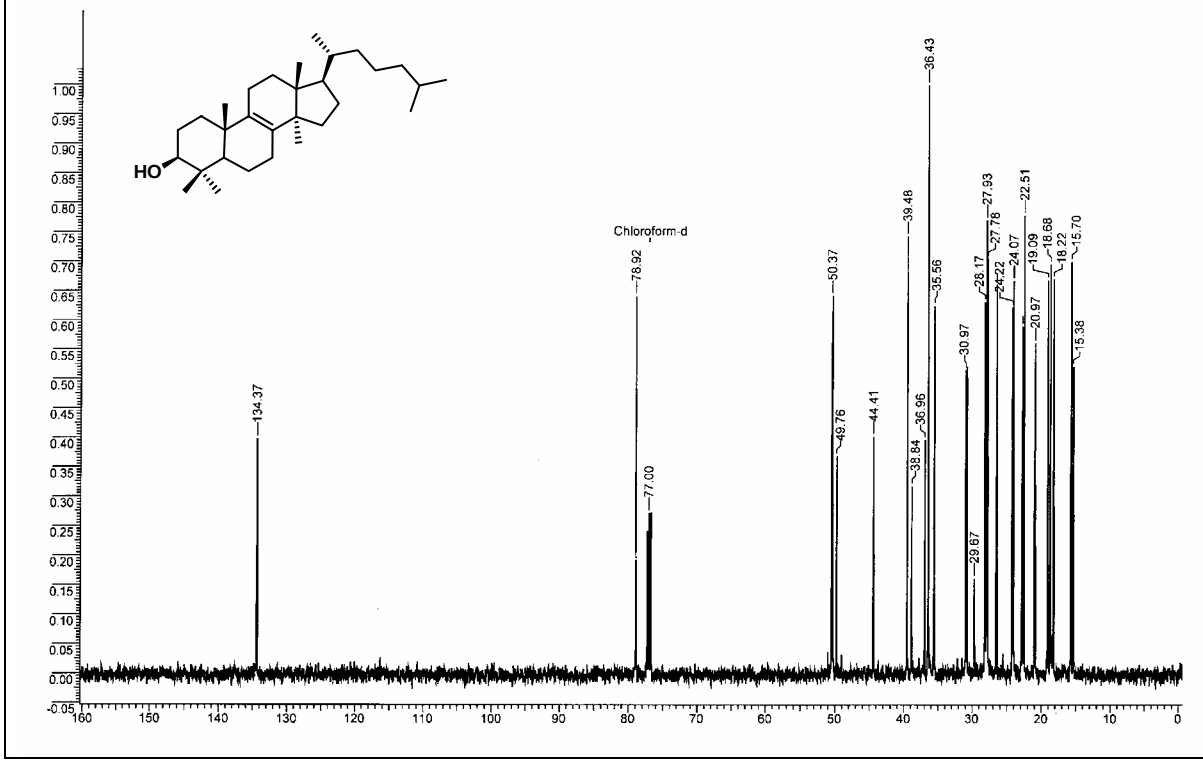
24: DEPT, 50 MHz, $CDCl_3$



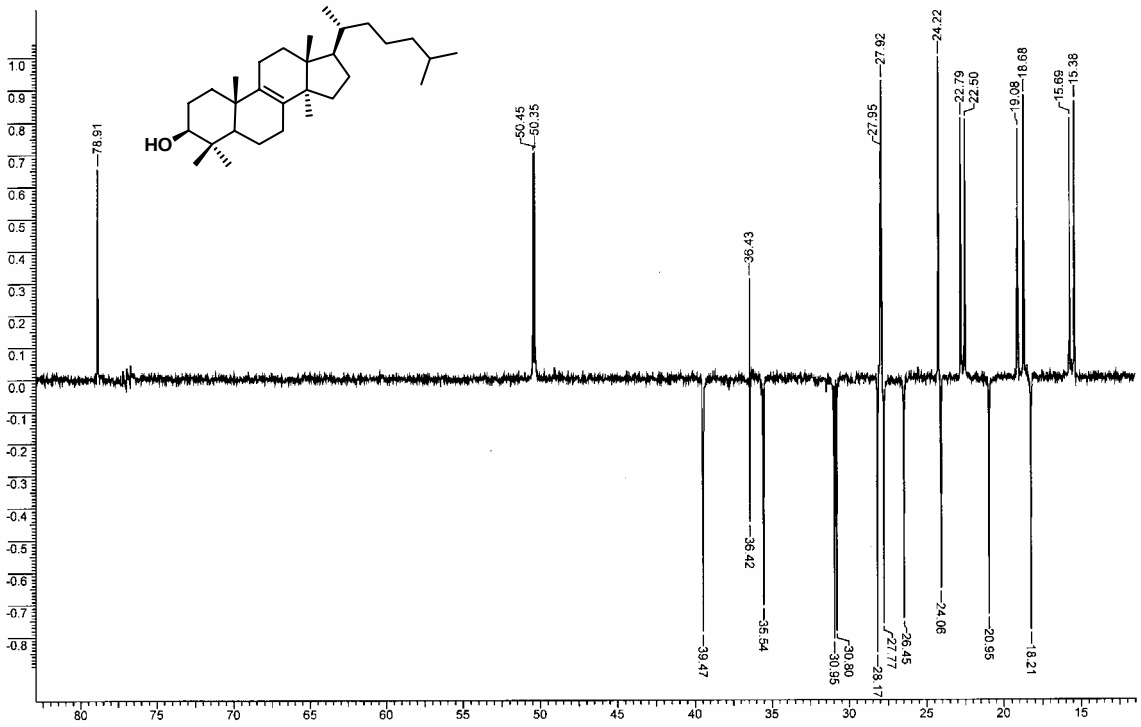
16: *(24,25-dihydro analogue)*: ^{1}H NMR, 300 MHz, $CDCl_3$



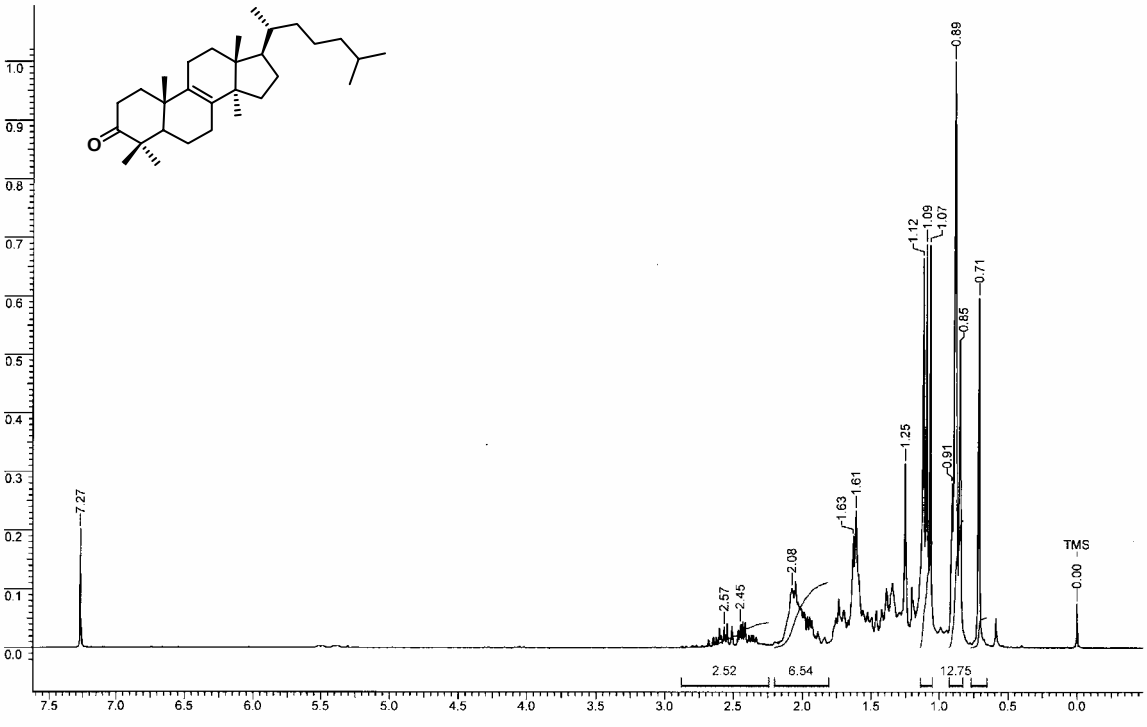
16: *(24,25-dihydro analogue): ^{13}C* NMR, 75 MHz, $CDCl_3$



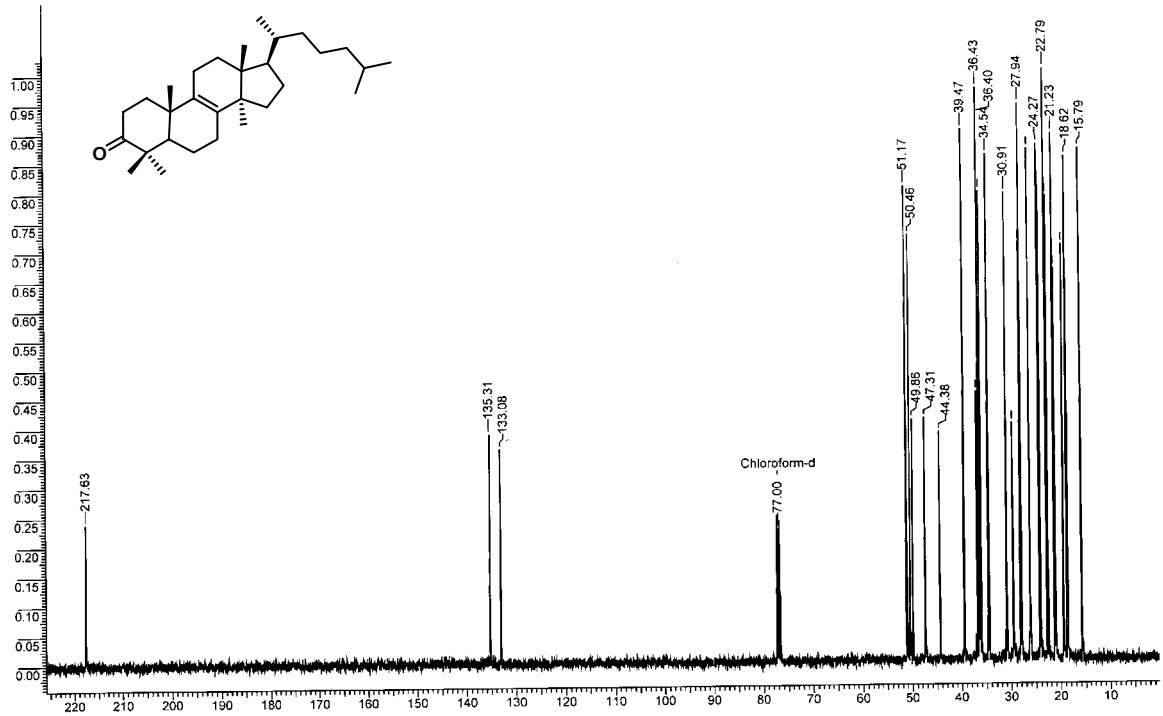
16: *(24,25-dihydro analogue)*: DEPT, 75 MHz, $CDCl_3$



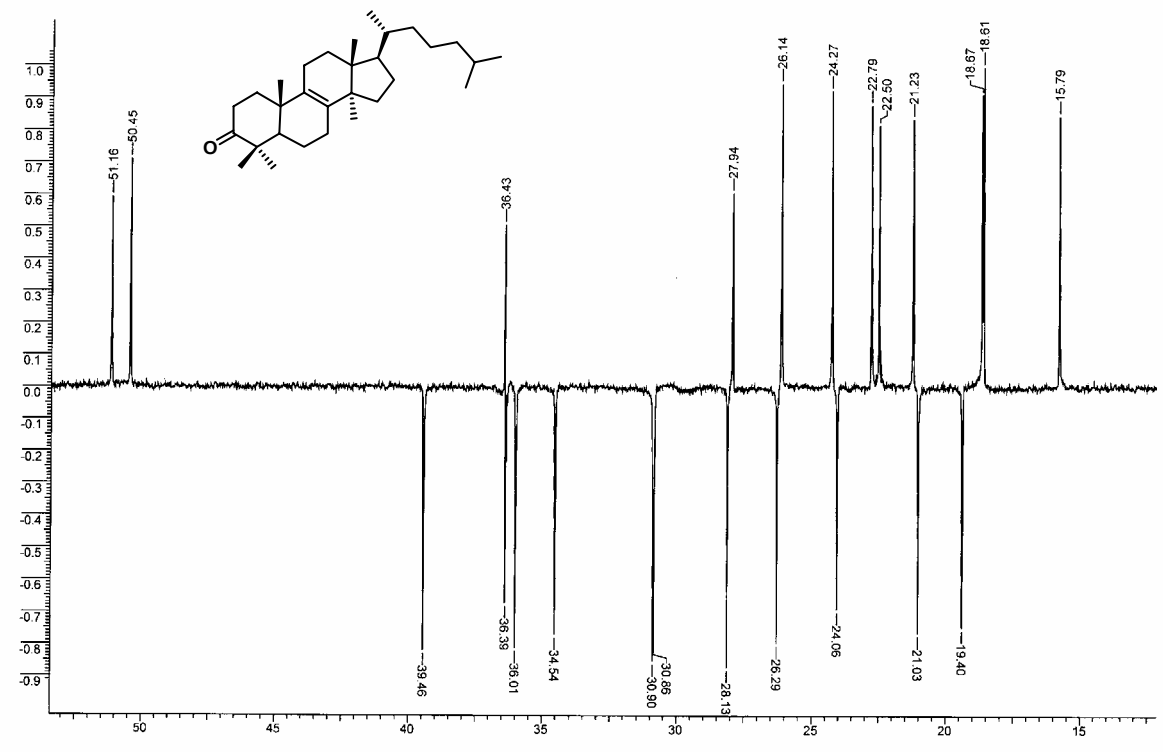
26: *(24,25-dihydro analogue)*: ^{1}H NMR, 300 MHz, $CDCl_3$



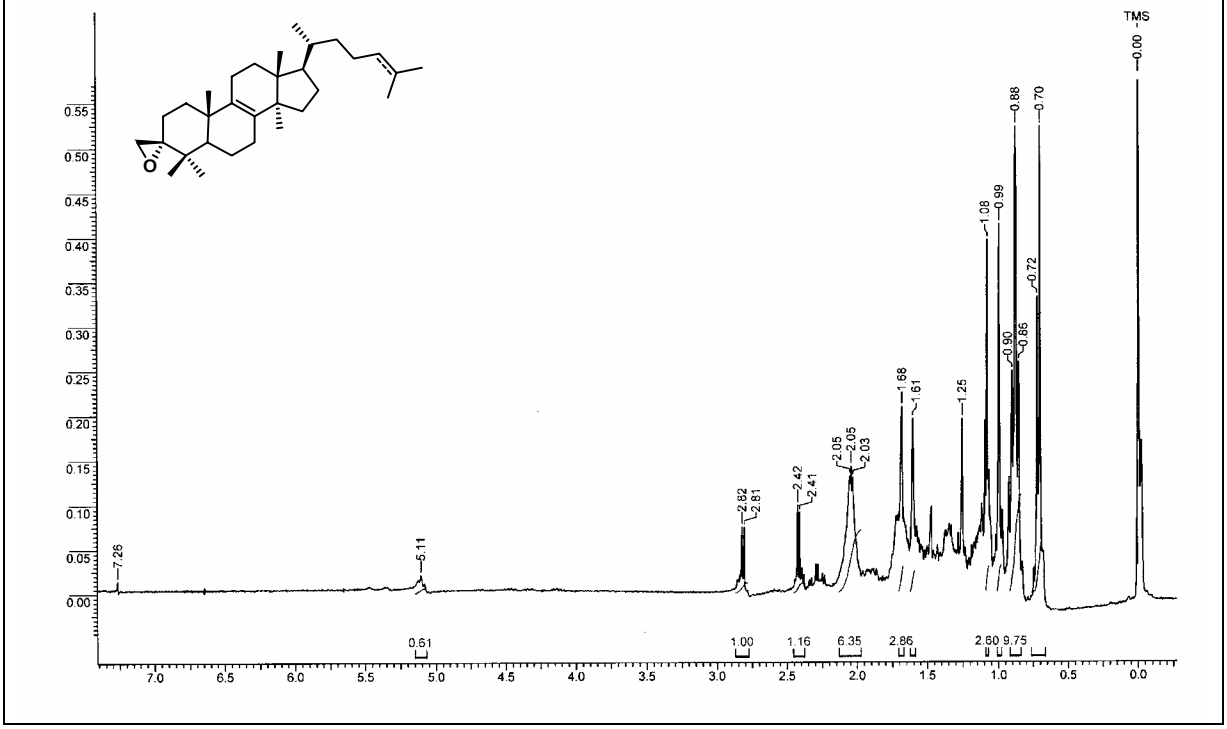
26: *(24,25-dihydro analogue)*: ^{13}C NMR, 75 MHz, $CDCl_3$



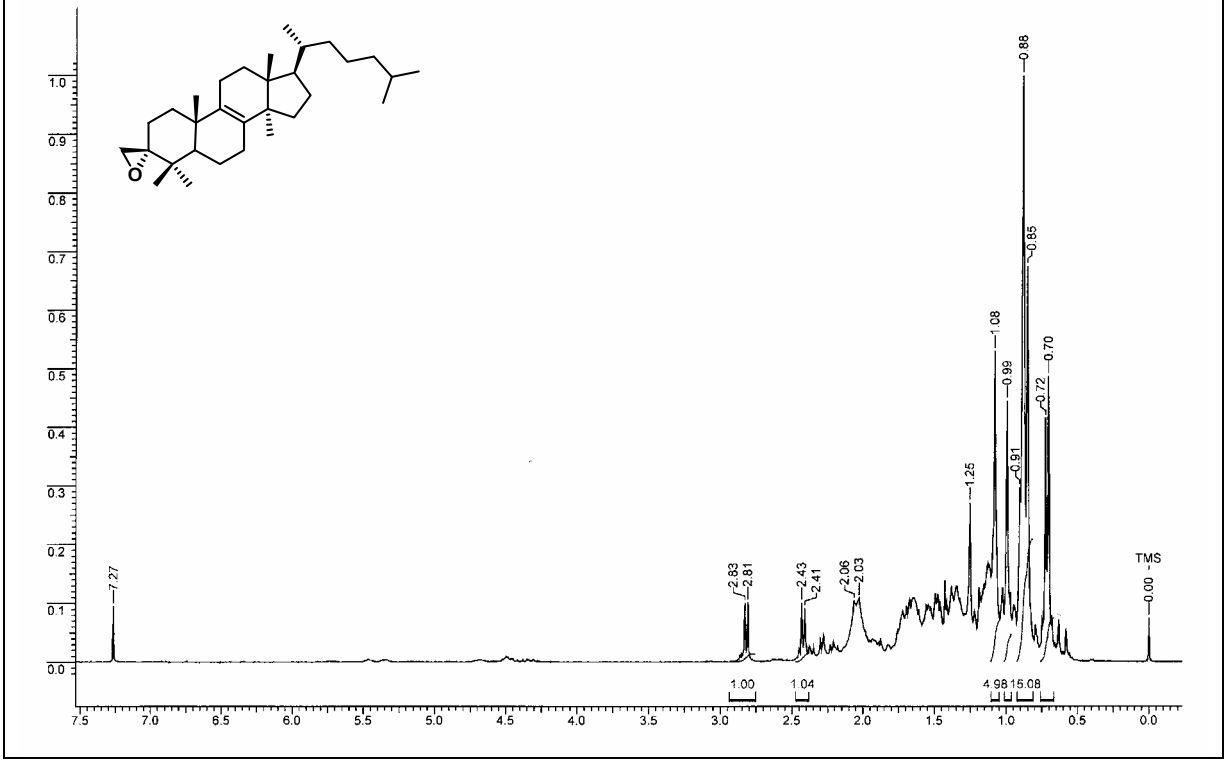
26: *(24,25-dihydro analogue)*: DEPT, 75 MHz, $CDCl_3$



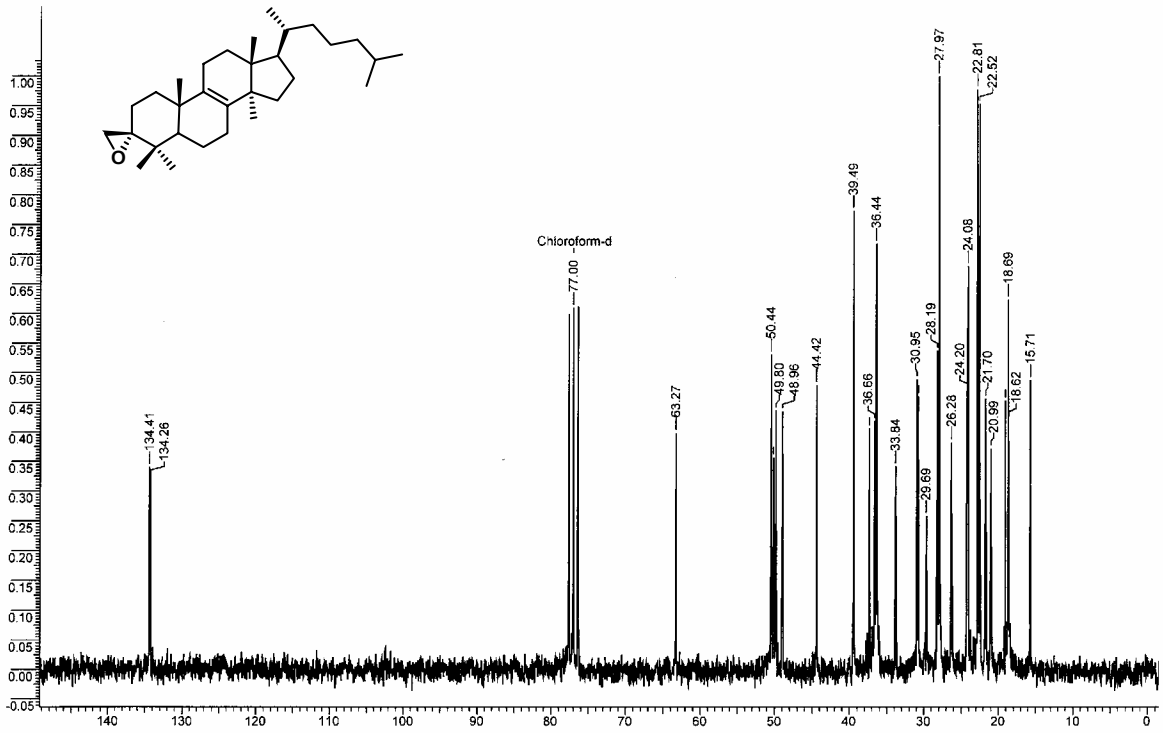
27: ^{1}H NMR, 200 MHz, $CDCl_3$



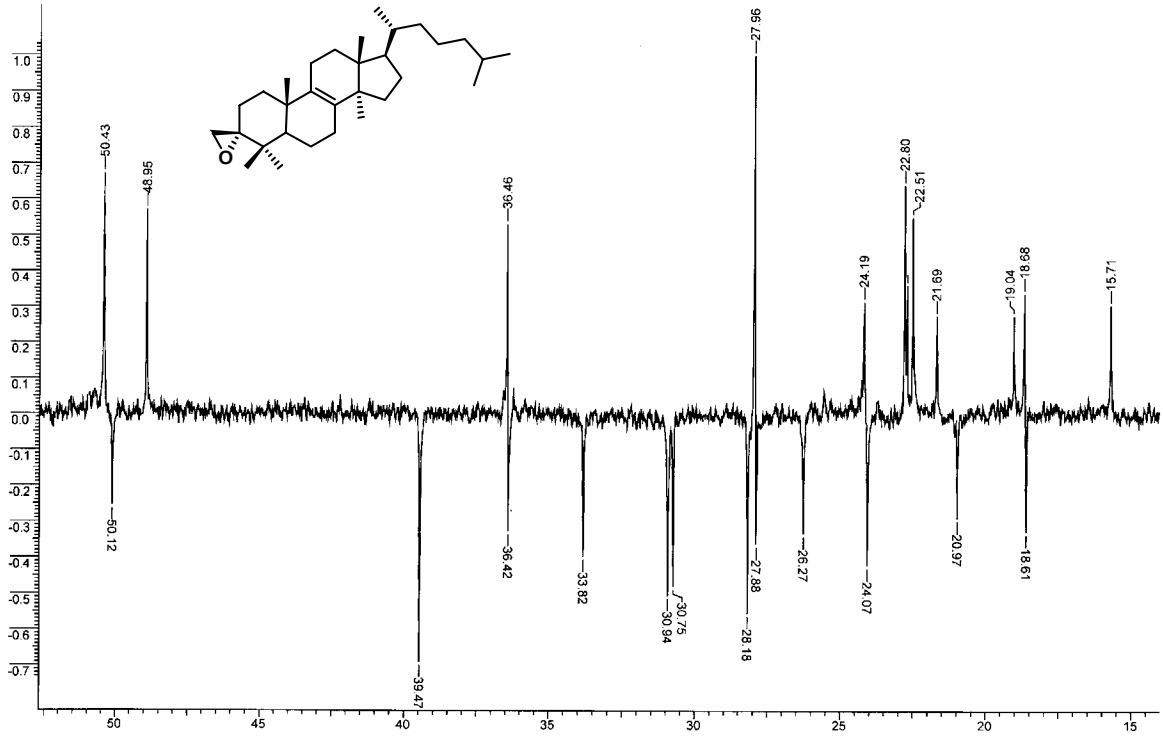
27: *(24,25-dihydro analogue)*: ^{1}H NMR, 200 MHz, $CDCl_3$



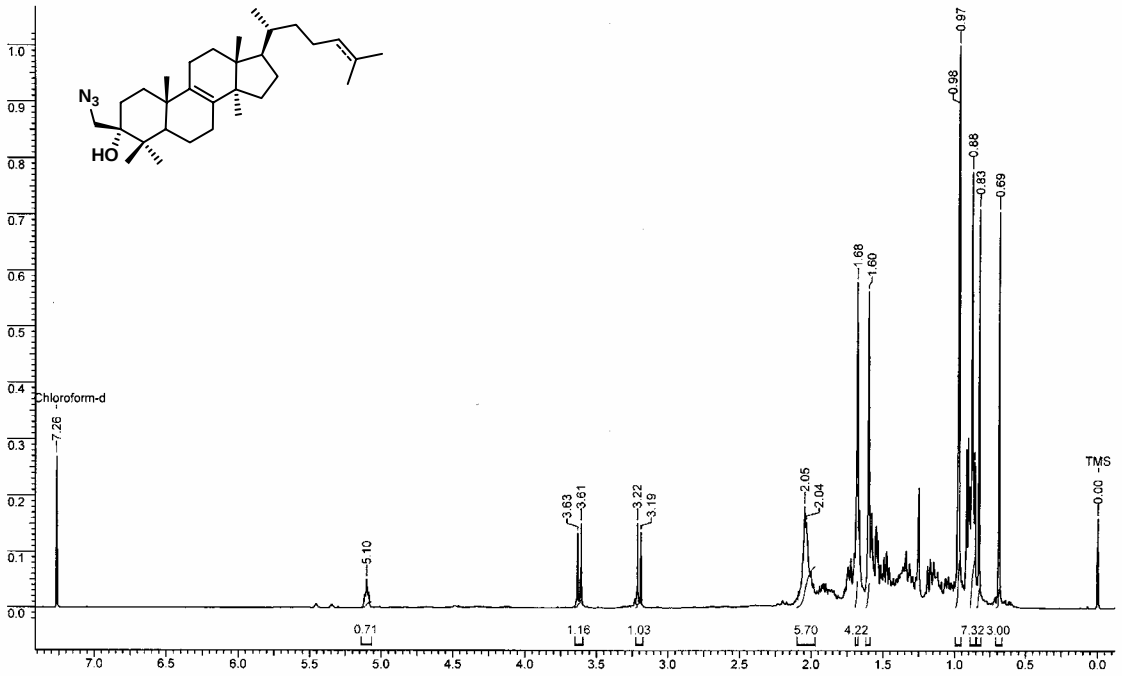
27: *(24,25-dihydro analogue)*: ^{13}C NMR, 50 MHz, $CDCl_3$



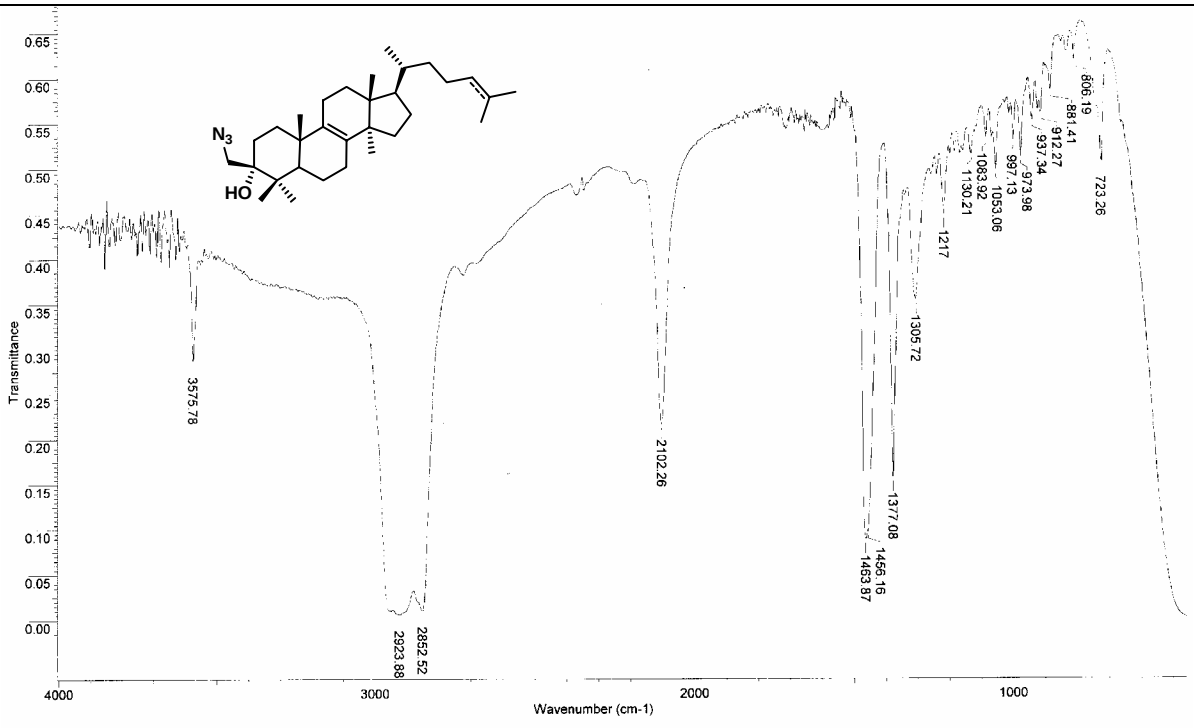
27: *(24,25-dihydro analogue)*: DEPT, 50 MHz, $CDCl_3$



28: ^{1}H NMR, 500 MHz, $CDCl_3$



28: IR



B4.8. References

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LIST OF RESEARCH PUBLICATIONS

1. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S. Bile acid-polyamine conjugates as synthetic ionophores. *Arkivoc* **2003**, *9*, 115-125.
2. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S.; Bhat, M. K.; Nahar, P. B. and Deshpande, M. V. New steroidal dimers with antifungal and antiproliferative activity. *J. Med. Chem.* **2004**, *47*, 1591-1594.
3. **Salunke, D. B.**; Hazra, B. G.; Gonnade, R. G.; Bhadbhade M. M. and Pore, V. S. An efficient method for the synthesis of methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate. *Tetrahedron* **2005**, *61*, 3605-3612.
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6. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S.; Gonnade, R. G.; Bhadbhade M. M. Molecular association *via* halogen bonding and other weak interactions in the crystal structures of 11-bromo-12-oxo-5β-cholan derivatives. *Tetrahedron* **2008** (*Communicated*).
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POSTER PRESENTATIONS

1. Hazra, B. G.; Pore, V. S.; **Salunke, D. B.**; Kurhade, S. H.; Thakur, A. P. Synthesis of new mimics of squalamine. Poster presented at CRSI's Fourth National Symposium in Chemistry, **2002**, Pune, India.
2. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S. Synthesis of squalamine analogues derived from bile acids. Poster presented at CRSI's Fifth National Symposium in Chemistry, **2003**, Chennai, India.
3. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S. New steroidal dimers with antifungal and antiproliferative activity. Poster presented at CRSI's Sixth National Symposium in Chemistry, **2004**, Kanpur, India.
4. Aher, N. G.; **Salunke, D. B.**; Pore, V. S. Design and synthesis of steroid based hybrid antifungal agents. Poster presented at CRSI's Seventh National Symposium in Chemistry, **2005**, Kolkata, India.
5. Aher, N. G.; **Salunke, D. B.**; Hazra, B. G.; Pore, V. S. Design and synthesis of steroid based potential inhibitors of ergosterol biosynthesis as antifungal agents. Poster presented at International Symposium on Advances in Organic Chemistry, INSOC **2006**, Kerala, India.

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1. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S.; Nahar, P. B.; Deshpande, M. V. Bile acid derived steroidal dimers with novel amphiphilic topology having antifungal activity. **2005** US Patent, US 2005222115 A1.
2. **Salunke, D. B.**; Hazra, B. G.; Pore, V. S.; Bhat, M. K. Bile acid derived steroidal dimers with amphiphilic topology having antiproliferative activity. **2006** US 2006003974 A1.

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