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Preparation of Divalent and Trivalent Antigens for Immunogical Studies on Degranulation of Mast Cells and Preparation of Ceragenins for Antiviral Studies Against Vaccinia Virus.

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PREPARATION OF DIVALENT AND TRIVALENT ANTIGENS FOR IMMUNOLOGICAL STUDIES ON DEGRANULATION OF MAST CELLS AND PREPARATION OF CERAGENINS FOR ANTIVIRAL ACTIVITY STUDIES AGAINIST VACCINIA VIRUS

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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List of abbreviation

MIRRs	Multichain immune recognition receptors
Ig	Immunoglobulin
IgE	Immunoglobulin E
FcεRI	Fc ε Receptor I
CTL	Cytotoxic T lymphocyte
DAG	diacylglycerol
IP_3	1,4,5-trisphosphate
BCR	B Cell Receptor
TCR	T Cell Receptor
RBL	Rat Basophilic leukemia
DNP	Dinitrophenyl
Tris	Tris-hydroxymethylaminomethane
DMF	Dimethylformamide
Ph_3P	Triphenylphosphine
EtOAc	Ethyl acetate
DCM	Dichloromethane
THF	Tetrahydrofuran
DMSO	Dimethyl sulfoxide
Boc	*tert*-Butoxycarbonyl
Fmoc	9-Fluorenylmethoxycarbonyl
DCC	Dicyclohexylcarbodiimide
HOBT	Hydroxybenztriazole
EDCl	1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride
DMAP	Dimethylaminopropylamine
AMPs	Antimicrobial Peptides
HBD-3	Human β-defensin 3
VV	Vaccinia Virus
OM	Outer Membrane
LPS	Lipopolysaccharide
EDTA	Ethylenediaminetetraacetic acid
EV	Eczema vaccination
AD	Atopic Dermatitis
Tr	Trityl
MOM	Methoxymethyl
p-TsOH	*p*-Toluenesulfonic acid
MsCl	Methanesulfonyl chloride
TFA	Trifluoroacetic acid

ABSTRACT

PREPARATION OF DIVALENT AND TRIVALENT ANTIGENS FOR IMMUNOLOGICAL STUDIES OF DEGRANULATION OF MAST CELLS AND PREPARATION OF CERAGENINS FOR ANTIVIRAL ACTIVITY STUDIES AGAINIST VACCINIA VIRUS

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CHAPTER 1

Aggregation of receptors for IgE (FcεRI) causes mast cells and basophils to release preformed contents of granules, including histamine and a variety of enzymes. This process, called degranulation plays a central role in allergic reactions. Methods to study this process are to create multivalent ligands which can interact with the receptors and, in turn, lead to aggregation of the receptors. We prepared a series of fluorophore-labeled divalent and trivalent antigens to

study the degranulation of mast cells. Trivalent antigens proved to be much better stimulators for degranulation of mast cells than divalent antigens. These results indicate that aggregates formed by trivalent antigens are more complicated than those of divalent antigens.

CHAPTER 2

Membrane-active antibiotics include antimicrobial peptides (AMPs) and a class of amphiphilic steroids termed ceragenins. Recent studies of membrane-active antibiotics show that cationic, facially amphiphilic molecules could disrupt bacterial membranes. It was found recently that some antibiotics, including AMPs and ceragenins, may share both antibacterial and antiviral activity. We prepared a series of ceragenins to optimize the antiviral activity of ceragenins against vaccinia virus (VV). The results show that ceragenins exhibit potent activity against VV, protect keratinocytes against VV-mediated cell death, and preferentially target the virus. It also shows that antibacterial and antiviral activities do not correlate with each other. Although ceragenins show good antiviral activity against VV, the mechanism for this activity still remains unclear.

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CHAPTER 1

PREPARATION AND IMMUNOLOGICAL STUDIES OF DIVALENT AND TRIVALENT ANTIGENS FOR DEGRANULATION OF MAST CELLS

1.1 Introduction:

Aggregation of cell surface receptors is an important mechanism for initiating signal transduction across cell membranes in many cellular systems.[1] Multichain immune recognition receptors (MIRRs) (Figure 1-1) are found on the surface of many cells in the immune system, including T cells and B cells. One of the most important MIRRs is the high affinity receptor for IgE (FcεRI) that is located on surface of mast cells and basophils. Aggregation of the receptors for IgE (FcεRI) causes mast cells and basophils to release preformed contents of granules, including histamine and a variety of enzymes. This process, termed degranulation plays a central role in allergic reactions.[2,3] When multivalent antigens crosslink IgE antibodies bound to FcεRI on the cell surface, aggregation of these receptors occurs, resulting in a signal transduction that lead to degranulation. The signaling pathway of aggregation is not very clear. The most accepted explanation is that phosphorylation of tyrosines may be a proximal event in the signal transduction pathway. The methods to study this process are to create multivalent ligands which can interact with the receptors and, in turn, lead to aggregation of the receptors.

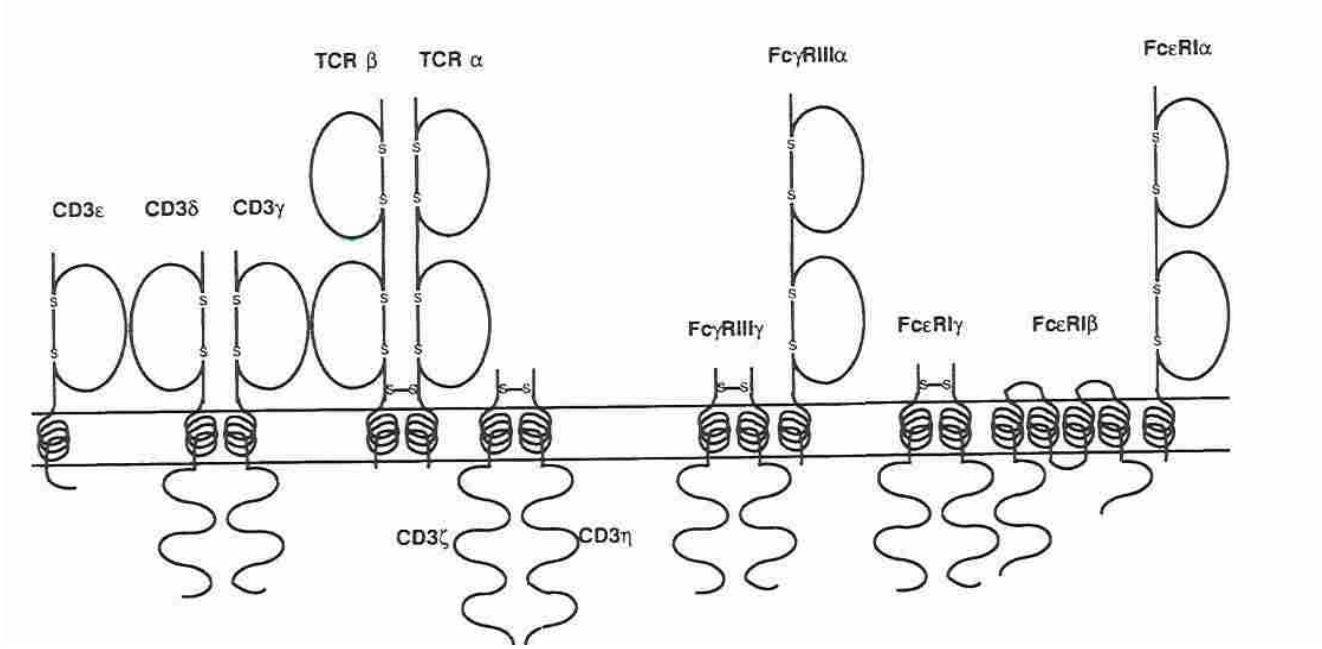


Figure 1-1. The structures of MIRRs. Adapted from ref. 2.

1.1.1 Transmembrane signaling: Aggregation of receptors on the surface of cells

About 80 years ago, Karl Landsteiner found that simple polyvalent silver could trigger allergic reactions whereas monovalent antigens ("haptens") were ineffective.[4] Further studies indicated that the reactions he found were initiated by transmembrane signaling of the IgE receptors (FcεRI) on the surface of mast cells. MIRRs are found on T and B cells, the classical mediators of the immune system. They are also found on mast cells and basophils where they bind with the Fc portion of immunoglobulins.[5] The T-cell receptor, the B-cell receptor and FcεRI are quite similar in structure. They consist of antigen- or Fc-binding chains which known as extracellular domains, transmembrane domains and cytoplasmic domains (Figure 1-2). The typical antibody includes three parts: Fab regions, hinge and Fc regions (Figure 1-2). A ligand can bind with the Fab

region of the antibody or the extracellular domain of the receptor which can stimulate a biochemical cascade through the Fc region and cytoplasmic domain.

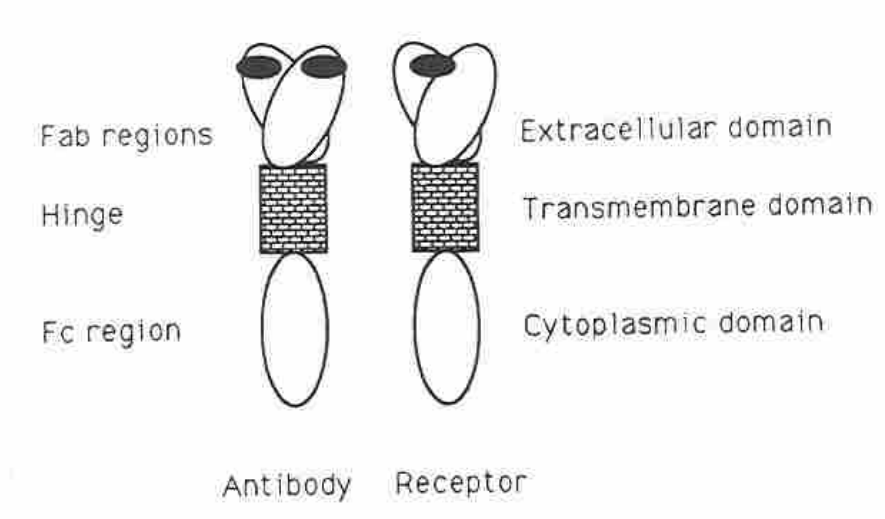


Figure 1-2. Comparative aspects of an antibody and a membrane receptor. Adapted from ref. 1.

How does silver interacting with the Fab region of an antibody stimulate biological responses that result from interaction between the Fc portion of the antibody and other components? One explanation is that there is an allosteric conformational change; however, it was difficult to reconcile this idea with what was known about the structure of antibodies as well as with functional data.[6,7] Further studies showed that the role of the silver is to aggregate the antibody.[8,9,10,11] The ligand-induced aggregation of receptors (Figure 1-3) which stimulates an immune response was also found in many other cellular systems, including T and B cells. The idea of molecular aggregation as a fundamental mechanism by which biological systems control metabolic pathways was accepted after many studies.[1]

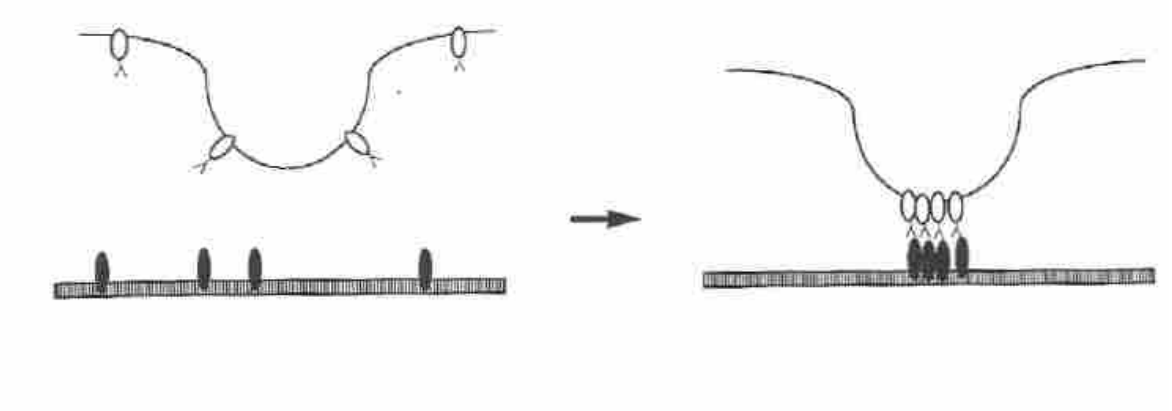


Figure 1-3. Typical ligand-induced aggregation of receptors. Adapted from ref. 1.

MIRRs are similar not only in their structure but also in their signal transduction pathways. Aggregation of FcεRI causes mast cells and basophils to release preformed contents of granules, including histamine, 5-hydroxytryptamine, and a variety of enzymes.[5] This process is called degranulation, and plays a central role in allergic reactions (Figure 1-4). However, this process is not limited to FcεRI-bearing cells. The degranulation of both cytotoxic T lymphocytes (CTL) and NK cells is also the result of receptor aggregation.[12,13] It has been known for some time that aggregation of MIRRs results in a rise in intracellular free Ca^{2+} concentration. It has also been known that aggregation results in the hydrolysis of phosphatidylinositols, leading to the generation of diacylglycerol (DAG) and inosito-1,4,5-trisphosphate (IP_3). More recently, it has become clear that the activation of tyrosine kinases occurs quickly after MIRRs aggregation. It may be a very proximal event in signal transduction. In each cell type that expresses MIRRs, aggregation of the receptors has been shown to cause rapid tyrosine phosphorylation of substrates, including components of the MIRRs.

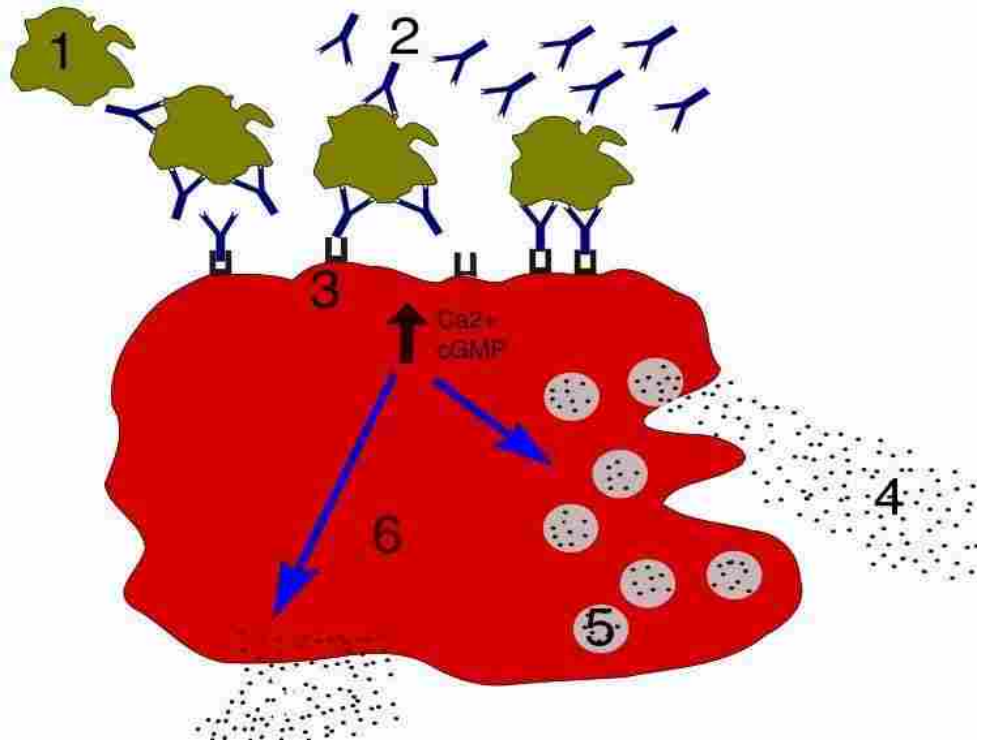


Figure 1-4. Activation of mast cell degranulation by IgE interaction with FcεRI. 1 = antigen; 2 = IgE; 3 = FcεRI; 4 = preformed mediators (histamine, proteases, chemokines, heparin); 5 = granules; 6 = mast cell. Adapted from Wikipedia online.

1.1.2 Antigen-mediated IgE receptor (FcεRI) aggregation and signaling on cell surface

Cell surface receptors in the immune system have evolved to respond to foreign substances for host defense. These foreign substances (antigens) have millions of different structures that must be recognized with high discrimination. Three types of immunoreceptors become activated when they are aggregated on the cell surface by ligands that bind two or more receptors simultaneously. First, B lymphocytes respond to multivalent soluble antigens or to bivalent anti-Ig antibodies that bind the surface Ig of the B-cell-receptor (BCR) complex.[14,15] Second, T-cell receptors recognize small peptides that are bound in the clefts of major histocompatability complex proteins when these peptides are presented

on the surfaces of other cells.[16]

The third type of immunoreceptor that mediates responses to foreign antigens is the Fc receptor for soluble Ig. A specific Fc receptor binds to the constant Fc segment of a particular Ig class (or subclass); the variable domains in each of the two Ig Fab segments provide specific antigen recognition. A particularly well-characterized Fc receptor is FcεRI, the high-affinity receptor for IgE (Figure 1-1 and Figure 1-5A) which is found on mast cells and basophils.[17] Among the immunoreceptors, the necessity of cross-linking for functional activation has been firmly established for FcεRI.[1]

Many factors influence the signals generated by FcεRI aggregation, including the size of aggregates,[18] the spacing of receptors in aggregates,[19] and the time that individual receptors spend within a complex.[20] Early studies indicated that small, symmetric, divalent ligands are sufficient to trigger the degranulation of mast cells. IgE has a binding site for haptens at the end of each of its two Fab segments; however, divalent ligands can make a large variety of complexes, including different lengths of linearly cross-linked IgE- FcεRI and cyclized complexes (Figure 1-5B). There can also be monomeric complexes in which the bivalent ligand binds simultaneously to both sites of IgE. In an initial study, Segal et al[21] found that divalent antigens could form large aggregates in stimulating degranulation of mast cells. In a subsequent study, however, Fewtrell and Metzger[22] showed that highly divalent antigens stimulated the rat basophilic leukemia (RBL) cells poorly, whereas trivalent antigens were

effective. Evidence shows that the aggregates generated by divalent antigens are predominantly in the form of cyclic dimers. The study of divalent antigens binding with receptors is limited because of their inability to activate strong cellular responses. Trivalent antigens are predicted to form more complicated aggregates compared to divalent antigens (Figure 1-5B). The highly branched aggregates formed by trivalent antigens lead to strong responses by IgE-FcεRI presenting cells (Figure 1-5C). Recently, Whitesides and co-workers[23] reported the preparation of a trivalent antigen capable of aggregating IgE. They found that aggregates formed with stoichiometries of 3:2 (IgE/trivalent ligand).

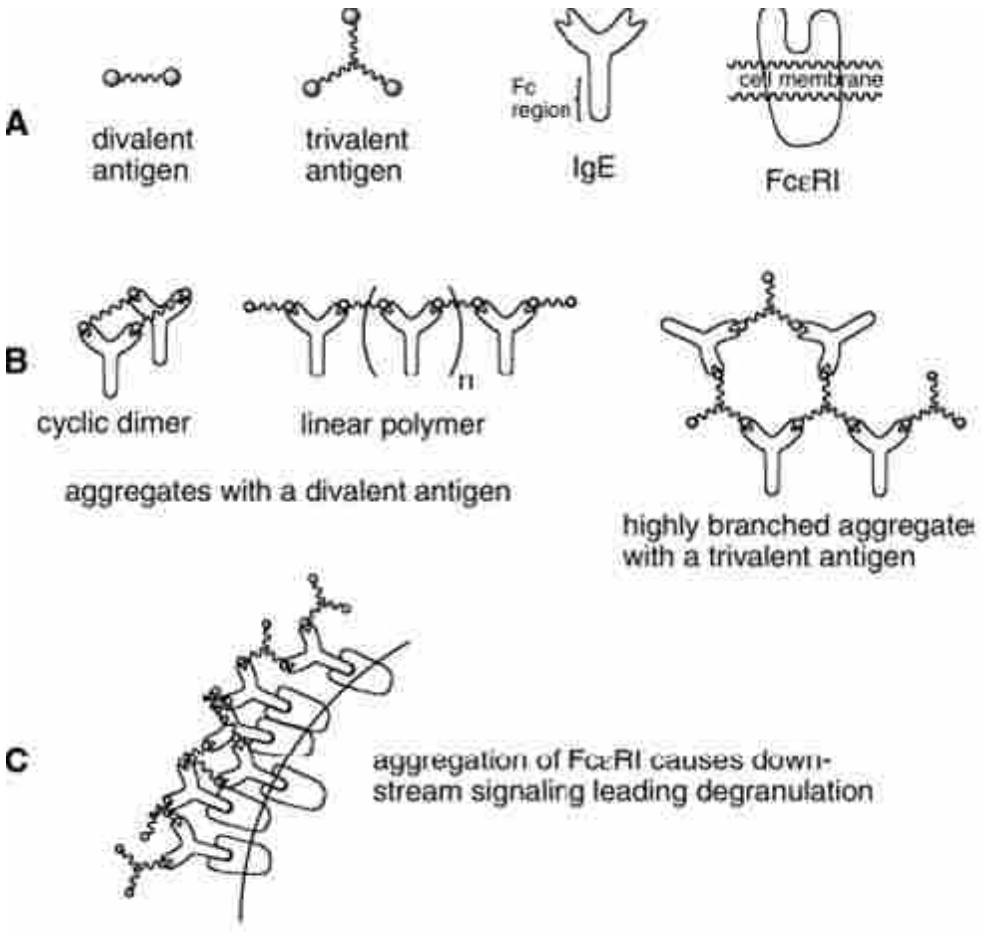


Figure 1-5. (A) Schematic representation of ligands, antibodies and receptors involved in degranulation; (B) IgE aggregates formed with di- and trivalent antigens; (C) Aggregation of FcεRI leading to degranulation. Adapted from 28.

In the activation process one interesting phenomenon is that the cellular response is rapidly halted with the addition of a large excess of monovalent hapten when multivalent antigen is used to aggregate IgE-FcεRI complexes. Two interpretations of the monovalent ligand effect are consistent with these results. First, the population of cross-linked IgE-FcεRI that is responsible for the functional response could represent a very small subset that is readily disaggregated by the addition of monovalent ligand. Second, activation could be maintained by ongoing formation of transiently active FcεRI. Both interpretations were supported by different evidence.[24]

As mentioned in the previous section, biochemical studies have shown that aggregation of FcεRI, TCR, and BCR activate a tyrosine kinase cascade. Pribluda and Metzger[25] found that aggregation-dependent tyrosine phosphorylation of the FcεRI β and γ subunits can be obtained with broken cell preparations but that phosphorylation of other cellular substrates is largely absent. Although the importance of tyrosine kinase activation in initiating such cellular responses such as degranulation is now well established, the biochemical results in this process and the participation of membrane structure are only partially understood.

1.2 Design of the trivalent and divalent antigens

IgE targets a nearly limitless variety of antigens in allergic responses. Dinitrophenyl (DNP) groups have been used to interact with IgE in previous studies because the DNP antibodies are readily available. In this study, we also

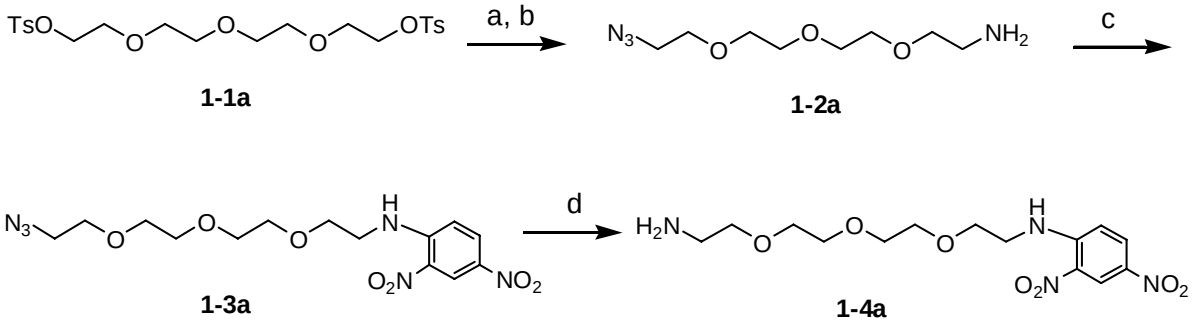
used the DNP groups as the binding sites interacting with the Fab domain of IgE. The length between DNP groups and core site was also considered. In earlier work in our group, we found that when separation of antigenic epitopes is more than 40 Å, it is enough to allow efficient binding to IgE. So we selected tetra- and pentaethylene glycol as the branches of these multivalent antigens, which are predicted a maximum separation of 43.3 and 49.5 Å (MMFF with Spartan). Tris-hydroxymethylaminomethane (Tris) and serinol were used as the core to generate the trivalent and divalent antigens.[26,27] In addition, the amine group in Tris and serinol provides a reactive group for attachment of fluorophores.

1.3 Results[28]

1.3.1 Synthesis of trivalent antigens

Compound **1-4a** is one of the dinitrophenyl-appended glycol linkers, and the synthesis of it is shown in Scheme 1-1. Compound **1-1a** was converted to diazide compound, followed by selective reduction of one azide group giving **1-2a**. The amine group of **1-2a** then reacted with 2,4-dinitrofluorobenzene affording compound **1-3a**. Reduction of the azide group of **1-3a** with triphenylphosphine gave **1-4a**.

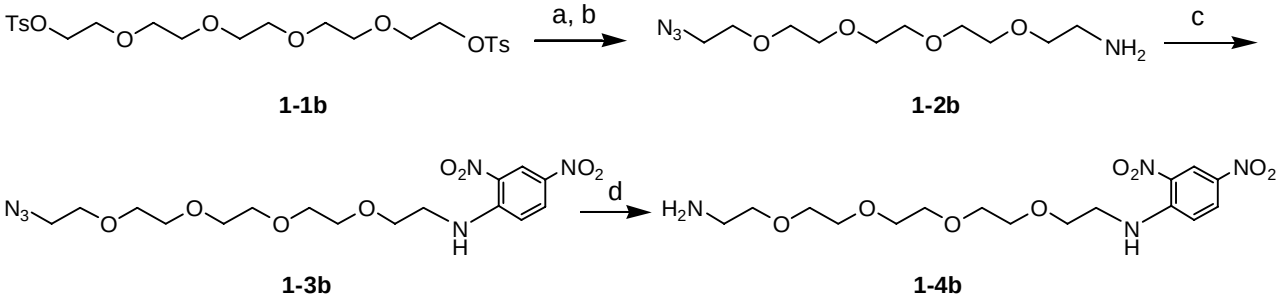
Scheme 1-1. Synthesis of **1-4a**



Reagents (yields in parentheses): a) NaN_3, DMF/H_2O (95%); b) Ph_3P, EtOAc/HCl/H_2O (70%); c) 2, 4-dinitrofluorobenzene, CH_2Cl_2 (85%); d) Ph_3P, EtOAc/Et_2O/HCl (70%).

The structure of compound **1-4b** is similar to **1-4a** and the procedure to make **1-4b** is also very similar to compound **1-4a**, which is started from **1-1b**. The synthesis of **1-4b** is shown in Scheme 1-2.

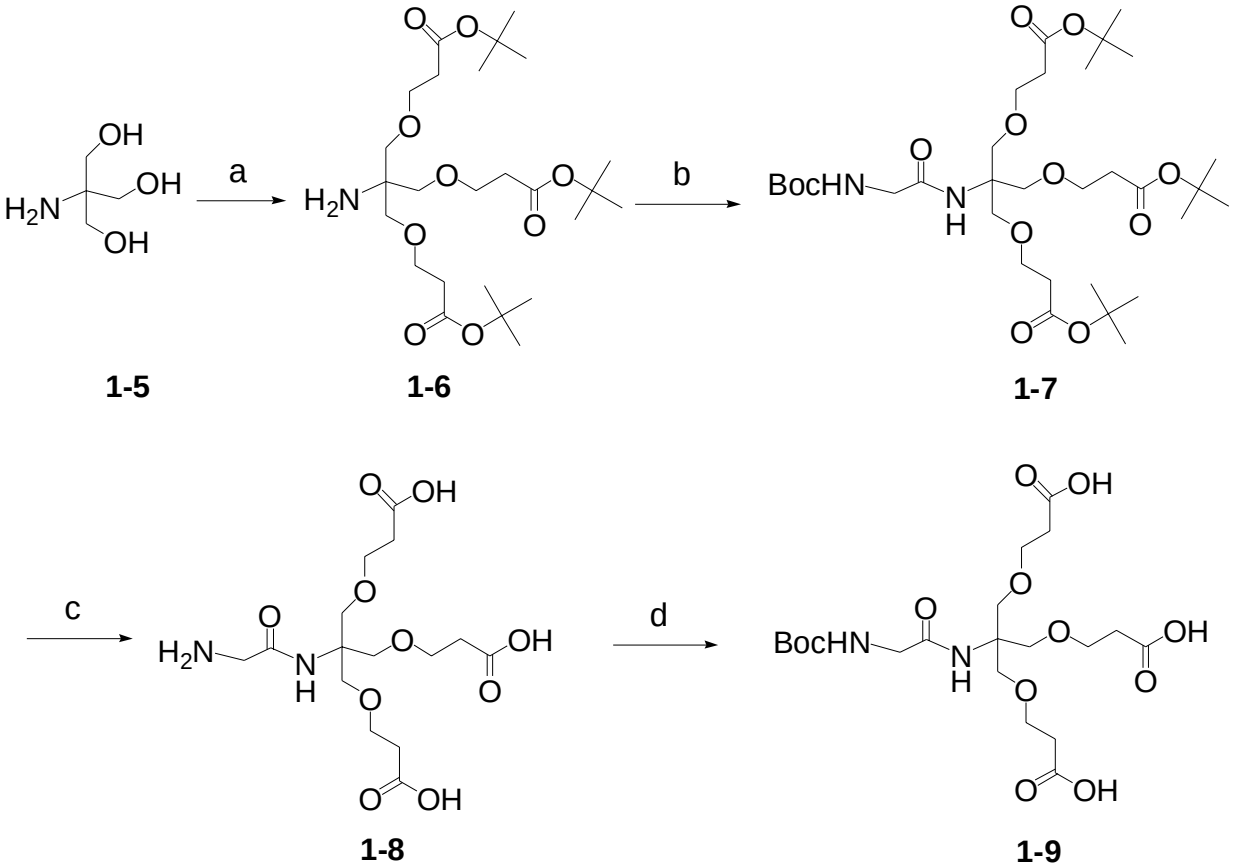
Scheme 1-2. Synthesis of **1-4b**



Reagents (yields in parentheses): a) NaN_3, DMF/H_2O (95%); b) Ph_3P, EtOAc/HCl/H_2O (70%); c) 2, 4-dinitrofluorobenzene, CH_2Cl_2 (85%); d) Ph_3P, EtOAc/Et_2O/HCl (70%).

Compound **1-9** is an important intermediate for making the trivalent antigens. The synthesis of it is shown in Scheme 1-3. Tris-hydroxymethylaminomethane (Tris) **1-5** reacted with *tert*-butyl acrylate under basic condition giving compound **1-6**. The free amine group of **1-6** coupled with Boc-glycine giving **1-7**. Treatment of **1-7** with formic acid gave the deprotected compound **1-8**. The free amine of **1-8** then was protected with Boc group giving **1-9**. Unfortunately, when compound **1-9** was concentrated, it decomposed to the free amine again. The reason may be result from the three carboxylic acid groups which increase the acidity of the system. So, a different strategy was used to get another similar key intermediate **1-10**.

Scheme 1-3. Attempted synthesis of **1-9**



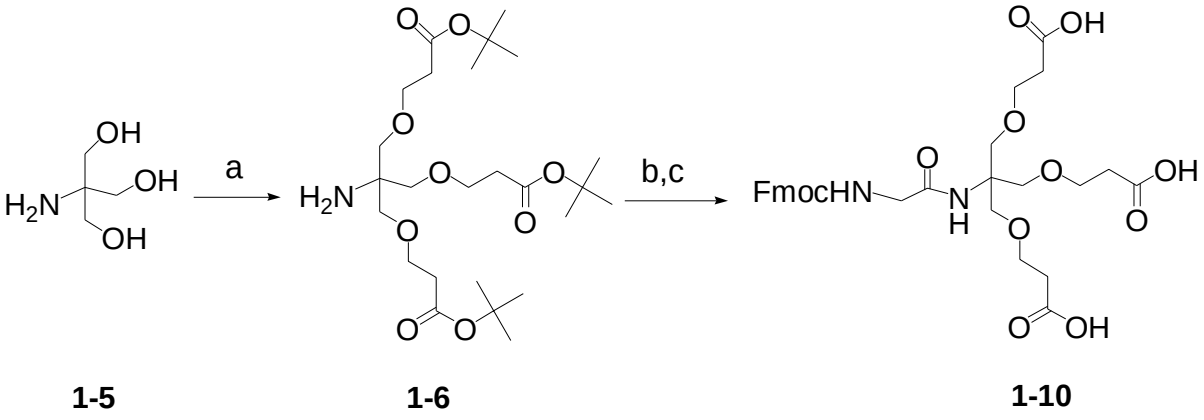
1-5 **1-6** **1-7**

1-8 **1-9**

Reagents (yields in parentheses): a) *tert*-butyl acrylate, NaOH, DMSO/H_2O (50%); b) Boc-glycine, DCC, CH_2Cl_2 (85%); c) HCOOH (95%); d) Boc_2O, dioxane/H_2O/NaOH.

Compound **1-10** was prepared using different procedure compared to **1-9**, which is shown in Scheme 1-4. In this procedure, the Boc-glycine was replaced by Fmoc-glycine to do the coupling reaction. The free amine group of compound **1-6** coupled with Fmoc-glycine, followed by deprotection of the ester group with formic acid giving compound **1-10**, which is the key intermediate for making trivalent antigens.

Scheme 1-4. Synthesis of **1-10**

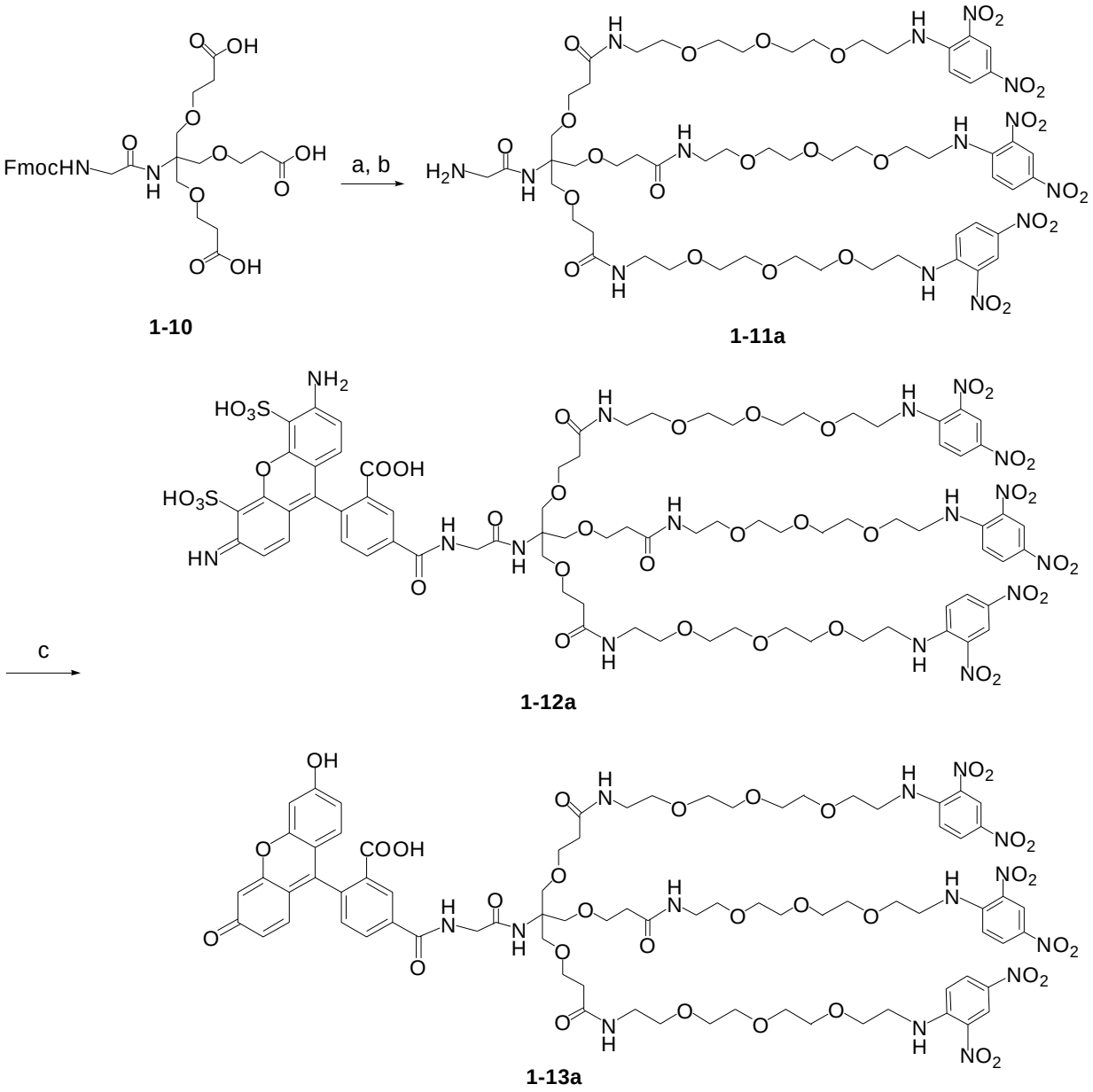


1-5 **1-6** **1-10**

Reagents (yields in parentheses): a) *tert*-butyl acrylate, NaOH, DMSO/H_2O (50%); b) Fmoc-glycine, DCC, CH_2Cl_2 (82%); c) HCOOH (95%).

With compound **1-10** in hand, fluorophore-labeled trivalent antigens were prepared. The synthesis of compound **1-12a** and **1-13a** is shown in Scheme 1-5. The coupling reaction was carried out between compound **1-10** and **1-4a**, followed by deprotection of Fmoc group giving compound **1-11a**. Reaction of **1-11a** with Alexa-488 succinimidyl ester or 5-(and-6)-carboxyfluorescein succinimidyl easter gave the fluorophore-labeled trivalent antigens **1-12a** and **1-13a**.

Scheme 1-5. Synthesis of **1-12a** and **1-13a**

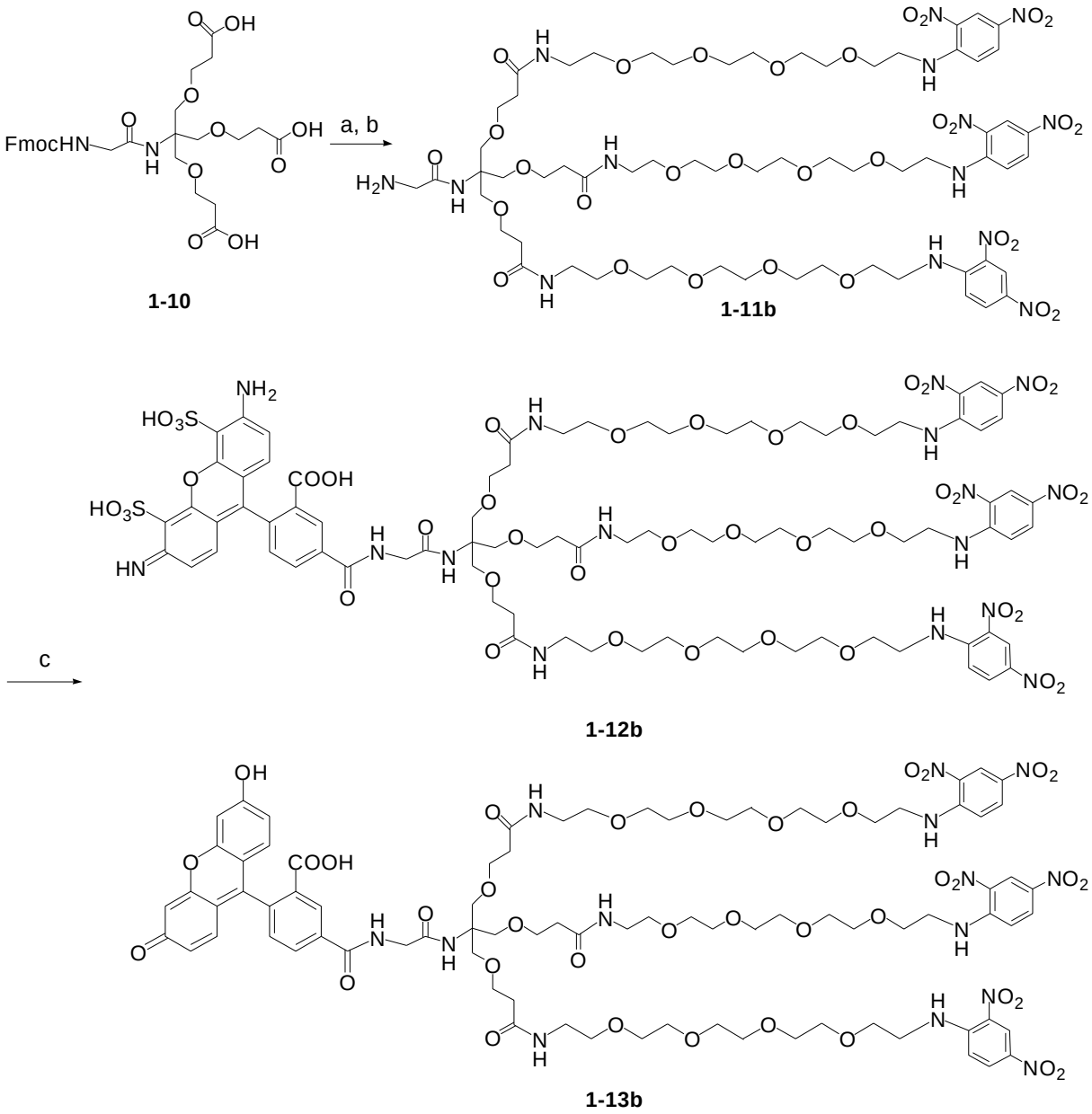


1-10 **1-11a** **1-12a** **1-13a**

Reagents (yields in parentheses): a) **1-4a**, HOBT, Et_3N, and EDCl, THF (60%); b) piperidine (90%); c) Alexa-488 succinimidyl easter or 5-(and-6)-carboxyfluorescein succinimidyl easter, DMAP, DMF (50%).

Fluorophore-labeled trivalent antigens **1-12b** and **1-13b** were prepared, whose structures are similar to **1-12a** and **1-13a**. The synthesis of **1-12b** and **1-13b** also started with compound **1-10** (Scheme 1-6). The coupling reaction between compound **1-10** and **1-4b**, followed by deprotection of Fmoc group gave compound **1-11b**. Reaction of **1-11b** with succinimidyl ester of Alexa-488 or fluorescein gave the labeled trivalent antigens **1-12b** and **1-13b**.

Scheme 1-6. Synthesis of **1-12b** and **1-13b**

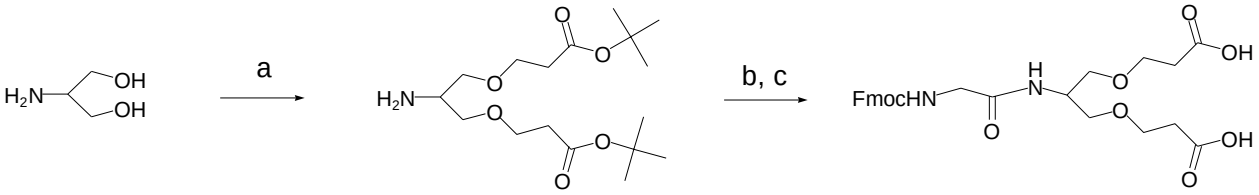


Reagents (yields in parentheses): a) **1-4b**, HOBT, Et_3N, and EDCl, THF (60%); b) piperidine (90%); c) Alexa-488 succinimidyl easter or 5-(and-6)-carboxyfluorescein succinimidyl easter, DMAP, DMF (50%).

1.3.2 Synthesis of divalent antigens

Compound **1-16** is the key intermediate for making the divalent antigens. The synthesis of it is shown in Scheme 1-7. Serinol **1-14** reacted with *tert*-butyl acrylate giving **1-15**. Coupling reaction between **1-15** and Fmoc-glycine followed by deprotection with formic acid afforded compound **1-16**.

Scheme 1-7. Synthesis of **1-16**

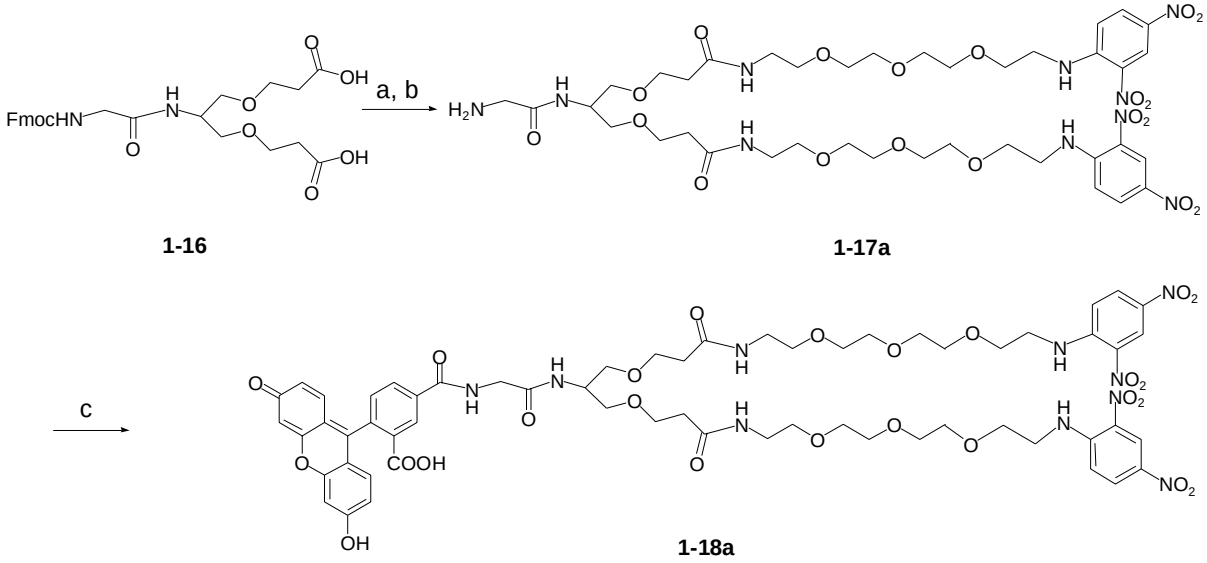


1-14 **1-15** **1-16**

Reagents (yields in parentheses): a) *tert*-butyl acrylate, NaOH, DMSO/H_2O (50%); b) Fmoc-glycine, DCC, CH_2Cl_2 (82%); c) HCOOH (95%).

Compound **1-18a** is a fluorophore-labeled divalent antigen, and the synthesis of it is shown in Scheme 1-8. The coupling reaction was carried out between compound **1-16** and **1-4a**, followed by deprotection of Fmoc group giving compound **1-17a**. Reaction of **1-17a** with succinimidyl ester of fluorescein gave the fluorophore-labeled divalent antigen **1-18a**.

Scheme 1-8. Synthesis of **1-18a**



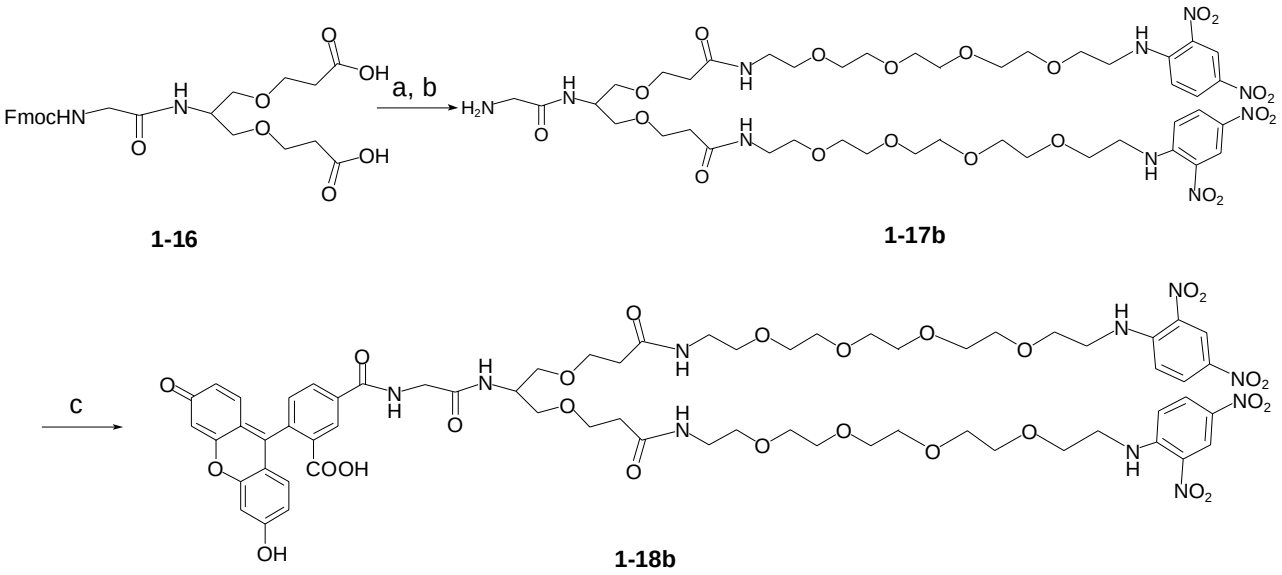
1-16 **1-17a** **1-18a**

Reagents (yields in parentheses) a) **1-4a**, HOBT, Et_3N, and EDCl, THF (65%); b) piperidine (90%); c) 5-(and-6)-carboxyfluorescein succinimidyl easter, DMAP, DMF (50%).

Fluorophore-labeled divalent antigen **1-18b** was prepared, whose structure is similar to **1-18a**. The synthesis of **1-18b** was also started with compound **1-16** (Scheme 1-9). The coupling reaction between compound **1-16** and **1-4b**, followed by deprotection of Fmoc group gave compound **1-17b**. Reaction of

1-17b with succinimidyl ester of fluorescein gave the fluorophore-labeled divalent antigen **1-18b**.

Scheme 1-9. Synthesis of **1-18b**



Reagents (yields in parentheses) a) **1-4b**, HOBT, Et_3N, and EDCl, THF (65%); b) piperidine (90%); c) 5-(and-6)-carboxyfluorescein succinimidyl easter, DMAP, DMF (50%)

1.3.3 The ability of tri- and divalent antigens to stimulate degranulation

This work was done by Seth Haymore and James Bogert in Dr. Richard G. Posner group at Northern Arizona University and Israel Pecht and Arieh Licht in Weizmann Institute of Science.

1.3.3.1 Materials for degranulation assays

Mouse monoclonal anti-DNP–IgE obtained from hybridoma H1 26.8 was purified with ion exchange chromatography and gel filtration. RBL-2H3 cells were grown in 75 cm^2 flasks at 37 °C and 5% CO_2 for 5 days. Cell Media included MEM 1× with Earle's salts without glutamine (Gibco-BRL), 20% fetal bovine serum (Hyclone), 1% (v/v) glutamine (Gibco-BRL) and 1% (v/v) penicillin and streptomycin (Gibco-BRL). After harvesting by first rinsing with trypsin–EDTA (Gibco-BRL), cells were incubated with trypsin–EDTA for 5

minutes at 37°C. Cells in culture were incubated with 10 μg of anti-DNP FITC–IgE at 37°C. After 12 hours, the cells were harvested and then resuspended in buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% gelatin, 20 mM Hepes, pH 7.4).

1.3.3.2 Degranulation assays

The degranulation of RBL cells was measured by the activity of the granule-stored enzyme β-hexosaminidase secreted into the supernatant. RBL-2H3 cells (1 x 105 cells/100 mL/well), which were suspended in medium containing saturating concentrations of DNP-specific IgE, were plated in 96-well plates. After 2 hours in the incubator, cells were washed with Tyrode's buffer three times and then exposed to the indicated concentration of ligand. After transferring twenty microliters of the supernatant to a new 96-well plate, it was then treated with 50 mL substrate solution (*p*-nitrophenyl-*N*-acethyl-β-D-glucosamine, 1.3 mg/mL, in 0.1 M citrate, pH 4.5). The mixture was kept at 37 °C for 1 hour before terminating by addition of 'stop' solution (150 mL; 0.2 M glycine, pH 10.7). The intensity of the yellow color formed at this pH is proportional to the concentration of the secreted enzyme because of the nitrophenol produced by the enzymatic hydrolysis of the substrate. The optical density of the color intensity was measured at 405 nm in an ELISA plate reader. All assays were repeated at least four times and their results are expressed as net percent of the cells' total enzyme activity contents ±5 to 10%.The results are shown in Figure 1-6.

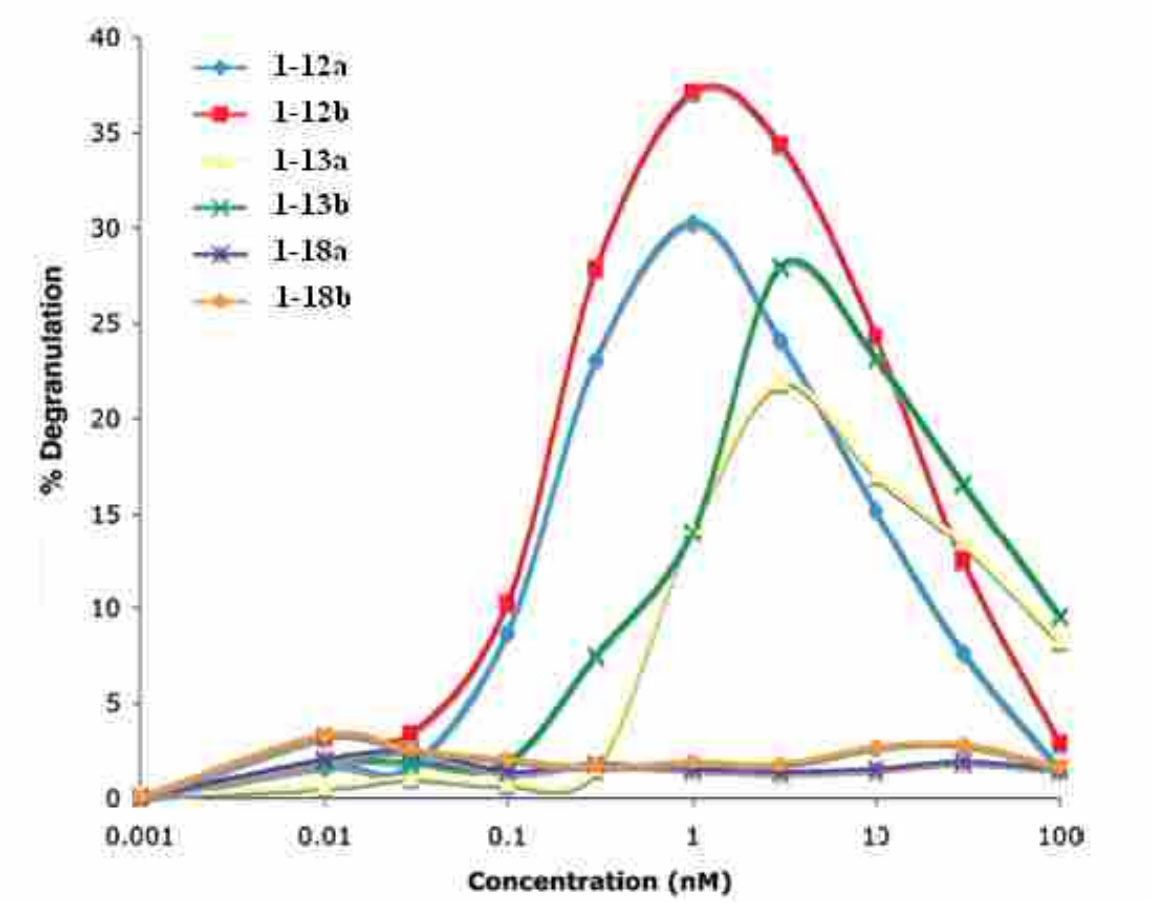


Figure 1-6. Degranulation of RBL cells in response to di- and trivalent antigens.

1.4 Discussion

It was shown that trivalent antigens **1-12a**, **1-12b**, **1-13a**, and **1-13b** all have potent ability to stimulate degranulation of RBL cells below the nM level of concentration of the antigens. The ability in stimulation between the trivalent antigens labeled with Alexa-488 and those labeled with fluorescein is different. The difference may be caused by the different solubility between Alexa-488 and fluorescein. The higher solubility of Alexa-488 may decrease self-aggregation, which in turn results in better stimulation for degranulation. In addition, the tether lengths also affect the ability of stimulating degranulation.

The antigens with longer tether lengths showed greater degranulation ability than those with shorter tether lengths.

It is notable that the curve in Figure 1-6 is bell-shaped. When the concentration of trivalent antigens increases, the ability of trivalent antigens for stimulating degranulation increases to a maximum value, and then it drops off after the optimal antigen concentration. The reason for this phenomenon is that the monovalent binding begins to predominate when the ligand is in excess. So it can be concluded that the nature of IgE aggregates is concentration dependent.

The divalent antigens **1-18a** and **1-18b** show no degranulation over the concentration range shown in Figure 1-6. Considering the structures of the divalent and trivalent antigens, the binding affinities and the space between their dinitrophenylamine groups and the IgE binding sites should be similar. The only difference between these divalent and trivalent antigens is that they have different ability for cross-linking the receptors. The aggregation formed by the trivalent antigens is much more complicated than those formed by divalent antigens, which means the trivalent antigens own much greater ability for stimulating degranulation than the divalent antigens. Therefore, in this concentration range at which the trivalent antigens begin to stimulate degranulation, it is apparent that the divalent antigens show no signals of degranulation.

1.5 Conclusion

A series of fluorophore-labeled divalent and trivalent antigens for degranulation of mast cells were prepared successfully. Trivalent antigens proved to be very good stimulators for degranulation of RBL cells. Divalent antigens did not show notable results for degranulation, which indicated that trivalent antigens had better ability for aggregation of receptors than those of divalent antigens. These small, potent, fluorophore-labeled antigens will facilitate study of receptor aggregation on cell surfaces and signaling pathway.

1.6 Experimental Section

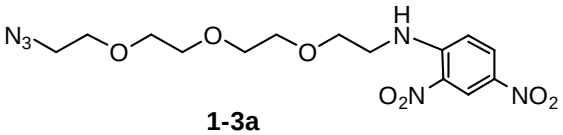
General: Reagents were purchased from Aldrich Chemical Co. unless otherwise noted. Methylene chloride, THF, DMF and DMSO were dried by passage through a Glass Contour solvent drying system containing a cylinder of activated alumina. Silica gel was used for chromatography unless otherwise noted.

Instrumentation: ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 500 (500 MHz) spectrometer. Proton chemical shift were referenced to tetramethylsilane (TMS) and *d*-DMSO. Carbon chemical shifts were referenced to carbon resonance of solvents ($CDCl_3$ and *d*-DMSO). High resolution electron impact mass spectra (HR-MS) were obtained on a JOEL SX 102A spectrometer.

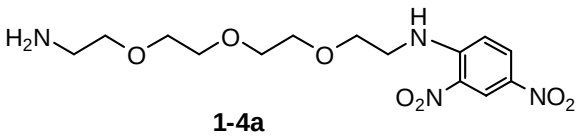
Experiments:

Compound 1-3a: Compound **1-2a** (0.24 g, 1.1 mmol) and 2,4-dinitrofluorobenzene (0.32 g, 1.7 mmol) were dissolved in CH_2Cl_2 (20 mL) and stirred for 20 h at 40℃. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:1 EtOAc/hexane) to yield **1-3a** (0.36 g, 85%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.05 (d, J = 2.5 Hz, 1 H), 8.78 (s, 1 H), 8.21 (dd, J = 2.0, 9.0, 1 H), 6.94 (d, J = 9.5 Hz, 1 H), 3.81 (t, J = 5.0 Hz, 2 H), 3.63-3.70 (m, 10 H), 3.59 (q, J = 5.5 Hz, 2 H), 3.35 (t, J = 5.0 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 148.3, 135.7, 130.1, 130.0, 123.9, 114.2, 70.5, 69.8, 68.4, 50.4, 43.1; FAB-MS: $[M+Na]^+$ 407.1293 (54.1%); cacld:

407.1291.



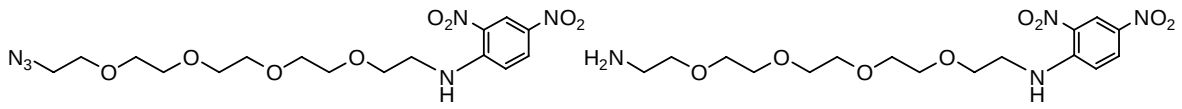
1-3a



1-4a

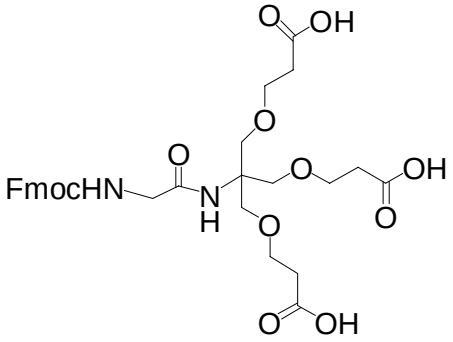
Compound 1-4a: Compound **1-3a** (0.34 g, 0.9 mmol) was stirred in EtOAc (10 mL) and 1 N HCl (5 mL) while Ph_3P (0.25 g, 9.5 mmol) in Et_2O (10 mL) was added dropwise over 1 h. After stirring under N_2 for 24 h, the separated aqueous layer was washed with Et_2O (4×40 ml), and pH was adjusted to 12 by addition of solid NaOH. The aqueous suspension was then extracted with CH_2Cl_2 (20 ×10 ml) and the combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to afford **1-4a** (0.22 g, 70%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.12 (d, *J* = 4.5 Hz, 1 H), 8.82 (s, 1 H), 8.26 (dd, *J* = 4.5, 16, 1 H), 6.97 (d, *J* = 16 Hz, 1 H), 3.85 (t, *J* = 8.5 Hz, 2 H), 3.63-3.73 (m, 10 H), 3.51 (t, *J* = 8.5 Hz, 2 H), 2.86 (s, 2 H), 1.53 (s, 2 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 148.6, 136.1, 130.5, 124.4, 114.6, 70.7, 70.6, 70.3, 69.2, 68.7, 43.4, 40.5; FAB-MS: $[M+H]^+$ 359.1569 (100%); cacld: 359.1567.

Compound 1-3b: Same procedure as for **1-3a**. The yield was 86%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.09 (d, *J* = 3.0 Hz,1 H), 8.79 (s, 1 H), 8.23 (dd, *J* = 2.5, 9.5 Hz, 1 H), 6.95 (d, *J* = 10.0 Hz, 1 H), 3.82 (t, *J* = 4.5 Hz, 2 H), 3.60-3.70 (m, 14 H), 3.59 (q, *J* = 4.5 Hz, 2 H), 3.36 (t, *J* = 4.5 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 148.5, 136.1, 130.6, 130.3, 124.4, 114.3, 70.9, 70.8, 70.7, 70.1, 68.7, 50.8, 43.4; FAB-MS: $[M+Na]^+$ 451.1507; cacld: 451.1548.



1-3b **1-4b**

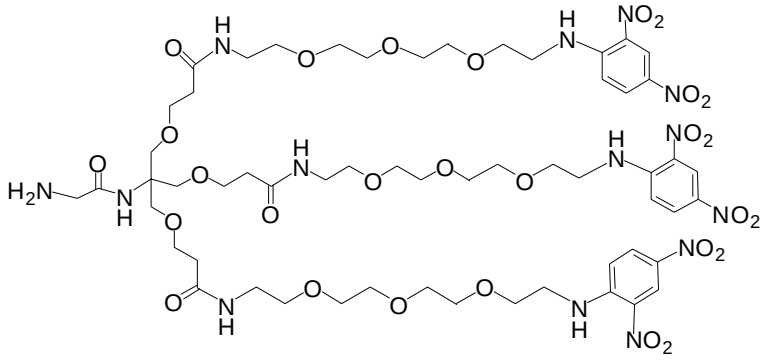
Compound 1-4b: Same procedure as for **1-4a**. The yield was 75%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.17 (d, *J* = 6.5 Hz, 1 H), 8.83 (s, 1 H), 8.29 (dd, *J* = 4.5, 16 Hz, 1 H), 6.99 (d, *J* = 16 Hz, 1 H), 3.88 (t, *J* = 8.5 Hz, 2 H), 3.63-3.72 (m, 14 H), 3.53 (t, *J* = 8.5 Hz, 2 H), δ 2.89 (s, 2 H), 1.66 (s, 2 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 148.6, 136.3, 130.6, 124.5, 114.6, 77.5, 77.3, 77.0, 70.9, 70.5, 70.4, 70.2, 68.8, 68.1, 43.4, 40.3; FAB-MS: $[M+H]^+$ 403.1815 (100%); cacld: 403.1829.



1-10

Compound 1-10: Compound **1-6** (2.4 g, 4.7 mmol) and Fmoc-glycine (1.4 g, 4.7 mmol) were dissolved in CH_2Cl_2 (200 ml) and cooled to 0℃. DCC (1.1 g, 5.3 mmol) in CH_2Cl_2 (20 mL) was added slowly over 1 h. The reaction mixture then was allowed to reach room temperature and left stirring for 24 h. The solvent was removed under vacuum, and the residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to afford a colorless oil (3.0 g, 82%). The colorless oil was dissolved in HCOOH (10 mL), and stirred 24 h at 60℃. The HCOOH then was removed under vacuum and the residue was purified by

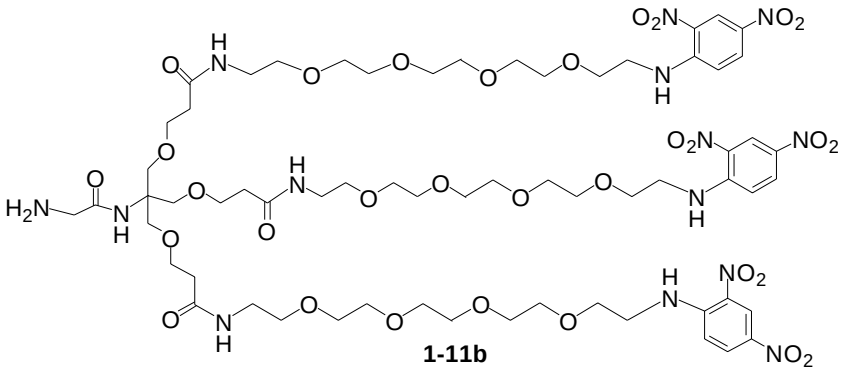
column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2 +0.5% v/v AcOH) to yield **1-10** (2.3 g, 95%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.68 (d, *J* = 7.0 Hz, 1 H), 7.57 (d, *J* = 7.0 Hz, 2 H), 7.36 (t, *J* = 7.5 Hz, 2 H), 7.28 (t, *J* = 7.5 Hz, 2 H), 6.57 (s, 1 H), 6.18 (s, 1 H), 4.33 (d, *J* = 7.5 Hz, 2 H), 4.17 (t, *J* = 6.5 Hz, 1 H), 3.88 (s, 2 H), 3.67 (m, 12 H), 2.53 (t, *J* = 6.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 175.8, 170.0, 157.5, 143.9, 141.4, 127.9, 127.3, 125.4, 120.2, 69.4, 67.7, 67.1, 60.4, 50.6, 47.2, 44.5, 35.1; FAB-MS: $[M+H]^+$ 617.2347 (100%); cacld: 617.2347.



1-11a

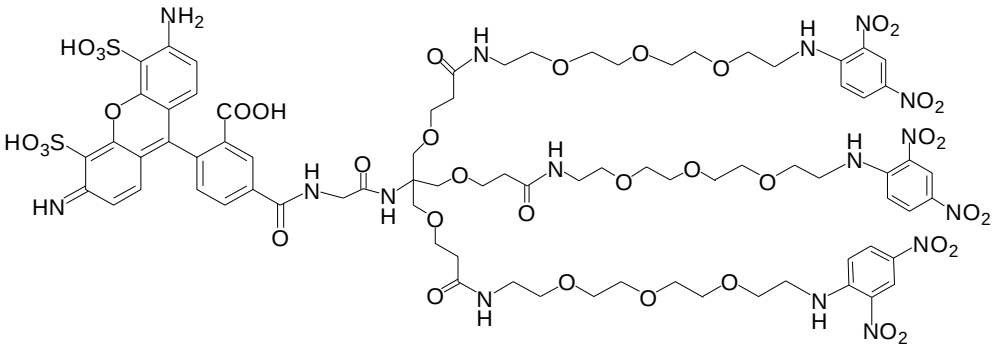
Compound 1-11a: 1-Hydroxybenzotriazole (HOBT, 24.9 mg, 0.18 mmol), Et_3N (0.08 mL, 0.58 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI, 112.3 mg, 0.58 mmol) were added to **5** (120.2 mg, 0.19 mmol) in dry THF (10 mL). Compound **1-4a** (210.0 mg, 0.58 mmol) dissolved in dry THF (5.0 mL) was added, and the reaction mixture was stirred under N_2 for 24 h. After removal of the solvent at reduced pressure, the residue was purified by column chromatography (SiO_2, 1:1 EtOAc/hexane) to afford a yellow oil (175 mg, 55%). The yellow oil (175 mg, 0.10 mmol) was dissolved in dry THF (5 mL),

followed by the addition pipiredine (1 mL). The reaction mixture was stirred for 10 min. The solvent was removed under vacuum, and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2 + 0.05% NH_4OH) to give **1-11a** (136 mg, 90%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.12 (t, *J* = 1.5 Hz, 3 H), 8.81(s, 3 H), 8.27 (dd, *J* = 2.5, 9.7 Hz, 3 H), 7.44(s, 1 H), 6.97 (d, *J* = 9.5 Hz, 3 H), 6.62 (t, *J* = 5.0 Hz, 3 H), 3.83 (t, *J* = 5.0 Hz, 6 H), 3.55-3.71 (m, 48 H), 3.44 (q, *J* = 5.5 Hz, 6 H), 3.28 (s, 2 H), 2.42 (t, *J* = 5.5 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 171.6, 148.6, 136.2, 130.6, 130.5, 124.5, 114.5, 70.9, 70.8, 70.7, 70.4, 70.1, 69.6, 68.7, 67.7, 59.9, 44.5, 43.427, 39.5, 36.9; FAB-MS: $[M+Na]^+$ 1437.5626 (100%); cacld: 1437.5634.



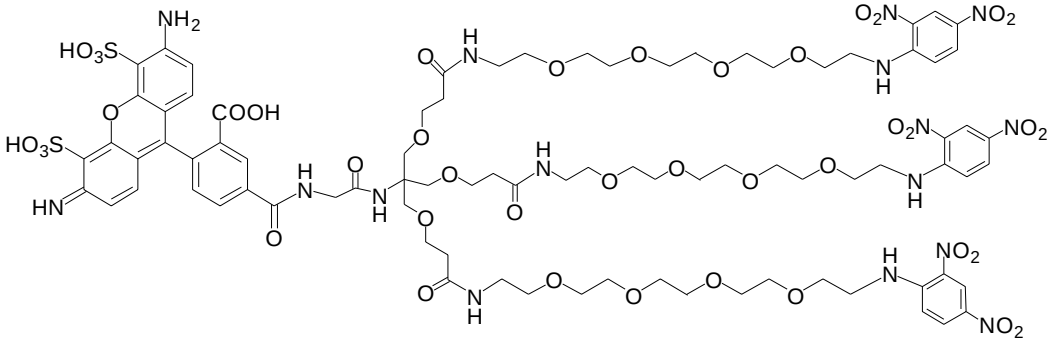
1-11b

Compound 1-11b: Same procedure as for **1-11a** using **1-4b**. The yield was 88%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.12 (d, *J* = 2.5 Hz, 3 H), 8.82(s, 1 H), 8.27 (dd, *J* = 2.5 Hz, 10.0, 3 H), 7.50(s, 1 H), 6.99 (d, *J* = 9.0 Hz, 3 H), 6.77(t, *J* = 6.0 Hz, 3 H), 3.84 (t, *J* = 5.0 Hz, 6 H), 3.61-3.73 (m, 60 H), 3.56(t, *J* = 5.5 Hz, 6 H), 3.43 (q, *J* = 5.5 Hz, 6 H), 3.28 (s, 2 H), 2.43 (t, *J* = 6.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 171.6, 148.6, 136.2, 130.6, 130.5, 124.4, 114.4, 70.9, 70.8, 70.7, 70.6, 70.3, 70.1, 69.6, 68.8, 67.6, 59.7, 44.9, 43.4, 39.4, 36.9; FAB-MS: $[M+Na]^+$ 1569.6423 (100%); cacld: 1569.6420.



1-12a

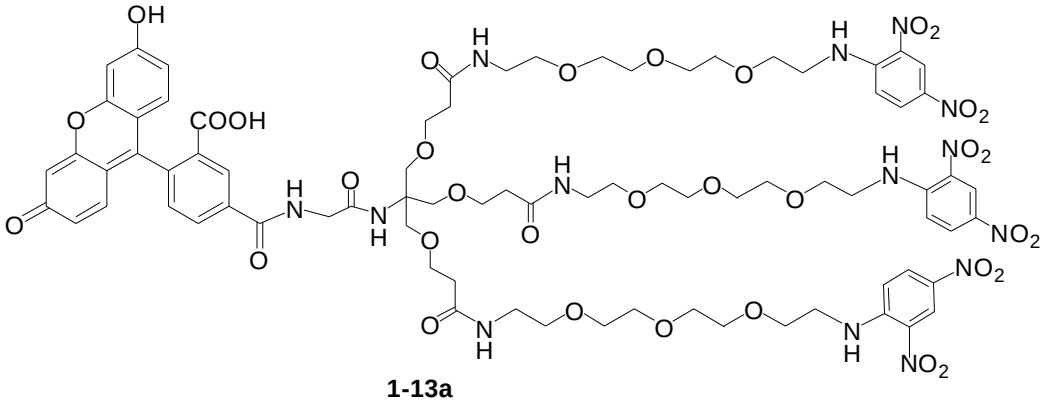
Compound 1-12a: To the solution of **1-11a** (4.0 mg, 0.0028 mmol) in DMF (2 mL) added Alexa fluor 488 (1 mg, 0.0015 mmol) and a piece of DMAP, the mixture was stirred for 20 h under N_2. After removal of the solvent under vacuum, the residue was purified by chromatography (SiO_2, 25:65:4 MeOH/CH_2Cl_2/H_2O) to get **1-12a** (1.8 mg, 60%). ^{1}H NMR (d-DMSO, 500 MHz) δ 8.84 (d, *J* = 3.5 Hz, 3 H), 8.24 (q, *J* = 10.0, 2.5 Hz, 3 H), 8.14 (d, *J* = 8.0 Hz, 1 H), 8.02 (d, *J* = 8.5 Hz, 1 H), 7.89 (s, 1 H), 7.65 (s, 1 H), 7.26 (d, *J* = 10.0 Hz, 3 H), 6.43 (d, *J* = 9.0 Hz, 1 H), 6.32 (d, *J* = 8.5 Hz, 1 H), 5.76 (s, 1 H), 4.06 (s, 2 H), 3.63-3.67 (m, 6 H), 3.32-3.57 (m, 48 H), 3.14-3.26 (m, 6 H), 2.26 (t, *J* = 6.5 Hz, 6 H); FAB-MS: $[M+Na]^+$ 1953.5564 (100%); cacld: 1953.5567.



1-12b

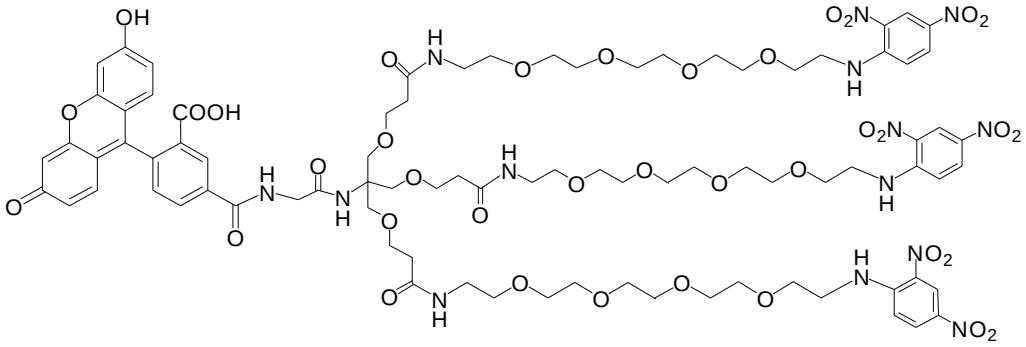
Compound 1-12b: Same procedure as for **1-12a** using **1-11b**. The yield was 55%. ^{1}H NMR (d-DMSO, 500 MHz) δ 8.83 (d, *J* = 3.0 Hz, 3 H), 8.23 (q, *J* = 10.0 Hz, 3 H), 8.13 (d, *J* = 7.5 Hz, 1 H), 8.01 (d, *J* = 8.0, 1 H), 7.89 (s, 1 H),

7.65 (s, 1 H), 7.26 (d, J = 9.5 Hz, 3 H), 6.43 (d, J = 9.0 Hz, 1 H), 6.32 (d, J = 8.5 Hz, 1 H), 5.40 (s, 1 H), 3.78(s, 2 H), 3.63-3.66 (m, 6 H), 3.31-3.56 (m, 60 H), 3.14-3.25 (m, 6 H), 2.26 (t, J = 6.5 Hz, 6 H); FAB-MS: $[M+Na]^+$ 2085.6343 (61.3%); cacld: 2085.6354.



1-13a

Compound 1-13a: 1-11a (5 mg, 0.0035 mmol) and 5-(and-6)-carboxyfluorescein succinimidyl ester (1.5 mg, 0.0031 mmol) were dissolved in dry THF (3 mL), and the mixture was stirred under N_2 for 24 h. After removal of the solvent under vacuum, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to afford **1-13a** (3 mg, 50%). ^{1}H NMR (d-DMSO, 500 MHz) δ 8.84 (t, J = 5.5 Hz, 3 H), 8.23 (dd, J = 2.5, 9.5 Hz, 3 H), 8.13 (d, J = 7.5 Hz, 1 H), 8.05 (d, J = 7.5 Hz, 1 H), 7.86 (t, J = 6.0 Hz, 3 H), 7.69 (s, 1 H), 7.24 (d, J = 9.5 Hz, 3 H), 6.65 (s, 1 H), 6.58 (d, J = 8.5 Hz, 1 H), 6.53 (s, 1 H), 3.80 (d, J = 5.5 Hz, 2 H), 3.61-3.67 (m, 12 H), 3.43-3.56 (m, 18 H), 3.29-3.35 (m, 24 H), 3.15 (q, J = 6.0 Hz, 6 H), 2.25 (t, J = 6.5 Hz, 6 H); ^{13}C NMR (d-DMSO, 500 MHz) δ 170.2, 148.3, 134.9, 129.9, 129.6, 123.5, 115.6, 69.8, 69.5, 69.1, 68.2, 67.3, 42.6, 38.5, 35.8; FAB-MS: $[M+H]^+$ 1773.6278 (100%); cacld: 1773.6292.

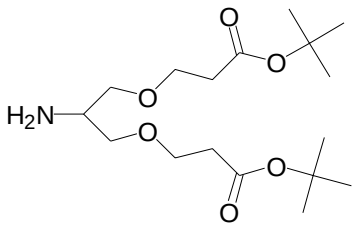


1-13b

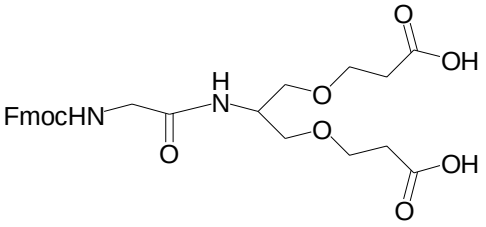
Compound 1-13b: Same procedure as for **1-13a** using **1-11b**. The yield was 52%. ^{1}H NMR (d-DMSO, 500 MHz) δ 8.85 (t, *J* = 5.5 Hz, 3 H), 8.50 (s, 1H), 8.24 (dd, *J* = 2.5, 9.5 Hz, 3 H), 8.14 (d, *J* = 7.0 Hz, 1 H), 7.95 (t, *J* = 5.5 Hz, 3 H), 7.32 (d, *J* = 8.0 Hz, 1 H), 7.26 (d, *J* = 9.5 Hz, 3 H), 6.59 (d, *J* = 9.0 Hz, 1 H), 6.51 (s, 1 H), 6.25 (s, 1 H), 3.92 (d, *J* = 5.5 Hz, 2 H), 3.63-3.69 (m, 12 H), 3.35-3.58 (m, 30 H), 3.34-3.39 (m, 24 H), 3.19 (q, *J* = 6.0 Hz, 6 H), 2.30 (t, *J* = 6.0 Hz, 6 H); ^{13}C NMR (d-DMSO, 500 MHz) δ 170.3, 148.4, 134.9, 129.9, 129.6, 123.5, 115.7, 69.8, 69.7, 69.5, 69.1, 68.2, 67.3, 42.6, 38.5, 35.9; FAB-MS: $[M+H]^{+}$ 1905.7061 (100%); cacld: 1905.7018.

Compound 1-15: The serinol (0.91 g, 10.0 mmol) in DMSO (2.0 mL) was cooled to 15℃ under N_2. Then, 5.0 M NaOH (0.2 mL) was injected while stirring. After that, the *tert*-butyl acrylate (3.2 mL, 22 mmol) was added dropwise. The reaction mixture was allowed to reach room temperature and left stirring for 24 h. After removal of the solvent under vacuum, the residue was purified by column chromatography (SiO_2, 2:1 EtOAc/hexane + 0.05% NH_4OH) to afford **1-15** (1.73 g, 50%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.56 (m, 4 H), 3.34 (m, 2 H), 3.21 (m, 2 H), 2.36 (t, *J* = 6.5 Hz, 4 H), 1.34 (s, 18 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 170.8, 80.4, 73.3, 71.2, 66.8, 61.4, 58.2, 50.8, 42.8, 36.5,

36.3, 36.1, 28.1; FAB-MS: $[M+Na]^+$ 370.22028; cacld: 370.22001.

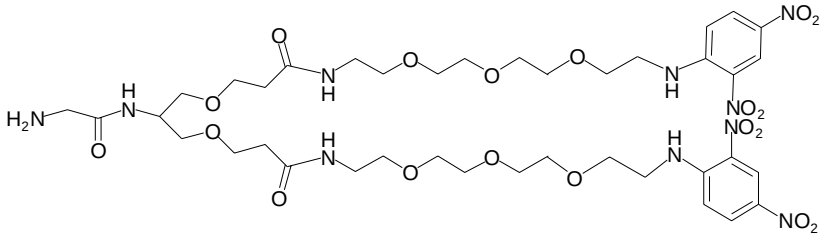


1-15



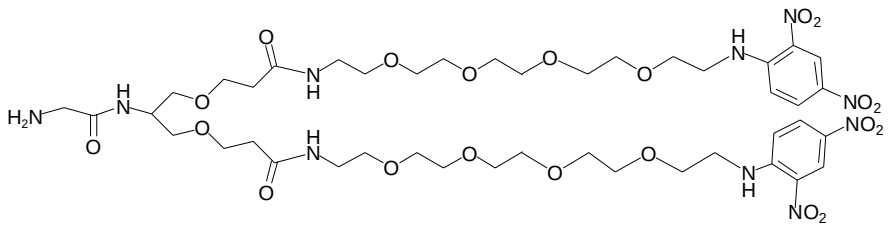
1-16

Compound 1-16: Compound **1-15** (1.7 g, 4.9 mmol) and Fmoc-glycine (1.46 g, 4.9 mmol) were dissolved in CH_2Cl_2 (200 ml) and cooled to 0℃. DCC (1.1 g, 5.3 mmol) in CH_2Cl_2 (20 mL) was added slowly over 1 h. The reaction mixture then was allowed to reach room temperature and left stirring for 24 h. The solvent was removed under vacuum, and the residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to afford a colorless oil (2.6 g, 85%). The colorless oil was dissolved in HCOOH (10 mL), and stirred 24 h at 60℃. The HCOOH then was removed under vacuum and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2 +0.5% v/v AcOH) to yield **1-16** (2.0 g, 95%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.72 (d, J = 7.0 Hz, 2 H), 7.57 (d, J = 7.5 Hz, 2 H), 7.36 (t, J = 7.5 Hz, 2 H), 7.26 (t, J = 6.5 Hz, 2 H), 6.92 (d, J = 7.5 Hz, 1 H), 6.22 (s, 1 H), 4.35 (d, J = 7.0 Hz, 2 H), 4.20 (m, 2 H), 3.92 (d, J = 5.0 Hz, 2 H), 3.55 (m, 8 H), 2.53 (m, 4 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 175.9, 170.1, 157.5, 144.0, 141.5, 128.0, 127.3, 125.4, 120.2, 69.5, 67.7, 66.8, 49.1, 47.2, 44.4, 35.1; FAB-MS: $[M+H]^+$ 515.20289; cacld: 515.20241.



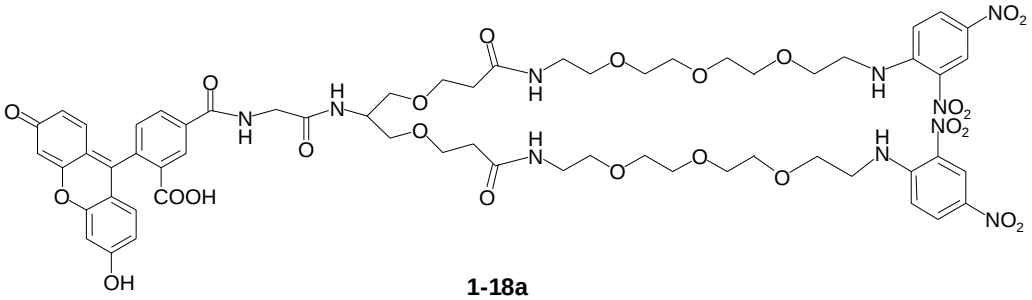
1-17a

Compound 1-17a: 1-Hydroxybenzotriazole (HOBT, 24.9 mg, 0.18 mmol), Et_3N (0.055 mL, 0.39 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCl, 75.5 mg, 0.39 mmol) were added to **1-16** (98.0 mg, 0.19 mmol) in dry THF (10 mL). Compound **1-4a** (141.0 mg, 0.39 mmol) dissolved in dry THF (5.0 mL) was added, and the reaction mixture was stirred under N_2 for 24 h. After removal of the solvent at reduced pressure, the residue was purified by column chromatography (SiO_2, 1:1 EtOAc/hexane) to afford a yellow oil (148 mg, 65%). The yellow oil (148 mg, 0.12 mmol) was dissolved in dry THF (5 mL), followed by the addition pipiredine (1 mL). The reaction mixture was stirred for 10 min. The solvent was removed under vacuum, and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2 + 0.05% NH_4OH) to give **1-17a** (108 mg, 90%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.81 (s, 2 H), 8.27 (dd, *J* = 2.5, 9.5 Hz, 2 H), 6.97 (d, *J* = 9.5 Hz, 2 H), 6.57 (t, *J* = 5.5 Hz, 2 H), 4.19 (m, 1 H), 3.84 (t, *J* = 5.0 Hz, 4 H), 3.55-3.76 (m, 28 H), 3.43-3.48 (m, 6 H), 3.36 (s, 2 H), 2.45 (m, 4 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 172.8, 171.4, 148.6, 136.3, 130.7, 130.5, 124.4, 114.4, 71.0, 70.9, 70.7, 70.4, 70.0, 69.5, 68.7, 67.5, 48.4, 45.1, 43.4, 39.5, 37.0; FAB-MS: $[M+Na]^+$ 995.39530 ; cacld: 995.39284.



1-17b

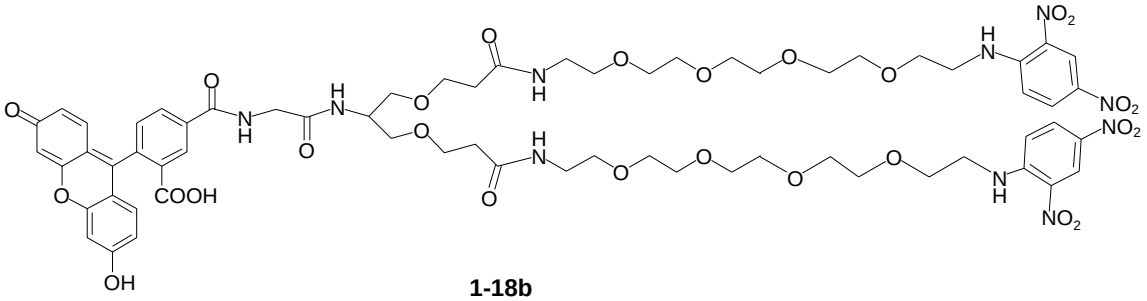
Compound 1-17b: Same procedure as for **1-17a** using **1-4b**. The yield was 55%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.81 (s, 2 H), 8.27 (dd, *J* = 2.5, 9.5 Hz, 2 H), 6.98 (d, *J* = 9.5 Hz, 2 H), 6.66 (t, *J* = 4.5 Hz, 2 H), 4.19 (m, 1 H), 3.83 (t, *J* = 5.0 Hz, 4 H), 3.54-3.76 (m, 38 H), 3.42-3.48 (m, 6 H), 3.36 (s, 2 H), 2.45 (m, 4 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 172.9, 171.4, 148.6, 136.3, 130.7, 130.4, 124.4, 114.4, 71.0, 70.9, 70.8, 70.7, 70.6, 70.4, 70.0, 69.5, 68.8, 67.4, 48.4, 45.1, 43.4, 39.4, 37.0; FAB-MS: $[M+Na]^+$ 1083.44519; cacld: 1083.44527.



1-18a

Compound 1-18a: **1-17a** (3.4 mg, 0.0035 mmol) and 5-(and-6)-carboxyfluorescein succinimidyl ester (1.5 mg, 0.0031 mmol) were dissolved in dry THF (3 mL), and the mixture was stirred under N_2 for 24 h. After removal of the solvent under vacuum, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to afford **1-18a** (2.3 mg, 50%). ^{1}H NMR (d-DMSO, 500 MHz) δ 8.82-8.85 (m, 3 H), 8.16-8.25 (m, 2 H), 7.78-7.79 (m, 3 H), 7.26 (d, *J* = 9.5 Hz, 2 H), 6.58-6.62 (m, 3 H), 6.50 (d, *J* = 8.0 Hz, 2 H), 3.93 (d, *J* = 6.0 Hz, 2 H), 3.82 (d, *J* = 5.5 Hz, 1 H), 3.64-3.69 (m,

6 H), 3.45-3.60 (m, 20 H), 3.27-3.45 (m, 10 H), 3.14-3.20 (m, 4 H), 2.29 (m, 4 H); ^{13}C NMR (d-DMSO, 500 MHz) δ 170.1, 168.5, 168.4, 165.1, 164.9, 148.3, 135.6, 134.8, 129.8, 129.6, 129.3, 123.5, 115.6, 109.4, 102.3, 69.7, 69.5, 69.2, 68.2, 66.9, 48.6, 42.6, 42.3, 38.5, 35.8; FAB-MS: $[M+H]^+$ 1331.45979 ; cacld: 1331.45863.



1-18b

Compound 1-18b: Same procedure as for **1-18a** using **1-17b**. The yield was 52%. ^{1}H NMR (d-DMSO, 500 MHz) δ 8.82-8.85 (m, 3 H), 8.23-8.26 (m, 2 H), 7.83-7.90 (m, 3 H), 7.26 (d, *J* = 9.5 Hz, 2 H), 6.68 (m, 2 H), 6.53-6.59 (m, 3 H), 3.93 (d, *J* = 5.5 Hz, 2 H), 3.81 (d, *J* = 5.5 Hz, 1 H), 3.63-3.69 (m, 6 H), 3.45-3.59 (m, 20 H), 3.28-3.40 (m, 18 H), 3.14-3.27 (m, 4 H), 2.30 (m, 4 H); ^{13}C NMR (d-DMSO, 500 MHz) δ 170.1, 168.4, 168.3, 164.9, 159.6, 148.3, 135.8, 134.8, 129.8, 129.6, 129.2, 126.4, 123.5, 115.6, 112.6, 109.1, 102.3, 69.7, 69.5, 69.2, 68.2, 66.9, 54.9, 48.6, 42.6, 42.3, 38.5, 35.8; FAB-MS: $[M+H]^+$ 1419.51502 ; cacld: 1419.51106.

Materials for degranulation assays:

Mouse monoclonal anti-DNP–IgE was obtained from hybridoma H1 26.8 and affinity purified. Final steps in the purification included ion exchange chromatography to remove bound DNP-glycine, then gel filtration to separate

monomeric IgE from small amounts of IgE aggregates. RBL-2H3 cells were grown adherent in 75 cm^2 flasks, kept at 37 °C and 5% CO_2 and generally used 5 days after passage. Cell Media consisted of MEM 1× with Earle's salts, without glutamine (Gibco-BRL), 20% fetal bovine serum (Hyclone), 1% (v/v) -glutamine (Gibco-BRL) and 1% (v/v) penicillin and streptomycin (Gibco-BRL). Cells are harvested by first rinsing with trypsin–EDTA (Gibco-BRL) and then incubating with trypsin–EDTA for 5 min at 37° C. Cells in culture were incubated overnight at 37° C with 10 μg of anti-DNP FITC–IgE. After harvesting, the cells were washed and then resuspended in buffered salt solution (BSS: 135 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 5.6 m*M* glucose, 0.1% gelatin, 20 m*M* Hepes, pH 7.4).

Degranulation assays:

The secretion response of RBL cells was monitored by measuring the activity of the granule-stored enzyme β-hexosaminidase secreted into the supernatant. RBL-2H3 cells (1 x 105 cells/100 mL/well), suspended in medium containing saturating concentrations of DNP-specific IgE were plated in 96-well plates. Cells were allowed to adhere for at least 2 h in the incubator followed by three washings with Tyrode's buffer and then were exposed to the indicated concentration of ligand. Twenty microliters of the supernatants was transferred to a new 96-well plate and reacted with 50 mL of substrate solution (*p*-nitrophenyl-*N*-acethyl-β-D-glucosamine, 1.3 mg/mL, in 0.1 *M* citrate, pH 4.5). The mixture was incubated at 37 °C for 1 h, and the reaction terminated

and developed by addition of 150 mL ‘stop’ solution (0.2 *M* glycine, pH 10.7). The intensity of the yellow color formed at this pH, due to the nitrophenol produced by the enzymatic hydrolysis of the substrate, is proportional to the concentration of the secreted enzyme. The optical density of the color intensity was measured at 405 nm in an ELISA plate reader. All assays were repeated at least four times and their results are expressed as net percent of the cells’ total enzyme activity contents ±5–10%.

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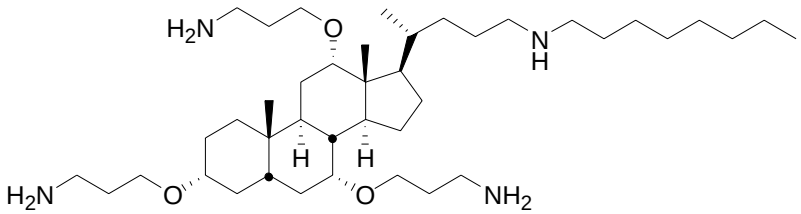
CHAPTER 2

PREPARATION AND STUDIES ON ANTIVIRAL ACTIVITY AGAINST VACCINIA VIRUS OF CERAGENINS

2.1 Introduction

Membrane-active antibiotics include antimicrobial peptides (AMPs) and a class of amphiphilic steroids termed ceragenins. Studies of membrane-active antibiotics show that cationic, facially amphiphilic molecules disrupt bacterial membranes.[1,2] Both AMPs and ceragenins have similar antibacterial activities including depolarization of the bacterial membranes, activation of the bacterial promoters and disintegration of the bacterial membranes.[3] Considering their similarity in the amphiphilic morphology, AMPs and ceragenins may share similar mechanisms in disrupting bacterial membranes. It was found recently that some antibiotics, including AMPs and ceragenins, may share both antibacterial and antiviral activity.[4,5,6] For example, both cathelicidin LL-37[4] and CSA-13 show good antiviral activity against vaccinia virus (VV). However, there are no published reports describing the mechanism by which viruses are disrupted by AMPs and ceragenins.



CSA-13

LL37 LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

Figure 2-1. Structures of CSA-13 and LL-37 (single letter for amino acids is used)

2.1.1 Mechanism of AMPs and ceragenins in disrupting Gram-negative bacterial membrane

2.1.1.1 Gram-negative bacterial membrane structure

The gram-negative bacterial membrane contains three layers: an outer membrane (OM), a cell wall and an inner membrane. The outer membrane of gram-negative bacteria contains lipopolysaccharide (LPS) as the essential component in the outer leaflet. LPS is an unusual compound found on the outer leaflet of the membrane, where it mediates contact with the environment. It is composed of three main regions: O-antigen, core polysaccharide and lipid A.[7] LPS is essential to the function of the OM and, as a structural component of the cell, it may also play several roles in the pathogenesis of gram-negative bacterial infections. First, it is a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. Second, it impedes destruction of the bacterial cells by serum components and phagocytic cells. Third, LPS plays an important role as a surface structure in the interaction between the pathogen and its host.[8]

The glycosidic diphosphate moiety of lipid A (Figure 2-2A) has affinity for metal ions such as Mg^{2+} and Ca^{2+}, which are proposed to stabilize the outer membrane by forming ionic cross bridging between LPS molecules. The cross bridging brings Gram-negative bacteria an additional permeability barrier to hydrophobic antibiotics, lysozymes and proteases (Figure 2-2B). Disruption of the lipid A cross bridging significantly increases the permeability of the OM. So

compounds that bind either lipid A or divalent cations disrupt the organization of the outer membrane, increase its permeability, and sensitize bacteria to hydrophobic antibiotics that ineffectively traverse the outer membrane. For example, cross bridging can be disrupted by metal ion chelators such as EDTA[9] which can remove metal ions from LPS molecules, by amines via electrostatic interaction with LPS, or by antibiotics that selectively bind with LPS.[10]

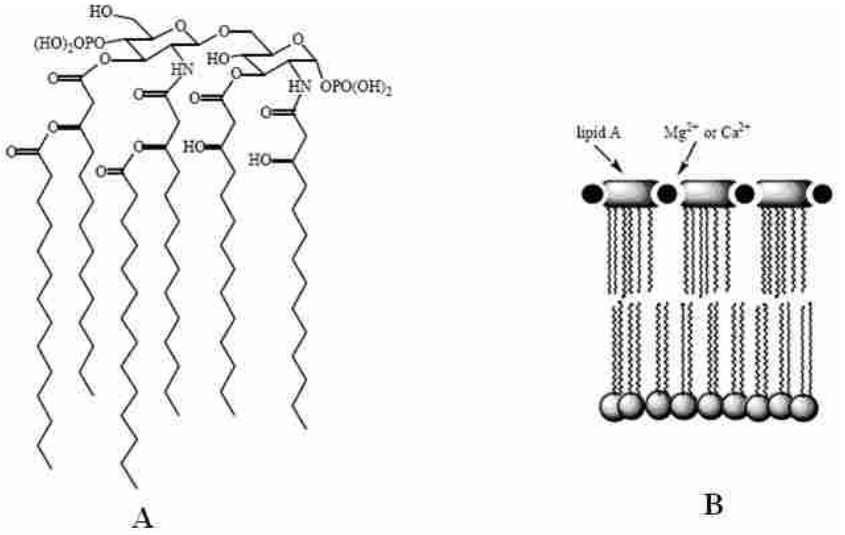


Figure 2-2. (A) Structure of lipid A and (B) Lipid A-divalent interactions. Adapted from ref. 15

2.1.1.2 Cationic facial amphiphiles

Recent studies of membrane-active antibiotics indicated that bacterial membranes can be disrupted by cationic, facially amphiphilic molecules.[1,2] Facial amphiphiles are different from typical amphiphilic compounds.[11,12] The typical amphiphiles possess a hydrophilic head group and a hydrophobic tail. Facial amphiphiles display separate hydrophilic groups and hydrophobic faces (Figure 2-3). Many membrane-active compounds are facial amphiphiles including most AMPs[13] and ceragenins.

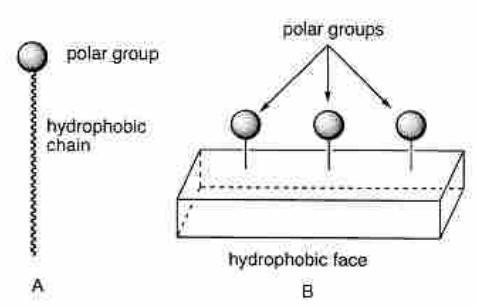


Figure 2-3. (A) A 'typical' amphiphile and (B) A facial amphiphile. Adapted from ref. 15.

2.1.1.3 Mechanism of cationic, facially amphiphilic molecule in disrupting OM

There are two ways to disrupt the OM: binding of divalent cations or binding of the phosphate groups of lipid A. Compounds that bind either lipid A or divalent cations increase the permeability of OM and sensitize bacteria to hydrophobic antibiotics. AMPs and ceragenins may have similar mechanisms of action in disrupting OM because of their similarity in the amphiphilic morphology. It is believed that the permeabilization of OM proceeds by the 'carpet' model.[14] In the 'carpet' model, the antibiotic associates with the cell surface (via ionic interactions), and when it reaches adequate concentration, the bacterial membrane is disrupted (Figure 2-4).[15]

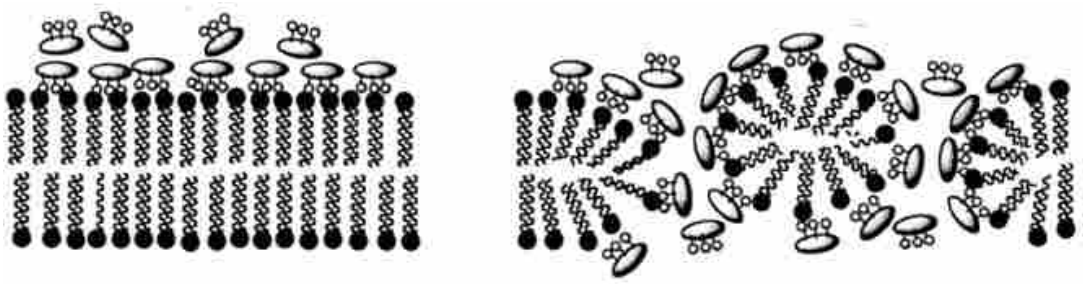


Figure 2-4. 'Carpet model' of action of cationic facial amphiphiles. Adapted from ref. 15.

2.1.2 Antiviral activities of AMPs and ceragenins against Vaccinia virus

Since the terrorist attacks of September 11, 2001, there is a concern that the next terrorism attack will include the use of bioterrorism agents such as the smallpox virus. This concern has risen because 119 million U.S. residents born after 1970's when the smallpox vaccination was terminated lack immunity to smallpox virus. Vaccinia virus (VV) is used in smallpox vaccination to make people have immunity to smallpox virus. Eczema vaccinatum (EV) is a potentially fatal adverse reaction that occurs in individuals with atopic dermatitis (AD) due to disseminated viral skin infection following exposure to VV.[16,17] Recent studies indicate that some AMPs show good activity against VV, such as LL-37, MIP-3α and HBD-3.[4,5,6] Ceragenins were designed to mimic the structure and function of AMPs,[15] which have been shown to exhibit potent activity against VV.

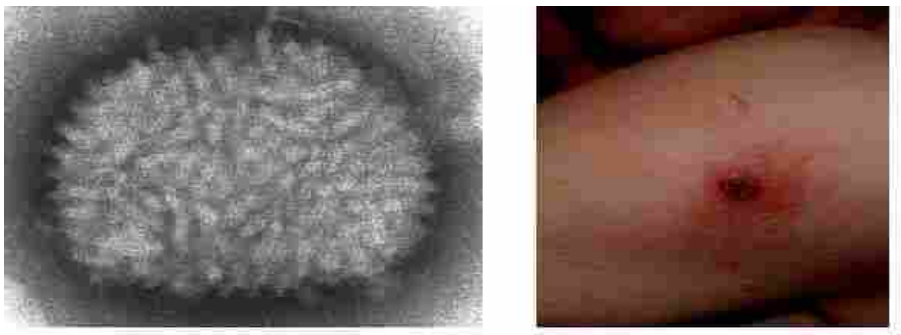
2.1.2.1 Vaccinia virus

Vaccinia virus is a large, complex, enveloped virus belonging to the poxvirus family (Figure 2-5A).[18] It has a linear, double-stranded DNA genome approximately 190 kbp in length, and which encodes for approximately 250 genes. The dimensions of the virion are roughly 360 × 270 × 250 nm. VV is well-known for its role as a vaccine that eradicated the smallpox disease, making it the first human disease to be successfully eradicated by mankind.

Vaccinia virus is unique among DNA viruses because it replicates only in the cytoplasm of the host cell, outside of the nucleus.[19] During its replication cycle,

VV produces four infectious forms which differ in their outer membranes: intracellular mature virion (IMV), the intracellular enveloped virion (IEV), the cell-associated enveloped virion (CEV) and the extracellular enveloped virion (EEV).[20] Although the issue remains contentious, the prevailing view is that the IMV consists of a single lipoprotein membrane, while the CEV and EEV are both surrounded by two membrane layers and the IEV has three envelopes. The IMV is the most abundant infectious form and is thought to be responsible for spread between hosts. On the other hand, the CEV is believed to play a role in cell-to-cell spread and the EEV is thought to be important for long range dissemination within the host organism.[20]

A VV infection (Figure 2-5B) is typically very mild and asymptomatic in healthy individuals. Immune responses generated from a VV infection protect the person against a lethal smallpox infection. For this reason, VV was, and is still being used as a live-virus vaccine against smallpox. However, certain complications and/or vaccine adverse effects occasionally arise. The chance of this happening is significantly increased in people who are immunocompromised.



A **B**

Figure 2-5. (A) Vaccinia virus and (B) Site of a vaccinia injection. Adapted from Wikipedia online.

2.1.2.2 Antiviral activity of AMPs against VV

The increased propensity of AD patients toward EV may be related to a deficiency of naturally occurring AMPs such as cathelicidins or human β defensins.[4,21] Therefore, recent studies of antiviral activity against VV focus on the cathelicidins and β-defensins. Howell and coworkers[4] found that LL-37 (Figure 2-1) showed potent antiviral activity against VV. LL-37 is the only human antimicrobial peptide in the cathelicidin family. It is widely expressed in both neutrophils and epithelial tissues. LL-37 is a multifunctional peptide with many important biological activities such as antimicrobial activity, chemoattraction, dendritic cell differentiation and wound healing.[22,23,24] In their study, a series of peptides was used to investigate the antiviral activities against VV. All the peptides used in their study have good antibacterial activities. They found only LL-37 and CRAMP (the murine homologue of LL-37) showed potent activities against VV. The results show that not all cationic and membrane active peptides have antiviral activity, nor are able to inhibit vaccinia replication. The mechanism by which VV is disrupted by AMPs is still unclear. They studied the process by electron microscopy and suggested that LL-37 had direct effects on the integrity of VV membrane structure in the IMV form.

Antiviral activity of human β-defensin 3 (HBD-3) against VV was also studied by Howell and coworkers.[5] HBD-3 is a 5-kd peptide produced primarily by keratinocytes after skin injury or inflammation. In addition, it has been shown to exhibit antibacterial activity against a variety of bacteria.[25] It was

shown that HBD-3 exhibited potent antiviral activity against VV, making it unique from HBD-1 and HBD-2, which had shown no antiviral activity against VV.[4]

2.1.2.3 Antiviral activity of ceragenins against VV

Recent studies in our group discovered that CSA-8 (Figure 2-7) and CSA-13 (Figure 2-1) showed antiviral activity similar to that of LL-37 against vaccinia virus. When CSA-8 and CSA-13 were used to treat vaccinia virus, both of them showed antiviral activities at concentrations comparable to LL-37. In contrast, CSA-54 (Figure 2-7) was only weakly active (Figure 2-6). Viral replication was also studied with CSA-8 and it showed that CSA-8 was efficient at restraining the viral replication. These results indicate that ceragenins share both antibacterial and antiviral activities.

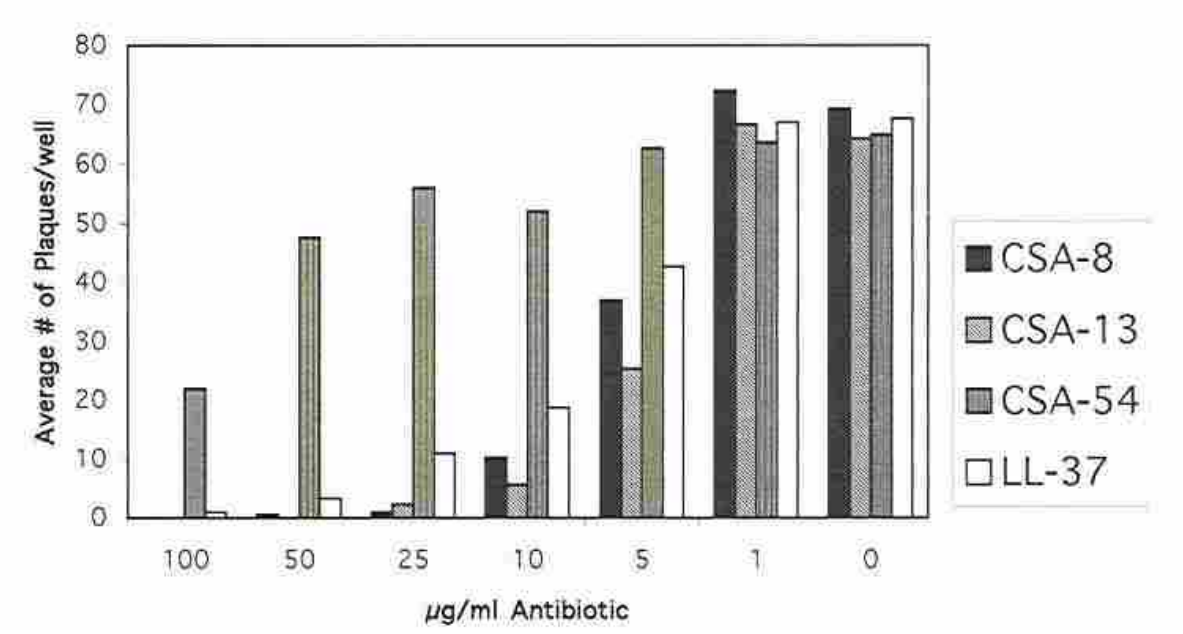
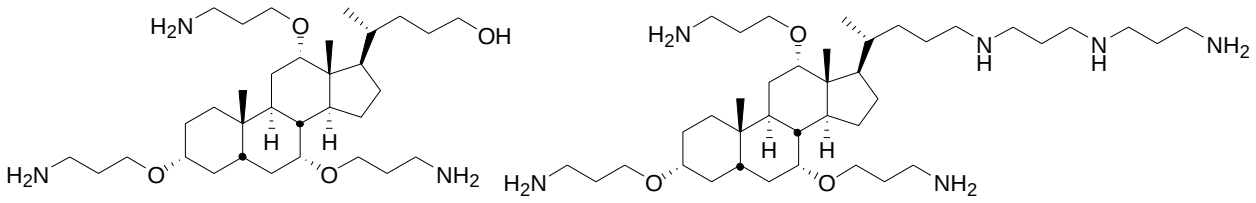


Figure 2-6. Antiviral properties of CSA-8, CSA-13, CSA-54 and LL-37 against vaccinia virus



CSA-8 **CSA-54**

Figure 2-7. Structures of CSA-8, CSA-54.

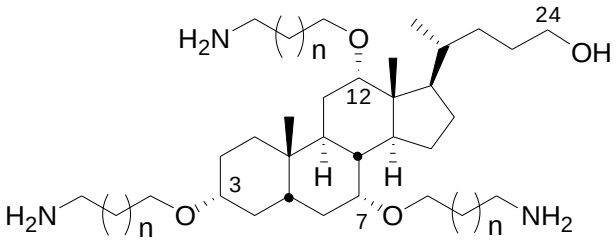
As mentioned above, the ceragenins have antiviral activities against VV and lower viral replication in infected keratinocytes. However, the mechanism of the antiviral activities of ceragenins against VV remains unknown. Studies of bactericidal activity with both peptide antibiotics and ceragenins indicate that the balance between cationic groups and hydrophobic face plays a key role in their antibacterial activities.[14,30] Prokaryotes generally display negative charges in their membrane, while eukaryotes display the zwitterionic membrane component. So it is reasonable that antibacterial activities of ceragenins will not correlate with antiviral activities. Because of the zwitterionic nature of viral envelopes, it may be targeted by antiviral agents bearing both cationic and anionic groups. However, this hypothesis has not yet been tested and the optimal balance between hydrophobic area and ionic character is not well-determined.

2.2 Research design of antiviral Ceragenins

Two aims have been designed to optimize antiviral properties of ceragenins. Aim 1 is to determine the optimal balance between hydrophobic and cationic character for antiviral properties. Three sets of variations to ceragenins have been explored: 1) changes in the lengths of the tethers linking cationic groups to

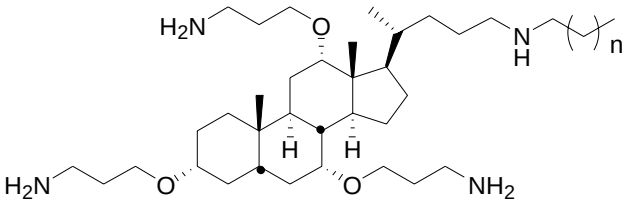
the steroid backbone; 2) variations in the length and nature of the group at C24; and 3) the use of deoxy cholic acid derivatives (Figure 2-8).

Variation Set 1

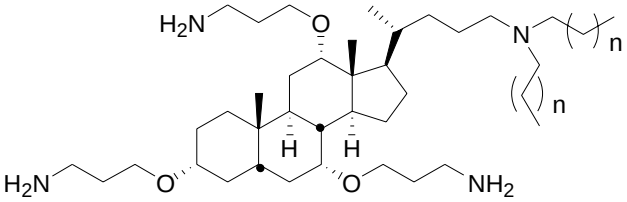


n=1, 2, 3

Variation Set 2

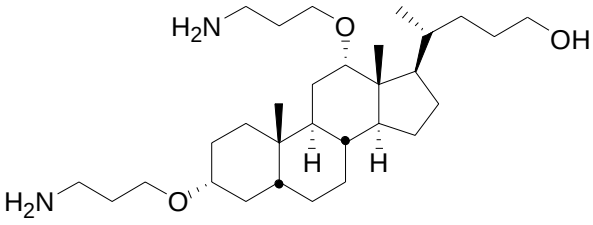


n=1, 3, 6, 14



n=3, 6

Variation Set 3



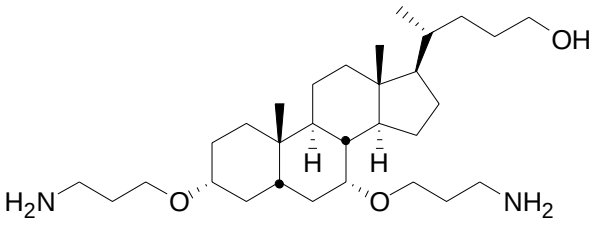


Figure 2-8. Structures of ceragenins for Aim 1

Aim 2 determines the antiviral abilities of zwitterionic ceragenins against VV. To test this hypothesis, ceragenins have been prepared with varied number of

anionic groups at C3 and C24 (Figure 2-9).

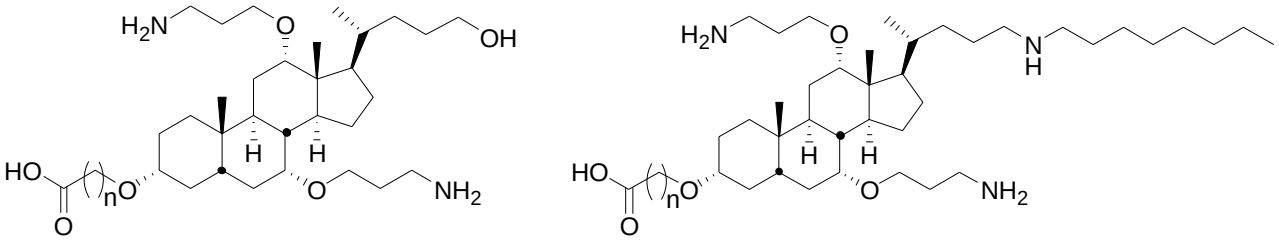
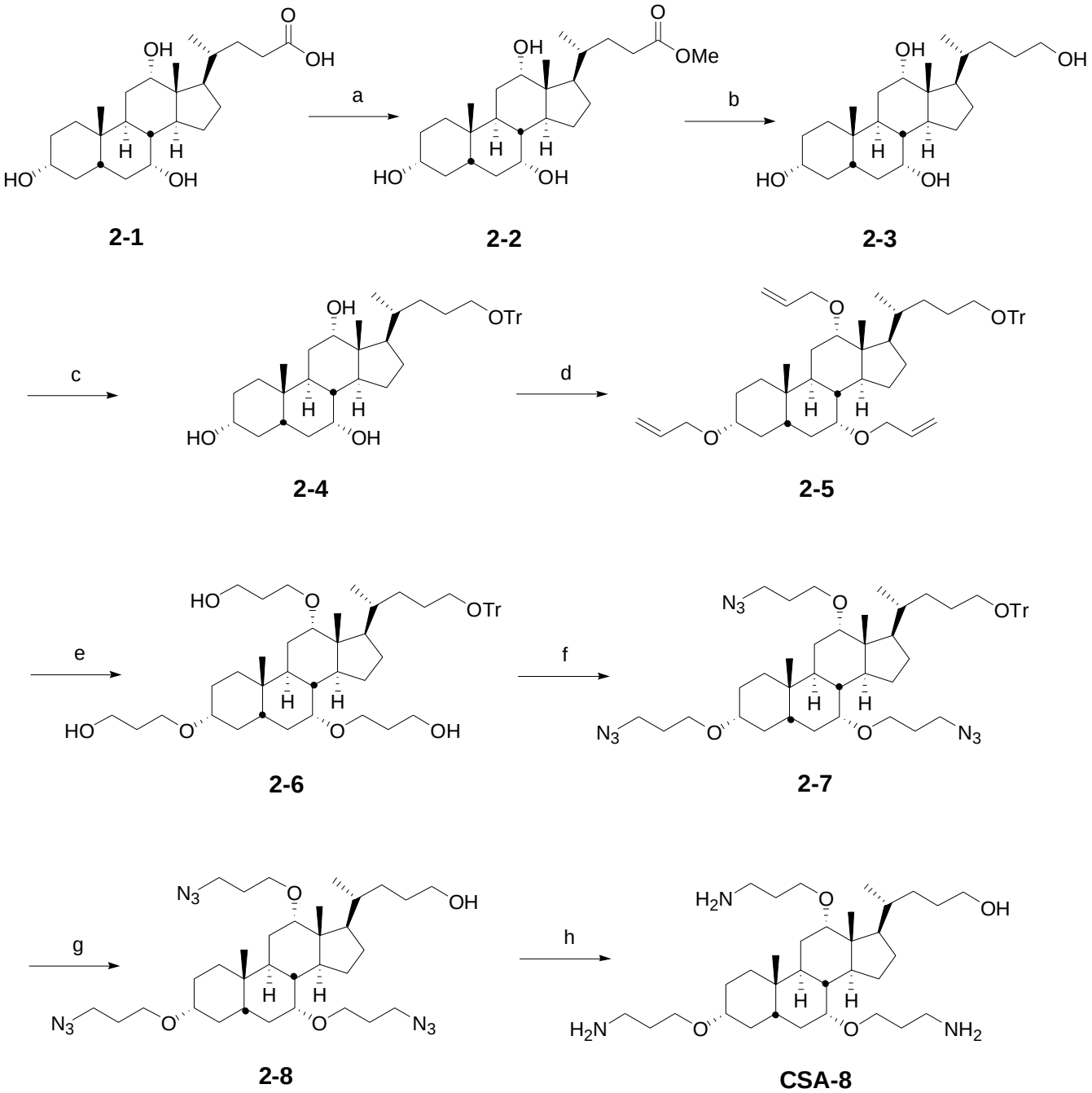


Figure 2-9. Structures of ceragenins for Aim 2

2.3 Results

2.3.1 Synthesis of ceragenins in Aim 1

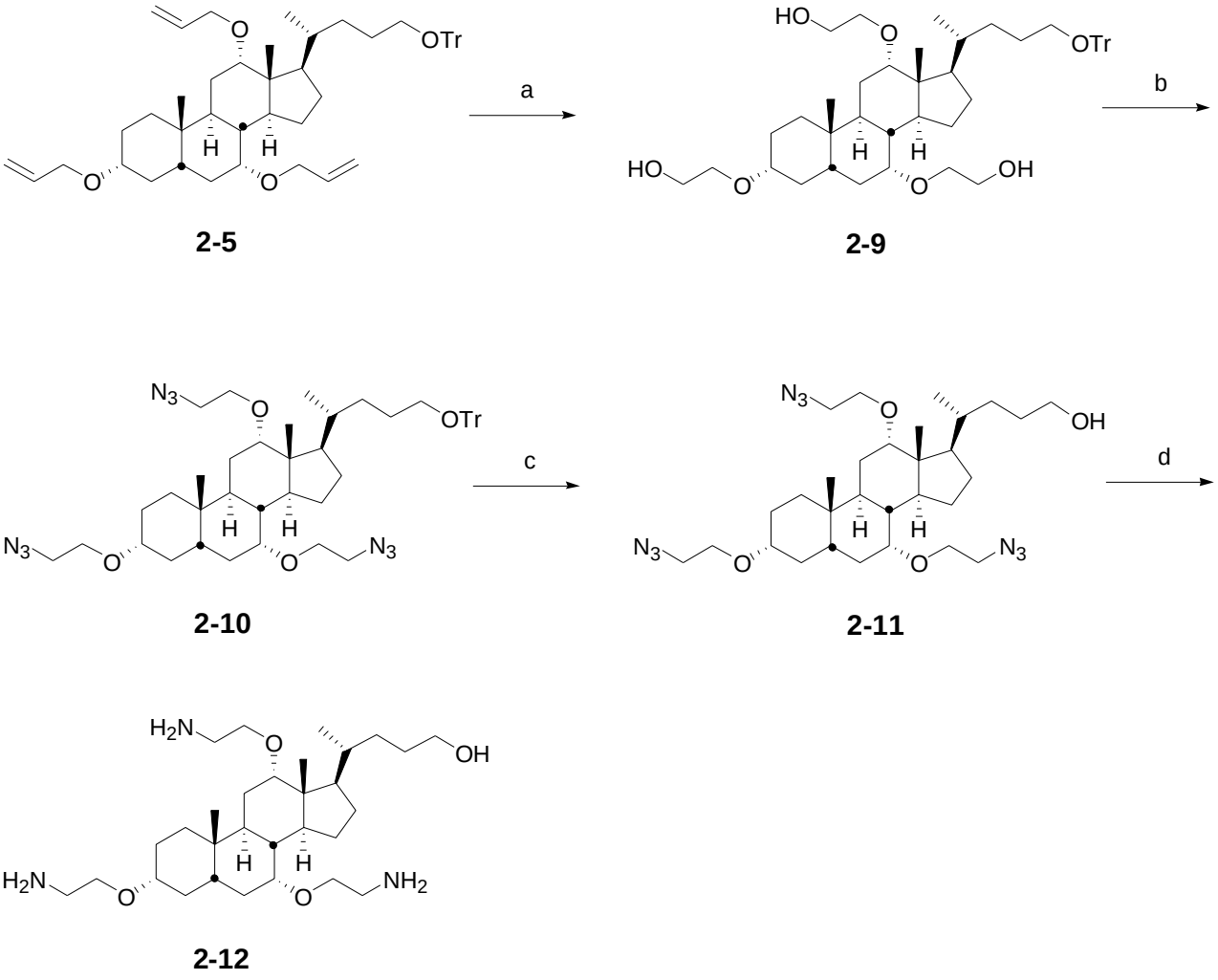
Scheme 2-1. Synthesis of **2-5**, **2-6** and **2-8**



Reagents (yields in parentheses): a) 2,2-dimethoxypropane, *p*-TsOH, methanol, 70℃ (97%); b) $LiAlH_4$, THF, reflux (93%); c) triphenylmethyl chloride, DMAP, Et_3N, THF, reflux (70%); d) allyl bromide, NaH, THF, reflux (80%); e) dicyclohexylborane, THF; H_2O_2, NaOH (80%); f) MsCl, Et_3N; NaN_3, DMF/H_2O (80%); g) *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (90%); h) Ph_3P, THF/H_2O (85%).

Compounds **2-5**, **2-6** and **2-8** are the intermediates for making the CSAs in Aim 1, and their synthesis is shown in Scheme 2-1. Cholic acid **2-1** was converted to its methyl ester **2-2** with 2,2-dimethoxypropane in methanol. Reduction of **2-2** by $LiAlH_4$ afforded **2-3**. The primary hydroxyl group of **2-3** was then protected with trityl group giving **2-4**. Treatment of **2-4** with allyl bromide provided **2-5**. Hydroboration-oxidation of **2-5** yielded alcohol **2-6**. Compound **2-6** was converted to azide compound **2-7**, followed by removal of the trityl group of **2-7** giving **2-8**.

Scheme 2-2. Synthesis of **2-12**



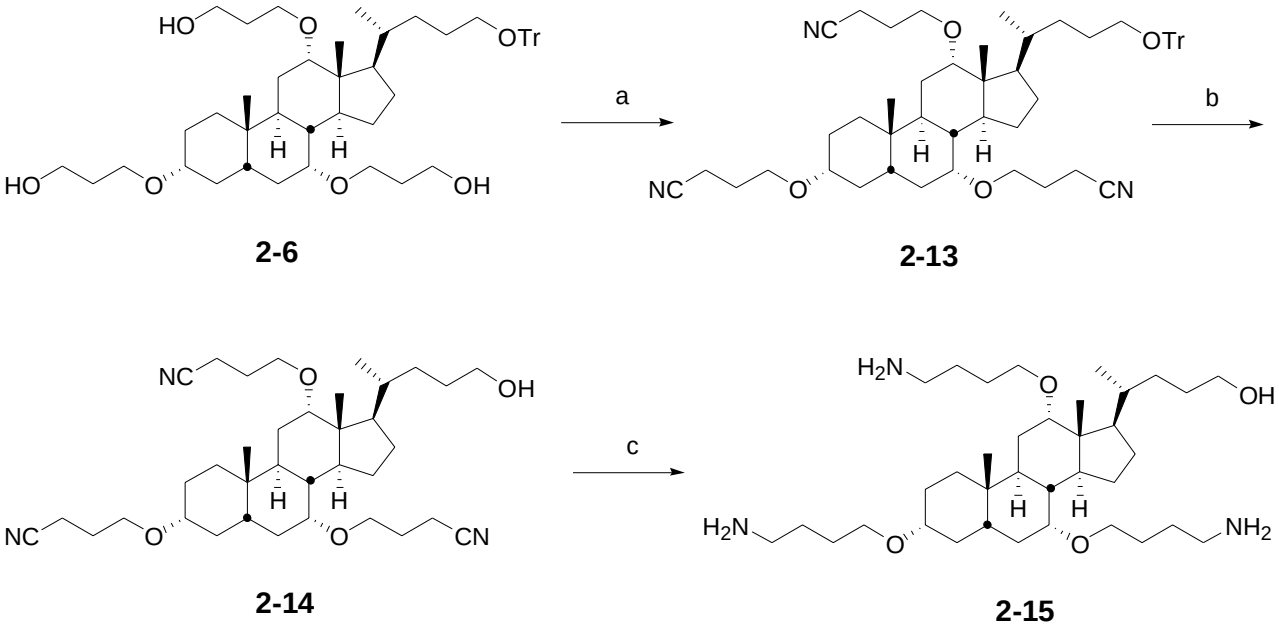
2-5 **2-9** **2-10** **2-11** **2-12**

Reagents (yields in parentheses): a) O_3, Me_2S; $NaBH_4$ (65%); b) MsCl, Et_3N; NaN_3, DMF/H_2O (80%); c) *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (90%); d) Ph_3P, THF/H_2O (85%).

The structure of **2-12** is similar to **CSA-8** except one carbon less on each tether branch. Treatment of **2-5** with ozone and dimethyl sulfide followed by reduction with sodium borohydride gave the alcohol **2-9**. Compound **2-9** was converted to azide **2-10**, followed by removal of the trityl group of **2-10** giving **2-11**. Reduction of **2-11** afforded compound **2-12** (Scheme 2-2).

The structure of **2-15** is also similar to **CSA-8** except one carbon more on each tether branch. Compound **2-6** was converted to nitrile compound **2-13**, followed by removal of the trityl group giving **2-14**. Amine **2-15** was obtained by reduction of nitrile group in **2-14** (Scheme 2-3).

Scheme 2-3. Synthesis of **2-15**

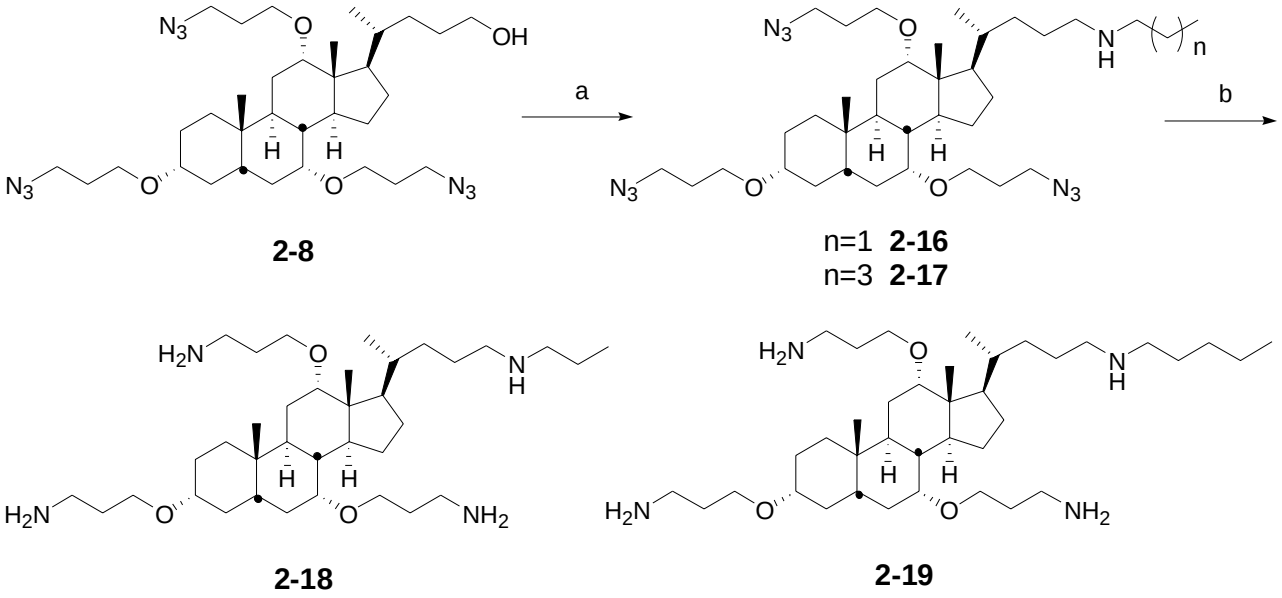


Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); KCN, DMF/H_2O (80%); b) *p*-TsOH, MeOH/CH_2Cl_2 (1:1), (90%); c) H_2, Raney Ni (85%).

To determine the effect of hydrophobic character of ceragenins for antiviral activities, compounds **2-18**, **2-12**, **CSA-13**, **2-22**, **2-25**, **2-26** were prepared (Scheme 2-6, 2-7 and 2-8). The only difference of these compounds is that different amines were used to connect with C24. The procedure of preparing

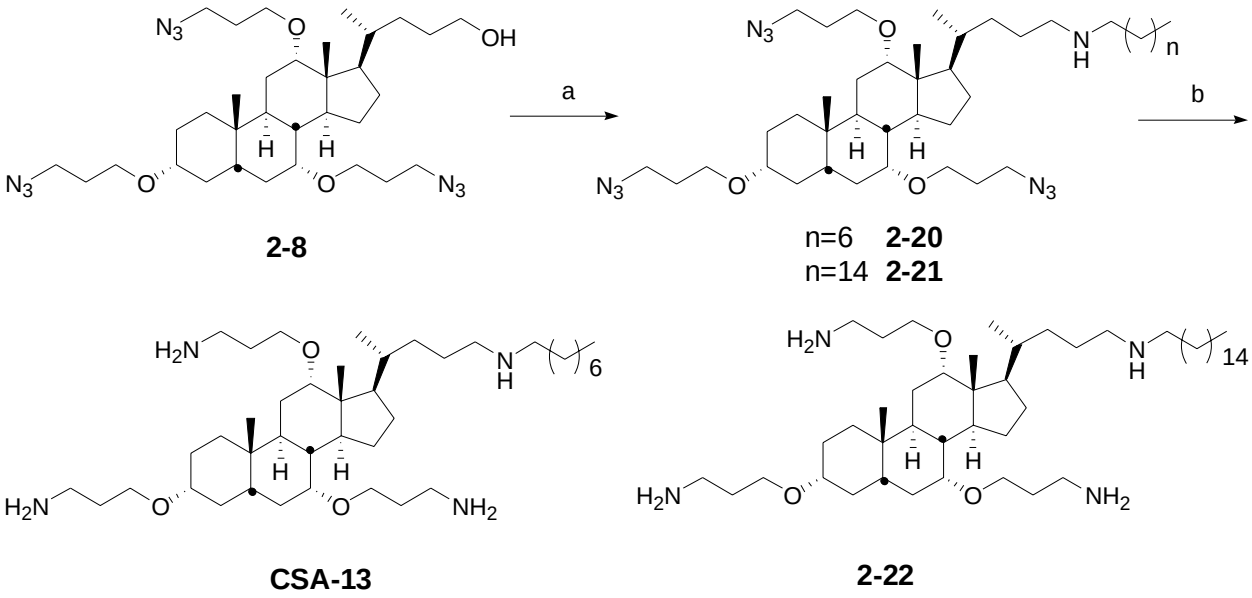
these compounds was similar. Taking **2-19** as example: compound **2-8** was converted to **2-17** by S_N2 reaction with pentylamine at C24, followed by reduction of **2-17** giving compound **2-19** (Scheme 2-6).

Scheme 2-6. Synthesis of **2-18**, **2-19**



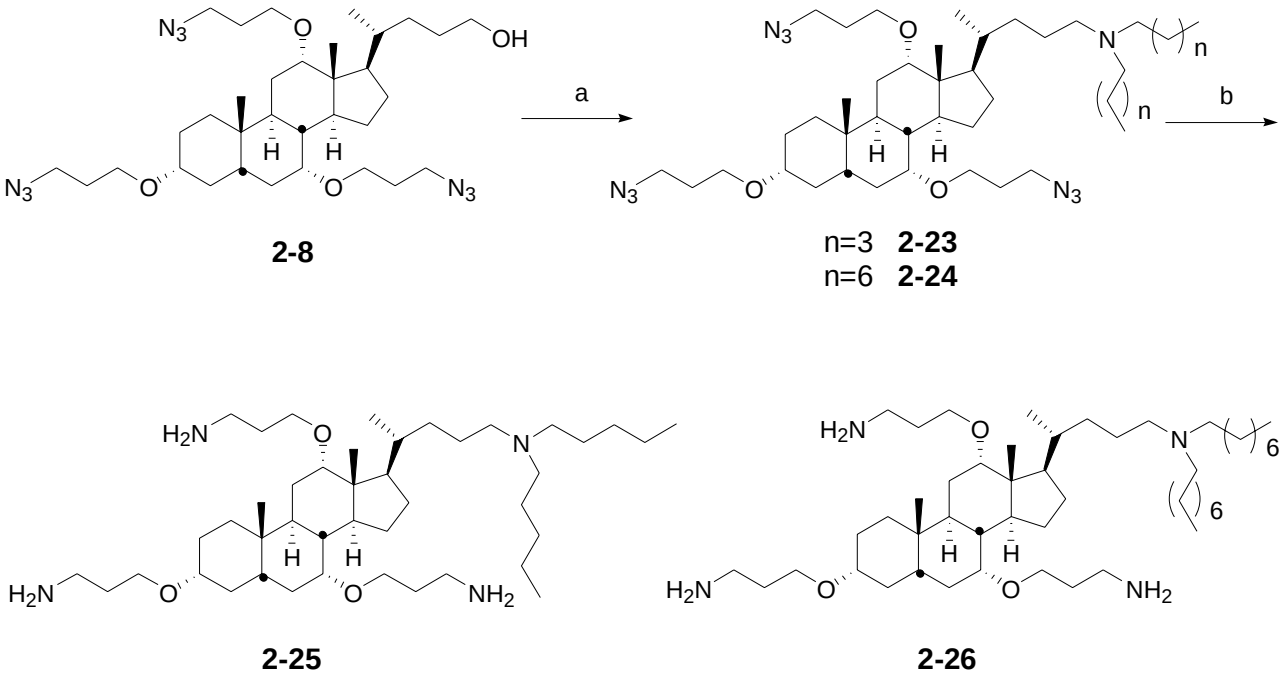
Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); propylamine or pentylamine (80%); b) Ph_3P, THF/H_2O (85%).

Scheme 2-7. Synthesis of **CSA-13**, **2-22**



Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); octylamine or hexadecylamine (80%); b) Ph_3P, THF/H_2O (85%).

Scheme 2-8. Synthesis of **2-25**, **2-26**



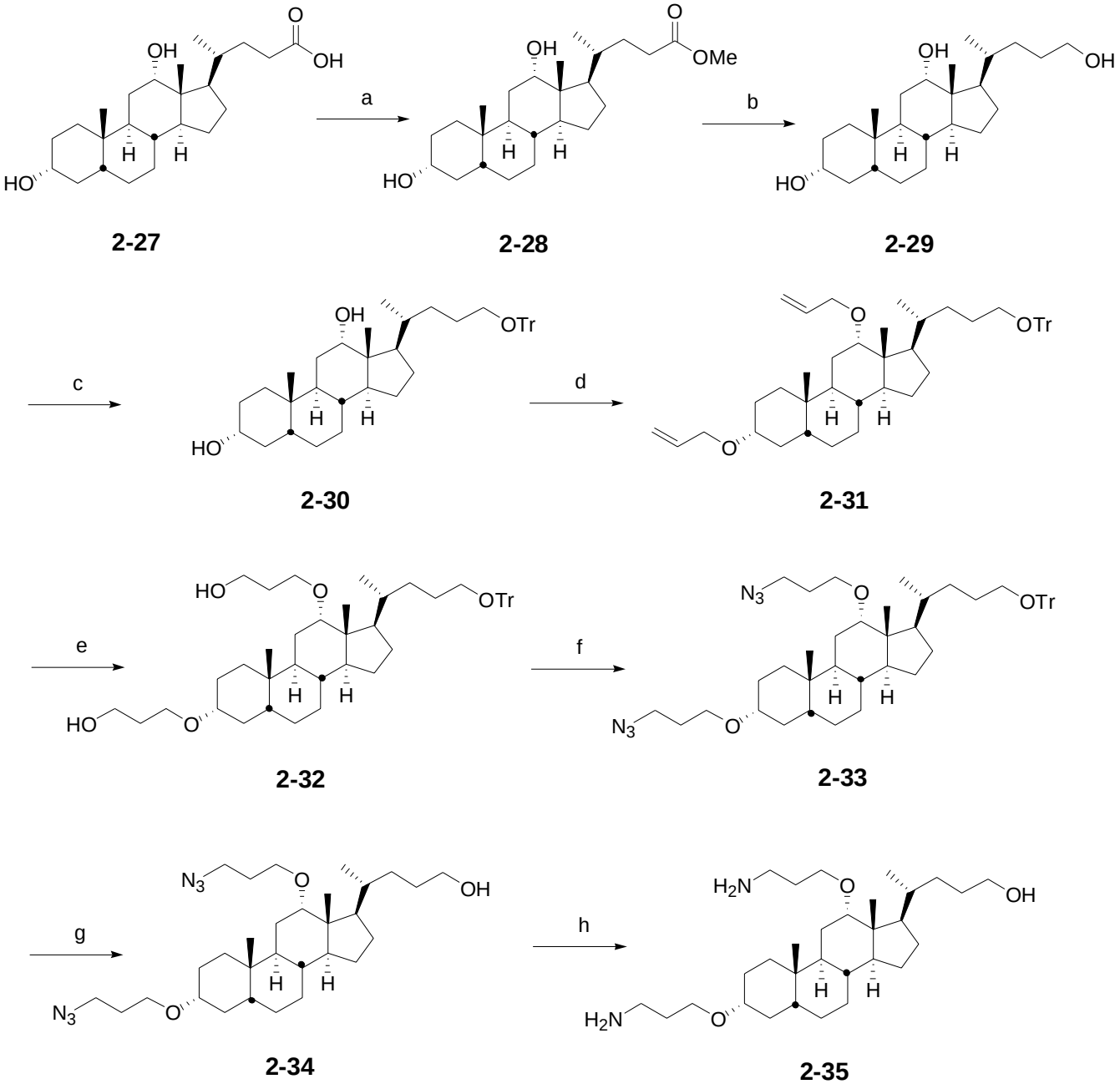
Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); dipentylamine or dioctylamine (80%); b) Ph_3P, THF/H_2O (85%).

Compound **2-35** was prepared from deoxycholic acid (Scheme 2-9). deoxycholic acid **2-27** was converted to its methyl ester **2-28** with 2,2-dimethoxypropane in methanol. Reduction of **2-28** by $LiAlH_4$ afforded **2-29**. The primary hydroxyl group of **2-29** was then protected with trityl group giving **2-30**. Treatment of **2-30** with allyl bromide provided **2-31**. Hydroboration-oxidation of **2-31** yielded alcohol **2-32**. Compound **2-32** was converted to azide **2-33**, followed by removal of the trityl group of **2-33** giving **2-34**. Reduction of **2-34** afforded compound **2-35**.

The preparation of **2-44** is very similar to that of **2-35**. It started with chenodeoxycholic acid **2-36**, which was converted to its methyl ester **2-37**. After reduction and protection with trityl group of **2-38**, compound **2-39** was obtained. Treatment of **2-39** with allyl bromide provided **2-40**.

Hydroboration-oxidation of **2-40** yielded alcohol **2-41**. Compound **2-41** was converted to azide **2-42**, followed by removal of the trityl group of **2-42** giving **2-43**. Reduction of **2-43** afforded compound **2-44** (Scheme 2-10).

Scheme 2-9. Synthesis of **2-35**



2-27 **2-28** **2-29**

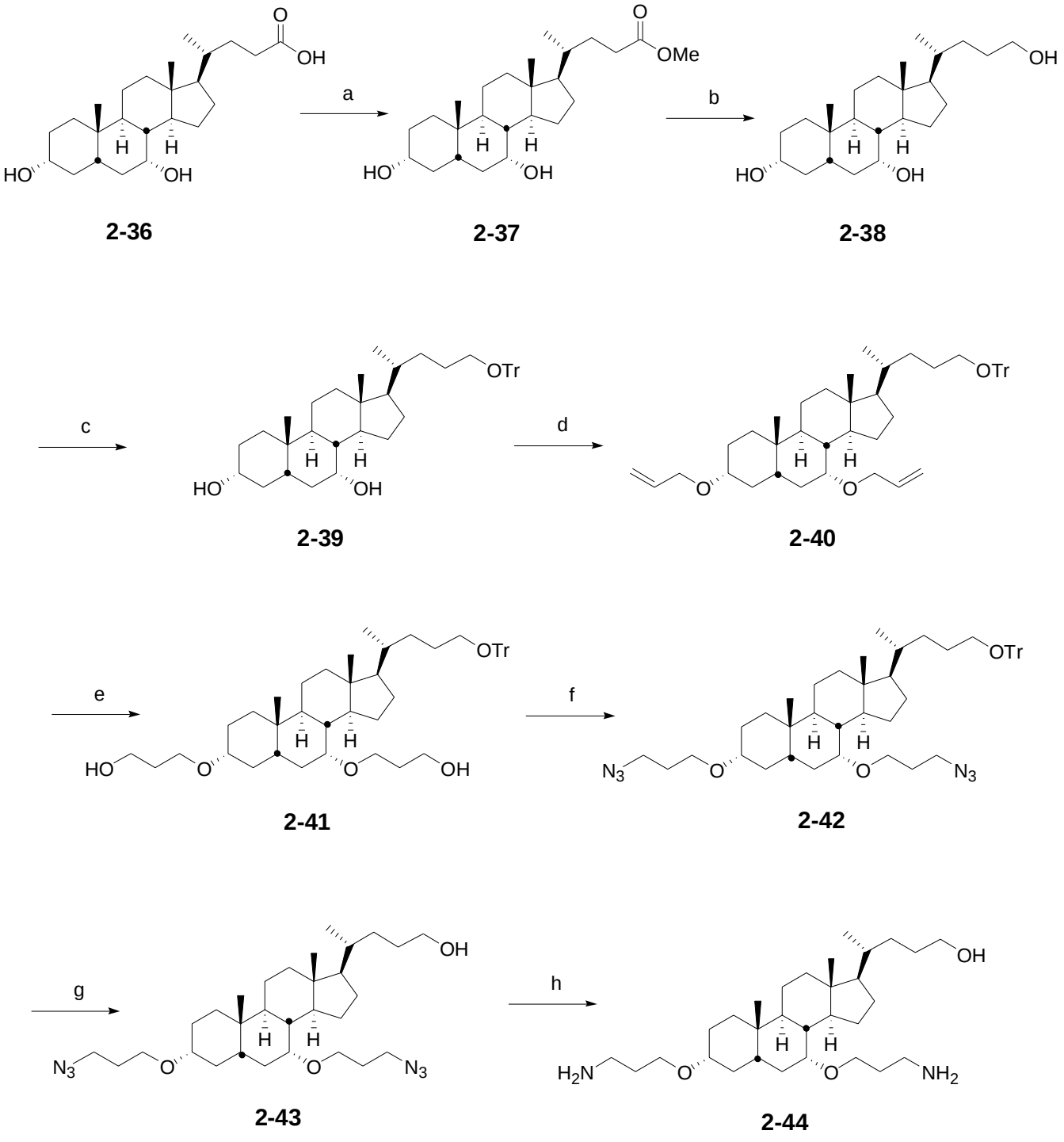
2-30 **2-31**

2-32 **2-33**

2-34 **2-35**

Reagents (yields in parentheses): a) 2,2-dimethoxypropane, *p*-TsOH, methanol, 70℃ (96%); b) $LiAlH_4$, THF, reflux (93%); c) triphenylmethyl chloride, DMAP, Et_3N, THF, reflux (72%); d) allyl bromide, NaH, THF, reflux (81%); e) dicyclohexylborane, THF; H_2O_2, NaOH (82%); f) MsCl, Et_3N; NaN_3, DMF/H_2O (81%); g) *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (91%); h) Ph_3P, THF/H_2O (85%).

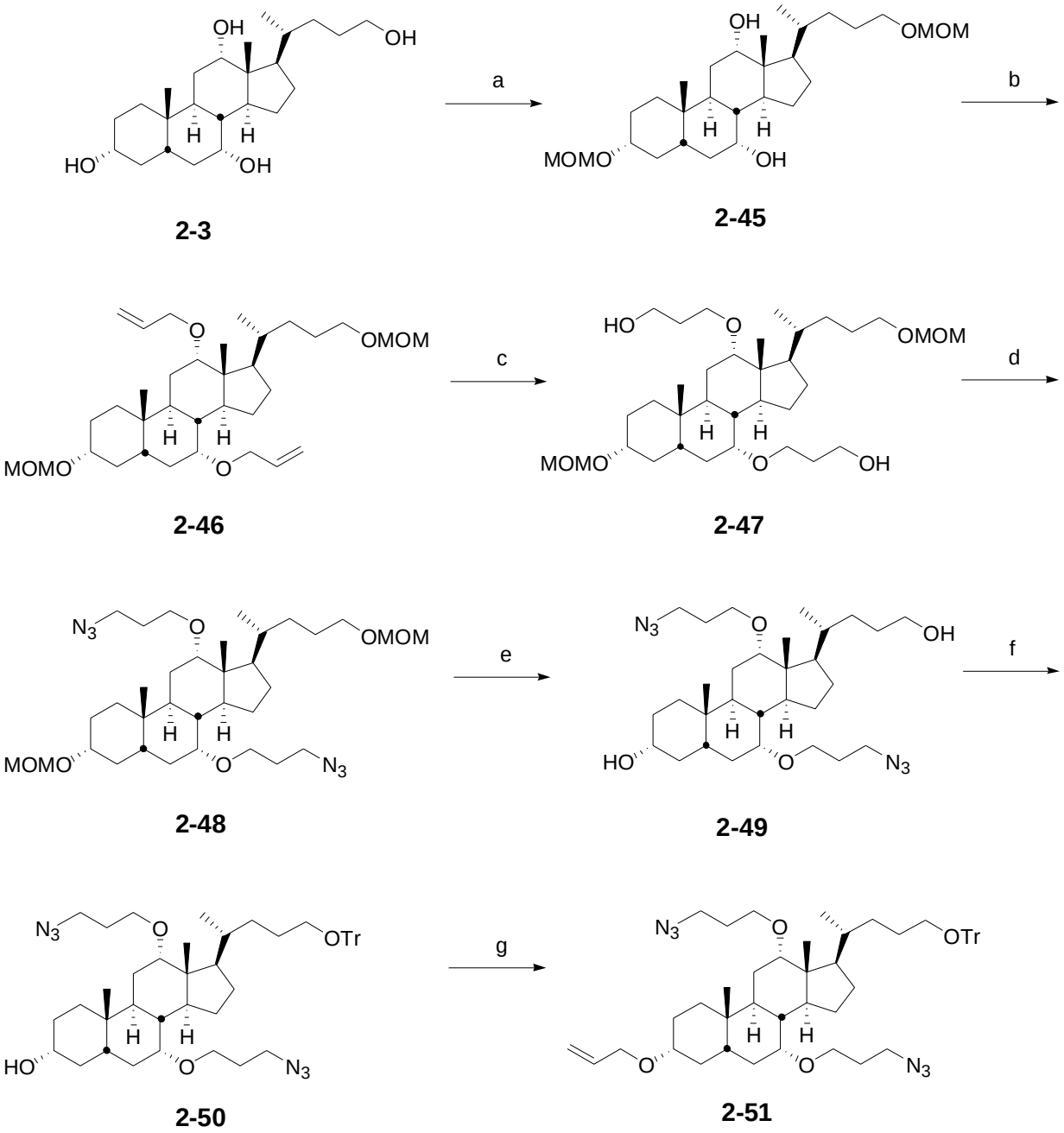
Scheme 2-10. Synthesis of **2-44**



Reagents (yields in parentheses): a) 2,2-dimethoxypropane, *p*-TsOH, methanol, 70℃ (98%); b) $LiAlH_4$, THF, reflux (90%); c) triphenylmethyl chloride, DMAP, Et_3N, THF, reflux (72%); d) allyl bromide, NaH, THF, reflux (85%); e) dicyclohexylborane, THF; H_2O_2, NaOH (83%); f) MsCl, Et_3N; NaN_3, DMF/H_2O (82%); g) *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (92%); h) Ph_3P, THF/H_2O (83%).

2.3.2 Synthesis of ceragenins in Aim 2

Scheme 2-11. Synthesis of **2-51**



2-3 **2-45** **2-46** **2-47** **2-48** **2-49** **2-50** **2-51**

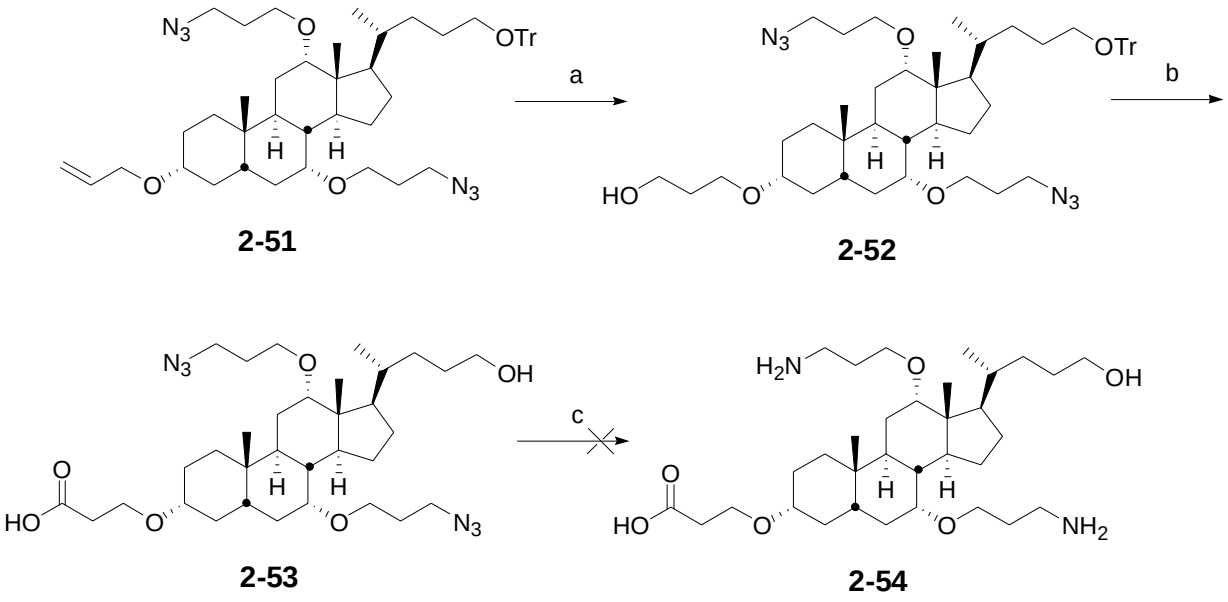
Reagents (yields in parentheses): a) MOMCl, Et_3N, THF (60%); b) allyl bromide, NaH, THF (80%); c) dicyclohexylborane, THF; H_2O_2, NaOH (80%); d) MsCl, Et_3N; NaN_3, DMF/H_2O (80%); e) HCl, MeOH (95%); f) TrCl, pyridine, THF (90%); g) allyl bromide, NaH, THF (90%);

Compounds **2-51** is the key intermediate for making the ceragenins in Aim 2, and the synthesis of it is shown in Scheme 2-11. The hydroxyl group at C3 and

C24 of compound **2-3** were selectively protected with MOM group giving **2-45**. Treatment of **2-45** with allyl bromide provided **2-46**. Hydroboration-oxidation of **2-46** yielded alcohol **2-47**. Compound **2-47** was converted to azide **2-48**, followed by removal of the MOM group of **2-48** giving **2-49**. The primary hydroxyl group of **2-49** was then protected with trityl group giving **2-50**. Treatment of **2-50** with allyl bromide provided **2-51**.

The attempt to synthesize **2-54** is shown in Scheme 2-12. Hydroboration-oxidation of **2-51** yielded alcohol **2-52**. The primary hydroxyl group was oxidized to carboxylic acid group with TEMPO/BAIB, followed by removal of trityl group giving **2-53**. But the reduction of **2-53** to **2-54** under H_2 failed because of the problem of catalyst.

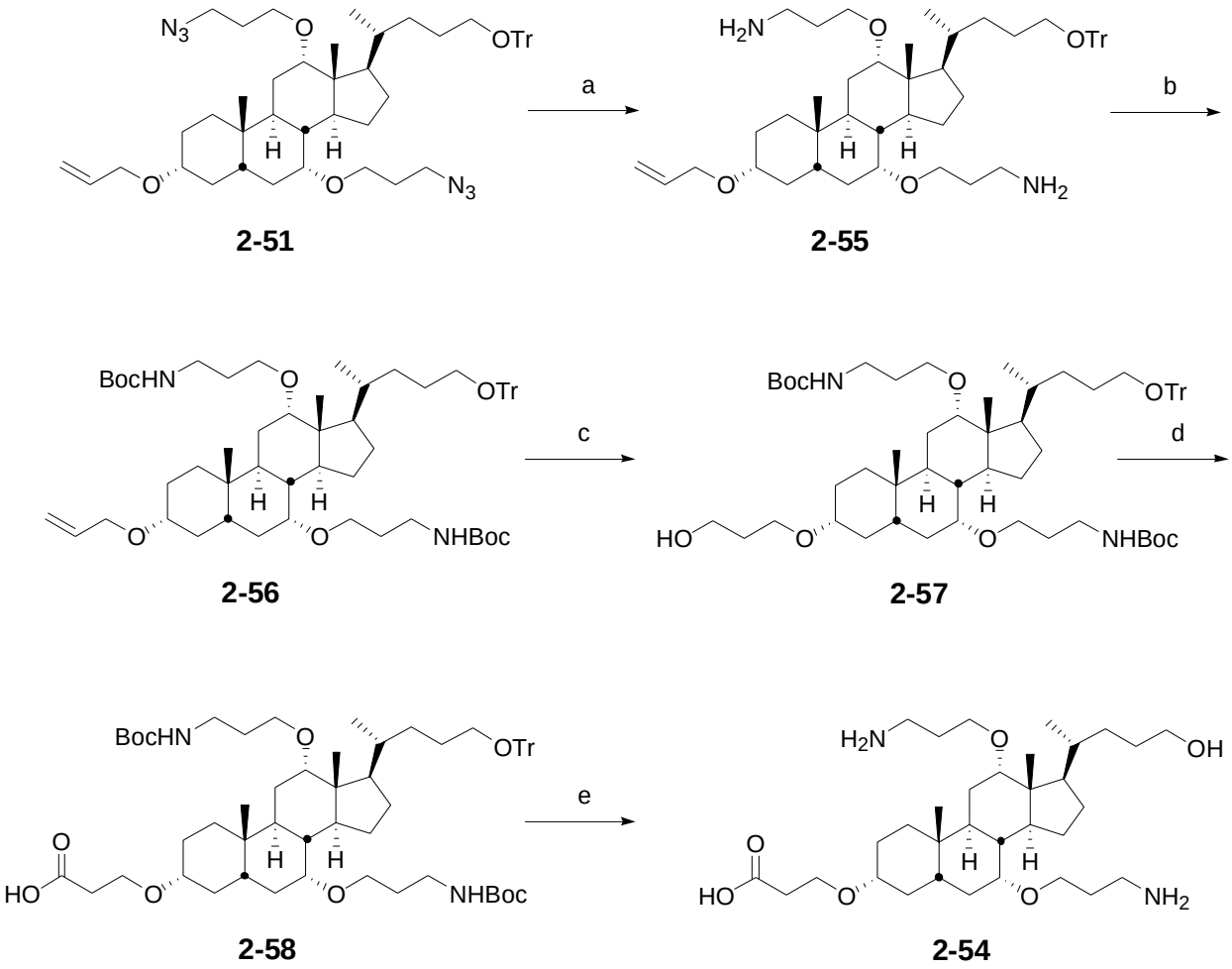
Scheme 2-12. Attempt synthesis of **2-54**



Reagents (yields in parentheses): a) dicyclohexylborane, THF; H_2O_2, NaOH (85%); b) TEMPO/BAIB, CH_2Cl_2/H_2O (75%); *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (90%); c) H_2, C/Pd.

In this case, another synthesis route was designed to get compound **2-54**, which is shown in Scheme 2-13. Reduction of the azide group of **2-51** with triphenylphosphine, followed by protection with Boc group afforded compound **2-56**. Hydroboration-oxidation of **2-56** yielded alcohol **2-57**. Oxidation of the hydroxyl group of **2-57** to carboxylic group with TEMPO/BAIB gave **2-58**. Removal of trityl and Boc group of **2-58** by TFA gave compound **2-54**.

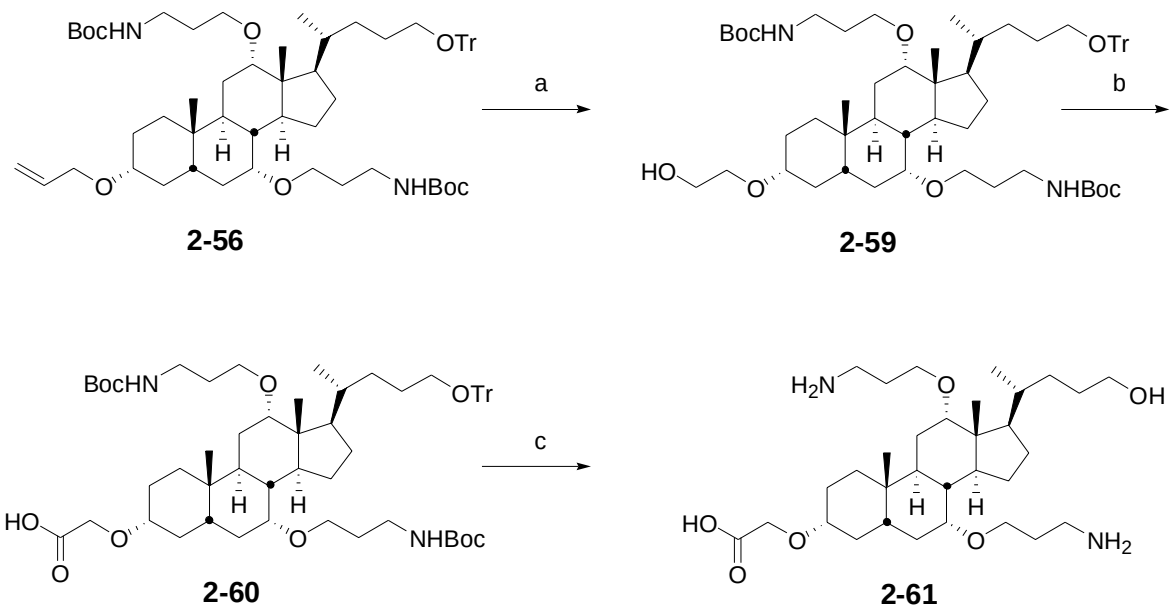
Scheme 2-13. Synthesis of **2-54**



Reagents (yields in parentheses): a) Ph_3P, THF/H_2O, (85%); b) Boc_2O, Et_3N, CH_2Cl_2 (95%); c) dicyclohexylborane, THF; H_2O_2, NaOH (85%); d) TEMPO/BAIB, CH_2Cl_2/H_2O (75%); e) TFA (85%).

The synthesis of compound **2-61** is similar to that of **2-54**, which is shown in Scheme 2-14. It started with compound **2-56**. Treatment of **2-56** with ozone and dimethyl sulfide followed by reduction with sodium borohydride gave the alcohol **2-59** which is one carbon less comparable to compound **2-57**. Oxidation of the hydroxyl group of **2-59** to carboxylic group with TEMPO/BAIB gave compound **2-60**. Removal of trityl and Boc group of **2-60** by TFA gave compound **2-61**.

Scheme 2-14. Synthesis of **2-61**

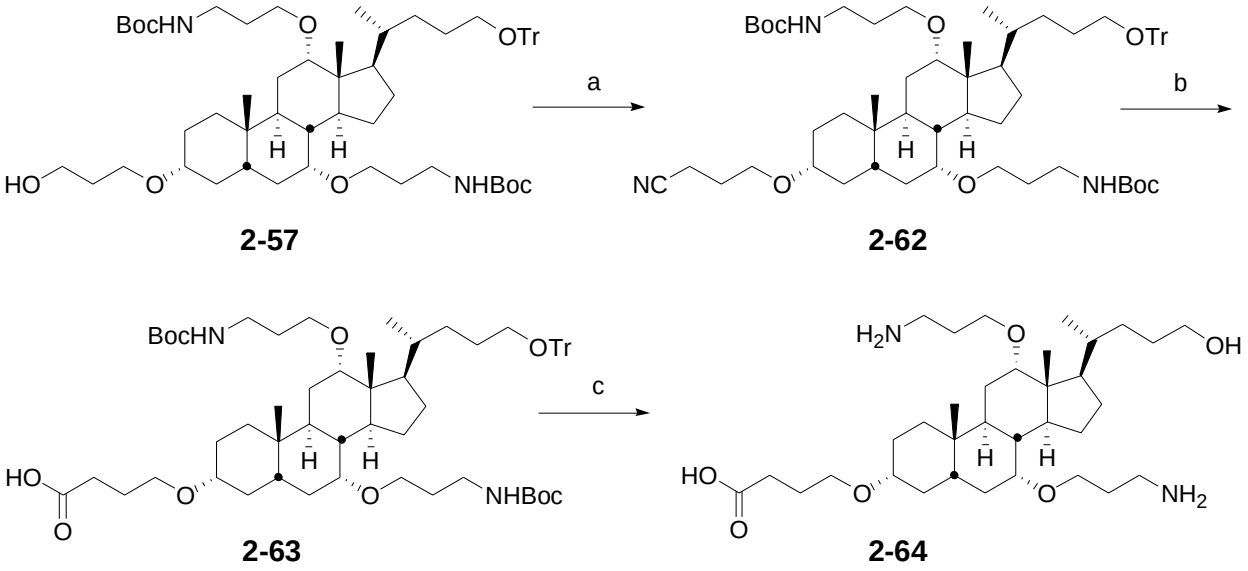


2-56 **2-59** **2-60** **2-61**

Reagents (yields in parentheses): a) O_3, Me_2S; $NaBH_4$ (65%); b) TEMPO/BAIB, CH_2Cl_2/H_2O (75%); e) TFA (85%).

The synthesis of **2-64** which is one more carbon than **2-54** started with **2-57** (Scheme 2-15). Compound **2-57** was converted to nitrile **2-62**, followed by hydrolysis of **2-62** giving carboxylic acid **2-63**. Removal of trityl and Boc group of **2-63** by TFA gave compound **2-64**.

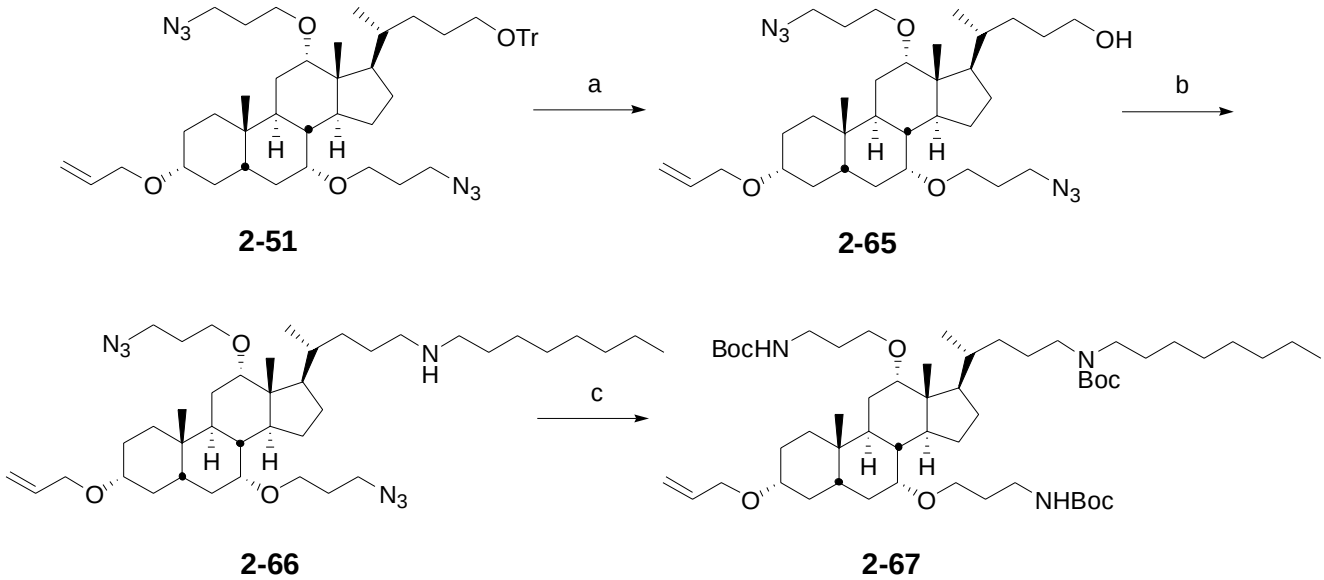
Scheme 2-15. Synthesis of **2-64**



Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); KCN, DMF/H_2O (80%); b) NaOH, EtOH/H_2O (75%); c) TFA (85%).

Compound **2-67** is the key intermediate for making **2-70**, **2-73** and **2-76**. The synthesis of it is shown is Scheme 2-16. Removal of trityl group of **2-51** gave **2-65**. **2-65** was then converted to **2-66** with octylamine. Reduction of the azide group of **2-66** followed by protection with Boc group afforded **2-67**.

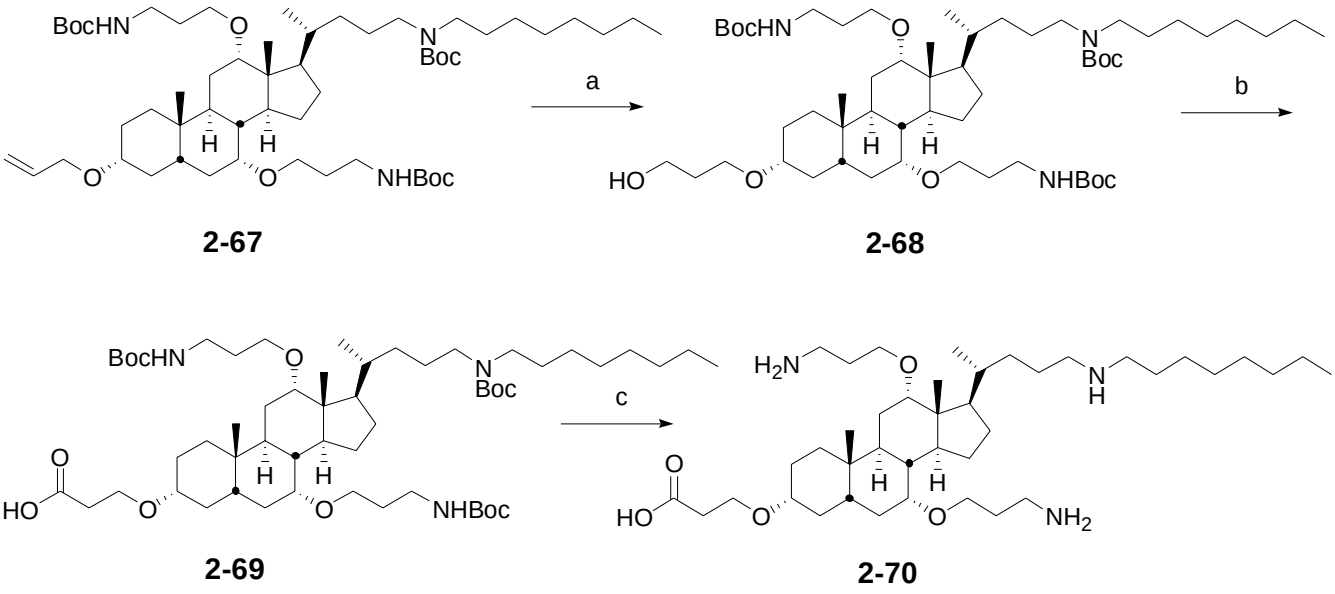
Scheme 2-16. Synthesis of **2-67**



Reagents (yields in parentheses): a) *p*-TsOH, MeOH/CH_2Cl_2 (1:1) (95%); b) MsCl, Et_3N, CH_2Cl_2 (95%); octylamine (80%); c) Ph_3P, THF/H_2O (85%); Boc_2O, Et_3N, CH_2Cl_2 (90%).

The synthesis of compound **2-70** started with compound **2-67**, which is shown is Scheme 2-17. Hydroboration-oxidation of compound **2-67** yielded alcohol **2-68**. Oxidation of the hydroxyl group of **2-68** to carboxylic group with TEMPO/BAIB gave compound **2-69**. Removal of Boc group of **2-69** by TFA gave compound **2-70**.

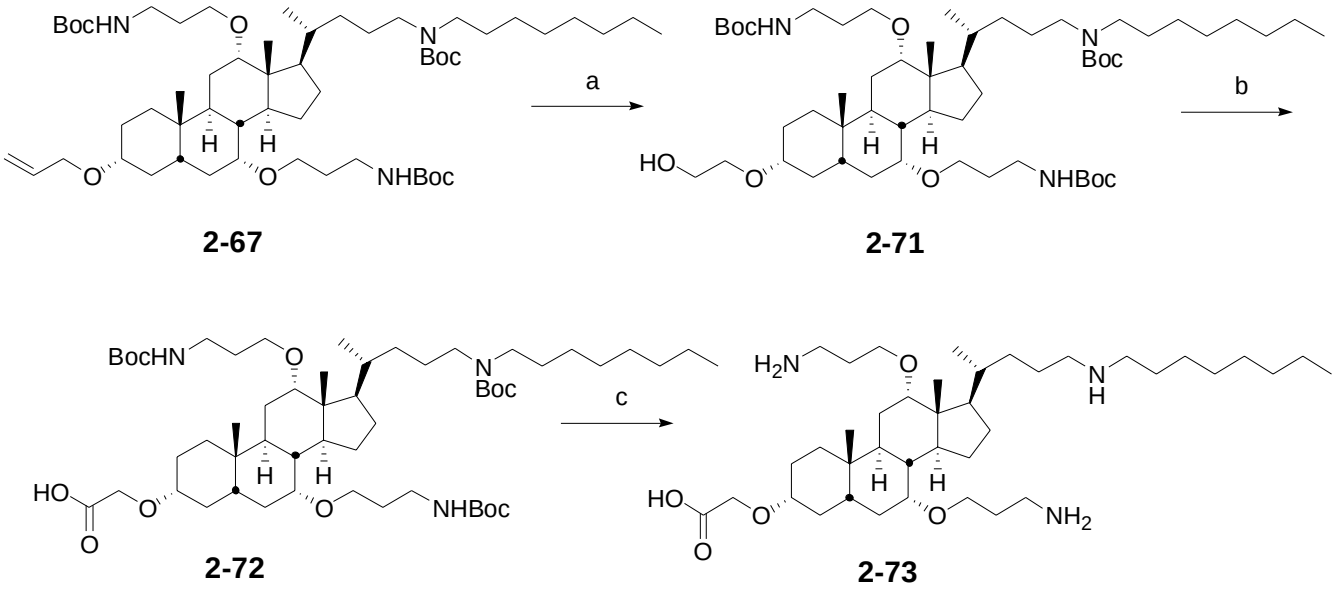
Scheme 2-17. Synthesis of **2-70**



Reagents (yields in parentheses): a) dicyclohexylborane, THF; H_2O_2, NaOH (85%); b) TEMPO/BAIB, CH_2Cl_2/H_2O (75%); c) TFA (85%).

The synthesis of compound **2-73** is shown in Scheme 2-18. Treatment of **2-67** with ozone and dimethyl sulfide followed by reduction with sodium borohydride gave the alcohol **2-71** which is one carbon less than compound **2-68**. Oxidation of the hydroxyl group of **2-71** to carboxylic group with TEMPO/BAIB gave compound **2-72**. Removal of Boc group of **2-72** by TFA gave compound **2-73**.

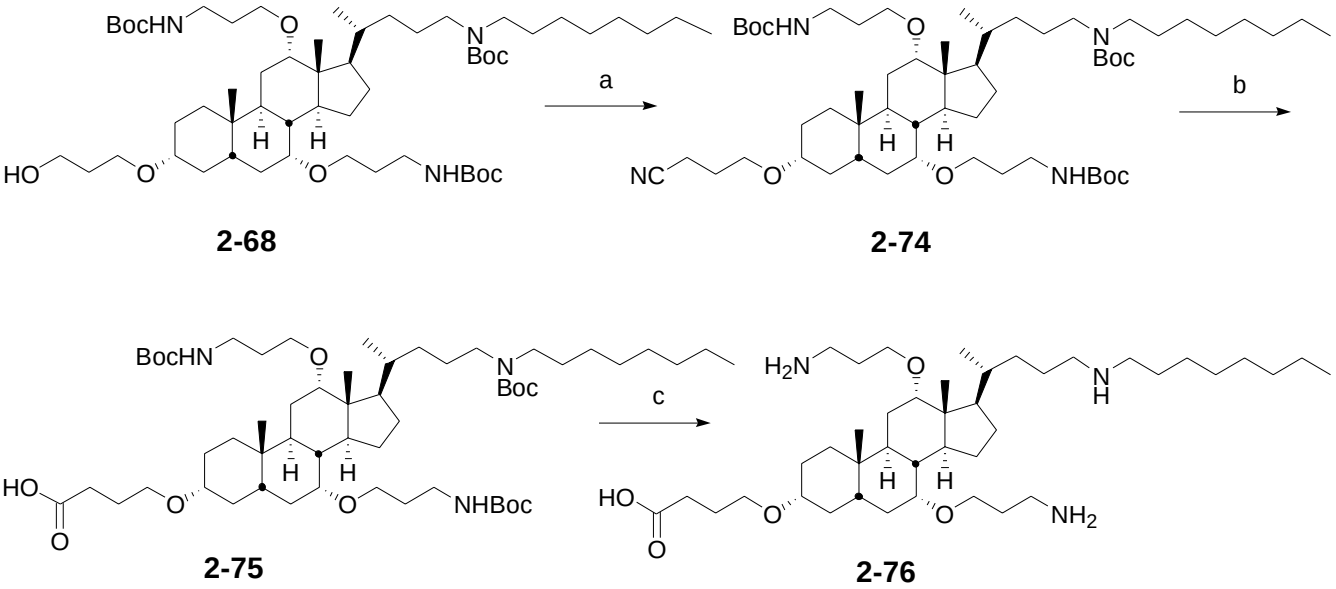
Scheme 2-18. Synthesis of **2-73**



Reagents (yields in parentheses): a) O_3, Me_2S; $NaBH_4$ (65%); b) TEMPO/BAIB, CH_2Cl_2/H_2O (75%); e) TFA (85%).

The synthesis of **2-76** which is one more carbon than **2-70** started with **2-68** (Scheme 2-19). Compound **2-68** was converted to nitrile **2-74**, followed by hydrolysis of **2-74** giving carboxylic acid **2-75**. Removal of Boc group of **2-75** by TFA gave compound **2-76**.

Scheme 2-19. Synthesis of **2-83**



Reagents (yields in parentheses): a) MsCl, Et_3N, CH_2Cl_2 (95%); KCN, DMF/H_2O (80%); b) NaOH, $EtOH/H_2O$ (75%); c) TFA (85%).

2.3.3 Studies of antiviral activities of ceragenins against VV

This work was done by Michael D. Howell and Joanne E. Streib in Dr. Donald Y. M. Leung's group in National Jewish Medical and Research Center and Department of Pediatrics of Division of Allergy and Immunology.

2.3.3.1 Virus preparation

The Wyeth strain of VV was obtained from the Centers for Disease Control and Prevention. Virus was propagated by inoculating confluent HeLa S3 human adenocarcinoma cells with 1 plaque forming unit (pfu) per cell. It was then incubated at 37°C in 5% CO_2 for three days. After forty-eight hours, media was removed and cells were fixed with formalin-crystal violet. Plaques were visualized on an Inverted Nikon Microscope under 1.3 x 10 magnification. Virus stocks were stored at -70°C.

2.3.3.2 Viral plaque assay

BS-C-1 African green monkey kidney cells were grown at 2 x 10^5/well in 24 well tissue culture plates in MEM (10% FCS, 1% penicillin/streptomycin). After overnight, the supernatant was removed and replaced with MEM (2.5% FCS) for virus incubation. Proper concentrations of ceragenins were prepared in tryptic soy broth containing 10 mM sodium phosphate buffer (pH=7.4). Virus was diluted in the same buffer and was added to the ceragenins. After incubation for 24 hours at 37°C, they were added by twenty microliters of the peptide/virus mixture in 0.5 ml MEM-2.5% FCS. After another 48 hours, medium was removed and wells were overlaid with 0.5 ml 4% buffered

formalin. They were then allowed to fix for 10 minutes at room temperature. After removal of formalin, 0.5 ml 0.1% crystal violet in PBS was added to the wells for 5 minutes at room temperature. The wells were then aspirated and air-dried for visualization of plaques. It was found that the most accurate results with the virus alone forming 50 to 80 plaques per well.

2.3.3.3 Antiviral activities of ceragenins against VV

Using a standard viral plaque assay, the antiviral activity of multiple structurally modified ceragenins was evaluated. The results are shown in Table 1. Data are expressed as percent killing. Significant antiviral activity was defined as greater than 50% viral killing.

Table 1. Antiviral activity of CSAs against VV

	CSA Concentration					
	1 μM	**5 μM**	**10 μM**	**25 μM**	**50 μM**	**100 μM**
2-26	**89.69±1.62**	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
2-22	**59.13±1.73**	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
2-25	35.27±3.48	**76.33±6.17**	85.51±2.21	92.27±1.28	95.17±1.28	100.00±0.00
CSA-13		**62.50±4.59**	91.18±1.10	96.69±0.64	100.00±0.00	100.00±0.00
CSA-8		38.13±2.02	**71.21±1.03**	95.33±0.67	97.67±0.67	98.44±0.39
2-64	27.70±1.24	43.66±9.16	49.30±4.07	**59.15±2.15**	68.08±1.69	86.38±3.29
2-61	<1	<1	8.74±4.85	49.03±2.52	49.03±2.52	**72.82±2.70**
2-54	2.71±3.17	11.76±2.83	21.72±6.57	15.84±3.59	33.03±2.75	**65.16±4.02**
2-73	13.94±2.54	11.06±1.92	8.41±3.16	16.35±2.20	32.69±1.27	36.06±2.68
2-76	11.01±4.08	17.43±4.20	16.06±0.79	14.22±1.65	23.39±2.79	31.19±2.86
2-70	1.94±4.63	<1	0.55±5.52	1.66±2.46	1.94±4.26	3.05±0.73
CSA-31		0.76±1.98	0.76±1.98	<1	<1	<1

The structures of these ceragenins and their abilities against VV were shown in Figure 2-12.

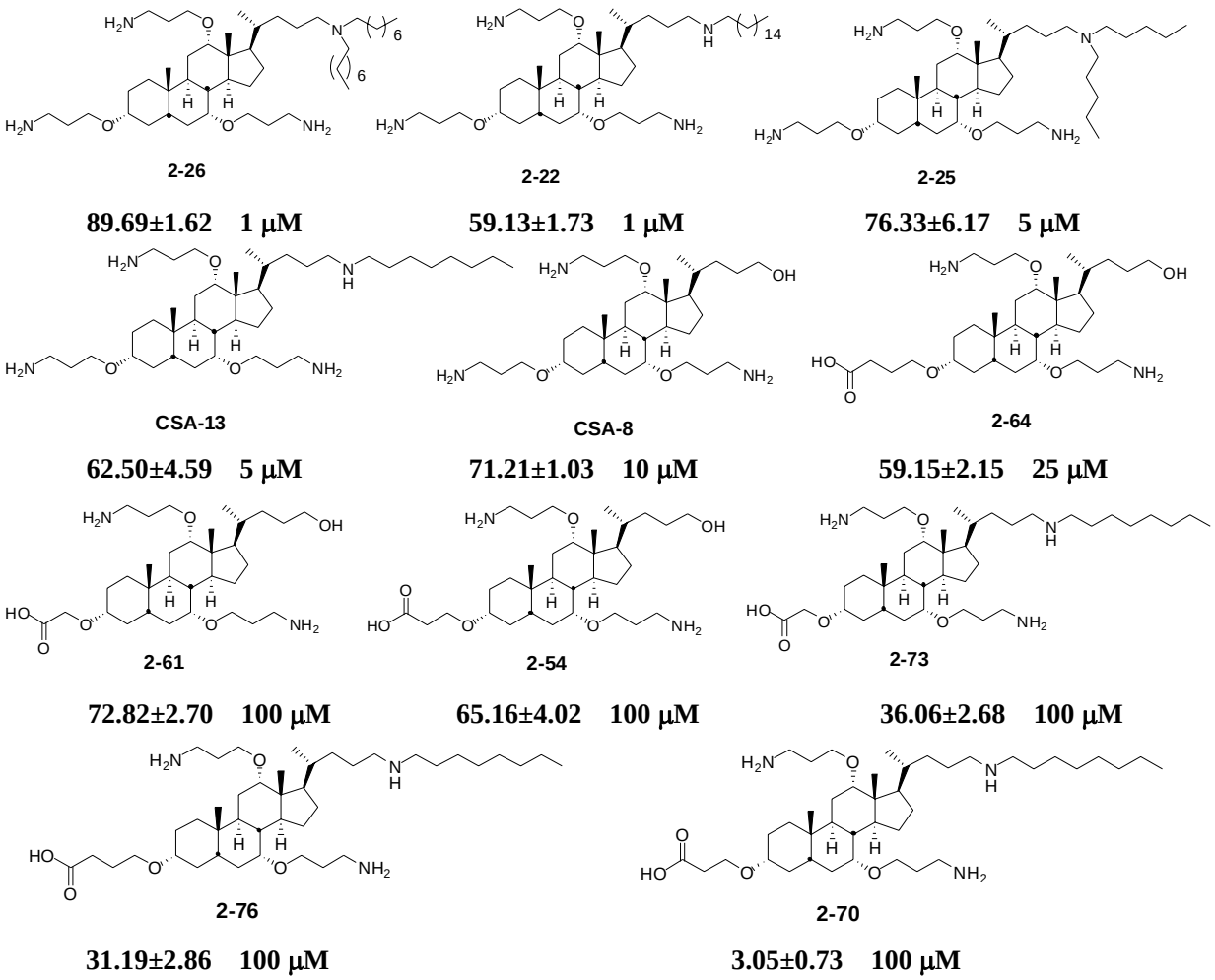


Figure 2-12. Structures of ceragenins and their abilities against VV

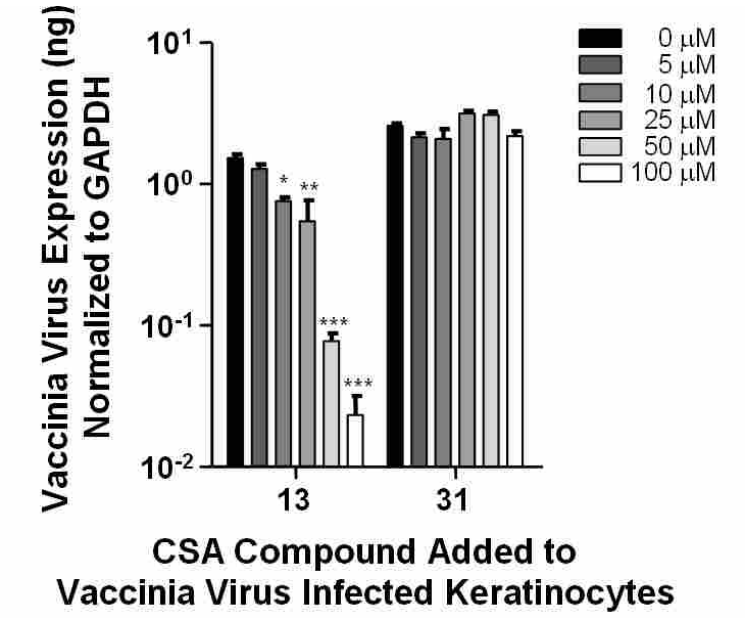
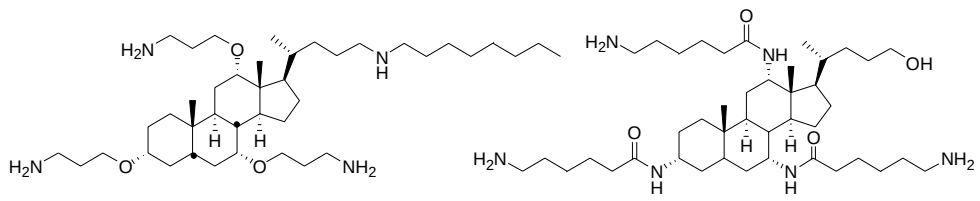


Figure 2-13. Comparison of antiviral activity of CSA-13 and CSA-31

Scheme 2-20. Structures of CSA-13 and CSA-31



CSA-13 CSA-31

In addition, CSA-13 also protected the viability of the keratinocytes (Figure 2-14).

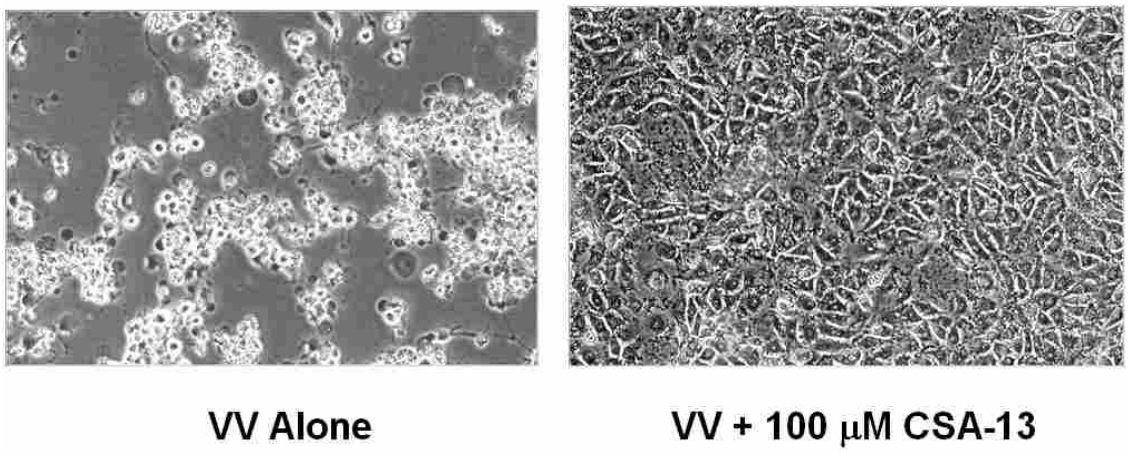


Figure 2-14. Viability of the keratinocytes

2.4 Discussion

It is believed that the facially amphiphilic structure of ceragenins plays a key role in the antibacterial activity. In CSA-13, relatively short tethers between the amines and the steroid scaffolding enforce orientation on one face. The tethers in CSA-31, being significantly longer, could possibly fold up on the hydrophobic face of the cholic acid nucleus resulting in a loss of facial amphiphilicity. The lack of antiviral activity of CSA-31 suggests that conformations necessary for antibacterial are similar to those for antiviral activity.

The structures of CSA-8, CSA-13, and CSA-54 are similar except different

groups attached at C24. CSA-13 bears the most hydrophobic character because of the long lipid chain. In contrast, CSA-54 shows almost no hydrophobic character due to the additional positive charges at the chain. CSA-13 shows maximum antiviral activity. However, CSA-54 is only weakly active for antiviral activity. This result suggests that the lipid chain at C24 plays a very important role in antiviral activity. This idea is also supported by the antiviral activity of compound **2-33** and **2-22** (Table 1). For both **2-33** and **2-22**, they possess more hydrophobic characters than CSA-13. Therefore, it is not surprising that they show much better antiviral activity than CSA-13.

Because of the weak antibacterial activity of **2-22**, it shows that antibacterial and antiviral activities do not correlate to each other. Compounds **2-54**, **2-61**, **2-64**, **2-70**, **2-73**, **2-76** were prepared to study the mechanism for antiviral activity of ceragenins. These compounds all bear both cationic and anionic groups. Considering the fact that lipid enveloped virus obtain their lipids from host cells, the zwitterionic nature of viral envelops may be best targeted by these compounds. However, all of these compounds show only weak antiviral activity or no activity (Table 1). So it can be anticipated that the mechanism of antiviral activity of ceragenins is different from that of antibacterial activity.

2.5 Conclusion

We prepared a series of ceragenins to optimize the antiviral activity of ceragenins against VV. The results show that ceragenins exhibit potent antiviral activity against VV, protect keratinocytes against VV-mediated cell death, and

preferentially target the virus. It also shows that antibacterial and antiviral activities do not correlate with each other. Although ceragenins show good antiviral activity, the mechanism for this activity still remains unclear.

2.6 Experimental Section

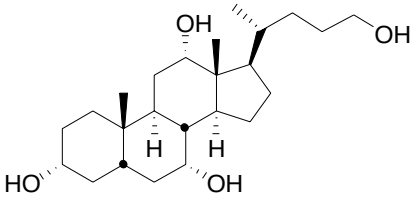
General: Reagents were purchased from Aldrich Chemical Co. unless otherwise noted. Methylene chloride, THF, DMF and DMSO were dried by passage through a Glass Contour solvent drying system containing a cylinder of activated alumina. Silica gel was used for chromatography unless otherwise noted.

Instrumentation: ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 500 (500 MHz) spectrometer. Proton chemical shift were referenced to tetramethylsilane (TMS) and *d*-MeOH. Carbon chemical shifts were referenced to carbon resonance of solvents ($CDCl_3$ and *d*-MeOH). High resolution electron impact mass spectra (HR-MS) were obtained on a JOEL SX 102A spectrometer.

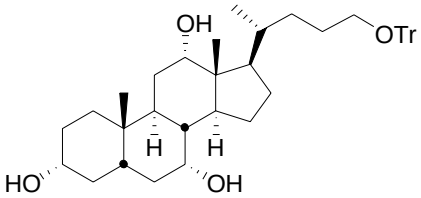
Experiments:

Compound 2-3: To a 1 L round-bottom flask were added **2-2** (30.67 g, 72.7 mmol) in dry THF (600 mL) and $LiAlH_4$ (4.13 g, 109 mmol). After reflux 48 hr, saturated aqueous Na_2SO_4 (100mL) was introduced slowly, and the resulted precipitate was filtered out and washed with hot THF and MeOH. Recrystalization from MeOH gave **2-3** (28.2 g, 98%). ^{1}H NMR ($CDC1_3$/MeOH-d4, 500 MHz) δ 3.98 (bs, 1 H), 3.83 (bs, 1 H), 3.60-3.46 (m, 2 H), 3.38 (bs, 5 H), 2.30-2.10 (m, 2H), 2.05-1.05 (m, 22 H), 1.03 (bs, 3 H), 0.92 (s, 3 H). 0.71 (s, 3 H); ^{13}C NMR ($CDCl_3$/MeOH-d4, 500 MHz) δ 73.89, 72.44, 68.99, 63.51, 48.05, 47.12, 42.49, 40.37, 39.99, 36.62, 36.12, 35.58, 35.40,

32.77, 30.69, 30.04, 29.02, 28.43, 27.27, 23.96, 23.08, 18.00, 13.02; FAB-MS: $[M+H]^+$ 417.2992; cacld: 417.2981.



2-3

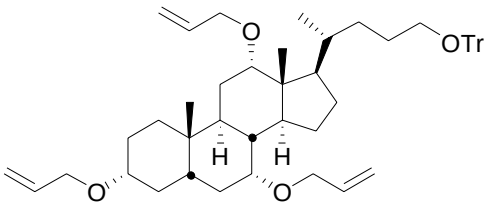


2-4

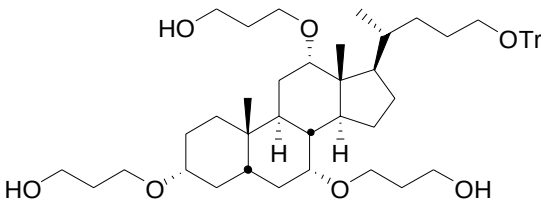
Compound 2-4: To a round-bottom flask were added **2-3** (28.2 g, 71.7 mmol) in THF (300 mL), Et_3N (20 mL, 143.4 mmol), trityl chloride (25.98 g, 93.2 mmol) and DMAP (0.13 g, 1.07 mmol). The mixture was refluxed under N_2 for 30 hr followed by the introduction of water (800 mL) and extraction with EtOAc (5 x 200 mL). The combined extracts were washed with water and brine and then dried over $MgSO_4$. After removal of solvent in vacuo, the residue was purified using column chromatography (SiO_2, EtOAc) to give **2-4** (31.0 g, 70%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.46-7.42 (m, 6 H), 7.32-7.17 (m, 9 H), 3.97 (bs, 1 H), 3.83 (bs, 1 H), 3.50-3.38 (m, 1 H), 3.01 (bs, 1 H), 2.94 (dd, J = 14.2, 12.2 Hz, 2 H), 2.64 (bs, 1 H), 2.51 (bs, l H), 2.36-2.10 (m, 2 H), 2.00-1.05 (m, 22 H), 0.96 (d, J = 5.8 Hz, 3 H), 0.87 (s, 3 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.77, 128.93, 127.91, 127.01, 86.43, 73.35, 72.06, 68.66, 64.28, 47.47, 46.53, 41.74, 41.62, 39.64, 35.57; 35.46, 34.91, 34.82, 32.40, 30.55, 28.21, 27.69, 26.80, 26.45, 23.36, 22.59, 17.83, 12.61; FAB-MS: $[M+Na]^+$ 659.4069; cacld: 659.4076.

Compound 2-5: To a round-bottom flask were added **2-4** (20.0 g, 31.4 mmol) in dry THF (600 mL) and NaH (60% in mineral oil, 6.3 g, l57.2 mmol). The

mixture was refluxed for 30 min under N_2 followed by addition of allyl bromide (27 mL, 314 mmol). After 24 h at reflux, water (20 mL) was introduced slowly. The mixture was then extracted with ether (3 x 100 mL) and the combined extracts were washed with brine (2 x 100 mL). The ether solution was dried over anhydrous Na_2SO_4, and after removal of solvent, the residue was purified using column chromatography (SiO_2, 1:8 EtOAc/hexane) to give **2-5** (22.76 g, 96%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.48-7.30 (m, 6 H), 7.32-7.14 (m, 9 H), 6.04-5.80 (m, 3 H), 5.36-5.04 (m, 6 H), 4.14-3.94 (m, 4 H), 3.74 (td, J = 13.8,5.8 Hz, 2 H), 3.53 (bs, 1 H), 3.20-2.94 (m, 3 H), 3.31 (bs, 1 H), 2.38-1.90 (m, 4 H), 1.90-0.96 (m, 20 H), 0.90 (d, J = 5.4 Hz, 3 H), 0.89 (s, 3 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.83, 136.27, 136.08, 128.94, 127.90, 126.98, 116.46, 115.70, 86.42, 80.94, 79.29, 74.98, 69.52, 69.39, 68.86, 64.39, 46.51, 46.42, 42.67, 42.14, 39.92, 35.63,3 5.51, 35.13, 32.45, 28.98, 28.09, 27.66, 27.57, 26.72, 23.32, 23.11, 17.92, 12.69; FAB-MS: $[M+Na]^+$ 779.5013; cacld: 779.5015.



2-5



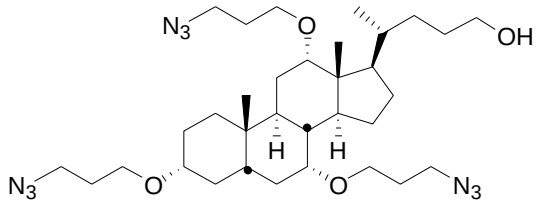
2-6

Compound 2-6: A round-bottom flask was charged with THF (80 mL) and cooled to 0℃, Borane-THF complex (150 mL, 0.15 mol) and cyclohexene(31 mL, 0.3 mol) were added sequentially, and the mixture was stirred at 0℃ for 3 h under N_2. **2-5** (15 g, 0.04 mol) in THF (50 mL) was added dropwise and the

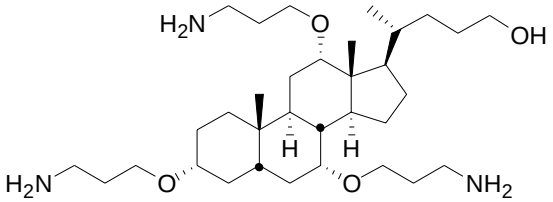
mixture was stirred for 12 h at room temperature. Aqueous NaOH (20%) (80 mL) and H_2O_2 (50 mL) were added in sequence. The mixture was refluxed for 3 h followed by the addition of brine (100 mL) and extracted with EtOAc (3× 100 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2) to afford **2-6** (10.3 g, 80%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.50-7.42 (m, 6 H), 7.34-7.16 (m, 9 H), 3.90-3.56 (m, 13 H), 3.50 (bs, 1 H), 3.40-2.96 (m, 6 H), 2.30-0.94 (m, 30 H), 0.90 (s, 3 H), 0.88 (d, J = 5.4 Hz, 3 H), 0.64 (s, 3 H); ^{13}C NMR($CDCl_3$, 500 MHz) δ 144.73, 128.88, 127.85, 126.94, 86.36, 80.52, 78.90, 76.36, 66.82, 66.18, 65.77, 64.22, 61.53, 6l.41, 61.34, 46.89, 46.04, 42.60, 41.59, 39.60, 35.37, 35.27, 34.88, 32.75, 32.44, 32.31, 28.82, 27.65, 27.48, 27.13, 26.77, 23.35, 22.74, 22.38, 18.08, 12.48; FAB-MS: $[M+Na]^+$ 833.5331; cacld: 833.5332.

Compound 2-8: To a round-bottom flask were added **2-6** (10 g, 0.01 mol) and Et_3N (10 mL, 0.06 mol) in CH_2Cl_2 (100 mL). The mixture was cooled to 0℃ followed by addition of methyl chloride (14.4 mL, 0.06 mol). After 1 hr, brine (20 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (3×40 mL) and the combined extracts was dried over anhydrous Na_2SO_4. The product was isolated as a colorless oil which was dissolved in DMF (100 mL) and H_2O (20 mL). NaN_3 (4.2 g, 0.8 mol) was added to the solvent and the mixture was heated to 80℃ and stirred for 16 hr. The mixture was then extracted with ether (5×20 mL) and the combined extracts was dried over anhydrous Na_2SO_4. After

removed the solvent under vacuum, the residue was dissolved in MeOH (30 mL) and CH_2Cl_2 (30 mL) followed by addition of catalytic amount of *p*-TsOH (20 mg, 0.1 mmol). The solution was stirred at room temperature for 3 hr before the addition of saturated $NaHCO_3$ solution (15 mL). The mixture was extracted with EtOAc (5×20 mL). The combined extracts were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. The residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-8** (7.1 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.68-3.56 (m, 3 H), 3.56-3.34 (m, 10 H), 3.28-3.00 (m, 4 H), 2.20-2.00 (m, 3 H) 1 98-1.55 (m, 15 H), 1.55-0.96 (m, 13 H), 0.92 (d, J = 6.6 Hz, 3 H), 0.89 (s, 3 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.63, 79.79, 76.04, 64.99, 64.45, 64.30, 63.72, 49.01, 48.94, 48.74, 46.49, 46.39, 42.70, 41.98, 39.80, 35.65, 35.42, 35.28, 35.08, 31.99, 29.78, 29.75, 29.70, 29.49, 29.06, 27.87, 27.79, 27.65, 23.53, 23.04, 22.85, 18.05, 12.59; FAB-MS: $[M+Na]^+$ 666.4415; cacld: 666.4431.



2-8



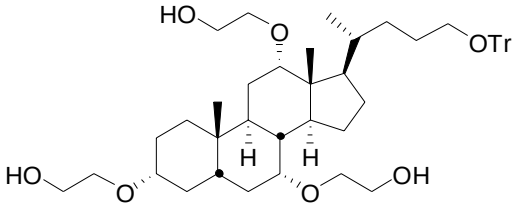
CSA-8

CSA-8: **2-8** (5.0g, 7.9 mmol) was dissolved in THF (25 mL) and H_2O (10 mL). Triphenylphosphine (10.4 g, 39.8 mmol) was added, and the solution was stirred for 12 hr. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to yield **CSA-**8 (4.0 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.73 (bs, 7 H), 3.74-3.70 (m, 1 H),

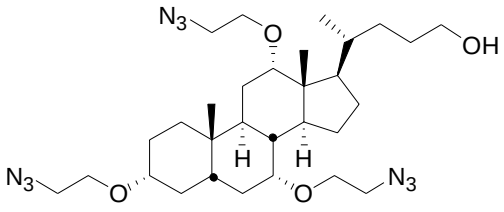
3.65-3.60 (m, 2 H), 3.56-3.52 (m, 4 H), 3.31-3.28 (m, 2 H), 3.16-3.09 (m, 2 H), 2.82- 2.71 (m, 6 H), 2.20-2.06 (m, 3 H) 1 98-1.66 (m, 15 H), 1.58-1.48 (m, 3 H), 1.38-0.98 (m, 7 H), 0.96 (d, J = 6.6 Hz, 3 H), 0.93 (s, 3 H), 0.71 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.80, 80.60, 77.17, 67.88, 67.86, 67.18, 60.73, 48.11, 47.28, 43.93, 42.99, 41.34, 40.76, 40.72, 40.24, 39.70, 36.33, 36.18, 35,86, 34.29, 33.99, 33.96, 33.83, 29.60, 29.00, 28.57, 28.54, 24.33, 23.59, 23.48, 18.86, 13.04; FAB-MS: $[M+H]^+$ 552.4756; cacld: 552.4772.

Compound 2-9: Ozone was bubbled into the solution of **2-5** (5.5 g, 7.29 mmol) in CH_2Cl_2 (80 mL) and MeOH (40 mL) until the solution turned a deep blue. Methylsulfide (5 mL) was added followed by the addition of $NaBH_4$ (2.0 g, 54 mmol) in 5% NaOH solution and methanol. The mixture was stirred overnight at rt and washed with brine until it became neutral. The brine was then extracted with CH_2Cl_2 (5×20 mL). The combined extracts were dried over Na_2SO_4. After removal of solvent in vacuo, the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2) to yield **2-9** (3.6 g, 65%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.46-7.42 (m, 6 H), 7.32-7.18 (m, 9 H), 3.77-3.57 (m, 10 H), 3.48-3.44(m, 2 H), 3.36-3.30 (m, 2 H), 3.26-3.20(m, 1 H), 3.04-2.99 (m, 2 H), 2.37-0.95 (m, 27 H), 0.92 (s, 3 H), 0.91 (d, J = 6.6 Hz, 3 H), 0.67 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.69, 128.87, 127.84, 126.94, 86.44, 81.05, 76.86, 74.65, 69.91, 69.22, 68.77, 64.24, 62.42, 62.26, 46.92, 46.54, 42.87, 39.73, 36.86, 35.52, 35.13, 32.82, 32.54, 30.36, 28.71, 27.61, 27.44, 26.79, 23.82, 23.51, 23.38, 23.31, 18.28, 12.74; FAB-MS: $[M+Na]^+$ 791.4844;

cacld: 791.4863.



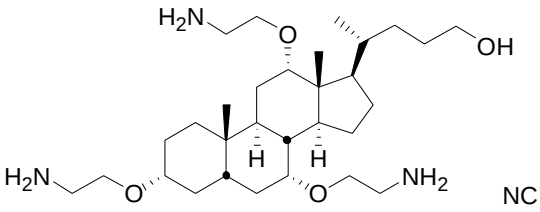
2-9



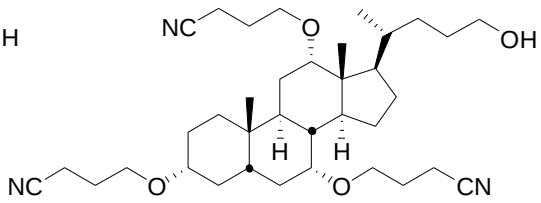
2-11

Compound 2-11: Same procedure as for **2-8** using **2-9**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.67-3.54 (m, 3 H), 3.54-3.33 (m, 6 H), 3.27-3.01 (m, 2H), 2.21-2.02 (m, 3 H) 1 97-1.54 (m, 15 H), 1.54-0.95 (m, 13 H), 0.91 (d, J = 6.6 Hz, 3 H), 0.88 (s, 3 H), 0.67 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.65, 79.81, 76.06, 64.98 64.46, 64.29, 63.75, 49.01, 48.95, 48.75, 46.50, 46.42, 42.71, 41.96, 39.85, 35.66, 35.41, 35.29,35.06, 31.97,29.77, 29.76, 29.71, 29.47, 29.05, 27.88, 27.78, 27.64, 23.55, 23.05, 22.84, 18.05, 12.60; FAB-MS: $[M+Na]^+$ 624.7715; cacld: 624.7728.

Compound 2-12: Same procedure as for **CSA-8** using **2-11**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.73 (bs, 7 H), 3.74-3.70 (m, 1 H), 3.65-3.60 (m, 2 H), 3.56-3.52 (m, 4 H), 3.31-3.28 (m, 2 H), 3.16-3.09 (m, 2 H), 2.82- 2.71 (m, 6 H), 2.20-2.06 (m, 3 H) 1 98-1.66 (m, 15 H), 1.58-1.48 (m, 3 H), 1.38-0.98 (m, 7 H), 0.96 (d, J = 6.6 Hz, 3 H), 0.93 (s, 3 H), 0.71 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.80, 80.60, 77.17, 67.88, 67.86, 67.18, 60.73, 48.11, 47.28, 43.93, 42.99, 41.34, 40.76, 40.72, 40.24, 39.70, 36.33, 36.18, 35,86, 34.29, 33.99, 33.96, 33.83, 29.60, 29.00, 28.57, 28.54, 24.33, 23.59, 23.48, 18.86, 13.04; FAB-MS: $[M+H]^+$ 546.7866; cacld: 546.7872.



2-12

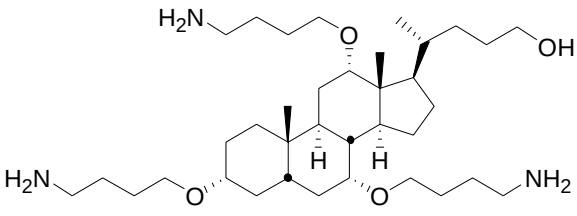


2-14

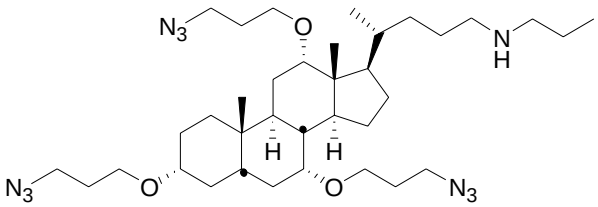
Compound 2-14: Same procedure as for **2-8** using KCN. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.82-3.70 (m, 2 H), 3.64-3.55 (m, 6 H), 3.47-3.31 (m, 3 H), 2.66-2.56 (m, 6 H), 2.33 (td, 1 H), 2.19-1.80 (m, 5 H), 1.77-1.01 (m, 25 H), 0.94 (d, J = 6.6 Hz, 3 H), 0.92 (s, 3 H), 0.68 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.11, 76.89, 76.06, 74.83 63.46, 62.74, 62.45, 46.25, 46.23, 42.24, 39.42, 35.50, 34.87, 32.20, 31.70, 30.07, 29.31, 28.52, 27.56, 27.14, 24.70, 23.44, 23.08, 23.04, 19.37, 19.33, 19.21, 18.06, 12.44; FAB-MS: $[M+Na]^+$ 618.8428; cacld: 618.8434.

Compound 2-15: **2-14** (2 g, 3.4 mmol) dissolved in THF (20 mL) in bump followed by addition Raney Ni (200 mg). H_2 was adjusted to 300 ppi and this reaction was stirred for 3 days. After removal of solvent in vacuo, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to yield **2-15** (0.9 g, 50%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.78 (bs, 7 H), 3.74-3.69 (m, 1 H), 3.67-3.60 (m, 2 H), 3.56-3.51 (m, 4 H), 3.29-3.26 (m, 2 H), 3.15-3.09 (m, 2 H), 2.82- 2.70 (m, 6 H), 2.20-2.07 (m, 3 H) 1.98-1.67 (m, 15 H), 1.58-1.47 (m, 3 H), 1.38-0.99(m, 13 H), 0.97 (d, J = 6.6 Hz, 3 H), 0.92 (s, 3 H), 0.70 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.79, 80.61, 77.16, 67.85, 67.83, 67.19, 60.73, 48.10, 47.27, 43.94, 42.99, 41.35, 40.75, 40.71, 40.25, 39.71, 36.34, 36.19, 35,86, 34.29, 33.98, 33.96, 33.82, 29.60, 29.01, 28.67, 28.54,

24.33, 23.59, 23.48, 18.86, 13.00; FAB-MS: $[M+H]^+$ 552.5249; cacld: 630.5256.



2-15

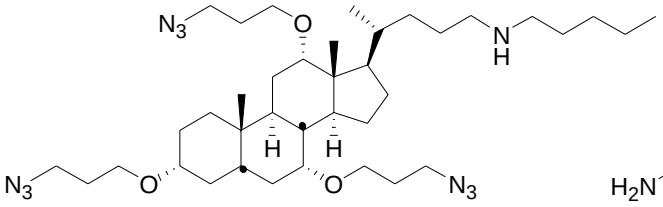


2-16

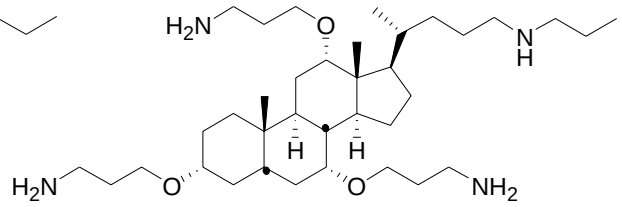
Compound 2-16: To a round-bottom flask were added **2-8** (0.6 g, 0.96 mmol) in CH_2Cl_2 (30 mL) and Et_3N (0.20 mL, 1.44 mmol). The mixture was put in ice-bath under N_2 followed by addition of mesyl chloride (0.089 mL, 1.15 mmol). After 30 min, water (20 mL) and brine (120 mL) were added. The CH_2Cl_2 layer was washed with brine (2 x 20 mL). The combined aqueous mixture was extracted with EtOAc (3 x 30 mL). The combined extracts were washed with brine and dried over anhydrous Na_2SO_4. The desired mesylate was isolated as a pale yellowish oil after removal of solvent. This mesylate was stirred with excess propylamine (2 mL) at 80℃ for 12 hr. After removal of propylamine in vacuo, the residue was chromatographed (SiO2, EtOAc / hexanes 1:4 with 2% Et_3N) to afford **2-16** (0.52 g, 85%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.69-3.37 (m, 11 H), 3.26-3.00 (m, 4 H), 2.61-2.53 (m, 4 H), 2.20-2.02 (m, 3 H), 1.98-0.99 (m, 30 H), 0.92-0.85 (m, 9 H), 0.65 (s, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.60, 79.74, 76.05, 64.97, 64.40, 64.28, 50.79, 50.25, 49.00, 48.90, 48.71, 46.47, 46.34, 42.65, 41.96, 39.80, 35.77, 35.41, 35.27, 35.05, 33.73, 31.96, 30.25, 29.76, 29.74, 29.67, 29.39, 29.05, 27.84, 27.61, 27.55, 26.70, 23.50, 23.00, 22.82, 22.79, 18.06, 14.23, 12.54; FAB-MS:

$[M+Na]^+$ 707.5128; cacld: 707.5134.

Compound 2-17: Same procedure as for **2-16** using pentylamine. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.67-3.37 (m, 13 H), 3.25-3.01 (m, 4 H), 2.61-2.52 (m, 4 H), 2.20-2.00 (m, 3 H), 1.99-0.99 (m, 32 H), 0.92-0.87 (m, 9 H), 0.66 (s, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.62, 79.75, 76.07, 64.97, 64.42, 64.28, 50.77, 50.27, 49.00, 48.91, 48.71, 46.46, 46.34, 42.66, 41.96, 39.81, 35.77, 35.40, 35.27, 35.06, 33.73, 31.95, 30.25, 29.77, 29.74, 29.68, 29.39, 29.06, 27.84, 27.65, 27.55, 26.72, 23.50, 23.01, 22.82, 22.79, 18.07, 14.23, 12.56; FAB-MS: $[M+Na]^+$ 735.5456; cacld: 735.5467.



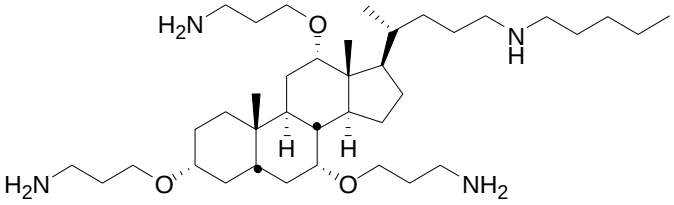
2-17



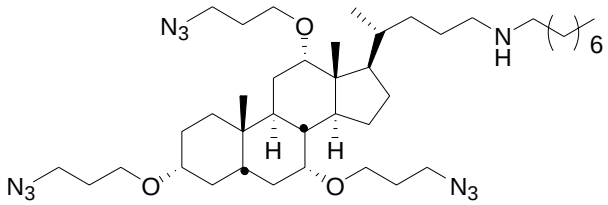
2-18

Compound 2-18: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.77 (bs, 5 H), 3.72-3.69 (m, 1 H), 3.65-3.60 (m, 1 H), 3.55-3.51 (m, 3 H), 3.32-3.29 (m, 2 H), 3.17-3.09 (m, 2 H), 2.87-2.72 (m, 6 H), 2.59-2.50 (m, 4 H), 2.18-2.09 (m, 3 H), 1.96-1.67 (m, 10 H), 1.62-0.99 (m, 21 H), 0.96 (d, J = 6.3 Hz, 3 H), 0.92 (s, 3 H), 0.89 (t, J = 6.8 Hz, 3 H), 0.69 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.82, 80.63, 77.23, 67.85, 67.19, 51.20, 50.69,47.82, 47.24, 43.92, 43.01, 41.30, 40.80, 40.68, 40.22, 36.74, 36.38, 36.20,35.87, 34.66, 34.15, 33.87, 32.90, 30.54, 30.39, 30.30, 29.64, 29.03, 28.59, 28.41, 26.96, 24.37, 23.65, 23.48, 18.75, 14.53, 13.09; FAB-MS: $[M+H]^+$ 607.5540; cacld: 607.5548.

Compound 2-19: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.82 (bs, 7 H), 3.81-3.74 (m, 1 H), 3.69-3.62 (m, 1 H), 3.59-3.53 (m, 3 H), 3.35-3.29 (m, 2 H), 3.19-3.09 (m, 2 H), 2.89-2.72 (m, 4 H), 2.63-2.51 (m, 4 H), 2.20-2.09 (m, 3 H), 1.99-1.68 (m, 10 H), 1.66-0.97 (m, 25 H), 0.97 (d, J = 6.3 Hz, 3 H), 0.94 (s, 3 H), 0.88 (t, J = 6.8 Hz, 3 H), 0.67 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.93, 80.70, 77.30, 67.85, 67.21, 51.20, 50.69, 47.90, 47.25, 43.96, 43.06, 41.30, 40.85, 40.68, 40.32, 36.74, 36.42, 36.24, 35.87, 34.69, 34.20, 33.87, 32.90, 30.57, 30.40, 30.30, 29.64, 29.06, 28.59, 28.44, 26.97, 24.39, 23.68, 23.48, 18.77, 14.55, 13.12; FAB-MS: $[M+H]^+$ 635.5864; cacld: 635.5867.



2-19

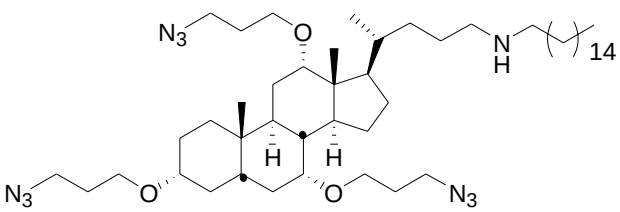


2-20

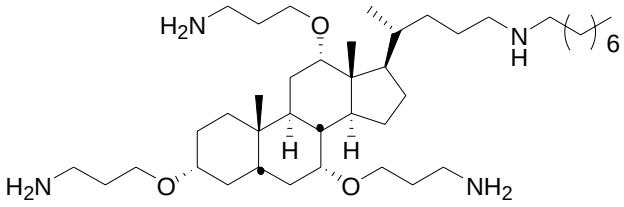
Compound 2-20: Same procedure as for **2-16** using octylamine. The yield was 80%. ^{I}H NMR ($CDCl_3$, 500 MHz) δ 3.69-3.37 (m, 11 H), 3.26-3.00 (m, 4 H), 2.61-2.53 (m, 4 H), 2.20-2.02 (m, 3 H), 1.98-0.99 (m, 40 H), 0.92-0.85 (m, 9 H), 0.65 (s, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.60, 79.74, 76.05, 64.97, 64.40, 64.28, 50.79, 50.25, 49.00, 48.90, 48.71, 46.47, 46.34, 42.65, 41.96, 39.80, 35.77, 35.41, 35.27, 35.05, 33.73, 31.96, 30.25, 29.76, 29.74, 29.67, 29.39, 29.05, 27.84, 27.61, 27.55, 26.70, 23.50, 23.00, 22.82, 22.79, 18.06, 14.23, 12.54; FAB-MS: $[M+H]^+$ 755.6012, cacld: 755.6024.

Compound 2-21: Same procedure as for **2-16** using hexadecylamine. The yield

was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.69-3.35 (m, 15 H), 3.26-3.00 (m, 4 H), 2.63-2.51 (m, 4 H), 2.20-2.02 (m, 3 H), 1.97-0.99 (m, 46 H), 0.92-0.85 (m, 9 H), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.67, 79.78, 76.07, 64.97, 64.42, 64.29, 50.76, 50.27, 49.01, 48.91, 48.76, 46.47, 46.34, 42.66, 41.98, 39.81, 35.75, 35.40, 35.28, 35.06, 33.78, 31.95, 30.26, 29.77, 29.74, 29.69, 29.39, 29.06, 27.83, 27.66, 27.55, 26.74, 23.50, 23.00, 22.82, 22.80, 18.07, 14.23, 12.54; FAB-MS: $[M+Na]^+$ 889.7128; cacld: 889.7134.



2-21

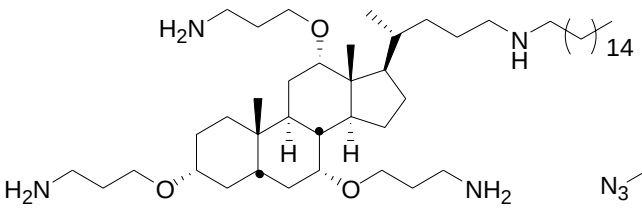


CSA-13

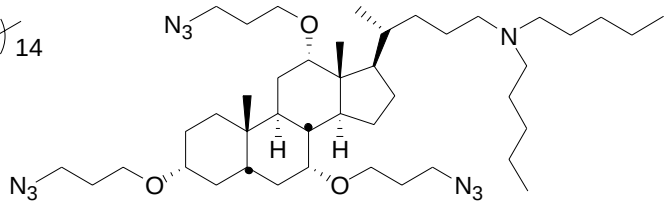
CSA-13: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.79 (bs, 7 H), 3.74-3.70 (m, 1 H), 3.66-3.61 (m, 1 H), 3.56-3.51 (m, 3 H), 3.31-3.29 (m, 2 H), 3.16-3.09 (m, 2 H), 2.88-2.72 (m, 6 H), 2.59-2.51 (m, 4 H), 2.18-2.07 (m, 3 H), 1.97-1.66 (m, 14 H), 1.62-0.97 (m, 25 H), 0.95 (d, J = 6.3 Hz, 3 H), 0.93 (s, 3 H), 0.89 (t, J = 6.8 Hz, 3 H), 0.70 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.82, 80.63, 77.23, 67.85, 67.19, 51.20, 50.69,47.82, 47.24, 43.92, 43.01, 41.30, 40.80, 40.68, 40.22, 36.74, 36.38, 36.20,35.87, 34.66, 34.15, 33.87, 32.90, 30.54, 30.39,30.30, 29.64, 29.03, 28.59, 28.41, 26.96, 24.37, 23.65, 23.48, 18.75, 14.53, 13.09; FAB-MS: $[M+H]^+$ 677.6309; cacld: 677.6309.

Compound 2-22: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.79 (bs, 9 H), 3.72-3.69 (m, 1 H), 3.65-3.60 (m, 1

H), 3.55-3.51 (m, 3 H), 3.32-3.29 (m, 2 H), 3.17-3.09 (m, 2 H), 2.87-2.72 (m, 12 H), 2.59-2.50 (m, 4 H), 2.18-2.09 (m, 3 H), 1.96-1.67 (m, 20 H), 1.62-0.99 (m, 21 H), 0.96 (d, J = 6.3 Hz, 3 H), 0.92 (s, 3 H), 0.89 (t, J = 6.8 Hz, 3 H), 0.69 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.90, 80.63, 77.30, 67.85, 67.19, 51.20, 50.69,47.82, 47.24, 43.92, 43.01, 41.30, 40.80, 40.68, 40.22, 36.74, 36.38, 36.20,35.87, 34.66, 34.15, 33.87, 32.90, 30.54, 30.39, 30.30, 29.64, 29.03, 28.59, 28.41, 26.96, 24.37, 23.65, 23.48, 18.75, 14.53, 13.09; FAB-MS: $[M+H]^+$ 789.7621; cacld: 789.7623.



2-22

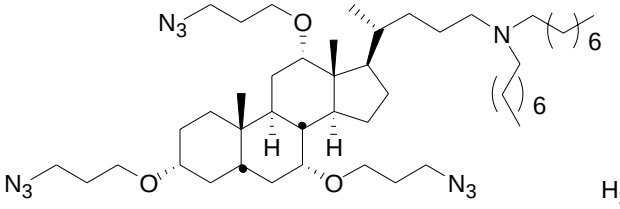


2-23

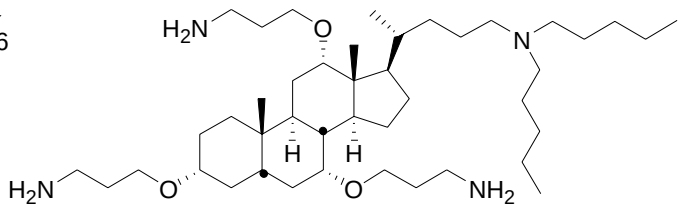
Compound 2-23: Same procedure as for **2-16** using dipentylamine. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.83 (bs, 5 H), 3.77-3.74 (m, 1 H), 3.65-3.61 (m, 1 H), 3.59-3.51 (m, 6 H), 3.35-3.28 (m, 2 H), 3.19-3.09 (m, 2 H), 2.89-2.74 (m, 6 H), 2.59-2.51 (m, 4 H), 2.18-2.07 (m, 3 H), 1.99-1.67 (m, 14 H), 1.62-1.00 (m, 21 H), 0.97 (d, J = 6.3 Hz, 3 H), 0.92 (s, 3 H), 0.89 (t, J = 6.8 Hz, 3 H), 0.70 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.90, 80.65, 77.24, 67.86, 67.19, 51.22, 50.69, 47.82, 47.24, 43.92, 43.01, 41.30, 40.81, 40.68, 40.22, 36.74, 36.36, 36.20, 35.87, 34.65, 34.15, 33.88, 32.90, 30.54, 30.45, 30.30, 29.64, 29.03, 28.62, 28.41, 26.96, 24.37, 23.65, 23.48, 18.77, 14.53, 13.11; FAB-MS: $[M+Na]^+$ 805.6250; cacld: 805.6256.

Compound 2-24: Same procedure as for **2-16** using dioctylamine. The yield

was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.80 (bs, 9 H), 3.85-3.77 (m, 1 H), 3.68-3.62 (m, 1 H), 3.58-3.53 (m, 3 H), 3.35-3.28 (m, 2 H), 3.19-3.09 (m, 2 H), 2.87-2.72 (m, 4 H), 2.66-2.51 (m, 4 H), 2.21-2.09 (m, 3 H), 1.98-1.68 (m, 16 H), 1.69-0.97 (m, 28 H), 0.98 (d, J = 6.3 Hz, 3 H), 0.92 (s, 3 H), 0.86 (t, J = 6.8 Hz, 3 H), 0.66 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.99, 80.76, 77.34, 67.85, 67.21, 51.22, 50.69, 47.91, 47.25, 43.96, 43.05, 41.30, 40.85, 40.67, 40.32, 36.74, 36.42, 36.26, 35.87, 34.69, 34.22, 33.87, 32.91, 30.57, 30.40, 30.33, 29.66, 29.07, 28.59, 28.44, 26.99, 24.39, 23.69, 23.48, 18.77, 14.57, 13.15; FAB-MS: $[M+H]^+$ 889.7125; cacld: 889.7129.



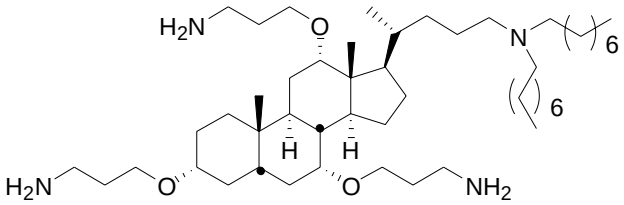
2-24



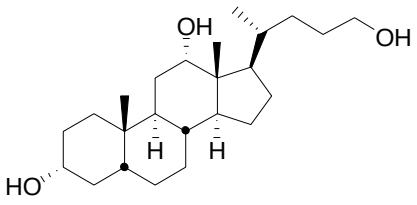
2-25

Compound 2-25: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.80 (bs, 7 H), 3.73-3.69 (m, 1 H), 3.67-3.61 (m, 1 H), 3.54-3.51 (m, 3 H), 3.33-3.29 (m, 2 H), 3.19-3.10 (m, 2 H), 2.88-2.72 (m, 8 H), 2.60-2.50 (m, 4 H), 2.17-2.09 (m, 3 H), 1.95-1.67 (m, 10 H), 1.61-0.99 (m, 23 H), 0.96 (d, J = 6.3 Hz, 3 H), 0.93 (s, 3 H), 0.88 (t, J = 6.8 Hz, 3 H), 0.67 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.86, 80.64, 77.25, 67.85, 67.19, 51.20, 50.69, 47.81, 47.24, 43.93, 43.01, 41.30, 40.83, 40.68, 40.21, 36.74, 36.38, 36.20, 35.88, 34.66, 34.15, 33.85, 32.90, 30.54, 30.39, 30.30, 29.64, 29.04, 28.59, 28.41, 26.97, 24.37, 23.65, 23.48, 18.75, 14.55, 13.10; FAB-MS: $[M+H]^+$ 705.6672; cacld: 705.6677.

Compound 2-26: Same procedure as for **CSA-10**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.82 (bs, 9 H), 3.81-3.74 (m, 1 H), 3.69-3.62 (m, 1 H), 3.59-3.53 (m, 3 H), 3.35-3.29 (m, 2 H), 3.19-3.09 (m, 2 H), 2.89-2.72 (m, 4 H), 2.63-2.51 (m, 4 H), 2.20-2.09 (m, 3 H), 1.99-1.68 (m, 14 H), 1.66-0.97 (m, 33 H), 0.97 (d, J = 6.3 Hz, 3 H), 0.94 (s, 3 H), 0.88 (t, J = 6.8 Hz, 3 H), 0.67 (s, 3 H); ^{13}C NMR ($CDC1_3$, 500 MHz) δ 81.99, 80.75, 77.40, 67.85, 67.24, 51.20, 50.69, 47.90, 47.24, 43.96, 43.05, 41.32, 40.85, 40.68, 40.33, 36.74, 36.42, 36.24, 35.87, 34.70, 34.20, 33.87, 32.90, 30.58, 30.40, 30.34, 29.64, 29.06, 28.59, 28.45, 26.97, 24.39, 23.68, 23.46, 18.78, 14.56, 13.11; FAB-MS: $[M+H]^+$ 789.7654; cacld: 789.7656.



2-26

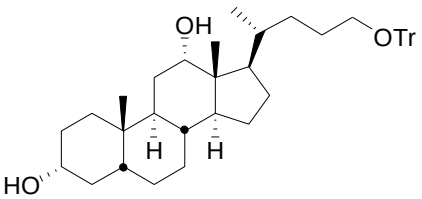


2-29

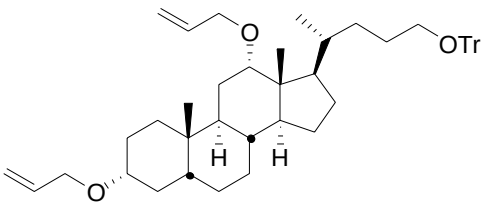
Compound 2-29: To a 1 L round-bottom flask were added **2-28** (30.0 g, 71.2 mmol) in dry THF (600 mL) and $LiAIH_4$ (4.13 g, 109 mmol). After reflux 48 hr, saturated aqueous Na_2SO_4 (100mL) was introduced slowly, and the resulted precipitate was filtered out and washed with hot THF and MeOH. Recrystalization from MeOH gave colorless crystals of **2-29** (27.2 g, 98%). ^{1}H NMR ($CDC1_3$/MeOH-d4, 500 MHz) δ 3.97 (bs, 2 H), 3.84 (bs, 1 H), 3.61-3.45 (m, 2 H), 3.39 (bs, 5 H), 2.30-2.11 (m, 2H), 2.05-1.07 (m, 22 H), 1.02 (bs, 3 H), 0.91 (s, 3 H). 0.72 (s, 3 H); ^{13}C NMR ($CDCl_3$/MeOH-d4, 500 MHz) δ 73.77, 72.57, 68.97, 63.51, 48.04, 47.12, 42.47, 40.37, 39.98, 36.62, 36.11, 35.58,

35.41, 32.77, 30.66, 30.04, 29.04, 28.43, 27.27, 23.94, 23.08, 18.00, 13.01; FAB-MS: $[M+H]^+$ 379.3243; cacld: 379.3245.

Compound 2-30: To a round-bottom flask were added **2-29** (27.2 g, 70.0 mmol) in THF (300 mL), Et_3N (20 mL, 143.4 mmol), trityl chloride (25.98 g, 93.2 mmol) and DMAP (0.13 g, 1.07 mmol). The mixture was refluxed under N_2 for 30 hr followed by the introduction of water (800 mL) and extraction with EtOAc (5 x 200 mL). The combined extracts were washed with water and brine and then dried over $MgSO_4$. After removal of solvent in vacuo, the residue was purified using column chromatography (SiO_2, EtOAc) to give **2-30** as a pale yellow solid (29.0 g, 70%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.44-7.42 (m, 6 H), 7.32-7.18 (m, 9 H), 3.97 (bs, 2 H), 3.82 (bs, 1 H), 3.51-3.39 (m, 1 H), 3.00 (bs, 1 H), 2.93 (dd, J = 14.2, 12.2 Hz, 2 H), 2.65 (bs, 1 H), 2.51 (bs, l H), 2.34-2.10 (m, 2 H), 2.00-1.06 (m, 22 H), 0.96 (d, J = 5.8 Hz, 3 H), 0.88 (s, 3 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.79, 128.91, 127.91, 127.04, 86.45, 73.35, 72.06, 68.67, 64.28, 47.48, 46.53, 41.74, 41.61, 39.64, 35.57; 35.46, 34.91, 34.85, 32.40, 30.54, 28.21, 27.69, 26.84, 26.45, 23.36, 22.58, 17.83, 12.62; FAB-MS: $[M+Na]^+$ 643.4176; cacld: 643.4178.



2-30



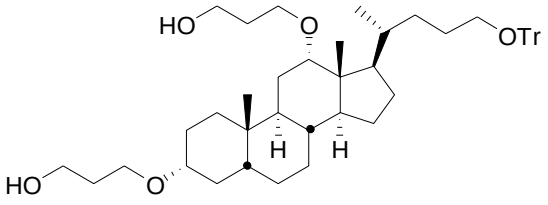
2-31

Compound 2-31: To a round-bottom flask were added **2-30** (29.0 g, 40.4 mmol) in dry THF (600 mL) and NaH (60% in mineral oil, 6.3 g, l57.2 mmol). The

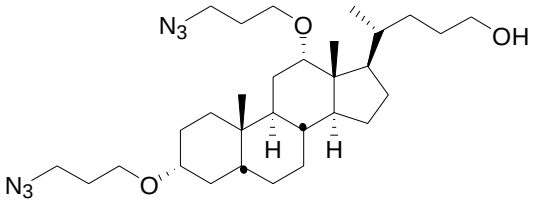
mixture was refluxed for 30 min under N_2 followed by addition of allyl bromide (27 mL, 314 mmol). After 24 hr at reflux, water (20 mL) was introduced slowly followed by addition of 1 % HCl until the aqueous layer became neutral. The, mixture was then extracted with ether (3 x 100 mL) and the combined extracts were washed with water (100 mL) and brine (2 x 100 mL). The ether solution was dried over anhydrous Na_2SO_4, and after removal of solvent, the residue was purified using column chromatography (SiO_2, 1:8 EtOAc/hexane) to give **2-31** (25.76 g, 90%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.48-7.30 (m, 6 H), 7.31-7.14 (m, 9 H), 6.03-5.80 (m, 3 H), 5.36-5.04 (m, 6 H), 4.14-3.94 (m, 4 H), 3.73 (td, J = 13.8,5.8 Hz, 2 H), 3.54 (bs, 1 H), 3.20-2.94 (m, 3 H), 3.31 (bs, 1 H), 2.38-1.90 (m, 4 H), 1.90-0.96 (m, 20 H), 0.91 (d, J = 5.4 Hz, 3 H), 0.89 (s, 3 H), 0.63 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.84, 136.26, 136.08, 128.95, 127.90, 126.98, 116.47, 115.70, 86.42, 80.94, 79.28, 74.98, 69.51, 69.39, 68.86, 64.39, 46.51, 46.43, 42.67, 42.14, 39.94, 35.63,3 5.51, 35.13, 32.46, 28.98, 28.09, 27.67, 27.57, 26.72, 23.32, 23.11, 17.92, 12.68; FAB-MS: $[M+Na]^+$ 709.4675; cacld: 709.4677.

Compound 2-32: A round-bottom flask was charged with THF (80 mL) and cooled to 0℃, Borane-THF complex (150 mL, 0.15 mol) and cyclohexene(31 mL, 0.3 mol) were added sequentially, and the mixture was stirred at 0℃ for 3 hr under N_2. **2-31** (25 g, 0.07 mol) in THF (50 mL) was added dropwise and the mixture was stirred for 12 hr at room temperature. Aqueous NaOH (20%) (80 mL) and H_2O_2 (50 mL) were added in sequence. The mixture was refluxed for 3

hr followed by the addition of brine (100 mL) and extracted with EtOAc (3× 100 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2) to afford **2-32** (20.0 g, 80%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.50-7.42 (m, 6 H), 7.34-7.16 (m, 9 H), 3.91-3.56 (m, 15 H), 3.51(bs, 1 H), 3.40-2.96 (m, 6 H), 2.30-0.94 (m, 30 H), 0.90 (s, 3 H), 0.89 (d, J = 5.4 Hz, 3 H), 0.66 (s, 3 H); ^{13}C NMR($CDCl_3$, 500 MHz) δ 144.72, 128.88, 127.84, 126.94, 86.36, 80.55, 78.90, 76.36, 66.82, 66.18, 65.75, 64.26, 61.53, 6l.43, 61.34, 46.89, 46.04, 42.60, 41.59, 39.60, 35.37, 35.27, 34.88, 32.75, 32.44, 32.32, 28.82, 27.65, 27.48, 27.13, 26.77, 23.34, 22.74, 22.39, 18.08, 12.46; FAB-MS: $[M+Na]^+$ 759.5031; cacld: 759.5034.



2-32



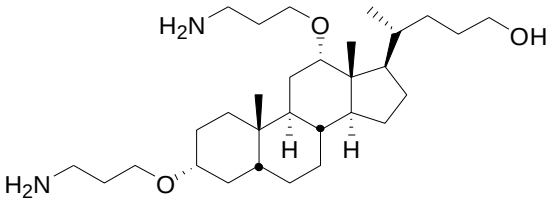
2-34

Compound 2-34: To a round-bottom flask were added **2-32** (20 g, 0.04 mol) and Et_3N (20 mL, 0.12 mol) in CH_2Cl_2 (100 mL). The mixture was cooled to 0 ℃ followed by addition of methyl chloride. After 1 hr, brine (40 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (3×40 mL) and the combined extracts was dried over anhydrous Na_2SO_4. The product was isolated as a colorless oil which was dissolved in DMF (100 mL) and H_2O (20 mL). NaN_3 (2.1 g, 0.4 mol) was added to the solvent and the mixture was heated to 80℃ and stirred for 16 hr. The mixture was then extracted with ether (5×20

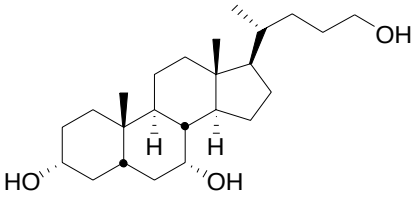
mL) and the combined extracts was dried over anhydrous Na_2SO_4. After removed the solvent under vacuum, the residue was dissolved in MeOH (30 mL) and CH_2Cl_2 (30 mL) followed by addition of catalytic amount of *p*-TsOH (10 mg, 0.05 mmol). The solution was stirred at room temperature for 3 hr before the addition of saturated $NaHCO_3$ solution (15 mL). The mixture was extracted with EtOAc (5×20 mL). The combined extracts were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. The residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-34** (10.0 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.69-3.54 (m, 5 H), 3.54-3.34 (m, 10 H), 3.28-3.00 (m, 4 H), 2.20-2.01 (m, 3 H) 1 98-1.56 (m, 15 H), 1.55-0.96 (m, 13 H), 0.92 (d, J = 6.6 Hz, 3 H), 0.88 (s, 3 H), 0.69 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.64, 79.78, 76.03, 64.99, 64.45, 64.31, 63.72, 49.01, 48.93, 48.74, 46.49, 46.39, 42.70, 41.96, 39.80, 35.66, 35.42, 35.28, 35.08, 31.99, 29.78, 29.75, 29.70, 29.49, 29.06, 27.87, 27.79, 27.65, 23.53, 23.04, 22.84, 18.09, 12.59; FAB-MS: $[M+Na]^+$ 567.4019; cacld: 567.4023.

Compound 2-35: **2-34** (5.0g, 8.2 mmol) was dissolved in THF (25 mL) and H_2O (10 mL). Triphenylphosphine (10.4 g, 39.8 mmol) was added, and the solution was stirred for 12 hr. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to yield **2-42** (4.0 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.75 (bs, 9 H), 3.77-3.70 (m, 1 H), 3.68-3.60 (m, 2 H), 3.56-3.52 (m, 4 H), 3.33-3.28 (m, 2 H), 3.16-3.08 (m, 2 H), 2.82- 2.70 (m, 6 H), 2.20-2.05 (m, 3 H), 1.98-1.66 (m, 15 H),

1.57-1.48 (m, 3 H), 1.39-0.98 (m, 7 H), 0.96 (d, J = 6.6 Hz, 3 H), 0.92 (s, 3 H), 0.70 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.81, 80.62, 77.17, 67.88, 67.86, 67.18, 60.71, 48.11, 47.28, 43.95, 42.99, 41.36, 40.76, 40.72, 40.24, 39.70, 36.33, 36.19, 35,86, 34.29, 33.99, 33.96, 33.87, 29.60, 29.00, 28.57, 28.54, 24.33, 23.59, 23.50, 18.88, 13.00; FAB-MS: $[M+H]^+$ 493.4450; cacld: 493.4458.



2-35

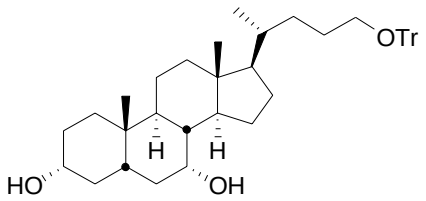


2-38

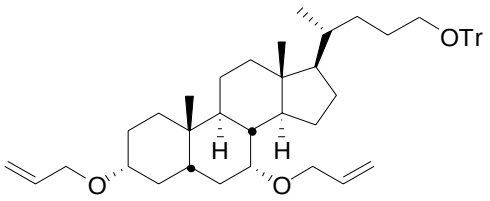
Compound 2-38: To a 1 L round-bottom flask were added **2-37** (30.0 g, 71.2 mmol) in dry THF (600 mL) and $LiAlH_4$ (4.13 g, 109 mmol). After reflux 48 hr, saturated aqueous Na_2SO_4 (100mL) was introduced slowly, and the resulted precipitate was filtered out and washed with hot THF and MeOH. Recrystalization from MeOH gave colorless crystals of **2-38** (27.2 g, 98%). ^{1}H NMR ($CDC1_3$/MeOH-d4, 500 MHz) δ 3.99 (bs, 2 H), 3.83 (bs, 1 H), 3.62-3.45 (m, 2 H), 3.40 (bs, 5 H), 2.30-2.12 (m, 2H), 2.05-1.08 (m, 22 H), 1.02 (bs, 3 H), 0.91 (s, 3 H). 0.71 (s, 3 H); ^{13}C NMR ($CDCl_3$/MeOH-d4, 500 MHz) δ 73.77, 72.57, 68.97, 63.51, 48.04, 47.12, 42.47, 40.37, 39.98, 36.62, 36.11, 35.58, 35.41, 32.77, 30.66, 30.04, 29.04, 28.43, 27.27, 23.94, 23.08, 18.00, 13.01; FAB-MS: $[M+H]^+$ 379.3244; cacld: 379.3245.

Compound 2-39: To a round-bottom flask were added **2-38** (27.2 g, 70.0 mmol) in THF (300 mL), Et_3N (20 mL, 143.4 mmol), trityl chloride (25.98 g, 93.2

mmol) and DMAP (0.13 g, 1.07 mmol). The mixture was refluxed under N_2 for 30 hr followed by the introduction of water (800 mL) and extraction with EtOAc (5 x 200 mL). The combined extracts were washed with water and brine and then dried over $MgSO_4$. After removal of solvent in vacuo, the residue was purified using column chromatography (SiO_2, EtOAc) to give **2-39** as a pale yellow solid (29.0 g, 70%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.45-7.42 (m, 6 H), 7.32-7.19 (m, 9 H), 3.98 (bs, 2 H), 3.81 (bs, 1 H), 3.51-3.40 (m, 1 H), 3.01 (bs, 1 H), 2.94 (dd, J = 14.2, 12.2 Hz, 2 H), 2.66 (bs, 1 H), 2.51 (bs, l H), 2.34-2.11 (m, 2 H), 2.00-1.08 (m, 22 H), 0.96 (d, J = 5.8 Hz, 3 H), 0.89 (s, 3 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.79, 128.91, 127.91, 127.04, 86.45, 73.35, 72.06, 68.67, 64.28, 47.48, 46.53, 41.74, 41.61, 39.64, 35.57; 35.46, 34.91, 34.85, 32.40, 30.54, 28.21, 27.69, 26.84, 26.45, 23.36, 22.58, 17.83, 12.62; FAB-MS: $[M+Na]^+$ 643.4175; cacld: 643.4178.



2-39



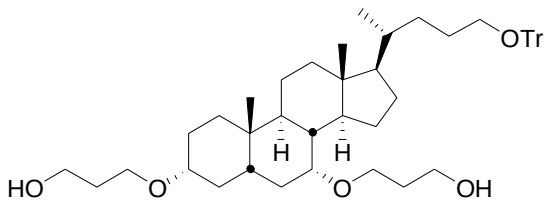
2-40

Compound 2-40: To a round-bottom flask were added **2-39** (29.0 g, 40.4 mmol) in dry THF (600 mL) and NaH (60% in mineral oil, 6.3 g, l57.2 mmol). The mixture was refluxed for 30 min under N_2 followed by addition of allyl bromide (27 mL, 314 mmol). After 24 h at reflux, water (20 mL) was introduced slowly followed by addition of 1 % HCl until the aqueous layer became neutral. The, mixture was then extracted with ether (3 x 100 mL) and the combined extracts

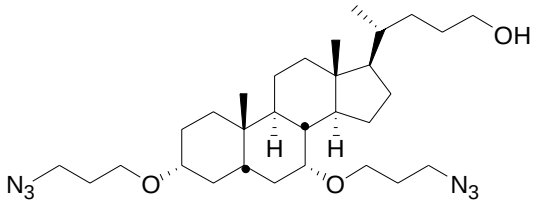
were washed with water (100 mL) and brine (2 x 100 mL). The ether solution was dried over anhydrous Na_2SO_4, and after removal of solvent, the residue was purified using column chromatography (SiO_2, 1:8 EtOAc/hexane) to give **2-40** (25.76 g, 90%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.48-7.31 (m, 6 H), 7.31-7.15 (m, 9 H), 6.03-5.81 (m, 3 H), 5.36-5.03 (m, 6 H), 4.14-3.96 (m, 4 H), 3.72 (td, J = 13.8,5.8 Hz, 2 H), 3.51 (bs, 1 H), 3.20-2.97 (m, 3 H), 3.30 (bs, 1 H), 2.38-1.91 (m, 4 H), 1.91-0.96 (m, 20 H), 0.92 (d, J = 5.4 Hz, 3 H), 0.89 (s, 3 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.84, 136.26, 136.08, 128.95, 127.90, 126.98, 116.47, 115.70, 86.42, 80.94, 79.28, 74.98, 69.51, 69.39, 68.86, 64.39, 46.51, 46.43, 42.67, 42.14, 39.94, 35.63,3 5.51, 35.13, 32.46, 28.98, 28.09, 27.67, 27.57, 26.72, 23.32, 23.11, 17.92, 12.68; FAB-MS: $[M+Na]^+$ 709.4676; cacld: 709.4677.

C**ompound 2-41**: A round-bottom flask was charged with THF (80 mL) and cooled to 0℃, Borane-THF complex (150 mL, 0.15 mol) and cyclohexene(31 mL, 0.3 mol) were added sequentially, and the mixture was stirred at 0℃ for 3 hr under N_2. Compound **2-40** (25 g, 0.07 mol) in THF (50 mL) was added dropwise and the mixture was stirred for 12 hr at room temperature. Aqueous NaOH (20%) (80 mL) and H_2O_2 (50 mL) were added in sequence. The mixture was refluxed for 3 h followed by the addition of brine (100 mL) and extracted with EtOAc (3×100 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2) to afford **2-41** (20.0

g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.50-7.45 (m, 6 H), 7.34-7.15 (m, 9 H), 3.91-3.56 (m, 15 H), 3.52 (bs, 1 H), 3.40-2.97 (m, 6 H), 2.30-0.94 (m, 30 H), 0.90 (s, 3 H), 0.88 (d, J = 5.4 Hz, 3 H), 0.67 (s, 3 H); ^{13}C NMR($CDCl_3$, 500 MHz) δ 144.73, 128.89, 127.86, 126.94, 86.37, 80.55, 78.90, 76.36, 66.82, 66.18, 65.75, 64.25, 61.53, 6l.43, 61.33, 46.89, 46.05, 42.60, 41.59, 39.60, 35.37, 35.28, 34.88, 32.75, 32.46, 32.32, 28.82, 27.65, 27.47, 27.13, 26.77, 23.34, 22.74, 22.39, 18.07, 12.47; FAB-MS: $[M+Na]^+$ 759.5031; cacld: 759.5034.



2-41



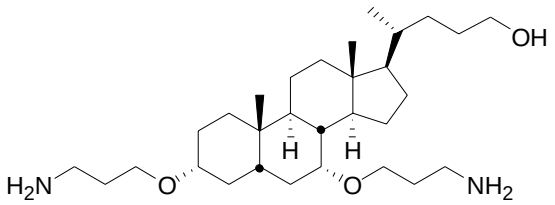
2-43

Compound 2-43: To a round-bottom flask were added **2-41** (20 g, 0.04 mol) and Et_3N (20 mL, 0.12 mol) in CH_2Cl_2 (100 mL). The mixture was cooled to 0 ℃ followed by addition of methyl chloride. After 1 hr, brine (40 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (3×40 mL) and the combined extracts was dried over anhydrous Na_2SO_4. The product was isolated as a colorless oil which was dissolved in DMF (100 mL) and H_2O (20 mL). NaN_3 (2.1 g, 0.4 mol) was added to the solvent and the mixture was heated to 80℃ and stirred for 16 h. The mixture was then extracted with ether (5×20 mL) and the combined extracts was dried over anhydrous Na_2SO_4. After removed the solvent under vacuum, the residue was dissolved in MeOH (30 mL) and CH_2Cl_2 (30 mL) followed by addition of catalytic amount of *p*-TsOH (10

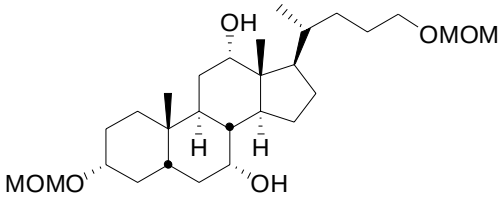
mg, 0.05 mmol). The solution was stirred at room temperature for 3 hr before the addition of saturated $NaHCO_3$ solution (15 mL). The mixture was extracted with EtOAc (5×20 mL). The combined extracts were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. The residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-43** (10.0 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.70-3.55 (m, 5 H), 3.54-3.35 (m, 10 H), 3.28-3.01 (m, 4 H), 2.20-2.00 (m, 3 H) 1.98-1.57 (m, 15 H), 1.55-0.97 (m, 13 H), 0.92 (d, J = 6.6 Hz, 3 H), 0.89 (s, 3 H), 0.68 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.66, 79.77, 76.03, 64.98, 64.45, 64.33, 63.72, 49.01, 48.97, 48.74, 46.49, 46.39, 42.70, 41.97, 39.80, 35.66, 35.44, 35.28, 35.08, 31.99, 29.78, 29.75, 29.70, 29.49, 29.05, 27.87, 27.79, 27.66, 23.53, 23.04, 22.84, 18.08, 12.60; FAB-MS: $[M+Na]^+$ 567.4020; cacld: 567.4023.

Compound 2-44: Compound **2-43** (5.0g, 8.2 mmol) was dissolved in THF (25 mL) and H_2O (10 mL). Triphenylphosphine (10.4 g, 39.8 mmol) was added, and the solution was stirred for 12 hr. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to yield **2-51** (4.0 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.77 (bs, 9 H), 3.77-3.71 (m, 1 H), 3.68-3.62 (m, 2 H), 3.56-3.51 (m, 4 H), 3.33-3.27 (m, 2 H), 3.16-3.09 (m, 2 H), 2.82- 2.71 (m, 6 H), 2.20-2.04 (m, 3 H), 1.98-1.68 (m, 15 H), 1.57-1.47 (m, 3 H), 1.39-0.99 (m, 7 H), 0.95 (d, J = 6.6 Hz, 3 H), 0.92 (s, 3 H), 0.69 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 81.80, 80.63, 77.17, 67.88, 67.86, 67.19, 60.71, 48.13, 47.26, 43.95, 42.98, 41.36, 40.76, 40.71, 40.24,

39.70, 36.34, 36.19, 35,85, 34.29, 33.99, 33.99, 33.87, 29.60, 29.01, 28.57, 28.54, 24.33, 23.58, 23.50, 18.87, 12.99; FAB-MS: $[M+H]^+$ 493.4454; cacld: 493.4458.



2-44

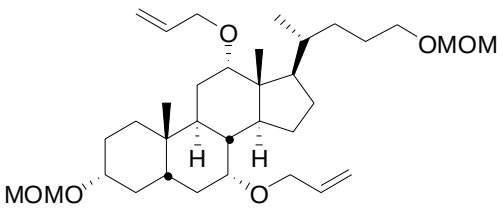


2-45

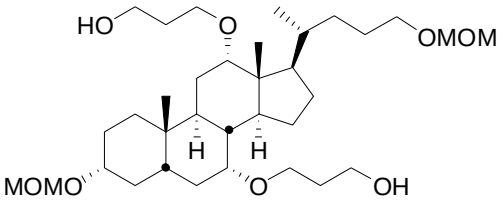
Compound 2-45: Compound **2-3** (10 g, 0.025 mol) and Et_3N (7.1 ml, 0.05 mol) were dissolved in THF (500 mL), MOMCl (5.5 mL, 0.5 mol) in THF (30 mL) then was added dropwise. After stirred for 48 hr, the solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 2:1 EtOAc/hexane) to yield **2-45** (6.0 g, 50%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.69-4.66 (bs, 2 H), 4.13-4.12 (m, 1 H), 3.90 (s, 1 H), 3.45 (s, 1 H), 3.43-3.32 (m, 10 H), 2.28-2.04 (m, 3 H), 1.97 (s, 2 H), 1.93-0.89 (m, 25 H), 0.69 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 96.59, 94.51, 73.14, 68.55, 68.51, 60.62, 55.33, 47.70, 46.73, 42.20, 41.73, 39.85, 36.78, 35.66, 35.44, 35.09, 34.80, 32.44, 28.55, 27.91, 27.78, 26.90, 23.40, 22.88, 21.28, 17.86, 14.42, 12.79; FAB-MS: $[M+Na]^+$ 505.3489; cacld: 505.3499.

Compound 2-46: To a round-bottom flask were added **2-45** (6.0 g, 0.0125 mol) and NaH (2.5 g, 0.0625 mol) in dry THF (300 mL). The mixture was refluxed 1 hr under N_2 followed by addition of allyl bromide. After 36 hr at reflux, water (50 mL) was introduced slowly. The mixture was then extracted with EtOAc (3 ×100 mL) and the combined extracts were dried over anhydrous Na_2SO_4. The

solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:4 EtOAc/hexane) to afford **2-46** (5.6 g, 80%). ^1H NMR ($CDCl_3$, 500 MHz) δ 5.95-5.86 (m, 2 H), 5.31-5.24 (m, 2 H), 5.14-5.00 (m, 2 H), 4.65 (d, J = 9.0 Hz, 2 H), 4.01-3.80 (m, 2 H), 3.80-3.77 (m, 2 H), 3.68-3.54 (m, 2 H), 3.54-3.31 (m, 4 H), 3.31-3.21 (m, 4 H), 2.29-2.18 (m, 2 H), 2.10-2.00 (m, 2 H), 1.85-0.88 (m, 27 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 136.21, 115.61, 96.60, 94.80, 80.97, 69.51, 69.41, 68.73, 55.30, 46.70, 46.54, 42.81, 39.99, 35.80, 35.71, 35.57, 35.03, 32.52, 28.99, 28.18, 27.84, 26.64, 23.42, 23.20, 17.99, 12.80; FAB-MS: $[M+Na]^+$ 585.4121; cacld: 585.4125.



2-46



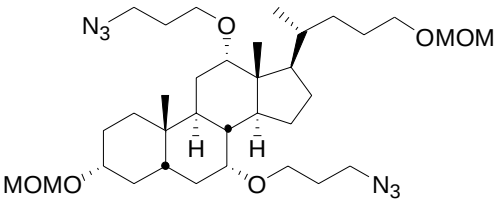
2-47

Compound 2-47: A round-bottom flask was charged with THF (20 mL) and cooled to 0℃, Borane-THF complex (30 mL, 0.03 mol) and cyclohexene(6.2 mL, 0.06 mol) were added sequentially, and the mixture was stirred at 0℃ for 3 hr under N_2. Compound **2-46** (5.6 g, 0.01mol) in THF (20 mL) was added dropwise and the mixture was stirred for 12 h at room temperature. Aqueous NaOH (20%) (20 mL) and H_2O_2 (20 mL) were added in sequence. The mixture was refluxed for 3 hr followed by the addition of brine (50 mL) and extracted with EtOAc (3×60 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified

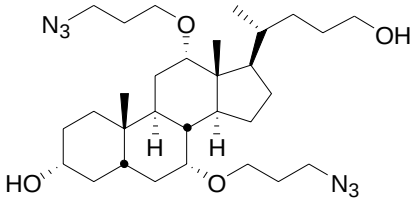
by column chromatography (SiO_2, 2:1 EtOAc/hexane) to afford **2-47** (4.8 g, 80%).^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.85-3.75 (m, 8 H), 3.55-3.50 (m, 4 H), 3.40-3.35 (m, 4 H), 3.15-3.25 (m, 4 H), 2.95-3.10 (m, 2 H), 2.20-2.30 (m, 4 H), 2.10-2.18 (m, 4 H), 2.00-2.08 (m, 4 H), 1.60-1.98 (m, 12 H), 1.20-1.60 (m, 10 H), 0.90-1.20 (m, 12 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 96.64, 94.65, 80.92, 68.67, 66.99, 66.92, 62.05, 61.59, 55.34, 47.13, 46.23, 42.88, 41.59, 39.83, 35.84, 35.69, 35.55, 35.03, 32.96, 32.72, 29.04, 28.15, 27.79, 27.75, 26.72, 23.56, 23.05, 22.66, 18.16, 12.61; FAB-MS: $[M+H]^+$ 599.4531; cacld: 599.4517.

Compound 2-48: To a round-bottom flask were added **2-47** (4.8 g, 0.008 mol) and Et_3N (1.2 mL, 0.0085mol) in CH_2Cl_2 (50 mL). The mixture was cooled to 0 ℃ followed by addition of methyl chloride. After 1 hr, brine (30 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (3×50 mL) and the combined extracts was dried over anhydrous Na_2SO_4. The product was isolated as a colorless oil which was dissolved in DMF (50 mL) and H_2O (10 mL). NaN_3 (1.95 g, 0.03 mol) was added to the solvent and the mixture was heated to 80℃ and stirred for 16 h. The mixture was then extracted with ether (5×50 mL) and the combined extracts was dried over anhydrous Na_2SO_4. After removed the solvent under vacuum, the residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-48** (3.8 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.67-4.62 (m, 2 H), 3.75-3.50 (m, 8 H), 3.50-3.25 (m, 10 H), 3.24-3.10 (m, 8 H), 2.25-2.05 (m, 6 H), 2.00-1.58 (m, 12 H), 1.58-0.88 (m, 20 H), 0.66

(s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 96.63, 94.69, 80.72, 68.68, 65.08, 64.45, 55.33, 55.30, 49.10, 49.01, 46.72, 46.50, 42.76, 41.09, 39.91, 35.80, 35.54, 35.01, 32.58, 29.92, 29.80, 28.24, 27.92, 27.87, 26.62, 23.62, 23.14, 22.95, 18.11, 12.69; FAB-MS: $[M+H]^+$ 649.4650; cacld: 649.4657.



2-48

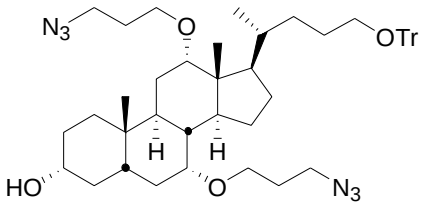


2-49

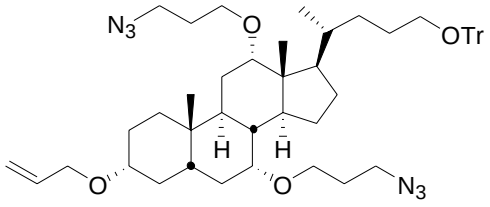
Compound 2-49: compound **2-48** (3.8 g, 0.0058 mol) was dissolved in CH_3OH (30 mL) and aqueous HCl (10 mL, 5%). The mixture was refluxed over 12 hr. The pH value was adjusted to about 8 by addition of solid NaOH. The mixture then was extracted with EtOAc (3×60 mL) and the combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:1 EtOAc/hexane) to afford **2-49** (3.2 g, 95%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.72-3.50 (m, 6 H), 3.52-3.25 (m, 10 H), 3.24-3.11 (m, 8 H), 2.27-2.05 (m, 6 H), 2.00-1.58 (m, 12 H), 1.55-0.88 (m, 20 H), 0.67 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 80.79, 72.21, 65.01, 64.33, 63.87, 48.99, 46.64, 46.45, 42.77, 42.04, 39.90, 38.93, 35.70, 35.52, 34.80, 32.07, 31.01, 29.83, 29.58, 29.00, 27.93, 27.86, 23.60, 23.01, 22.92, 18.17, 12.66; FAB-MS: $[M+Na]^+$ 583.3956; cacld: 583.3942.

Compound 2-50: To a round-bottom flask were added compound **2-49** (3.2 g, 0.0057 mol), pyridine (0.48 mL, 0.006 mol) and TrCl (1.67 g, 0.006 mol) in CH_2Cl_2 (50 mL). The mixture was refluxed over 12 hr. After removed the

solvent under vacuum, the residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-50** (4.1 g, 90%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.40-7.30 (m, 6 H), 7.24-7.20 (m, 9 H), 3.70-3.55 (m, 6 H), 3.51-3.24 (m, 10 H), 3.24-3.11 (m, 8 H), 2.29-2.06 (m, 6 H), 2.00-1.57 (m, 12 H), 1.55-0.89 (m, 20 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.73, 128.89, 127.86, 126.96, 86.44, 80.72, 77.48, 77.23, 76.97, 76.18, 72.15, 64.97, 64.36, 64.29, 48.95, 46.75, 46.41, 42.68, 42.00, 39.86, 38.89, 35.66, 35.50, 34.77, 32.60, 30.96, 29.80, 29.71, 28.97, 27.88, 27.72, 26.86, 23.57, 22.98, 22.88, 18.15, 12.65; FAB-MS: $[M+Na]^+$ 825.5041; cacld: 825.5037.



2-50

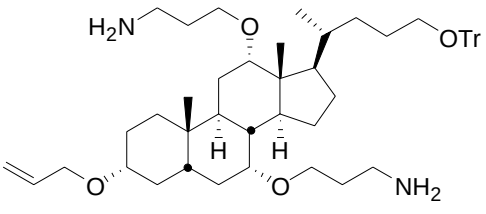


2-51

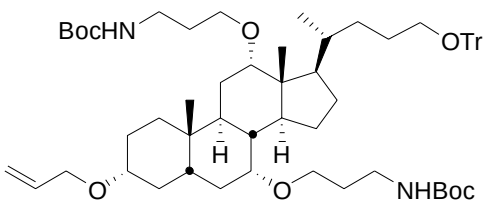
Compound 2-51: To a round-bottom flask were added **2-50** (4.1 g, 0.005 mol) and NaH (1 g, 0.025 mol) in dry THF (200 mL). The mixture was refluxed 1 hr under N_2 followed by addition of allyl bromide. After 12 hr at reflux, water (40 mL) was introduced slowly. The mixture was then extracted with EtOAc (3× 100 mL) and the combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:6 EtOAc/hexane) to afford **2-51** (3.3g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.42-7.30 (m, 6 H), 7.28-7.20 (m, 9 H), 5.91-5.90 (m, 1 H), 5.28-5.24 (m, 1 H), 5.10 (d, J = 6.5 Hz, 1 H), 4.00 (s, 2 H), 3.70-3.55 (m, 2 H), 3.51-3.24 (m, 8H), 3.24-3.11 (m, 4 H), 2.29-2.06 (m, 8 H), 2.00-1.57 (m, 12 H),

1.55-0.89 (m, 20 H), 0.63 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.71, 135.92, 128.86, 127.82, 126.91, 116.41, 86.40, 80.77, 79.05, 77.48, 77.23, 76.97, 76.08, 68.98, 65.03, 64.52, 64.32, 48.99, 48.92, 46.61, 42.42, 42.04, 39.85, 35.71, 35.47, 35.33, 35.12, 34.82, 32.58, 31.74, 29.84, 27.92, 27.74, 27.69, 26.83, 25.44, 23.57, 23.06, 22.82, 18.07, 12.64; FAB-MS: $[M+Na]^+$ 865.5419; cacld: 865.5350.

Compound 2-55: **2-51** (3.0g, 3.0 mmol) was dissolved in THF (25 mL) and H_2O (10 mL). Triphenylphosphine (1.4 g, 5.0 mmol) was added, and the solution was stirred for 12 hr. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, 10% CH_3OH in CH_2Cl_2) to yield **2-55** (2.4 g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.42-7.30 (m, 6 H), 7.28-7.20 (m, 9 H), 5.93-5.90 (m, 1 H), 5.29-5.24 (m, 1 H), 5.11 (d, J = 6.5 Hz, 1 H), 4.00 (s, 2 H), 3.70-3.56 (m, 2 H), 3.51-3.26 (m, 8H), 3.24-3.10 (m, 4 H), 2.29-2.06 (m, 8 H), 2.00-1.59 (m, 12 H), 1.55-0.89 (m, 24 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.77, 135.96, 128.87, 127.84, 126.97, 116.41, 86.40, 80.79, 79.05, 77.46, 77.23, 76.93, 76.06, 68.98, 65.04, 64.52, 64.32, 48.99, 48.94, 46.61, 42.42, 42.01, 39.85, 35.75, 35.47, 35.37, 35.12, 34.82, 32.58, 31.74, 29.89, 27.92, 27.74, 27.69, 26.87, 25.44, 23.56, 23.06, 22.82, 18.09, 12.69; FAB-MS: $[M+Na]^+$ 813.5535; cacld: 813.5540.



2-55

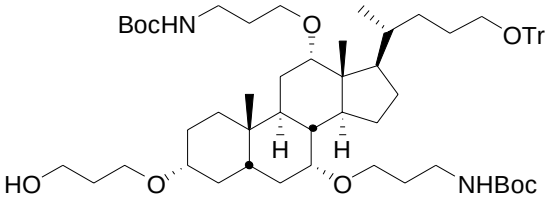


2-56

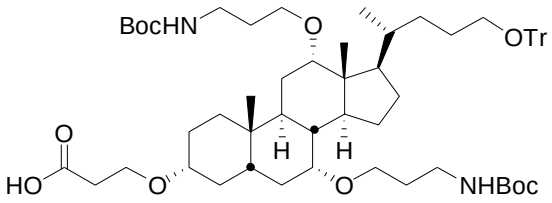
Compound 2-56: **2-55** (1.0 g, 1.2 mmol) dissolved in CH_2Cl_2 (30 mL) followed by addition Et_3N (0.5 mL, 3.0 mmol) and Boc_2O (0.65 g, 3.0 mmol). The mixture was stirred for 12 hr. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, 1:2 EtOAc/hexane) to yield **2-56** (1.2 g, 95%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.42-7.31 (m, 6 H), 7.29-7.20 (m, 9 H), 5.95-5.90 (m, 1 H), 5.29-5.23 (m, 1 H), 5.13 (d, J = 6.5 Hz, 1 H), 4.01 (s, 2 H), 3.70-3.55 (m, 2 H), 3.51-3.25 (m, 8H), 3.24-3.10 (m, 4 H), 2.29-2.06 (m, 8 H), 2.00-1.60 (m, 12 H), 1.55-0.89 (m, 34 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.75, 135.89, 128.77, 127.72, 126.66, 116.45, 86.41, 80.80, 79.06, 77.48, 77.23, 76.92, 76.06, 68.97, 65.04, 64.55, 64.31, 48.99, 48.96, 46.61, 42.40, 42.00, 39.85, 35.76, 35.47, 35.36, 35.12, 34.85, 32.58, 31.73, 29.89, 27.91, 27.74, 27.67 26.87, 25.46, 23.56, 23.09, 22.82, 18.06, 12.66; FAB-MS: $[M+Na]^+$ 991.6870; cacld: 991.6875.

Compound 2-57: A round-bottom flask was charged with THF (40 mL) and cooled to 0℃, Borane-THF complex (1.5 mL, 1.5 mol) and cyclohexene(0.31 mL, 3.0 mmol) were added sequentially, and the mixture was stirred at 0℃ for 3 hr under N_2. **2-56** (1.1 g, 1.1 mmol) in THF (20 mL) was added dropwise and the mixture was stirred for 12 hr at room temperature. Aqueous NaOH (20%) (20 mL) and H_2O_2 (12 mL) were added in sequence. The mixture was refluxed for 3 h followed by the addition of brine (10 mL) and extracted with EtOAc (3 ×20 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column

chromatography (SiO_2, 5% CH_3OH in CH_2Cl_2) to afford **2-57** (0.95 g, 84%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.44-7.31 (m, 6 H), 7.30-7.20 (m, 9 H), 5.99-5.90 (m, 1 H), 4.05 (s, 2 H), 3.70-3.65 (m, 2 H), 3.51-3.25 (m, 10H), 3.26-3.11 (m, 4 H), 2.27-2.06 (m, 6 H), 2.00-1.61 (m, 18 H), 1.55-0.88 (m, 32 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.78, 135.90, 128.79, 127.69, 126.59, 116.50, 86.42, 80.83, 79.08, 77.49, 77.21, 76.92, 76.09, 68.96, 65.08, 64.53, 64.31, 48.99, 48.93, 46.61, 42.42, 42.00, 39.85, 35.71, 35.47, 35.33, 35.12, 34.85, 32.58, 31.73, 29.85, 27.91, 27.73, 27.67 26.87, 25.47, 23.56, 23.09, 22.89, 18.09, 12.68; FAB-MS: $[M+Na]^+$ 1031.6699; cacld: 1031.6695.



2-57

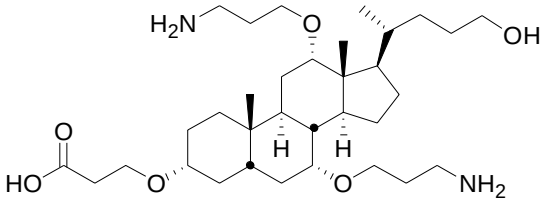


2-58

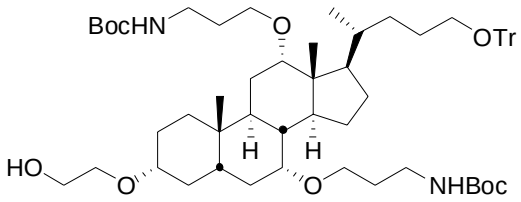
Compound 2-58: **2-57** (0.9 g, 0.8 mmol) was dissolved in CH_2Cl_2 (1 mL) and H_2O (0.5 mL) followed by the addition of TEMPO (2.5 eq) and BAIB (0.2 eq). The mixture was stirred for 12 hr and was extracted with CH_2Cl_2 (3×5 mL). The combined extracts were dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:2:0.05 EtOAc/hexane/AcOH) to afford **2-58** (0.55g, 65%). ^1H NMR ($CDCl_3$, 500 MHz) δ 7.45-7.31 (m, 6 H), 7.29-7.20 (m, 9 H), 5.20-5.14 (m, 2 H), 3.60-3.52 (m, 5 H), 3.41-3.05 (m, 10H), 2.44 (s, 2 H), 2.20-2.05 (m, 4 H), 1.89-0.89 (m, 54 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.12, 156.59, 156.33, 144.63, 86.33, 80.76, 79.32, 78.92, 77.48,

77.23, 76.97, 75.87, 66.59, 65.81, 64.18, 46.99, 46.23, 42.68, 41.90, 39.81, 35.32, 34.95, 32.56, 31.52, 30.64, 29.79, 28.91, 28.58, 27.74, 27.58 26.87, 25.27, 23.41, 22.61, 18.04, 12.62; FAB-MS: $[M+Na]^+$ 1059.6648; cacld: 1059.6644.

Compound 2-54: **2-58** (0.55 g, 0.5 mmol) dissolved in TFA and the mixture was stirred for 2 hr. The solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 65:35:5 CH_2Cl_2/MeOH/H_2O) to afford **2-54** (0.25g, 80%). ^1H NMR ($CDCl_3$, 500 MHz) δ 3.66-3.52 (m, 5 H), 3.44-3.05 (m, 10H), 2.90-2.55 (m, 10 H), 1.89-0.79 (m, 24 H), 0.60 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 175.92, 159.90, 159.65, 159.24, 138.02, 129.53, 128.83, 125.94, 121.20, 80.85, 79.07, 76.04, 66.66, 65.52, 65.30, 61.91, 46.82, 46.48, 43.10, 41.79, 40.39, 40.22, 40.06, 39.89, 39.55, 39.40, 37.55, 35.72, 35.33, 35.00, 32.43, 31.27, 29.85, 28.13, 28.07, 27.75, 25.83 , 23.46, 23.22, 23.11, 21.58, 18.42, 12.87; FAB-MS: $[M+Na]^+$ 603.4335; cacld: 603.4343.



2-54

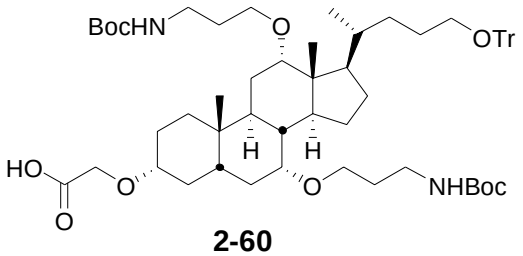


2-59

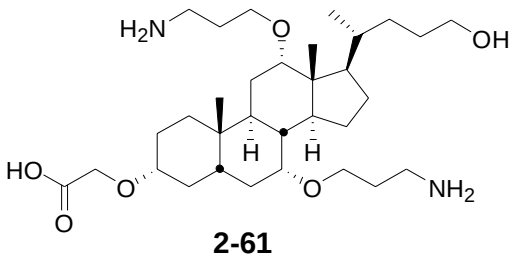
Compound 2-59: Same procedure as for **2-9**. The yield was 60%. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.44-7.31 (m, 6 H), 7.31-7.20 (m, 9 H), 5.97-5.90 (m, 1 H), 4.06 (s, 2 H), 3.72-3.65 (m, 2 H), 3.51-3.25 (m, 8H), 3.26-3.15 (m, 4 H), 2.27-2.05 (m, 6 H), 2.00-1.61 (m, 16 H), 1.55-0.88 (m, 32 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 145.01, 135.88, 128.70, 127.66, 126.55, 116.50,

86.42, 80.84, 79.08, 77.43, 77.21, 76.91, 76.09, 68.97, 65.08, 64.55, 64.31, 48.98, 48.93, 46.62, 42.42, 42.04, 39.88, 35.71, 35.49, 35.33, 35.14, 34.85, 32.56, 31.77, 29.86, 27.90, 27.73, 27.68 26.88, 25.47, 23.55, 23.09, 22.90, 18.08, 12.66; FAB-MS: $[M+Na]^+$ 1017.6532; cacld: 1017.6538.

Compound 2-60: Same procedure as for **2-58**. The yield was 65%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.45-7.30 (m, 6 H), 7.29-7.21 (m, 9 H), 5.22-5.14 (m, 2 H), 3.62-3.55 (m, 5 H), 3.44-3.07 (m, 8H), 2.46 (s, 2 H), 2.21-2.05 (m, 4 H), 1.89-0.87 (m, 54 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.22, 156.57, 156.43, 144.66, 86.31, 80.79, 79.41, 78.99, 77.46, 77.26, 76.97, 75.87, 66.59, 65.85, 64.18, 46.94, 46.22, 42.68, 41.92, 39.84, 35.32, 34.99, 32.56, 31.52, 30.66, 29.79, 28.95, 28.55, 27.73, 27.58 26.88, 25.29, 23.44, 22.61, 18.09, 12.69; FAB-MS: $[M+Na]^+$ 1031.6358; cacld: 1031.6367.



2-60

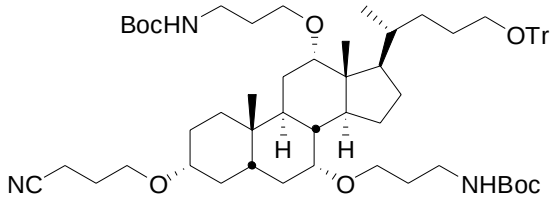


2-61

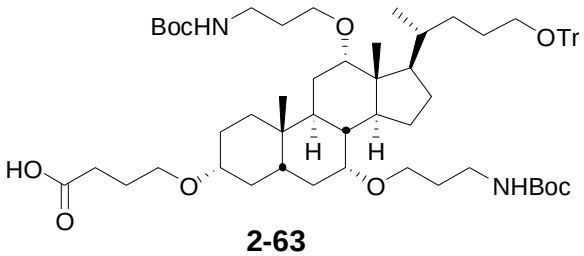
Compound 2-61: Same procedure as for **2-54**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.70-3.52 (m, 5 H), 3.46-3.06 (m, 8H), 2.93-2.57 (m, 10 H), 1.87-0.77 (m, 22 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 175.99, 159.93, 159.61, 159.26, 138.06, 129.53, 128.85, 125.96, 121.23, 80.85, 79.07, 76.02, 66.68, 65.52, 65.31, 61.91, 46.85, 46.48, 43.12, 41.79, 40.39, 40.24, 40.06, 39.85, 39.55, 39.43, 37.55, 35.772, 35.33, 35.00, 32.41, 31.27, 29.85, 28.12, 28.08, 27.75, 25.84 , 23.46, 23.27, 23.11, 21.59, 18.41, 12.89; FAB-MS:

$[M+Na]^+$ 589.4250; cacld: 589.4255.

Compound 2-62: Same procedure as for **2-13**. The yield was 75%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.45-7.31 (m, 6 H), 7.31-7.20 (m, 9 H), 5.95-5.90 (m, 1 H), 4.04 (s, 2 H), 3.74-3.65 (m, 2 H), 3.52-3.25 (m, 10H), 3.26-3.11 (m, 4 H), 2.27-2.07 (m, 6 H), 2.02-1.61 (m, 18 H), 1.55-0.89 (m, 32 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 144.80, 135.88, 128.81, 127.68, 126.59, 116.51, 86.44, 80.84, 79.08, 77.47, 77.22, 76.92, 76.05, 68.96, 65.09, 64.53, 64.31, 48.99, 48.96, 46.61, 42.45, 42.04, 39.86, 35.71, 35.49, 35.36, 35.12, 34.85, 32.56, 31.77, 29.85, 27.93, 27.73, 27.65, 26.86, 25.47, 23.56, 23.08, 22.89, 18.12, 12.77; FAB-MS: $[M+Na]^+$ 1040.6760; cacld: 1040.6765.



2-62

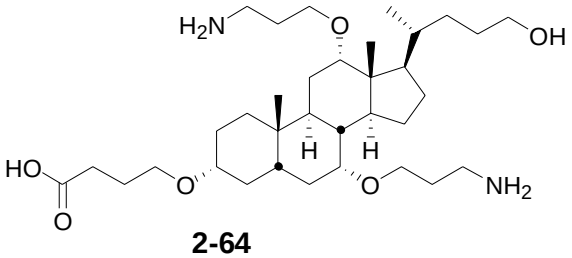


2-63

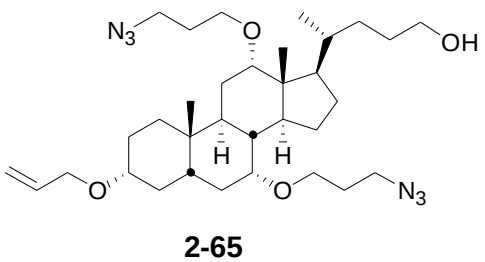
Compound 2-63: **2-62** (0.5 g, 0.5 mmol) was dissolved in EtOH (20 mL) and NaOH (5 mL, 20%). The mixture refluxed for 24 hr. The pH value of this solution was adjusted to 3 using AcOH. After extracted with EtOAc, the solvent was removed under vacuum and the residue was purified by column chromatography (SiO_2, 1:2:0.05 EtOAc/hexane/AcOH) to afford **2-63** (0.42g, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.40-7.30 (m, 6 H), 7.28-7.21 (m, 9 H), 5.22-5.12 (m, 2 H), 3.62-3.56 (m, 5 H), 3.44-3.08 (m, 12H), 2.47 (s, 2 H), 2.21-2.04 (m, 4 H), 1.89-0.89 (m, 54 H), 0.68 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.26, 156.59, 156.47, 144.65, 86.31, 80.77, 79.45, 78.97, 77.46,

77.26, 76.99, 75.88, 66.59, 65.86, 64.18, 46.97, 46.22, 42.66, 41.92, 39.88, 35.39, 34.94, 32.55, 31.52, 30.67, 29.79, 28.98, 28.59, 27.73, 27.55 26.83, 25.26, 23.46, 22.61, 18.12, 12.71; FAB-MS: $[M+Na]^+$ 1059.6628; cacld: 1059.6637.

Compound 2-64: Same procedure as for **2-54**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.62-3.52 (m, 5 H), 3.44-3.09 (m, 14H), 2.95-2.55 (m, 10 H), 1.89-0.77 (m, 24 H), 0.60 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 175.95, 159.97, 159.62, 159.24, 138.03, 129.57, 128.81, 125.94, 121.22, 80.85, 79.07, 76.01, 66.66, 65.52, 65.33, 61.92, 46.82, 46.49, 43.10, 41.79, 40.38, 40.24, 40.06, 39.89, 39.56, 39.40, 37.52, 35.72, 35.33, 35.04, 32.43, 31.27, 29.85, 28.15, 28.06, 27.75, 25.88 , 23.46, 23.25, 23.14, 21.59, 18.47, 12.90; FAB-MS: $[M+Na]^+$ 617.4452; cacld: 617.4500.



2-64

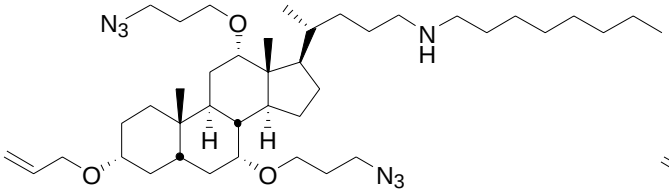


2-65

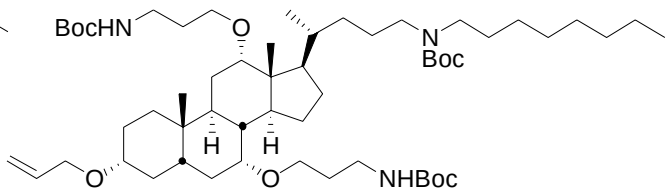
Compound 2-65: Same procedure as for **2-8**. The yield was 90%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.93-5.90 (m, 1 H), 5.29-5.24 (m, 1 H), 5.11 (d, J = 6.5 Hz, 1 H), 4.01 (s, 2 H), 3.70-3.56 (m, 2 H), 3.51-3.25 (m, 8H), 3.24-3.11 (m, 4 H), 2.29-2.07 (m, 8 H), 2.00-1.57 (m, 12 H), 1.55-0.88 (m, 20 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 86.41, 80.79, 79.06, 77.48, 77.24, 76.97, 76.08, 68.99, 65.03, 64.54, 64.32, 48.99, 48.92, 46.61, 42.42, 42.04, 39.85, 35.71, 35.44, 35.34, 35.12, 34.82, 32.57, 31.74, 29.86, 27.92, 27.77, 27.69, 26.83,

25.44, 23.58, 23.06, 22.83, 18.09, 12.64; FAB-MS: $[M+Na]^+$ 623.4274; cacld: 623.4255.

Compound 2-66: Same procedure as for **2-20**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.99-5.90 (m, 1 H), 5.30-5.25 (m, 1 H), 5.15 (d, J = 6.5 Hz, 1 H), 4.03 (s, 2 H), 3.73-3.58 (m, 2 H), 3.51-3.25 (m, 16H), 3.24-3.11 (m, 4 H), 2.29-2.07 (m, 8 H), 2.05-1.57 (m, 12 H), 1.55-0.88 (m, 30 H), 0.67 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 156.40, 156.23, 155.81, 135.98, 116.42, 94.98, 80.93, 78.99, 78.85, 77.48, 77.24, 76.97, 75.88, 68.99, 66.71, 65.82, 47.75, 47.25, 46.35, 42.79, 42.04, 39.90, 39.53, 38.42, 35.72, 35.44, 35.09, 33.21, 32.00, 29.57, 29.48, 28.99, 28.70, 27.77, 27.09, 25.88, 23.52, 22.84, 18.09, 12.69; FAB-MS: $[M+Na]^+$ 734.5758; cacld: 734.5769.



2-66

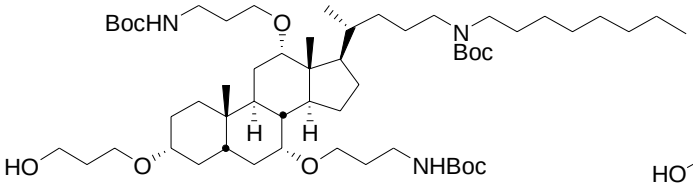


2-67

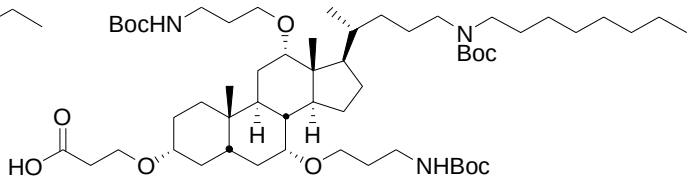
Compound 2-67: Same procedure as for **2-56**. The yield was 95%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.95-5.91 (m, 1 H), 5.33-5.21 (m, 1 H), 5.17 (d, J = 6.5 Hz, 1 H), 4.01 (s, 2 H), 3.73-3.59 (m, 2 H), 3.50-3.26 (m, 22H), 3.24-3.12 (m, 4 H), 2.27-2.08 (m, 8 H), 2.05-1.59 (m, 14 H), 1.55-0.89 (m, 40 H), 0.65 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 156.67, 156.35, 155.78, 135.93, 116.42, 94.98, 80.91, 78.92, 78.85, 77.43, 77.25, 76.96, 75.88, 68.98, 66.71, 65.82, 47.75, 47.24, 46.35, 42.77, 42.04, 39.92, 39.53, 38.43, 35.72, 35.44, 35.09, 33.21, 32.01, 29.57, 29.48, 28.93, 28.70, 27.75, 27.09, 25.86, 23.52, 22.86, 18.09,

12.67; FAB-MS: $[M+Na]^+$ 982.7419; cacld: 982.7430.

Compound 2-68: Same procedure as for **2-57**. The yield was 85%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.99-5.90 (m, 2 H), 3.70-3.65 (m, 8 H), 3.51-3.00 (m, 14H), 2.27-2.06 (m, 3 H), 2.00-1.11 (m, 70 H), 1.55-0.88 (m, 12 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 156.55, 155.84, 80.69, 79.42, 79.01, 75.90, 66.77, 65.96, 62.03, 47.29, 46.33, 42.70, 42.00, 41.91, 39.89, 39.62, 38.60, 35.68, 33.21, 32.36, 30.85, 29.60, 28.97, 28.72, 27.75, 27.10, 23.51, 23.02, 22.86, 18.14, 14.31, 12.69; FAB-MS: $[M+Na]^+$ 1000.7532; cacld: 1000.7536.



2-68

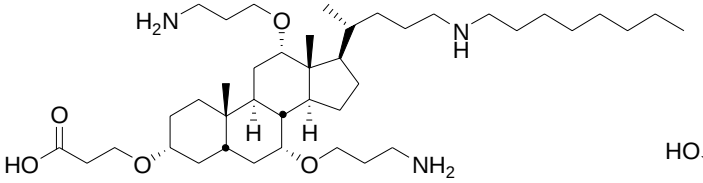


2-69

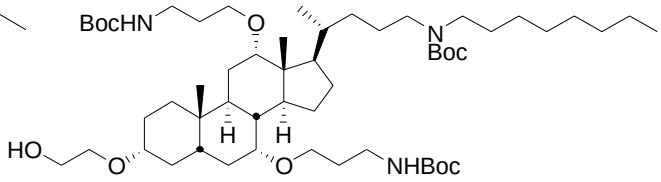
Compound 2-69: Same procedure as for **2-58**. The yield was 65%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.20-5.97 (m, 2 H), 4.20 (s, 2 H), 3.74-3.68 (m, 8 H), 3.51-3.04 (m, 14H), 2.27-2.09 (m, 3 H), 2.05-1.12 (m, 70 H), 1.66-0.89 (m, 12 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 157.32, 155.67, 80.77, 79.38, 79.00, 75.94, 66.78, 65.96, 62.10, 47.29, 46.35, 42.70, 42.09, 41.93, 39.87, 39.61, 38.60, 35.64, 33.21, 32.37, 30.85, 29.66, 28.98, 28.76, 27.75, 27.15, 23.51, 23.03, 22.81, 18.14, 14.41, 12.80; FAB-MS: $[M+Na]^+$ 1014.7355; cacld: 1014.7362.

Compound 2-70: Same procedure as for **2-54**. The yield was 90%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.56-3.40 (m, 8 H), 3.39-3.02 (m, 10H), 2.90-2.55 (m, 8 H), 2.37 (s, 2 H), 2.00-0.91 (m, 52 H), 0.61 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500

MHz) δ 173.28, 159.02, 158.77, 158.52, 158.26, 120.53, 118.38, 116.01, 113.63, 80.19, 78.51, 75.42, 64.28, 64.79, 63.18, 47.24, 46.83, 46.21, 45.96, 42.39, 41.15, 40.39, 40.02, 39.85, 39.68, 39.51, 39.18, 36.97, 36.83, 35.59, 34.87, 34.67, 34.43, 32.38, 31.20, 28.48, 28.21, 28.03, 27.54, 27.40, 27.10, 25.96, 22.88, 22.71, 22.50, 22.14, 17.62, 13.98 12.34; FAB-MS: $[M+Na]^+$ 714.5759; cacld: 714.5755.



2-70

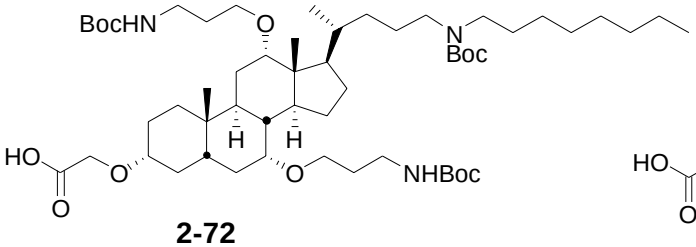


2-71

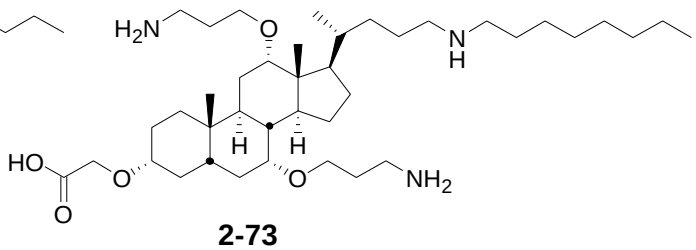
Compound 2-71: Same procedure as for **2-59**. The yield was 60%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.97-5.90 (m, 2 H), 3.72-3.66 (m, 6 H), 3.55-3.03 (m, 14H), 2.27-2.08 (m, 3 H), 2.00-1.10 (m, 70 H), 1.55-0.89 (m, 12 H), 0.61 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 171.34, 156.78, 155.84, 80.69, 79.58, 79.01, 77.48, 77.23, 76.97, 75.86, 69.23, 66.42, 65.61, 62.30, 60.58, 47.26, 46.31, 42.77, 42.05, 41.99, 39.83, 39.62, 38.64, 35.68, 33.26, 32.37, 30.85, 29.63, 28.99, 28.70, 27.75, 27.15, 23.51, 23.01, 22.86, 21.23, 18.10, 14.29, 12.65; FAB-MS: $[M+Na]^+$ 986.7383; cacld: 986.7379.

Compound 2-72: Same procedure as for **2-60**. The yield was 65%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.18-5.99 (m, 2 H), 4.22 (s, 2 H), 3.72-3.69 (m, 8 H), 3.55-3.03 (m, 14H), 2.27-2.09 (m, 3 H), 2.05-1.10 (m, 68 H), 1.69-0.89 (m, 12 H), 0.69 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 174.28, 157.35, 155.68, 80.77, 79.38, 79.02, 75.94, 66.78, 65.94, 62.10, 47.28, 46.35, 42.73, 42.09, 41.93,

39.87, 39.64, 38.61, 35.65, 33.21, 32.38, 30.85, 29.64, 28.98, 28.76, 27.76, 27.15, 23.52, 23.03, 22.88, 18.14, 14.45, 12.87; FAB-MS: $[M+Na]^+$ 1000.7247; cacld: 1000.7253.



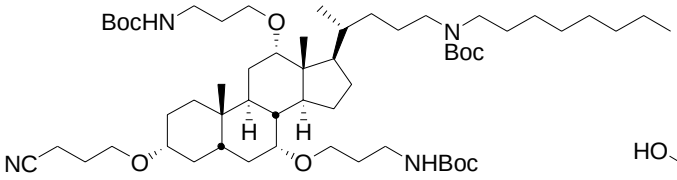
2-72



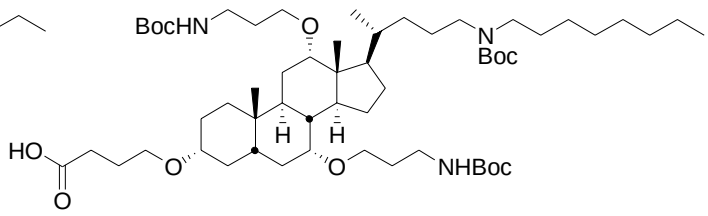
2-73

Compound 2-73: Same procedure as for **2-70**. The yield was 85%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.96-3.80 (m, 2 H), 3.79-3.02 (m, 12H), 2.90-2.55 (m, 8 H), 2.37 (s, 2 H), 2.00-0.91 (m, 50 H), 0.61 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 173.66, 158.81, 158.56, 158.31, 120.76, 118.37, 116.03, 113.63, 80.15, 78.51, 75.42, 64.28, 64.79, 63.15, 47.27, 46.83, 46.24, 45.96, 42.36, 41.15, 40.39, 40.02, 39.85, 39.68, 39.51, 39.15, 36.97, 36.83, 35.59, 34.87, 34.69, 34.43, 32.38, 31.20, 28.48, 28.25, 28.03, 27.55, 27.40, 27.10, 25.96, 22.88, 22.71, 22.50, 22.14, 17.68, 12.28, 11.35; FAB-MS: $[M+Na]^+$ 700.5586; cacld: 700.5598.

Compound 2-74: Same procedure as for **2-13**. The yield was 80%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.99-5.93 (m, 2 H), 3.72-3.65 (m, 8 H), 3.51-3.04 (m, 14H), 2.27-2.03 (m, 3 H), 2.00-1.11 (m, 68 H), 1.58-0.89 (m, 14 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 157.56, 155.87, 80.69, 79.45, 79.01, 75.90, 66.79, 65.96, 62.06, 47.29, 46.33, 42.72, 42.00, 41.95, 39.89, 39.67, 38.60, 35.68, 33.21, 32.38, 30.85, 29.61, 28.97, 28.72, 27.75, 27.12, 23.51, 23.02, 22.83, 18.19, 14.39, 12.62; FAB-MS: $[M+Na]^+$ 1009.7530; cacld: 1009.7538.

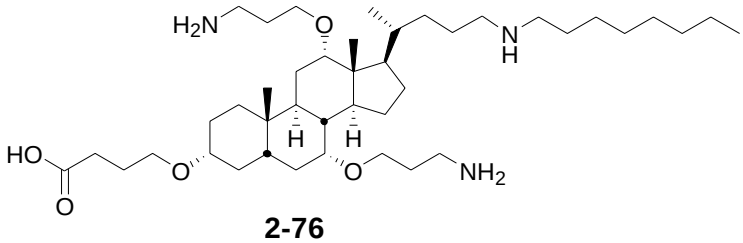


2-74



2-75

Compound 2-75: Same procedure as for **2-63**. The yield was 70%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.99-5.77 (m, 2 H), 4.22 (s, 2 H), 3.78-3.68 (m, 8 H), 3.51-3.03 (m, 16H), 2.27-2.09 (m, 3 H), 2.05-1.10 (m, 70 H), 1.66-0.87 (m, 12 H), 0.64 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 156.31, 155.62, 80.77, 79.39, 79.00, 75.98, 66.78, 65.98, 62.10, 47.29, 46.34, 42.70, 42.09, 41.93, 39.87, 39.61, 38.61, 35.64, 33.24, 32.37, 30.88, 29.66, 28.98, 28.78, 27.75, 27.15, 23.51, 23.03, 22.89, 18.23, 14.50, 12.74; FAB-MS: $[M+Na]^+$ 1028.7534; cacld: 1028.7539.



2-76

Compound 2-76: Same procedure as for **2-73**. The yield was 85%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.66-3.41 (m, 10 H), 3.37-3.05 (m, 10H), 2.91-2.55 (m, 8 H), 2.40 (s, 2 H), 2.00-0.91 (m, 52 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 173.26, 159.00, 158.78, 158.53, 158.26, 120.56, 118.39, 116.01, 113.63, 80.18, 78.51, 75.46, 64.28, 64.77, 63.18, 47.24, 46.83, 46.21, 45.96, 42.37, 41.15, 40.38, 40.02, 39.85, 39.64, 39.51, 39.18, 36.99, 36.83, 35.59, 34.87, 34.68, 34.43, 32.38, 31.22, 28.48, 28.23, 28.05, 27.54, 27.41, 27.10, 25.96, 22.88, 22.77, 22.55, 22.14, 17.68, 13.95 12.35; FAB-MS: $[M+Na]^+$ 729.5908;

cacld: 729.5913.

Virus Preparation

The Wyeth strain, currently the strain of VV used for vaccinations in the United States, was obtained from the Centers for Disease Control and Prevention. The Western Reserve VV strain was obtained from ATCC (VR1354). Virus was propagated by inoculating confluent HeLa S3 (ATCC#CCK-2.2) human adenocarcinoma cells with 1 plaque forming unit (pfu) per cell and incubating at 37°C in 5% CO_2 for three days. For virus titration, ten-fold dilutions of stock were made and 0.1 ml of each dilution was added to the fibroblast cell sheets in 24 well tissue culture plates. Adsorption was allowed to take place for one hour at 37° in 5% CO_2 and was followed by the addition of Earle's-minimal essential media (MEM) with 2.5% fetal calf serum (FCS; Gemini Bio Products, Woodland, CA). Forty-eight hours following infection, media was removed and cells fixed with formalin-crystal violet. Plaques were visualized on an Inverted Nikon Microscope under 1.3 x 10 magnification. Virus stocks were stored at -70°C.

Viral Plaque Assay

BS-C-1 (ATCC #CCL-26) African green monkey kidney cells were seeded at 2 x 10^5/well in 24 well tissue culture plates in MEM containing 10% FCS, 1% penicillin/streptomycin and allowed to grow overnight before having the supernatant removed and replaced with MEM with 2.5% FCS for virus incubation. The BS-C-1 cells were used for the quantitative estimates because

they present uniform plaques. HeLa S3 cells are routinely used for preparations of virus stock as they give consistently high yields of virus, but due to their rounded morphology, do not present uniform plaques.

Ceragenin stocks were hydrated at 5 mg/ml in DMSO, mixed well, aliquotted, and stored at –80°C. Ceragenins were diluted to the proper concentrations in 0.01x tryptic soy broth containing 10 mM sodium phosphate buffer, pH=7.4. Virus diluted in the same buffer was added to the compounds, and they were incubated for 24 hours at 37°C. Twenty microliters of the peptide/virus mixture was added to the cells in 0.5 ml MEM-2.5% FCS and allowed to infect for 48 hours for plaque development. Medium was removed and wells were overlaid with 0.5 ml 4% buffered formalin, allowed to fix for 10 minutes at room temperature. The formalin was removed and 0.5 ml 0.1% crystal violet in PBS was added to the wells for 5 minutes at room temperature. Wells were then aspirated and air-dried for visualization of plaques. We found the most accurate results with the virus alone forming 50-80 plaques per well. Results are expressed the percent killing in order to allow comparisons between compounds.

Keratinocyte Cell Culture

Primary human keratinocytes (Cascade Biologics, Portland, OR) were grown in serum-free keratinocyte growth medium (EpiLife®, Cascade Biologics), supplemented with 1% human keratinocytes growth supplement V2 (Cascade Biologics), 0.06 mM $CaCl_2$ and 1% of penicillin and streptomycin.

To investigate the anti-viral activity of ceragenins, cells were seeded in 24 well plates at a concentration of 2 x 10^5 cells/well. Cells were infected with VV (0.05 plaque forming units/cell) for 6 hours. Following the incubation, virus was removed and the cells were washed with media to remove remaining virus. Ceragenins (0 – 100 μM) were added to the infected keratinocytes and allowed to incubate for an additional 18 hours. RNA was isolated for analysis of VV gene expression.

Murine Experiments

CBy.SmnCB17-Prkdc <SCID>/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All protocols with these animals were approved by the Institutional Animal Care and Use Committee at National Jewish Medical and Research Center. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection and Research Risks.

The dorsal thoracic and lumbar regions of mice were clipped and treated with the depilatory agent Nair® to remove all hair. Seventy-two hours following hair removal, mice were anesthetized and inoculated with 5 x 10^6 pfu of Western Reserve VV by scarification. Two hours following the inoculation, CSA-13, -92, or control cream was applied to the dorsal thoracic and lumbar regions. Topical application was repeated daily until day 10 post inoculation. Mice were monitored for weight loss and mortality.

Statistical Analyses

All statistical analysis was conducted using Graph Pad Prism, version 4.01

(San Diego, CA). Statistical differences in the number of viral plaques and viral replication (by real-time RT-PCR) were determined using a student's T test or one-way analysis of variance (ANOVA) and significant differences determined by a Tukey-Kramer test.

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