Synthesis and Characterization of New Polyamino-Cholic Acid Dimers

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Cholic acid was selectively protected by trifluoroacetic anhydride, then selectively deprotected at C-3 in order to synthesize 3-keto-diprotected methyl cholate. Dimerization *via* A-A ring connection was achieved by reductive amination of 3-keto-diprotected methyl cholate with different di and polyamines. Diamines chosen to build SAR were n = 3, 4, 6. In addition, polyamine (spermidine) was chosen to study the effect of increasing the distance between the two sides of dimer in bioactivity. Several dimeric methyl cholate derivatives were synthesized to evaluate their biological activities.

Key Words: Synthesis, Characterization, Polyamino-cholic acid, Dimers.

INTRODUCTION

The cephalostatins that are a group of dimeric steroids are among the most potent natural cytotoxins. This exceptional activity of cephalostatins has led to interest in the synthesis of these compounds and analogues as potential anti-tumor agents. It is thought that DNA is the likely target of the steroidal diamine dimers. Similar studies on steroidal diamine indicate that this family of compounds has a moderate biological activity. In addition, SAR showed that as the number of hydroxyl groups increases in steroidal diamine dimers, the biological activity increases[1-3].

Bile acids are naturally occurring amphiphilic compounds, stored in the gall bladder that plays an important physiological role in all mammals. Bile acids and their salt forms serve as emulsifiers for the solubilization and digestion of fats and lipids in food[4]. Cholic acid is also an ideal building block for artificial enzymes because the two faces of the steroid differ dramatically in their properties, where the alpha-face displays three hydrogen bonding groups, while the beta-face is entirely hydrophobic[5]. In addition, the three hydroxyl groups are directed convergent toward the centre of the concave face that is expected to increase the solubility of cholic acid derivatives in the human tissues.

Recently, Davis and co-workers[6] reported the well-planned synthesis of a new cholic acid derivative (triamino-analogue) of methyl cholate used as antibiotic,

via a multi-step procedure. Chunhong and co-workers[7] have been interested in compounds similar to the previous one that are considered to be antibiotics and permeabilizers of the outer membrane of gram-negative bacteria. In addition to that, Regan *et al.*[8] reported the so-called "molecular umbrella" from the reaction of cholic acid and triamine spermidine, creating structures that can mask an attached agent (dansyl as drug mimetic) from the surrounding environment.

Polyamino-steroid dimerization is one of the most important subjects in the new chemical synthesis, because of the effective bioactivities of both the steroids and the polyamines. This dimerization could be carried out taking two major forms. The first form is a steroid-polyamine amide[6]. For example, a new class of supramolecular transmembrane ion channels was prepared by linking two amphiphilic cholic acid methyl ethers through biscarbamate bonds. The connection of two cholic acid methyl ether derivatives was done to obtain a membrane penetrating component creating supramolecular ion channels. Basic properties of these artificial ion channels are characterized *via* measurement of single ion channel currents[9]. Another example is a conjugate derived from cholic acid, spermidine and Ellman's reagent, bearing covalently attached glutathione that readily crosses phospholipid bilayers[10].

The second form of dimerization occurs by reductive amination of cholic acid with different polyamines to produce polyamino steroid dimers. There is no single example in literature up to our knowledge for the type of dimerization of cholic acid with di- or polyamines. This led us to build cholic acid derivatives that can be coupled to di- or polyamines *via* reductive amination.

RESULTS AND DISCUSSION

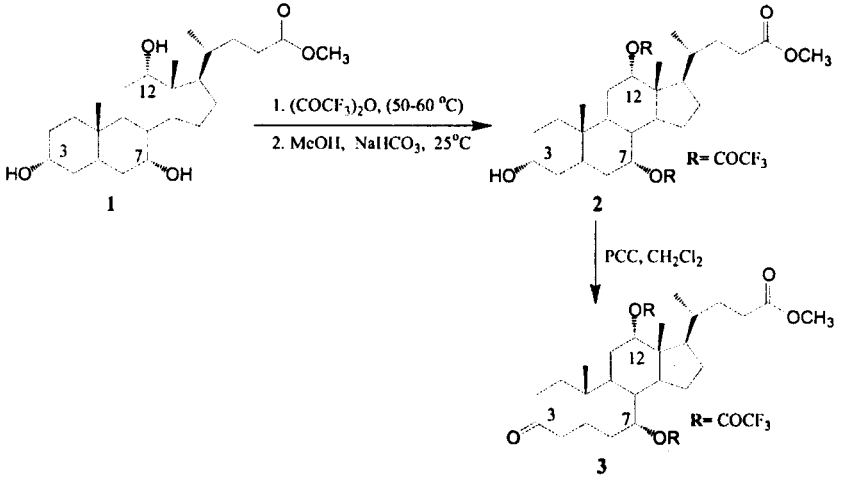
Retrosynthetic analysis indicates that the dimers could be synthesized from protected 3-ketocholic acid with diamine (1,3-diaminopropane, 1,4-diaminobutane and 1,6-diaminohexane) and polyamine (spermidine).

Polyamines are positively charged at neutral pH and occur as organic cations in all cells. It has long been recognized that polyamines, in the form of polycations, bind to polyanions. In view of their high positive charge, polyamines most likely exert many of their effects through direct interaction with negatively charged DNA and RNA[11, 12]. It is also known that the length of these polyamines is very important for binding ability to DNA. For all these reasons, different polyamine chain lengths have been used in the present studies.

According to our strategy all methyl cholate-polyamine dimers could be synthesized from 3-keto-7,12-diprotected methyl cholate; thus, the first step was to choose a suitable protecting group. It is well known that the three secondary hydroxyl groups, at positions 3, 7 and 12 in methyl cholate, have approximately the same chemical properties but different reactivity. The hydroxyl group at position 3 is more reactive than that at position 7 and this is more reactive than that at position 12. This small difference in reactivity is due to the geometrical shape of methyl cholate, where position 3 is less sterically hindered than positions 7 and 12. According to this point, a selective route was designed in which the

hydroxyl groups at positions 7 and 12 are protected, leaving hydroxyl group at position 3 unprotected and available for oxidation to the corresponding ketone.

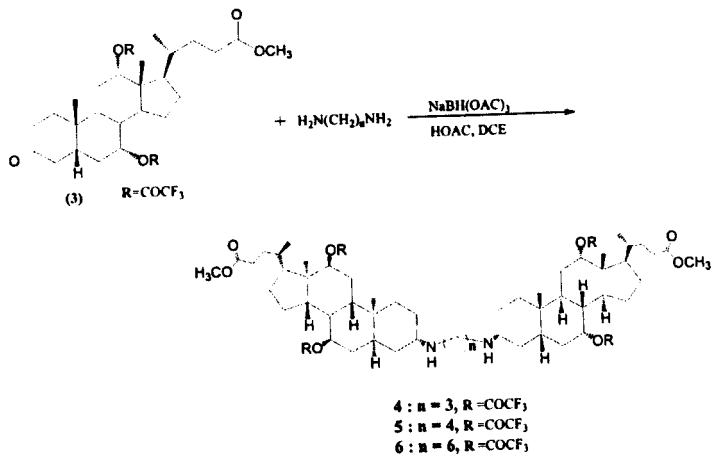
In order to achieve our goal, it was decided to try trifluoroacetic anhydride as protecting group[13]. Compound **(2)** was synthesized first by the reaction of methyl cholate **(1)** with excess amount of trifluoroacetic anhydride at 50–60°C followed by selective deprotection[13] which was carried out using absolute methanol in a weakly basic medium $NaHCO_3$ **(Scheme-1)**. The hydroxyl group at position 3 was selectively deprotected, while that at positions 7 and 12 still protected. This reaction is time dependent. If the reaction time is increased, all hydroxyl groups will be deprotected, and methyl cholate will be reproduced. Compound **(2)** was characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy. The ^{1}H-NMR spectrum showed two peaks at 5.15 and 5.33 ppm characteristic of the protons of C-7 and C-12, respectively. The peak at 4.85 ppm in the starting material disappeared and a new signal at 3.46 ppm characteristic for C-3 proton was displayed. A peak at 3.66 ppm is characteristic for methyl ester of C-24. The ^{13}C-NMR spectrum showed three peaks at 71.2, 76.1, 80.0 ppm characteristic of C-3, C-7 and C-12 respectively, this also proved the deprotection at C-3. Another peak at 51.6 ppm characteristic of C-24 methyl ester is observed. Also, two peaks at 174.4, 156 are characteristics for C-24 carbonyl group and carbonyl group of trifluoroacetyl protecting group respectively.



Scheme-1. Synthesis of protected cholic acid methyl ester

The next goal is to oxidize the hydroxyl group at C-3 into 3-keto that is essential for reductive amination process. Oxidation of compound **(2)** using pyridinium chlorochromate (PCC) furnished 3-ketocholic acid methyl ester **(3)**, which is considered as the principal backbone for A-A-ring dimerization. The ^{1}H-NMR showed two characteristic peaks at 5.25 and 5.38 ppm for C-7 and C-12 protons respectively, while the peak at 3.46 ppm disappeared. The ^{13}C-NMR showed a characteristic peak at 210 ppm for the keto group at C-3 and another two peaks at 75.77 and 79.8 ppm of protected C-7 and C-12 respectively.

The dimerization of protected 3-ketocholic acid methyl ester (3) with 1,3-diaminopropane was carried out by reductive amination using triacetoxyborohydride as reducing agent to give dimer **4** in 66% yield after chromatographic separation as shown in **Scheme-2**. The ^{1}H-NMR of dimer (4) showed two characteristic peaks at 5.16 and 5.34 ppm for C-7 and C-12 protons respectively. A peak at 3.1 ppm for β-proton on the carbon bearing a nitrogen at position 3 and a peak at 2.85 ppm for α-proton on the carbon bearing a nitrogen at position 3 of the other side of the dimer. A sharp singlet at 3.66 ppm was observed for the methyl ester. The ^{13}C-NMR showed two peaks at 75.4 and 80.0 ppm for C-7 and C-12, respectively and a characteristic peak at 53.8 ppm of the C—N at position 3. The molecular mass spectrum displayed a molecular ion peak at 1267 (M) and a peak at 1171 due to the loss of one $COCF_3$ group and another peak at 1075, for the loss of the second $COCF_3$ group. Also a peak at 834 representing the cleavage of all $COCF_3$ protecting groups was observed. In addition, the elemental analysis data were in good agreement.



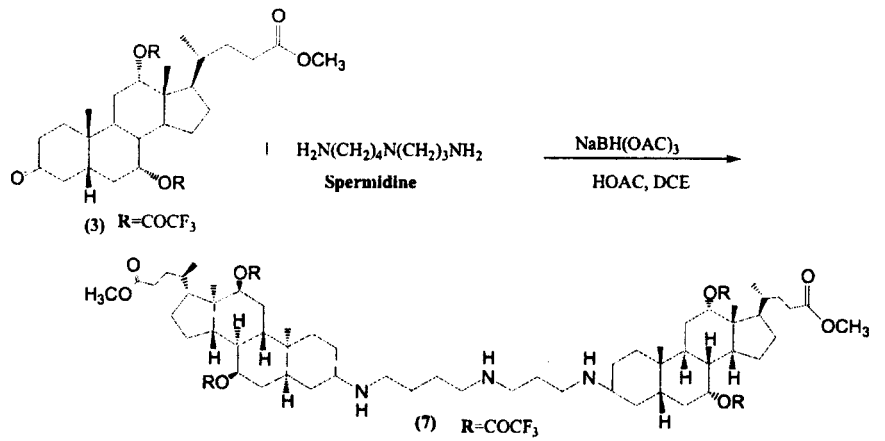
Scheme-2. Dimerization of protected cholic acid methyl ester with diamines

Using the same methodology, the dimer **5** was synthesized by reductive amination of protected 3-ketocholic acid methyl ester (3) with 1,4-diaminobutane (putrescine) in an excellent yield (90%) as shown in **Scheme-2**. The ^{1}H-NMR spectrum of dimer **5** showed again two characteristic peaks at 5.19 and 5.34 ppm of C-7 and C-12 protons respectively, while that at 2.9 ppm of β-proton at C-3 bearing a nitrogen atom and a peak at 3.11 ppm for the β-proton at C-3 bearing a nitrogen in the other side of the dimer. The mass spectrum showed a molecular ion peak at 1281 (M) and another peak at 685 which indicates the loss of all $COCF_3$ protecting groups and the loss of all oxygen atoms bearing them at positions 7 and 12 in each side.

Another analogue, compound **6** was synthesized by reductive amination of 1,6-diaminohexane with protected 3-ketocholic acid methyl ester (3) in 88% yield as shown in **Scheme-2.** The ^{1}H-NMR showed two characteristic peaks at

5.17 and 5.30 ppm of C-7 and C-12 protons respectively, while the peak at 2.9 ppm is of the α-proton at C-3 bearing nitrogen and the peak at 3.3 ppm is of the β-proton at C-3 bearing a nitrogen in the other side of the dimer. In mass spectrum a strong peak appeared at 925 unit, which indicates the loss of all protecting groups ($COCF_3$).

The final dimer **7** was synthesized by reductive amination with a polyamine (spermidine) in 90% yield as shown in **Scheme-3**. The ^{1}H-NMR showed two peaks at 5.16 and 5.27 ppm typical for C-7 and C-12 respectively, while a peak at 2.9 ppm is characteristic of the α-proton at C-3 and a peak at 3.27 ppm is for the β-proton at C-3 in the other side of the dimer. A characteristic sharp singlet peak at 3.60 ppm for C-24 methyl ester and a peak at 6.07 ppm is characteristic of N-H proton of spermidine. The expected molecular ion peak at 1338 in the mass spectrum was not observed. However, a peak at 1146 indicates the loss of two protecting groups ($COCF_3$), and another peak at 954 indicates the cleavage of all protecting groups.



Scheme-3. Dimerization of protected cholic acid methyl ester with spermidine

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Nicolet-Impact 410 FT-IR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using a 200 MHz or 300 MHz Brucker. Spectra were recorded in deuteriochloroform $CDCl_3$ with chemical shift values in ppm relative to the solvent peak (7.26 ppm). All reductive amination reactions were carried out under nitrogen atmosphere. Cholic acid methyl ester (Fluka) was purified before using by recrystallization. The following chemicals was used as received: sodium triacetoxyborohydride (Fluka), $CaCO_3$ (GPR), ether (Aldrich) and 1,2-dichloroethane (DCE) (Aldrich), pyridine chlorochromate (PCC) (Aldrich).

Methyl-3α-hydroxy-7α,12α-bis(trifluoroacetyloxy)-5β-cholan-24-oate (2): Trifluoroacetic anhydride (10 mL, 70 mmol) was added to methyl cholate (**1**)

(1.64 g, 3.8 mmol). The mixture was heated gently (50–60°C) under nitrogen for 20 min. The reaction mixture was then added to 100 g of ice water and the product was extracted with chloroform (3 × 20 mL). The combined organic layers were washed with water, then twice with brine (15 mL), dried over Na_2SO_4 and filtered. The filtrate was evaporated under vacuum to give a yellow product. Distilled methanol (40 mL) was added to the yellow product (1.0 g, 1.4 mmol). The mixture was stirred under nitrogen for 5 min., then $NaHCO_3$ (1.07 g, 13 mmol) was added and the reaction mixture was stirred under nitrogen at temperature for 2 h. The solvent was evaporated under vacuum to give a white solid. The product was extracted with chloroform (3 × 20 mL), then the combined organic layers were washed with water, then twice with brine (15 mL), dried over Na_2SO_4 and filtered. The filtrate was evaporated under vacuum to give a white solid, which was purified by column chromatography (1 : 3 ethylacetate : hexane), to yield the product (**2**) as a white solid (0.6 g, 70%); m.p. = 137.5–138.5°C. IR (KBr, cm^{-1}): 1732, 1777, 3519. ^{1}H-NMR (ppm): 0.79 (s, 3H, C-18), 0.95 (s, 3H, C-19), 3.46 (m, 1H, C-3), 3.66 (s, 3H, OCH_3), 5.15 (m, 1H, C-7), 5.33 (m, 1H, C-12). ^{13}C-NMR (ppm): 11.96 (C-18), 22.6 (C-19), 51.6 (OCH_3), 71.2 (C-3), 76.1 (C-7), 80.0 (C-12), 108-122(CF_3), 156 ($COCF_3$), 174.3(C-24). MS : 614.5 (M), 501.5 (M-$COCF_3$-OH).

Methyl-3-oxo-7α,12α-bis(trifluoroacetyloxy)-5β-cholan-24-oate (3): Pyridinium chlorochromate (PCC) (0.3 g, 1.4 mmol) was added to a solution of (2) (0.2 g, 0.33 mmol) in 20 mL of chloroform, containing $CaCO_3$ (0.32 g, 3.2 mmol). The reaction mixture was stirred under nitrogen at room temperature. After 1 h diethyl ether (20 mL) and charcoal (0.3 g) were added. The mixture was allowed to reflux for 30 min. After that, it was filtered through florisil, and the bad of florisil was washed with ethyl acetate (4 × 20 mL). The organic solvent was removed under vacuum to produce (**3**) as a white solid. (0.18 g, 90%), m.p. = 160–161°C. IR (KBr cm^{-1}): 1713, 1732, 1777. ^{1}H-NMR (ppm): 0.84 (s, 3H, C-18), 1.0 (s, 3H, C-19), 3.66 (s, 3H, OCH_3), 5.25 (m, 1H, C-7), 5.38 (m, 1H, C-12).

General procedure for reductive amination of protected 3-ketocholic acid methyl ester (3) with diamines: To a solution of protected 3-ketocholic acid methyl ester (**3**) (0.31 g, 0.5 mmol) in DCE (20 mL) diamines (0.65 mmol) was added, the mixture was stirred at temperature under nitrogen for 24 h. Sodium triacetoxyborohydride (0.21 g, 1 mmol) and glacial acetic acid (0.5 mL) were added respectively. The reaction mixture was stirred for 72 h. The reaction mixture was neutralized with 1 N NaOH and the product was extracted with chloroform (4 × 40 mL). The combined organic layers were washed twice with brine (25 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated under vacuum to give a pale yellow solid that was purified by column chromatography (2 : 3 ethylacetate : hexane) to yield the dimeric product.

Dimer 4 (Diamine is 1,3-diaminopropane): Yield 66%; m.p. 86°C (d). IR (KBr cm^{-1}): 1732, 1777, 2940, 3417. ^{1}H-NMR (ppm): 0.79 (s, 3H, C-18), 0.97 (s, 3H, C-19), 2.85 (m, 1H, C-3α), 3.1 (m, 1H, C-3β) 3.66 (s, 3H, OCH_3), 5.16 (m, 1H, C-7), 5.34 (m, 1H, C-12). ^{13}C-NMR (ppm): 12.1 (C-18), 22.70 (C-19), 51.6 (OCH_3), 53.8 (C-3), 75.4 (C-7), 80.0 (C-12), 108-122 (CF_3), 156.3 ($COCF_3$), 174.8 (C-24). MS: 1267 (M), 1171 (M-$COCF_3$), 1075 (M-$COCF_3$-$COCF_3$), 834

(M-4($COCF_3$). Anal. calcd. for $C_{61}H_{86}F_{12}N_2O_{12}$: C, 57.81; H, 6.84; F, 17.99; N, 2.21, found: C, 57.20; H, 6.77; F, 17.79 N, 2.34.

Dimer 5 (Diamine is 1,4-diaminobutane): Yield 90%; m.p. 95°C (d). IR (KBr, cm^{-1}): 1732, 1777, 2950, 3417. ^{1}H-NMR (ppm): 0.66 (s, 3H, C-18), 0.88 (s, 3H, C-19), 2.9 (m, 1H, C-3α), 3.1 (m, 1H, C-3β), 3.66 (s, 3H, OCH_3), 5.19 (m, 1H, C-7), 5.34 (m, 1H, C-12). MS: 1281 (M), 685 (M-4($OCOCF_3$)-$C_6H_{10}O_4$). Anal. calcd. for $C_{62}H_{88}F_{12}N_2O_{12}$: C, 58.12; H, 6.92; F, 17.79; N, 2.19, found: C, 57.82; H, 6.77; F, 17.70; N, 2.28.

Dimer 6 (Diamine is 1,6-diaminohexane): Yield; 88%; m.p. 102°C (d). IR (KBr, cm^{-1}): 1735, 2940, 3417. ^{1}H-NMR (ppm): 0.66 (s, 3H, C-18), 0.96 (s, 3H, C-19), 2.9 (m, 1H, C-3α), 3.3 (m, 1H, C-3β), 3.65 (s, 3H, OCH_3), 5.17 (m, 1H, C-7), 5.30 (m, 1H, C-12). ^{13}C-NMR (ppm): 12.1 (C-18), 22.70 (C-19), 51.8 (OCH_3), 55.3 (C-3), 68.0 (C-7), 73.4 (C-12), 175.8 (C-24). Anal. calc. for: $C_{64}H_{92}F_{12}N_2O_{12}$: C, 58.71; H, 7.08; F, 17.41; N, 2.14, found: C, 58.64; H, 6.97; F, 17.40; N, 2.28.

Dimer 7 (Polyamine is spermidine): Yield; 90%; m.p. 98°C (d). IR (KBr, cm^{-1}): 1735, 1777, 2945, 3435. ^{1}H-NMR (ppm): 0.60 (s, 3H, C-18), 0.90 (s, 3H, C-19), 2.9 (m, 1H, C-3α), 3.3 (m, 1H, C-3β), 3.60 (s, 3H, OCH_3), 5.16 (m, 1H, C-7), 5.27 (m, 1H, C-12), 6.07 (br, 1H, NH). ^{13}C-NMR (ppm): 12.0 (C-18), 22.80 (C-19), 51.6 (OCH_3), 73.0 (C-7), 77.4 (C-12), 109-123 (CF_3), 161.2 ($COCF_3$), 174.9 (C-24). Anal. calcd. for $C_{65}H_{95}F_{12}N_3O_{12}$: C, 58.33; H, 7.15; F, 17.03; N, 3.14, found: C, 58.44; H, 7.07; F, 15.40; N, 3.31.

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