Synthesis and Evaluation of the Cholic Acid Derivatives with Multi-trifluoroacetylbenzoyl (TFAB) Groups as Carbonate Ionophores

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Several cholic acid derivatives containing 1-3 trifluoroacetylbenzoyl (TFAB) moieties were synthesized using selective acetylations, hydrolysis and/or oxidation of cholic acid derivatives and tested as receptors for a carbonate ion through solvent extraction method. The compounds having two and three TFAB moieties exhibited enhanced binding affinities toward a carbonate ion in comparison with those with one TFAB group and the extent of complex formation also depended on the position of TFAB group attached.

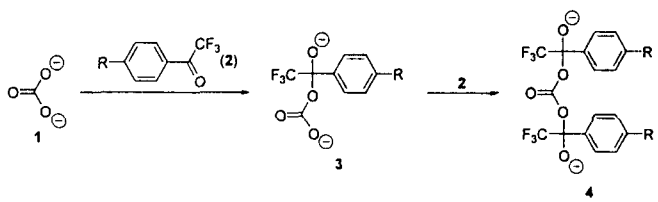
Introduction

Since crown ethers were found to have an ability of complex formation with cations,[1] there has been a great deal of interest in ion-complexing agents.[2] Especially, uncharged hydrophobic receptors for ions have attracted much attention since they may serve as transmembrane ion carriers or ionophores of ion-selective electrode membranes.[3] Although there have been published numerous articles on receptors for cations, only a few anion ionophores have been reported.[2]

Among the anion ionophores, trifluoroacetophenone (TFAP) derivatives are a few known examples of neutral carriers for anions in ion-selective membranes that exhibit an unusual preference for carbonate over other anions.[4] Thus, analytical chemists have attempted to use these compounds as the ionophores of a carbonate-selective electrode determining the total CO_2 species in blood serum.[5] One of the known problems associated with the trial is the interference by salicylate that is abundant in blood serum for the patients who take aspirin. One possible solution may be the design of a new ionophore with enhanced binding affinity to the carbonate over salicylate.

Previously, the mechanism on interactions between carbonate ions and TFAP derivatives was suggested by Meyerhoff *et al.*[6] According to this mechanism, one carbonate ion is covalently bound to the carbonyl carbons of two TFAP moieties and, consequently, a carbonate ion and two TFAP derivatives form a 1 : 2 complex (**4**). (Scheme 1) Thus, we expected that compounds with two TFAP moieties in a molecule could capture a carbonate ion more favorably if their linker could donate appropriate conformation. However, several compounds with two TFAP groups linked by saturated alkyl chains did not show promising binding affinity to a carbonate ion based on the test conducted by a carbonate-selective electrode.[7] It was ascribed to the fact that the binding energy between a carbonate and the two TFAP groups is not enough to overcome the required conformational energy to bring two TFAP groups in a molecule. In addition, it has been suggested that an anion pocket for a carbonate ion in liquid polymeric membrane might be formed by two or three TFAP derivatives.[8] Thus, we hope to synthesize compounds with two or three TFAP moieties, which are oriented at the same side of a molecule using an appropriate rigid linker.

We thought that bile acids could be used as possible rigid linkers. Recently, these compounds have attracted great attention to several researchers, because of their easy availability and unique functional group distribution on a rigid steroidal skeleton.[9] Previously, we reported that a deoxycholic acid derivative (**7**) with two trifluoroacetylbenzoyl (TFAB) groups attached to the hydroxyl groups exhibited enhanced binding affinity compared to that of the compounds **5** and **6**, based on the solvent extraction experiment; thus it seems to act as a carbonate ion tweezer.[10] Therefore we expected that the other bile acid might act as a better linker for two or more TFAB moieties. Among bile acids, cholic acid is another well known compound which possesses three (3α, 7α, and 12α) hydroxyl groups on one side of the conformationally rigid steroidal ring structure. The distance between 3α-7α and 7α-12 hydroxyl groups is about 4.5-5.0 Å,[11] which is similar to that between two methyl carbons of dimethyl carbonate. (~4.8 Å) and is closer than that between 3α-12α hydroxyl groups in deoxycholic acid or cholic acid structure. (about 6 Å) Therefore, we envisioned that the cholic acid derivatives with two TFAB groups on 3,7- or 7,12-hydroxyl groups might be better ionophores of a carbonate ion than **7**. Here we wish to report the synthesis of



Scheme 1. The proposed mechanism of interaction between carbonate ion and TFAP derivatives.

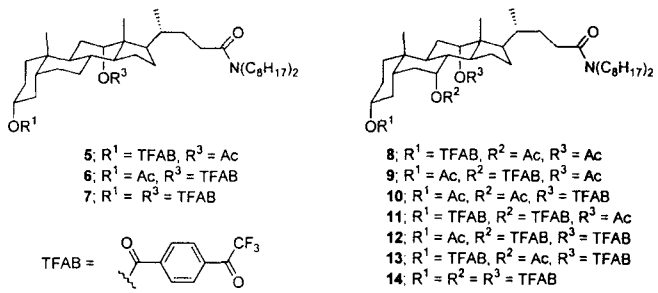


Figure 1. Deoxycholic acid and cholic acid based carbonate ionophores.

seven cholic acid derivatives (**8-14**) containing 1-3 TFAB group(s) on the three hydroxyl groups (Fig. 1) and compare them as receptors for a carbonate ion by solvent extraction method.

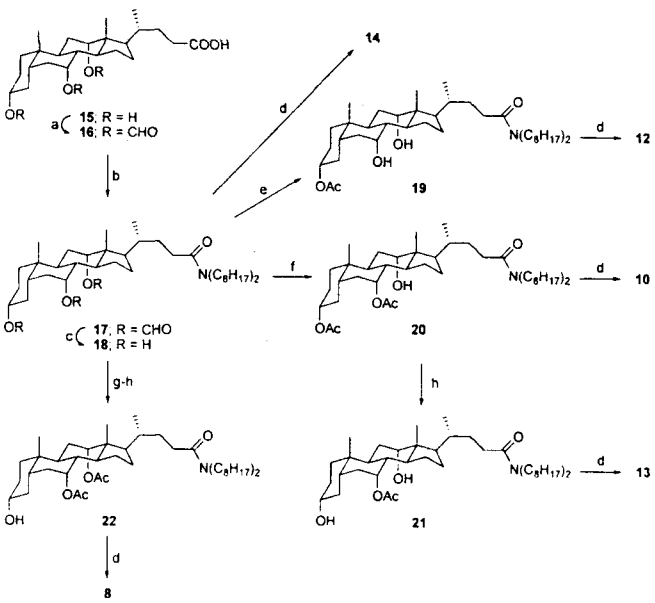
Results and Discussion

Synthesis of the target compounds. The synthesis of these target molecules was performed by the selective mono- or diacetylation of the three hydroxyl groups of cholic acid derivatives and trifluoroacetylbenzoylation of the remaining hydroxyl groups. (Scheme 2) The carboxyl group of cholic acid (**15**) was first converted to long chain dialkylamide 18 before the manipulation of hydroxyl groups. Two long alkyl chains are required for enough hydrophobicity of the compounds in ion-selective membranes.

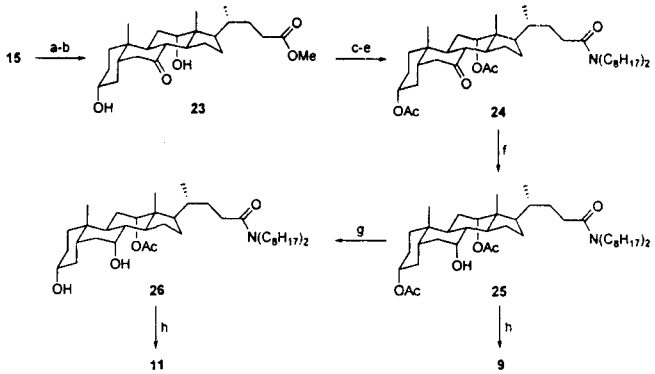
Subsequently, selective mono- or diacetylation of **18** was executed. It has been previously known that the reactivity of the three hydroxyl groups of methyl cholate for acetylation decreases in the order of 3-OH, 7-OH and 12-OH groups.[12] However, the reactivities were not sufficient enough to differentiate these hydroxyl groups so that the reaction conditions, the amount of reagents, and catalyst should be optimized. Through these modifications, we could obtain 3-acetylated and 3,7-diacetylated compounds (**19** and **20**) in moderate yields. Since selective hydrolysis of 3-acetate moiety in acetylated cholic acid derivatives is possible,[12] the 7-monoacetylated compound **21** was obtained by the treatment of **20** with K_2CO_3 in methanol. Similarly, the 7,12-diacetylated compound **22** was obtained by triacetylation of **18** followed by selective hydrolysis.

Among the six partially acetylated cholic acid derivatives, the selective acetylation and/or hydrolysis could not afford the 3,12-diacetylated and the 12-monoacetylated compounds (**25** and **26**). Thus, cholic acid (**15**) was treated with NBS to obtain 7-ketocholic acid, which was esterified to yield methyl ester **23** for easy purification.[13] (Scheme 3) After hydrolysis of the ester, it was converted to the diacetyl amide **24**. Reduction of **24** by $NaBH_4$ regenerated 7α-hydroxyl group to give **25** and subsequent hydrolysis of 3-acetoxy group provided **26** in good yield.

Finally, TFAB groups were introduced on the remaining hydroxyl groups in partially acetylated compounds and **18**. Trifluoroacetylbenzoyl chloride (TFAB-Cl) was prepared from 1,4-dibromobenzene in 63% yield by the procedure of



Scheme 2. (a) HCOOH, cat. $HClO_4$, Ac_2O, 55 °C, (b) ClCOOMe, NEt_3, $HN(C_8H_{17})_2$, CH_2Cl_2, 0 °C, (c) K_2CO_3, aq. MeOH, 60 °C, (d) CaH_2, Bu_4NBr, ex. TFAB-Cl, toluene, reflux, (e) 3 eq. Ac_2O, 3 eq. NEt_3, CH_2Cl_2, rt, (f) 3 eq. Ac_2O, 3 eq. NEt_3, 0.1 eq. DMAP, CH_2Cl_2, -10 °C, (g) 5 eq. Ac_2O, 5 eq. NEt_3, 0.5 eq. DMAP, CH_2Cl_2, rt, (h) K_2CO_3, aq. MeOH, rt.



Scheme 3. (a) *N*-Bromosuccinimide, $NaHCO_3$, rt, (b) HCl, MeOH, rt, (c) 2 eq. LiOH, aq. MeOH, rt, (d) 5 eq. Ac_2O, 5 eq. NEt_3, 0.5 eq. DMAP, CH_2Cl_2, rt, (e) ClCOOMe, NEt_3, $HN(C_8H_{17})_2$, CH_2Cl_2, 0 °C, (f) $NaBH_4$, MeOH, rt, (g) K_2CO_3, aq. MeOH, rt, (h) CaH_2, Bu_4NBr, ex. TFAB-Cl, toluene, reflux.

Simon *et al.*[14] The introduction of TFAB groups was performed by Oppenauer procedure (CaH_2, toluene, Bu_4NBr) with excess amount of TFAB-Cl.[15] Thus, final target molecules **8-14** were obtained in 38 through 93% yields.[16]

Evaluation of the compounds as carbonate ion receptors. These compounds were tested and compared as receptors for a carbonate ion by solvent extraction method and the complex formation with a carbonate ion was determined by UV absorption at 260 nm.[18] Since the molar absorptivities of TFAB groups on the 3, 7, and 12 position of the steroidal ring might not be exactly the same, precise quantitative analyses were impossible. In addition, the compounds were easily contaminated with the corresponding hydrated form (*gem*-diol) of the TFAB groups. However, the tendencies of

these molecules as a carbonate ion binder could be estimated qualitatively by this procedure.

Thus, the solution of these compounds in dichloromethane were extracted with buffer solutions (0.10 M Tris-H_2SO_4, pH 8.6) containing 0.002 M tetrabutylammonium chloride (Bu_4NCl) as a source of a hydrophobic counter ion with or without 0.030 M $NaHCO_3$. The concentration of these compounds was adjusted to have the same concentration (8.0×10^{-5} M) of TFAB groups regardless of the number of TFAB groups in these compounds. The buffer solution with $NaHCO_3$ was maintained to be pH 8.6.[19] According to the previous results with ion-selective membrane containing TFAP derivatives, these compounds are believed to be poor ionophores for sulfate and chloride ($\log K^{pot}_{CO_3^{2-},SO_4^{2-}}$ and $\log K^{pot}_{CO_3^{2-},Cl^-}$ are less than -2.0).[14,20] In fact, when we performed the same experiments with Tris-HCl buffer (pH 8.6), the results were the same. It has also been previously reported that TFAP derivatives act as carbonate ionophores, not as bicarbonate ionophores, based on the ISE experiments.[21]

After extraction with buffer solution without $NaHCO_3$, the UV absorption spectra of the compounds with one TFAB group (**8-10**) showed a main absorption at 260 nm. A small shoulder at 230 nm indicated the existence of some hydrated species. Previously, it has been reported that TFAP derivatives might exist as *gem*-diol species depending on the substituents on the phenyl ring and the pH of the solution.[17] In addition, TFAP derivatives have been used as additives in solvent polymeric membranes for humidity[22] or ethanol[23] sensors. The absorption of these compounds at 260 nm, after extraction with buffer solution containing a carbonate ion, were almost the same as those extracted with buffer solution without a carbonate ion, implying that the complex formation with a carbonate ion by **8-10** is negligible in these conditions.[24] (Table) The similar result was also observed with *n*-heptyl 4-trifluoroactylbenzoate (ETH6010) and 4-*n*-decyltrifluoroacetophenone(TFADB), the commercially available carbonate ionophores.

Contrasted to the above, the compounds containing two TFAB groups (**11-13**) showed significant decrease in UV absorption at 260 nm after extraction with buffer solution without $NaHCO_3$. This phenomenon was previously observed in the case of deoxycholic acid derivative **7** and it is believed that a small hydroxide ion tends to bind to a TFAB group more favorably to some extent if the other TFAB moiety exists in the molecule. In addition, when we compare **7** with **13**, the portion of hydrated species in **13** is more than that in **7**. It is ascribed that the 7α-acetate group in **13** affects the hydration of TFAB groups considerably. Not surprisingly, compound **14** containing three TFAB groups showed even more decrease of the absorption.

When the solutions of these compounds were extracted with a carbonate ion containing buffer, the UV absorption at 260 nm was further decreased. These results seem to demonstrate that two TFAB groups in a molecule with a rigid linker cooperatively bind a carbonate ion. Interestingly enough, the extent of the decrease of absorption was quite distinctive among three compounds with two TFAB groups

Table. Absorbances at 260 nm after extraction with buffer solutions with or without $NaHCO_3$

Compounds	A_0[a]	A[b]	% decreased
ETH6010	1.64	1.63	1
5	1.53	1.52	1
6	1.48	1.47	1
7	1.26	1.15	9
8	1.59	1.58	1
9	1.58	1.58	0
10	1.52	1.53	-1
11	1.05	0.95	10
12	0.93	0.42	55
13	1.10	1.02	7
14	0.79	0.23	71

[a] After extraction with buffer solution without $NaHCO_3$. [b] After extraction with buffer solution containing 0.030 M $NaHCO_3$.

(**11-13**). In the cases of compound **11** and **13**, the absorptions decreased by 10% and 7%, respectively, after extraction with 0.03 M $NaHCO_3$ containing buffer. On the contrary, **12** and **14** showed significantly large decrease of UV absorption (55% and 71% decrease, respectively. see Figure 2). In fact, the absorption of **14** seemed to show no existence of TFAB chromophore at all after extraction with 0.03 M $NaHCO_3$ containing buffer. It was evidenced by the fact that its absorption spectrum after extraction with 0.10 M $NaHCO_3$ containing buffer was the same.

The reason for the predominant binding affinity of **12** to a carbonate ion among **11-13** can not be explained clearly. However, it is apparent that distance between two electrophilic carbons on the three hydroxyl groups of cholic acid derivatives is different. A tentative explanation for the difference of binding affinity of **12** among **11-13** might be due to the fact that the two hydroxyl groups at 7- and 12-positions are placed at about the same distances from the rigid

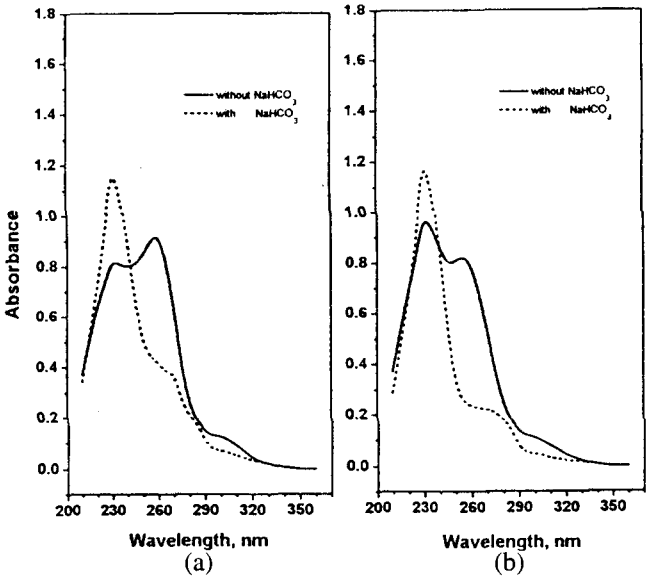


Figure 2. Ultraviolet absorption spectra of compound **12** and **14** after extraction with buffer solutions. (a) compound **12** (b) compound **14**.

steroidal plane. Whereas the hydroxyl group at 3-position is located further apart from the skeleton. As a result, we believe that **12** is conformationally more appropriate to bind a carbonate ion cooperatively than either **11** or **13**.

However, molecular dynamic simulation using HyperChem™ showed that the distance between two electrophilic carbons in **12** could be much further (more than 7 Å) than the optimal distance. (about 4.8 Å) Thus, it is still an open question at this point whether a carbonate ion actually binds simultaneously to the two TFAB groups in these molecules with multi-TFAB moieties through covalent bonds as proposed by Meyerhoff *et al.*[6] In addition, the reason that the compounds containing multi-TFAB groups have an enhanced ability to bind to a hydroxide ion more favorably than those with one TFAB group is not clear at this point. Thus, although the multi-TFAB derivatives showed enhanced binding abilities to a carbonate ion, selectivities to a carbonate ion over other anions should be tested by the other method.[26] Use of these compounds as ionophores in ion-selective membrane for a carbonate ion-selective electrode will be tested and reported in due course.

Experimental Section

General comments. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer. Chemical shifts (δ) are reported as ppm downfield from tetramethylsilane internal standards or using residual solvent peak as a standard. ^{19}F NMR spectra were recorded on a Varian UNITYplus-300 NMR spectrometer and chemical shifts (δ) are reported using fluorotrichloromethane as an internal standard. IR spectra were recorded on a Nicolet 205 FT-IR spectrophotometer. Mass spectra were obtained by Jeol HX110/HX110 mass spectrometer by fast atom bombardment (FAB) ionization method. UV spectra were obtained on Shimadzu UV-240 UV-Vis spectrophotometer.

All anhydrous reactions were carried out under nitrogen atmosphere. THF and ether were distilled from sodium ketyl of benzophenone. CH_2Cl_2 was distilled from CaH_2. Toluene was purified as described in a reference[25] and stored in the presence of Type 4A grade of molecular sieves. Absolute methanol obtained from Aldrich Chemical Co. was used without further purification. CH_2Cl_2 for UV spectroscopy and tetrabutylammonium chloride (Bu_4NCl) for ion pair chromatography from Fluka Chemical Co. were used for spectroscopic experiments. All the other reagents were purchased from either Aldrich or Fluka Chemical Co. unless noted otherwise. 4-Trifluoroacetylbenzoyl chloride (TFAB-Cl) was prepared by the procedure of Simon *et al.*[14] and purified by bulb-to bulb distillation before use.

***N,N*-Dioctyl-3α,7α,12α-triformyloxy-5β-cholan-24-amide (17)**. To a stirred solution of the cholic acid triformate **16**[27] (2.46 g, 5.0 mmol) and NEt_3 (0.77 mL, 5.50 mmol) in CH_2Cl_2 (45 mL) was added methyl chloroformate (428 μL, 5.50 mmol) at 0 °C. After 2 h, *N,N*-dioctylamine (1.81 mL, 6.0 mmol) was added and the solution was further stirred at 0 °C for 2 h. The solution was diluted with CH_2Cl_2 (100 mL), washed with water (100 mL), dried, and evaporated under reduced pressure. The residue was purified by column chromatography on silica using ethyl acetate-hexane (3 : 17) as eluent to give the amide **17** (2.4 g, 67%) as an waxy solid; IR (film) ν_{max} 2940, 2861, 2736, 1729, 1637, 1473, 1374, 1190, 762 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) δ 0.76 (s, 3H, 18-CH_3), 1.01 (s, 3H, 19-CH_3), 0.84-2.39 (m, 57H), 3.4-3.18 (m, 4H, $N(CH_2R)_2$), 4.85 (m, 1H, 3β-H), 5.06 (s, 1H, 7β-H), 5.31 (s, 1H, 12β-H), 8.02 (s, 1H, OCHO), 8.11 (s, 1H, OCHO), 8.17 (s, 1H, OCHO).

***N,N*-Dioctyl-3,7,12-trihydroxy-5-cholan-24-amide (18)**. To a stirred solution of **17** (850 mg, 1.19 mmol) in THF (17 mL) was added K_2CO_3 solution (3%) in 80% aq. MeOH (15 mL). The solution was stirred for 3 days at 60 °C and concentrated to a small volume. The residual solution was diluted with CH_2Cl_2 (100 mL), and washed with saturated NH_4Cl (40 mL) and water (100 mL). After the organic fraction was dried and evaporated under reduced pressure, the residue was purified by column chromatography on silica using ethyl acetate-hexane (13 : 7) as eluent to give the triol **18** (570 mg, 76%) as an waxy solid; IR (film) ν_{max} 3388, 2934, 2861, 1624, 1473, 1374, 1085, 1045, 762 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) δ 0.67 (s, 3H, 18-CH_3), 0.88 (s, 3H, 19-CH_3), 0.85-2.24 (m, 60H), 3.20-3.37 (m, 4H, $N(CH_2R)_2$), 3.43 (m, 1H, 3β-H), 3.84 (s, 1H, 7β-H), 3.99 (s, 1H, 12β-H).

***N,N*-Dioctyl-3α,7α,12α-tris(4-trifluoroacetylbenzoxy)-5β-cholan-24-amide (14)**. To a suspension of **15** (190 mg, 0.30 mmol), CaH_2 (132 mg, 3.00 mmol), Bu_4NBr (25 mg, 0.08 mmol) in toluene (4 mL) was added TFAB-Cl (1.49 g, 6.30 mmol). The suspension was refluxed for 24 h and filtered through celite (5 g) pad after cooling. After the celite pad was washed with ethyl acetate (80 mL), the combined filtrate and washing were concentrated, and the residue was dissolved in toluene (60 mL). To the solution was added silica gel (30 g) and water (0.1 mL) and the mixture was stirred for 2 h at rt. After the mixture was filtered and the filter cake was washed with ethyl acetate (150 mL), the combined filtrate and washing were washed with saturated $NaHCO_3$ (2 x 70 mL), dried ($MgSO_4$), and concentrated. Purification of the residue by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **14** (230 mg, 63%); IR (film) ν_{max} 2927, 2861, 1729, 1624, 1473, 1289, 1216, 1190, 1150, 1117, 946, 736 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) δ 0.89 (s, 3H, 18-CH_3), 1.08 (s, 3H, 19-CH_3), 0.83-2.20 (m, 57H), 3.05-3.31 (m, 4H, $N(CH_2R)_2$), 4.79 (m, 1H, 3β-H), 5.38 (s, 1H, 7β-H), 5.51 (s, 1H, 12β-H), 7.88-8.23 (m, 12H, $3C_6H_4$); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.25, 14.01, 17.80, 22.39, 22.56, 22.65, 23.00, 25.51, 26.55, 26.78, 26.93, 27.10, 27.64, 28.98, 29.09, 29.16, 29.18, 29.30, 29.66, 30.02, 31.22, 31.36, 31.65, 31.71, 34.41, 34.86, 34.94, 38.23, 40.53, 43.78, 45.56, 45.93, 47.98, 48.32, 72.63, 75.15, 116.22 (q, *J*=291 Hz, CF_3), 116.26 (q, *J*=291 Hz, CF_3), 129.40, 129.50, 129.80, 129.87, 129.97, 132.90, 133.17, 133.24, 136.02, 136.31, 136.33, 163.61, 164.12, 172.70, 172.92, 179.52 (q, *J* =36 Hz, $\underline{C}OCF_3$), 179.62 (q, *J*=36 Hz, $\underline{C}OCF_3$), 179.80 (q, *J* =36 Hz, $\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.42,

-72.48, -72.51; LRFABMS (NBA) *m/z* 1232.6 (M+H), 1250.6 (M+H_2O+H), 1268.6 (M+2H_2O+H), 1385.8 (M+NBA+H), 1403.8 (M+NBA+H_2O+H), 1421.7 (M+NBA+2H_2O+H); HRFABMS (NBA) Calcd for $C_{67}H_{83}F_9NO_{10}$ (M+H); 1232.5873 Found; 1232.5880.

***N,N*-Dioctyl-3α-acetoxy-7α,12α-dihydroxy-5β-cholan-24-amide (19).** To a solution of **18** (580 mg, 0.92 mmol) and NEt_3 (0.385 mL, 2.76 mmol) in CH_2Cl_2 (3 mL) was added acetic anhydride (0.260 mL, 2.76 mmol) at 0 °C. The solution was stirred at rt for 65 h, diluted with ether (30 mL), washed with 1 N HCl (30 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography on silica using ethyl acetate-hexane (1:3) as eluent to afford the 3-monoacetate **19** (350 mg, 56%) as an waxy solid; IR (film) ν_{max} 3434, 2934, 2861, 1736, 1624, 1473, 1387, 1368, 1249, 1045, 1025, 762 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.69 (s, 3H, 18-CH_3), 0.89 (s, 3H, 19-CH_3), 0.99 (d, 3H, *J*=6.2 Hz, 21-CH_3), 0.84-2.21 (m, 56H), 2.03 (s, 3H, OAc), 3.19-3.24 (m, 4H, N($CH_2R)_2$), 3.99 (s, 1H, 7β-H), 4.06 (m, 1H, 3β-H), 4.10 (s, 1H, 12β-H).

***N,N*-Dioctyl-3α-acetoxy-7α,12α-bis(4-trifluoroacetylbenzoxy)-5β-cholan-24-amide (12)** was synthesized by the same procedure for the synthesis of **14** using **19** (520 mg, 0.78 mmol), CaH_2 (344 mg, 7.80 mmol), Bu_4NBr (66 mg, 0.20 mmol) and TFAB-Cl (2.58 g, 10.9 mmol). Purification of the product by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **12** (480 mg, 57%); IR (film) ν_{max} 2934, 2861, 1729, 1618, 1473, 1374, 1282, 1190, 1065, 736 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.87 (s, 3H, 18-CH_3), 1.03 (s, 3H, 19-CH_3), 0.81-2.37 (m, 57H), 2.18 (s, 3H, OAc), 3.06-3.27 (m, 4H, N($CH_2R)_2$), 4.51 (m, 1H, 3β-H), 5.32 (s, 1H, 7β-H), 5.48 (s, 1H, 12β-H), 8.10-8.26 (m, 8H, 2C_6H_4); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.12, 13.95, 17.67, 21.04, 21.31, 22.34, 22.49, 22.90, 25.43, 26.54, 26.68, 26.85, 27.03, 27.55, 28.89, 29.02, 29.10, 29.23, 29.59, 29.85, 31.12, 31.29, 31.58, 31.65, 31.81, 34.33, 34.43, 34.74, 34.93, 38.10, 40.41, 43.64, 45.44, 45.83, 47.85, 48.19, 72.54, 73.44, 77.00, 116.22 (q, *J*=291 Hz, 2CF_3), 129.75, 129.81, 130.00, 133.00, 133.10, 136.21, 163.63, 163.95, 170.24, 172.60, 179.58 (q, *J*=35 Hz, 2$\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.35, -72.37; LRFABMS (NBA) *m/z* 1074.58 (M+H), 1092.59 (M+H_2O+H), 1110.57 (M+2H_2O+H), 1227.62 (M+NBA+H), 1245.7 (M+NBA+H_2O+H); HRFABMS (NBA) Calcd for $C_{60}H_{82}F_6NO_9$ (M+H); 1074.5894 Found; 1074.5870.

***N,N*-Dioctyl-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-amide (20).** To a solution of **18** (1.58 g, 2.50 mmol), *N,N*-dimethylaminopyridine (DMAP; 15 mg, 0.25 mmol), NEt_3 (1.01 mL, 7.50 mmol) in CH_2Cl_2 (10 mL) was added acetic anhydride (0.710 mL, 7.50 mmol) at -78 °C. After 1 h, the solution was stored in freezer for 20 h and the solution was diluted with ether (50 mL). The diluted solution was washed with 1 N HCl (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography on silica using ethyl acetate-hexane (1 : 5) to obtain the 3,7-diacetate **20** (1.10 g, 62%); IR (film) ν_{max} 3447, 2934, 2855, 1736, 1631, 1466, 1387, 1368, 1256, 1032, 762 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.67 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 0.98 (d, 3H, *J*=5.5 Hz, 21-CH_3), 0.73-2.20 (m, 55H), 2.00 (s, 3H, OAc), 2.04 (s, 3H, OAc), 3.17-3.19 (m, 4H, N($CH_2R)_2$), 3.99 (s, 1H, 12β-H), 4.56 (m, 1H, 3β-H), 4.87 (s, 1H, 7β-H).

***N,N*-Dioctyl-3α,7α-diacetoxy-12α-(4-trifluoroacetylbenzoxy)-5β-cholan-24-amide (10)** was synthesized by the same procedure for the synthesis of **14** using **20** (240 mg, 0.34 mmol), CaH_2 (78 mg, 1.70 mmol), Bu_4NBr (22 mg, 0.07 mmol) and TFAB-Cl (279 mg, 1.19 mmol). Purification of the product by chromatography on silica using ethyl acetate-hexane (1 : 3) gave **10** (280 mg, 91%); IR (film) ν_{max} 2940, 2861, 1723, 1624, 1473, 1387, 1282, 1183, 1071, 1025, 769 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.82 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 0.61-2.08 (m, 57H), 1.92 (s, 3H, OAc), 2.12 (s, 3H, OAc), 3.11-3.27 (m, 4H, N($CH_2R)_2$), 4.52 (m, 1H, 3β-H), 4.98 (s, 1H, 7β-H), 5.41 (s, 1H, 12β-H), 8.19-8.24 (m, 4H, C_6H_4); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.21, 13.99, 17.71, 21.23, 21.49, 22.41, 22.52, 22.55, 22.87, 25.53, 26.67, 26.79, 26.95, 27.24, 27.69, 28.83, 29.02, 29.08, 29.16, 29.18, 29.30, 29.60, 30.03, 31.16, 31.26, 31.64, 31.71, 34.31, 34.55, 34.64, 34.94, 37.78, 40.75, 43.59, 45.45, 45.82, 47.86, 48.28, 70.72, 73.74, 77.23, 116.37 (q, *J*=291 Hz, 2CF_3), 129.92, 130.13, 132.99, 136.58, 164.28, 170.03, 170.39, 172.50, 179.99 (q, *J*=36 Hz, 2$\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.22; LRFABMS (NBA) *m/z* 916.49 (M+H), 934.50 (M+H_2O+H), 1069.50 (M+NBA+H); HRFABMS (NBA) Calcd for $C_{53}H_{81}F_3NO_8$ (M+H); 916.5914 Found; 916.5922; HRFABMS (NBA) Calcd for $C_{53}H_{83}F_3NO_9$ (M+H_2O+H); 934.6019 Found; 934.6047.

***N,N*-Dioctyl-7α-acetoxy-3α,12α-dihydroxy-5β-cholan-24-amide (21).** A solution of **20** (136 mg, 0.19 mmol) and K_2CO_3 (52 mg, 0.38 mmol) in methanol (2 mL) was stirred for 6 h at rt and acetic acid (1.3 mL, 21.9 mmol) was added to the solution. After 30 min stirring, the solution was concentrated and the residue was dissolved in ether (30 mL). The solution was washed with brine (30 mL) and water (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography on silica using ethyl acetate-hexane (2 : 1) to give the 7-monoacetate **21** (110 mg, 86%); IR (film) ν_{max} 3408, 2927, 2861, 1736, 1631, 1466, 1387, 1256, 1078, 762 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.68 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 0.99 (d, 3H, *J*=6.1 Hz, 21-CH_3), 0.84-2.25 (m, 56H), 2.06 (s, 3H, OAc), 3.15-3.37 (m, 4H, N($CH_2R)_2$), 3.48 (m, 1H, 3β-H), 4.01 (s, 1H, 12β-H), 4.89 (s, 1H, 7β-H).

***N,N*-Dioctyl-7α-acetoxy-3α,12α-bis(4-trifluoroacetylbenzoxy)-5β-cholan-24-amide (13)** was synthesized by the same procedure for the synthesis of **14** using **21** (323 mg, 0.48 mmol), CaH_2 (212 mg, 4.80 mmol), Bu_4NBr (40 mg, 0.12 mmol) and TFAB-Cl (1.59 g, 6.72 mmol). Purification of the crude product by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **13** (195 mg, 38%); IR (film) ν_{max} 2934, 2855, 1729, 1624, 1466, 1387, 1289, 1190, 1124, 1071, 1025, 946, 736 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.77 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 0.79-2.26 (m,

57H), 2.05 (s, 3H, OAc), 3.02-3.23 (m, 4H, $N(CH_2R)_2$), 4.72 (m, 1H, 3β-H), 4.93 (s, 1H, 7β-H), 5.20 (s, 1H, 12β-H), 7.91-8.03 and 8.08-8.21 (m, 8H, $2C_6H_4$); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.21, 13.99, 17.73, 21.46, 22.38, 22.55, 22.62, 22.89, 25.53, 26.67, 26.79, 26.95, 27.24, 27.67, 28.85, 29.01, 29.10, 29.18, 29.30, 29.62, 30.06, 31.15, 31.26, 31.66, 31.72, 31.86, 34.33, 34.53, 34.61, 34.98, 37.81, 40.78, 43.57, 45.43, 45.88, 47.91, 48.26, 70.76, 75.44, 77.18, 116.32 (q, *J*=291 Hz, $2CF_3$), 129.68, 129.89, 130.04, 132.78, 133.01, 136.33, 136.63, 164.18, 164.37, 169.89, 172.61, 179.87 (q, *J*=36 Hz, $\underline{C}OCF_3$), 179.91 (q, *J*=35 Hz, $\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.28, -72.34; LRFABMS (NBA) *m/z* 1074.50 (M+H), 1092.54 ($M+H_2O+H$), 1110.52 ($M+2H_2O+H$), 1227.5 (M+NBA+H), 1245.5 (M+NBA+H_2O+H); HRFABMS (NBA) Calcd for $C_{60}H_{82}F_6NO_9$ (M+H); 1074.5894 Found; 1074.5907.

***N,N*-Dioctyl-7α,12α-diacetoxy-3α-hydroxy-5β-cholan-24-amide (22)**. A solution of **18** (632 mg, 1.00 mmol), NEt_3 (0.70 mL, 5.00 mmol), and DMAP (61 mg, 0.50 mmol) in CH_2Cl_2 (3 mL) was stirred at 0 °C as acetic anhydride (0.47 mL, 5.00 mmol) was added. The solution was stirred for 20 h at rt, diluted with ether (30 mL), washed with 1 N HCl (30 mL), dried ($MgSO_4$), and concentrated. The crude triacetate product was treated with K_2CO_3 (276 mg, 2.00 mmol) in MeOH (5 mL) for 3 h at rt. To the solution was added acetic acid (3.0 mL, 50.5 mmol) and the resulting solution was stirred for 30 min at rt and concentrated. The residue was dissolved in ether (40 mL), washed with brine (40 mL) and water (40 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography on silica using ethyl acetate-hexane (1 : 1) to afford the 7,12-diacetate **22** (490 mg, 69%); IR (film) ν_{max} 3427, 2934, 2861, 1736, 1637, 1473, 1381, 1249, 1085, 1032, 755 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.70 (s, 3H, 18-CH_3), 0.80 (d, 3H, *J*=6.3 Hz, 21-CH_3), 0.88 (s, 3H, 19-CH_3), 0.82-2.20 (m, 55H), 2.06 (s, 3H, OAc), 2.09 (s, 3H, OAc), 3.13-3.28 (m, 4H, $N(CH_2R)_2$), 3.56 (m, 1H, 3β-H), 4.87 (s, 1H, 7β-H), 5.07 (s, 1H, 12β-H).

***N,N*-Dioctyl-7α,12α-diacetoxy-3α-(4-trifluoroacetyl-benzoxy)-5β-cholan-24-amide (8)** was synthesized by the same procedure for the synthesis of **14** using **22** (240 mg, 0.34 mmol), CaH_2 (46 mg, 1.00 mmol), Bu_4NBr (22 mg, 0.07 mmol) and TFAB-Cl (199 mg, 0.85 mmol). Purification of the product by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **8** (242 mg, 78%); IR (film) ν_{max} 2934, 2861, 1729, 1637, 1473, 1381, 1282, 1256, 1183, 1117, 1065, 1019, 946, 762 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.75 (s, 3H, 18-CH_3), 0.96 (s, 3H, 19-CH_3), 0.83-2.20 (m, 57H), 2.07 (s, 3H, OAc), 2.13 (s, 3H, OAc), 3.21-3.28 (m, 4H, $N(CH_2R)_2$), 4.85 (m, 1H, 3β-H), 4.93 (s, 1H, 7β-H), 5.12 (s, 1H, 12β-H), 8.15-8.17 (m, 4H, C_6H_4); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.21, 14.06, 17.68, 21.35, 21.58, 22.56, 22.57, 22.59, 22.77, 25.56, 26.86, 26.98, 27.22, 27.71, 28.89, 29.16, 29.20, 29.23, 29.26, 29.34, 29.63, 30.20, 31.16, 31.38, 31.69, 31.74, 34.28, 34.49, 34.59, 34.97, 37.64, 40.88, 43.33, 45.00, 45.83, 47.74, 47.94, 70.60, 75.35, 75.73, 116.29 (q, *J*=289 Hz, CF_3), 129.89, 132.64, 136.48, 164.44, 170.16, 170.38, 172.65, 179.91 (q, *J* =36 Hz, $\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.21; LRFABMS (NBA) *m/z* 916.49 (M+H), 934.50 ($M+H_2O$+H), 1069.50 (M+NBA+H); HRFABMS (NBA) Calcd for $C_{53}H_{81}F_3NO_8$ (M+H); 916.5914 Found; 916.5922; HRFABMS (NBA) Calcd for $C_{53}H_{83}F_3NO_9$ ($M+H_2O$+H); 934.6019 Found; 934.6047.

***N,N*-Dioctyl-3α,12α-diacetoxy-7-oxo-5β-cholan-24-amide (24)**. A solution of methyl 7-ketocholate[13] (**23**; 186 mg, 0.442 mmol) and $LiOH{\cdot}H_2O$ (37.1 mg, 0.884 mmol) in 50% aq. MeOH (24 mL) was stirred at rt for 4 h and concentrated to about 1 mL. The residual solution was diluted with water (7 mL) and the crude acid was solidified by slow addition of 1 N HCl. After the solid was filtered, washed with water (30 mL), and dissolved in methanol (10 mL), the solution was concentrated.

The residual solid (180 mg) and DMAP (4.9 mg, 0.04 mmol) were dissolved in NEt_3 (1.8 mL, 12.4 mmol) and cooled at 10 °C as acetic anhydride (0.9 mL, 9.6 mmol) was added dropwise. The solution was stirred for 3 h at -10 °C, diluted with CH_2Cl_2 (35 mL) and water (35 mL), and acidified with acetic acid. The organic layer was washed with water (35 mL×2), dried ($MgSO_4$), and concentrated. To a solution of this residue in CH_2Cl_2 (12 mL) were added silica gel (10 g) and water (4 drops) and the mixture was stirred for 2 h at rt. After the mixture was filtered and the filter cake was washed with ethyl acetate (50 mL), the combined filtrate and washing were washed with saturated $NaHCO_3$ (2× 70 mL), dried ($MgSO_4$), and concentrated.

A solution of the residue (173 mg) and NEt_3 (0.070 mL, 0.50 mmol) in CH_2Cl_2 (4 mL) was stirred and cooled at -10 °C as methyl chloroformate (0.034 mL, 0.43 mmol) was added. After 1 h, *N,N*-dioctylamine (0.163 mL, 0.54 mmol) was added and the solution was stirred for 1 h. The solution was diluted with CH_2Cl_2 (30 mL), washed with water (30 mL), dried ($MgSO_4$), and concentrated. Purification of the product by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **24** (176 mg, 55%); IR (film) ν_{max} 2934, 2861, 1743, 1716, 1644, 1473, 1381, 1368, 1249, 1032, 762 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.74 (s, 3H, 18-CH_3), 0.83-2.48 (m, 57H), 2.00 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.87 (dd, 1H, *J*=12.6 and 6.0 Hz, 8-H), 3.16-3.36 (m, 6H, $C(6)H_2$ and $N(CH_2R)_2$), 4.60 (m, 1H, 3-H), 5.11 (s, 1H, 12-H).

***N,N*-Dioctyl-3α,12α-diacetoxy-7α-hydroxy-5β-cholan-24-amide (25)**. A solution of **24** (176 mg, 0.246 mmol) in THF/methanol (2 : 1; 4.5 mL) was stirred at 0 as $NaBH_4$ (10.6 mg, 0.295 mmol) was added. The resulting solution was stirred for 30 min at rt, concentrated, and the residue was dissolved in ethyl acetate (30 cm^3) before washing with saturated $NaHCO_3$ (30 mL), brine (50 mL), and water (70 mL). After the organic fraction was dried ($MgSO_4$) and concentrated, the residue was purified by chromatography on silica using ethyl acetate-hexane (1 : 2) to obtain **25** (149 mg, 85%); IR (film) ν_{max} 3441, 2927, 2861, 1736, 1631, 1473, 1387, 1368, 1256, 1032, 762 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.74 (s, 3H, 18-CH_3), 0.82-2.30 (m, 61H), 2.00 (s, 3H, OAc), 2.09 (s, 3H, OAc), 3.15-3.28 (m, 4H,

$N(CH_2R)_2$), 3.86 (s, 1H, 7β-H), 4.54 (m, 1H, 3β-H), 5.08 (s, 1H, 12β-H).

***N,N*-Dioctyl-3α,12α-diacetoxy-7α-(4-trifluoroacetyl-benzoxy)-5β-cholan-24-amide (9)** was synthesized by the same procedure for the synthesis of **14** using **25** (240 mg, 0.34 mmol), CaH_2 (78 mg, 1.70 mmol), Bu_4NBr (22 mg, 0.07 mmol) and TFAB-Cl (479 mg, 2.04 mmol). Purification of the crude product by chromatography on silica using ethyl acetate-hexane (1 : 4) gave **9** (290 mg, 93%); IR (film) ν_{max} 2934, 2861, 1729, 1631, 1473, 1374, 1282, 1256, 1183, 1117, 1065, 1025, 755 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.77 (s, 3H, 18-CH_3), 0.99 (s, 3H, 19-CH_3), 0.82-2.26 (m, 57H), 1.92 (s, 3H, OAc), 2.17 (s, 3H, OAc), 3.16-3.28 (m, 4H, $N(CH_2R)_2$), 4.56 (m, 1H, 3β-H), 5.17 (s, 1H, 7β-H), 5.24 (s, 1H, 12β-H), 8.20 (m, 4H, C_6H_4); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.22, 14.08, 17.81, 21.34, 22.53, 22.62, 22.69, 22.95, 25.43, 26.82, 26.91, 27.03, 27.16, 27.76, 28.89, 29.18, 29.14, 29.23, 29.29, 29.38, 29.65, 29.69, 30.24, 31.39, 31.73, 31.78, 31.95, 34.37, 34.59, 34.97, 35.04, 38.16, 40.64, 43.35, 45.10, 45.88, 47.83, 48.00, 72.72, 73.70, 75.32, 116.38 (q, *J*=291 Hz, CF_3), 129.97, 130.05, 132.94, 136.47, 163.92, 170.20, 170.43, 172.64, 180.00 (q, *J*=36 Hz, $\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.22; LRFABMS (NBA) *m/z* 916.49 (M+H), 934.48 ($M+H_2O$+H), 1069.50 (M+NBA+H); HRFABMS (NBA) Calcd for $C_{53}H_{81}F_3NO_8$ (M+H); 916.5914 Found; 916.5914; HRFABMS (NBA) Calcd for $C_{53}H_{83}F_3NO_9$ ($M+H_2O$+H); 934.6019 Found; 934.6028.

***N,N*-Dioctyl-12α-acetoxy-3α,7α-dihydroxy-5β-cholan-24-amide (26)**. A solution of **25** (765 mg, 1.07 mmol) and K_2CO_3 (295 mg, 2.14 mmol) in methanol (15 mL) was stirred for 4 h at rt. After acetic acid (1.5 mL, 26.2 mmol) was added, the solution was further stirred for 30 min at rt and concentrated. The residue was dissolved in ether (30 mL), washed with brine (30 mL) and water (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography on silica using ethyl acetate-hexane (1 : 1) to afford **26** (640 mg, 89%); IR (film) ν_{max} 3421, 2934, 2855, 1736, 1637, 1466, 1374, 1249, 1091, 1038, 755 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.75 (s, 3H, 18-CH_3), 0.89 (s, 3H, 19-CH_3), 0.82-2.36 (m, 59H), 2.09 (s, 3H, OAc), 3.12-3.36 (m, 4H, $N(CH_2R)_2$), 3.47 (m, 1H, 3β-H), 3.89 (s, 1H, 7β-H), 5.09 (s, 1H, 12β-H).

***N,N*-Dioctyl-12α-acetoxy-3α,7α-bis(4-trifluoroacetyl-benzoxy)-5β-cholan-24-amide (11)** was synthesized by the same procedure for the synthesis of **14** using **26** (260 mg, 0.39 mmol), CaH_2 (172 mg, 3.90 mmol), Bu_4NBr (33 mg, 0.10 mmol) and TFAB-Cl (942 mg, 3.99 mmol). After the reaction, the crude residue was dissolved in toluene (50 mL) with water (0.1 mL) and silica gel (20 g), and the suspension was stirred for 2 h at rt. The suspension was filtered and the filter cake was washed with ethyl acetate (150 mL). After the filtrate and the washing were combined and concentrated, the residue was dissolved in ether (70 mL), washed with saturated $NaHCO_3$ (2×70 mL), dried ($MgSO_4$), and concentrated. Purification of the crude product by chromatography on silica using ethyl acetate-hexane (1 : 3) gave **11** (288 mg, 69%); IR (film) ν_{max} 2940, 2861, 1723, 1618, 1473, 1387, 1282, 1183, 1117, 1065, 1025, 946, 755 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 0.79 (s, 3H, 18-CH_3), 0.83-2.30 (m, 60H), 2.18 (s, 3H, OAc), 3.16-3.29 (m, 4H, $N(CH_2R)_2$), 4.85 (m, 1H, 3β-H), 5.27 (s, 1H, 7β or 12β-H), 5.30 (s, 1H, 7β or 12β-H), 7.75-8.24 (m, 8H, $2C_6H_4$); ^{13}C NMR (75 MHz; $CDCl_3$) δ 12.22, 14.06, 17.81, 21.29, 22.50, 22.61, 22.97, 25.42, 26.75, 26.90, 27.01, 27.15, 27.75, 28.91, 29.12, 29.17, 29.22, 29.28, 29.36, 30.05, 30.20, 31.36, 31.71, 31.77, 34.38, 34.49, 34.96, 38.17, 40.64, 43.32, 45.10, 45.88, 47.84, 47.99, 72.68, 73.53, 75.30, 116.30 (q, *J*=288 Hz, CF3), 116.32 (q, *J*=291 Hz, CF_3), 129.65, 129.93, 132.86, 132.94, 136.28, 136.47, 163.71, 164.24, 170.08, 172.61, 179.78 (q, *J*=36 Hz, $\underline{C}OCF_3$), 179.88 (q, *J*=36 Hz, $\underline{C}OCF_3$); ^{19}F NMR (282 MHz; $CDCl_3$) δ -72.33, -72.38; LRFABMS (NBA) *m/z* 1074.50 (M+H), 1092.54 ($M+H_2O$+H), 1110.52 ($M+2H_2O$+H), 1227.5 (M+NBA+H), 1245.5 (M+NBA+H_2O+H); HRFABMS (NBA) Calcd for $C_{60}H_{82}F_6NO_9$ (M+H); 1074.5894 Found; 1074.5870.

Method of solvent extraction and spectroscopic evaluation. Tris-H_2SO_4 buffer solution (0.10 M; pH 8.6) was prepared just before the experiment and *n*-Bu_4NCl (2.0×10^{-3} M) and $NaHCO_3$ (3.0×10^{-2} M) were dissolved in this buffer if necessary. The solution of the compounds **8-10**, **11-13**, and **14** were prepared by dissolving in CH_2Cl_2 for UV spectroscopy and the concentration of the solutions were 8.0×10^{-5} M, 4.0×10^{-5} M, 2.7×10^{-5} M, respectively.

For extraction, 4 mL of each organic solution of each compound and 4 mL of Tris-H_2SO_4 buffer, with or without TBAC, were thoroughly mixed and the solution was centrifuged for 1 min. The lower organic layer was taken to obtain UV spectrum. The experiments were triplicated and averaged.

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