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Supramolecular ion channels from a transmembrane bischolic acid derivative showing two discrete conductances

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Bischolic acid derivative **1** linked by *m*-xylylene dicarbamate at the 3,3′-position was synthesized and the single ion channel properties were examined. Compound **1** showed two clearly distinct conductances, 9.5 (type A) and 25.3 pS (type B), under 500 mM KCl symmetric salt conditions, whereas various conductances, ranging from 5–20 pS, were observed in the tetramethylether analogue reported previously. Results indicate that the replacement of four methylether groups with hydroxyls at the 7, 7′, 12, and 12′ positions in the bischolic acid framework influences the stability of aggregated (supramolecular) ion channel structures. Ion permeability ratios (potassium/chloride and potassium/sodium) for each type of channel were also determined.

Introduction

Each biological cell is separated by a lipid bilayer membrane through which hydrophilic substances are generally impermeable. However, on demand a tremendous number of ions are transferred with significant order across the membrane. Ion channels play a central role in the generation of such ionic fluxes as well as control the concentrations of various ions such as potassium, sodium, calcium, and chloride ions, both inside and outside the membrane.[1] This function is of interest not only from a biological viewpoint but also as an ionic device that works in artificial membranes. A number of scientists have been interested in mimicking these functions by synthesizing artificial,[2] peptidic[3–15] and non-peptidic[16–35] ion channels. There has been increasing interest in the numerous functions of natural ion channels. In an effort to explore these functional mimics, supramolecular as well as molecular artificial ion channels were synthesized and successful control of ion selectivity[36] and voltage dependent properties[37–40] was demonstrated. Further, transmembrane bischolic acid methylethers **2** were recently reported to generate stable ion channel currents.[41] The cholic acid framework in the transmembrane channels is known to afford rigid and amphiphilic structures. Therefore stable ion channels can be constructed by aggregation of certain numbers of the transmembrane components. Once a structure is formed in the lipid bilayer membrane, the structure can be maintained during the measurement of ion channel currents. However, in the case of supramolecular channels, different conductances were usually observed in different runs of current measurements. This observation is thought to be associated with the formation of ion channels with different aggregation numbers and states, which can not be arbitrarily controlled in the case of **2**. This is one of the unique properties of supramolecular channels. However, this unique property can sometimes be troublesome in fully characterizing ion channel properties such as continuously changing the salt conditions in order to differentiate single-ion channels from multiple-ion channels. Here, through our continuous efforts to elucidate the relationship between ion channel structure and function, we unintentionally discovered unique ion channels of constant conductance. In order to increase the hydrophilicity of the interior pore, hydroxy groups were left unsubstituted rather than converted to methoxy groups. The dependence of conductance on salt concentration was analyzed in these unsubstituted compounds. Two specific conductances were clearly identified and their single channel properties were analyzed in full detail in order to confirm the cation selective single-ion channels. The channel was determined to be selective for potassium over sodium ions.

Results and discussion

Two cholic acids were designed to be connected through bisurethane bonds in order to construct transmembrane ion channel **1**, in which hydroxy groups were left unmodified in order to increase the hydrophilicity and interaction among components. The synthetic scheme of **1** is shown in Scheme 1. 3-Hydroxy-7,12-diacetoxy cholanic acid methyl ester **4** was synthesized in 16% yield from methyl cholate by selective THP protection of the 3-hydroxy group and acetylation followed by deprotection of the tetrahydropyranyl (THP) group. Two equivalents of **4** were treated with xylylenediisocyanate to give a 41% yield of **5**. Finally, hydrolysis of **5** with 1 N NaOH afforded transmembrane bischolic acid derivative **1** in 39%. The structure was characterized by ^{1}H and ^{13}C NMR and mass spectrometry. The purity was checked using high resolution NMR (600 MHz). Since no other signal was observed in the NMR spectra, the sample was subjected to ion channel measurements.

Single ion channel currents were measured using the established method for a planar bilayer membrane under 500 mM KCl symmetric conditions.[41] A typical ion channel record at +65 mV is shown in Fig. 1. In this record, two ion channels with different current levels were simultaneously observed. The open states for both channels were determined to be very stable and the duration reached over 2 s. The data were collected at various applied voltages, and the current–voltage relationship was analyzed. Interestingly, two discrete states were observed for all the experimental runs. The probabilities of observing the two different channel currents were almost the same. Current–voltage plots of all the collected data are shown in Fig. 2A. These were fitted by two straight lines to afford conductances of 9.52 ± 1.0 pS and 25.3 ± 1.6 pS. For convenience, the former state (smaller conductance) was defined as type A, and the latter as type B. The smaller conductance value was similar to the values observed for the methyl ether derivative **2** (5–20 pS). However, the conductance range of **1** for all the experiments is quite narrow compared with those observed for **2**. On the other hand, the larger conductance state (25.3 pS) was hardly observed in **2**. The appearance of only two specific conducting states is a unique characteristic of ion channels composed of **1**.

Since type A and B channels are clearly distinguished, the ion selectivity for each channel was examined. The current–voltage plots under asymmetric salt conditions (*cis* 100 mM KCl/*trans* 500 mM KCl and *cis* 500 mM KCl/*trans* 500 mM NaCl) are shown in Figs. 2B and C. Reversal potentials were determined from these data as 26.2 and 24.6 mV (Fig. 2B), and 14.6 and 19.7 mV (Fig. 2C)[42] for type A and B, respectively. The permeability ratios

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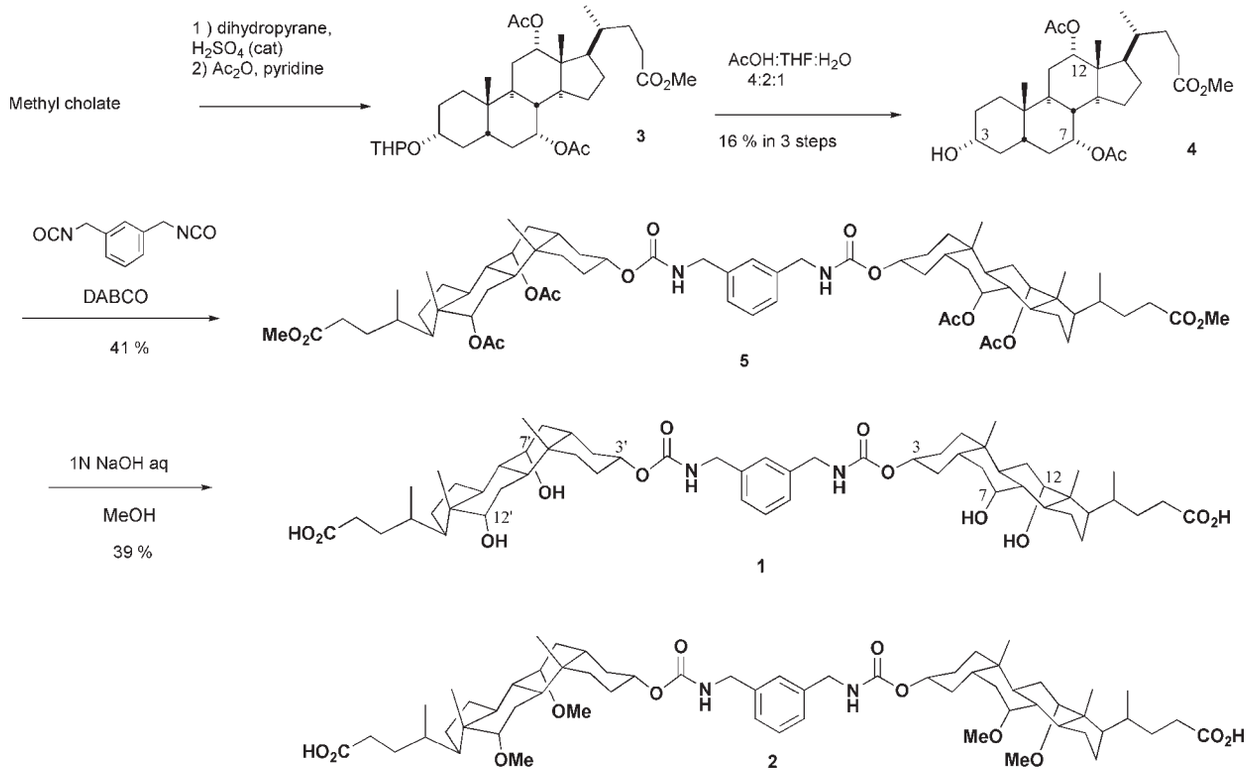

Scheme 1 Synthetic scheme of bischolic acid derivative **1**.

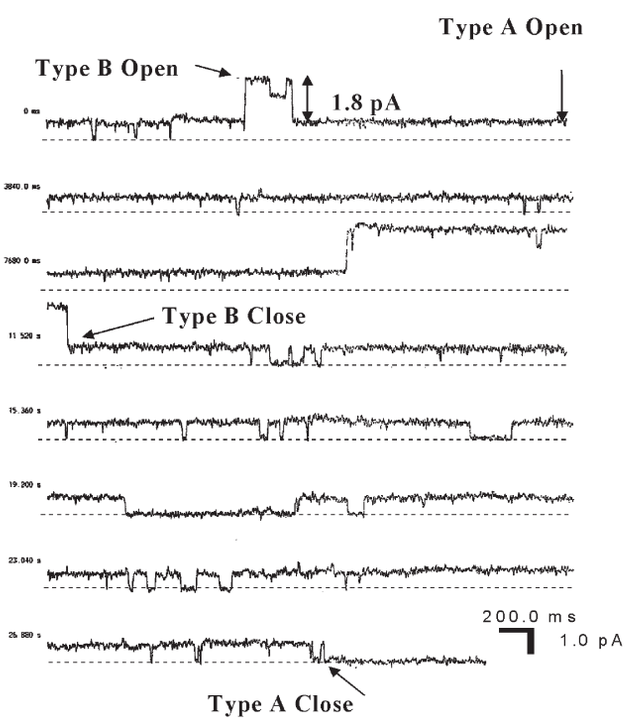

Fig. 1 Single ion channel record of **1** under symmetric 500 mM KCl at pH 7.2.

were obtained according to the Goldman–Hodgkin–Katz equation, and are listed in Table 1.

The permeability ratios (P_{K^+}/P_{Cl^-}) for types A and B were almost the same, with values of 7, and less than half of the value of **2** (17). This result suggests that the permeability of the chloride ion is increased by changing the methoxy substituent to a hydroxyl. Similarly, the permeability ratios (P_{K^+}/P_{Na^+}) of types A and B decreased to 2.07 and 2.53, respectively, compared with the value of 3.1 observed for **2**. All previously reported artificial ion channels from our laboratory showed potassium ion selectivity over chloride and sodium ions. This property is based on the relatively narrow pore and the hydrophobic environment of the pore. Replacement of

Table 1 Conductances and permeability ratios of ion channels **1** and **2**

Run	Ion channel	Conductance/pS ± SD[a]	P_{K^+}/P_{Cl^-}[b]	P_{K^+}/P_{Na^+}[c]
1	**1** (Type A)	9.52 ± 1.0	7.91	2.07
2	**1** (Type B)	25.3 ± 1.6	6.55	2.53
3[d]	**2**	5–20	17	3.1

[a] Under 500 mM KCl symmetric conditions at pH 7.2. [b] Obtained under 500 mM/100 mM KCl asymmetric conditions at pH 7.2. [c] Obtained under 500 mM KCl/500 mM NaCl asymmetric conditions at pH 7.2. [d] Ref. 41.

methylether to hydroxy groups (from **2** to **1**) decreased both of the permeability ratios of P_{K^+}/P_{Cl^-} and P_{K^+}/P_{Na^+}.

The dependence of conductance on the salt concentration is a fundamental property of investigation for single channel measurements. However, the existence of several conductance states makes it very difficult to carry out such experiments. Here, activity/conductance plots were obtained as shown in Fig. 3. Since these plots can be fitted to theoretical curves calculated from the Michaelis–Menten equation (V_{max} 10.0 pS and K_m 0.03 M for type A, V_{max} 25.3 pS and K_m 0.007 M for type B), both channels were considered single-ion channels.

It is noteworthy that only two specific ion channel states were identified from the supramolecular assemblies. At least three components are thought to be required in order to make a hydrophilic pore in a lipid membrane (Fig. 4a). At present, the number of components of cholic acid derivatives for types A and B cannot be specified. However, we propose the assembly numbers of three and four for channel types A and B, respectively, as the first rough approximations. In the case of channel **2**, various conductances were observed for each measurement run and different states may be allowed to exist for a state with the same assembly number. These differences are thought to originate from the different conformational states present to form the pore (Fig. 4b). Water molecules may also contribute to the stabilization of the hydrophilic pore in the membrane. Therefore, its number and various modes of interaction may be additional influencing factors. Methoxy groups may exert only weak structure-stabilizing interactions with themselves and water molecules, and therefore different states appear randomly for different experimental runs. Contrastingly, hydroxyl groups have relatively strong interactions among components and water mol-

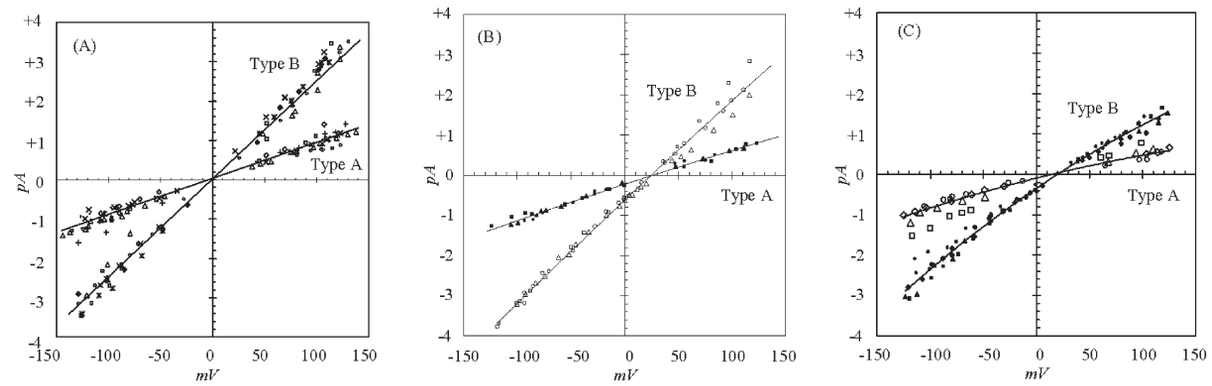

Fig. 2 Current–voltage plots of channel **1** under (A) symmetric 500 mM KCl, (B) *cis* 100 mM KCl/*trans* 500 mM KCl, and (C) *cis* 500 mM NaCl/*trans* 500 mM KCl conditions at pH 7.2.

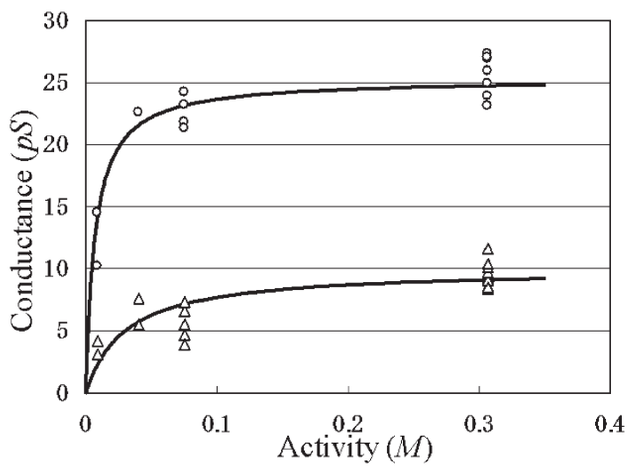

Fig. 3 Conductance–activity relationship of **1**. Open triangles: type A. Fitted solid line was obtained from the Michaelis–Menten equation (V_{max} = 10.0 pS, K_m = 0.03 M). Open circles: type B. Fitted solid line (V_{max} = 25.3 pS, K_m = 0.007 M).

ecules in order to restrict the mode of interaction at the most stable form. The two observed forms may be the two of the most stable forms, types A and B (Fig. 4c). The difference in the conductances of type A and B (9.5 pS *vs.* 25.3 pS) was attributed to the number of components, because the difference was significantly large. Increasing the number of components increases the size of the pores and the conductance. Considering that the permeability ratios P_{K^+}/P_{Cl^-} and P_{K^+}/P_{Na^+} remain similar to each other, the hydrophilic character inside the pore is thought to be similar.

Conclusions

Bischolic acid derivatives with hydroxy groups **1** provided ion channels exhibiting two specific conductances, and the properties of each individual single channel current were analyzed. The conductance of type A (9.5 pS) was close to typical ones of cholic acid derivative **2** with methoxy groups, whereas the conductance of type B was larger (25.3 pS). The difference could best be accounted for by the difference in the assembly number of the components constructing the channel. The permeability selectivity ratios P_{K^+}/P_{Cl^-} and P_{K^+}/P_{Na^+} of compound **1** decreased compared with those observed for **2**. This result indicates that the presence of hydroxy groups increases the hydrophilicity inside the pore. Even so, type A and B channels of **1** sustain the potassium preference to both chloride and sodium ions. Therefore, the pore environment is regarded as moderately hydrophilic. Further, the permeability selectivity ratios P_{K^+}/P_{Cl^-} and P_{K^+}/P_{Na^+} for type A and B channels of compound **1** closely resemble each other and their pore environments are thought to be very similar in spite of the conductance differences. In summary, two discrete ion channels were successfully prepared by introducing hydroxy groups. The ion channel properties could be analyzed independently because the random appearance of different states was avoided. Such fuctionalization methodology is thought to be useful for the further development of artificial ion channels with specific states.

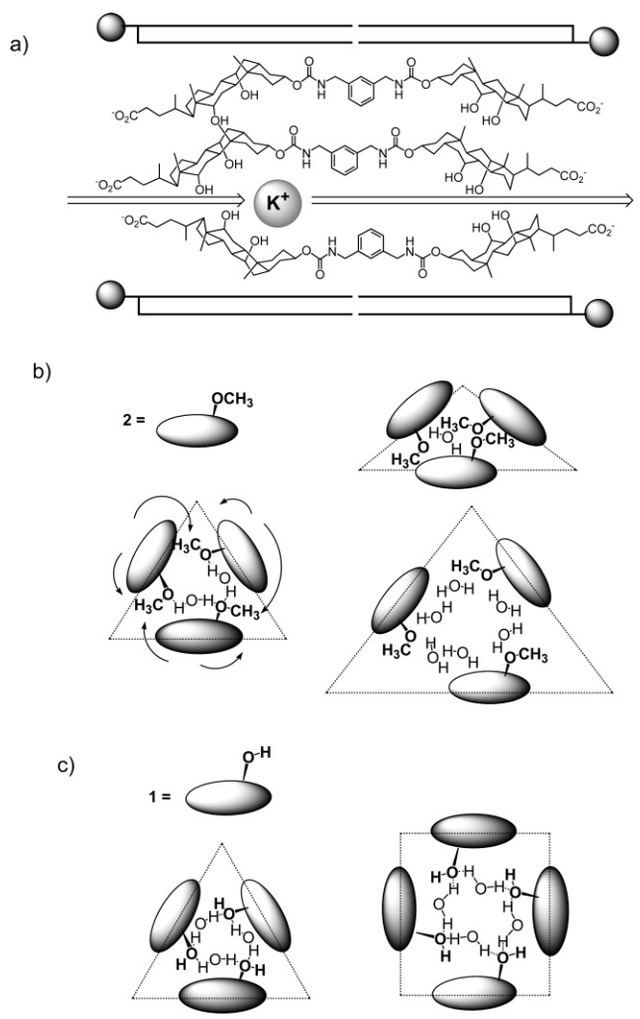

Fig. 4 Expected structure of aggregated channels **2** and **1**. a) An aggregated form of three bischolic acid derivatives **1** in a lipid bilayer membrane. b) Cross section images of three different states of trimer $(\mathbf{2})_3$ with water molecules. c) Cross section images of stiff trimer (left) and tetramer (right) of **1** with water molecules.

Experimental

General procedure

^{1}H NMR spectra were recorded in $CDCl_3$ or CD_3OD on a JEOL ECP 400 or 600 spectrometer with tetramethylsilane (δ = 0 ppm) as an internal reference. MALDI-TOF mass spectra were measured on a Perseptive Voyager DE STR through laser ionization with dithranol, or α-CHCA as matrices. Soybean lecithin (type-IIS) was purchased from Sigma Chemical Co. Ltd. and used without purification. Sol-

vents and chemicals were used as received unless otherwise noted. Thin-layer chromatography (TLC) was performed on a glass plate precoated with silica gel (E. Merck Kieselgel 60 F254). Column chromatography was performed with silica gel 60 (E. Merck, particle size 0.063–0.200 mm, 60–230 mesh).

Methyl 3α-(2′-tetrahydropyranyl)-7α,12α-dihydroxy cholanoate (6)

Cholic acid methyl ester (5 g, 0.012 mol) was dissolved in $CHCl_3$ (50 mL), and conc. H_2SO_4 (1 drop) was added to the solution. A solution of 3,4-dihydro-2*H*-pyran (DHP) (0.84 g, 0.010 mol) in $CHCl_3$ (10 mL) was added dropwise to the solution. The mixture was stirred for 5 h at 0 °C. Anhydrous K_2CO_3 was added to the reaction mixture, and the reaction mixture was extracted with $CHCl_3$ and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography (SiO_2, benzene/AcOEt = 1 : 1) to give **6** (1.41 g, 23%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.70–4.78 (m, –O–CH–O–, 1H), 3.94–4.00 (m, –CH–OTHP, 1H), 3.88–3.94 (m, –CH–OH, 1H), 3.80–3.88 (m, –CH–OH, 1H), 3.67 (s, $-CO_2CH_3$, 3H), 3.42–3.52 (m, –O–CH_2–, 2H), 1.10–2.34 (m, 30H), 0.98 (d, J = 6.4 Hz, $-CH_3$, 3H), 0.90 (s, $-CH_3$, 3H), 0.66 (s, $-CH_3$, 3H).

Methyl 3α-(2′-tetrahydropyranyl)-7α,12α-diacetoxy cholanoate (3)

Diol **6** (200 mg, 0.39 mmol) was dissolved in pyridine (1 mL) and acetic anhydride (0.4 mL, 3.90 mmol) was added to the solution at 0 °C. The solution was allowed to warm to 40 °C and stirred under argon atmosphere. Further acetic anhydride (0.8 mL, 7.80 mmol) was added to the solution and the solution was stirred for 3 days. The reaction mixture was poured onto water at 0 °C. The mixture was extracted with $CHCl_3$ and the organic solution was washed with saturated aqueous $NaHCO_3$ and dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure to give **3** (230 mg) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02–5.12 (m, –CH–OCO–, 1H), 4.80–4.98 (m, –O–CH–O–, 1H), 4.60–4.74 (m, –CH–OCO–, 1H), 3.85–3.97 (m, –CH–OTHP, 1H), 3.67 (s, $-CO_2CH_3$, 3H), 3.42–3.52 (m, –O–CH_2, 2H), 2.12 (s, –OCO–CH_3, 3H), 2.07 (s, –OCO–CH_3, 3H), 0.93–2.34 (m, 30H), 0.90 (s, $-CH_3$, 3H), 0.80 (d, J = 6.4 Hz, $-CH_3$, 3H), 0.72 (s, $-CH_3$, 3H).

Methyl 3α-hydroxy-(7α,12α-diacetoxy) cholaonate (4)

The crude **2** was dissolved in a mixture of AcOH : THF : H_2O (4 : 2 : 1, 10 mL), and the solution was stirred for 20 h at 40 °C. After evaporation under reduced pressure, the organic layer was extracted with ethyl acetate, and the combined organic layers were washed with saturated aqueous $NaHCO_3$ and dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure and the residue was purified by column chromatography (SiO_2, benzene/AcOEt = 1 : 1) to give **4** (193 mg, 83%, 2 steps from **6**) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02–5.14 (m, –CH–OCO–, 1H), 4.85–4.95 (m, –CH–OCO–, 1H), 3.66 (s, $-CO_2CH_3$, 3H), 3.40–3.60 (m, –C<u>H</u>–OH, 1H), 2.17 (s, –OCO–CH_3, 3H), 2.12 (s, –OCO–CH_3, 3H), 0.95–2.34 (m, 25H), 0.91 (s, $-CH_3$, 3H), 0.80 (d, J = 6.4 Hz, $-CH_3$, 3H), 0.73 (s, $-CH_3$, 3H).

Bis[(7α,12α-diacetoxy-24-methoxycarbonyl)-3-cholanyl] *N*,*N*′-xylylene dicarbamate (5)

Xylylene diisocyanate (93 µl, 0.59 mmol) and 1,4-diazabicyclo-[2.2.2]octane (DABCO, 25 mg, 0.23 mmol) were added to a solution of **4** (594 mg, 1.17 mmol) in dry dioxane (10 ml) at room temperature. The solution was heated at 60 °C for 21 h, then the solvent was evaporated under reduced pressure. Aqueous 1 N HCl solution was added and DABCO was removed by decantation. The supernatant was concentrated and the residue was purified by silica gel column chromatography (Benzene : AcOEt = 1 : 1) to give **5** (290 mg, 41%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.30 (t, 1H, J = 7.2 Hz, H_d), 7.22–7.17 (m, 3H, H_a, H_c, $H_{c'}$), 5.10–5.07 (m, 2H, H_{12}), 4.96–4.92 (m, 2H, H_7), 4.92–4.88 (m, 2H, NH), 4.52–4.45 (m, 2H, H_3) 4.36–4.30 (m, 4H, H_{32}, $H_{32'}$), 3.65 (s, 6H, CO_2Me), 2.10 (s, 6H, OCOMe), 2.06 (s, 6H, OCOMe), 2.37–1.04 (m, 61H), 0.90 (s, 6H, H_{19}, $H_{19'}$), 0.80 (d, 6H, J = 6.6 Hz, H_{25}, $H_{25'}$), 0.72 (s, 6H, H_{18}, $H_{18'}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.3 (CH_3, C_{18}), 17.6 (CH_3, C_{25}), 21.4, 21.7 (CH_3, C_{27}, C_{29}), 22.6 (CH_3, C_{19}), 22.9 (CH_2), 25.6 (CH_2), 27.3 (CH_2), 27.4 (CH_2), 28.9 (CH), 30.9 (CH_2), 31.0 (CH_2), 31.3 (CH_2), 34.4 (C), 34.6 (CH), 34.7 (CH_2), 35.2 (CH_2), 37.8 (CH), 41.0 (CH), 43.5 (CH), 45.0 (CH_2), 45.1 (C), 47.5 (CH), 51.6 (CH_3, C_{23}), 70.8 (CH, C_{28}), 74.8 (CH, C_3), 75.4 (CH, C_{12}), 76.8 (CH, C_7), 126.6, 128.3 (CH, C_a, C_c), 129.1 (CH, C_d), 139.2 (C, C_b), 156.2 (C, C_{30}), 170.3, 170.4 (C, C_{26}, C_{28}), 174.5 (C, C_{23}). MALDI-TOF (positive mode, matrix: α-CHCA): *m*/*z* calcd for $C_{68}H_{100}N_2O_{16}Na$: 1223.7 (100), 1224.7 (78), 1225.7 (34) Found: 1223.9 (100), 1224.8 (90), 1225.8 (36) $[M + Na]^+$.

Bis(7α,12α-dihydroxy-24-carboxyl-3-cholanyl) *N*,*N*′-xylylene dicarbamate (1)

Dimethyl ester **5** (13 mg, 11.4 µmol) was dissolved in MeOH (2.7 ml), and 1 N NaOH solution (0.8 ml) was added to the solution. The mixture was stirred at 50 °C for 48 h, and 1 N HCl solution was added to the reaction mixture to adjust the pH at 2–3. Solvent was removed under reduced pressure, and the yellow residue was washed with $CHCl_3$ and H_2O until a colorless solid was obtained. The colorless solid was dissolved in MeOH, and undissolved solid was separated by filtration. The filtrate was concentrated under reduced pressure and purified by column chromatography (SiO_2, $CHCl_3$/MeOH = 3 : 1) to give **1** as a colorless solid (4.5 mg, 39%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.25 (t, 1H, J = 7.3 Hz, H_d), 7.19 (s, 1H, H_a), 7.14 (d, 2H, J = 7.3 Hz, H_c, $H_{c'}$), 4.46–4.36 (m, 2H, H_3, $H_{3'}$), 4.30–4.20 (m, 4H, H_{26}, $H_{26'}$), 3.98–3.92 (m, 2H, H_{12}, $H_{12'}$), 3.82–3.76 (m, 2H, H_7, $H_{7'}$) 2.44–2.15 (m, 8H), 2.35–1.02 (m, 48H), 1.01 (d, 6H, J = 6.6 Hz, H_{24}, $H_{24'}$), 0.93 (s, 6H, H_{19}, $H_{19'}$), 0.91, 0.71 (s, 6H, H_{18}, $H_{18'}$). ^{13}C NMR (150 MHz, CD_3OD) δ 11.7 (CH_3, C_{18}), 16.3 (CH_3, C_{24}), 21.7, 21.8 (CH_3, C_{19}, $C_{19'}$), 22.9 (CH_2), 26.6 (CH), 27.3 (CH_2), 28.3 (CH_2), 31.3 (CH_2), 34.4 (CH_2), 34.6 (C), 34.9 (CH_2), 35.2 (CH_2), 35.5 (CH), 35.7 (CH_2), 39.2 (CH_2), 39.7 (CH), 41.7 CH), 41.9 (CH), 44.0 (CH_2, C26), 46.2 (C), 46.8 (CH), 67.7 (CH, C_7), 72.7 (CH, C_{12}), 75.2 (CH, C_3),125.6, 125.7 (CH, C_a, C_c), 128.2 (CH, C_d), 139.7 (C, C_b), 157.7 (C, C_{25}), 178.3 (C, C_{23}) MALDI-TOF (positive mode, matrix: α-CHCA): *m*/*z* calcd for $C_{58}H_{88}N_2O_{12}Na$: 1027.6 (100), 1028.6 (67), 1029.6 (24), 1030.6 (6); Found:1027.4 (100), 1028.4 (75), 1029.4 (39), 1030.4 (22) $[M + Na]^+$. HRMS (FAB, matrix: NBA) calcd for $C_{58}H_{88}N_2O_{12}Na$: 1027.6235; Found:1027.6235.

Measurement of single channel currents

Single ion channel currents were measured by the planar bilayer lipid membrane method. Details were described in previous papers.[39–41]

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