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Space-Filling Effects in Membrane Disruption by Cationic Amphiphiles

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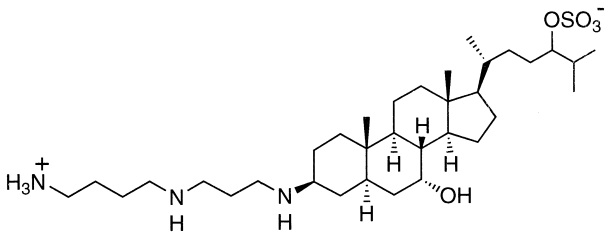
Abstract—We studied the hemolytic activity towards bovine erythrocytes of novel synthetic steroid–polyamine conjugates consisting of a rigid hydrophobic steroid unit, and a flexible hydrophilic polyamine unit connected by a linker. The steroid structure, polyamine chain length, and the presence of a hydrophobic substituent on the steroid, all influenced the activity. Analysis of the time dependence of hemolysis suggested that these structurally related cationic amphiphiles have different mechanisms of membrane perturbation.

Introduction

Molecular interactions of amphiphilic molecules with lipid membranes are ubiquitous, being involved in a wide range of biological events. An understanding of bilayer interactions with natural and synthetic compounds would be beneficial in many areas, for example, in developing novel antimicrobial agents which can specifically perturb bacterial cell membranes.[1,2] Many mechanisms of bilayer interactions have been proposed so far, including channel formation,[3] direct entry of compounds into lipid membranes to form holes[4] and others. For example, polyene macrolide antibiotics, such as amphotericin B, are proposed to form ion channels in biomembranes.[2] On the other hand, naturally occurring saponins such as digitonin are surface-active, inducing hemolysis and having other biological activities.[3,5] It has been proposed that digitonin and amphotericin B enter lipid membranes and form complexes with sterols such as cholesterol and ergosterol.[2,4] Recently a naturally occurring sterol–polyamine conjugate, squalamine, was isolated from stomach tissue of shark and its antimicrobial and hemolytic properties were evaluated (Chart 1).[6] Squalamine includes a long and rigid hydrophobic sterol unit, a hydrophilic polyamine, and a zwitterionic (sulfate anion) head group.[7] In the present work, we synthesized novel polyamine–steroid conjugates, and evaluated structural effects on their membrane disruption activity, using bovine erythrocytes. These steroid–polyamine conjugates (**A** and **B**, Chart 2) consist of a hydrophobic, structurally rigid steroid, a flexible hydrophilic polyamine, the nitrogen atoms of which can be protonated under physiological conditions, and a linker which connects the hydrophobic and hydrophilic units. The steroid moieties used were cholestane and lithocholic acid, of which the former has a *trans*-decalin structure and the latter, a *cis*-decalin structure. In the case of lithocholic acid-polyamine conjugates (**B**, Chart 2), a hydrophobic substituent (R_2) was also introduced. The polyamine chain length was varied. Thus, in the present series of synthetic cationic amphiphiles, the space-filling characters of the hydrophobic and hydrophilic units are different (Chart 2). We found that the membrane-perturbing effect of the steroid–polyamine conjugates is influenced by both polyamine chain length and steroid structure, and we postulate that different mechanisms of action



Squalamine

Chart 1. Structure of squalamine.

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are involved in the hemolytic activities of these structurally related compounds. These structural features relevant to hemolytic activities are apparently different from those of gene transfection activities of these compounds as cationic liposomal vehicles.[8]

Results

Synthesis of cationic amphiphiles

Polyamines were synthesized in a modified manner of Nakanishi et al.[9] Michael addition of the terminal

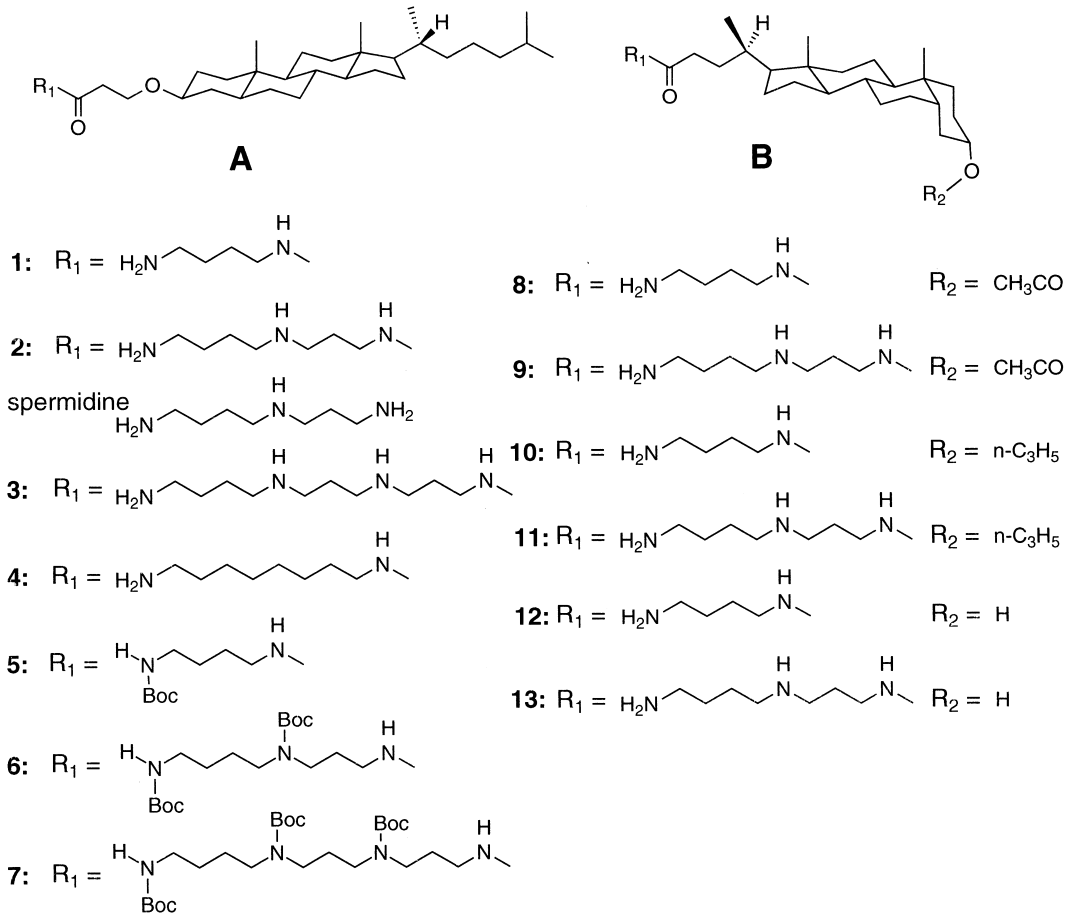
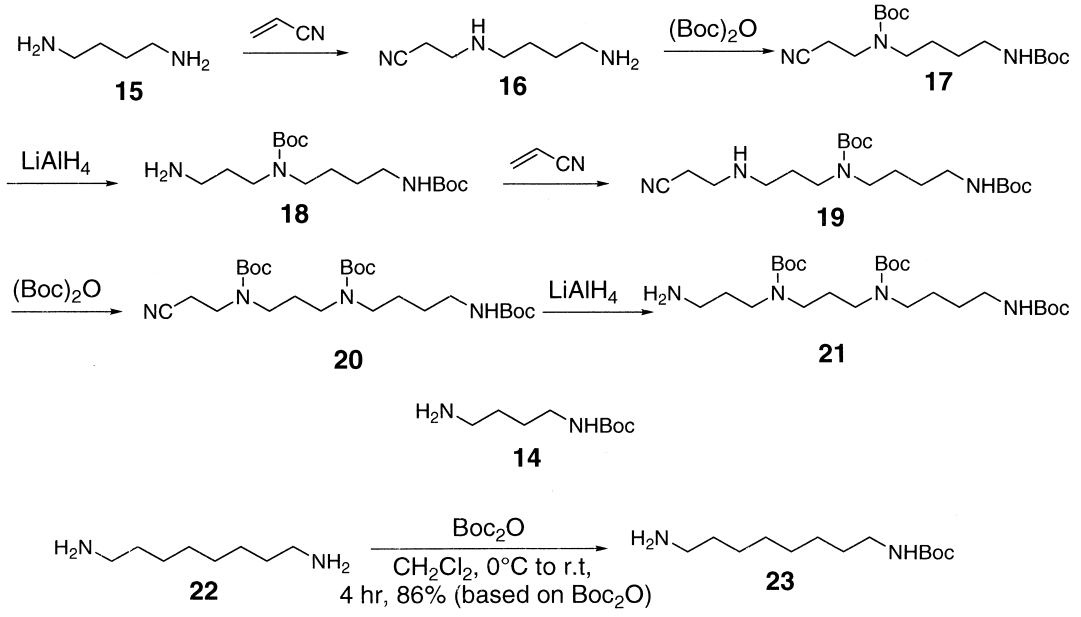


Chart 2. Cationic Amphiphiles.

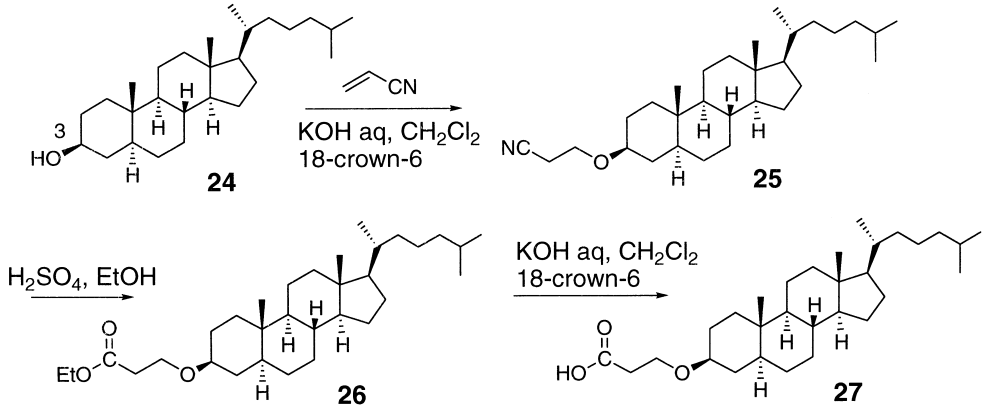


Scheme 1. Preparation of polyamines.

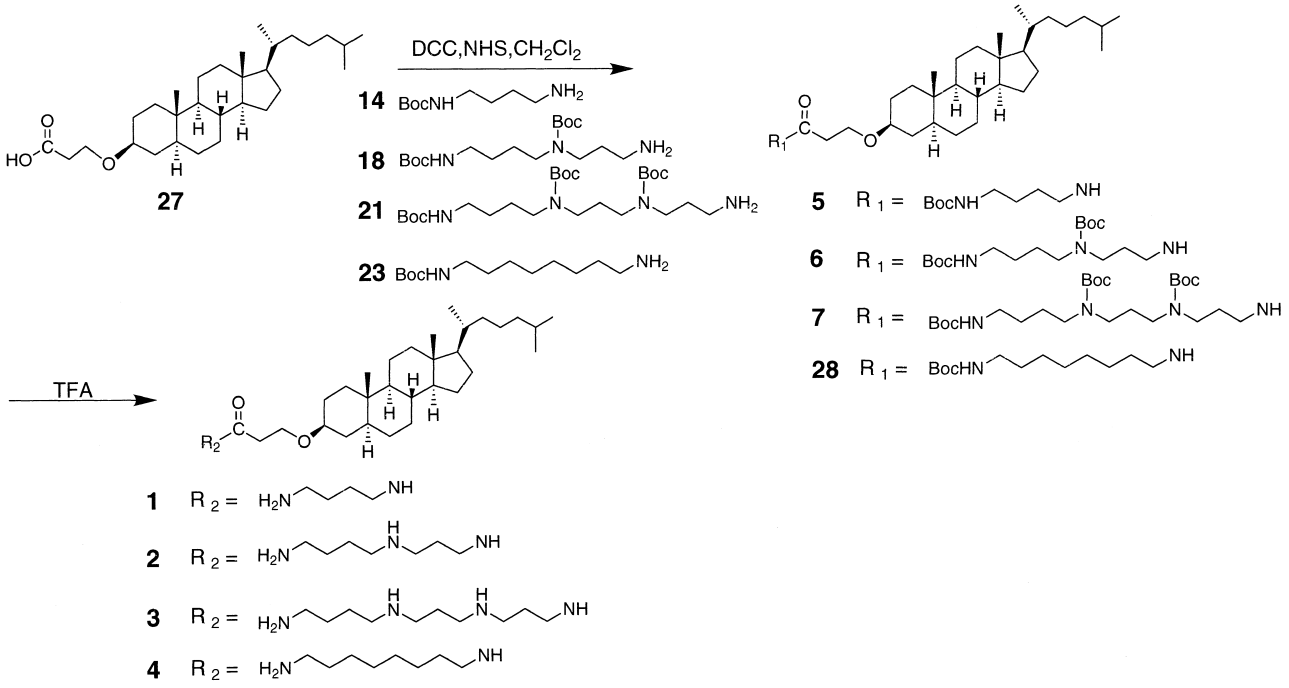
amine of diamine **15** to acrylonitrile, followed by *N*-Boc protection, reduction of the nitrile with lithium aluminum hydride led to extension of a propylamine unit (Scheme 1). Addition of a linker moiety to cholestane **24** was carried out by Michael reaction of acrylonitrile with the 3β-hydroxyl group of **24**, acid-catalyzed alcoholysis of the nitrile **25** to give the ester (**26**) and alkaline hydrolysis of the ester **26** to the acid **27** (Scheme 2).[10] Amide coupling of the cholestane acid **27** and an *N*-Boc (t-butoxycarbonyl) protected polyamine (**14**, **17**, **20** and **23**) was carried out with the conventional *N*-hydroxysuccinimide (NHS)-DCC method. The polyamine chain length was varied. Deprotection of the Boc group was carried out in trifluoroacetic acid (TFA). The cationic amphiphiles (**1**–**4**) were obtained as TFA salts (Scheme 3). 3-α-Acetyl lithocholic acid **30** was readily obtained by acetylation of lithocholic acid **29**. Amide coupling of **30** with the *N*-Boc-protected polyamine (**14** or **18**) was performed with the NHS-DCC chemistry, followed by deprotection of the BOC group in TFA to give **8** and **9** (Scheme 4). Allylation of 3α-hydroxy group of the methyl ester (**33**) of lithocholic acid with allyl bromide in the presence of Hünich base (*N*,*N*-diisopropylethylamine) was performed to give **34**. Catalytic hydrogenation over Pd/C and alkaline hydrolysis gave *O*-propyl lithocholic acid **35** (Scheme 5). Amide coupling of the acid **36** with the *N*-protected polyamine (**14** or **18**) gave **37** and **38**, respectively followed by deprotection of the Boc group in TFA to give **10** and **11** (Scheme 6).

Hemolytic activities of polyamine–cholestane conjugates towards bovine erythrocytes

We prepared four derivatives of cholestane–polyamine conjugates (**1**–**4**) and the corresponding *N*-Boc-protected counterparts (**5**–**7** and **28**) (Chart 2). The steroid moiety is cholestane and the cationic hydrophilic moiety (R_1) is a polyamine. The polyamine moiety of **1** was diaminobutane, that of **2** was spermidine, and that of **3**

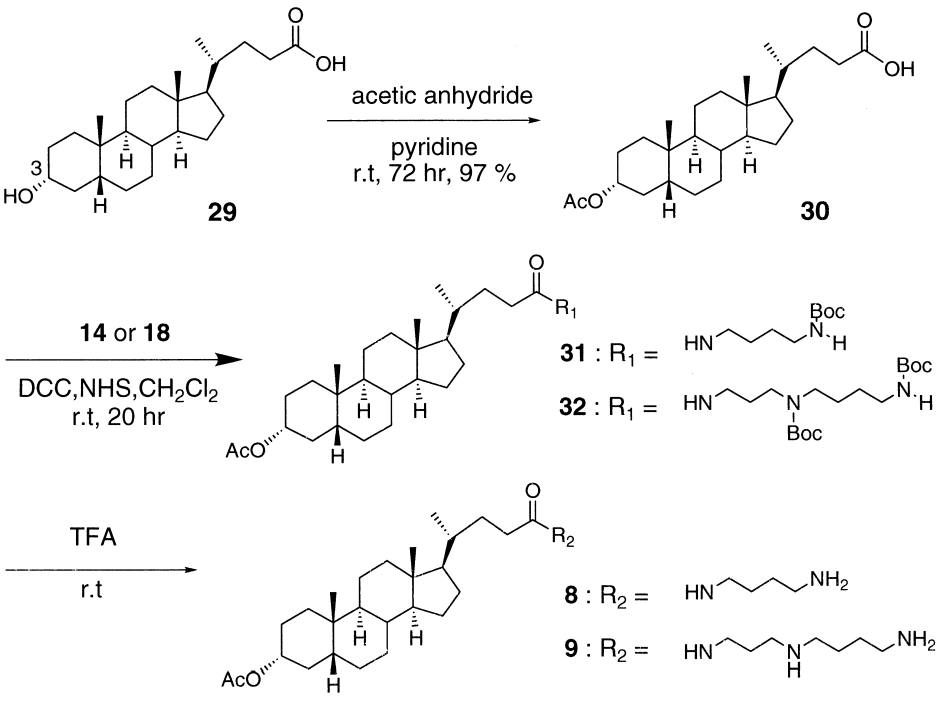


Scheme 2. Preparation of cholestane bearing a linker.

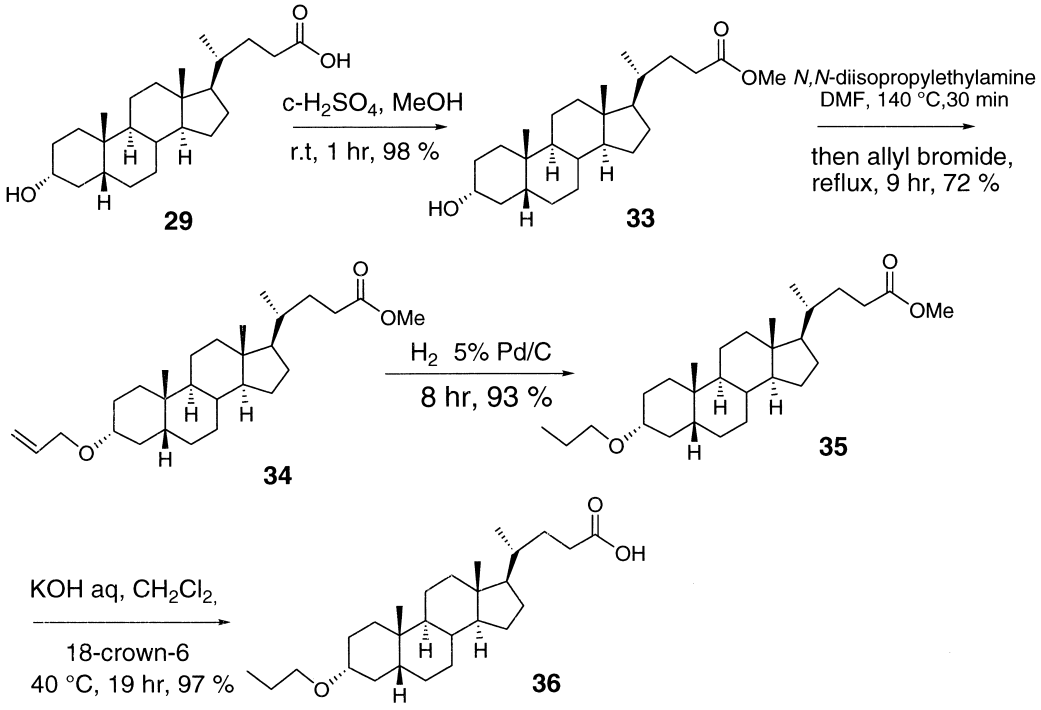


Scheme 3. Preparation of cationic amphiphiles based on cholestane.

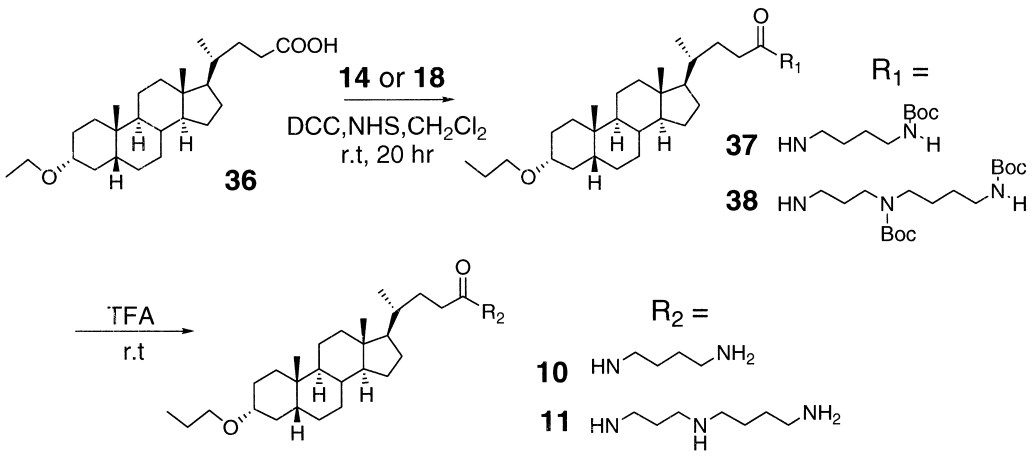
was a non-natural isomer of spermine. The linker bond of these cholestane–polyamine conjugates is an amide group. In order to evaluate the membrane-disrupting ability of these cholestane–polyamine conjugates, we measured their dose-dependent hemolytic activity towards bovine erythrocytes at 37 °C at 60 min after the addition of compounds (Fig. 1). The conjugates **2** and **3** showed dose-dependent hemolytic activity while the short polyamine analogue **1** showed no significant activity at concentrations below 100 μM. The effective concentrations (EC_{50}) of **2** and **3** that induce 50% hemolysis under the present experimental conditions are 15.8 and 20.1 μM, respectively. Basicity of the amino nitrogen atoms of the polyamine chain is crucial for the hemolytic activity, because the *N*-Boc-protected polyamine analogues **5**–**7** (Chart 2) showed no significant hemolytic activity (Fig. 1). A polyamine by itself may not induce hemolysis because spermidine showed no hemolytic activity at concentrations below 100 μM (Fig. 2). The importance of the two amine



Scheme 4. Cationic amphiphiles derived from 3-acetyl lithocholic acid.



Scheme 5. Alkylation of 3-hydroxy group of lithocholic acid.



Scheme 6. Cationic amphiphiles derived from 3-propyl lithocholic acid.

nitrogen atoms of **2** for hemolytic activity is suggested by the observation that the carbon analogue **4**, in which the secondary amine of **2** is replaced with a methylene group, has little hemolytic activity (Fig. 2).

Membrane perturbation arising from polyamine–lithocolic acid conjugates. Effect of steroid structure on hemolytic activity

We prepared six derivatives of the lithocholic acid–polyamine conjugates (**8**–**13**) (Chart 2). The polyamine moiety was diaminobutane (in **8** and **10**) or spermidine (in **9** and **11**), both of which are surface-active in the case of the cholestane–polyamine conjugates. The hydroxyl group at C-3 of lithocholic acid (substituent R_2, Chart 2) was substituted with an acetyl group (**8** and **9**), or a propyl group (**10** and **11**). Hemolytic activities of these cationic amphiphiles were measured in the same manner as those of the cholestane–polyamine conjugates, i.e. 60 min incubation after the addition of the compounds at 37 °C (Fig. 3). The short-polyamine conjugates **8** and **10** showed no significant hemolytic activity, while **9** and **11** showed activity (Fig. 3), the latter being as active as **2**. The effective concentrations (EC_{50}) of **8**, **9** and **11** are 216.8, 32.3 and 8.6 μM, respectively. The polyamine chain lengths of **9** and **11** are the same, but the hemolytic activity of **11** is stronger than that of **9**. Thus, the substituent R_2 (Chart 2) is another factor which modifies the hemolytic activity, that is, the non-polar hydrophobic *n*-propyl group is more effective than the polar acetyl group.

Time-dependence of membrane perturbation

In order to shed light on the kinetics of hemolysis, the magnitude of hemolysis was recorded over 120 min after the addition of compounds. The results at various concentrations (1, 25 and 100 μM) of compounds **2**, **9** and **11** are shown in Figure 4A–C. Compounds **2**, **9** and **11** all have the same polyamine chain, while the hydrophobic units and the substituents (R_2) are different, i.e. the steroid unit is cholestane in **2** and lithocholic

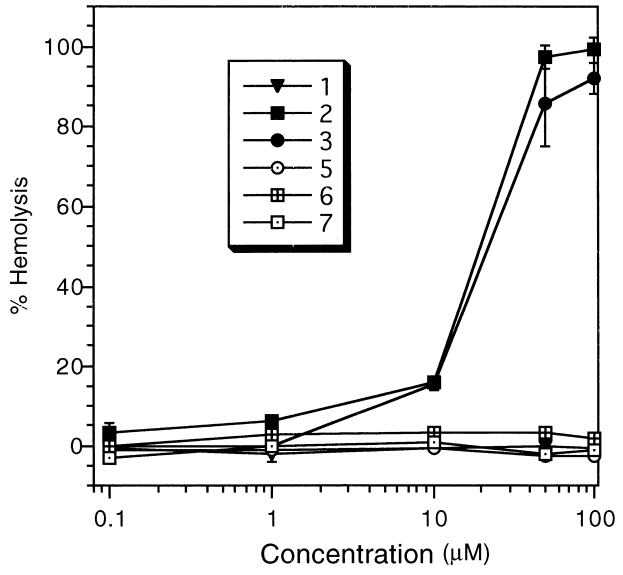


Figure 1. Dose-dependent hemolysis induced by cholestane–polyamine conjugates. Incubation at 37 °C for 60 min. Each value is the mean ± S.E. ($n = 3$).

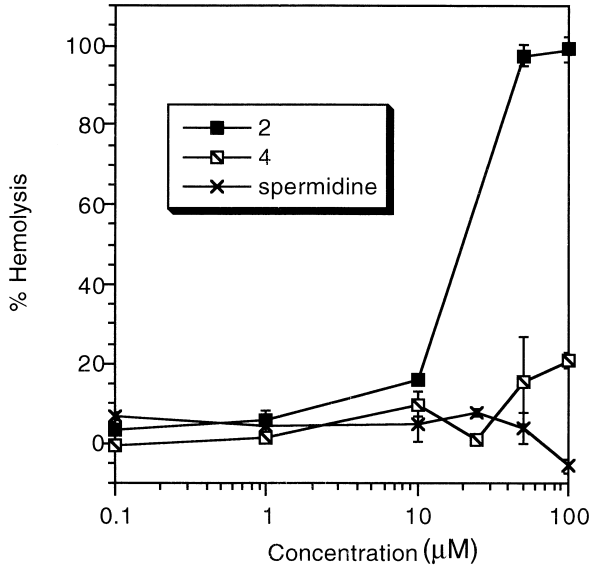


Figure 2. Structural requirements for hemolytic activity of cholestane–polyamine conjugates. Incubation at 37 °C for 60 min. Each value is the mean ± S.E. (n = 3).

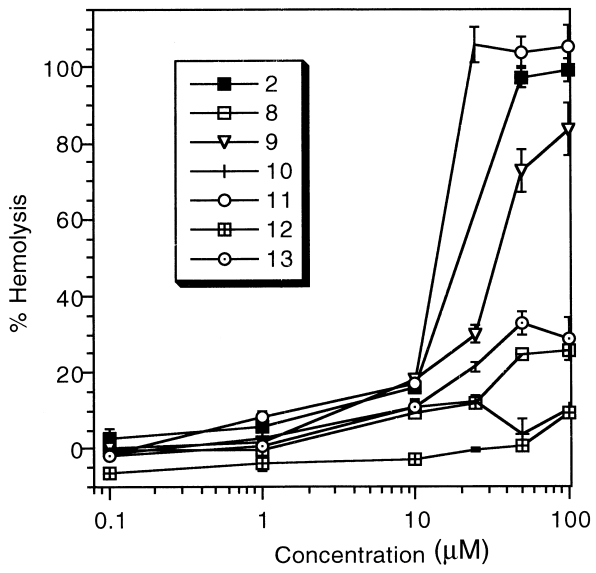


Figure 3. Hemolytic activity of lithocholic acid–polyamine conjugates. incubation at 37 °C for 60 min. Each value is the mean ± S.E. (n = 3).

acid in **9** and **11**, while the hydrophobic substituent (R_2) is acetyl (**9**) or *n*-propyl (**11**). At the concentration of 1 μM, these compounds showed practically no hemolysis (Fig. 4A). Addition of **11** to the cells at the final concentration of 25 μM induced 100% hemolysis within 20 min (Fig. 4B). In contrast, compounds **2** and **9** at the concentration of 25 μM induced a plateau level of 60% hemolysis after about 60 min (Fig. 4B). The times required to reach the half-maximum level of hemolysis (t_{50}) with 25 μM of **2**, **9** and **11** were 9.2, 27.8, and 3.7 min, respectively. Even at the concentration of 100 μM, hemolysis induced by **9** was slow, and reached a plateau at 80% after 120 min. Compounds **2** and **11** both induced complete hemolysis within 20 min at the concentration of 100 μM (Fig. 4C). At 100 μM, the t_{50} values of **2**, **9** and **11** were 2.3, 21.2, and 3.7 min, respectively. The hemolysis curves of **2** and **11** are very similar. In the case of **2**, the t_{50} value depends inversely on the concentration of the reagent, i.e., as the concentration of the reagent was increased four-fold (25 to 100 μM), the t_{50} value decreased to one-fourth (9.2 to 2.3 min). On the other hand, the t_{50} value

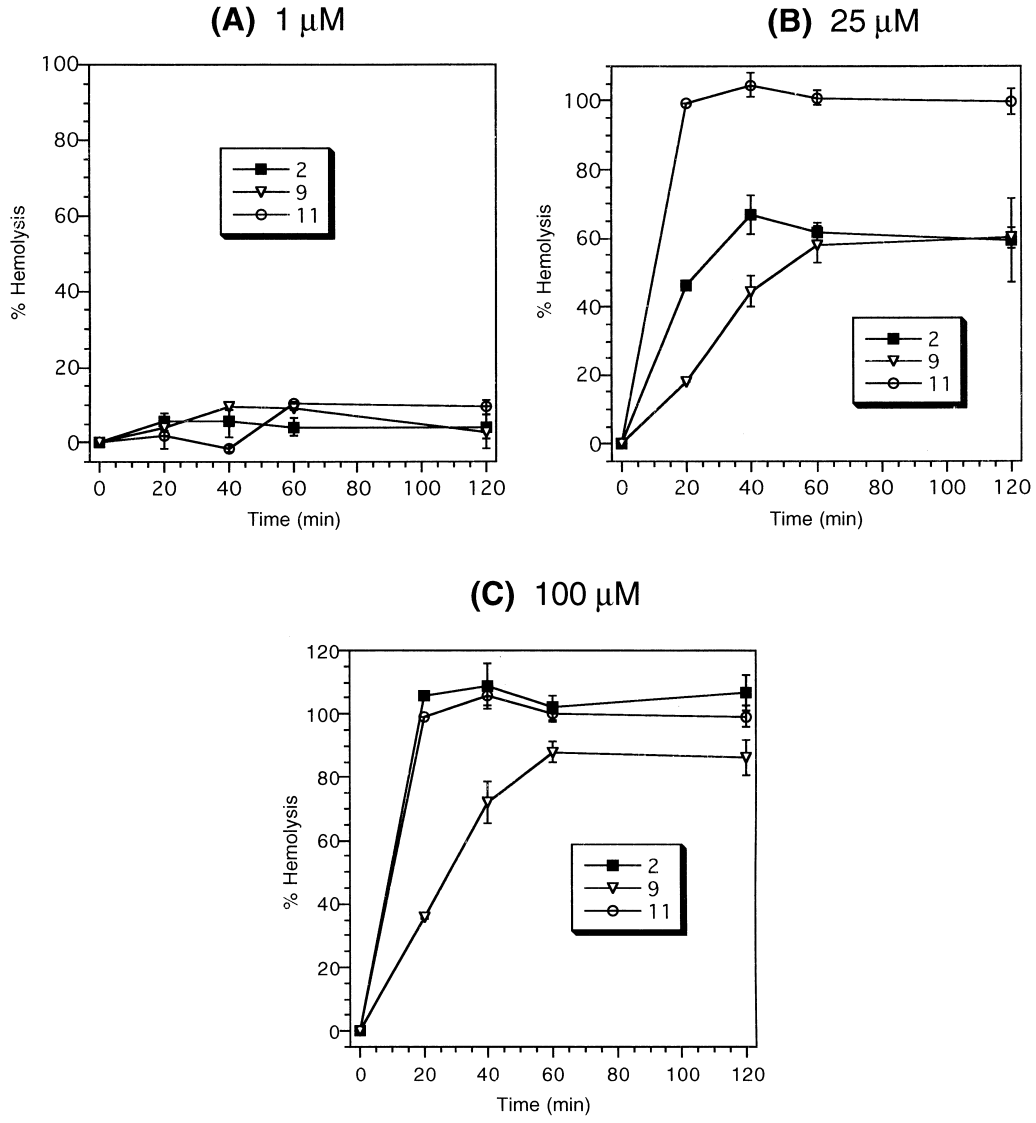


Figure 4. Time-dependency of hemolytic activity of steroid–spermidine conjugates. Incubation at 37 °C. Each value is the mean ± S.E. (*n* = 3).

of **11** seems to be insensitive to the concentration of the reagent. Differences in the rate of partition of the compounds into the plasma membrane of the erythrocytes may account for the difference of kinetics.

Discussion

Although the hemolytic activities of some steroid-saponins and triterpenoid-saponins have been well studied,[4,5] structural effects remain controversial, i.e., it is unclear whether differences of hemolytic activity and time-dependency are attributable to the hydrophilic sugar moiety or the hydrophobic aglycon structure. Intuitively, a hydrophilic moiety should be inert for membrane disruption[11] while the hydrophobic moiety of a cationic amphiphile should be crucial for membrane disruption, because anchoring of the hydrophobic moiety to the cell membrane would be the initial step of hemolysis. However, we found that the polyamine chain length has a striking effect on hemolytic activity: the analogue bearing a short polyamine chain (**1**) showed no hemolytic activity whereas the analogues (**2** and **3**) with longer polyamine chains are surface-active. Furthermore, the time-dependent hemolytic behavior at the concentration of 25 μM highlighted the difference in activities of the lithocholic acid–spermidine conjugate **11** and the cholestane–spermidine conjugate **2** (Fig. 4B): i.e., the order of hemolytic activity is **11** > **2**. Thus, space-filling of the hydrophobic steroid unit (shape and orientation) also affects the hemolytic activities. In the time-dependency experiments at the concentration of 25 μM (Fig. 4B), hemolysis due to the lithocholic acid–polyamine conjugate **11** was very rapid being completed in 20 min, whereas the lithocholic acid–polyamine derivative **9** induced slow hemolysis, which reached a plateau. The only difference in structural units between **9** and **11** is the substituent R_2 (Chart 2). In addition, lithocholic acid–polyamine derivatives (**12** and **13**, Chart 2) bearing a hydroxyl group ($R_2 = H$) showed significantly reduced hemolytic activities (Fig. 3), the ED_{50} value of **13** being 150.3 μM, much larger than those of **9** and **11**. Thus a small hydrophobic appendant (i.e. the R_2 substituent) can substantially influence the interaction with the bilayer.

Conclusion

We prepared cationic amphiliphiles based on the cholestane and lithocholic acid structures. These present novel steroid–polyamine conjugates can serve as useful probes to study space-filling effects, i.e., the effects of shape and orientation, of hydrophilic and hydrophobic structural units upon interactions with a bilayer.

The hemolytic activity of a structurally similar compound, squalamine (Chart 1), was measured after 10 min incubation with erythrocytes and no significant hemolysis was observed at 25 μM even though some compounds tested in this study showed distinct hemolytic activities at this concentration (Fig. 4B).[6] Considering that squalamine exerts antibiotic activity against both Gram-positive and Gram-negative bacteria at lower concentrations than those at which hemolytic activity is observed, the present study may provide a new approach for the development of novel, potent, broad-spectrum antibiotics.[6,12]

Experimental

General methods

All the melting points were measured with a Yanaco Micro Melting Point Apparatus (MP-500D) and are uncorrected. Proton (400 MHz) NMR spectra were measured on a JEOL Caliber-GX400 NMR spectrometer with TMS as an internal reference in $CDCl_3$ as the solvent, except otherwise specified. Chemical shifts are shown in ppm. Coupling constants are given in hertz. High-resolution mass spectra (HRMS, EI) and FAB mass spectra (FABMS) were recorded on a JEOL JMS-SX 102A instrument. High-performance liquid chromatography (HPLC) was run on a Shimadzu LC-10A system and a Shimadzu SPD-M10A system equipped with a UV–vis photo diode array detector on ODS gel (RP-18 GP, Mighty sil, Kanto Chemicals, Japan) packing (20 mm × 25 cm) with the specified eluent. The eluents were 0.1% (v/v) TFA–99.9% H_2O solution and 0.08% (v/v) TFA–99.92% CH_3CN. Column chromatography was performed on silica gel [silica gel 60 (63–200 μm, Merck], and flash column chromatography was carried out on silica gel [silica gel 60 (40–63 μm), Merck]. The combustion analyses were carried out in the microanalytical laboratory of this faculty.

Preparation of the steroid–polyamines conjugates

4-Aza-8-amino-octacarbonitrile (16). To a solution of 1,4-diaminobutane (**15**) [1.32 g (15.0 mmol)] in 0.35 mL of methanol, acrylonitrile [1.25 g (1.54 equiv)] was added at 0 °C (in an ice-water bath) over 2 min.[9] The mixture was stirred at ambient temperature for 17 h. The organic solvent was evaporated to give the residue which was column-chromatographed (*i*$PrNH_2$:MeOH:$CHCl_3$ = 1:5:15) to give the nitrile **16** (1.21 g (57% yield) as colorless liquid. ^1H NMR: 2.930 (2H, t, J = 6.60 Hz), 2.711 (2H, t, J = 6.78 Hz), 2.654 (2H, t, J = 6.97 Hz), 2.529 (2H, t, J = 6.60 Hz), 1.542 (3H, m), 1.509 (4H, m). MS (EI): (M^+) 141.

***N*⁴,*N*⁸-di-Boc-4-Aza-8-amino-octacarbonitrile (17).** To a solution of **16** [282.0 mg (2.0 mmol)] in 2.5 mL of CH_2Cl_2, a solution of $(Boc)_2O$ (di *t*-butyl dicarbonate) [894.8 mg (4.1 mmol)] in 2.5 mL of CH_2Cl_2 was added at 0 °C (in an ice-water bath).[10] The whole was stirred at ambient temperature for 4 h. The whole was poured into 30 mL of water, and was extracted with ethyl acetate (20 mL × 2). The organic layer was washed with brine, and was dried over magnesium sulfate. The organic solvent was evaporated to give the residue which was flash-chromatographed ($CHCl_3$, and then $CHCl_3$:MeOH = 99:1) to give **17** [88.7 mg (86% yield)] as colorless liquid. ^1H NMR: 4.585 (1H, br s), 3.461 (2H, t, J = 6.60 Hz), 3.280 (2H, t, J = 7.24 Hz), 2.564 (2H, br s),

1.598–1.441 (6H, m), 1.468 (9H, s), 1.441 (9H, s). MS (EI): 341 (M^+); HRMS (EI): calcd for $C_{17}H_{31}N_3O_4$, 341.2316. Found: 341.2317.

N^4,N^8-Di-Boc-4-aza-octane-1,8-diamine (18). To a suspension of $LiAlH_4$ (1.26 g) in 150 mL of dry ether, a solution of **17** [3.16 g (9.27 mmol)] in 50 mL of dry ether was added at 0 °C (in an ice-water bath) with stirring over 30 min. After 1 h, aqueous 1 N NaOH solution was added, and the inorganic salts were filtered off. The obtained organic layer was washed with brine, and was dried over magnesium sulfate. The organic solvent was evaporated to give the amine **18** [2.86 g (90% yield)] as colorless liquid. ^{1}H NMR: 4.570 (1H, br s), 3.252 (2H, br s), 3.139 (4H, m), 2.683 (2H, t, J = 6.70 Hz), 1.638 (2H, m), 1.542–1.242 (6H, m), 1.453 (9H, s), 1.440 (9H, s). HRMS (EI, M^+): calcd for $C_{17}H_{35}N_3O_4$, 345.2627. Found: 345.2622.

N^8,N^{12}-di-Boc-4,8-diaza-12-amino-dodecacarbonitrile (19). The nitrile **19** was prepared from **18** in a similar manner of **16**. Yield: 58%. Colorless liquid. ^{1}H NMR: 4.561 (1H, br s), 3.247 (1H, br s), 3.137 (4H, m), 2.912 (2H, t, J = 6.69 Hz), 2.618 (2H, t, J = 6.60 Hz), 2.510 (2H, t, J = 6.69 Hz), 1.692 (2H, quintet, J = 6.87 Hz), 1.596–1.440 (6H, m), 1.452 (9H, s), 1.440 (9H, s). MS(EI): 398 (M^+). HRMS (EI, M^+): calcd for $C_{20}H_{38}N_4O_4$: 398.2893. Found: 398.2908.

N^4,N^8,N^{12}-tri-Boc-4,8-diaza-12-amino-dodecacarbonitrile (20). The tri-Boc protected nitrile **20** was prepared from **19** in a similar manner to **17**. Yield: 94%. Colorless liquid. ^{1}H NMR ($CDCl_3$): 4.635 (1H, br s), 3.478 (4H, t, J = 6.60 Hz), 3.265 (2H, t, J = 7.42 Hz), 3.172 (6H, m), 2.635 (2H, br s), 1.763 (2H, quintet, J = 6.87 Hz), 1.573–1.440 (6H, m), 1.471 (9H, s), 1.452 (9H, s), 1.439 (9H, s). MS(EI): 498 (M^+). HRMS (EI, M^+): calcd for $C_{25}H_{46}N_4O_6$: 498.3417. Found: 498.3416.

N^4,N^8,N^{12}-tri-Boc-4,8-diaza-12-amino-dodecane-1,12-diamine (21). The amine **21** was prepared in a similar manner of **18**. Yield: 68%. ^{1}H NMR: 4.650 (1H, br s), 3.279 (2H, br s), 3.153 (6H, br s), 2.729 (2H, t, J = 7.24 Hz), 1.534–1.438 (6H, m), 1.455 (9H, s), 1.449 (9H, s), 1.438 (9H, s). MS(EI): 502 (M^+); HRMS (EI, M^+): calcd for $C_{25}H_{50}N_4O_6$: 502.3730. Found: 502.3731.

N^8-Boc-octane-1,8-diamine (23). To a solution of 1,8-diaminooctane (**22**) [5.77 g (40.0 mmol)] in 60 mL of 1,4-dioxane, a solution of $(Boc)_2O$ 1.09 g (5 mmol) in 30 mL of 1,4-dioxane was added at 25 °C over 2 h. The whole was stirred at 25 °C for 24 h. The solvent was evaporated, and to the residue 100 mL of water was added. Undissolved materials were separated by filtration with suction, and the filtrate was extracted with carbon tetrachloride (30 mL×3). The organic layer was washed with water (20 mL×4), and was dried over sodium sulfate. The organic solvent was evaporated to give **23** [756.4 mg (63% yield)] as colorless solid. ^{1}H NMR: 4.499 (1H, br s), 3.014 (2H, m), 2.673 (2H, t, J = 6.98 Hz), 1.443 (9H, br s), 1.299 (8H, br s). MS (EI): 224 (M^+). HRMS (EI, M^+): calcd for $C_{13}H_{28}N_2O_2$: 224.2152. Found: 224.2152.

3α-*O*-Cyanoethyl cholestane (25). To a solution of 7.77 g (1.0 mmol) of α-cholestane **24** in 30 mL of CH_2Cl_2, a mixture of 6.0 mL of aqueous KOH (w/w 40%) and 4.0 mL of acrylonitril was added at ambient temperature, followed by the addition of 18-crown-6 (520.0 mg). The whole was stirred at ambient temperature for 18 h. The whole was diluted with 100 mL of H_2O, and was acidified with aqueous 1 N HCl solution. The mixture was extracted with $CHCl_3$ (50 mL×2), and the organic layer was washed with brine, and was dried over magnesium sulfate. The organic solvent was evaporated to give 10.17 g of the crude **25**. Recrystallization from methanol gave 7.53 g (85% yield) of pure nitrile **25** as colorless plates. Mp 102.5–104.5 °C. ^{1}H NMR: 3.684 (2H, d,d, J = 1.65, 6.42 Hz), 3.289 (1H, m), 2.569 (2H, t, J = 6.51 Hz), 1.962 (1H, m), 0.897 (3H, d, J = 6.60 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.860 (3H, d, J = 6.60 Hz) 0.795 (3H, s), 0.646 (3H, s), 1.829–0.577 (total 31H, m). Anal. calcd for $C_{30}H_{51}NO$: C, 81.57; H, 11.64; N, 3.17. Found: C, 81.41; H, 11.91; N, 3.23.

Ethyl cholestane-3-*O*-propylate (26). A solution of nitrile **25** [110.3 mg (0.25 mmol)] in 95% EtOH (1 mL) in the presence of 0.5 mL of concentrated H_2SO_4, was refluxed for 5 h. The whole was diluted with 50 mL of water, followed by extraction with $CHCl_3$ (30 mL×2). The organic layer was washed with 10% aqueous $NaHCO_3$ (20 mL×2) and brine. The organic layer was dried over magnesium sulfate. The organic solvent was evaporated to give the residue which was flash-chromatographed (hexane:AcOEt = 19:1) to give 63.4 mg (52% yield) of the ester **26**. Mp 57.5–59.5 °C (recrystallized from ethanol, colorless plates). ^{1}H NMR: 4.147 (2H, q, J = 7.15 Hz), 3.730 (2H, dd, J = 2.02, 6.60 Hz), 3.232 (1H, m), 2.547 (2H, t, J = 6.51 Hz), 1.956 (1H, m), 1.256 (3H, t, J = 7.15 Hz), 0.896 (3H, d, J = 6.60 Hz), 0.863 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.60 Hz), 0.781 (3H, s), 0.641 (3H, s), 1.826–0.572 (total 31H, m). Anal. calcd for $C_{32}H_{56}O_3$: C, 78.63; H, 11.55; N, 0.00. Found: C, 78.48; H, 11.69; N, 0.00.

Cholestane-3-*O*-propionic acid (27). To a solution of **26** [500.2 mg (0.976 mmol)] and 18-crown-6 (50.8 mg) in 3.0 mL, 1.5 mL of 40% (w/w) aqueous NaOH solution was added. The whole was stirred at ambient temperature for 17 h, and then was poured into 30 mL of water. The whole was acidified with aqueous 1 N HCl solution, and was extracted with $CHCl_3$ (30 mL×2). The organic layer was washed with water (20 mL), and was dried over magnesium sulfate. The organic solvent was evaporated to give crude **27** (470.5 mg), followed by recrystallization from methanol to give **27** (421.4 mg, 90% yield) as colorless plates. Mp 139.5–141.0 °C. ^{1}H NMR: 3.754 (2H, dd, J = 1.65, 6.23 Hz), 3.310 (1H, m), 2.625 (2H, t, J = 6.14 Hz), 1.962 (1H, m), 0.897 (3H, d, J = 6.42 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.856 (3H, d, J = 6.60 Hz), 0.795 (3H, s), 0.646 (3H, s), 1.876–0.580 (total 31H, m). Anal. calcd for $C_{30}H_{52}O_3$: C, 78.21; H, 11.38; N, 0.00. Found: C, 78.03; H, 11.54; N, 0.00.

***N*-Boc-Diamine–cholestane conjugate (5).** To a stirred mixture of **27** [172.5 mg (0.375 mmol)], *N*-Boc-1,4-diaminobutane (**14**) (70.5 mg (1.0 equiv)) and NHS (*N*-

hydroxysuccinimide) [48.3 mg (1.1 equiv)] in 2.0 mL of dry CH_2Cl_2, DCC (*N*,*N*-dicyclohexylcarbodiimide) [77.1 mg (1.0 equiv)] was added at ambient temperature, and the whole was stirred for 20 h. After removal of the precipitate of DCurea by filtration with suction, and the filtrate was washed with saturated aqueous $NaHCO_3$, water, and brine. The organic phase was dried over magnesium sulfate. The residue after evaporation of the organic solvent was flash-chromatographed (hexane: AcOEt = 2:1 and then 1:3) to give **5** (202.0 mg (85% yield)). Mp 96.0–98.0 °C (recrystallized from CH_2Cl_2/n-hexane). ^{1}H NMR: 6.520 (1H, br s), 4.756 (1H, br s), 3.690 (2H, m), 3.257 (3H, m), 2.428 (2H, t, J = 5.68 Hz), 1.440 (9H, s), 0.898 (3H, d, J = 6.60 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.860 (3H, d, J = 6.60 Hz), 0.795 (3H, s), 0.646 (3H, s), 2.045–0.582 (total 37H, m). FABMS (MH^+): 631. HRMS (FAB^+, MH^+): calcd for $C_{39}H_{71}N_4O_2$: 631.5414. Found: 631.5435. Anal. calcd for $C_{39}H_{70}N_2O_4$: C, 74.24; H, 11.18; N, 4.44. Found: C, 74.31; H, 11.06; N, 4.22.

Diamine–cholestane conjugate (1). Under Ar atmosphere a solution of **5** [90.0 mg (0.143 mmol)] in 2.0 mL of TFA was stirred at ambient temperature for 30 min. The acid was evaporated to give the residue which was flash-chromatographed ($CHCl_3$:MeOH = 9:1 and then $CHCl_3$: MeOH:*i*PrNH$_2$ = 30:5:1), followed by freeze-drying in vacuum to give pale yellow amorphous **1** (129.9 mg). The product was purified with HPLC (retention time 20.25 min (ODS analytical column. Eluent: 0.1% TFA–H_2O:0.08% TFA–CH_3CN = 10:90) ^{1}H NMR: 3.746 (2H, t, J = 5.59 Hz), 3.325 (3H, m), 3.163 (2H, m), 2.555 (2H, t, J = 5.32 Hz), 0.897 (3H, d, J = 6.60 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.60 Hz), 0.789 (3H, s), 0.647 (3H, s), 2.045–0.582 (total 37H, m). FABMS (MH^+): 531. HRMS (FAB^+, MH^+): calcd for $C_{34}H_{63}N_2O_2$: 531.4889. Found: 531.4905.

***N*,*N*′-DiBoc triamine–cholestane conjugate (6).** A similar coupling of **27** and **18** was carried out in a similar manner of **1** (43% yield). Colorless liquid. ^{1}H NMR: 4.559 (1H, br s), 3.712 (2H, t, J = 5.50 Hz), 3.300–3.000 (9H, m), 2.437 (2H, t, J = 5.89 Hz), 1.452 (9H, s), 1.440 (9H, s), 0.896 (3H, d, J = 6.60 Hz), 0.863 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.60 Hz), 0.785 (3H, s), 0.643 (3H, s), 1.975–0.607 (total 37H, m). FABMS: 789 (MH^+)

Triamine–cholestane conjugate (2). Deprotonation of the Boc group of **6** was carried out in a similar manner of **1**. Yield: 73%. The product was purified with HPLC [retention time: 8.21 min (ODC analytical column: 0.1% TFA–H_2O:0.08% TFA–CH_3CN = 10:90)]. ^{1}H NMR: 3.712 (2H, m), 3.480 (2H, m), 3.257 (1H, m), 2.857–2.802 (6H, m), 2.455 (2H, t, J = 5.87 Hz), 0.897 (3H, d, J = 6.42 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.60 Hz), 0.783 (3H, s), 0.645 (3H, s), 1.975–0.607 (total 37H, m). FABMS: 588 (M^+), 589 (MH^+).

***N*,*N*′,*N*″-Tri-Boc tetramine–cholestane conjugate (7).** The *N*-Boc protected amide **7** was prepared from **27** and **21** in a similar manner of **5** (59% yield). Colorless liquid. ^{1}H NMR: 6.998 (1H, br s), 4.632 (1H, br s), 3.711 (2H, t, J = 5.87 Hz), 3.258–3.135 (13H, m), 2.435 (2H, t, J = 5.87 Hz), 1.455 (9H, s), 1.447 (9H, s), 1.438 (9H, s), 0.896 (3H, d, J = 6.60 Hz), 0.863 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.60 Hz), 0.785 (3H, s), 0.643 (3H, s), 1.974–0.586 (total 39H, m). FABMS: 946 (MH^+).

Tetramine–cholestane conjugate (3). The amine conjugate **3** was prepared from **7** in a similar manner of **1** (60% yield). Pale yellow oil. The crude product was purified by HPLC [retention time: 6.48 min (ODS analytical column: 0.1% TFA–H_2O: 0.08% TFA–CH_3CN = 10:90)]. ^{1}H NMR: 6.885 (1H, br s), 3.704 (2H, t, J = 5.04 Hz), 3.326 (1H, m), 3.257 (1H, m), 2.863 (2H, t, J = 6.23 Hz), 2.769 (6H, m), 2.676 (2H, t, J = 6.32 Hz), 2.434 (2H, t, J = 5.96 Hz), 0.897 (3H, d, J = 6.60 Hz), 0.864 (3H, d, J = 6.60 Hz), 0.859 (3H, d, J = 6.42 Hz), 0.789 (3H, s), 0.645 (3H, s), 1.978–0.592 (total 31H, m). FABMS: 646 (MH^+).

***N*-Boc-diamine–cholestane conjugate (28).** The amide **28** was prepared from **27** and **23** in a similar manner of **5** (82% yield). Mp 80.3–81.9 °C (recrystallized from CH_2Cl_2/*n*-hexane, colorless cubes). ^{1}H NMR: 6.468 (1H, m), 4.498 (1H, br s), 3.693 (2H, t, J = 6.23 Hz), 3.254 (1H, m), 3.229 (2H, q, J = 5.68 Hz), 3.091 (2H, m), 2.429 (2H, t, J = 5.50 Hz), 1.443 (9H, s), 0.897 (3H, d, J = 6.42 Hz), 0.859 (3H, d, J = 6.42 Hz), 0.795 (3H, s), 0.647 (3H, s), 1.949–0.620 (total 43H, m). FABMS: 687 (MH^+). HRMS (FAB^+, MH^+): calcd for $C_{43}H_{79}N_2O_4$: 687.6030. Found: 687.6019. Anal. calcd for $C_{43}H_{78}N_2O_4 + 1/4\ H_2O$: C, 74.67; H, 11.44; N, 4.05. Found: C, 74.64; H, 11.40; N, 4.15.

Diamine–cholestane conjugate (4). The conjugate **4** was prepared from **28** (86% yield). Further purification was performed on HPLC (retention time: 26.50 min (ODC analytical column: 0.1% TFA–99.9%H_2O:0.08% aqueous TFA–CH_3CN = 10:90)). ^{1}H NMR: 7.542 (2H, br s), 7.113 (1H, br s), 3.627 (3H, br m), 3.192 (2H, br s), 2.881 (2H, br s), 2.448 (2H, br s), 0.896 (3H, d, J = 5.68 Hz), 0.860 (3H, d, J = 6.23 Hz), 0.787 (3H, s), 0.645 (3H, s), 1.976–0.645 (total 46H, m). FABMS: 587 (MH^+); HRMS (FAB^+, MH^+): calcd for $C_{39}H_{71}N_2O_2$: 587.5515. Found: 587.5510.

3-*O*-acetyl lithocholic acid (30). A solution of lithocholic acid (**29**) [1.00 g (2.66 mmol)] and 5.0 mL of acetic anhydride in 5.0 mL of pyridine was stirred at ambient temperature for 72 h. The whole was poured into 100 mL of water, followed by addition of saturated aqueous $NaHCO_3$. The whole was extracted with $CHCl_3$ (30 mL × 3). The organic layer was washed with 1N aqueous HCl and with water, and was dried over magnesium sulfate. The organic solvent was evaporated to give 1.07 g (97% yield). Mp 50.5–52.5 °C (recrystallized from ethanol; colorless plates); ^{1}H NMR: 4.720 (1H, m), 2.031 (3H, s), 0.927 (3H, s), 0.926 (3H, d, J = 5.50 Hz), 0.649 (3H, s), 2.401–0.996 (total 32 H, m). Anal. calcd for $C_{25}H_{42}O_4 + 1/4\ C_2H_5OH$: C, 73.81; H, 10.12; N, 0.00. Found: C, 73.65; H, 10.40; N, 0.00.

***N*-Boc-Diamine-3-acetyl-lithocholic acid conjugate (31).** To a stirred mixture of **30** (203.0 mg [0.486 mmol)], **14** (94.0 mg (1.0 equiv)) and NHS [64.5 mg (1.1 equiv)] in

3.0 mL of CH_2Cl_2, DCC 102.5 mg (1.0 equiv) was added, and the whole was stirred at ambient temperature for 24 h. After removal of DCurea by filtration with suction, the filtrate was washed with saturated aqueous sodium bicarbonate, water and brine, and was dried over sodium sulfate. The residue obtained after evaporation was flash-chromatographed (*n*-hexane: AcOEt = 2:1, and then 3:2) to give **31** (253.6 mg (89% yield)). Mp 40.5–42.4 °C (recrystallized from CH_2Cl_2/*n*-hexane). ^{1}H NMR: 5.653 (1H, br s), 4.720 (1H, m), 4.598 (1H, br s), 3.495 (1H, d, J = 4.77 Hz), 3.267 (2H, q, J = 5.87 Hz), 3.137 (2H, d, J = 6.05 Hz), 2.233 (1H, m), 2.047 (1H, m), 2.033 (3H, s), 1.455 (9H, s), 0.925 (3H, s), 0.917 (3H, d, J = 6.05 Hz), 0.639 (3H, s), 1.977–0.957 (total 35 H, m). FABMS: 589 (MH^+). Anal. calcd for $C_{35}H_{60}N_2O_5 + 3/4\ CH_2Cl_2$: C, 65.80; H, 9.50; N, 4.29. Found: C, 65.97; H, 9.49; N, 4.01.

Diamine-3-acetyl-lithocholic acid conjugate (8). Under Ar atmosphere, a solution of **31** [72.2 mg (0.123 mmol)] in 2.0 mL of TFA was stirred at ambient temperature for 3.5 h. The acid was evaporated to give the residue which was flash-chromatographed ($CHCl_3$:MeOH = 9:1, and then 8:2), followed by freeze-drying in vacuum to give **8** [60.8 mg (82% yield)] as colorless liquid. Further purification was performed on HPLC (retention time 10.36 min (analytical column: 0.1% TFA–H_2O:0.08% TFA–CH_3CN = 40:60)). ^{1}H NMR (CD_3OD): 4.854 (1H, m), 3.194 (2H, t, J = 6.87 Hz), 2.936 (2H, t, J = 7.42 Hz), 2.213 (1H, m), 2.107 (1H, m), 1.997 (3H, s), 0.963 (3H, s), 0.961 (3H, d, J = 6.42 Hz), 0.688 (3H, s), 2.073–0.953 (total 34 H, m). FABMS: 490 (MH^+).

***N*,*N*′-diBoc-triamine-3-acetyl-lithocholic acid conjugate (32).** The *N*-Boc protected conjugate **32** was prepared from **29** and **18** in a similar manner of **31**. Yield: 89%. Colorless liquid. ^{1}H NMR: 6.724 (1H, brs), 4.179 (1H, m), 4.548 (1H, brs), 3.277 (2H, br s), 3.204 (2H, br), 3.140 (4H, m), 2.248 (1H, m), 2.063 (1H, m), 2.030 (3H, s), 1.463 (9H, s), 1.441 (3H, s), 0.925 (3H, s), 0.924 (3H, d, J = 6.23 Hz), 0.639 (3H, s), 1.980–1.027 (total 32H, m). FABMS: 746 (MH^+). HRMS (FAB^+, MH^+): calcd for $C_{43}H_{76}N_3O_7$: 746.5729. Found: 746.5706.

Triamine-3-acetyl-lithocholic acid conjugate (9). The polyamine conjugate **9** was prepared from **32** through deprotection of the Boc group in TFA. Yield: 58% yield. Colorless liquid. Further purification was performed on HPLC [retention time 4.89 min (ODS analytical column: 0.1% TFA–H_2O: 0.08% TFA–CH_3CN = 40:60)]. ^{1}H NMR (CD_3OD): 4.670 (1H, m), 3.282 (2H, t, J = 6.42 Hz), 2.995 (6H, m), 2.286 (1H, m), 2.251 (1H, m), 1.998 (3H, s), 0.966 (3H, d, J = 6.42 Hz), 0.963 (3H, s), 0.690 (3H, s), 2.034–0.958 (total 36 H, m). FABMS: 546 (MH^+). HRMS (FAB^+, MH^+): calcd for $C_{33}H_{60}N_3O_3$: 546.4634. Found: 546.4611.

Methyl lithocholate (33). A mixture of lithocholic acid [(**29**) 4.01 g (10.7 mmol)] and 4.05 mL of concd sulfuric acid in 30 mL of methanol was stirred at ambient temperature for 1 h. After evaporation of methanol, the residue was dissolved in 60 mL of $CHCl_3$, and the organic layer was washed with water (40 mL×2), and was dried over magnesium sulfate. The organic solvent was evaporated to give the crude **33** (4.08 g (98%)). Mp 129.0–130.4 °C (recrystallized from AcOEt, colorless needles). ^{1}H NMR: 3.664 (3H, s), 3.624 (1H, m), 2.354 (1H, m), 2.327 (1H, m), 0.917 (3H, s), 0.907 (3H, d, J = 4.03 Hz), 0.641 (3H, s), 1.974–0.970 (total 26 H, m). FABMS: 390 (M^+). Anal. calcd for $C_{25}H_{42}O_3$; C, 76.87; H, 10.84; N, 0.00. Found: C, 76.77; H, 10.92; N, 0.00.

Methyl 3-*O*-allyl-lithocholate (34). A solution of methyl lithocholate [(**33**) 4.68 g (12.0 mmol)] and *N*,*N*-diisopropylethylamine 4.66 g (3.0 equiv) in 40 mL of dry DMF was heated to reflux. After reflux for 30 min, allyl bromide (5.0 mL) was added to this solution over 5 min. The whole was refluxed for 9 h. The whole was poured into 30 mL of H_2O, and was acidified with 1 N aqueous HCl. The mixture was extracted with Et_2O (30 mL×3) and the organic layer was washed with brine, and was dried over magnesium sulfate. Evaporation of the solvent gave the residue which was flash-chromatographed (*n*-hexane:AcOEt = 30:1) to give **34** (3.72 g (72% yield) as pale yellow solid. Mp 76.0–77.9 °C (recrystallized from methanol, colorless plates). ^{1}H NMR: 5.936 (1H, m), 5.275 (1H, dd, J = 1.23, 17.41 Hz), 5.155 (1H, d, J = 10.26 Hz), 4.019 (2H, d, J = 5.50 Hz), 3.664 (3H, s), 3.306 (1H, m), 2.349 (1H, m), 2.214 (1H, m), 0.910 (3H, s), 0.903 (3H, d, J = 5.50 Hz), 0.632 (3H, s), 1.956–0.957 (total 26H, m). FABMS: 429 ($M-1^+$). Anal. calcd for $C_{28}H_{46}O_3$: C, 78.09; H, 10.77; N, 0.00. Found: C, 77.88; H, 10.72; N, 0.00.

Methyl 3-*O*-propyl-lithocholate (35). The allyl group of **34** was hydrogenated over 5% Pd-C in ethyl acetate at ambient temperature for 8 h. Yield: 93%. Mp 60.3–62.1 °C (recrystallized from MeOH, colorless plates). ^{1}H NMR: 3.663 (1H, m), 3.411 (2H, t,d, J = 2.02, 6.97 Hz), 3.224 (1H, m), 2.353 (1H, m), 2.213 (1H, m), 1.579 (2H, q, J = 7.33 Hz), 0.918 (3H, t, J = 7.33 Hz), 0.909 (3H, s), 0.903 (3H, d, J = 5.32 Hz), 0.632 (3H, s), 1.953–0.895 (total 26 H, m). FABMS: 431 ($M-1^+$). Anal. calcd for $C_{28}H_{48}O_3$: C, 77.72; H, 11.18; N, 0.00. Found: C, 77.64; H, 11.31; N, 0.00.

3-*O*-Propyl-lithocholic acid (36). A mixture of **35** (1.50 g (3.47 mmol)), 18-crown-6 (700.0 mg) and 1 N aqueous KOH solution in 30 mL of dioxane was heated at 40 °C for 19 h. The whole was diluted with 50 mL of water (50 mL), acidified with 1 N aqueous HCl solution, and extracted with diethyl ether (30 mL×3). The organic layer was washed with water, and with brine, and was dried over sodium sulfate. Evaporation of the organic solvent gave the solid **36** (1.41 g, 97% yield). Mp 126.1–128.0 °C (recrystallized from methanol, colorless plates). ^{1}H NMR: 3.415 (2H, t,d, J = 2.02, 6.97 Hz), 3.229 (1H, m), 1.581 (2H, q, J = 7.15 Hz), 0.918 (3H, t, J = 7.33 Hz), 0.918 (3H, d, J = 6.42 Hz), 0.910 (3H s), 0.637 (3H, s), 1.958–0.884 (total 27 H, m). Anal. calcd for $C_{27}H_{46}O_3$: C, 77.46; H, 11.07; N, 0.00. Found: C, 77.19; H, 10.96; N, 0.00.

***N*-Boc-Diamine-3-*O*-propyl lithocholic acid conjugate (37).** A mixture of **36** [209.0 mg (0.50 mmol)], and **14** (94.0 mg (1.0 equiv)), NHS [64.5 mg (1.1 equiv)] and

DCC 102.8 mg (1.0 equiv) in methylene chloride was stirred at ambient temperature for 18 h. After addition of another portion of DCC (30.2 mg), the whole was stirred for another 42 h. After removal of DCurea by filtration with suction, the organic layer was washed with saturated aqueous sodium bicarbonate, water and brine, and was dried over sodium sulfate. The residue obtained after evaporation was flash-chromatographed (*n*-hexane:AcOEt = 3:2, and then 2:3) to give **37** (282.1 mg (96%)) as colorless liquid. ^{1}H NMR: 5.657 (1H, br s), 4.599 (1H, br s), 3.412 (2H, t,d, J = 1.65, 6.97 Hz), 3.265 (2H, d, J = 5.87 Hz), 3.241 (1H, m), 3.135 (2H, br d, J = 5.31 Hz), 2.219 (1H, m), 2.043 (1H, m), 1.580 (2H, quartet, J = 7.15 Hz), 1.444 (9H, s), 0.918 (2H, t, J = 7.33 Hz), 0.913 (3H, d, J = 4.22 Hz), 0.908 (3H, s), 0.629 (3H, s), 1.953–0.908 (total 32H, m). FABMS: 589 (MH^+). HRMS (FAB^+, MH^+): calcd for $C_{36}H_{65}N_2O_4$: 589.4944. Found: 589.4957.

Diamine-3-O-propyl lithocholic acid conjugate (10). Under Ar atmosphere, a solution of **37** [100.1 mg (0.170 mmol)] in 2.0 mL of TFA was stirred at ambient temperature for 1 h. The acid was evaporated to give the residue which was flash-chromatographed ($CHCl_3$:MeOH = 9:1), followed by freeze-drying in vacuum to give **10** [90.2 mg (88%)] as colorless liquid. Further purification was performed with HPLC (Retention Time: 8.95 min (ODS analytical column: 0.1% TFA–H_2O: 0.08% TFA–CH_3CN = 40:60). ^{1}H NMR: 7.680 (2H, br s), 6.890 (1H, brs), 3.477 (2H, t, J = 6.96 Hz), 3.38 (1H, m), 3.266 (2H, br s), 3.042 (2H, br s), 2.311 (1H, m), 2.111 (1H, m), 1.789 (2H, q, J = 7.14 Hz), 0.913 (3H, t, J = 7.42 Hz), 0.913 (3H, s), 0.889 (3H, d, J = 4.22 Hz), 0.622 (3H, s), 1.945–0.884 (total 30H, m). FABMS: 489 (MH^+).

***N*,*N*′-diBoc-Triamine-3-*O*-propyl lithocholic acid conjugate (38).** The *N*-Boc protected amine conjugate **38** was prepared from **36** and **18** in a similar manner of **37**. Yield: 66%. Colorless liquid. ^{1}H NMR: 6.718 (1H, brs), 4.543 (1H, brs), 3.411 (2H, t, d, J = 6.78, 2.20 Hz), 3.215–3.125 (9H, m), 1.464 (9H, s), 1.411 (9H, s), 0.918 (3H, t, J = 7.33 Hz), 0.922 (3H, d, J = 6.78 Hz), 0.908 (3H, s), 0.629 (3H, s), 1.975–0.900 (total 36H, m). FABMS: 746 (MH^+).

Triamine-3-*O*-propyl lithocholic acid conjugate (11). Deprotection of the Boc group of **38** was carried out in a similar manner of **37** to give **11**. Yield: 84%. Colorless liquid. Further purification was performed with HPLC [retention time 14.37 min (ODS analytical column: 0.1% TFA–H_2O: 0.08% TFA–CH_3CN = 40:60)]. ^{1}H NMR: 6.736 (1H, t, J = 5.02 Hz), 3.411 (2H,td, J = 2.20, 6.90 Hz), 3.355 (2H, q, J = 6.42 Hz), 3.225 (1H, m), 2.848 (4H, m), 2.800 (2H, t, J = 6.05 Hz), 2.232 (1H, m), 2.082 (1H, m), 1.580 (2H, q, J = 6.70 Hz), 0.918 (3H,t, J = 7.33 Hz), 0.913 (3H, d, J = 4.03 Hz), 0.908 (3H, s), 0.628 (3H, s), 1.955–0.908 (total 35 H, m). FABMS: 546 (MH^+). HRMS (FAB^+, MH^+): calcd for $C_{34}H_{64}N_3O_2$: 546.4998. Found: 546.4976.

Triamine–lithocholic acid conjugate (13). Alkaline hydrolysis of the acetyl group of **32**, followed by deprotection of the Boc group in TFA gave **13**. Mp 93.5–97.1 °C. ^{1}H NMR($CDCl_3 + D_2O$): 3.647–3.575 (1H, m), 3.337 (2H, t, J = 6.23 Hz), 2.717 (4H, t, J = 6.23 Hz), 2.625 (2H, t, J = 6.23 Hz), 2.252–2.175 (1H, m), 2.087–2.010 (1H, m), 1.971–0.916 (total 38H, m), 0.640 (3H, s). HRMS (ESI^+, MH^+): calcd for $C_{31}H_{58}N_3O_2$: 504.4529. Found: 504.4510.

Biological materials

Triton X-100 was purchased from Sigma (St. Louis, MO, USA). Spermidine was purchased from Aldrich (Milwaukee, WI, USA), and was used without further purification. Bovine erythrocytes were purchased from Nippon Bio-Supply Center (Japan).

Hemolytic activity towards bovine erythrocytes

Bovine erythrocytes were centrifuged at 800×*g* (2200 rpm) for 10 min and the plasma and buffy coat were discarded. To the precipitated cells was added phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO4{\cdot}12H_2O$, 1.47 mM KH_2PO_4; pH:7.4), followed by centrifugation to collect the cells. This process was repeated twice. The cells were suspended in PBS to obtain 1×10^8 cells/mL of erythrocyte suspension. To 1-mL aliquots of the above suspension, 1 μL of 0.1, 1,10, 50 or 100 mM solution of the compound in dimethylsulfoxide (DMSO; the final concentration of DMSO was 0.1% v/v) was added, and the whole was mixed gently by using a vortex mixer, then the whole was incubated for 60 min at 37 °C. The supernatant, after centrifugation at 800×*g* (2200 rpm) for 5 min, was collected. The absorbance (I_x) at 576 nm of an aliquot of the supernatant (750 μL) was measured with a spectrophotometer U-2000 (Hitachi; Tokyo, Japan). The absorbance of the supernatant of the cell suspension treated with vehicle only (0.1% (v/v) DMSO) was used as a control value ($I_{control}$). The intensity of absorption corresponding to 100% hemolysis (I_{100}) was determined by adding 50 μL of a 10% (v/v) aqueous solution of Triton X-100 to a 1-mL aliquot of the cell suspension in each hemolysis experiment. The percentage hemolysis was then obtained according to eq 1:

$$\text{hemolysis } \% = 100\ (I_x - I_{control})/\ (I_{100} - I_{control}) \qquad (1)$$

No significant hemolysis was observed when the cells were incubated with 0.1% (v/v) DMSO. All absorption measurements were carried out at ambient temperature. Non-linear curve fitting of concentration (x)−percent hemolysis (y) curves (Figs 1–3) was carried out with the assumption of the relation $y = 100/(1 + EC_{50}/x)$ wherein EC_{50} is an effective concentration that induces 50% hemolysis under the present experimental conditions. Time-dependent hemolysis curves (Fig. 4 B,C) were also fitted non-linearly with the assumption of the relation % hemolysis (y) = $y_{max}(1-e^{-kt})$ wherein t represents time (min). Fitting was carried out with PRISM (GraphPad, CA, U.S.A).

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