Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2897–2901

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Anchoring and Bola Cationic Amphiphiles for Nucleotide Delivery. Effects of Orientation and Extension of Hydrophobic Regions

Tomonobu Yoshimura,[a] Seiji Hasegawa,[a] Naohide Hirashima,[a] Mamoru Nakanishi[a] and Tomohiko Ohwada[b,*]

[a]*Graduate School of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan*
[b]*Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

Received 27 June 2001; accepted 7 August 2001

Abstract—Novel cationic amphiphiles, based on lithocholic acid derivatives with two structural motifs, anchoring lipids and bola lipids, were designed and synthesized. Both bear extended hydrophobic space-filling substituents. A significant effect of the orientation and extension of hydrophobic regions around the ether linkage at the 3-position was found on the efficiency of DNA delivery.

There is considerable interest in novel self-assembling systems based on liposomes as carriers of pharmaceutical agents[1] and for targeted DNA delivery[2–4] aimed at replacing a defective or adding a missing gene.[5] Although the potential of cationic liposomes as DNA delivery vehicles has been demonstrated, more effective and less cytotoxic cationic liposomes are needed.[6–8] Cationic liposomes consist of binary mixtures of lipids that contain DOPE (dioleoyl-phosphatidylethanolamine) as a neutral co-lipid and a cationic lipid (cationic amphiphile).[9] The process of lipid-based DNA delivery is proposed to involve multiple steps, such as DNA condensation due to electrostatic interaction with cationic liposomes, cell targeting, endosomolysis and nuclear translocation.[2,3] The physico-chemical nature and biological behavior of DNA–cationic liposome complexes are not well defined.[10,11] A recent structural study suggested a multilamellar structure with alternating lipid bilayer and DNA monolayer.[12] Therefore, the structures of the lipid bilayers of the cationic liposomes are probably conserved upon complexation with DNA. The width of the bilayer is estimated to be 36 Å.[12] In this paper, we describe the design and synthesis of novel cationic amphiphiles based on lithocholic acid derivatives with two structural motifs, anchoring lipids and bola lipids,[13] both of which bear extended hydrophobic space-filling substituents (Chart 1). We found a significant effect of the orientation and extension of hydrophobic regions around the ether linkage at the 3-position on the efficiency of DNA delivery.

Among synthetic DNA delivery agents, cationic lipid based on the cholesterol structure (e.g., 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] chlolesterol (DC-Chol, **1**)) is a representative commercially available example (Chart 2).[14] The hydrophobic moiety of **1** is cholesterol, and the hydrophilic region is polyamine. Our previous study highlighted the effects of the structure of the steroid moiety on the efficiency of DNA delivery.[15] That is, comparisons were made between cholestane-based and lithocholic acid-based cationic amphiphiles (cholestane has a *trans*-decalin structure and lithocholic acid, a *cis*-decalin structure). Lithocholic acid-polyamine conjugate (e.g., **2b** and **2c**) showed

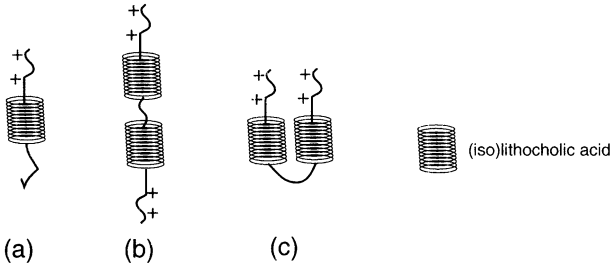

Chart 1. Anchoring (a) cationic lipids and (b,c) bola cationic lipids.

*Corresponding author. Tel.: +81-3-5841-4730; fax: +81-3-5841-4735; e-mail: ohwada@mol.f.u-tokyo.ac.jp

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00563-7

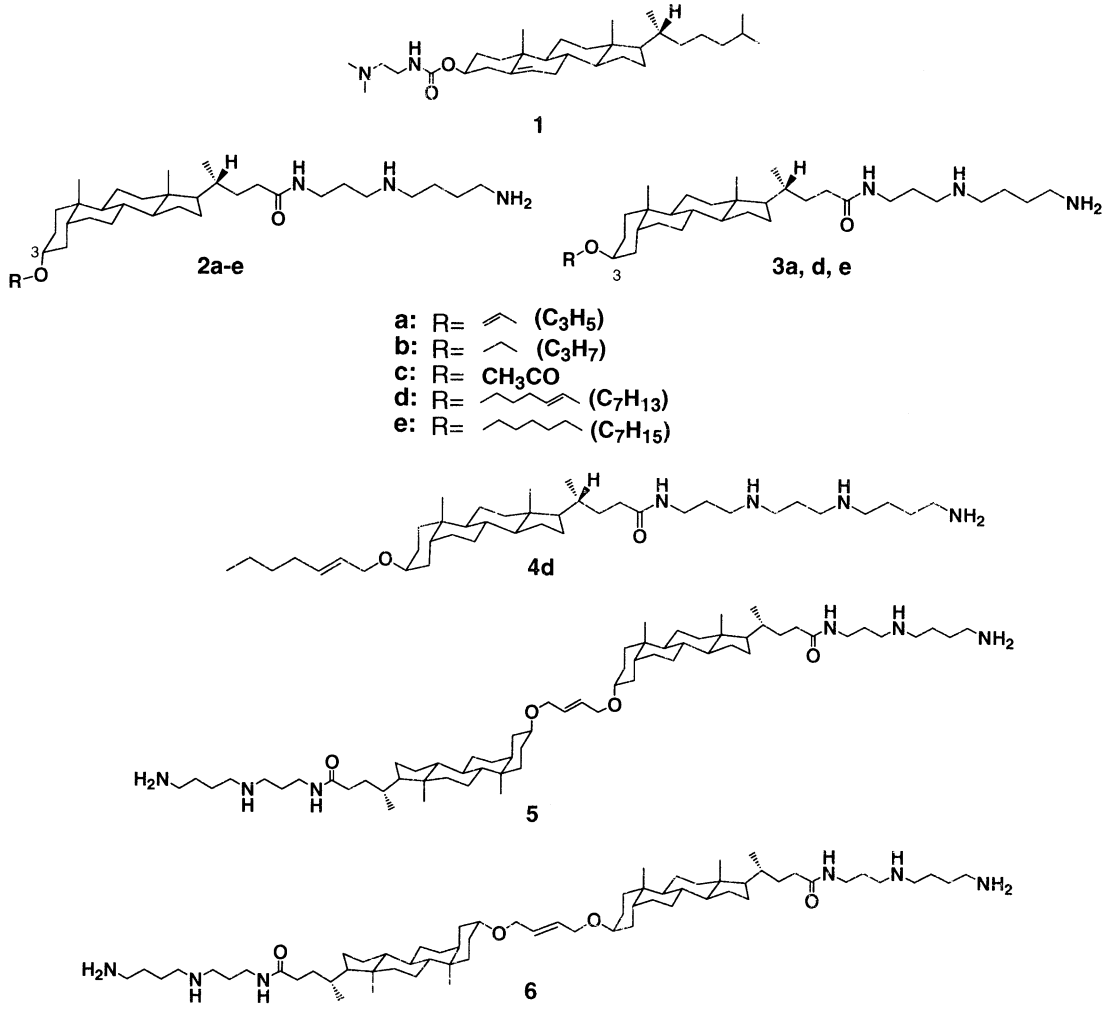

Chart 2. Cationic amphiphiles in this study.

transfection activity much lower than that of the corresponding cholestane–polyamine conjugate, though the polyamine chains are identical.[15] These results suggested a significant effect of the shape of the steroid moiety upon gene transfection activity.

In fact, the *O*-allyl lithocholic acid derivative **2a** showed significantly reduced gene transfection activity as compared with that of DC-Chol **1** (Fig. 1). Cationic liposomes prepared by mixing lithocholic acid–polyamine conjugate and DOPE in a mol ratio of 2:3 were used to transfect NIH3T3 cells with plasmid pGL3 (encoding luciferase). An excess amount of the liposomes with respect to the plasmid DNA was used,[15] that is, 50 nmol of the liposomes with 5.4 μg of plasmid DNA, which corresponds to a charge ratio (±) of 2.4 when the two amino nitrogen atoms of the amphiphile are fully protonated.[16,17] These conditions are similar to those described previously.[15,18] The efficiency of the gene transfection is represented in terms of relative light unit (RLU) per mg protein in luciferase assay. When the appendant R group is a heptenyl group (**2d**) or a heptanyl group (**2e**), the gene transfection activity is apparently increased as compared with the short-chain analogue **2a** (Fig. 1).[19,20]

On the other hand, the stereochemistry of the hydrophobic appendant at the 3-hydroxyl group also has a significant effect on the gene transfection activities. Although the *O*-allyl counterpart of the isolithocholic acid derivative **3a** is still an inefficient vehicle for DNA (Fig. 1), the heptenyl (**3d**) and heptanyl (**3e**) analogues show a higher activity than **1** (DC-Chol) or the corresponding isomers **2d** and **2e** (Fig. 2).[19,20]

The additional hydrophobic region at the 3-hydroxy group may enforce hydrophobic interaction in the assembly, resulting in anchoring of the amphiphiles to the bilayer [Chart 1 (a)].

The previous study showed that an optimal chain length of the polyamine exists in a series of cholestane-polyamine conjugates,[15] although increasing the number of basic amine nitrogen atoms seemingly would enhance electrostatic attraction upon protonation with DNA. The liposomes derived from the analogue **4d** with a longer polyamine chain showed increased transfection activity as compared with **3d** (Fig. 2).

In order to expand the hydrophobic region of the cationic amphiphiles we designed bola cationic amphiphiles,[13]

which are bipolar double-headed lipids (Chart 1). The hydrophobic region consists of homodimers of lithocholic acid (**5**) and isolithocholic acid (**6**) (Chart 2).[19,20] In the gene transfection assay, the effect of composition of cationic liposomes was examined using cationic lipid **5**. A mixture of liposomes (50 nm, a mixture of **5** and DOPE) and 5.4 μg of plasmid DNA was used. Thus, the ratio of liposomes/DNA was fixed. The mol ratio of **5**/DOPE in the liposomes was changed to 5:45, 10:40, 20:30, 30:20 and 40:10 (Fig. 3). The optimal ratio of **5**/DOPE seems to be 30:20. Furthermore, the liposome/DNA ratio was changed. The amount of liposomes was set at 12.5, 25, 50 and 100 nmol with a fixed amount of 5.4 μg of plasmid DNA (data not shown). The optimal amount of the liposomes is 25 nmol. In terms of cytotoxicity of the cationic liposomes/DNA complex, the present optimal composition is satisfactory (Fig. 4). Viability of NIH3T3 cells in the presence of liposome/DNA complexes was evaluated by using trypan blue exclusion assay. Under these optimal conditions the gene transfection activities of **5** and **6** were examined. The cationic liposomes prepared from **5** or **6** and DOPE showed transfection activity, and the efficiency seemed to be comparable to that of **1** (DC-Chol) (Fig. 5). In particular the homodimer **5** showed apparent transfection activity in contrast to the monomer **2a**, which is inferior to **1** (DC-Chol) (see Fig. 1). Therefore, the present work showed that bola structures can serve as a structure motif for building of novel cationic lipids for gene delivery. Molecular modeling suggested that if the extended conformation is assumed, the hydrophobic region of **5** and **6** is 30 Å long, which is comparable to

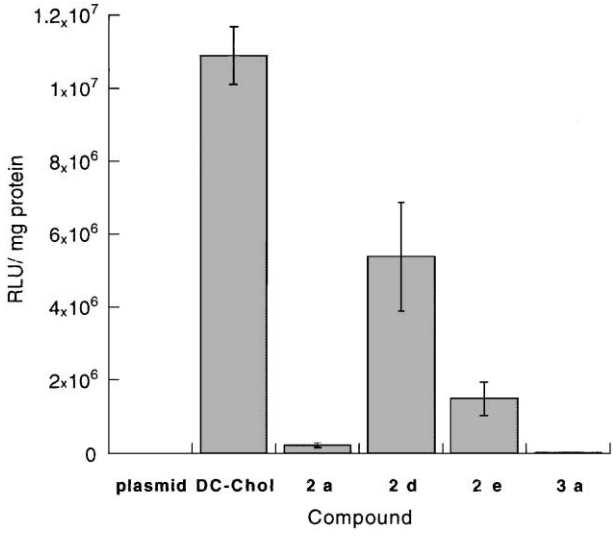

Figure 1. Transfection activity of short-appendant derivative determined by luciferase assay. Transfection activity without lipids is shown as 'plasmid'. Each value is the mean ± SE ($n = 3$).

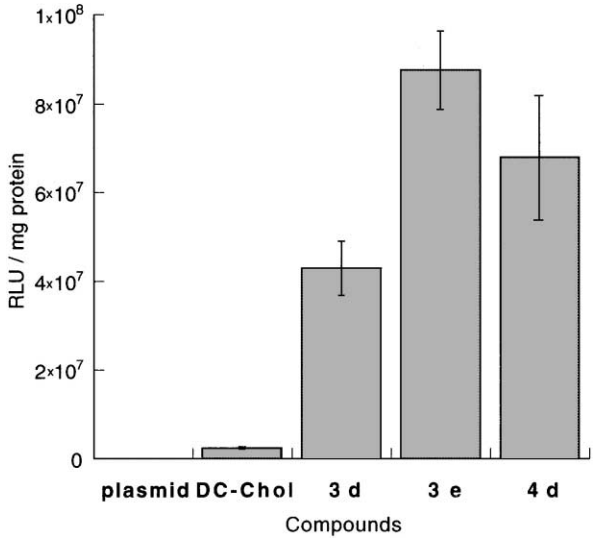

Figure 2. Transfection activities of cationic liposomes based on isolithocholic acid derivatives. Transfection activity without lipids is shown as 'plasmid'. Each value is the mean ± SE ($n = 3$).

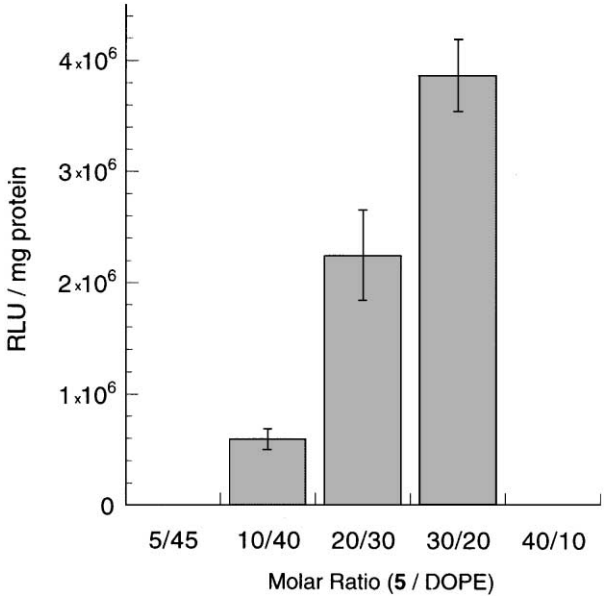

Figure 3. Effects of the composition of cationic liposomes on transfection activity. The dose of the lipids was fixed at 50 nmol. The molar ratio of **5** and DOPE was changed. Each value is the mean ± SE ($n = 3$).

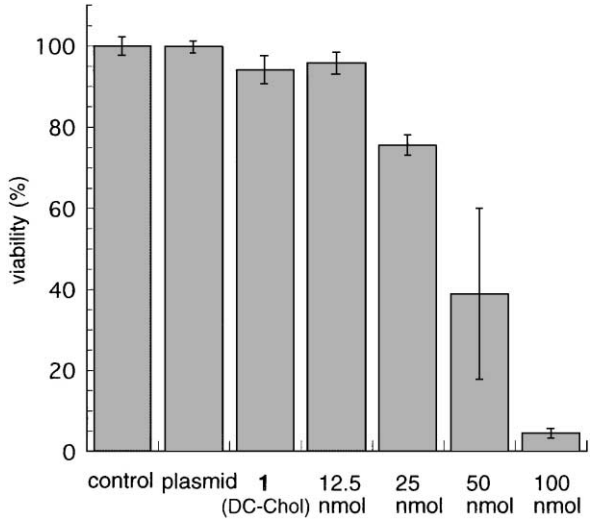

Figure 4. Cytotoxicity of liposome–plasmid DNA complex derived from **5**. The amount of the liposomes was changed with a fixed amount of 5.4 μg of plasmid DNA. Liposomes derived from **1** (DC-Chol) were used in 50 nmol to the same amount of the plasmid DNA. Each value is the mean ± SE ($n = 3$).

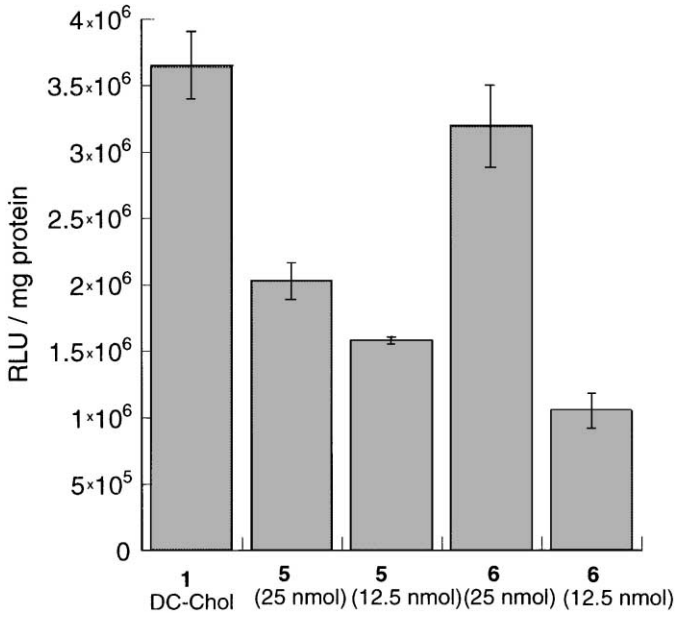

Figure 5. Transfection activity of the liposomes derived from bola amphiphiles **5** and **6** determined by luciferase assay. Luciferase activities at different doses of the liposomes (25 and 12.5 nmol) were measured and compared to that using DC-Chol (50 nmol liposomes). Each value is the mean ± SE ($n = 3$).

the width of lipid bilayers. Thus, a membrane-spanning arrangement [Chart 1 (b)] of **5** and **6**, as well as a bending conformation [Chart 1(c)], is possible.[21] This idea will be examined in due course.

In summary, two new types of structures of cationic amphiphiles, anchoring lipids and bola lipids were shown to be potent motifs for the design of highly efficient gene delivery agents. Changes of stereochemistry in the hydrophobic regions, leading to change of space-filling of hydrophobic moieties, can modify the nature of the assembling system, such as cationic liposomes and liposomes/DNA complex, resulting in a significant impact on the transfection efficiency.

Assay

Materials

3β-[*N*-(Dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) were purchased from Sigma (St. Louis, MO, USA). Plasmid DNA (pGL3) was purchased from Promega (Madison, WI, USA).

Gene transfection activities

Liposome preparation. A solution of DOPE in chloroform was added to a solution of a steroid–polyamine conjugate[19,20] in chloroform to give a mixture of DOPE/synthetic conjugate in a mol ratio of 3:2. The solvent was evaporated under reduced pressure to give a thin film of lipids. To this lipid film, phosphate-buffered saline (PBS) was added, and the sample was allowed to stand for 60 min under protection from sunlight. Then the samples were sonicated by using a bath-type sonicator (model 5210J, Branson) for 2–3 min, followed by a sonication with a probe-type sonicator (Sonifier 250, Branson) for 10 min to give small unilamellar vesicles (SUVs).

Cell culture and transfection. NIH3T3 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Bio-Whittaker; Walkerville, MD, USA). Plasmid pGL3 DNA (5.4 μg) in TE buffer (the stock solution of the DNA was 1 mg/mL) was complexed with the above liposomes (prepared from 30 nmol of DOPE and 20 nmol of the synthetic conjugate (in the cases of **1**–**4**) in PBS) in SFM101 (Nissui, Japan) at ambient temperature by pipetting well, and the whole was incubated at 37 °C for 15 min to allow formation of the DNA–liposome complex. In the cases of **5** and **6**, liposomes, prepared from 10 nmol of DOPE and 15 nmol of the synthetic conjugate (**5** or **6**), was treated with 5.4 μg of plasmid pGL3 DNA. Then this DNA–liposome complex was incubated with the cells (1×10^6 cells/dish) in the SFM101 medium in a culture dish (Corning; 60×15 mm) for 4 h at 37 °C. The cells were washed and cultured in growth medium (DMEM) for another 40 h at 37 °C, followed by luciferase assay after cell lysis.

Luciferase assay. The luciferase assay was carried out using a picagene luciferase assay kit (Toyo Ink, Tokyo, Japan). The cells were washed three times with PBS and lysed in a cell lysis buffer for 15 min at room temperature. The lysate was centrifuged at 12,000×*g* at 4 °C for 1 min and the supernatant was subjected to luciferase assay. Light emission was measured with a luminometer (TD-20/20, Turner Designs; Sunnyvale, CA, USA) and normalized to the amount of protein of each sample, determined with BCA assay.

Viability assay. After incubation with liposome/DNA complexes for 4 h, cell suspension was mixed with an equal volume of trypan blue buffer (Trypan Blue Stain 0.4%; Gibco BRL). The number of cells that excluded trypan blue was divided by the total number of cells to calculate the viability.

Acknowledgements

Part of the work was financially supported by Suzuken Memorial Foundation (Nagoya, Japan).

References and Notes

1. Gregoriadis, G. *Trends Biotechnol*. **1995**, *13*, 527.
2. Behr, J.-P. *Acc. Chem. Res*. **1993**, *26*, 274.
3. Miller, A. D. *Angew. Chem., Int. Ed*. **1998**, *37*, 1768.
4. Lasic, D. D.; Templeton, N. S. *Adv. Drug Deliv. Rev*. **1996**, *20*, 221.
5. Crystal, R. G. *Science* **1995**, *270*, 404.
6. Eaton, M. A. W.; Baker, T. S.; Catterall, C. F.; Crook, K.; Macaulay, G. S.; Mason, B.; Norman, T. J.; Parker, D.; Perry, J. J. B.; Taylor, R. J.; Turner, A.; Weir, A. N. *Angew. Chem., Int. Ed*. **2000**, *39*, 4063.
7. Zhu, J.; Munn, R. J.; Nantz, M. H. *J. Am. Chem. Soc*. **2000**, *122*, 2645.
8. Walker, S.; Sofia, M. J.; Kakarla, R.; Kogan, N. A.; Wierichs, L.; Longley, C. B.; Bruker, K.; Axelrod, H. R.; Midha, S.; Babu, S.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 1585.
9. Farhood, H.; Bottega, R.; Epand, R. M.; Hung, L. *Biochim. Biophys. Acta* **1992**, *1111*, 239.
10. Lasic, D. D.; Strey, H.; Stuart, M. C. A.; Podgornik, R.; Frederik, P. M. *J. Am. Chem. Soc*. **1997**, *119*, 832.
11. Bronich, T. K.; Nguyen, H. K.; Eisenberg, A.; Kabanov, A. V. *J. Am. Chem. Soc*. **2000**, *122*, 8339.
12. Rädler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. *Science* **1997**, *275*, 810.
13. Moss, R. A.; Li, J.-M. *J. Am. Chem. Soc*. **1992**, *114*, 9227.
14. Gao, X.; Huang, L. *Biochem. Biophys. Res. Commun*. **1991**, *179*, 280.
15. Fujiwara, T.; Hasegawa, S.; Hirashima, N.; Nakanishi, M.; Ohwada, T. *Biochim. Biophys. Acta* **2000**, *1468*, 396.
16. Stewart, K. D. *Biochem. Biophys. Res. Commun*. **1988**, *152*, 1441.
17. Stewart, K. D.; Gray, T. *J. Phys. Org. Chem*. **1992**, *5*, 461.
18. Takeuchi, K.; Ishihara, M.; Kawaura, C.; Noji, M.; Furuno, T.; Nakanishi, M. *FEBS Lett*. **1996**, *397*, 207.
19. Isolithocholic acid was prepared by Mitsunobu inversion reaction (*p*-nitrobenzonic acid/Ph_3P/DEAD) of the 3-hydroxyl group of lithocholic acid (in a form of methyl ester). The synthesis of *O*-allyl lithocholic acid derivative **2a** was reported previously.[20] Synthesis of **2d**, **2e**, **3d** and **3e** was accomplished by olefin metathesis of **2a** (or **3a**) and 1-hexene in the presence of the ruthenium-alkylidene catalyst developed by Grubbs (see review: Shuster, M.: Blechert, S., *Angew. Chem., Int. Ed. Engl*. **1997**, *36*, 2036). Preparation of polyamines and amide coupling were also described previously.[20] Synthesis of **5** and **6** was accomplished by dimerization of **2a** or **3a** in the olefin metathesis. All new compounds are satisfactory with ^1H NMR, and High-resolution mass spectra (HRMS, EI^+) or FAB mass spectra (FABMS).
20. Fujiwara, T.; Hirashima, N.; Hasegawa, S.; Nakanishi, M.; Ohwada, T. *Bioorg. Med. Chem*. **2001**, *9*, 1013.
21. Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl* **1988**, *27*, 113.