Proc. Indian Acad. Sci. (*Chem. Sci.*), Vol. 114, No. 3, June 2002, pp 197–201

Synthesis of novel cationic lipids with fully or partially non-scissile linkages between the hydrocarbon chains and pseudoglyceryl backbone

SANTANU BHATTACHARYA* and SAUBHIK HALDAR
Department of Organic Chemistry, Indian Institute of Science,
Bangalore 560 012, India
e-mail: sb@orgchem.iisc.ernet.in

MS received 4 April 2002

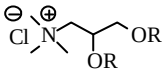
Abstract. Five novel cationic lipids with fully or partially *non-scissile* linkage regions between the pseudoglyceryl backbone and the hydrocarbon chains have been synthesized. The membrane-forming properties of these new lipids are briefly presented.

Keywords. Cationic lipids; non-scissile linkages; glycerol backbone; membranes; gene transfection.

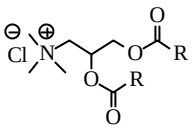
Cationic lipids are attracting a lot of current attention owing to their applications in gene therapy [1,2]. The functional group that links the backbone bearing the polar head group with the hydrocarbon chains of these lipid molecules plays an important role in their utilization in gene transfer events. For instance, DOTMA, which contains a hydrolytically stable *ether* linkage between the head group and the long alkyl chain is shown to have much greater *in vivo* transfection efficiency than the corresponding cationic lipid with an *ester* linkage (DOTAP) [3]. Other instances are also known where the choice of linkage type between the head group with the hydrophobic segment is crucial for achieving efficient transfection [4]. It is, however, not clear why such small difference in the linkage region affects the gene transfer efficiency. One reason for lack of understanding of this difference in transfection activity at molecular level stems from the fact that membrane-forming properties of the cationic lipid molecules that differ at the linkage region are not known. For the last few years, we have been investigating the role of various molecular level modifications on the properties of membranes formed from different lipids [5]. Herein we present a facile route to the synthesis of a novel series of cationic lipids, that possess either fully or partially *non-scissile* linkage regions between the pseudoglyceryl backbone and the hydrocarbon chains. We also briefly report the characterization of the membranes formed from these types of lipids.

The synthesis of these *vicinally anchored*, trimethylammonium lipids, which contain either identical or dissimilar linkages in both the chains at the C-1 and C-2 positions of the pseudoglyceryl backbone, is outlined in scheme 1. Alkylation of diethyl malonate with either *n*-octadecyl bromide or *n*-hexadecyl bromide in dry THF in the presence of NaH under

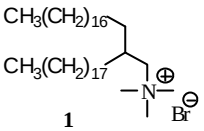
*For correspondence



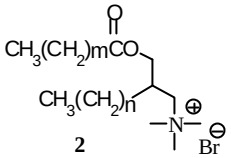
DOTMA: R = *n*-(9*z*)-$C_{18}H_{35}$



DOTAP: R = *n*-(9*z*)-$C_{17}H_{33}$



1

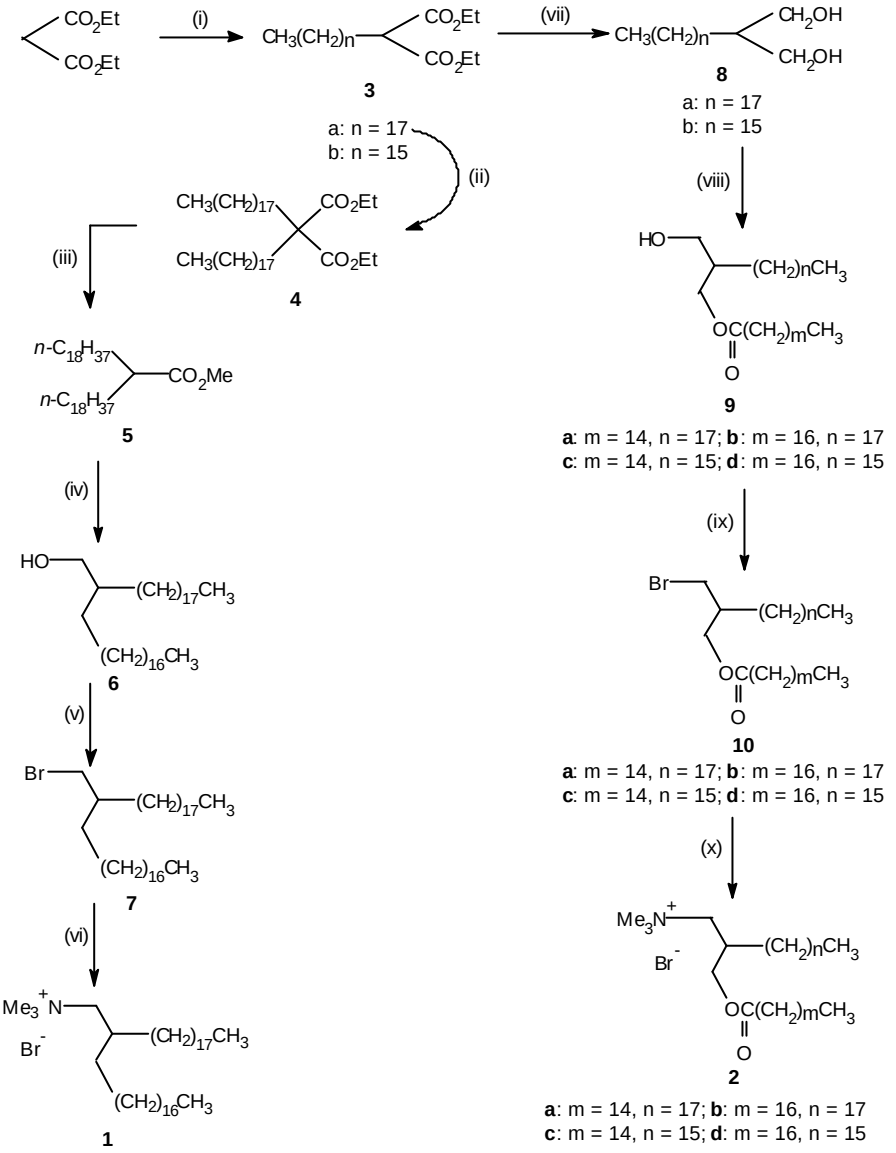


2

a: m = 14, n = 17; **b**: m = 16. n = 17;
c: m = 14, n = 15; **d**: m = 16; n = 15.

inert atmosphere afforded the mono-alkylated diethyl malonate derivatives, **3a**–**b**. These were isolated in reasonably good yields upon purification by gravity-driven column chromatography over silica gel. One part of *n*-octadecyl-diethyl malonate, **3a** was then subjected to a second alkylation using *n*-octadecyl bromide in dry toluene in the presence of NaH under refluxing conditions. Upon purification over silica gel column, the *bis*-octadecyl-diethyl malonate, **4** was isolated as a waxy solid in ~72% yield. The diester, **4** was then saponified under reflux in a mixed solvent system of MeOH–THF–H_2O using KOH. The solvent from the resulting mixture was evaporated and the residue was acidified with 6N HCl and taken up in $CHCl_3$. Organic layer from this mixture was separated, dried over anhydrous Na_2SO_4 and concentrated to produce a residue. This neat mass was subjected to a ***b***-ketoacid decarboxylation upon heating at 150°C for 16 h. Then the resulting material was converted to the corresponding methyl ester, **5** and isolated in 66% yield upon column chromatography over silica gel. The ester, **5** was reduced with $LiAlH_4$ in dry THF to furnish the corresponding alcohol, **6** in 88% yield. The bromination of **6** under Appel conditions (CBr_4/PPh_3 in CH_2Cl_2) afforded the bromide, **7** in 94% isolated yield. The resulting bromide, **7** was then converted to the cationic trimethyl ammonium lipid, **1**, by Menshutkin reaction with Me_3N in dry acetone at 80°C in a screw-top pressure tube for 14 h. Lipid, **1** which separated as insoluble material from the reaction mixture was filtered and was purified by recrystallizations from dry acetone.

For the synthesis of mixed-chain cationic lipids **3a** and **b** were individually subjected to reduction in the presence of $LiAlH_4$ in dry THF under N_2 atmosphere. This afforded the respective 2-alkyl-1,3-propanediols, **8a**–**b** in 88 and 76% yields respectively (scheme 1). Each of these were then esterified with one equivalent of fatty acid of appropriate chain length using DCC in the presence of catalytic amount of DMAP in dry $CHCl_3$. Work-up followed by purification over silica gel column afforded monoesters, **9a**–**d** in moderate yields. The OH group in each of **9a**–**d** was then converted to corresponding bromides in excellent yields with PPh_3 and CBr_4 at room temperature in dry CH_2Cl_2. The resulting bromo-compounds, **10a**–**d**, were directly used for the final step of quarternization with trimethylamine in dry acetone in screw top pressure tubes to make the resulting pseudo-glyceride cationic lipids, **2a**–**d**. Each of these lipids were purified by column chromatography over silica gel and finally isolated as white solids upon repeated crystallizations from dry EtOAc. All numbered intermediates and the final compounds were characterized by their IR, NMR spectra and elemental analysis [6].



Scheme 1. *Reagents, conditions and yields*: (i) $CH_3(CH_2)_nBr$ (n = 17, 15), NaH, THF, −20°C, 15 min, 8 h at room temp., reflux 6 h (74 and 68% for **3a–b**, respectively); (ii) n-$C_{18}H_{37}Br$, NaH, toluene, reflux for 48 h (72%); (iii) KOH, THF–MeOH–H_2O (4 : 1 : 1), reflux, 24 h, cool, add 6N HCl, extract in $CHCl_3$, concentrate, heat to 150°C for 16 h, take up residue in THF–MeOH (1 : 1) with 0·5N H_2SO_4, reflux 12 h (66%); (iv) LAH, THF (N_2) reflux, 12 h (88%); (v) PPh_3, CBr_4, CH_2Cl_2, 0°C, 0·5 h, then 8 h at room temp. (94%); (vi) NMe_3, acetone, 80°C, pressure tube, 14 h (52%); (vii) LAH, THF (N_2) reflux, 16 h (83 and 76% for **8a–b**, respectively); (viii) $CH_3(CH_2)_nCO_2H$, DCC, DMAP, $CHCl_3$, room temp., 48 h (69, 46, 51 and 52% for **9a–d**, respectively); (ix) PPh_3, CBr_4, CH_2Cl_2, 0°C, 0·5 h, then 8 h at room temp. (95, 88, 94 and 98% for **10a–d**, respectively); (x) NMe_3, acetone, 80°C, pressure tube, 24 h (48, 45, 52 and 49% for **2a–d**, respectively).

All the newly synthesized cationic lipids, **1**, **2a**–**d**, produced stable membrane vesicles under the given conditions. For the preparation of vesicles, a known amount of lipid (2–3 mg) was dissolved in $CHCl_3$. Lipid film, produced first by evaporation of $CHCl_3$ under a stream of N_2 and then upon storage under high vacuum for ~ 5 h, was subjected to hydration with water (Millipore). Sonication (~ 10 min) of each lipid suspension in water above 60°C afforded stable translucent solutions. Electron micrography confirmed the presence of vesicular aggregates in these suspensions (not shown). Then lipid suspensions doped with 1,6-diphenylhexatriene (DPH) (0·1 mol%) were prepared and the fluorescence anisotropy values (*r*) as 'sensed' by DPH were measured at various temperatures from 25 to 60°C. The *r* vs *T* plots for **1**, and **2a–d** gave the respective thermal solid-to-fluid phase transition temperatures (T_m). T_m values of vesicular **1**, **2a**–**d** were found to be ca. 50, 45, 54, 42 and 43°C respectively. The *r*-values (0·27 to 0·34) of these membranes at 25°C compared well with that of the membranes prepared from naturally occurring lipid, dipalmitoyl phosphatidylcholine (0·31). This indicates that similar lipid order was preserved in the bilayer membranes of the newly synthesized lipids with that of the natural phospholipids.

Like DOTMA, which is one of the effective gene transfer agents, lipid **1** also maintains a hydrolytically stable link at the chain-backbone junction. Although less pronounced than ester-linked DOTAP, the chain-backbone region in DOTMA, may however, still participate in H-bonding with water molecules adhering to the interfaces of bilayer and bulk water. No such association is possible with lipid **1** making it and its analogues attractive candidates for gene transfer studies.

In summary, we have been able to synthesize a series of novel cationic lipids, which will help in understanding the effect of modulation of hydration at the linkages of lipid molecules in model membranes. Subsequent synthetic extension to the corresponding phosphocholine lipids to utilize them as inhibitors of enzymes such as phospholipases is in progress.

Acknowledgments

This work was supported by the Swarnajayanti Fellowship Grant of the Department of Science & Technology to SB.

References

1. For general reviews, see: (a) Miller A D 1998 *Angew. Chem., Int. Ed. Engl.* **37** 1768; (b) Deshmukh H M and Huang L 1997 *New. J. Chem.* **21** 113; (c) Behr J P 1993 *Acc. Chem. Res.* **26** 274
2. (a) Bhattacharya S and Mandal S S 1998 *Biochemistry* **37** 7764; (b) Wheeler C J *et al* 1996 *Proc. Natl. Acad. Sci. USA* **93** 114544
3. (a) Song Y K, Liu F, Chu S and Liu D 1997 *Human Gene Therapy* **8** 1585; (b) Felgner J H *et al* 1994 *J. Biol. Chem.* **269** 2550
4. (a) Ghosh Y K, Visweswaraiah S S and Bhattacharya S 2000 *FEBS Lett.* **473** 341; (b) Fichert T, Regelin A and Massing U 2000 *Bioorg. Med. Chem. Lett.* **10** 787
5. (a) Bhattacharya S and Dileep P V 1999 *Tetrahedron Lett.* **40** 8167; (b) Bhattacharya S and De S 1999 *Chem. Eur. J.* **5** 2335; (c) Bhattacharya S and De S 1998 *J. Org. Chem.* **63** 7640; (b) Bhattacharya S, Ghosh S and Easwaran K R K 1998 *J. Org. Chem.* **63** 9232; (c) Bhattacharya S, De S and George S K 1997 *Chem. Commun.* 2287; (d) Ghosh S, Easwaran K R K and Bhattacharya S 1996 *Tetrahedron Lett.* **37** 5769; (e) Bhattacharya S and De S 1996 *Chem. Commun.* 1283; (f) Bhattacharya S and De S 1995 *Chem. Commun.* 651

6. All the new compounds exhibited spectral characteristics consistent with their given structures. Selected spectral data for the final compounds are as follows: (**1**) ^{1}H-NMR (300 MHz, $CDCl_3$): *d*: 0·88 (*t*, 2 X-C$\underline{H}_3$, 6H), 1·25 (*br m*, 2X (C$\underline{H}_2$)$_{16}$, 64H), 1·44 (*m*, (C$\underline{H}_2$)$_2$CHCH$_2$N$^+$, 4H), 1·9 (*m*, CH$_2$C$\underline{H}$CH$_2$N$^+$, 1H), 3·3 (*d*, CH$_2$CHC$\underline{H}_2$N$^+$, 2H), 3·35 (*s*, (C$\underline{H}_3$)$_3$N$^+$, 9H). Anal. calcd. for $C_{41}H_{86}NBr$: C, 73·17; H, 12·88; N, 2·08; Found: C, 73·36; H, 13·04; N, 2·19. (**2a**): IR (nujol). 1740 cm^{-1}. ^{1}H-NMR (300 MHz) *d*: 0·88 (*t*, 2 X-CH$_3$, 6H), 1·25 (*br m*, 2X(C$\underline{H}_2$)$_{15}$, 60H), 1·58–1·66 (*m*, OCH$_2$–C$\underline{H}$–CH$_2$–N$^+$, *b*-C$\underline{H}_2$ to C=O, 3H), 2·3 (*t*, C$\underline{H}_2$C=O, 2H), 3·42–3·53 (*m* + *s*, C$\underline{H}_2$N$^+$(C$\underline{H}_3$)$_3$, 11H), 4·15 (*m*, O–C$\underline{H}_2$–CH, 2H). Anal. calcd. for $C_{40}H_{82}O_2NBr$, 0·5 H_2O: C, 68·83; H, 11·98; N, 2·0; Found: C, 69·06; H, 12·21; N, 2·29. (**2b**) IR (nujol). 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz,) *d*: 0·88 (*t*, 2X-C$\underline{H}_3$, 6H), 1·25 (*br m*, 2X(C$\underline{H}_2$)$_{16}$, 64H), 1·58–1·66 (*m*, OCH$_2$–C$\underline{H}$–CH$_2$–N$^+$, *b*-C$\underline{H}_2$ to C=O, 3H), 2·3 (*t*, C$\underline{H}_2$C=O, 2H), 3·42–3·54 (*m* + *s*, C$\underline{H}_2$N$^+$(C$\underline{H}_3$)$_3$, 11H), 4·15 (*m*, O–C$\underline{H}_2$–CH, 2H). Anal. calcd. for $C_{42}H_{88}O_2NBr$, 0·5 H_2O: C, 69·1; H, 12·59; N, 1·99; Found: C, 69·28; H, 12·32; N, 1·99. (**2c**) IR (nujol). 1740 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) *d*: 0·88 (*t*, 2X-C$\underline{H}_3$, 6H), 1·25 (*br m*, 2X (C$\underline{H}_2$)$_{14}$, 56H), 1·58–1·68 (*m*, OCH$_2$–C$\underline{H}$–CH$_2$–N$^+$, *b*-C$\underline{H}_2$ to C=O, 3H), 2·3 (*t*, C$\underline{H}_2$C=O, 2H), 3·42–3·55 (*m* + *s*, C$\underline{H}_2$N$^+$(C$\underline{H}_3$)$_3$, 11H), 4·15 (*m*, O–C$\underline{H}_2$–CH, 2H). Anal. calcd. for $C_{38}H_{78}O_2NBr$: C, 65·05; H, 11·89; N, 2·12; Found: C, 65·07; H, 11·89; N, 2·17. (**2d**) IR (nujol). 1735 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) *d*: 0·88 (*t*, 2X-CH$_3$, 6H), 1·25 (*br m*, 2X (C$\underline{H}_2$)$_{15}$, 60H), 1·56–1·64 (*m*, OCH$_2$–C$\underline{H}$–CH$_2$–N$^+$, *b*-C$\underline{H}_2$ to C=O, 3H), 2·3 (*t*, C$\underline{H}_2$C=O, 2H), 3·42–3·53 (*m* + *s*, C$\underline{H}_2$N$^+$(C$\underline{H}_3$)$_3$, 11H), 4·15 (*m*, O–C$\underline{H}_2$–CH, 2H). Anal. calcd. for $C_{40}H_{82}O_2NBr$, H_2O: C, 67·95; H, 11·97; N, 1·98; Found: C, 67·72; H, 12·03; N, 2·23.