

A Simple Approach to DC-Cholesterol, its Analogues and Vitamin D-Based Cationic Lipids for Gene Therapy

Armandodoriano Bianco*, Francesco Bonadies, Raffaella Napolitano and Giancarlo Ortaggi

Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza" and Istituto di Chimica Biomolecolare del CNR, Piazzale Aldo Moro, 5 00185 Roma, Italy

Received April 8, 2004; Accepted July 23, 2004

Abstract: An improved synthesis of DC-Cholesterol, its analogues and Vitamin D-based lipids for the preparation of liposomes in gene therapy, is described. This synthetic strategy affords significative increase in the yields of DC-Cholesterol and its analogs.

Keywords: Cationic lipids, gene therapy, DC-Cholesterol, modified Curtius reaction, DC-Vitamin D-based cationic lipids.

INTRODUCTION

In the past decade, gene therapy is definitely one of the fastest developing fields of biomedical research. It offers a conceptually novel therapeutic strategy for the treatment and cure of acquired diseases like cancer [1] and inherited diseases such as cystic fibrosis [2]. Although a wide array of physical, chemical and biological methods are available for transferring genes into the cells. An ideal vector should be highly efficient in delivering the gene in a target-specific manner, stable *in vitro* as well as *in vivo*, non toxic, non immunogenic, easy prepared in large quantities and it should protect the gene from nuclease degradation.

The DC-Chol **1**, (3 β -[N-(N',N'-dimethylaminoethane) carbamoyl]cholesterol) (see Fig. (1)), is one of cationic lipid more used in the preparation of liposomes in combination with DOPE **2**, (dioleoyl-phosphatidyl-ethanolamine), for the delivery of nucleic acids fragments in the gene therapy [3].

There are useful features that make DC-Chol high in the list of cationic lipids for gene delivery. It forms a stable liposomal formulation with DOPE which can be stored for months at 4°C without any change in size or lipid degradation [4]. It is synthesised using a coupling reaction and the product can be easily isolated and purified in a not expensive way. US FDA and the regulatory authorities of

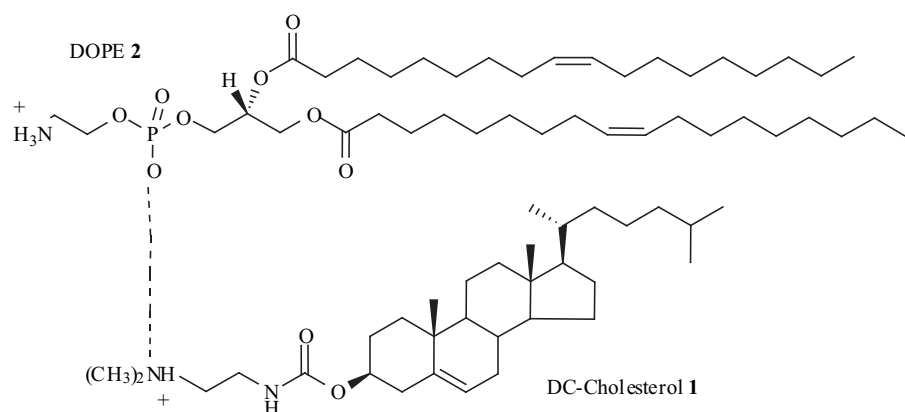


Fig. (1).

The cationic liposomes are non-viral most widely used vectors, owing to their capacity in packaging well the DNA fragments with their positive charge. They have many important qualities such as being much less or non immunogenic and non toxic, have no know limitation in the size of DNA, can be custom-synthesised for targeting and easy scalable for large scale production.

other countries, for use in clinical trials, approved it. DC-Chol/DOPE liposomes show a better transfection activity than other cationic lipids formulations *in vivo* [5].

RESULTS AND DISCUSSIONS

The DC-Chol is commercially available. Previously, the routes of synthesis for steroidal cationic lipids have been focussed on a combination of cationic aminic groups with limited variations of steroidal backbone. For these reasons we have described [6] an improvement in the synthesis of DC-Chol, using a modified Curtius reaction, according to the methodology used for the synthesis of methyl

*Address correspondence to these authors at the Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza" and Istituto di Chimica Biomolecolare del CNR, Piazzale Aldo Moro, 5 00185 Roma, Italy; E-mail: armandodoriano.bianco@uniroma1.it

carbamates [7]. This procedure consists in a one pot reaction between a halogeno-acyl chloride and an alcohol in the presence of sodium azide. The carbamic linker between the hydrophilic head group and the hydrophobic moiety was obtained with high yields.

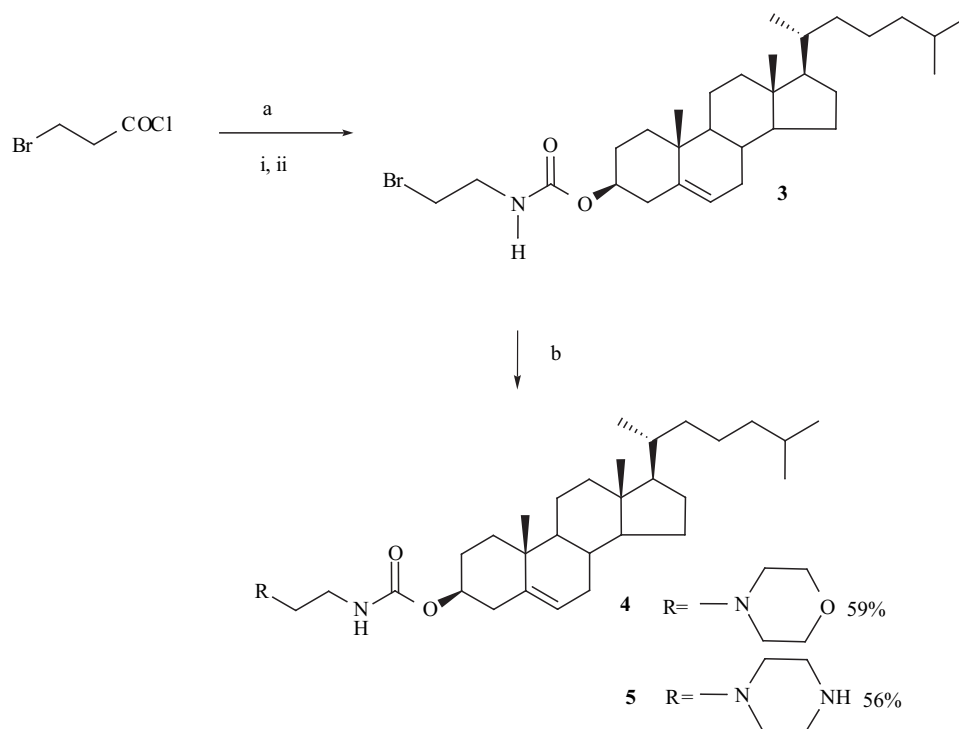
Nowadays, the industrial synthesis of DC-Chol is based on the reaction of cholesterol with phosgene derivatives. The cholesteryl chloroformate obtained, is treated with *N,N*-dimethylethylenediamine to give the final product with a 21% overall yield.

Our synthetic strategy presents a good flexibility and we here describe the synthesis of a wide variety of the DC-Chol derivatives with different secondary amines.

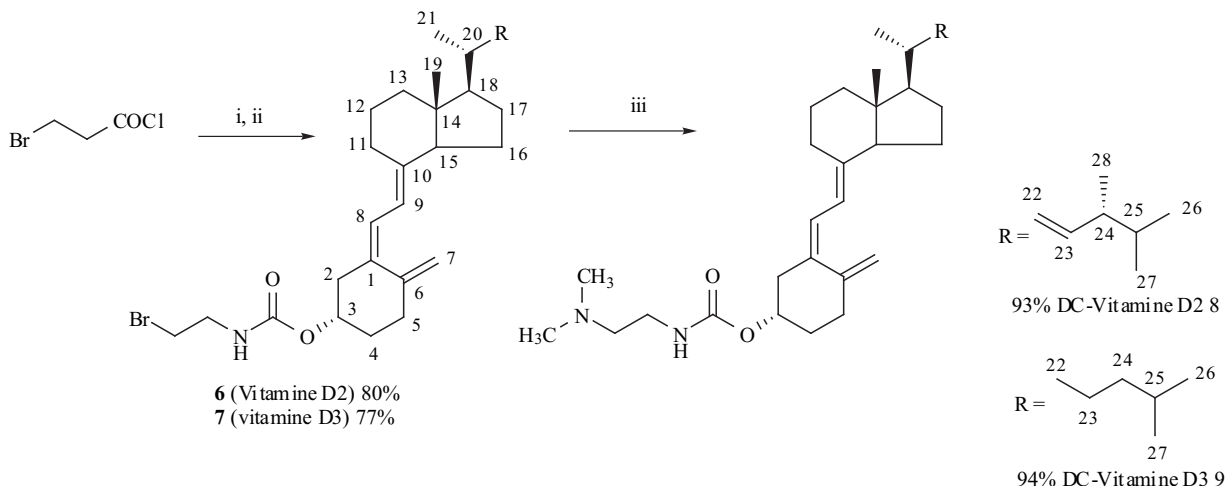
It was possible to synthesise DC-Chol analogues by substituting the bromine linked to the chain of DC-Chol precursor 3. A suitable halogenoacyl chloride, in this case 3-bromo propionyl chloride, was coupled with cholesterol. The described protocol allowed us to obtain the intermediate key (3) in 74% yield in one pot.

The cationic lipids prepared by this synthetic pathway are compounds 4 (where a morpholine residue is present) and 5 (where there is a piperazine one). These secondary amines were selected because the same cationic lipids were reported in literature and they have a good transfection activity [8,9].

This synthetic procedure was efficient not only to obtain cationic lipids with different polar head groups but also to



Scheme 1. a) i) NaN_3 , benzyl-triethyl-ammonium chloride, toluene, 80 °C. 1 hr; ii) cholesterol, overnight, r.t. b) morpholine or piperazine, CH_3CN , r.t., overnight.



Scheme 2. a) i) NaN_3 , benzyl-triethyl-ammonium chloride, toluene, 80 °C. 1 hr; ii) vitamine D2 or D3, overnight, r.t. b) dimethylamine 2M solution in THF, THF, r.t., overnight.

reach cationic lipids with different hydrophobic heads. The Vitamine D2 and the Vitamine D3 were secosteroids with 9,10 carbon-carbon bond breakage of B ring on the steroidal backbone, which would serve as hydrophobic domain in the cationic lipid-mediated gene transfection. In literature was reported the synthesis of Vitamin D-Based Cationic Lipids [10] using, to obtain the carbamic linker, the 1,1'-carbonyldiimidazole. We have synthesised the same cationic lipids using our synthetic procedure. The synthetic procedure is an analog to that described in Scheme 1. Bromopropionyl chloride was coupled with our methodology with Vitamine D2 or Vitamine D3, affording the key intermediates **6** and **7** respectively (see Scheme 2).

We have performed the substitution of bromine with dimethylamine, obtaining lipids **8** and **9** in very satisfactory yields (93% and 94% respectively). So, we have improved the synthetic strategy to obtain the DC-Cholesterol analogues and Vitamine D based cationic lipids.

EXPERIMENTAL

Materials and Methods

General Remarks

All reactions were carried out under dry argon using anhydrous solvents of Sigma- Aldrich. Glassware was flame-dried prior to use. Commercial reagents were purchased from Sigma-Aldrich or Fluka and were used without further purification. Reactions were monitored by thin layer chromatography (TLC) on Merck silica gel plates (0.25 mm); spots were visualised using UV light or by spraying with phosphomolybdic reagent. Reaction temperature was measured externally. Flash chromatography was performed on Merck silica gel 60 (particle size 0.040-0.063 mm). Yields refer to chromatographically and spectroscopically (^1H NMR) pure materials. NMR spectra were recorded in CDCl_3 solution on a Varian Gemini 200 spectrometer at room temperature. Chemical shifts are reported relative to the residual solvent peak (CHCl_3 : $\delta_{\text{H}}=7.26$, CDCl_3 : $\delta_{\text{C}}=77.00$).

Synthesis of Intermediate 3

3-Bromopropionyl chloride (1 mmol) was dissolved in dry toluene (5 ml) and dry benzyltriethylammonium chloride (0.1 mmol) was added with stirring. Dry sodium azide (2.4 mmol) was added in portions (4 x 0.6 mmol) over 1 h keeping the reaction temperature at 60°C under stirring. The solution was kept for 15 min at 80°C . Cholesterol (5 mmol) was then added and the reaction mixture allowed to stand over night at room temperature. The solution was diluted with ethyl ether (50 mL) washed with water and dried with anhydrous sodium sulphate. Solvent removal gave intermediate **3** that can be used in the next step without purification. For analytical purposes, crude **3** was purified on silica gel, eluting with $\text{CHCl}_3/\text{MeOH}$ 98:2 (80% yield, amorphous solid) and characterised by ^1H - and ^{13}C -NMR. ^1H NMR δ : 0.69 (3H, s, CH_3 -18), 0.86-0.89 (6H, d, $J=6$ Hz, CH_3 , 26-27), 0.91-0.94 (3H, d, $J=6$ Hz, CH_3 -21), 1.03 (3H, s, CH_3 -19), 3.45-3.48 (2H, m, $J=6$ Hz, CH_2NH), 3.58-3.61 (2H, t, $J=6$ Hz, CH_2Br), 4.60-4.64 (1H, m, H-3), 5.04-5.08 (1H, m, NH), 5.37-5.40 (1H, d, $J=6$ Hz, H-6). ^{13}C NMR δ : 11.93, 14.18, 22.75, 22.89, 26.80, 27.30,

28.09, 28.27, 29.26, 29.40, 29.75, 29.84, 31.98, 35.88, 36.27, 36.65, 38.68, 39.59, 39.82, 42.40, 45.57, 45.86, 50.11, 53.21, 53.93, 36.22, 56.77, 129.79, 130.12, 156.22. Anal. Calcd for $\text{C}_{30}\text{H}_{50}\text{BrNO}_2$: C 67.15%, H 9.39%, Br 14.89%, N 2.61%. **Found**: C 66.99%, H 9.43%, Br 14.85%, N 2.59%.

Synthesis of Lipids 4 and 5

The amine (1.5 mL, 10 mmols) was added to a solution of **3** (1 mmol) dissolved in CH_3CN (10 mL). The reaction mixture was refluxed overnight, concentrated *in vacuo* to remove CH_3CN , extracted with ethyl acetate, dried (Na_2SO_4), and the organic layer was concentrated *in vacuo*. The residue was purified by flash chromatography (100% chloroform for morpholine derivative **4** and 2% methyl alcohol in chloroform for piperazine derivative **5**) to give the lipid **4** and the lipid **5**.

Compound **4**: Yield: 74%, amorphous solid. ^1H NMR δ : 0.69 (3H, s, CH_3 -18), 0.86-0.89 (6H, d, $J=6$ Hz, CH_3 , 26-27), 0.91-0.94 (3H, d, $J=6$ Hz, CH_3 21), 1.03 (3H, s, CH_3 19), 2.28 (6H, s, $\text{N}(\text{CH}_3)_2$), 2.47 (2H, t, $J=6$ Hz, $\text{CH}_2\text{N}(\text{CH}_3)_2$), 3.15-3.18 (2H, m, $J=6$ Hz, CH_2NH), 3.40 (2H, t, $J=6$ Hz, CH_2Br), 4.60-4.64 (1H, m, H-3), 5.18-5.22 (1H, m, NH), 5.37-5.40 (1H, d, $J=6$ Hz, H-6). ^{13}C NMR δ : 11.93, 14.18, 22.75, 22.89, 26.80, 27.30, 28.09, 28.27, 29.26, 29.40, 29.75, 29.84, 31.98, 35.88 (x2), 36.65, 38.68, 39.59, 39.82, 42.40, 45.57, 45.86, 50.11, 53.21, 53.93, 36.22, 56.77, 129.79, 130.12, 156.22. Anal. Calcd for $\text{C}_{34}\text{H}_{58}\text{N}_2\text{O}_3$: C 75.23%, H 10.77%, N 5.16%. **Found**: C 75.01%, H 11.43%, N 5.51%.

Compound **5**: Yield: 70%, amorphous solid. ^1H NMR δ : 0.69 (3H, s, CH_3 -18), 0.86-0.89 (6H, d, $J=6$ Hz, CH_3 26-27), 0.91-0.94 (3H, d, $J=6$ Hz, CH_3 21), 1.03 (3H, s, CH_3 19), 2.83 (4H, m, CH_2NH), 2.92-2.95 (2H, t, $J=6$ Hz, CH_2N), 3.15-3.18 (2H, bq, $J=6$ Hz, CH_2NCO), 3.23 (4H, m, CH_2N), 4.62 (1H, m, H-3), 4.95 (1H, m, NH), 5.20 (1H, m, CONH), 5.37-5.40 (1H, d, $J=6$ Hz, H-6). ^{13}C -NMR δ : 11.98, 14.19, 22.76, 22.82, 26.82, 27.30, 28.11, 28.23, 29.26, 29.39, 29.74, 29.83, 31.92, 35.81, 38.69, 39.53, 39.81, 42.44, 45.57, 45.86, 50.21, 51.12 (x2), 53.23, 53.83, 56.72, 57.73(x2), 129.76, 130.16, 156.46. Anal. Calcd for $\text{C}_{34}\text{H}_{59}\text{N}_3\text{O}_2$: C 75.37%, H 10.98%, N 7.75%. **Found**: C 75.28%, H 11.01%, N 7.72%.

Synthesis of Intermediates 6 and 7

Compound **6** and **7** were prepared from vitamins D2 and D3, according to the same procedure used for the synthesis of **3**, on a 3 mmol scale referred to terpenoid moiety.

Compound **6**, 77% yield, amorphous solid. ^1H NMR δ : 0.82-0.85 (6H, d, $J=7$ Hz, CH_3 26-27), 0.87 (3H, d, $J=6$ Hz, CH_3 28), 0.89 (3H, d, $J=7$ Hz, CH_3 21), 3.50 (1H, m, H-3), 3.64 (2H, m, CH_2NH), 3.75 (2H, m, CH_2Br), 5.28-5.30 (3H, m, H-7, H-9), 5.45 (1H, bt, NH), 6.12 (1H, m, H-8). ^{13}C -NMR δ : 18.10, 18.78, 20.26, 22.66, 22.95, 23.97, 28.20, 28.59, 31.56, 32.16, 32.65, 34.29, 38.56, 39.61, 40.50, 40.68, 40.99, 43.50, 46.47, 53.62, 56.07, 62.48, 106.44, 121.07, 123.68, 145.39, 150.97, 154.28, 156.15. Anal. Calcd for $\text{C}_{31}\text{H}_{48}\text{BrNO}_2$: C 68.12%, H 8.85%, N 2.56%. **Found**: C 67.89%, H 8.99%, N 2.60%.

Compound **7**, 80% yield, amorphous solid. ^1H NMR δ : 0.80-0.83 (6H, d, $J=7$ Hz, CH_3 26-27), 0.87 (3H, d, $J=7$

Hz, CH_3 21), 3.48 (1H, m, H-3), 3.62 (2H, m, CH_2NH), 3.73 (2H, m, CH_2Br), 5.26-5.28 (3H, m, H-7, H-9), 5.40 (1H, bt, NH), 6.03 (1H, m, H-8). ^{13}C -NMR δ : 18.07, 18.74, 20.22, 22.63, 22.91, 23.93, 28.18, 28.55, 31.54, 32.12, 32.63, 34.27, 38.53, 39.59, 40.46, 40.65, 40.97, 43.48, 46.45, 53.60, 56.04, 62.44, 106.43, 121.05, 123.65, 145.37, 150.93, 154.25, 156.08. Anal. Calcd for $C_{30}H_{48}BrNO_2$, C 67.40%, H 9.05%, N 2.62%. Found C 67.28%, H 9.15%, N 2.67%.

Synthesis of DC-Vitamines 8 and 9

DC-Vitamine D2, **8** and DC-Vitamine D3, **9** were prepared from **6** or **7**, according to the same procedure used for the synthesis of **4** and **5**, on a 0.5 mmol scale referred to terpenoid moiety.

DC-Vitamine D2, **8**, 93% yield, amorphous solid. 1H NMR δ : 0.82-0.85 (6H, d, $J = 7$ Hz, CH_3 26-27), 0.86 (3H, d, $J = 6$ Hz, CH_3 28), 0.88 (3H, d, $J = 7$ Hz, CH_3 21), 2.26 (6H, s, $N(CH_3)_2$), 2.56 (2H, t, $CH_2N(CH_3)_2$), 3.50 (1H, m, H-3), 3.63 (2H, m, CH_2NH), 5.28-5.30 (3H, m, H-7, H-9), 5.42 (1H, bt, NH), 6.05 (1H, m, H-8). ^{13}C -NMR δ : 18.07, 18.75, 20.22, 22.65, 22.97, 23.92, 28.10, 28.55, 31.54, 32.13, 32.65, 36.10, 38.58, 39.60, 40.46, 40.68, 40.97, 41.24 (x2), 43.50, 46.45, 53.62, 56.10, 62.48, 106.45, 121.05, 123.66, 145.40, 150.97, 154.28, 156.16. Anal. Calcd for $C_{33}H_{54}N_2O_2$, C 77.60%, H 10.66%, N 5.48%. Found C 77.48%, H 10.80%, N 5.59%.

DC-Vitamine D3, **9**, 94% yield, amorphous solid. 1H NMR δ : 0.80-0.83 (6H, d, $J = 7$ Hz, CH_3 26-27), 0.87 (3H, d, $J = 7$ Hz, CH_3 21), 2.24 (6H, s, $N(CH_3)_2$), 2.54 (2H, t, $CH_2N(CH_3)_2$), 3.48 (1H, m, H-3), 3.62 (2H, m, CH_2NH), 5.26-5.28 (3H, m, H-7, H-9), 5.40 (1H, bt, NH), 6.03 (1H,

m, H-8). ^{13}C -NMR δ : 18.05, 18.72, 20.20, 22.62, 22.90, 23.89, 28.08, 28.52, 31.51, 32.10, 32.60, 36.04, 38.52, 39.58, 40.44, 40.63, 40.94, 41.20 (x2), 43.46, 46.42, 53.58, 56.01, 62.42, 106.42, 121.03, 123.63, 145.35, 150.91, 154.22, 156.06. Anal. Calcd for $C_{32}H_{54}N_2O_2$, C 77.06%, H 10.91%, N 5.62%. Found: C 77.00%, H 11.00%, N 5.75%.

ACKNOWLEDGEMENTS

This work was supported by FIRB project of the Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica.

REFERENCES

- [1] Nabel, E.G.; Yang, Z.; Muller, D.; Chang, A.E.; Gao, X.; Huang, L.; Cho, K.J.; Nabel, G.J. *Hum. Gen. Ther.*, **1994**, *5*, 1089-1094.
- [2] Caplen, N.J.; Alton, E.W.F.W.; Middleton, P.G.; Dorin, J.R.; Stevenson, B.J.; Gao, X.; Durham, S.R.; Jeffery, P.K.; Hodson, M.E.; Coutelle, C.; Huang, L.; Porteous, D.J.; Williamson, R.; Geddes, D.M. *Nat. Med.*, **1995**, *1*, 39-46.
- [3] Gao, X.; Huang, L. *Biochem. Biophys. Res. Commun.*, **1991**, *179*, 280-285.
- [4] Sorgi, F.L.; Huang, L. *Int. J. Pharm.*, **1996**, *144*, 131-139.
- [5] Egilmez, N K.; Iwanuma, Y.; Bankert, R.B. *Biochem. Biophys. Res. Commun.*, **1996**, *221*, 169-173.
- [6] Bianco, A.; Bonadies, F.; Napolitano, R.; Ortaggi, G. *C. R. Chimie*, **2003**, *6*, 613-615.
- [7] Lo Scalzo, R.; Mascitelli, L.; Scarpati, M.L. *Gazz. Chim. Ital.*, **1988**, *118*, 819-820.
- [8] Gao, H.; Hui, K.M. *Gene Ther.*, **2001**, *8*, 855-863.
- [9] Deshmukh, H. M.; Huang, L. *PCT Int. Appl.*, **1997**, 45 pp. WO 9739019 A1 19971023 CAN 127:331637 AN 1997:696779
- [10] Ren, T.; Zang, G.; Liu, F.; Liu, D. *Biorg. Med. Chem. Lett.*, **2000**, *10*, 891-894.