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Synthesis of novel cationic lipids for preparation of liposomes to be used in gene therapy of glioma

Armandodoriano Bianco [a,*], Francesco Bonadies [a], Raffaella Napolitano [a], Giancarlo Ortaggi [a], Claudio Esposito [b], Giuseppe Mossa [b], Cesare Cametti [c]

[a] *Dipartimento di Chimica, Università degli Studi di Roma 'La Sapienza' and Istituto di Chimica Biomolecolare del CNR, Piazzale Aldo Moro 5, 00185 Roma, Italy*
[b] *Istituto di Neurobiologia e Medicina Molecolare del CNR,Via Fosso del Cavaliere 100, 00133 Roma, Italy*
[c] *Dipartimento di Fisica, Università La Sapienza, Roma and INFM, Unità Roma1, Piazzale Aldo Moro 5, 00185 Roma, Italy*

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Abstract

The synthesis of a series of cationic lipids for the preparation of liposomes to be used in gene therapy is described. The synthetic strategy is designed in few steps and affords good yields. ***To cite this article: A. Bianco et al., C. R. Chimie 6 (2003).***

Résumé

La synthèse d'une série de lipides cationiques pour la préparation of liposomes destinés à être utilisés en thérapie génique est décrite. Le protocole de synthèse comprend peu d'étapes et permet d'obtenir de bons rendements. ***Pour citer cet article : A. Bianco et al., C. R. Chimie 6 (2003).***

Keywords: cationic lipids; liposomes

Mots clés : lipides cationiques ; liposomes

1. Introduction

Gene therapy is a promising approach for the treatment of cancer and inherited diseases [1]. In gene therapy, the most important is the vector which efficiently packages the DNA, carries it trough the membranes and deliver the gene to the nucleus for expression. An ideal vector should be highly efficient in delivering the gene in a target-specific manner, stable in vitro as well as in vivo, protect the gene from nuclease degradation, non toxic, non immunogenic and easily prepared in large quantities.

The most widely used types of vehicles for gene delivery are viral vectors and non-viral ones, such as liposomes.

* Corresponding author.
E-mail address: armandodoriano.bianco@uniroma1.it (A. Bianco).

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Viral vectors are more efficient than their non-viral counterparts, but they have the disadvantage of being immunogenic, potentially mutagenic, with low viral titres and limited loading capacity in terms of DNA size.

Although less efficient in delivering gene fragments than virus, cationic liposomes have many important qualities, such as being much less or non-immunogenic and non-toxic. For this reason, we have decided to synthesise new cationic lipids for the preparation of liposomes able to vehicle nucleic acids for malign glioma gene therapy.

The most known cationic lipids, that formed cationic liposomes with a good delivering activity, are the *N*-[1-(2,3-dioleoyloxy)propyl]*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA) [2], or 1,2 dioleoyloxy-3-(trimethylammonio) propane (DOTAP) [2] and 3β[*N*-*(N′*,*N′*-dimethylammino-ethane)carbamoyl]-cholesterol (DC-Chol) [3] (Fig. 1).

DC-Chol has a tertiary amino group and a spacer that offers a good transfection activity, a stable carbamoyl bond that provides excellent stability and transfection efficiency as well as potential biodegradability, and hence reduced toxicity compared with the widely used cationic lipid with an ether bond. This means that is was deliberately designed with a relatively labile carbamoyl linker, which is not hydrolysed easily like the ester bond but, once inside the cell, is eventually biodegradable, probably by the cellular esterase.

2. Results and discussion

In an attempt to improve the delivering property of liposomes obtained from DOTAP mixed with dioleoyl-fosfatidyl-etanolamine (DOPE), we have synthesised analogues of DOTAP.

In all the structures that we designed, the bond between the lipophilic chain and the cationic head was preferably of carbammic nature. In fact, the relatively labile carbamoyl linker of Dc-Chol and analogues is one of the reasons for their good pharmaceutical characteristics [4].

The cationic lipids obtained are different, not only for the polar head groups, but also for the linkers.

We have used the modified Curtius reaction [5, 6] to obtain these lipids. The reaction consists in a one-pot reaction between the acyl chloride and the selected hydroxyl compound in the presence of sodium azide. In particular, we employed 3 bromo-1,2-propandiol 1 for the synthesis of a series of cationic glycerolipids. The contemporaneous presence of both primary and secondary alcoholic functions on the hydroxyl compound allows directing the reaction towards the mono- or di-carbamate, simply by varying the stoichiometry of the sodium azide.

In fact, the primary alcoholic function appears more reactive in this reaction and a mono-carbamate type **2** was obtained when equimolecular quantities of substrate and sodium azide are used. By a simple esterification reaction, the urethane/ester compound of type

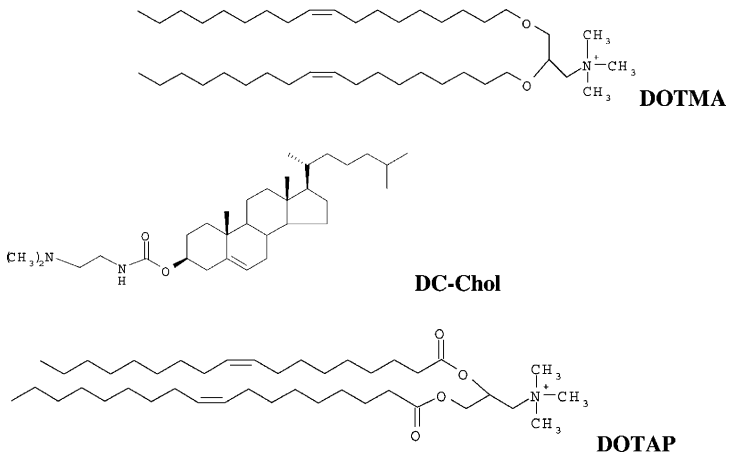

Fig. 1. Structures of *N*-[1-(2,3-dioleoyloxy)propyl]*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA) [2], 3β[*N*-*(N′*,*N′*-dimethylammino-ethane)carbamoyl]-cholesterol (DC-Chol.), and 1,2 dioleoyloxy-3-(trimethylammonio) propane (DOTAP).

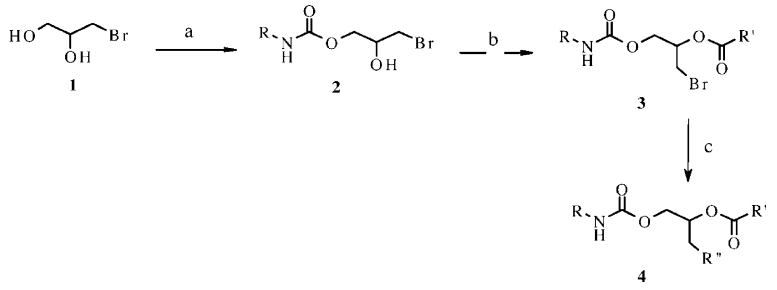

Fig. 2. (**a**) 1.2 equiv NaN_3, palmitoyl chloride or oleoyl chloride, benzyl-triethyl-ammonium chloride, toluene, 60 °C; (**b**) palmitoyl chloride or oleyl chloride, pyridine, DMAP, CH_2Cl_2; (**c**) morpholine or piperazine, CH_3CN, 24 h. **2a**: R = palmitoyl, yield 60%; **2b**: R = oleyl, yield 52%; **3a**: R = palmitoyl, R' = palmitoyl, yield 90%; **3b**: R = palmitoyl, R' = oleyl, yield 85%; **3c**: R = oleyl, R' = oleyl, yield 87%; **3d**: R = oleyl, R' = palmitoyl, yield 85%; **4a**: R = palmitoyl, R' = palmitoyl, R"= morpholine, yield 70%; **4b**: R = palmitoyl, R' = palmitoyl, R"= piperazine, yield 65%; **4c**: R = palmitoyl, R' = oleyl, R"= morpholine, yield 77%; **4d**: R = palmitoyl, R' = oleyl, R"= piperazine, yield 69%; **4e**: R = oleyl, R' = oleyl, R"= morpholine, yield 78%; **4f**: R = oleyl, R' = oleyl, R"= piperazine, yield 67%; **4g**: R = oleyl, R' = palmitoyl, R"= morpholine, yield 86%; **4h**: R = oleyl, R' = palmitoyl, R" = piperazine, yield 56%.

3 is obtained. Finally a S_N2 reaction gives the final product of type **4**, as shown in Fig. 2.

A fatty acid, the commercially available oleyl and palmitoyl chlorides are the hydrophobic moiety; as polar head, we have chosen an amine, in particular piperazine and morpholine. This synthetic methodology is shown to be versatile, because with simple synthetic steps and by modifying the reagents, it is possible to obtain a series of cationic lipids, with several polar head groups and linkers.

Both primary and secondary alcoholic functions are reactive when the quantities of sodium azide are doubled, affording in satisfactory yields the dicarbamate type **5**. The cationic lipids types **6** are obtained using the previously described procedure, as reported in Fig. 3.

Cationic lipids with a non-cyclic polar head group were also synthesised. We selected the dimethylamine instead of piperazine and morpholine and synthesized the corresponding lipids **6e** and **6f**. The synthetic procedure is the same for all cationic lipids synthesised and is reported in Fig. 3.

All before-reported cationic lipids form liposomes in combination with DOPE in 1:1 molar ratio, with the procedure reported in the 'Experimental' section.

The dimensions were measured by a light scattering apparatus and are suitable for transfection experiments [7]. In the dynamic light scattering technique, we measure the normalized time autocorrelation of the intensity of the scattered light which, for a dilute suspension of monodisperse particles decays exponentially with a decay rate $G = 2\ q^{2D}$, where q is the magnitude of the scattering wave vector and D the diffusion coefficient. The hydrodynamic radius of the diffusing particles is obtained through the Stokes–Einstein relationship. For a polydisperse system, when the single exponential decay is no longer valid, the size and size distribution of the diffusing particles were found using the standard data analysis program CONTIN, in terms of a continuous distribution of exponential decay times. A typical

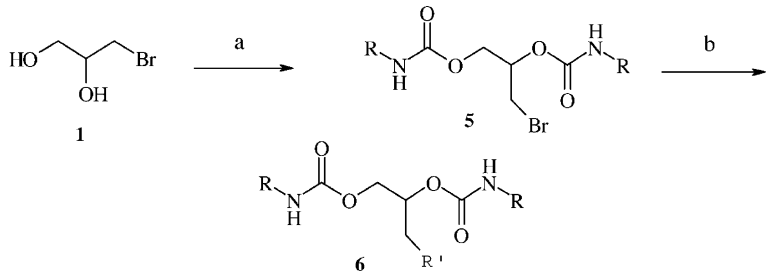

Fig. 3. (**a**) 2.4 equiv NaN_3, palmitoyl chloride or oleoyl chloride; (**b**) benzyl-triethyl-ammonium chloride, toluene, 60 °C, (**c**) morpholine or piperazine, CH_3CN, 24 h, or dimethyl-amine, THF, 24 h. **5a:** R = palmitoyl, yield 53%; **5b:** R = oleyl, yield 46%; **6a**: R = palmitoyl, R' = morpholine, yield 60%; **6b**: R = oleyl, R' = morpholine, yield 57%; **6c**: R = palmitoyl, R' = piperazine, yield 52%; **6d**: R = oleyl, R' = piperazine, yield 72%; **6e**: R = oleyl, R' = dimethyl-amine, yield 82%; **6f**: R = palmitoyl, R' = dimethyl-amine, yield 76%.

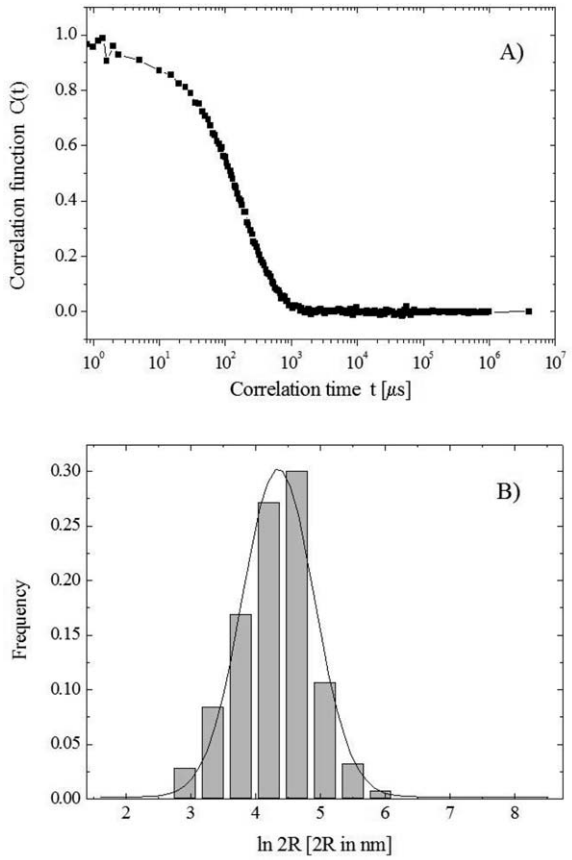

Fig. 4. Autocorrelation function measured by dynamic light scattering and the CONTIN analysis.

example of the autocorrelation function measured by dynamic light scattering and the CONTIN analysis giving the size distribution is shown in Fig. 4.

For all the samples investigated, the relaxation time distribution gives a single population (monomodal distribution), with some degree of polydispersity (a measurement of the reduced second moment of the distribution), ranging from 0.08 to 0.13, as reported in Table 1.

The cationic lipid **6e** (code name: MM54) resulted to be the most interesting one for its transfection good properties [7] in C6 glyoma cells.

3. Experimental

3.1. 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 visualized 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 (^{1}H NMR) pure materials. NMR spectra were recorded in a $CDCl_3$ solution on a Varian Gemini 200 spectrometer at room temperature. Chemical shifts are reported relative to the residual solvent peak ($CHCl_3$: δ_H = 7.26, $CDCl_3$: δ_c = 77.0).

3.2. Liposome preparation

Liposomes were prepared by the lipid hydration method. Appropriate amounts of lipids, in 1:1 molar ratio with DOPE, were dissolved in ethanol, followed by injection in sterile deionised water under vigorous stirring [8] to reach a final concentration of 1 mg ml^{-1}, at a temperature above the phase-transition temperature. The resulting aqueous lipid mixture was sonicated at pulsed power mode followed by extrusion through polycarbonate filters of varying size. A homogeneous liposomal suspension of unilamellar vesicles was obtained, as reported in Fig. 1 and Table 1.

Table 1
Selected liposome characteristics.

Lipid	Polar head group	Liposome mean diameter (nm)	Liposome polydispersity
6c	Piperazine	122	0.10
4d	Piperazine	145	0.10
4c	Morpholine	214	0.08
6b	Morpholine	420	0.13
4e	Morpholine	240	0.09
6e	Dimethyl-amine	290	0.10

3.3. Dynamic light-scattering measurements

Light-scattering experiments were performed using a classic set-up with a correlator (BI9000AT, Brookhaven) operating with a logarithmic sample time spacing. The light source is a 10-mW He–Ne laser (wavelength 632.8 nm) and the measurements have been made at a scattering angle of 90°.

4. Synthesis of intermediate 2

The acyl chloride (1 mmol) and dry benzyltriethylammonium chloride (0.1 mmol) were added to dry toluene (2 ml) stirring for 5 min at 60 °C. Dry sodium azide (1.2 mmol) was added in portions (4 × 0.3 mequiv) over 1 h at 60 °C under stirring. The solution was kept for 15 min at 80 °C. The alcohol (5 mmol) was then added at room temperature and allowed to stand overnight. The solution was diluted with ethyl ether (50 ml), washed with water and dried with anhydrous sodium sulphate. Solvent removal gave the product that was purified on a column of flash silica gel, eluting with 0–2% MeOH in chloroform.

4.1. Product **2a**

^{1}H NMR δ: 3.15 (2H, bq, J = 6 Hz, CH_2N), 3.40 (2H, m, CH_2Br), 4.02 (1H, m, C*H*O), 4.16 (1H, m, C*H*OH), 5.60 (1H, m, N*H*). ^{13}C–NMR $CDCl_3$ 200 MHz δ: 14.04, 18.08, 22.66, 26.80, 29.12, 29.47 (× 2), 29.70 (× 2), 29.85 (× 2), 31.92, 34.16, 41.23, 57.94, 66.42, 69.53, 156.90.

4.2. Product **2b**

^{1}H NMR δ: 3.19–3.23 (2H, bq, J = 12 Hz, CH_2NH), 3.45–3.49 (2H, dd, J = 2.2 Hz, J' = 6 Hz, CH_2Br), 4.02–4.044 (1H, m, C*H*OH), 4.24–4.26 (2H, bd, J = 4 Hz, CH_2OC=O), 4.88 (1H, m, N*H*), 5.33–5.38 (2H, m, C*H* = C*H*).

4.3. Synthesis of intermediate **3**

To a solution of **2** (1 mmol) in CH_2Cl_2 anhydrous (1 ml) is added the acyl chloride (3 mmol), pyridine (3 mmol) and DMAP (0.1 mmol). The reaction was allowed to stand at room temperature overnight. Then solvents were evaporated in vacuo and the residue purified by flash chromatography (104–20% EtOAc in petroleum ether), to give **3.**

4.4. Product **3a**

^{1}H NMR δ: 2.40 (1H, t, J = 8 Hz, CH_2CO), 3.15 (2H, bq, J = 6 Hz, CH_2N), 3.40 (2H, m, CH_2Br), 4.20 (1H, m, C*H*OCONH), 4.10 (1H, m, C*H*OH), 4.80 (1H, m, NH), 5.20 (1H, m, C*H*OCO).

4.5. Product **3b**

^{1}H NMR δ: 2.30–2.39 (2H, m, CH_2C=O), 3.142–3.17 (2H, m, CH_2NH), 3.46–3.52 (2H, m, CH_2Br), 4.24–4.28 (CH_2OC=ONH), 4.80 (1H, m, N*H*), 5.20–5.22 (1H, m, C*H*OC=O), 5.31–5.36 (2H, m, C*H* = C*H*)

4.6. Product **3c**

^{1}H NMR δ: 2.37–2.40 (2H, m, CH_2C=O) 3.19–3.23 (2H, bq, J = 12 Hz, CH_2NH), 3.45–3.49 (2H, dd, J = 2.2 Hz, J' = 6 Hz, CH_2Br), 4.24–4.26 (2H, bd, J = 4 Hz, CH_2OC=O), 4.88 (1H, m, N*H*), 5.09 (1H, m, C*H*OC=O), 5.33–5.38 (2H, m, C*H* = C*H*).

4.7. Product **3d**

^{1}H NMR δ: 2.30–2.39 (2H, m, CH_2C=O), 3.12–3.18 (2H, m, CH_2NH), 3.47–3.53 (2H, m, CH_2Br), 4.25–4.30 (CH_2OC=ONH), 4.80 (1H, m, N*H*), 5.20–5.22 (1H, m, C*H*OC=O), 5.32–5.37 (2H, m, C*H* = C*H*)

4.8. Synthesis of intermediate **5**

The acyl chloride (1 mmol) and dry benzyltriethylammonium chloride (0.1 mmol) were added to dry toluene (2 ml) under stirring for 5 min at 60 °C. Dry sodium azide (2.4 mmol) was added in portions (4 × 0.6 mmol) over 1 h at 60 °C under stirring. The solution was kept for 15 min at 80 °C. The alcohol (5 mmol) was then added at room temperature and allowed to stand overnight. The solution was diluted with ethyl ether (50 ml) washed with water and dried with anhydrous sodium sulphate. Solvent removal gave the product, which was purified on a column of flash silica gel, eluting with 0–2% MeOH in chloroform.

*4.9. Product **5a***

^{1}H NMR δ: 3.15 (4H, bq, J = 6 Hz, CH_2N), 3.40 (2H, m, CH_2Br), 4.02 (2H, m, CH_2OCONH), 4.10 (1H, m, C*H*OH), 5.20 (1H, m, C*H*OCONH), 5.60 (1H, m, N*H*).

C^{13} $CDCl_3$ 200 MHz δ: 14.13 (× 2), 22.74 (× 2), 26.80 (× 2), 29.35 (× 2), 29.41 (x4), 29.64 (x4), 29.73 (x4), 30.08 (× 2), 30.85 (× 2), 31.98 (× 2), 41.23, 51.96, 63.95, 70.88, 76.50, 155.26, 166.90.

*4.10. Product **5b***

^{1}H NMR δ: 3.19–3.23 (4H, bq, J = 12 Hz, CH_2NH), 3.45–3.49 (2H, dd, J = 2.2 Hz, J' = 6 Hz, CH_2Br), 4.24–4.26 (2H, bd, J = 4 Hz, CH_2OC=O), 4.88 (1H, m, N*H*), 5.20 (1H, m, CHOC=O), 5.33–5.38 (2H, m, C*H* = C*H*).

*4.11. Synthesis of type-**4** and type-**6** lipids*

To a solution of **3** (1 mmol) in CH_3CN (10 ml), morpholine or piperazine (20 mmol) is added. The reaction mixture was refluxed overnight, concentrated in vacuo to remove CH_3CN, and the residue was flash-chromatographed (chloroform for morpholine derivatives and 2% MeOH in chloroform for piperazine derivatives), to give **5**.

To a solution of **5** (1 mmol) in THF (10 ml), 2 M dimethylamine in THF (20 mmol) is added. The reaction mixture was refluxed overnight concentrated in vacuo to remove THF, and the residue was flash chromatographed on 5% MeOH in chloroform.

*4.12. Product **4a***

^{1}H NMR δ: 2.40 (1H, t, J = 8 Hz, CH_2CO), 2.65 (4H, m, CH_2N), 3.15 (2H, bq, J = 6 Hz, CH_2N), 3.72 (4H, m, CH_2O), 4.20 (1H, m, C*H*OCONH), 4.10 (1H, m, C*H*OH), 4.80 (1H, m, NH), 5.20 (1H, m, C*H*OCO).

*4.13. Product **4b***

^{1}H NMR δ: 2.29–2.32 (2H, t, J = 6 Hz, CH_2C=O), 2.60 (4H, m, CH_2N), 3.19–3.22 (2H, bq, CH_2NHC=O), 3.77 (4H, m, CH_2O), 4.19–4.22 (2H, bq, CH_2OC=O), 4.80 (1H, m, N*H*), 5.20 (1H, m, C*H*OC=O).

*4.14. Product **4c***

^{1}H NMR δ: 2.29–2.33 (2H, t, J = 6 Hz, CH_2C=O), 2.50–2.54 (4H, m, CH_2N), 3.15–3.18 (2H, bq, CH_2NHC=O), 3.67–3.72 (4H, t, J = 4 Hz, CH_2O), 4.19–4.22 (2H, bq, CH_2OC=O), 4.80 (1H, m, N*H*), 5.20 (1H, m, C*H*OC=O), 5.30 (2H, m, C*H* = C*H*).

*4.15. Product **4d***

^{1}H NMR δ: 2.30–2.39 (2H, m, CH_2C=O), 3.10 (2H, m, CH_2N), 3.14–3.17 (2H, m, CH_2NH), 3.40 (4H, m, CH_2N), 3.62 (4H, m, CH_2NH), 4.24–4.28 (CH_2OC=ONH), 4.80 (1H, m, N*H*), 5.20–5.22 (1H, m, C*H*OC=O), 5.31–5.36 (2H, m, C*H* = C*H*).

*4.16. Product **4e***

^{1}H NMR δ: 2.29–2.33 (2H, t, J = 6 Hz, CH_2C=O), 2.50–2.54 (4H, m, CH_2N), 3.15–3.18 (2H, bq, CH_2NHC=O), 3.67–3.72 (4H, t, J = 4 Hz, CH_2O), 4.19–4.22 (2H, bq, CH_2OC=O), 4.80 (1H, m, N*H*), 5.20 (1H, m, C*H*OC=O), 5.30 (4H, m, C*H* = C*H*).

*4.17. Product **4f***

^{1}H NMR δ: 2.30–2.39 (2H, m, CH_2C=O), 3.10 (2H, m, CH_2N), 3.14–3.17 (2H, m, CH_2NH), 3.40 (4H, m, CH_2N), 3.62 (4H, m, CH_2NH), 4.24–4.28 (CH_2OC=ONH), 4.80 (1H, m, N*H*), 5.20–5.22 (1H, m, C*H*OC=O), 5.31–5.36 (4H, m, C*H* = C*H*)

*4.18. Product **4g***

^{1}H NMR δ: 2.29–2.33 (2H, t, J = 6 Hz, CH_2C=O), 2.50–2.54 (4H, m, CH_2N), 3.15–3.18 (2H, bq, CH_2NHC=O), 3.67–3.72 (4H, t, J = 4 Hz, CH_2O), 4.19–4.22 (2H, bq, CH_2OC=O), 4.80 (1H, m, N*H*), 5.20 (1H, m, C*H*OC=O), 5.30 (4H, m, C*H* = C*H*).

*4.19. Product **4h***

^{1}H NMR δ: 2.30–2.39 (2H, m, CH_2C=O), 3.10 (2H, m, CH_2N), 3.14–3.17 (2H, m, CH_2NH), 3.40 (4H, m, CH_2N), 3.62 (4H, m, CH_2NH), 4.24–4.28 (CH_2OC=ONH), 4.80 (1H, m, N*H*), 5.20–5.22 (1H, m, C*H*OC=O), 5.31–5.36 (2H, m, C*H* = C*H*).

*4.20. Product **6a***

^{1}H NMR δ: 3.15 (4H, bq, J = 6 Hz, CH_2NHCO), 2.60 (4H, m, CH_2NH), 3.40 (4H, m, CH_2N), 3.70 (4H, m, CH_2O), 4.02 (2H, m, CH_2OCONH), 4.10 (1H, m, C*H*O), 4.90 (1H, m, NH), 5.20 (2H, m, C*H*OCONH).

*4.21. Product **6b***

^{1}H NMR δ: 3.15 (4H, bq, J = 6 Hz, CH_2NHCO), 2.90 (4H, m, CH_2NH), 3.20 (2H, m, CH_2N), 3.40 (4H, m, CH_2N), 4.02 (2H, m, CH_2OCONH), 4.10 (1H, m, C*H*O), 4.90 (1H, m, NH), 5.20 (2H, m, C*H*OCONH).

*4.22. Product **6c***

^{1}H NMR δ: 3.15 (4H, bq, J = 6 Hz, CH_2NHCO), 2.60 (4H, m, CH_2NH), 3.40 (4H, m, CH_2N), 3.70 (4H, m, CH_2O), 4.02 (2H, m, CH_2OCONH), 4.10 (1H, m, C*H*O), 4.90 (1H, m, NH), 5.20 (2H, m, C*H*OCONH).

*4.23. Product **6d***

^{1}H NMR δ: 3.15 (4H, bq, J = 6 Hz, CH_2NHCO), 2.90 (4H, m, CH_2NH), 3.20 (2H, m, CH_2N), 3.40 (4H, m, CH_2N), 4.02 (2H, m, CH_2OCONH), 4.10 (1H, m, C*H*O), 4.90 (1H, m, NH), 5.20 (2H, m, C*H*OCONH).

*4.24. Product **6e***

^{1}H NMR δ: 2.32–2.37 (2H, m, CH_2N), 2.38 (6H, s, N (CH_3)$_2$), 3.13–3.16 (2H, bq, CH_2N), 3.85–3.99 (2H, m, CH_2OC=O), 5.20 (1H,m, C*H*OC=O), 5.30–5.36 (4H, m, C*H* = C*H*).

*4.25. Product **6f***

^{1}H NMR δ: 2.36–2.39 (2H, m, CH_2N), 2.41 (6H, s, N (CH_3)$_2$), 3.15–3.19 (2H, bq, CH_2N), 3.92–3.99 (2H, m, CH_2OC=O), 5.25 (1H,m, C*H*OC=O).

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