# SYNTHESIS AND CHARACTERIZATION OF A CARBOXYLIC ACID DERIVATIVE FOR LIPOSOMAL PREPARATIONS

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**Abstract**

A new compound with cholesteryl-derived structure containing a carboxylic acid functional group has been prepared in four steps starting from cholesterol. The product has been characterized by $^{1}H$, $^{13}C$ NMR and mass spectrometry analysis. It will be used for liposomal preparations, in order to evaluate the interaction of liposomes functionalized with carboxylic acid functional groups and hydroxyapatite.

**Rezumat**

Un nou compus cu structură derivată de la colesterol, care conține o grupare funcțională de acid carboxilic, a fost preparat în patru etape plecând de la colesterol. Produsul a fost caracterizat prin analiza spectrală $^{1}H$ și $^{13}C$-RMN și analizat prin spectrometrie de masă. Compusul va fi folosit pentru obținerea de preparate lipozomale, în vederea evaluării afinității dintre lipozomii funcționalizați cu grupări de acid carboxilic și hidroxiapatită.

**Keywords**: liposomes, cholesterol, carboxylic acid, spectral analysis

## Introduction

Bone tissue pathology includes diseases like osteoporosis, osteomyelitis, Paget's disease, etc. Classical medication is commercially available for these affections, but serious side effects (nausea, diarrhea, indigestion, constipation, headaches, etc.) are known to accompany these treatments. The unpleasant effects appear as a result of the lack of selectivity of the drugs. Creating modern drug delivery systems that can specifically target the bones, represents a very interesting perspective in treating bone-related diseases. Along with the decrease of systemic toxicity, osteotropic drug delivery systems allow the specific accumulation of the active principles in their therapeutic sites of action, increasing the drugs' efficacy.

Studies showed that various compounds posses HA (hydroxyapatite) affinity [1]. Among these compounds, the most important are tetracycline

[2] and its derivatives [3,4], phosphonates, bisphosphonates, hydroxybisphosphonates [5,6], derivatives of polymalonic acid [7], heterocycles with acidic functional groups [8], aromatic derivatives able to chelate $Ca^{2+}$ ions from HA [9], etc.

A great amount of attention has lately been devoted to liposomes, artificial lipid vesicles intensively studied because of their capacity of incorporating active principles with lipophilic, hydrophilic and amphiphilic structures [10, 11]. Several recent published papers have reported the preparation of liposomes functionalized with hydroxybisphosphonic acid (HBPA) functional groups and proved that they can bind to the inorganic part of bone (hydroxyapatite) with high affinity [12, 13, 14]. These liposomes posses negative zeta potential at physiologic pH due the presence of HBPA groups on their surface. Hengst et al. stated that it is not clear yet if specific HA-HBPA interactions are involved in the affinity of these liposomes for HA [13]. In order to verify if the affinity is due to specific HA-HBPA interactions or to the negative charge of the liposomes, we have prepared a ligand that does not contain the HBPA moiety, but which has negative charge at physiologic pH. The only difference between the new ligand and the previous tested compound is the presence of a carboxylic acid group instead of HBPA (Figure 1).

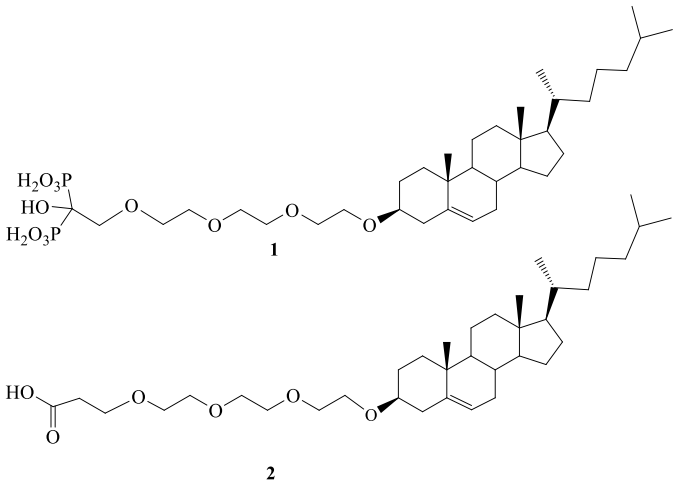



**Figure 1**

Structures of ligands designed for liposomal preparations with HA affinity: **1**-ligand proposed by Hengst et al. [13]; **2**-new ligand proposed in this study.

These liposomes may possess applications in the treatment of bone-related diseases, for example in the regeneration of bone tissue [15]. Therefore, in this study, we present the synthesis of 12-cholesteryloxi-

4,7,10-trioxadodecanoic acid **2** and its characterization by $^{1}H$, $^{13}C$-NMR and mass spectrometry analysis.

## Materials and Methods

General methods. All reagents were Merck products and were used as received. Thin layer chromatography was carried out with pre-coated silica gel plates (E. Merck, Silica gel 60 F254) and the spots were detected by immersing the plates in 10% phosphomolybdic acid solution in ethanol followed by heating. Column chromatography was performed on silica gel 60 Å (Carlo Ebra, 35-70 μm). The NMR spectra were recorded in $CDCl_3$ at 20°C on a Bruker DRX-400 spectrometer working at 400.13 MHz for $^{1}H$ and 100.62 MHz for $^{13}C$. The chemical shifts (δ) of $^{1}H$ and $^{13}C$ spectra are reported in ppm. The coupling constants (*J*) are reported in Hz. NMR spectra were recorded with the standard BRUKER sequences. The numbering system used in the assignments of the NMR spectra is presented in figure 2. The numbering system is not conventional; it was chosen in order to conserve the known numbering system of cholesterol. Mass spectra were registered on a Waters Q/Tof mass spectrometer in methanol.

Experimental.

**Cholesteryl-p-toluenesulfonate (4)** p-toluenesulfonyl chloride (TsCl) (10g, 0.026 mol) was added to a solution of cholesterol (5.42g, 0.028 mol) in pyridine (23mL) and the resulting mixture was stirred at room temperature overnight (12h). The crude mixture was diluted with $CH_2Cl_2$ and washed successively with HCl 1N (until pH=1), $NaHCO_3$ (until pH=8), distilled water and brine. Each aqueous phase was washed with $CH_2Cl_2$. The reunited organic phases were dried over anhydrous $Na_2SO_4$, filtered and concentrated. The product can be used directly in the next step without any further purification, or it can be precipitated using a $CH_2Cl_2$/MeOH mixture and recrystallized from petroleum ether to obtain pure cholesteryl-p-toluenesulfonate as white crystals in 90% yield; Rf: 0.68 (petroleum ether/methylene chloride 5/5).

**8-cholesteryloxy-3,6-dioxaoctan-1-ol (5)** Triethylene glycol (32 mL, 257 mmol) was added to a suspension of cholesteryl-p-toluenesulfonate (**4**) (3.5g, 6,46 mmol) in dioxane (23 mL) and the mixture was stirred under reflux for 2 h. The solution was cooled, diluted with water and extracted with ethyl acetate (3 times). The reunited organic phases were washed with 5 % $NaHCO_3$ solution, water and brine, dried over anhydrous $Na_2SO_4$, filtered and concentrated. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 1/9) to obtain **5** as opaque gum (72%). Rf: 0.22 (petroleum ether/ethyl acetate 1/9).

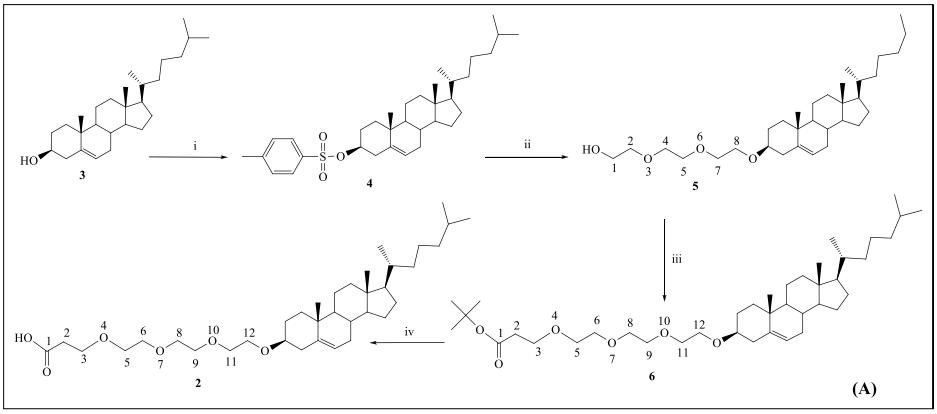
**Tert-butyl 12-cholesteryloxi-4,7,10-trioxadodecanoate (6)** A 50% NaOH solution (2.6 mL) was added to a solution formed by compound **5** (0.502 g, 0.97 mmol), n-butylsulfamic acid (0.041 g, 0.27 mmol) and tert-butyl 3-bromopropionate (0.22 mL, 1.36 mmol) in toluen (2.6 mL). The resulting mixture was stirred at room temperature for 2 hours and then it was diluted with ethyl acetate and acidified with 2N HCl solution. The aqueous phase was washed with diethyl ether and the reunited organic phases were washed with NaCl saturated solution, dried over anhydrous $Na_2SO_4$, filtered and concentrated. The residue was purified by chromatography on silica gel column (petroleum ether/ethyl acetate 6.5/3.5 v/v) to yield **6** as colourless oil (60%). Rf: 0.61 (EP/AcOEt 1/9 v/v); $^1$H NMR (400.13 MHz, $CDCl_3$): 0.67 (s, 3H, $H_{18}$); 0.85 (d, 3H, $^3J_{H26-H25}$=6.6 Hz, $H_{26}$); 0.86 (d, 3H, $^3J_{H27-H25}$=6.6 Hz, $H_{27}$); 0.90 (d, 3H, $^3J_{H21-H20}$=6.6 Hz, $H_{21}$); 0.99 (s, 3H, $H_{19}$); 0.83-2.38 (m, 28H, protons of cholesteryl group); 1.44 (s, 9H, t-Bu); 2.49 (t, 2H, $^3J_{H35-H34}$=6.6 Hz, H35); 3.17 (m, 1H, $H_3$); 3.58-3.67 (m, 12H, H28-H33); 3.70 (t, 2H, $^3J_{H34-H35}$=6.6 Hz, H34); 5.32-5.33 (m, 1H, $H_6$); $^{13}$C NMR (100.62 MHz, $CDCl_3$) δ (ppm): 11.8 (C18); 18.7 (C21); 19.4 (C19); 21.0 (C11); 22.5 and 22.8 (C26 and C27); 23.8 (C23); 24.3 (C15); 27.9 (C25); 28.1 (3C, *C*$H_3$-t Bu); 28.2 and 28.4 (C2 and C16); 31.9 (2C, C8 and C7); 35.8 (C20); 36.2 (C22); 36.3 (C35); 36.9 (C10); 37.2 (C1); 39.0 (C4); 39.5 (C24); 39.8 (C12); 42.3 (C13); 50.2 (C9); 56.1 (C17); 56.8 (C14); 66.9 (C34); 67.3, 70.4, 70.5, 70.6 (2C), 70.9 (C28-C33); 79.5 (C3); 80.5 ($C_{IV}$-t Bu); 121.5 (C6); 141.0 (C5); 170.9 (C36); MS: (ESI$^+$/MeOH) m/z: 669.7 $[M+Na]^+$.

**12-cholesteryloxi-4,7,10-trioxadodecanoic acid (2)**. p-toluene-sulfonic acid (p-TsOH) (0.05 g, 0.261 mmol) was added to a solution of compound **6** (0.563 g, 0.870 mmol) in toluene (25 mL) and the resulting mixture was refluxed for 40 minutes. After cooling, a saturated $NaHCO_3$ solution was added to the reaction mixture; the upper layer was separated, dried over anhydrous $Na_2SO_4$, filtered and concentrated. The residue was purified by chromatography on silica gel column (petroleum ether/ethyl acetate 6/4 v/v as eluent until complete elution of impurities, then pure ethyl acetate) to obtain **2** as pale yellow oil (75%). Rf: 0.23 ($CH_2Cl_2$/MeOH 9.5/0.5). $^1$H NMR (400.13 MHz, $CDCl_3$): 0.66 (s, 3H, $H_{18}$); 0.85 (d, 3H, $^3J_{H26-H25}$=6.6 Hz, $H_{26}$); 0.86 (d, 3H, $^3J_{H27-H25}$=6.6 Hz, $H_{27}$); 0.90 (d, 3H, $^3J_{H21-H20}$=6.5 Hz, $H_{21}$); 0.98 (s, 3H, $H_{19}$); 0.83-2.39 (m, 28H, protons of cholesteryl group); 2.61 (t, 2H, $^3J_{H35-H34}$=6.1 Hz, H35); 3.20 (m, 1H, $H_3$); 3.58-3.68 (m, 12H, H28-H33); 3.76 (t, 2H, $^3J_{H34-H35}$=6.2 Hz, H34); 5.32-5.33 (m, 1H, $H_6$); 5.34 (broad s, 1H, -COO*H*); $^{13}$C NMR (100.62 MHz, $CDCl_3$) δ (ppm): 11.8 (C18); 18.7 (C21); 19.4 (C19); 21.0 (C11); 22.5 and

22.8 (C26 and C27); 23.4 (C23); 24.3 (C15); 28.0 (C25); 28.2 (C2 and C16); 31.9 (C8 and C7); 35.0 (C35); 35.7 (C20); 36.2 (C22); 36.8 (C10); 37.2 (C1); 38.9 (C4); 39.5 (C24); 39.8 (C12); 42.3 (C13); 50.2 (C9); 56.1 (C17); 56.8 (C14); 66.5 (C34); 67.2, 70.2, 70.4, 70.5, 70.6, 70.7 (C28-C33); 79.7 (C3); 121.6 (C6); 141.8 (C5); 174.8 (C36); MS ($ESI^+$/MeOH) m/z: 613.7 $[M+Na]^+$; MS ($ESI^-$/MeOH) m/z: 589.7 $[M-H]^-$, 625.7 $[M+Cl]^-$.

## Results and Discussion

12-cholesteryloxi-4,7,10-trioxadodecanoic acid (**2**) has been prepared in four steps starting from cholesterol (Figure 2).



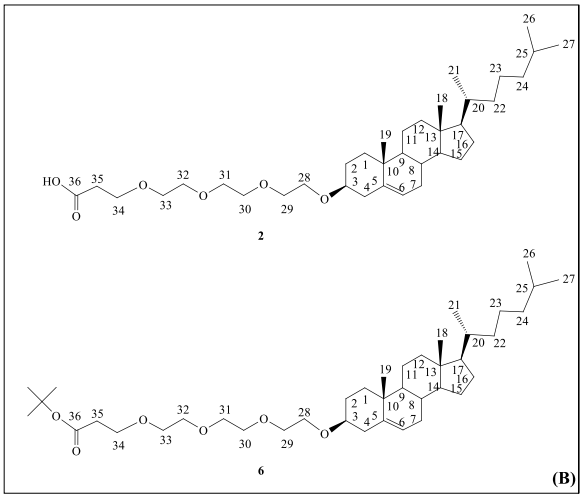




**Figure 2**

Frame A: Synthesis of **2**. Reagents, conditions and yields: (i) TsCl, pyridine, 12h at room temperature; 90%; (ii) Triethyleneglycol, dioxan, 2h at refluxing temperature; 72%; (iii) t-butyl 3-bromopropionate, n-butylsulfamic acid, Toluene/NaOH 50%; 60%; (iv) p-TsOH, toluene, 40 min at refluxing temperature; 75% (overall yield: 29% - calculated starting from cholesterol). Frame B: Numbering system used in the NMR assignments.

In the first step, cholesterol has been activated as tosylate, followed by the reaction of the obtained derivative with triethylene glycol in dioxane, using Davis' procedure [16]. The NMR data obtained for the two compounds (**4**, **5**) are in agreement with previously published results [17-19].

Starting from compound **5**, product **2** has been obtained in two steps. The conversion of the hydroxyl group of **5** to a carboxylic acid could be realized by oxidation, using published procedures [20], but the product would have had one carbon atom less than the desired compound **2**. A possible conversion of **5** into **2** was the reaction of **5** with lithiumbromopropionate in THF at -78ºC, using a method similar to that described by Greb [21]. We have chosen to apply the method of Wu et al. [22], which consists in two steps: (a) reaction of **5** with t-butyl 3-bromopropionate in toluene/water as solvent, in the presence of n-butylsulfamic acid as phase transfer catalyst; (b) deprotection of the t-butyl ester **6** in toluene with p-toluensulfonic acid as catalyst.

The $^{1}H$ NMR spectrum of **2** (Figure 3) proved that all the signals typical for compound **2** were present:

- the peaks characteristic for the cholesteryl group: the singlets of $H_{18}$ and $H_{19}$; three doublets corresponding to $H_{21}$, $H_{26}$ and $H_{27}$; a massif of peaks due to the four cholesteryl rings and two multiplets associated to $H_3$ and $H_6$, the most deshielded proton of the cholesterol residue.
- the peaks of the triethylene glycol-derived spacer. $H_{28}$-$H_{33}$ give as signal a massif of peaks (3.58-3.68 ppm), while $H_{34}$ and $H_{35}$ give as signals two triplets, at 3.76 and 2.61, respectively. The coupling constant $^{3}J_{H35\text{-}H34}$ is 6.1 Hz. Because of the absence of oxygen atoms in alpha position of $H_{35}$, these protons are the most shielded, when compared to the other protons of the oxygenated spacer. In the $^{1}H$-$^{1}H$ correlation (COSY) spectrum, the coupling of protons $H_{34}$ and $H_{35}$ can be observed (red lines in Figure 4).
- The proton of the carboxylic acid group has as signal a typical broad singlet overlapped with the signal of $H_6$.

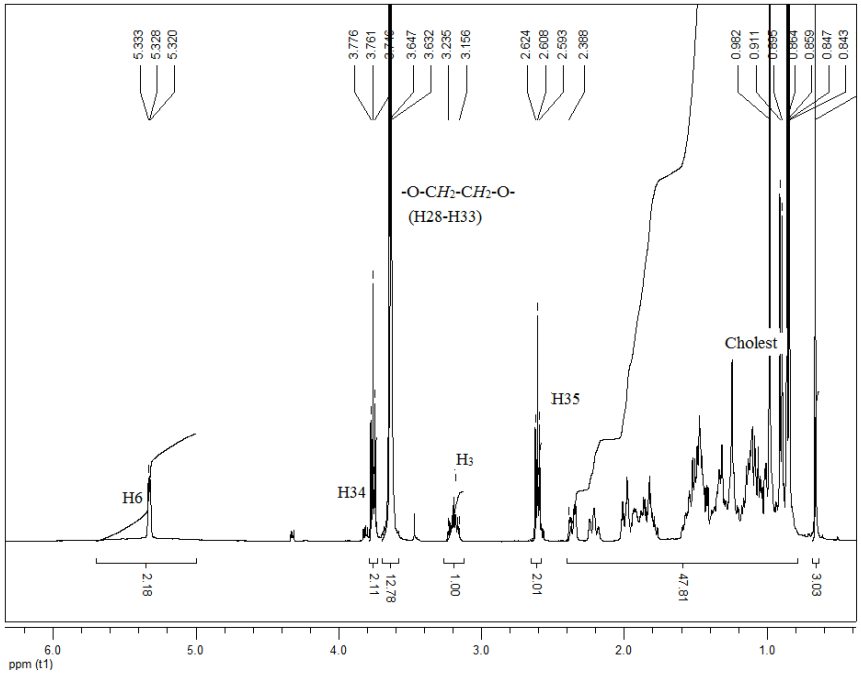



**Figure 3**

$^{1}$H-NMR spectrum of 12-cholesteryloxi-4,7,10-trioxadodecanoic acid **(2)**

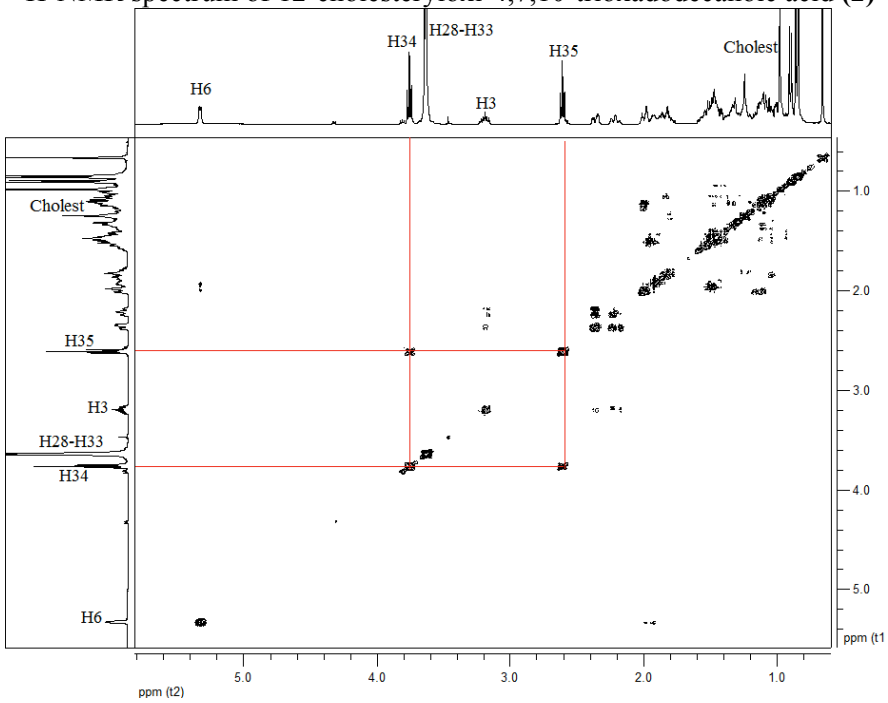



**Figure 4**

COSY spectrum of 12-cholesteryloxi-4,7,10-trioxadodecanoic acid **(2)**

In Figure 5, a comparison between the NMR spectra of compounds **6** (details A and B) and **2** (details C and D) has been realized. The deprotection of the t-butyl ester is reflected in:

- The absence of the signal specific for the t-butyl group (1.44 ppm in compound **6**-detail A) in the spectrum of the carboxylic acid **2** (detail C).
- The presence of a broad singlet due to the proton of the carboxylic acid group (detail D). This signal was absent in the spectrum of the t-butyl ester **6** (detail B).

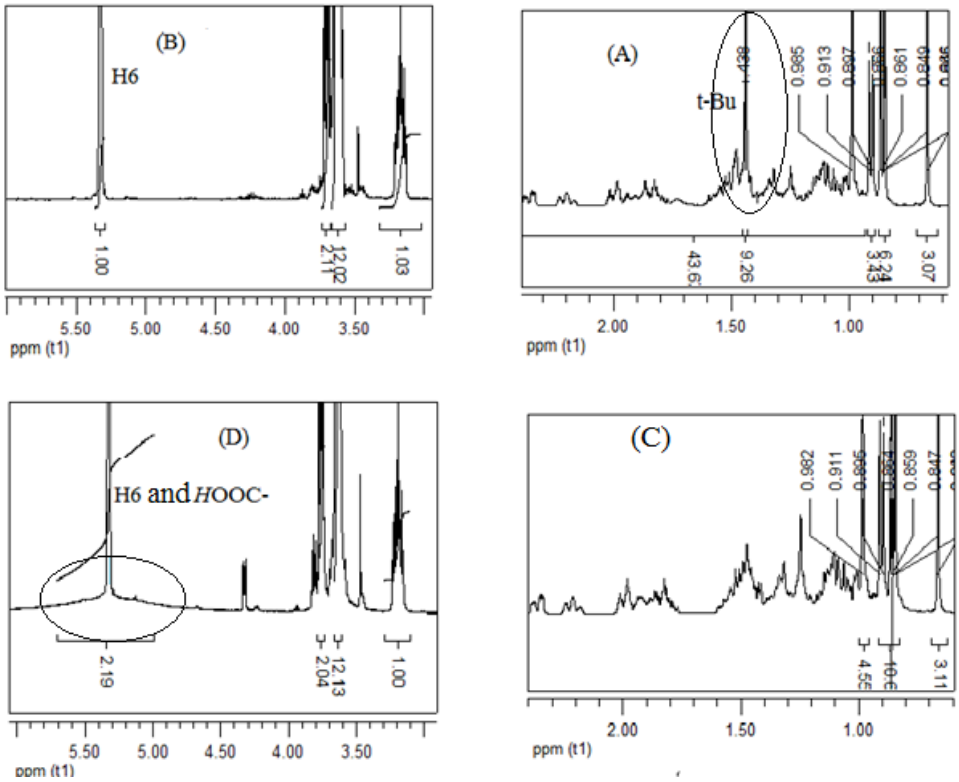



**Figure 5**

Details of the $^{1}$H NMR spectra of compounds **6** (A, B) and **2** (C,D).

Similar observations can be realized when the $^{13}$C NMR spectra of two products **2** and **6** are compared (Figure 6). In the frames B, C (compound **2**) and D, E (compound **6**), details of the regions which confirm the deprotection of the carboxylic acid are presented. For an easier understanding of the spectra, it has to be firstly noticed that in the $^{13}$C-APT (Attached-Proton-Test) NMR spectrum of compound **6,** the negative phase

of the peaks corresponds to $–CH_2-$ and to quaternary carbon atoms and the positive phase to –CH- and $–CH_3-$ atoms, while in the $^{13}$C-APT NMR spectrum of the acid **2**, the negative phase corresponds to odd carbon atoms and the positive phase to even atoms. This can be quickly noticed in details B and D, where it can be observed that $CDCl_3$ peaks have positive and negative phases, respectively. Compared to the spectrum of compound **6**, the spectrum of the carboxylic acid **2** doesn't contain the signals of the t-butyl group: (a) the peak due to the three primary carbon atoms, at 28.1 ppm in detail E; (b) the peak due to the quaternary carbon atom, at 79.5 ppm in detail D.

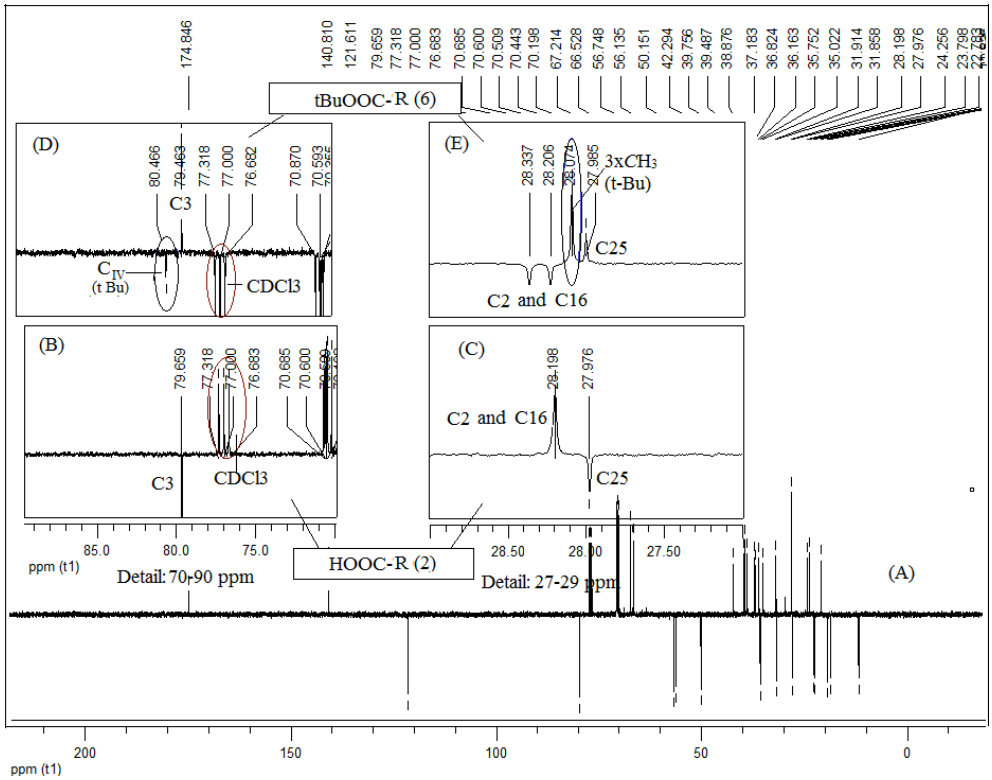



**Figure 6**

Full $^{13}$C-APT NMR spectrum of compound **2** (spectrum A) and details of the regions: 70-90 ppm (B) and 27-29 ppm (C). In frames (D) and (E), details of the corresponding regions of the $^{13}$C-APT NMR spectrum of compound **6** are presented for comparison.

The structure of the desired carboxylic acid **2** has also been confirmed by mass spectrometry. In Figure 7, it can be observed that the compound ionises in both positive (m/z=613.69 $[M+Na]^+$; spectrum A) and negative (m/z=589.68 $[M-H]^-$ and m/z=625.70 $[M+Cl]^-$ spectum B) modes.

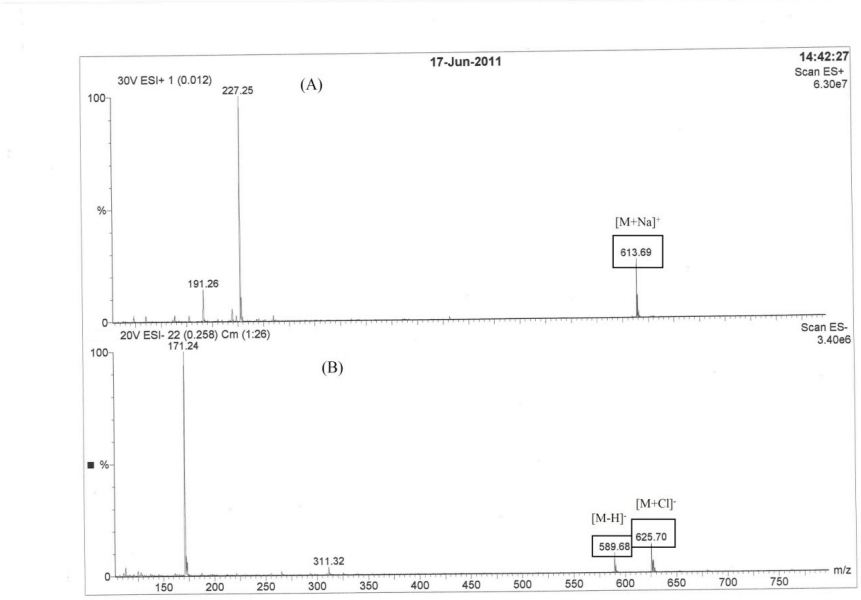



**Figure 7**
Electrospray ionization mass spectra of compound **2** in positive (spectrum A) and negative (spectrum B) modes.

## Conclusions

In this paper, we have presented the synthesis of a new carboxylic acid. The compound has been obtained in four steps starting from cholesterol, in 29% overall yield. Two of the intermediates obtained in this study (**4** and **5**) have previously been described, [17-19] while two of them are reported for the first time in this paper (**6** and **2**). The preparation and structural characterization of these compounds has been realized, with a detailed presentation of the former two. In perspective, quantitative binding assays will be realized, in order to evaluate the affinity of liposomes functionalized with the new ligand and HA.

**Acknowledgements**
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