Original Paper

Characterization of a tosylated cholesteryl derivative

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ABSTRACT: Purpose. This study presents the synthesis of 11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate and its detailed structural characterization. Bearing a p-toluenesulfonyl group, it is a very versatile compound that can be used in the synthesis of a wide variety of derivatives that can be incorporated in liposomes, with various applications in modern drug delivery systems. Material/Methods. The compound has been prepared in a sequence of three reactions, using cholesterol as starting compound. After purification, the tosylated product has been analysed by 1H, 13C NMR and mass spectrometry. Results. The performed analysis confirmed the structure of the desired derivative. Conclusions.11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate has been successfully obtained as a pure compound in 58.26 % overall yield starting from cholesterol.

KEYWORDS: *liposomes, ligands, cholesterol, NMR analysis, mass spectrometry*

Introduction

Liposomes are spherical artificial vesicles formed by one or more lipid double-layers that entrap an equal number of water compartments in their interior. Liposomes were first mentioned in 1964 by A. Bangman and R. Horne [1] and were initially studied as artificial mimics of cellular membranes. Since then, they received an increasing amount of attention from scientists, being nowadays adapted for cosmetic formulations and as drug transporting systems. One of the main advantages of liposomes as drug carriers is their ability of targeting specific cell lines, tissues or organs.[2] In passive targeting, liposomes can extravasate the vascular endothelium and selectively accumulate at infection, inflammation and tumour sites through the EPR effect (« The enhanced permeability and retention effect ») [3]. Nowadays, liposomal formulations are commercially available for cancer therapy (ovarian and breast cancer, Kaposi sarcoma, using doxorubicin or daunorubicin as active principles) and fungal infections (Amphotericin B as active principle), while other formulations are in different phases of clinical trials. [4], [5] Meanwhile, active targeting has been intensively studied. It relies on specific interactions between molecules that decorate the surface of liposomes and the targeted cells. Usually, they involve antigen-antiboby and ligand-receptor interactions. Liposomes must therefore be functionalized with molecules able to selectively bind to target.

One of the strategies used for decorating liposomes with specific functional groups consists in integrating compounds containing a lipophilic part in the lipid bilayer of liposomes (Figure 1). Cholesteryl residues are often preferred as anchors, because they easily integrate in the lipid layers through hydrophobic interactions. Chlesterol is an important constituent of cellular membranes, playing an important role in their stabilisation and modulating their physical and chemical properties.[6, 7] Usually, an oxygenated spacer separates the cholesteryl part from the targeting moiety. Oligoethyleneglycols are used as spacers because of their high stability in biologic environment compared for example with esters.

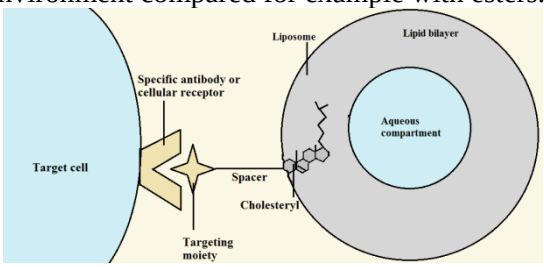

Fig.1.Schematic representation of the interaction of a liposome functionalized with a targeting group and the target cell.

Therefore, methods that can easily afford functionalized ligands with cholesteryl-olygoehtylenglycol-derived structure are of great importance in nanomedicine research. In this study, we present the synthesis and detailed characterization of 11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate TsO-TetEG-C (1). The compound is known [8,9] but

it is for the first time that its detailed characterization by NMR and mass spectrometry is reported.

The p-toluenesulfonate is a good leaving group that can be displaced by a variety of nucleophiles (ions like: hydrogen sulfide, cyanide, azide, halides, alkynes, thiolates, anions of carboxylic acids, anions of active methylene compounds, neutral nucleophiles, etc.) in order to introduce different functional groups. Thus, TsO-TetEG-C is a versatile intermediate that can be used in the synthesis of ligands for liposomal preparations.

Material 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 ^1H and 100.62 MHz for ^{13}C. The chemical shifts (δ) of ^1H 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 scheme 1. Mass spectra were registered on a Waters Q/Tof mass spectrometer in methanol.

Experimental. Cholesteryl-p-toluenesulfonate (3) and 11-cholesteryloxy-3,6,9-trioxaundecan-1-ol (**4**) have been prepared using known procedures [9, 10, 11, 12]. The synthesis of 11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate (1) has been realized using Bhattacharya's modified procedure of tosylation, as described below.

11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate (1). p-toluenesulfonyl chloride (3.01 g, 15.47 mmol) was added to a solution of 4 (3.96 g, 7.03 mmol) in a mixture of CH_2Cl_2 (55 mL) and pyridine (8 mL). The solution was stirred at room temperature overnight and then the mixture was partitioned between CH_2Cl_2 and HCl 1N. The aqueous phase was washed with CH_2Cl_2 and the reunited organic phases were washed with $NaHCO_3$, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 7.5/2.5) to obtain 1 as colorless oil (90.86 %); Rf: 0.60 (petroleum ether/ethyl acetate 1/9). For ^1H and ^{13}C NMR and mass spectrometry analysis, see the results and discussion part.

Results and discussions

11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate (1) has been prepared in three steps starting from cholesterol (2) (Fig.2.).

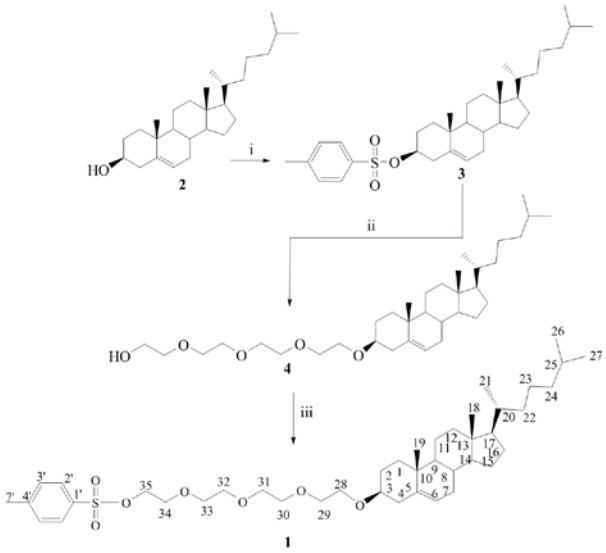

Fig.2.Synthesis of pTsO-TetEG-C (1). Reagents, conditions and yields: (i) tosyl chloride, pyridine, 12h at r. t., (ii) tetraethyleneglycol, dioxane, 2h at refluxing temperature; Yield: 64.13% over the two steps (i) and (ii); (iii) tosyl chloride, pyridine, CH_2Cl_2, 12h at room temperature, Yield: 90.86 %; Overall yield: 58.26%.

A known protocol of tosylation using David's procedure followed by reaction of the obtained p-toluenesulfonate ester with tetraethyleneglycol in dioxane have been applied in order to obtain 11-cholesteryloxy-3,6,9-trioxaundecan-1-ol (**4**). The two steps have been realized without purification of the intermediate cholesteryl p-toluenesulfonate (3), leading to 11-cholesteryloxy-3,6,9-trioxaundecan-1-ol (**4**) in 64% yield over the two steps. Subsequent activation of the hydroxyle group of 4 as p-toluenesulfonyl ester afforded the pure desired compound 1 in 90.86% yield.

^1H and ^{13}C NMR spectra of pure TsO-TetEG-C have been recorded. The ^1H NMR spectrum (Fig. 3) contains the following signals:

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 tetraethyleneglycol-derived spacer. H_{28}-H_{33} gives as signal a massif of peaks integrating for 12 protons (3.56-3.65 ppm), while H_{34} and H_{35}, being in the proximity of the p-toluenesulfonyl ester, are more deshilded, at 3.48 and 4.16, respectively.

the peaks of the p-toluenesulfonyl group: $H_{2'}$, $H_{3'}$, $H_{5'}$ and $H_{6'}$ give signals specific for para-subtituted aromatic rings while the methyl group gives a singlet integrating for three protons at 2.44 ppm.

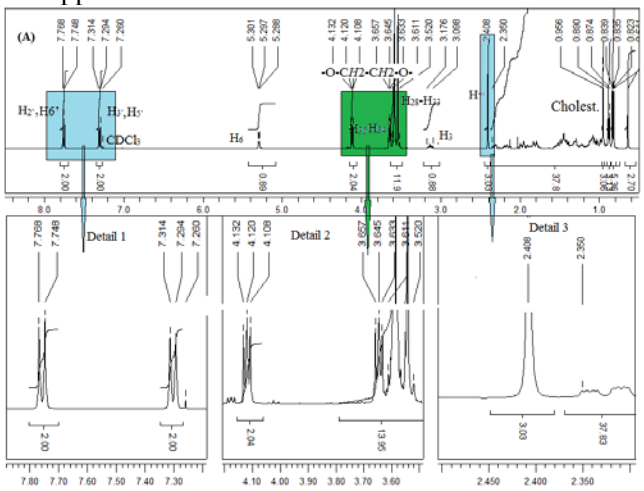

Fig.3.Full ^{1}H NMR spectrum of pTsO-TetEG-C (spectrum A) and details of the regions: 7.2-7.9 ppm (detail 1); 3.5-4.2 ppm (detail 2); 2.3-2.5 ppm (detail 3). The peaks of the p-toluenesulfonyl group are indicated in blue windows; the peaks of tetraethylenglycol residue are marked in a green window; the remaining peaks belong to cholesteryl.

The ^{1}H NMR data of pTsO-TetEG-C are gathered in Table 1.

Table 1: ^{1}H NMR chemical shifts and coupling constants of pTsO-TetEG-C

Hydrogen atom	δ (ppm), multiplicity and coupling constants	Hydrogen atom	δ (ppm), multiplicity and coupling constants
H_1,H_2,H_4, H_7,H_8,H_9, H_{11},H_{12}, H_{14},H_{15}, H_{16},H_{17},H_{20}, H_{22},H_{23},H_{24},H_{25}	0.83-2.39 (m, 28H)	H_{27}	0.86 (d, 3H, $^3J_{H27-H25}$=6.6 Hz)
H_3	3.13-3.20 (m, 1H)	H_{28}-H_{33}	3.56-3.65 (m, 12H)
H_6	5.32-5.34 (m, 1H)	H_{34}	3.68 (m, 2H)
H_{18}	0.67 (s, 3H)	H_{35}	4.16 (m, 2H)
H_{19}	0.99 (s, 3H)	$H_{3'}$ and $H_{5'}$	7.27 (d, 2H, $^3J_{H3'-H2'}$=8.0 Hz)
H_{21}	0.90 (d, 3H, $^3J_{H21-H20}$=6.5 Hz)	$H_{2'}$ and $H_{6'}$	7.73 (d, 2H, $^3J_{H2'-H3'}$=8.3 Hz)
H_{26}	0.85 (d, 3H, $^3J_{H26-H25}$=6.6 Hz)	$H_{7'}$	2.44 (s, 3H, $H_{7'}$)

For the ^{13}C NMR analysis, ^{13}C APT experiment has been recorded. In this experiment, -CH and -CH_3 atoms have positive phases, while –CH_2 and carbons with no attached protons have negative phases (Figure 3). The spectrum contains:

The peaks of cholesterol, in good agreement with literature data [13,14];

The peaks of the secondary carbon atoms belonging to the tetraethyleneglycol-derived spacer, having negative phases;

The peaks of the p-toluenesulfonyl group: C7' (primary C atom, with positive phase); C2', C3', C5', C6' (CH, with positive phases); C1' and C4' (quaternary atoms with negative phases).

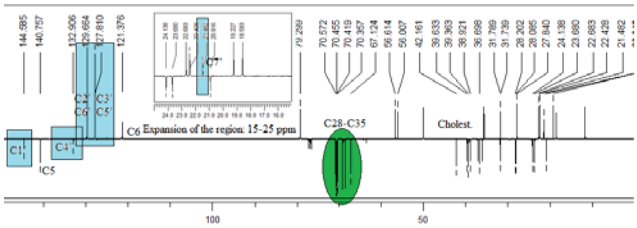

Fig.4. ^{13}C APT spectrum of pTsO-TetEG-C. The peaks of the p-toluenesulfonyl group are indicated in blue windows; the peaks of tetraethylenglycol residue are marked in a green window; the remaining peaks belong to cholesteryl.

The ^{13}C NMR data of pTsO-TetEG-C are presented in Table 2.

Table 2: ^{13}C NMR chemical shifts of pTsO-TetEG-C

Carbon atom	δ (ppm)	Carbon atom	δ (ppm)
C_1	37.1	C_{19}	19.2
C_2, C_{16}	28.1 and 28.2	C_{20}	35.6
C_3	79.3	C_{21}	18.7
C_4	38.9	C_{22}	36.0
C_5	140.6	C_{23}	23.7
C_6	121.4	C_{24}	39.4
C_7	31.8	C_{25}	27.8
C_8	31.7	C_{26}, C_{27}	22.4 and 22.7
C_9	50.0	C28-C33	67.1, 70.4 (2C),

			70.5, 70.6, 70.7
C_{10}	36.7	C34	68.5
C_{11}	20.9	C35	69.7
C_{12}	39.6	$C_{1'}$	144.6
C_{13}	42.2	$C_{2'}$; $C_{6'}$	129.7
C_{14}	56.6	$C_{3'}$; $C_{5'}$	128.7
C_{15}	24.1	$C_{4'}$	132.9
C_{17}	56.0	$C_{7'}$	21.5
C_{18}	11.8		

Bidimensional spectra of homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}C) correlations have also been recorded. They confirmed the assignments presented above and provided accurate attribution of peaks in the ^{13}C spectrum.

The structure of TsO-TetEG-C has also been proved by mass spectrometry (Figure 6). TsO-TetEG-C ionises in positive mode using electrospray ionization at m/z=739.78 $[M+Na]^+$.

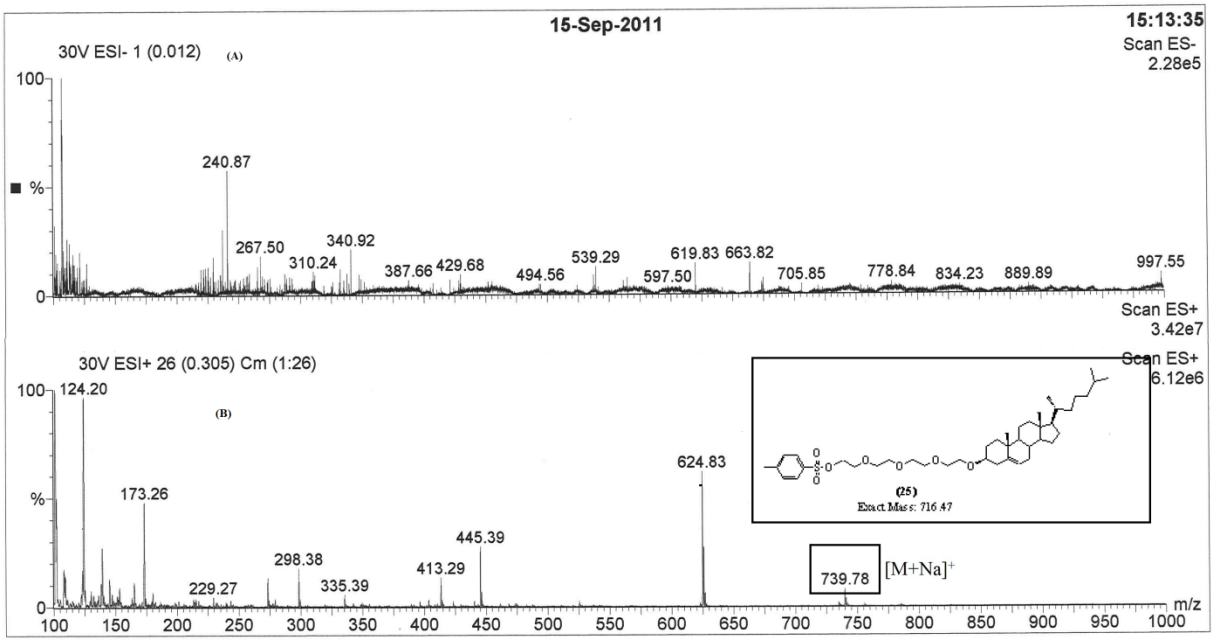

Fig.5.Mass spectra of pTsO-TetEG-C in methanol in negative mode (spectrum A) and positive mode (spectrum B).

Conclusion

In this study, we have presented the preparation of 11-cholesteryloxy-3,6,9-trioxaundecyl-1-p-toluenesulfonate with an overall yield of 58.26 % (calculated starting from cholesterol). A detailed presentation of the ^{1}H and ^{13}C NMR spectra of the compound has been realized and the mass spectra of the compound have been presented. Being a tosylate and having a cholesterol-derived structure, the product can be used in the synthesis of various compounds with applications in liposomal preparations.

Acknowledgements

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