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Letters in Organic Chemistry, **2016**, *13*, 71-75

New Chemoenzymatic Synthesis of (±)-N-(2-hydroxyethyl)-*N*,*N*-dime thyl-2,3-bis(tetradecyloxy)-1-propanammonium Bromide (DMRIE)

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Received February 24, 2015: Revised September 10, 2015: Accepted September 30, 2015

Abstract:

Background: Cytofectins are a class of positively charged lipid molecules that can facilitate the functional entry of polynucleotides, macromolecules, and small molecules into living cells, escaping lysosomal degradation. One of the most potent cytofectins, dimyristoyl Rosenthal inhibitor ether (DMRIE), is presently being used to deliver differents active molecules into animal and human diseased tissues. To our knowledge, two synthetic routes for the preparation of DMRIE have so far been reported. One of them is based on the reaction of epichlorhydrin with either dimethylamine chloride or dimethylamine using an alkaline solution as solvent. The main disadvantage of these routes is the formation of 3-(N,N-diamino)-1,2-propanediol. In summary, the synthetic routes mentioned use epichlorohydrin or glycidol as starting materials, both of which have been described as carcinogenic agents. Additionally, sometimes the reaction conditions are drastic. An alternative route for the preparation of other cytofectins is the use of glycerol as the starting material. Glycerol is a non-carcinogenic compound, but its use as starting material results in an increased number of steps of this reaction, generating an increase in the total time of synthesis and the formation of a greater amount of waste and scrap. Based on the above, here we propose a new chemoenzymatic synthetic strategy using glycerol as a starting material.

Methods: Initially, glycerol and vinyl benzoate were mixed in presence of *Mucor miehei* lipase (Lipozyme), it is converted into corresponding mono-benzoate (±)-1. Finally, DMRIE was obtained by the reaction of bromide (±)-3 with 2-dimethylaminoethanol (DMAE).

Results: We studied the stability of compound (±)-1 under the working conditions applied in the preparation of intermediate alcohol (±)-2. Additionally, we study different experimental parameters.

Conclusion: In summary, a new chemoenzymatic synthetic route was developed for the DMRIE using glycerol as the starting material. We studied and optimized various experimental parameters

of the various synthetic steps, and reached to develop the methodology to multigram scale, which compared to processes reported so far, has the advantages of fewer synthetic steps, the use of less aggressive reagents environment and generating fewer waste allowed to obtain the desired product; which results in a viable method for synthesizing analogs to DMRIE.

Keywords: Alkylation of tertiary amines, cationic lipid, cytofectin, DMRIE, glycerol, lipase.

INTRODUCTION

Cytofectins are a class of positively charged lipid molecules that can facilitate the functional entry of polynucleotides, macromolecules, and small molecules into living cells, escaping lysosomal degradation [1]. One of the most potent cytofectins, dimyristoyl Rosenthal inhibitor ether (DMRIE) [2], is presently being used to deliver differents active molecules into animal and human diseased tissues [3a].

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Normally, cytofectins are combined with an activity-augmenting phospholipid such as dioleoylphosphatidyl ethanolamine (DOPE) [4], and a film of dried, mixed lipid is prepared and hydrated to form cationic liposomes. The liposome solution is then mixed with a plasmid DNA solution to afford cytofectin-DNA complexes which, when presented to living cells, are internalized and the transgene is expressed [3a, 5, 6].

DMRIE, like all cationic lipids, is composed of three parts (Fig. **1**): a cationic head group (A), a lipophilic tail group (B), and a linker that tethers the hydrophilic head group and the hydrophobic tail group (C) [7].

To our knowledge, two synthetic routes for the preparation of DMRIE have so far been reported. One of them is

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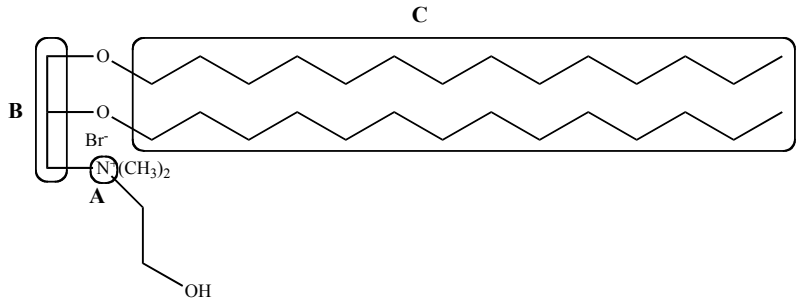
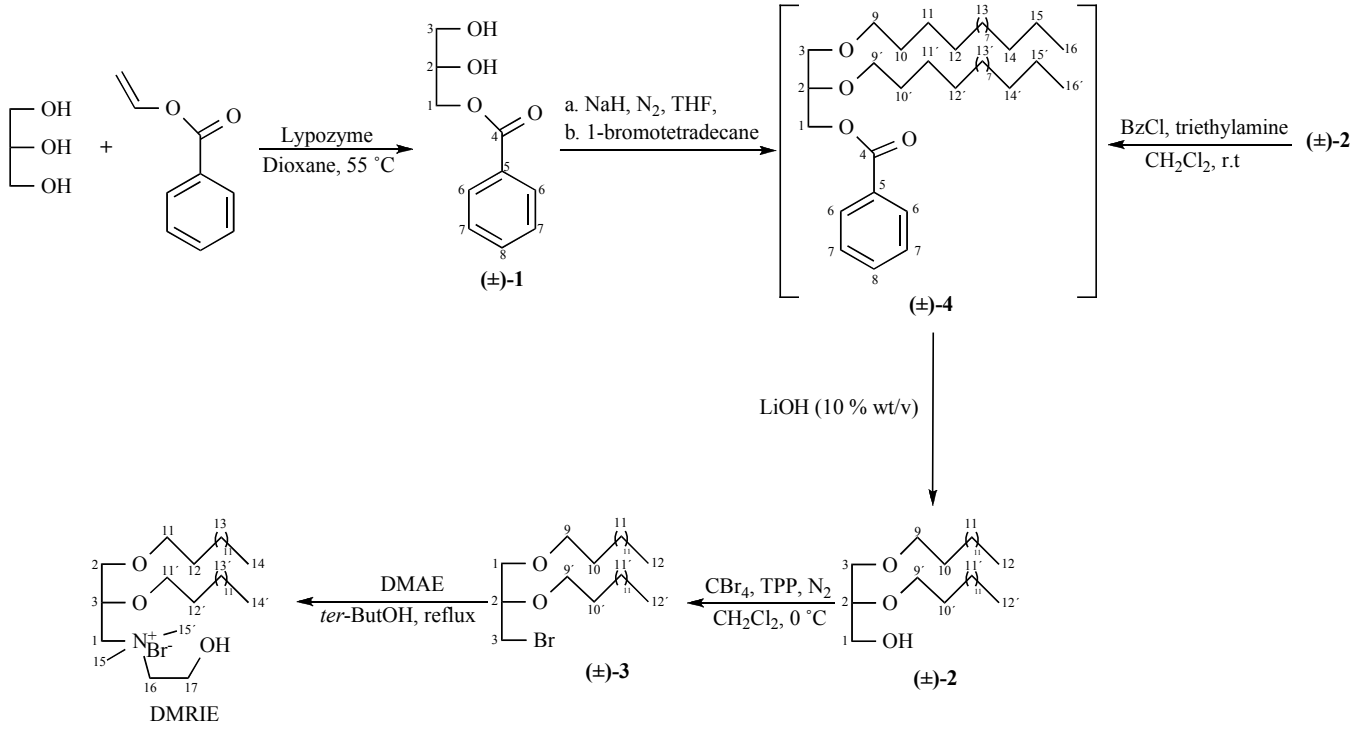


Fig. (1). Structure of DMRIE.



Scheme 1. Synthetic strategy of DMRIE using glycerol.

based on the reaction of epichlorhydrin with either dimethylamine chloride or dimethylamine using an alkaline solution as solvent [8].

Whereas the other [9], involves the protection of the hydroxyl group of the glycidol with the tosyl group and the opening of the epoxide in acid medium. Two hydrophobic tails are then formed, the tosyl group is hydrolyzed and the tertiary amino group is formed. The main disadvantage of these routes is the formation of 3-(*N*,*N*-diamino)-1,2-propanediol. In summary, the synthetic routes mentioned use epichlorohydrin or glycidol as starting materials, both of which have been described as carcinogenic agents [10-13]. Additionally, sometimes the reaction conditions are drastic.

An alternative route for the preparation of other cytofectins is the use of glycerol as the starting material, which is converted into the corresponding acetonide using acid catalysis. Subsequently, the free hydroxyl group is protected in the presence of benzyl bromide, hydrolyzed acetonide group being two free hydroxyl groups to react with the corresponding either tosyl or bromide alkyl, thereby forming the hydrophobic tails. Then, the benzyl group is removed by catalytic hydrogenolysis at high pressure if it is desired to shorten the reaction times (reaction times, 12 h at 50 psi, yield: 92% [7]; 36 h, 14.7 psi, yield: 70% [14]).

Glycerol is a non-carcinogenic compound, but its use as starting material results in an increased number of steps of this reaction, generating an increase in the total time of synthesis and the formation of a greater amount of waste and scrap.

Based on the above, here we propose a new synthetic strategy using glycerol as a starting material (Scheme **1**).

RESULTS AND DISCUSSION

Initially, equimolar amounts of glycerol and vinyl benzoate were mixed in presence of *Mucor miehei* lipase (Lipozyme), it is converted into (±)-2,3-dihydroxypropyl benzoate **(±)-1** (yield 75%). Thereafter, reaction with 1-bromotetradecane gives the benzoylated compound, which is hydrolyzed *in situ* by addition of a solution of LiOH (compound **(±)-2**). Compound **(±)-3** was obtained by treatment of **(±)-2** with carbon tetrabromide. Finally, DMRIE was obtained by the reaction of bromide **(±)-3** with 2-dimethylaminoethanol (DMAE).

The synthesis of compound **1** has been described in three reports [15-17]. However, these are focused on the stereoselective and regioselective acylation of glycerol to obtain compound **(*R*)-1** as the main product. The best results in this respect are obtained using CHIRAZYME as biocatalyst and relatively low temperatures (15°C). Here we present the regioselective acylation of glycerol, but eliminating the stereoselectivity observed in these reports, working at a higher temperature (55°C) and using Lipozyme as biocatalyst. We also performed assays using CAL B, but the results were not satisfactory and gave 2-hydroxypropyl-1,3-di benzoate as by-product.

We studied the stability of compound **(±)-1** under the working conditions applied in the preparation of intermediate **(±)-2**, without addition of teradecanyl mesylate After 12 h of reaction, ^{1}H NMR spectroscopic data showed no signals corresponding to glycerol or any other by-product, thus indicating that compound **(±)-1** is not decomposed. However, in the last 12 h of the addition of alkyl mesylate, we observed the presence of 1,2,3-tris-tetradecyloxypropane, evidence of hydrolysis of compound **(±)-1**, which could be the reason for the decrease in the yield of product **(±)-2**.

To study the hydrolysis reaction of compound **(±)**-4 and the *in situ* formation of alcohol **(±)-2**, the corresponding benzoate was prepared and then water and a solution of LiOH (10%) was added. The best results were obtained in the latter case, reaching the yield reported in the experimental section.

We also analyzed the decrease in the enzymatic activity of the lipase and observed that, after eight biocatalyst reuse cycles, this parameter ranged from 75 to 69 %. The study of the results of the biocatalyst /substrate ratio (wt: wt) showed that the optimum value was 1.6. Regarding the reaction solvent used, we found that the best performance was achieved using 1,4-dioxane.

For the step in which DMRIE is obtained, we studied the effects of the reaction medium and the **(±)-3**/dimethylaminoethanol molar ratio. Tests were conducted using a large excess of amine, dimethylformamide (DMF), ethanol, acetonitrile and *ter*-butanol; in each of the experiments, we used reflux, except for DMAE and DMF, in which case we worked at 105°C. In the latter two cases, the work up was complex and DMRIE required more complex treatment. The results obtained using the other solvents indicated that, both operationally and in terms of performance, the most appropriate solvent was *ter*-butanol. Regarding the study of the effect of the **(±)-3** / DMAE molar ratio, the value was 1:2.

EXPERIMENTAL

General

Melting points were determined at atmospheric pressure and uncorrected. ^{1}H NMR spectra were recorded on Bruker NMR spectrometers at 200 (compounds **(±)-1**, **(±)-2** and **(±)-3** and DMRIE) and 500MHz (**(±)-4**). Chemical shifts are reported in ppm (δ) relative to TMS and coupling constants (*J*) are reported in Hz. Infrared spectra were recorded on a Thermo Scientific Nicolet iS50 FT-IR spectrometer. Low-resolution mass spectra resulting from ionization by electronic impact (EI-LR-MS) were acquired on a Shimadzu QP-5000 mass spectrometer. Microanalyses were performed by the Elemental Analyzer (Exeter CE 440). Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates (250 μm) and developed with the appropriate solvents. The TLC spots were visualized either by UV light or by heating plates sprayed with a solution of phosphomolybdic acid (5% ethanolic solution). Flash column chromatography was carried out on silica gel (230-400 mesh). All chemicals and anhydrous solvents were purchased from Aldrich Chemical. All the extracts were dried over anhydrous Na_2SO_4 [7]. Spectroscopic data for **(±)-1**, **(±)-2**, **(±)-3** and DMRIE have been previously reported [18, 19, 20, 3a].

Procedure for the Synthesis of DMRIE

Preparation of (±)-2,3-Dihydroxypropyl benzoate (±)-1

To a 1,4-dioxane solution (200 mL) containing glycerol (18.4 g, 0.20 mol) and vinyl benzoate (29.6 g, 0.20 mol), Lipozyme (30 g) was added, and the mixture was stirred for 48 h at 55°C. After removal of the enzyme by filtration, the reaction mixture was evaporated under reduced pressure and the residue was partitioned between saturated brine and dichloromethane (100 mL each), and extracted with dichloromethane (3 x 60 mL). The combined organic layer was washed with saturated brine, and dried over anhydrous Na_2SO_4. The extract was washed with hexane to remove unreacted vinyl benzoate. Subsequently, the extract was vacuum dried, affording 36 g of **(±)-1** (75% yield) as a colorless oil. IR (KBr), cm^{-1}: 3600 - 3100, 3050, 1700, 1300, 690; ^{1}H NMR (200 MHz, CDCl3) δ 3.64-3.86 (2H, m, C3H$_2$), 4.04-4.14 (1H, m, C2H), 4.39-4.57 (2H, m, C1H$_2$), 7.40-7.52 (2H, m, 2C7H), 7.56 (1H, m, C8H), 8.02-8.13 (2H, m, 2C6H); ^{13}C NMR ($CDCl_3$, 200MHz) δ 63.8 (C3), 65.5 (C1), 70.6 (C2), 128.6 (2C7), 129.4 (2C6), 130.7 (C5), 133.1 (C8), 166.5 (C4). EI-LR-MS, m/z, M$^+$: 196.

Preparation of (±)-2,3-bis-Tetradecyloxypropan-1-ol (±)-2

To a stirred suspension of sodium hydride (0.273 g, 0.68 mol, 60% in oil) in anhydrous THF (100 mL) under a nitrogen atmosphere at 0°C, a solution of **(±)-**1 (33.3 g, 0.17 mol) in anhydrous THF (150 mL) was added over a period of 1 h maintaining the internal temperature below 5°C. After stirring at room temperature for 12 h, 1-bromotetradecane (187.7 g, 0.68 mol) was added at 0°C over a period of 1 h. After complete addition, the reaction mixture was stirred for 2 h at room temperature and this was gradually increased when reaching reflux, and then stirred for 24 h. A LiOH solution (10% wt/v,100 mL) was added, and stirring and the temperature were maintained for 12 h. The mixture was diluted with saturated ammonium chloride (300 mL). The aqueous layer was extracted with dichloromethane (700 mL), washed with water (3 x 150 mL), and dried over Na_2SO_4. The unreacted 1-bromotetradeane was removed by distillation under reduced pressure (1 mm of Hg and 125 ºC). The residue was purified by column chromatography eluting with hexane to obtain **(±)-**2 (39.5 g, 48%) as a white solid, mp. 43-45 ºC (lit. 42.5-43.5°C) [18]. ^{1}H NMR ($CDCl_3$, 200MHz) δ 0.87 (t, *J* = 6.7 Hz, 6H, C12-C12′H$_3$), 1.25 (br s, 44H,

C11-C11′H_2), 1.58-1.61 (m, 4H, C10-C10′H_2), 3.37-3.73 (m, 9H; C9-C9′-C3-C2H_2, C1H); ^{13}C NMR ($CDCl_3$, 200MHz) δ 14.1 (C16-16′), 22.8 (C15-15′), 27.6 (C12-12′), 29.1-29.5 (C13-13′), 29.7 (C11-11′), 32.5 (C14-14′), 32.8 (C10-10′), 64.3 (3C), 65.5 (C1), 71.0 (C9), 70.9(C9′),72.0 (C3), 78.4 (C2). EI-LR-MS, m/z, M^+: 484.

Preparation of (±)-1,2-bis-Tetradecyloxy-3-bromopropane (±)-3

To a solution of (±)-1,2-bis-tetradecyloxypropan-3-ol (26 g, 0.05 mol) in anhydrous dichloromethane (100 mL) under a nitrogen atmosphere at 0°C, triphenylphosphine (TPP) (17.6 g, 0.07 mol) was added. A solution of carbon tetrabromide (23.6 g, 0.07 mol) in dichloromethane (70 mL) was added to the reaction mixture dropwise in a period of 1 h and further stirred at 0°C for 3 h. The reaction mixture was diluted with water (200 mL) and the organic layer was separated, and dried over Na_2SO_4. The organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography over a silica gel with hexane to obtain (±)-1,2-bis-tetradecyloxy-3-bromopropane (26.5 g, 90%) as colorless oil. ^{1}H NMR ($CDCl_3$, 200MHz) δ 0.87 (t, J = 6.7Hz, 6H, C12-12′H_3), 1.25 (br s, 44H, C11-11′H_2), 1.51-1.61 (m, 4H, C10-10′H_2), 3.39-3.61 (m, 9H; C9-9′-C3-C1H_2, C2H); ^{13}C NMR ($CDCl_3$, 200MHz) δ 14.1 (C16-16′), 22.8 (C15-15′), 27.8 (C12-12′), 29.3-29.6 (C13-13′), 29.7 (C11-11′), 32.7 (C14-14′), 32.8 (C10-10′), 34.3 (C1) 66.3 (C3), 71.0 (C9), 71.5 (C9′), 77.8 (C2),. EI-LR-MS, m/z, M^+: 546.

Preparation of (±)-2,3-bis-(Tetradecyloxy)propyl benzoate (±)-4

A solution of benzoyl chloride (0.14 mL, 0.002 mol) in dichloromethane (10 mL) was added slowly to an ice-cooled solution of **(±)-2** (0.484 g, 0.001 mol) and triethylamine (1.3 mL, 0.010 mmol) in dichloromethane (10 mL) with stirring. After 1 h, the reaction mixture was poured into water, the organic layer was separated and the aqueous phase extracted with dichloromethane (2 x 20 mL). After evaporation of the solvent, purification by chromatography column on silica gel with hexane afforded 0.470 g (yield: 80%) of **(±)-4** as a viscous liquid. ^{1}H NMR ($CDCl_3$, 500MHz) δ 0.87 (t, J = 6.7Hz, 6H, C14-14′H_3), 1.29 (br s, 44H, C13-13′H_2), 1.54-1.61 (m, 4H, C12-12′H_2), 3.50-3.64 (m, 7H; C3-11-11′H_2, C2H)), 4.15-4.35 (2H, m, C1H_2), 7.39-7.53 (2H, m, 2C7H), 7.54 (1H, m, C8H), 8.04-8.17 (2H, m, 2C6H); ^{13}C NMR ($CDCl_3$, 500MHz) δ 14.2 (C16-16′), 22.8 (C15-15′), 27.8 (C12-12′), 29.1-29.6 (C13-13′), 29.7 (C11-11′), 32.5 (C14-14′), 32.8 (C10-10′), 64.3 (3-C), 70.7 (2-C), 71.8 (1-C), 65.5 (C1), 71.0 (C9), 71.5 (C9′),72.0 (C3), 78.8 (C2), 128.6 (2C7), 129.4 (2C6), 130.7 (C5), 133.1 (C8), 165.8 (C4). Anal. Calcd for $C_{38}H_{68}O_4$: C, 77.50; H, 11.64; Found: C, 77.60; H, 11.66.

Preparation of (±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanammonium bromide (DMRIE)

(±)-1,2-Bis-Tetradecyloxy-3-bromopropane (47.3 g, 0.08 mol) was dissolved in a solution of dimethylaminoethanol (14.3 g, 0.16 mol) in *ter*-butanol (200 mL) and heated at reflux for 46 h. The mixture was cooled to room temperature. Removal of the solvents and recrystallization of the residue from chloroform/acetone afforded 29.6 g (yield: 58 %) of DMRIE as a white solid, mp 70.3-71.7 ºC. ^{1}H NMR ($CDCl_3$, 200MHz): δ 0.88 (t, J = 6.7Hz, 6H, C14-14′), 1.25 (br s, 44H, C13-13′H_2, 1.52-1.56 (m, 4H, C12-12′H_2), 3.33 (br s, 6H, C15-15′), 3.43-3.85 (m, 12H; C11-11′-17H_2,), 4.05 (m, 1H, C2H); ^{13}C NMR ($CDCl_3$, 500MHz) δ 14.2 (C16-16′), 22.8 (C15-15′), 27.8 (C12-12′), 29.3-29.6 (C13-13′), 29.7 (C11-11′), 32.7 (C14-14′), 32.8 (C10-10′), 58.8 (C17), 60.8-61.0 (C15-15′), 65.2 (C1), 67.5 (C3), 71.0 (C9), 71.2 (C9′), 79.8 (C2),. Anal. Calcd for $C_{35}H_{74}BrNO_3$: C, 66.01; H, 11.71; N, 2.20; Found: C, 65.99; H, 11.79; N, 2.23.

CONCLUSION

In summary, a new chemoenzymatic synthetic route was developed for the DMRIE using glycerol as the starting material. We studied and optimized various experimental parameters of the various synthetic steps, and reached to develop the methodology to multigram scale, which compared to processes reported so far, has the advantages of fewer synthetic steps, the use of less aggressive reagents environment and generating fewer waste allowed to obtain the desired product; which results in a viable method for synthesizing analogs to DMRIE.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledges UMYMFOR-CONICET for the financial support of this study. In addition, authors are very thankful to the authorities of DQO-FCEyN-UBA for providing laboratory facilities.

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