SYNTHESIS OF CHOLESTERYL ESTERS OF LABELLED FATTY ACIDS

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SUMMARY

Cholesteryl esters of variously labelled fatty acids have been synthesized in high yield by 1,1'-carbonyldiimidazole activation of the acyl group and reaction of the resulting acylimidazole derivative with cholesterol. The method has been used to synthesize cholesteryl esters on micro and semimicro scales. Syntheses of the following esters are reported: cholesteryl palmitate-1-^{14}C, cholesteryl palmitate-d_{31}, cholesteryl palmitate-2,2-d_2, cholesteryl palmitate-3,3,4,4,5,5,6,6,7,7,8,8,9,9,-10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-d_{29}, cholesteryl 5-doxylpalmitate and cholesteryl 16-doxylstearate.

Key Words: Cholesteryl Palmitate, Carbon-14, Deuterium, 1,1'-Carbonyldiimidazole, Spin Labels.

INTRODUCTION

Esters of cholesterol are an active form of this important steroid in the transport process from liver to various body tissues via serum lipoprotein (1). This has created interest in the metabolism of cholesteryl esters and in the physical state of the esters in the lipoprotein complexes (1). The effect of the presence of cholesteryl esters on the physical properties of membranes (2-5) is also of interest. Thus, there is growing demand for a variety of labelled cholesteryl esters.

Previous syntheses of cholesteryl esters used labelled cholesterol and were carried out with excess acyl chlorides or acyl anhydrides (6). The yields in these methods are good if calculated on the basis of the amount of cholesterol

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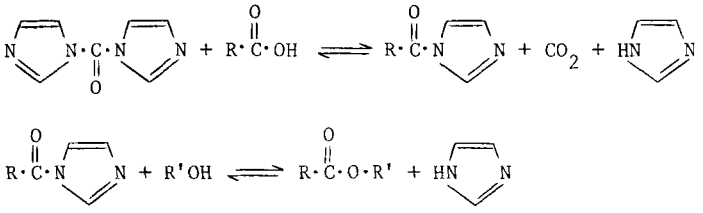
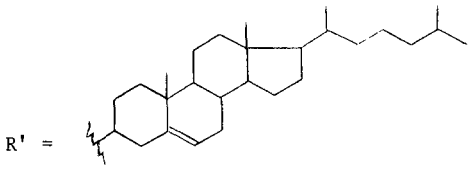
used, but the yields calculated on the basis of the amount of fatty acids used range from 0.5 - 15%.

In the present communication, a method is presented for the synthesis, in high yield, of cholesteryl esters with variously labelled aliphatic acids. The method involves synthesis of an acylimidazole derivative by reaction of 1,1'-carbonyldiimidazole (CDI) with long chain fatty acids (7). The resulting acylimidazole is directly reacted with cholesterol to yield cholesteryl esters. This is, we believe, the first application of the CDI method to cholesteryl ester synthesis.

The esters of cholesterol prepared in the present work were: palmitate-d_{31} (1), palmitate-2,2-d_2 (2), palmitate-3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,-12,13,13,14,14,15,15,16,16,16-d_{29} (palmitate-d_{29}) (3), palmitate-1-^{14}C (4), and the spin-labelled esters, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (5; 16-doxylstearate) and 2-(3-carboxypropyl)-2-undecyl-4,4-dimethyl-3-oxazolidinyloxyl (6; 5-doxylpalmitate) (see Figure 1). The latter two





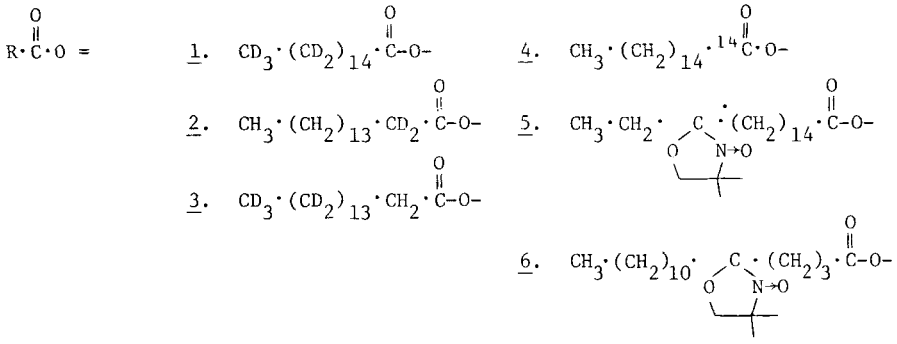


Figure 1. Synthetic scheme.

compounds, bearing the nitroxide moiety, were used for electron spin resonance studies.

RESULTS AND DISCUSSION

Cholesteryl esters of isotopically labelled (1-4) or spin-labelled (5,6) fatty acids were synthesized in good yields using the imidazole active ester procedure. The synthesis using deuterium labelled fatty acids (1-3) was conducted on a semi-micro scale in 70 - 82% yield. The synthesis of unlabelled fatty acids was used to establish optimum conditions. During the course of such trials, activation of the fatty acids with dicyclohexylcarbodiimide (DCC) was also attempted. However, the procedure resulted in only 30% yield. Thus, the method of activation of the acids using CDI appears superior to the use of DCC and is far superior to the methods involving acyl chlorides or anhydrides.

The micro-scale synthesis of cholesteryl palmitate-1-^{14}C (4) was achieved in 38% yield, the resulting material being > 99% pure. Thus, the method is also useful for micro-scale preparation of radiolabelled esters.

For compounds 1-4 the synthetic conditions consisted of reacting equimolar amounts of CDI and the appropriate acid in dry benzene, at room temperature, for approximately 1 hr. Then, a one-fold excess of cholesterol was added, the solvent removed in vacuo, the system sealed under dry N_2 and heated to 85°C for 3-4 hr.

In the case of the spin-labelled acids 5 and 6, heating the reaction mixture to 85°C resulted in very low yields due to the thermal instability of the doxyl moiety. Therefore, synthesis of the spin-labelled esters was conducted using the CDI activation followed by reaction of the activated acid with cholesterol in benzene, at room temperature, for 3-4 days. Since this reaction had to be conducted in the presence of benzene, it was critical that the benzene used was meticulously dried. Yields of the spin-labelled esters obtained under these conditions were 51% for 5 and 38% for 6. The resulting material was pure as determined by thin-layer chromatography and by ESR spectroscopy.

The unreacted acids were also recovered and, thus, there is essentially no loss of labelled material.

When the condensation of 5 and 6 was attempted using DCC several by-products were formed in the reaction, hence, the CDI method is preferred for spin-labelled ester synthesis as well.

EXPERIMENTAL

Materials

Cholesterol was purchased from Fisher Chemical Co., CDI from Aldrich Chemical Co. and cholesteryl palmitate from Sigma Chemical Co. Palmitic-1-^{14}C acid (Specific activity = 53 Ci/mol) and Liquifluor were purchased from New England Nuclear of Canada. Palmitic-d_{31} acid was a generous gift of Professor M. Bloom, Physics Department, University of British Columbia. The spin-label 16-doxyl-stearic acid was bought from Syva, Palo Alto, Ca. and 5-doxylpalmitic acid was a generous gift from Dr. A.C. Oehlschlager, Chemistry Department, Simon Fraser University. All other chemicals were purchased from standard commercial sources.

Radioactivity measurements were carried out by placing the samples in a scintillation cocktail and counting using a Beckman LS-200B Liquid Scintillation System. The scintillation cocktail contained 100 ml methanol, 20 ml ethylene glycol, 60 g naphthalene, 42 ml Liquifluor and dioxane to 1 ℓ. ^{1}H nmr spectra were determined, using a Varian A-60 spectrometer, in $CDCl_3$ solution with tetramethylsilane as internal standard. Mass spectra were run on a Hitachi Perkin-Elmer RMU-6E Mass Spectrometer.

Palmitic-2,2-d_2 acid. This compound was synthesized from palmitic acid by a published procedure (8). From 550 mg of palmitic acid was obtained 520 mgm of palmitic-2,2-d_2 acid (93% crude yield). ^{1}H NMR: complete absence of triplet δ 2.35. m/e 258 (100%, $M^{+\cdot}$) 215 (13.5%, M-43) 214 (19.1%, M-44) 187 (15.9%, M-71) 173 (10.1%, M-85) 157 (13.7%, M-101).

Palmitic-d_{29} acid. The starting material for this reaction was a donated sample of "perdeuterated" palmitic acid (Mass spectral analysis showed a mixture of fully deuterated, palmitic-d_{31} acid, 52%, with 37% mono-protonated acid, palmitic-d_{30} acid, plus 11% palmitic-d_{29} acid). The procedure was identical to the published one (8) except that D_2O was replaced by H_2O and NaOD by NaOH. The reaction was run for 22 hrs in a glass ampule (bath temp - 245°). Yield 90%.

^{1}H nmr: singlet (2H) δ 2.34. m/e 285 (100%, $M^{+\cdot}$) 284 (55.8%, M-1) 283 (16.4%, M-2) 237 (12.4%, M-48) 236 (22.2%, M-49) 235 (14.4%, M-50) 203 (12.1%, M-82) 173 (13.7%, M-112).

Cholesteryl palmitate-d_{31} (1). Palmitic-d_{31} acid (480 mg, 1.67 mmol) was dried under vacuum, dissolved in 10 ml dry benzene and stirred, along with 271 mg (1.7 mmol) of CDI under dry N_2, at room temperature. The reaction lead to CO_2 liberation, the cessation of which marked the completion of the activation reaction. The activation process leading to the synthesis of the acylimidazole derivative (Figure 1) was completed in 1 hour, at which time 1.3 g (3.37 mmol) of cholesterol was added. The contents were stirred and the solvent removed by flushing with dry N_2. Final traces of solvent were removed under vacuum (0.5 mm Hg) and then the system was sealed under dry N_2 and heated for 3-4 hours at 85°C. The reaction of the acylimidazole derivative with cholesterol resulted in formation of imidazole which crystallizes in the top of the reaction flask. The crystal formation was taken as an indication of the success of the esterification reaction. The resulting material was cooled and suspended in 20-30 ml $CHCl_3$ and 50 ml H_2O. The chloroform phase was separated and washed again with H_2O. The chloroform extracts were rotoevaporated to 2-3 ml volume. Purification of the cholesteryl ester was carried out using a 280 ml (45 x 2.8 cm) silicic acid column. $CHCl_3$ was used for packing the column as well as for elution. The resulting fractions were analyzed by chromatography on Silica Gel G TLC plates in $CHCl_3$. Fractions containing pure 1 were pooled, rotoevaporated, dried under vacuum and stored at -20°C. The cholesteryl palmitate-d_{31} gave a single spot (R_f = 0.58) on Silica Gel G TLC plates, in $CHCl_3$. The R_f values of various standards in this solvent system were: cholesteryl palmitate (0.58), cholesterol (0.25) and palmitic acid (0.05). Yield: 894 mg (1.35 mmoles, 82%), m.p. 66-68°C, m/e: 655 (0.4%, $M^{+\cdot}$), 368 (100%) and 353 (14.4%).

Cholesteryl palmitate-2,2-d_2 (2). To 262 mg (1.014 mmol) palmitic-2,2-d_2 acid in 10 ml of dry benzene was added 165 mg (1.018 mmol) CDI with stirring and under dry N_2. After one hour at room temperature 783 mg (2.025 mmol) cholesterol was added. The procedure from this point was identical to that described

for ester 1 except the silicic acid column was 40 x 2.2 cm. Ester 2 melted at 67-69°C. m/e: 626 (0.4%, $M^{+\cdot}$), 368 (100%) and 353 (13.0%). Yield: 482 mg (0.77 mmol, 76%).

Cholesteryl palmitate-d_{29} (3). Ester 3 was prepared using a procedure identical to the one used to prepare 1. Reactants were: palmitic-d_{29} acid (210 mg, 0.77 mmol), CDI (1.30 mg, 0.80 mmol) and cholesterol (800 mg, 2.07 mmol); m.p. 60-62°C, m/e: 653 (0.2% $M^{+\cdot}$), 368 (100%) and 353 (13.7%). Yield: 350 mg (0.54 mmol, 70%).

Cholesterol palmitate-1-^{14}C (4). Palmitic-1-^{14}C acid (0.38 µmol, 0.25 mCi) in a 25 ml rb flask was placed in vacuo (5 mm Hg) for 1.5 hours, then, 2 ml of dry benzene and 2 mg (12.3 µmol) of CDI were added. The contents were stirred under dry N_2 for 1 hour and 5 mg (12.9 µmol) cholesterol was added. Solvent was removed at 5 mm Hg (1.5 hours), the reaction mixture sealed under dry N_2 and heated to 70°C for 12 hours. Purification of 4 was effected using Silica Gel G coated plastic plates developed in $CHCl_3$. Unlabelled cholesteryl ester was spotted on one side of the plate, and, after chromatography, detected with I_2 vapor. The radioactive material corresponding to the R_f value of the unlabelled ester was then obtained by scraping off the silica and eluting it successively with $CHCl_3$:CH_3OH(1:3), $CHCl_3$:CH_3OH(3:1) and $CHCl_3$. The resulting material was repurified in the same manner and 99+ % of the original activity was found in the ester spot. Yield: 38%.

Cholesteryl 16-doxylstearic acid (5). To a solution of 49 mg (130 µmol) of 16-doxylstearic acid, dried under vacuum, in 2.5 ml dry benzene, was added 22 mg (136 µmol) of CDI. Upon cessation of CO_2 evolution (∿ 1 hr) 100 mg (260 µmol) of cholesterol was added. All the steps were carried out in containers masked by black tape to exclude light, and under dry N_2. After 3 days the product was streaked onto six 0.25 mm x 20 cm x 20 cm Silica Gel G coated plastic plates. The elution was performed as described for cholesteryl palmitate-1-^{14}C. The resulting ester 5 was pure as determined by TLC on Silica Gel G in chloroform and in hexane-benzene (2:3). A chloroform solution of 5 showed a three line ESR spectrum typical of nitroxide spin-labels (g = 2.008, T = 14.65 gauss). m/e: 752 (0.3%, $M^{+\cdot}$), 368 (20%), 167 (49%) and 149 (100%). Yield: 384 mg (51%).

Cholesteryl 5-doxylpalmitate (6). The 5-doxylpalmitate (6) ester was synthesized by the same method as used for ester 5. However, purification was affected using silicic acid column chromatography in $CHCl_3$, as described for the deuterated derivatives. m/e: 724 (0.04%, $M^{+\cdot}$), 368 (40%), 149 (100%). ESR spectrum in $CHCl_3$ gave three lines (g = 2.0085, T = 14.6 gauss). Yield, based on 160 mg (450 µmol) 5-doxylpalmitic acid used, was 275 mg (38%).

Unreacted spin-labelled acids were recovered in both instances. When a column procedure was used, elution was carried out with $CHCl_3$ to obtain the ester and the free cholesterol, and then with CH_3OH to elute the unreacted acid.

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