SHORT COMMUNICATION

Synthesis of Spin-Labeled Neutral Lipids: Nitroxyl Derivatives of Triglycerides and Sterol Esters

ABSTRACT

Methods for the preparation of useful spin-labeled neutral lipids are described. A spin-labeled triglyceride has been prepared by acylation of 1,3-distearoylglycerol with stearic acid anhydride bearing the 4′,4′-dimethyloxazolidine-N-oxyl ring at carbon-12. The same fatty acid anhydride has been used to acylate the 3-hydroxy group of cholesterol to obtain a cholesteryl ester with the nitroxyl function in the fatty acyl chain. The 4′,4′-dimethyloxazolidinyl-1-oxyl derivative of 5α-androstan-3-one-17β-ol has been esterified with stearic acid anhydride to obtain a steroid ester with the paramagnetic center in the steroid nucleus.

INTRODUCTION

The application of the spin-label method (1,2) to the structure and function of membranes (3-5) has depended heavily upon the design and synthesis of lipid molecules which could be introduced into the system of interest. Some of the earliest lipids prepared for this purpose included nitroxyl derivatives of the cholestane (6) and androstane (7) steroid nuclei. The synthesis of these derivatives was made possible by the development of a method for attaching the 4′,4′-dimethyloxazolidinyl-1-oxyl (doxyl) moiety to a ketonic carbon atom (6). An important feature of such derivatives is that their oxazolidine rings possess no motion independent of the carbon skeleton to which they are attached. Consequently, these rings have a fixed geometry, and the orientation of the 2 $p\pi$ orbital (which contains the free electron) relative to the host molecule is well defined. Oxazolidinyl derivatives of numerous keto fatty acids have been synthesized, and these derivatives have been incorporated subsequently into phosphatidylcholine (8) and phosphatidylethanolamine (9). Most of the spin-labeled lipids used to date have been relatively polar. Our need for chemically well defined neutral lipids of the triglyceride and sterol ester classes for the study of the more nonpolar regions of plasma lipoproteins (10) necessitated the preparation of these materials which we now report.

EXPERIMENTAL PROCEDURES

Spin-labeled fatty acids were obtained by hydrolysis of the corresponding esters in dioxane/aqueous sodium hydroxide and converted to their anhydrides by dehydration with dicyclohexylcarbodiimide in carbon tetrachloride (8). Spin-labeled derivatives of fatty acid methyl esters were prepared by m-chloroperbenzoic acid oxidation of the corresponding oxazolidine (6). Starting ketoesters were synthesized by standard methods (8). Spin-labeled androstol was prepared as described by Hubbell and McConnell (7).

The spin-labeled triglyceride (I) (Fig. 1) was prepared by the following three step synthesis. Stearic acid (28.4 g, 0.1 mole) and dihydroxyacetone (4.5 g, 0.05 mole) were dissolved in 150 ml dry, freshly distilled pyridine. To this solution was added dicyclohexylcarbodiimide (20.6 g, 0.1 mole) dissolved in 50 ml freshly distilled chloroform. Dicyclohexylurea precipitated within sec of mixing. After 48 hr, the precipitate was removed by filtration. The filtrate was subjected to rotary evaporation to remove chloroform, then shell frozen, and lyophilized to remove pyridine. The resulting solid mass was broken up, dissolved in hot acetone, and allowed to crystallize at room temperature. The colorless, crystalline 1,3-distearoxyacetone (19.8 g, 74%) had an R_f on thin layer chromatography (TLC) (Eastman chromagram sheets) of 0.90 in chloroform and 0.51 in benzene-chloroform (3:1), melted at 84-86 C (lit [11], 87.0-87.5 C), and gave an IR spectrum (KBr

wafer) exhibiting carbonyl absorption at 1730 cm^{-1}. In 20 ml dry tetrahydrofuran was dissolved 1.25 g (2 mmoles) 1,3 distearoxyacetone. Occasionally, it was necessary to heat the mixture gently to obtain complete solution. To this stirred solution at room temperature was added 2.5 ml (2.5 mmoles) diborane in tetrahydrofuran (1.0 molar in BH_3, Alfa Inorganics, Beverly, Mass.). The reaction was stirred for 18 hr after which time excess diborane was destroyed by adding 3 ml water. Tetrahydrofuran was removed by rotary evaporation leaving a white residue in water. Acetone (20 ml) was added, the residue was dissolved by heating, then set aside for crystallization at 20 C. The product (1,3-distearoylglycerol, 0.98 g, 79%) melted at 76-78 C (lit [12] 79.5 C) migrated as a single spot on TLC with an R_f of 0.75 in chloroform and 0.38 in hexane-ethyl acetate (6:1). No indication of the presence of 1,2-distearoylglycerol was observed with the latter solvent system which resolves the 1,3- and 1,2-isomers (13). The IR spectrum (KBr wafer) exhibited absorption bands at 3400 cm^{-1} (OH) and 1735 cm^{-1} (C=O). An alternative route to symmetrical 1,3-diglycerides has been published (13). This material (225 mg, 0.36 mmoles) was acylated with 12-doxyl stearic (m = 5, n = 10) anhydride (225 mg, 0.33 mmoles) in the presence of sodium oxide by a method similar to that of Robles and Van den Berg (14). (The procedure described by Robles and Van den Berg [14] calls for a three- to four-fold excess of anhydride. In the present work, equimolar anhydride and alcohol were used which, though giving lower yields, results in better overall incorporation of the spin-labeled fatty acyl chains present.) The crude product was purified partially by chromatography on a column of silica gel (Brinkmann Instruments, Westbury, N.Y.) equilibrated and eluted with methylene chloride. Purification to homogeneity was accomplished by prepatative TLC on plates of silica gel containing a fluorescent binder. The developing solvent was diethyl ether-hexane (3:7). The desired material was located by visualization with UV light. The appropriate band was scraped from the plate, transferred to a small column, and the desired material eluted from the absorbent with chloroform. The yellow product (5,10-I, 49 mg, 15%) migrated on TLC with an R_f of 0.39 in ether-hexane (3:7). Its IR spectrum (film on sodium chloride plates) exhibited strong absorption at 1735 cm^{-1} (C=O), but none at 3400 cm^{-1} (no OH). It became semicrystalline below ~10 C. Anal. calculated: $C_{61}H_{116}NO_8$: C, 73.88; H, 11.79; N, 1.41; 0, 12.91, Found: C, 73.84; H, 11.36; N, 1.75.

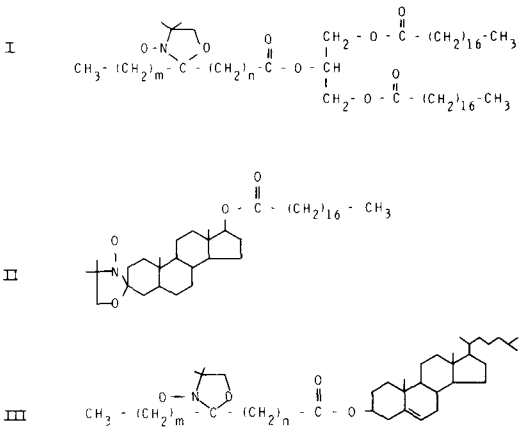

FIG. 1. Chemical structures of spin-labeled neutral lipids: 4',4'-dimethyloxazolidinyl-N-oxyl derivatives of (I) tristearin, (II) androstan-17-yl stearate, and (III) cholesteryl stearate.

The stearic acid ester of spin-labeled androstol (II) was prepared by acylation of spin-labeled androstol with stearic anhydride in anhydrous pyridine. The alcohol (95 mg, 0.25 mmole) and anhydride (275 mg, 0.50 mmole) were transferred to a 25 ml round bottom flask. Upon addition of dry pyridine (4 ml), the reaction mixture was stirred at room temperature and monitored by TLC. After 72 hr, the rate of ester formation appeared minimal. Pyridine was removed by rotary evaporation and the residue was dissolved in toluene and applied to a column of SilicAR CC-4 (Mallinckrodt, St. Louis, Mo.) equilibrated with toluene. Excess anhydride was eluted with toluene. The desired ester was eluted with methylene chloride while the alcohol remained adsorbed. The IR spectrum (KBr water) of the light yellow solid exhibited a strong band at 1727 cm^{-1}, migrated on TLC with an R_f of 0.82 in chloroform, melted at 122-124 C, and was obtained in a yield of 87 mg (54%). Anal. calculated: $C_{41}H_{72}NO_4$: C, 76.57; H, 11.29; N, 2.18; 0, 9.95, Found: C, 76.64; H, 11.01; N, 2.15.

A spin-labeled fatty acid cholesteryl ester (III; m = 5, n = 10) was synthesized by acylation of cholesterol with the fatty acid anhydride in pyridine. The anhydride (1.55 g, 2.06 mmoles) and cholesterol (1 g, 2.59 mmoles) were dissolved in 5 ml dry pyridine and stirred at room temperature. The slow formation of the desired ester was monitored for 7 days after which time solvent was removed by rotary evaporation and the residue was dried in vacuo overnight. The solid orange mass was dissolved in hexane and applied to a 2.5 x 30 cm column of silica gel (Brinkmann Instruments) equilibrated with hexane. The desired ester was

eluted with hexane:ether (95:5). TLC gave one spot with R_f = 0.58 (hexane:ether, 7:3). The viscous golden liquid exhibited an IR absorption band at 1740 cm^{-1} and was obtained in a yield of 0.87 g (36.5%). Anal. calculated: $C_{39}H_{86}NO_4$: C, 78.13; H, 11.51; N, 1.86; 0, 8.50, Found: C, 78.32; H, 11.62; N, 1.63.

JOEL D. MORRISETT
Department of Medicine
The Methodist Hospital
Baylor College of Medicine
Houston, Texas 77025

ACKNOWLEDGMENT

M. McNamee, R. Kornberg, and H. McConnell provided helpful discussions. Work was initiated during a U.S. Public Health Service postdoctoral fellowship at Stanford University.

REFERENCES

1. McConnell, H.M., and B.G. McFarland, Quart. Rev. Biophys. 3:91 (1970).
2. Hamilton, C.L., and H.M. McConnell, in "Structural Chemistry and Molecular Biology," Edited by A. Rich and N. Davidson, Freeman Press, San Francisco, Calif. 1968, p. 115.
3. Jost, P., A.S. Waggoner, and O.H. Griffith, in "Structure and Function of Biological Membranes," Edited by L. Rothfield, Academic Press, New York, N.Y., 1971, p. 83.
4. Jost, P., and O.H. Griffith, in "Methods of Pharmacology," Edited by C. Chignell, Vol. II, Appleton-Century-Crofts, New York, N.Y., 1972, p. 223.
5. Schreier-Muccillo, S., and I.C.P. Smith, in "Progress in Surface and Membrane Science," Vol. 9, Edited by J.F. Danielli, M.D. Rosenberg, and D.A. Cadenhead, Academic Press, New York, N.Y., (In press).
6. Keana, J.F.W., S.B. Keana, and D. Beetham, J. Amer. Chem. Soc. 89:3055 (1967).
7. Hubbell, W.L., and H.M. McConnell, Proc. Nat. Acad. Sci. U.S. 63:16 (1969).
8. Hubbell, W.L., and H.M. McConnell, J. Amer. Chem. Soc. 93:314 (1971).
9. Devaux, P., and H.M. McConnell, Ann. N.Y. Acad. Sci. 222:489 (1973).
10. Tauton, O.D., J.D. Morrisett, H.J. Pownall, R.L. Jackson, and A.M. Gotto, Circulation (In press).
11. Barry, P.J., and B.M. Craig, Can. J. Chem. 33:716 (1955).
12. Jackson, F.L., B.F. Daubert, C.G. King, and H.E. Longnecker, J. Amer. Chem. Soc. 66:289 (1944).
13. Bentley, P.H., and W. McCrae, J. Org. Chem. 35:2082 (1970).
14. Robles, E.C., and D. Van den Berg, Biochim. Biophys. Acta 187:520 (1969).

[Received March 27, 1974]