Inhibitors of sterol synthesis. Synthesis and spectral characterization of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one

Jan St. Pyrek and George J. Schroepfer, Jr.[1]

Departments of Biochemistry and Chemistry, Rice University, P. O. Box 1892, Houston, TX 77251

Abstract Reported herein are the chemical syntheses of 29 fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one. These compounds were characterized by the results of ultraviolet, ^{1}H nuclear magnetic resonance, and mass spectral studies.—**St. Pyrek, J., and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Synthesis and spectral characterization of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one. *J. Lipid Res.* 1987. **28:** 1308–1312.

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5α-Cholest-8(14)-en-3β-ol-15-one has been shown to be a potent inhibitor of sterol biosynthesis in cultured mammalian cells and to lower the levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in these cells (1–3). Moreover, this 15-oxygenated sterol has been found to have marked hypocholesterolemic activity in rats (4, 5), mice (4), baboons (6), and rhesus monkeys (7). An additional unique feature of this compound is that it serves as an efficient precursor of cholesterol as demonstrated both in homogenates of rat liver (8) and in intact rats ((9) and Brabson, J. S., and G. J. Schroepfer, Jr., unpublished data) and baboons (Schroepfer, G. J., Jr., T. N. Pajewski, M. Hylarides, K-S. Wang, and A. Kisic, unpublished results). In addition to its metabolism to cholesterol, the formation of fatty acid esters of the 15-ketosterol has been demonstrated in homogenates of rat liver (Monger, D. J., and G. J. Schroepfer, Jr., unpublished data) in cultured mammalian cells (10), in intact rats (Brabson, J. S., and G. J. Schroepfer, Jr., unpublished data), and in intact baboons (Schroepfer, G. J., Jr., T. N. Pajewski, M. Hylarides, K-S. Wang, and A. Kisic, unpublished results). The purpose of the present study was to prepare a series of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one for determination of their spectral properties and for use in a number of metabolic and analytical studies.

EXPERIMENTAL

General

Melting points were measured with a Thomas-Hoover melting point apparatus using evacuated capillaries. Ultraviolet spectra were recorded on an IBM 9430 spectrophotometer (Danbury, CT) using hexane as the solvent. Proton nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz on an IBM AF300 spectrometer (Danbury, CT) using deuterated chloroform as the solvent. Peaks are reported as ppm (δ) downfield from tetramethylsilane (internal standard). Mass spectra were measured with solid probe introduction under electron impact conditions at 15, 20, and 70 eV using the following instruments: LKB-9000 and QP-1000 (both Shimadzu Corp., Kyoto). Thin-layer chromatography (TLC) was performed using either precoated 0.2-mm high performance TLC plates (silica gel 60, EM Science (Merck), Cherry Hill, NJ) or precoated 0.25-mm silica gel G plates (Analtech, Inc., Newark, DE). Substances were visualized by spraying with 5% ammonium molybdate in 10% sulfuric acid. The synthesis of 5α-cholest-8(14)-en-3β-ol-15-one (15-ketosterol) was described previously (2, 11). Fatty acids (>99% pure) were purchased from Nu Check Prep., Inc. (Elysian, MN). Other chemicals were purchased from Aldrich (Milwaukee, WI) or Mallinckrodt (Los Angeles, CA). Methylene chloride used in the syntheses of the esters of the 15-ketosterol was freshly distilled from phosphorus pentoxide.

Abbreviations: UV, ultraviolet; NMR, nuclear magnetic resonance; MS, mass spectra; SC, the alkyl side chain (C_8H_{17}) of the sterol.

[1]To whom inquiries should be directed.

[2]Detailed spectral data will be provided by the authors upon request.

Stearic acid ester of 5α-cholest-8(14)-en-3β-ol-15-one

To an ice-cooled solution of the 15-ketosterol (0.80 g, 2 mmol) and stearic acid (0.70 g, 2.46 mmol) in methylene chloride (10 ml) was added a solution of dicyclohexylcarbodiimide (500 mg, 2.42 mmol) and 4-dimethylaminopyridine (50 mg) in methylene chloride (10 ml). The mixture was slowly warmed up to room temperature. Crystalline 1,3-dicyclohexylurea appeared within several minutes. On the next day, the reaction mixture was diluted with hexane (200 ml), filtered, and washed with 5% H_2SO_4 (50 ml), brine (50 ml), and 5% $NaHCO_3$ (50 ml). The hexane solution was filtered through alumina (activity III; ~50 g) and evaporated to dryness under reduced pressure. The residue was dissolved in methylene chloride (5 ml) and recrystallized from ethanol (50 ml) (after removal of methylene chloride on a steam bath) to give the stearate ester of the 15-ketosterol (1.257 g; 94% yield), which melted at 82.5–84.0°C.

A total of 29 esters of 5α-cholest-8(14)-en-3β-ol-15-one **(Table 1)** were prepared using this procedure. In the case of some esters, small amounts of fatty acid methyl esters were formed as by-products due to the presence of methanol of crystallization present in one lot of the 15-ketosterol. In order to remove these impurities, chromatographic purification was performed using alumina (activity III) columns with hexane–ethyl ether stepwise gradient elution. Esters of 15-ketosterol always preceded fatty acid methyl esters in the elution order. Esters of 15-ketosterol with lower fatty acids (C_{10}–C_{14}) were recrystallized from aqueous ethanol. Higher esters, including unsaturated C_{18} esters, were recrystallized from ethanol. Almost quantitative yields were obtained in all cases.

RESULTS AND DISCUSSION

A series of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one was required for use in a variety of metabolic and analytical studies in progress in this laboratory. We have previously utilized acyl chlorides for the preparation of a number of fatty acid esters of 15-oxygenated sterols ((12, 13) and Wang, K-S., and G. J. Schroepfer, Jr., unpublished results). In some cases only moderate yields of the desired esters were obtained under the conditions employed, and fairly extensive chromatography was required to obtain the products in a high state of purity.

TABLE 1. Properties of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one obtained by the dicyclohexylcarbodiimide method

Fatty Acid		Common Name	Preparation Scale	Melting Point (°C) (from Ethanol)
			mmol	
10:0		capric	3	94.5–95.5
11:0			3	82.5–84.0
12:0		lauric	3	90.0–91.0
13:0			3	93.5–94.0
14:0		myristic	3	92.0–92.5
15:0			3	83.0–83.5
16:0		palmitic	3	78.5–79.5
16:1	Δ^{9c}	palmitoleic	0.3	50.0–51.5
17:0		margaric	3	80.0–81.0
18:0		stearic	3	82.5–84.0
18:1	Δ^{6c}	petroselinic	0.3	oil[a]
18:1	Δ^{6t}	petroselaidic	0.3	(59)–75.0
18:1	Δ^{9c}	oleic	3	56.0–57.0
18:1	Δ^{9t}	elaidic	3	66.5–67.5
18:1	Δ^{11c}	*cis*-vaccenic	0.3	oil[a]
18:1	Δ^{11t}	*trans*-vaccenic	0.3	54.5–56.0
18:2	$\Delta^{9c, 12c}$	linoleic	2.9	50.0–51.0
18:3	$\Delta^{9c, 12c, 15c}$	linolenic	2.9	46.0–48.0
19:0			1	83.5–84.5
20:0		arachidic	3	87.0–88.0
20:3	$\Delta^{11c, 14c, 17c}$		0.3	oil[a]
20:3	$\Delta^{8c, 11c, 14c}$	homo-γ-linolenic	0.03	oil[a]
20:4	$\Delta^{5c, 8c, 11c, 14c}$	arachidonic	3.6	~15
21:0			0.5	87.5–88.5
22:0		behenic	3.0	89.0–90.5
22:1	Δ^{13c}	erucic	3.0	54.5–55.5
23:0			0.3	89.0–90.0
24:0		lignoceric	1.0	92.0–93.0
24:1	Δ^{15c}	nervonic	0.3	57.5–59.0

[a]Purified only by chromatography; not obtained in crystalline form; see Discussion.

Moreover, the use of acyl chlorides requires their synthesis and purification. Accordingly, we directed our efforts towards the use of methods utilizing the more readily available carboxylic acids. Initial efforts involved exploration of Brewster's method which involves treatment of an organic acid with an aromatic sulfonyl halide in pyridine to generate an anhydride which is then allowed to react with the appropriate alcohol (14–17). When the recommended stoichiometric ratio of *p*-toluenesulfonyl chloride to carboxylic acid was employed, the formation of variable amounts of the *p*-toluenesulfonate ester of the 15-ketosterol was observed, thereby requiring the separation of the sulfonate ester of the 15-ketosterol from the desired fatty acid ester of the 15-ketosterol. The results of preliminary experiments indicated that this problem could be obviated by modification of the reaction conditions so as to provide a three- to fourfold excess of the carboxylic acid.

The method that was utilized in this study involves the use of dicyclohexylcarbodiimide and dimethylaminopyridine to catalyze the formation of an ester from an alcohol and a carboxylic acid (18–20). Using a slight excess of dicyclohexylcarbodiimide and catalytic amounts of dimethylaminopyridine and employing equimolar amounts of the 15-ketosterol and stearic acid in dry dichloromethane at 0°C, an almost quantitative yield of the desired stearate ester of the 15-ketosterol was obtained. This method was employed for the esterification of the 15-ketosterol with 29 fatty acids of varying chain length and degrees of unsaturation (Table 1). The esters were, after an initial purification on a short deactivated alumina column, recrystallized from ethanol or (in the cases of lower saturated acids) from ethanol–water.

Presented in Table 1 is a listing of the individual esters that were prepared by this method along with their melting points and the scale of the syntheses. The esters showed a λ_{max} (in hexane) of ~250 nm and an average value of 14,780 ± 300 for the extinction coefficient. Four noncrystalline esters, obtained from fatty acids available in only small quantities, were purified by chromatography alone. These esters showed lower values for the extinction coefficient than those observed for all of the other esters which were obtained in crystalline form. The NMR spectra of the former esters indicated the presence of impurities of low molecular weight. All of the crystalline esters, including the highly unsaturated fatty acid esters, gave correct combustion analytical data with the notable exception of the relatively unstable linolenate (18:3) ester.

Proton NMR spectra were obtained for nine C_{18} esters and five other esters of the 15-ketosterol.[2] The characteristic resonance peaks due to the 3α-proton (4.73–4.76 m), the 7β-proton (4.09–4.14 d), and the 16β-proton (2.35–2.37 dd) were all easily detected as were the methyl protons on C-18 (0.97–0.98 s), C-19 (0.73 s), C-21 (0.99–1.00 d), and C-26 and 27 (0.86–0.88 d). The following resonances were also observed: olefinic protons (5.3–5.4) of the fatty acid moiety, as were allylic protons (2.0 for monounsaturated esters and 2.0 and 2.8 for esters of the 15-ketosterol with acids containing two or more isolated double bonds), protons on the α-carbon of the

TABLE 2. Electron impact probe mass spectra of selected fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one[a]

Fragment Ion, *m/z* (% rel. int.)	16:0 (Palmitic)	16:1 (Palmitoleic)	18:0 (Stearic)	18:1 (Oleic)	18:2 (Linoleic)	18:3 (Linolenic)	20:0 (Arachidic)	20:4 (Arachidonic)
M+	638 (78)	636 (95)	666 (41)	664 (60)	662 (55)	660 (23)	694 (80)	686 (33)
M-CH_3	623 (6)	621 (6)	651 (3)	649 (4)	647 (2)	645 (2)	679 (4)	671 (1)
M-H_2O	620 (6)	618 (6)	648 (4)	646 (4)	644 (4)	642 (2)	676 (5)	668 (1)
M-SC	525 (5)	523 (5)	553 (5)	551 (3)	549 (1)	547 (1)	581 (5)	573 (0.3)
M-SC-H_2O	507 (22)	505 (27)	535 (15)	533 (20)	531 (15)	529 (17)	563 (17)	555 (4)
M-SC-28	497 (3)	495 (2)	525 (2)	523 (2)			553 (1)	
RCOO+[b]	255 (3)				279 (33)	277 (100)		303 (34)
383 (M-RCOO·)	(32)	(32)	(34)	(78)	(100)	(99)	(41)	(100)
382 (M-RCOOH)	(23)	(26)	(24)	(29)	(36)	(35)	(25)	(22)
380	(5)	(6)	(6)	(6)	(31)	(46)	(3)	(13)
367 (M-RCOOH-CH_3)	(100)	(100)	(100)	(100)	(81)	(65)	(100)	(33)
365 (M-RCOO·-H_2O)	(8)	(10)	(8)	(12)	(19)	(21)	(5)	(12)
364 (M-RCOOH-H_2O)	(5)	(4)	(4)	(3)	(5)	(5)	(3)	(2)
355 (M-RCOO·-28)	(21)	(32)	(21)	(34)	(64)	(65)	(25)	(54)
341	(7)	(9)	(7)	(12)	(20)	(21)	(9)	(18)
287	(10)	(10)	(9)	(14)	(11)	(11)	(10)	(6)
276	(8)	(10)	(9)	(12)	(15)	(13)	(11)	(7)
275	(6)	(8)	(8)	(15)	(13)	(14)	(12)	(7)
269 (M-SC-RCOOH)	(38)	(57)	(36)	(61)	(65)	(48)	(39)	(18)
261	(15)	(17)	(13)	(20)	(19)	(17)	(14)	(8)
251 (M-SC-RCOOH-H_2O)	(60)	(66)	(55)	(75)	(65)	(46)	(59)	(20)
241	(8)	(9)	(9)	(12)	(19)	(18)	(9)	(7)

[a] Spectra in the mass range 200–900 amu were measured using direct inlet introduction at 70 eV. Ion source temperature was 250°C.
[b] $RCOO^+$ ion assigned to the fatty acid moiety.

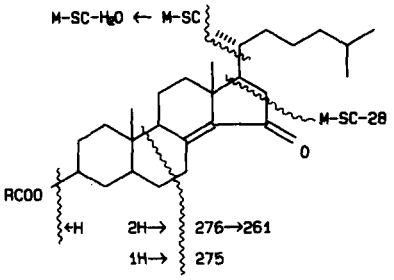

Fig. 1. Major modes of fragmentation of fatty acid esters of 5α-cholest-8(14)-en-3β-ol-15-one upon electron impact.

fatty acid moiety (2.24–2.27 t), protons on internal methylene carbons (1.3), and protons of the ω-methyl group of esters (0.87–0.99).

Electron impact mass spectral data were obtained for all of the esters of the 15-ketosterol by probe introduction at 20 eV and at 70 eV. A tabulation of a summary of the mass spectral data for eight of the esters is provided in **Table 2**. Most of the major fragment ions could be ascribed to relatively simple cleavage processes **(Fig. 1)** of the steroidal moiety of the molecule and were assigned on the basis of their strict analogy with published spectral data for the parent compound and other derivatives of the 15-ketosterol (1,12,21–27) and on the basis of the results of a more recent study dealing with spectral properties of isotopically labeled derivatives of the 15-ketosterol (28). The esters of the 15-ketosterol displayed strong molecular ions, even at 70 eV. This finding is in sharp contrast to the case of fatty acid esters of cholesterol for which molecular ions can be detected with difficulty even at low electron energies. Other major ions included those due to eliminations of water, a methyl radical, the alkyl side chain (C_8H_{17}), the C-3 substituent, and ions representing the loss of more than one of the above. Other minor fragment ions included those due to the loss of the alkyl side chain plus C-16 and C-17 and those due to a ring B cleavage process leading to the formation of ions at *m/z* 276 (and 275) and at *m/z* 261 (Fig. 1). Intense fragment ions corresponding to the fatty acid moiety ($RCOO^+$) were present only in the case of polyunsaturated acids.

Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Mass spectra, melting points and ultraviolet spectra were done with the help of Ms. M. Martin. Nuclear magnetic resonance spectra were measured by Dr. William K. Wilson. The synthetic studies and the mass spectral studies were supported by grants HL-22532 and HL-15376, respectively, from the National Institutes of Health. The Arco Foundation provided funds for the purchase of the NMR spectrometer. The support of the Ralph and Dorothy Looney Endowment Fund and the American Cyanamid Company is gratefully acknowledged.

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