[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, QUEEN'S UNIVERSITY]

Preparation and Certain Physical Properties of Some Plant Steryl Esters[1,2]

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The even numbered C_2-C_{22} saturated and some C_{18} unsaturated fatty acid esters of β- and γ-sitosterol, stigmasterol, their saturated analogs, and ergosterol were prepared by treating the corresponding acid chloride or anhydride with the sterol in the presence of pyridine in an inert solvent. Adsorption chromatographic purification of the steryl esters was essential if contamination with free sterols was to be avoided. In all cases the melting points of the esters were relatively sharp and decreased with increasing molecular weight of the fatty acid till a minimum was reached in the myristate-palmitate region, after which they increased. The introduction of a double bond into the fatty acid residue produced a 37 to 45° drop in the melting point when compared with the corresponding saturated ester. Additional double bonds brought about a further decrease but of lower magnitude. During melting the anisotropic solids were observed to change cleanly into isotropic liquids, and the temperature range required for this transformation broadened with increasing chain length of the fatty acid. Only the temperatures at which the samples became completely liquid and the polarized light field dark were reproducible. Little evidence was obtained for the formation of any other well defined and discrete transition states. A number of possibilities that might account for the hitherto reported mesomorphic behavior of steryl esters of long chain fatty acids are discussed. The specific rotations of the esters decreased with lengthening of the fatty acid chain; the molecular rotations remained essentially constant; and unsaturation of the fatty acid moiety had little or no effect. With the exception of the steryl esters of the lower fatty acids, these physical constants could not be used for a reliable identification of these compounds. The construction of a special heating stage for use in conjunction with a polarizing microscope is described in detail.

The isolation[3] of relatively large quantities of mixed plant steryl esters from the molecular distillates of corn oil appeared to offer an excellent opportunity for the study of this little known lipid class. Previous studies on the unsaponifiable matter of corn oil had revealed the presence of a complex mixture of plant sterols from which stigmasterol, β- and γ-sitosterol and their saturated analogs had been isolated.[4] The presence of ergosterol had been implied on the basis of the ultraviolet absorption spectrum of corn oil.[5] Though it could be readily demonstrated[3] that the ester mixture was made up of different fatty acid esters of several of these sterols, the absence of suitable means for their fractionation prevented the isolation and identification of any individual compounds. To facilitate the development of such means for the separation and possible identification of these esters, it was necessary that a number of reference compounds of unquestionable purity be prepared and their physical and chemical characteristics determined.

Although the acetates, benzoates, and dinitrobenzoates of these sterols are readily prepared and purified, and their physical properties well known, only rarely have the longer chain fatty acid esters of the plant sterols been prepared synthetically[6] or isolated from natural sources[7] and their properties studied. An examination of the esterification methods, such as fusion,[6b,8] heating in a closed vessel,[6d] strong acid catalysis,[9] and acid chloride alcoholysis in pyridine solution,[8c,9b,10] used for the syntheses of the few known plant steryl and the more frequently studied cholesteryl long chain fatty acid esters, indicated that significant modifications in the existing techniques would have to be made if maximum yields of high purity products were to be obtained.

From this survey it also became obvious that much of the present uncertainty[9b,11] in the physical properties of the steryl esters in general was due either to the use of unsuitable methods for their

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(2) Supported in part by a research grant from the Nutrition Foundation, Inc.

(3) A. Kuksis and J. M. R. Beveridge, J. Lipid Research, in press.

(4) R. J. Anderson and R. L. Shriner, *J. Am. Chem. Soc.*, **48**, 2976 (1926).

(5) I. M. Heilbron, E. D. Kamm, and R. A. Morton, *Biochem. J.*, **21**, 1279 (1927).

(6) (a) C. Tanret, *Compt. rend.*, **147**, 75 (1889), *Ann. chim. phys.* **15**, 313 (1908); (b) A. Heiduschka and H. W. Gloth, *Arch. Pharm.*, **253**, 415 (1915); (c) C. E. Bills and E. M. Honeywell, *J. Biol. Chem.*, **80**, 15 (1928); (d) A. Windaus and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math. physik. Kl.*, 202 (1928), cited in footnote 7a; (e) L. Swell, H. Field, and C. R. Treadwell, *Proc. Soc. Exptl. Biol. Med.*, **87**, 216 (1954).

(7) (a) A. E. Oxford and H. Raistrick, *Biochem. J.*, **27**, 1176 (1933); (b) A. Angeletti, G. Tappi, and G. Biglino, *Ann. chim. (Rome)*, **42**, 502 (1952); (c) F. E. King, T. J. King, K. G. Neill, and L. Jurd, *J. Chem. Soc.*, 1192 (1953); (d) V. Benesova, V. Herout, and F. Sorm, *Chem. listy*, **48**, 882 (1954); (e) J. Krepinsky, V. Herout, and F. Sorm, *Chem. listy*, **52**, 1784 (1958).

(8) (a) F. M. Jaeger, *Rec. trav. chim.*, **25**, 334 (1906), **26**, 311 (1907); (b) P. Gaubert, *Bull. soc. franc. mineral.*, **32**, 62 (1909), *Compt. rend.*, **149**, 608 (1909); (c) I. H. Page and H. Rudy, *Biochem. Z.*, **220**, 305 (1930).

(9) (a) E. L. Cataline, L. Worrell, S. F. Jeffries, and S. A. Aronson, *J. Am. Pharm. Assoc.*, **33**, 107 (1944); (b) D. Kritchevsky and M. E. Anderson, *J. Am. Chem. Soc.*, **74**, 1857 (1952).

(10) L. Swell and C. R. Treadwell, *J. Biol. Chem.*, **212**, 141 (1955).

(11) (a) C. E. Bills, *The Vitamins*, W. H. Sebrell, Jr., R. S. Harris, eds., Academic Press, Inc., New York, N. Y., 1954, Vol. 2, p. 183 ff.; (b) G. W. Gray, *J. Chem. Soc.*, 3733 (1956).

preparation or to incomplete subsequent purification or to both. Thus, in addition to the large discrepancies noted in some of the physical constants, it has been reported that the melting points of the long chain saturated cholesteryl esters decrease[10,11b,12] or increase[13] with increasing chain length of the fatty acid, and that some[8c,14] or all[11b,15] show mesomorphic transition states. Extended turbidity phases have also been observed[6c,8a] for some saturated fatty acid esters of plant sterols. In the case of the unsaturated fatty acid esters the situation has been equally confusing. Thus, Labarrere *et al.*[13] reported that the melting point of cholesteryl oleate was about 33° below that of the stearate, and that additional unsaturation decreased it still further. Page and Rudy[8c] and Bladon,[12] however, listed for cholesteryl linolenate a melting point higher than either that for oleate or linoleate, with an isotropic transition point approaching that for the stearate. The unsuitability of thionyl chloride for the preparation of unsaturated fatty acid chlorides now appears to be generally recognized[10,13] yet oxalyl chloride as commonly employed may also cause difficulties. For instance, Klein and Janssen[16] noted that the arachidonate ester of cholesterol prepared using the oxalyl chloride method of Wood *et al.*[17] gave low recoveries on adsorption chromatography, while the recoveries of natural arachidonate esters were considerably higher under the same chromatographic conditions. It is difficult to evaluate the significance of these reports, since the purity of the starting materials employed has seldom been defined.

In order to contribute to a more satisfactory knowledge of the physical and chemical properties of this class of compounds the original study was extended beyond the limited needs of our particular problem to include all the even numbered C_2-C_{22} saturated and the C_{18} unsaturated fatty acid esters of the corn oil sterols mentioned above. In developing improved techniques for the syntheses and purification of these compounds, recent observations on the preparation of acid chlorides in solution,[18] the beneficial effect of inert solvents on the esterification with acid chlorides and pyridine,[19] and the adsorption chromatographic behavior of steryl esters[20] have been extensively utilized.

RESULTS AND DISCUSSION

Starting materials. The difficulty of obtaining high quality starting material for the preparation of pure steryl esters can be readily appreciated. Both sterols and long-chain fatty acids are notoriously difficult to purify because of the similar physical and chemical characteristics of isomers and near homologs. Melting point depressions are often small and optical rotation differences slight. These criteria of purity, often unreliable, have been supplemented in this work by chromatography and spectrophotometry.

Using such means all the fatty acids, with the exception of linolenic acid, which contained minor amounts of *trans* and conjugated isomers and some linoleic acid, were demonstrably of high purity. The purity of ergosterol was verified by ultraviolet absorption measurements. The preparation of the acetate tetrabromide served to ensure the uniformity of stigmasterol. The β-sitosterol isolated from cottonseed oil, shown by Wallis and Chakravorty[21] to be almost free from other sterols, was considered sufficiently reliable.

No methods of a similar nature were available for double checking the purity of the γ-sitosterol preparations. Even the physical constants attributed to this sterol have been questioned by several workers. Thus, a comparison[22] of the molecular rotation differences of the γ-sitosterol derivatives with those obtained for the corresponding derivatives of cholesterol and stigmasterol shows considerable discrepancies. As a result it has been suggested that either γ-sitosterol is not the C_{24} epimer of 22,23-dihydrostigmasterol, or, more likely, that the preparations thus far obtained have been contaminated with a more levorotatory component such as 22,23-dehydro-γ-sitosterol. A compound giving molecular rotations corresponding approximately to those expected for a C_{24} epimer of β-sitosterol has been recognized[22] in the clionasterol isolated from sponges, and a recent review[12] on the chemistry of sterols lists for γ-sitosterol the constants of clionasterol. Further complicating the problem is the question of the correct molecular weight. On the basis of the recently observed[23] slightly greater polarity of γ-sitosterol in reversed phase partition systems, where there supposedly should not be any significant

(12) P. Bladon, *Cholesterol*, R. P. Cook, ed., Academic Press, Inc., New York, N. Y., 1958, p. 15 ff.

(13) J. A. Labarrere, J. R. Chipault, and W. O. Lundberg, *Anal. Chem.*, **30**, 1466 (1958).

(14) G. Friedel, *Ann. phys.*, **17**, 273 (1922).

(15) O. Lehmann, *Z. phys. Chem.*, **56**, 750 (1906).

(16) P. D. Klein and E. T. Janssen, *J. Biol. Chem.*, **234**, 1417 (1959).

(17) T. R. Wood, F. L. Jackson, A. R. Baldwin, and H. E. Longenecker, *J. Am. Chem. Soc.*, **66**, 287 (1944). Since the halogenation proceeds *via* the anhydride, 2.5 moles of oxalyl chloride are required per mole of acid.

(18) C. G. Youngs, A. Epp, B. M. Craig, and H. R. Sallans, *J. Am. Oil Chemists' Soc.*, **34**, 107 (1957).

(19) J. A. Mills, *J. Chem. Soc.*, 2332 (1951).

(20) (a) D. L. Fillerup and J. F. Mead, *Proc. Soc. Exptl. Biol. Med.*, **83**, 574 (1953); (b) E. J. Barron and D. J. Hanahan, *J. Biol. Chem.*, **231**, 493 (1958).

(21) E. S. Wallis and P. N. Chakravorty, *J. Org. Chem.*, **2**, 335 (1937).

(22) W. Bergmann and E. M. Low, *J. Org. Chem.*, **12**, 67 (1947).

(23) (a) H. Sulser and O. Hoegl, *Mitt. Gebiete Lebensm. u. Hyg.*, **48**, 245 (1957); (b) J. C. Riemersma and W. Stoutjesdijk, *Mitt. Gebiete Lebensm. u. Hyg.*, **49**, 115 (1958).

differences between truly epimeric pairs, it has been suggested[23b] that γ-sitosterol might not be the C_{29} system as presently believed,[24] but possibly a C_{28} compound as implied earlier.[25] This difference in the molecular weight, though accounting for the greater mobility and explaining a number of other observations,[26] would still not bring the molecular rotation differences for γ-sitosterol and derivatives into line with those for other sterols.

The possibility that γ-sitosterol isolations might have resulted in a product contaminated with some higher melting and more levorotatory component, such as 22,23-dehydro-γ-sitosterol, as suggested by Bergmann and Low,[22] or even stigmasterol, is quite probable in view of the relatively recent recognition[27] of the failure of the Windaus-Hauth process,[28] as commonly conducted, to remove all the 22,23-dehydrosterol. The use of exhaustive acetate tetrabromide precipitations or the insolubilization of stigmasterol[29] and possibly any other 22,23-dehydrosterols as their α-naphthyl carbamates, should then produce γ-sitosterol preparations with lower melting points and less negative optical rotations. In the preparations described in the present experiments conducted with the above considerations in mind, lower melting and less levorotatory samples were in fact obtained. These γ-sitosterol preparations, however, though almost identical with clionasterol in specific rotations, differed significantly from it in the melting points, and agreed fairly closely in such constants with certain descriptions of γ-sitosterol.[4,30]

In the procedures used for the isolation of γ-sitosterol, which were based on the work of Bonstedt[31] and Dirscherl and Nahm,[32] and were carried out on material freed from stigmasterol by exhaustive precipitation of the acetate tetrabromides, the α-naphthyl carbamates, or both, additional improvements were introduced. Among these the most important was believed to be the preliminary removal of the difficultly soluble campesterol by the method of Fernholz and MacPhillamy.[33] Paper partition[23a] and adsorption chromatographic techniques[30,34] capable of some degree of segregation of sterol mixtures served as a further check on the uniformity of the final sample.

As a result of a careful application of the above methods and a failure to demonstrate any residual 22,23-*trans* unsaturation in the γ-sitosterol preparations by an infrared assay,[27b] it was concluded that the physical constants obtained would not necessarily represent material contaminated with the 22,23-dehydrosterols, but might be those of the pure compound. This belief is strengthened by the observation that numerous isolations of γ-sitosterol from a wide variety of plant and marine invertebrate sources[35] performed since the contamination was first suggested have resulted in preparations with closely similar physical constants, which, however, differed greatly from clinoasterol in melting points. It would be difficult to imagine that all of these preparations could have been contaminated with the same 22,23-dehydrosterols and in about the same proportions.

Preparation of steryl esters. The preparation of low molecular weight fatty acid esters of sterols proceeds readily and the high reactivity of the fatty acids and their derivatives commonly employed in esterifications ensures an almost quantitative yield under most conditions. Esterifications with the relatively inert longer chain fatty acids and their derivatives, however, require the presence of catalysts and elevated temperatures and even then only moderate yields are obtained.[6b,6d,8a,8c,9a] The undesirable side effects (isomerization, dehydration, polymerization, etc.) exercised upon the starting materials and the final products by such catalysts and high temperatures, prohibit the utilization of these techniques in the preparation of high purity compounds.

The use of more reactive fatty acid derivatives such as the acid chlorides has permitted the application of lower temperatures[36,9b,10] resulting in higher yields of better quality products. While this method gives excellent results with stable

(24) C. W. Shoppe, *Chemistry of the Steroids*, Butterworths Scientific Publications, Ltd., London, 1958, p. 63.

(25) H. Sandqvist and E. Bengtsson, *Ber.*, **64**, 2169 (1931).

(26) For a discussion and repudiation of this possibility, see K. Bonstedt, *Z. physiol. Chem. Hoppe-Seyler's*, **205**, 137 (1932).

(27) (a) R. Neu and P. Ehrbacher, *Arch. Pharm.*, **283**, 227 (1950); (b) J. L. Johnson, M. F. Grostic, and A. O. Jensen, *Anal. Chem.*, **29**, 468 (1957).

(28) A. Windaus and A. Hauth, *Ber.*, **39**, 4378 (1906).

(29) J. A. Campbell, O. H. Shepherd, B. A. Johnson, and A. C. Ott, *J. Am. Chem. Soc.*, **79**, 1127 (1957).

(30) Hung-Shuin Ting, Yu Wang, Wei-Yi Ting, and Yao-Tseng Huang, *Hua Hsüeh Hsüeh Pao*, **22**, 288 (1956); *Chem. Abstr.*, **52**, 6463f.

(31) K. Bonstedt, *Z. physiol. Chem. Hoppe-Seyler's*, **176**, 269 (1928).

(32) W. Dirscherl and H. Nahm, *Ann.*, **555**, 57 (1944); **558**, 231 (1947).

(33) E. Fernholz and H. B. MacPhillamy, *J. Am. Chem. Soc.*, **63**, 1155 (1941).

(34) (a) L. F. Fieser, *J. Am. Chem. Soc.*, **75**, 4395 (1953); (b) A. Weizmann, A. Meisels, and Y. Mazur, *J. Org. Chem.*, **20**, 1173 (1955).

(35) (a) C. Djerassi, M. Gorman, A. L. Nussbaum, and J. Reynoso, *J. Am. Chem. Soc.*, **76**, 4463 (1954); (b) T. Matsumoto and T. Tamura, *Nippon Kagaku Zasshi*, **76**, 951 (1955); *Chem. Abstr.*, **51**, 18356*h*; (c) R. N. Chakravarty, D. Chakravarty, M. N. Mitra, B. Dasgupta, and P. C. Maiti, *J. Sci. Ind. Research (India)*, **15C**, 86 (1956); (d) M. Dymicky and R. L. Stedman, *Tobacco*, **148**, 48 (1959); (e) T. Tsukamoto, I. Nishioka, K. Mihashi, and H. Miyara, *Yakugaku Zasshi*, **78**, 1099 (1958); *Chem. Abstr.*, **53**, 5337*g*; (f) S. R. Bhattacharjee and D. K. Mullick, *Sci. and Culture (Calcutta)*, **24**, 293 (1958).

(36) E. Abderhalden and K. Kautsch, *Z. physiol. Chem. Hoppe-Seyler's*, **65**, 69 (1910).

materials, the preliminary treatment with the acid chloride, and the excess pyridine, usually used as solvent, present difficulties when working with acid sensitive compounds. The fatty acid anhydrides are also more reactive than the free acids themselves, but hitherto only those of the lower fatty acids have been widely employed.[6a,6c,8c,10, 11b]

Since the anhydrides are known[37] to be the mildest esterifying agents, they appeared to be particularly suited for the esterification of such acid sensitive sterols as ergosterol.[11a,38] Experimentation with these under a variety of conditions[39] indicated that their reactivity decreased progressively with increasing molecular weight. Thus, while the acetic to caproic acid anhydrides gave yields varying from quantitative to 70% when refluxed for one hour in a benzene solution with a trace of pyridine, caprylic to lauric acid anhydrides under similar conditions gave only 30 to 40% yields. Palmitic, stearic, arachidic, and behenic acid anhydrides appeared almost completely inert at the boiling point of benzene. At higher temperatures (boiling toluene or xylene) they were observed to produce the steryl esters in about the same yields as with the free fatty acids. This latter observation suggests that the esterification of the sterols by the free acids probably proceeds through the anhydrides which are continuously formed under the dehydrating conditions prevailing at elevated temperatures. These observations on the behavior of the long chain fatty acid anhydrides in sterol esterification are in line with certain accounts[40] of the behavior of these compounds towards water and aqueous alkali, which decompose them only with difficulty.

The lack of success with the long chain acid anhydrides led us to use the acid chlorides. For the elimination or at least reduction of the acid effect during esterification with acid chlorides, it was found profitable to perform the reaction in an inert solvent, such as benzene or toluene. These solvents precipitate most of the pyridine hydrochloride as soon as it is formed, and suppress the ionization of the rest. Mills[19] suggested this modification as an essential prerequisite for a successful esterification of a number of labile alcohols. Utilization of inert solvents is advantageous also when acid sensitivity is not a problem. In such cases the use of polar chlorocarbon solvents (*e.g.*, chloroform, ethylene chloride) which promote the reaction by immediately dissolving the reactants may result in improved yields and purer products. The use of such solvents may be advantageously combined with the preparation of acid chlorides in solution,[18] utilizing either phosphorus pentachloride for saturated acid chlorides or oxalyl chloride for unsaturated acid chlorides.

The ergosteryl esters were the most difficult to prepare. While the acetate and butyrate could be prepared in reasonable yields in the cold or at room temperature by treating the free sterol with the corresponding anhydride in the presence of pyridine, the higher esters had to be made by going through the acid chlorides. When the caproic, caprylic, and capric acid chlorides were allowed to react overnight with ergosterol at room temperature in the presence of the calculated amount of pyridine, yields of 70, 40, and 30%, respectively, were obtained. These yields could be further improved by extending the time of reaction to about three days. The ergosteryl esters of the longer chain fatty acids could not be made in satisfactory yields under these conditions. Heating was necessary, and it was accompanied by some destruction and isomerization of the sterol as indicated by the discoloration of the reaction products. The highest yields (50–70%) with the least destruction were obtained by performing the reaction in boiling benzene for up to thirty minutes under nitrogen. The preparation of the saturated fatty acid esters of the other sterols under similar conditions presented little difficulty, yields of 70 to 95% being obtained. The use of the higher boiling solvents such as toluene and xylene appeared to give somewhat higher yields with the saturated C_{16}, C_{18}, C_{20}, and C_{22} acid chlorides. The preparation of the unsaturated fatty acid esters in all cases required special care, and it was necessary to choose between a fair yield of a partially degraded product and a poor yield of a somewhat better product. The use of benzene to ensure the continuous removal of the pyridine hydrochloride formed and a short period of heating appeared to provide an acceptable compromise. Esterifications performed at temperatures lower than that of boiling benzene gave impractical yields.

The purification of the esters by recrystallization from the common organic solvents generally employed to free them from unchanged starting materials and by-products of the reaction was found to be unsatisfactory. The removal of the free sterols was particularly difficult, as they possess solubilities closely similar to those of their long chain fatty acid esters in these solvents. Recrystallization from glacial acetic acid has been described[11b] with apparently successful results. Since the sterols are very readily[34a] esterified by this reagent the final product is liable to be contaminated with the acetic acid ester. Samples giving no immediate precipitation with digitonin,[41] as commonly tested, could be demonstrated to contain residual free sterol when subjected to chromatography. The addition of aluminum chloride[42] to such test

(37) K. Kulka, *Am. Perfumer*, **51**, 141, 251, 253, 255, 342 (1948).

(38) (a) C. E. Bills and W. M. Cox, *J. Biol. Chem.*, **84**, 455 (1929); (b) C. E. Bills and F. G. McDonald, *J. Biol. Chem.*, **88**, 337 (1930).

(39) A. Kuksis and J. M. R. Beveridge, unpublished results.

(40) (a) D. Holde and R. Gentner, *Ber.*, **58B**, 1424 (1925); (b) N. O. V. Sonntag, J. R. Trowbridge, and I. J. Krems, *J. Am. Oil Chemists' Soc.*, **31**, 151 (1954).

TABLE I

CERTAIN PHYSICAL PROPERTIES OF SOME β-SITOSTERYL ESTERS

Ester	Method of Preparation[a]	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$[b] Obsd.	Specific Rotations, $(\alpha)_D$[b] Lit.	Specific Rotations, $(\alpha)_D$[b] Calcd.	Molecular Weights[c] Obsd.	Molecular Weights[c] Calcd.
Acetate	1A	98	125	127 [44][d]	−41	−42 [44]	−42 [44]	450	457
Butyrate	1A	95	111	93 [6][e]	−40		−39.6		485
Caproate	2B	95	105.5		−38		−37.5	520	513
Caprylate	2B	95	99		−36		−35.5	535	541
Caprate	2B	80	92		−33		−33.8	580	569
Laurate	2B	82	84		−31		−32.2		597
Myristate	2C	85	86.5		−30		−30.8	630	625
Palmitate	2C	80	85.5	83.5 [7][e]	−28	−7.3 [7][e]	−29.4		653
Stearate	2C	80	89	89 [7][e]	−28		−28.2	685	681
Oleate	2B	70	52	39 [6][e]	−28		−28.3		679
Linoleate	2B	60	43		−28		−28.4	670	677
Linolenate	2B	60	36		−28		−28.5	670	675
Arachidate	2D	65	92		−27		−27.2		709
Behenate	2D	65	95		−27		−26.1	745	737

[a] Methods 1 and 2 as described in the text: A refers to benzene used as solvent, room temperature, and an overnight reaction time. B refers to boiling benzene and 60- and 30-min. reaction times for the anhydrides and chlorides, respectively. C refers to boiling toluene and a 30-min. reaction time. D refers to boiling xylene and a 30-min. reaction time. [b] Specific rotations calculated from acetates by Tschugaeff's method. [c] Molecular weights of the esters synthesized were calculated from their saponification equivalents determined by standard methods. [d] Numbers in brackets indicate references.

TABLE II

CERTAIN PHYSICAL PROPERTIES OF SOME γ-SITOSTERYL ESTERS[a]

Ester	Method of Preparation	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$ Obsd.	Specific Rotations, $(\alpha)_D$ Lit.	Specific Rotations, $(\alpha)_D$ Calcd.	Molecular Weights Obsd.	Molecular Weights Calcd.
Acetate	1A	90	140	144[44]	−43	−45[44]	−45[44]	449	457
Butyrate	1A	90	121.5				−42.4		485
Caproate	2B	90	114.5				−40.0	520	513
Caprylate	2B	92	108		−39		−38.0		541
Caprate	2B	85	101				−36.2		569
Laurate	2B	75	95				−34.4		597
Myristate	2B	75	91				−32.9		625
Palmitate	2B	77	95		−30		−31.5	641	653
Stearate	2C	79	98				−30.2		681
Oleate	2B	63	55				−30.3		679
Linoleate	2B	55	51		−30		−30.4		677
Linolenate	2B	51	39				−30.5		675
Arachidate	2D	63	101		−28		−29.0	701	709
Behenate	2D	61	104				−27.9		737

[a] See notes at the foot of Table I.

solutions improved the sensitivity, but when sufficient ester was present, the water in the aqueous reagents caused its partial precipitation. Purification of the esters on silica or alumina columns was found to be essential. This permitted the removal of any nonpolar by-products, free sterols, and any peroxidized material formed during the esterification.

Performance of the esterification in the less polar solvents such as benzene or toluene or their mixtures with petroleum ether had also certain advantages for subsequent purification. When excessive amounts of pyridine were avoided, and the cooled reaction mixture poured on to the adsorbent column, all the pyridine was immobilized as its difficultly soluble hydrochloride or the unchanged acylpyridinium chloride at the top. The use of appropriate dilutions of benzene or ethyl ether in petroleum (b.p. 60–80°) removed selectively the desired reaction mixture component. Such an esterification in an inert solvent combined with the preparation of the acid chloride in solution, and followed by a direct adsorption chromatographic fractionation of the total reaction mixture, avoids unnecessary handling and time-consuming processing of the materials through distillations, washings, dryings, and numerous recrystallizations. Adsorption chromatographic methods for the purification of

(41) The steryl ester preparations were tested for the presence of free sterol by treating up to 10 mg. of the material dissolved in 5 cc. of acetone-ethanol (1:1) with a few drops (1 cc. max.) of 1% digitonin in ethanol-water (1:1). If no turbidity developed within a few hours 1 drop of a 30% aqueous solution of aluminum chloride hexahydrate[42] was added.

(42) H. H. Brown, A. Zlatkis, B. Zak, and A. J. Boyle, *Anal. Chem.*, 26, 397 (1954).

TABLE III

CERTAIN PHYSICAL PROPERTIES OF SOME STIGMASTERYL ESTERS[a]

Ester	Method of Preparation	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$ Obsd.	Specific Rotations, $(\alpha)_D$ Lit.	Specific Rotations, $(\alpha)_D$ Calcd.	Molecular Weights Obsd.	Molecular Weights Calcd.
Acetate	1A	95	142	144[44]	−54	−56[44]	−56[44]	462	455
Propionate[b]				122[28]					
Butyrate	1A	90	123	113[6e]	−53		−52.8		483
Caproate	2B	95	115		−51		−49.9	497	511
Caprylate	2B	93	112		−48		−47.3		539
Caprate	2B	91	106		−46		−44.9		567
Laurate	2B	73	102.5		−44		−42.8	600	595
Myristate	2B	71	101.5		−41		−40.9		623
Palmitate	2B	74	99.5	99[6b]	−41		−39.2	638	651
Stearate	2B	70	102	101[6b]	−39		−37.6		679
Oleate	2B	57	57	44[6b]	−39		−37.7		677
Linoleate	2B	54	50.5				−37.8		675
Linolenate	2B	49	38				−37.9		673
Arachidate	2D	63	104		−37		−36.1	693	707
Behenate	2D	67	106		−35		−34.7		735

[a] See notes at the foot of Table I. [b] Included for comparison.

TABLE IV

CERTAIN PHYSICAL PROPERTIES OF SOME STIGMASTANYL ESTERS[a]

Ester	Method of Preparation	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$ Obsd.	Specific Rotations, $(\alpha)_D$ Lit.	Specific Rotations, $(\alpha)_D$ Calcd.	Molecular Weights Obsd.	Molecular Weights Calcd.
Acetate	2B	99	136	138[44]	+13.3	+14[44]	+14[44]		459
Butyrate	2B	87	117				+13.2	500	487
Caproate	2B	84	109		+12.7		+12.5		515
Caprylate	2B	86	102		+12		+11.8		543
Caprate	2B	83	96				+11.2	557	571
Laurate	2C	80	91		+10		+10.7		599
Myristate	2C	77	89.5		+10		+10.2	613	627
Palmitate	2C	74	90.5				+9.8		655
Stearate	2D	76	92				+9.4		683
Oleate	2B	67	53		+9.5		+9.45	667	681
Linoleate	2B	59	48				+9.5		679
Linolenate	2B	47	42				+9.53		677
Arachidate	2D	62	95		+8.5		+9.05	706	711
Behenate	2D	53	98		+8.5		+8.7		739

[a] See notes at the foot of Table I.

steryl ester preparations have been used occasionally[43] before, but apparently it has not been generally appreciated that some such technique is essential to ensure removal of free sterol.

The methods used and the yields obtained in the preparation of the individual steryl esters together with the molecular weights calculated from the saponification equivalents determined are listed in Tables I–VI.

Properties of steryl esters. The melting points and the specific rotations of the steryl esters prepared are recorded in Tables I–VI. For the esters described previously the reported values are given for comparison. Since on heating, the crystalline steryl esters, because of their large and flat molecules, cannot pass into the isotropic state with a suddenness characteristic of the sharp melting low molecular weight compounds, but retain a considerable degree of order during the early stages of melting, difficulty is experienced in ascertaining the temperature at which this material starts to melt. After numerous observations it was noted that the most reliable temperature to record was that at which the material became fully liquid and the field of polarized light dark. These isotropic transitions were reproducible and the corresponding temperatures represent the melting points in the above tables. The rate of heating was critical, rapid rates giving lower melting points. For all steryl esters these melting points decreased with increasing molecular weight of the saturated fatty acids, until a minimum was reached in the myristate-palmitate region, after which the melting points tended to increase. For the esters of the β- and γ-sitosterols and of their saturated analogs the minima in the melting point curves were located at the myristates, while for the

(43) (a) R. O. Clinton, H. C. Neumann, S. C. Laskowski, and R. G. Christiansen, *J. Org. Chem.*, **22**, 473 (1957); (b) D. Gould, L. Finckenor, E. B. Hershberg, J. Cassidy, and P. L. Perlman, *J. Am. Chem. Soc.*, **79**, 4472 (1957).

TABLE V

CERTAIN PHYSICAL PROPERTIES OF SOME γ-SITOSTANYL ESTERS[a]

Ester	Method of Preparation	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$ Obsd.	Specific Rotations, $(\alpha)_D$ Lit.	Specific Rotations, $(\alpha)_D$ Calcd.	Molecular Weights Obsd.	Molecular Weights Calcd.
Acetate	2B	93	139	144[44]	+12	+10[44]	+10[44]	451	459
Butyrate	2B	91	120		+10.3		+9.4	482	487
Caproate	2B	90	112.5		+9.5		+8.9		515
Caprylate	2B	89	104.5				+8.5		543
Caprate	2C	77	97		+7.5		+8.0		571
Laurate	2C	75	92		+7.5		+7.7	607	599
Myristate	2C	73	90				+7.3		627
Palmitate	2C	75	93				+7.0		655
Stearate	2C	69	96		+7.0		+6.7	693	683
Oleate	2B	70	52		+6.5		+6.75		681
Linoleate	2B	61	44				+6.8		679
Linolenate	2B	57	39				+6.83		677
Arachidate	2D	76	101		+6.0		+6.45		711
Behenate	2D	78	103				+6.2	747	739

[a] See notes at the foot of Table I.

TABLE VI

CERTAIN PHYSICAL PROPERTIES OF SOME ERGOSTERYL ESTERS[a]

Ester	Method of Preparation	Yield, %	M.P., ° Obsd.	M.P., ° Lit.	Specific Rotations, $(\alpha)_D$ Obsd.	Specific Rotations, $(\alpha)_D$ Lit.	Specific Rotations, $(\alpha)_D$ Calcd.	Molecular Weights Obsd.	Molecular Weights Calcd.
Formate[b]				161.5[6a]		−97.9[6a]			
Acetate	1A	95	177	181[44]	−88	−90[44]	−90[44]		439
Butyrate	1A	90	134	129.5[6a]	−85		−84.7	451	467
Isobutyrate[b]				162[6e]		−84[6e]			
Isovalerate[b]				160[6e]		−82[6e]			
Caproate	1B	75	125.5				−79.8		495
Caprylate	1B	65	121		−80		−75.5	505	523
Caprate	1B	50	117.5				−71.7	550	551
Laurate	2B	70	116				−68.3		579
Myristate	2B	70	115		−67		−65.2	619	607
Palmitate	2B	72	110	107[7b]		−50.0[7b]	−62.2		635
Stearate	2B	60	113	110.5[7d]	−59	−53.4[7d]	−59.3	655	663
Oleate	2B	50	68	42[6e]			−59.7	647	661
Linoleate	2B	40	58		−58		−60.0		659
Linolenate	2B	40	47				−60.3		657
Arachidate	2C	50	116		−58		−57.2	703	691
Behenate	2C	50	115				−55.0		719

[a] See notes at the foot of Table I. [b] Included for comparison.

esters of the higher melting sterols (stigmasterol and ergosterol) the minima were at the palmitates. The decrease in the melting point per ethylene unit varied within an ester series and from one series to the other, but in all cases it was found to diminish progressively as the minimum was approached, after which there was a smaller but regular increase. The oleates melted 37 to 45° lower than the corresponding stearates and the introduction of additional double bonds in the fatty acid part of the steryl ester brought about further though considerably smaller lowering in the melting point.

In the cholesteryl ester series, Labarrere *et al.*[13] observed the minimum at myristate, after which the melting points increased regularly by about 5° per each ethylene unit added. Their observations were limited to the sequence laurate, myristate, palmitate, and stearate. They also reported the oleates to melt about 33° lower than the stearates, and each additional double bond was observed to lower the melting point by another 7°. The contamination of the linolenates with the linoleates in the present preparations did not permit such an exact evaluation of the effect of unsaturation upon the melting behavior of these series of steryl esters. The isotropic transition points reported by Gray[11b] for the complete series of the lower cholesteryl esters, however, indicate considerably more variation in the melting points and include a maximum at the acetate, a minimum at the caproate, and another maximum at the caprylate after which a progressive decrease was observed with the palmitates and the stearates having the same and the lowest isotropic transition points. In addition, a variety of other well defined transition states and temperatures corresponding to the smectic and nematic or cholesteric phases have been reported for cholestryl esters.[8a,11b,14,15] In the present investigation no such

definite and discrete mesomorphic transition states were observed. Though the alcohol parts of the esters are different it is doubtful whether one would be dealing here with a new phenomenon.

It appears very likely that the cholesteryl[8a,14,15] and other steryl[8a,8g] esters in which the mesomorphic melting transformations were first noted were impure. Recently, however, Gray[11b] was able to confirm these observations to some extent, and explained any differences in terms of purity of the samples and the conditions of examination. It is unfortunate that this report does not indicate the sources and properties of the starting materials and that the final products were characterized only by carbon and hydrogen analyses. Evidently the transition temperatures varied with impurity as the author continued the recrystallizations of the esters from glacial acetic acid and ethanol until these became constant.

When the rather sharply melting esters prepared in the present study were combined in various proportions, the mixtures melted over a wider range of temperatures, often with irregularities characteristic of the mesomorphic transitions, and with or without an actual change in the isotropic transition point of the major component. Similar effects have also been observed with other long chain compounds. Small amounts of octadecane have been found to cause the formation of two crystalline forms in hexadecane.[44] It may be of interest to note that the presence of a homolog has been reported to stabilize the metastable form of higher alcohols and nitriles.[45,46] Furthermore, work on the dimorphism of long chain secondary amines has shown that the relative stabilities of the polymorphic forms are influenced by extremely small amounts of impurities.[47] This influence of traces of contaminants on the polymorphic behavior of long chain compounds[48] raises the question whether even some of the supposedly well established observations upon the meso- and polymorphic states of pure compounds may actually relate to the behavior of mixtures.[48a] In the sterol series this dramatic effect of foreign material upon the melting point is particularly exaggerated. Thus, besides the esters, there have been described several loose combinations, for example, of ergosterol with glycerol, orcinol, urea, and substituted ureas, all of which exhibit more or less extended turbid phases in their melting.[8b] The possibility has also been expressed that the phenomenon of mesomorphism might be related to the turbidity associated with moisture.[11a] This latter effect is particularly pronounced when the melting points are determined under conditions where the escape of water is hindered, *e.g.* sealed capillaries.[6c] Since many of the compounds exhibiting mesomorphism are also easily oxidized, the impurity necessary for the stabilization of these transition states may come from air oxidation of the sample during melting. Melting points taken in evacuated capillaries have been demonstrated to result in sharp transitions.[34a]

Dr. Gray very kindly examined samples of β-sitosteryl laurate and myristate provided by us and also found that they melted sharply passing directly into the isotropic phase without going through any mesomorphic transition state. On cooling (including rapid chilling), however, though sometimes the melts crystallized directly, Dr. Gray was able to detect a mesophase resembling the smectic state, which was monotropic with respect to the crystals. That is, the mesomorphic-isotropic transition occurred at a temperature below the melting point of the crystals. Jaeger[8a] also noted that equilibrium disturbances such as sudden cooling of the sample facilitated the observation of certain mesomorphic transitions. In view of the above mentioned possibility of air oxidation, particularly on repeated remelting of the sample, the transitions observed on cooling should be viewed with caution. It may be further noted that neither the instrument of Gray[49] nor that described here may have been capable of recording the true transition temperatures. Thus, the reversion of the smectic state formed on cooling, to the isotropic, on raising the temperature again, may not be said with certainty to have taken place at the corresponding block temperature, as the melt may not have been in equilibrium with it. It is well known that when supercooled melts crystallize, the temperature immediately rises to the true melting or freezing point. In these apparatuses such a rise in temperature within the sample alone could not be determined. The rising temperature bringing about the reversion may have provided little more than a disturbance of the metastable supercooled melt.

The specific rotations decreased slightly with increasing molecular weight of the fatty acid residue, and the molecular rotations remained essentially constant. The introduction of unsaturation into the fatty acid moiety had little or no effect on the rotation. Similar observations have been made before[10,13] in the cholesteryl ester series and are in agreement with theory.[50] The measurements, however, were not sufficiently accurate to distinguish between the hypothesis of Tschugaeff[50a] and of Gerson.[50b] As a result of the high purity of the start-

(44) W. Bergmann, *Ann. Rev. Plant Physiol.*, **4**, 383 (1953).

(45) J. C. Smith, *J. Chem. Soc.*, 737 (1932); Annual Reports on the Progress of Chemistry for 1938, The Chemical Society, London, 1939, Vol. **35**.

(46) E. J. Hoffman, C. W. Hoerr, and A. W. Ralston, *J. Am. Chem. Soc.*, **67**, 1542 (1945).

(47) C. W. Hoerr, H. J. Harwood, and A. W. Ralston, *J. Org. Chem.*, **11**, 199 (1946).

(48) (a) A. W. Ralston, *Fatty Acids and Their Derivatives*, John Wiley & Sons, New York, N. Y., 1948, p. 322 ff.; (b) G. H. Brown and W. G. Shaw, *Chem. Revs.*, **57**, 1049 (1957).

(49) G. W. Gray, *Nature*, **172**, 1137 (1953).

(50) (a) L. Tschugaeff, *Ber.*, **1**, 360 (1898); (b) T. Gerson, *Nature*, **179**, 310 (1957).

ing materials, the relatively sharp melting points, and the failure to demonstrate the presence of impurities in any of the esters other than the linolenates by the chromatographic methods described, the physical constants reported are believed to be reliable. Because of the characterisic melting point curves of these homologous series, the melting temperatures of the longer chain fatty acid esters differ little, and cannot be used for a reliable identification of unknown plant steryl esters.

EXPERIMENTAL[51]

Ordinarily melting points were taken either in open capillary tubes using a modified Hershberg type melting point apparatus or the Fisher-Johns hot stage and were corrected. Sealed capillaries and overnight equilibration at −25° were used for the determination of the melting points of the lower melting fatty acids and their derivatives. For the detection of the mesomorphic transition stages of the steryl esters a specially constructed heating stage mounted on a polarizing microscope was used.[52] The rate of heating in all instruments was about 0.5° per min. in the region of melting.

The infrared measurements were made on a Perkin Elmer Infracord Spectrophotometer equipped with sodium chloride optics. Unless otherwise specified 10% solutions in carbon disulfide and 0.1-mm. cells were used. The ultraviolet measurements were made on a Beckman Model DK2 recording spectrophotometer with 1-cm. matched silica cells. Optical rotations were measured at 25° on 2% solutions in chloroform with a Hilger Model M 412 polarimeter equipped with a sodium vapor lamp. A 4-cc. top-filled cell was used.

Fatty acids. The acids, acetic to myristic (reagent grade), arachidic (synthetic, highest purity), and behenic (practical) were purchased from Fisher Scientific Co. Linolenic acid was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Palmitic, stearic, oleic, and linoleic acids were gifts from E. F. Drew and Co., Inc., Boonton, N. J. The acids were purified by low temperature crystallization[53] until correct[54] melting points of free acids and anilides, and satisfactory iodine (Yasuda) numbers were obtained. The recrystallized materials, with the exception of acetic, butyric, and caproic acids, were checked for purity using reversed phase paper partition chromatography.[55] The acids were stained either with iodine,[55a] the copper acetate–ferrocyanide[55b] or the mercuric acetate-*s*-diphenylcarbazide[55c] reagents. The only material to contain readily detectable amounts of contaminants was linolenic acid. It was estimated[55a] to contain not more than 10% linoleic acid, but in the absence of any ready means for the separation of the two acids, no attempt was made at further purification. The oleic and the linoleic acids were the natural isomers and appeared[56] to be free from *trans* isomers. The linolenic acid contained *trans* double bonds corresponding to an estimated maximum of about 15% of a *cis cis trans* isomer. In addition this acid also contained an estimated 2% of the conjugated form when assayed as described by Wood *et al.*[17]

Fatty acid chlorides. The acid chlorides, acetyl to myristyl (highest purity) were obtained from Fisher Scientific Co. and except for the lower three were demonstrated to be essentially free from homologs when chromatographed as the free acids.[55] The higher saturated fatty acid chlorides were prepared from the corresponding fatty acids by the method of Youngs *et al.*,[18] and the unsaturated acid chlorides by a modification of the procedure of Wood *et al.*[17] The unsaturated fatty acids (5 g.) were dissolved in 100 cc. of light petroleum (b.p. 30–60°) and the solution dried by distilling a small portion of the solvent. To the dried solution were added 2.5 to 3.0 molecular equivalents of oxalyl chloride and the solution refluxed for 1 hr. The reaction mixture was then cooled and the excess oxalyl chloride destroyed by extracting the solution with ice water. To elimi-

(51) All samples were dried in an Abderhalden drying pistol over phosphorus pentoxide for several hours at 60 or 80° and 2 mm. before being analyzed.

(52) The apparatus was similar in principle to that described by Gray[49] but differed in certain essential features. The heating stage consisted of an aluminum block 90 mm. in diameter and 26 mm. thick. A hole 13 mm. in diameter was bored through the center of the block and a recess 18 mm. in diameter and 7 mm. deep was provided at the top, into which an ordinary cover glass could be lowered. A small slot in the top connected with the circular recess to enable special bent forceps to be used when inserting or removing cover glasses. At the side of the block, just below the bottom of the recess, a hole was drilled to take a thermometer, the bulb of which protruded slightly into the 13 mm. diameter central hole. An aluminum tube was pressed part way into the side hole, leaving a clear space around the thermometer bulb. The tube extended far enough from the main block to provide ample support for the thermometer body (Fisher Scientific Co., Cat. No. 14-985). At the bottom of the main block a large diameter shallow recess accommodated a standard 100 watt Chromalox Ring Type heating element (Canadian Chromalox Co., Ltd., 251 Queen Street East, Toronto, Ontario), which was held in contact with the aluminum block by an aluminum plate with a 6-millimeter thick asbestos millboard in between. The plate was larger in diameter than the main block and was arranged to carry a tubular shield for protecting the block from air currents. The center of the plate was provided with a recess in which a glass disk was held by means of a split ring. Three Bakelite supporting posts were used to prevent heat dissipation through the microscope stage and frame. The posts were attached to another aluminum plate which was fastened to the microscope stage by knurled screws. The whole assembly was about 60 mm. high and could be readily accommodated on the rotating stage of a Leitz Model III M (E. Leitz, Wetzlar) polarizing microscope. Using a 30 mm. objective (12×) there is, after focusing, enough air space left between the heating stage and the lens (6 or 10×) to provide satisfactory insulation for temperatures up to 150°. A Leitz "Ultralux" illuminator served as an effective light source. The accuracy of the instrument was determined by taking melting points of purified organic substances covering a range of temperatures from 40 to 150°. The corrected values obtained with this instrument agreed within less than ±0.5° with those obtained in the Hershberg type melting point apparatus. For the determination, the sample was spread out evenly between two 18-mm. diameter cover slips and lowered into the central hole of the heating block. A microscope slide was then placed over the top of the hole and the melting behavior examined in the appropriate temperature range. Removal of the knurled screws (see above) permitted limited movement of the heating stage allowing examination of any part of the melt.

(53) J. B. Brown and D. K. Kolb, *Progress in the Chemistry of Fats and Other Lipids*, R. T. Holman, W. O. Lundberg, and T. Malkin, eds., Pergamon Press, New York, N. Y., 1955, Vol. 3, p. 57.

(54) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, *The Systematic Identification of Organic Compounds*, John Wiley and Sons, Inc., New York, N. Y., 1956, p. 274 ff.

(55) (a) H. Schlenk, J. L. Gellerman, J. A. Tillotson, and H. K. Mangold, *J. Am. Oil Chemists' Soc.*, **34**, 377 (1957); (b) H. P. Kaufmann and W. H. Nitsch, *Fette u. Seifen*, **56**, 154 (1954); (c) H. Wagner, L. Abisch, and K. Bernhard, *Helv. Chim. Acta.*, **38**, 1536 (1955).

(56) D. Swern, H. B. Knight, O. D. Shreve, and M. R. Heether, *J. Am. Oil Chemists' Soc.*, **27**, 17 (1950).

nate any significant hydrolysis of the acid chlorides excessive shaking was avoided and each time the water layer was removed as soon as it was formed. Usually such preparations of the fatty acid chlorides, as estimated by the method of Youngs *et al.*,[18] contained 2 to 6% of free acid. This contamination was not considered serious as the relatively inert fatty acids did not interfere with the esterification and the 20 to 50% excess of acid chloride used in most preparations was enough to ensure a complete esterification of the sterol. The acid chlorides gave anilides with correct melting points.[54]

Fatty acid anhydrides. The acid anhydrides, acetic, butyric, and caproic (highest purity) were purchased from Fisher Scientific Co. The other anhydrides, caprylic to behenic, were prepared as described by Wallace and Copehaver[57] from the corresponding fatty acids and acetic anhydride. These anhydrides possessed physical constants in good agreement with those reported in the literature.[40b]

Ergosterol. Purified ergosterol was prepared from a commercial product (m.p. 160°, $[\alpha]_D$ −137°) purchased from the Mann Research Laboratories, Inc., New York, by adsorption chromatography on aluminum oxide deactivated by ethyl acetate as described by Weizmann *et al.*[34b] The sterol was adsorbed from a benzene solution and the column developed by successive treatments with benzene containing increasing concentrations of ethyl ether. The major portion of the material was eluted as a continuous band with benzene:ether, 4:1 by volume. After discarding the front and the end fractions, which deviated considerably in their properties from ergosterol, the middle fractions were rechromatographed. The material (m.p. 163°; $[\alpha]_D$ −132°) consisting of the newly obtained front and middle fractions was pooled and recrystallized four times from methanol-ether, m.p. 164°; $[\alpha]_D$ −131.5°, lit.,[6e] m.p. 165°; $[\alpha]_D$ −130°. The ultraviolet absorption maxima were at 271, 282, and 293 mμ with ε of 11,100, 11,700, and 6600, respectively, identical with the literature.[58]

Stigmasterol (Method 1). This method was essentially the same as that described by Windaus and Hauth.[28] The zinc dust used in the debrominations was freshly prepared.[59] In this way 5 g. of stigmasterol was isolated from 100 g. of crude soy sterols (Distillation Products Industries, Rochester, N. Y.). Repetition of this treatment two more times yielded 4 more g. of stigmasterol, m.p. 168°, $[\alpha]_D$ −48°. On aluminum oxide chromatography (*cf.* procedure for ergosterol) a small amount of more polar material was removed. The recovered stigmasterol after three recrystallizations from methanol-ether melted at 168°, $[\alpha]_D$ −48°; lit.,[60] m.p. 170°; $[\alpha]_D$ −49°.

Stigmasterol (Method 2). This method was similar to that described by Campbell *et al.*[29] and permitted a nearly quantitative[27b] removal of stigmasterol from the crude soy sterol mixture. A total of 20 g. of stigmasterol, m.p. 164°, $[\alpha]_D$ −46°, was isolated from about 100 g. of the soy sterols. Repeated adsorbtion chromatographic purification (*cf.* procedure for ergosterol) of this preparation with repeated removal of the lower melting front fractions yielded stigmasterol which after four recrystallizations from methanol-ether melted at 168.5° and $[\alpha]_D$ −48.5°. A more effective way for the final purification of this stigmasterol preparation was by the precipitation of the acetate tetrabromides.

γ-Sitosterol (Method 1). The method of Dirscherl and Nahm[32] based on an earlier description by Bonstedt[31] was followed. The mixed soy steryl acetate dibromides, freed from the stigmasteryl acetate tetrabromides by repeated (five times) application of the Windaus-Hauth process, were debrominated and saponified. The free sterols were recrystallized from absolute ethanol to remove the dihydrositosterols. After twenty recrystallizations by the diamond type of triangulation,[61] the top 5 to 10% of the material was discarded. The rest of the material was pooled, acetylated, and the acetates recrystallized from absolute ethanol. The γ-sitosteryl acetate crystallized preferentially. After thirty-five recrystallizations the top 10% of the acetate (m.p. 142°; $[\alpha]_D$ −43.5°) was pooled, saponified, and the sterol recrystallized six times from methanol-ether, m.p. 146.5; $[\alpha]_D$ −41.9°; lit.,[62] m.p. 147–148°; $[\alpha]_D$ −43°. No change in these constants was observed when the γ-sitosterol preparation was subjected to adsorption chromatographic fractionation on aluminum oxide.

γ-Sitosterol (Method 2). The sitosteryl α-naphthyl carbamates free from stigmasterol were hydrolyzed[29] and the sterols recovered. These were freed from any dihydrositosterols by recrystallization from absolute ethanol.[31] The diamond type of triangulation was performed on 100-g. batches of the mixed sitosterols and after twenty recrystallizations the top 5% of each batch, enriched in the saturated sterols, were discarded. The rest of the material was pooled and the solvent removed. The dry sterols were taken up into acetone and any campesterol removed as described by Fernholz and MacPhillamy.[33] After twenty-five recrystallizations the top 5% of the material enriched in campesterol was discarded. The remaining material was pooled, the solvent removed, and the sterols acetylated. The acetates were recrystallized from absolute ethanol by triangulation when the more difficultly soluble γ-sitosteryl acetate accumulated in the top fractions. After about twenty-five recrystallizations the top fractions melted at 143°, $[\alpha]_D$ −41°. The recrystallization was continued and the top fractions pooled. After a total of thirty-five recrystallizations about 10% of the original acetate had reached the physical constants specified above. This material was saponified and the sterols recrystallized six times from absolute ethanol. Bromination-debromination or adsorption chromatography of this γ-sitosterol preparation on deactivated aluminum oxide (*cf.* procedure for ergosterol) effected no further segregation. The recovered sterol after two recrystallizations from methanol-ether melted at 143°, $[\alpha]_D$ −41.3°. Mixed melting point with the γ-sitosterol preparation from Method 1 resulted in a value (144–145°) intermediate between the melting points of the two preparations. Bergmann and Low[22] report a melting point of 138°, $[\alpha]_D$ −42° for the γ-sitosterol or clionasterol prepared from sponges.

β-Sitosterol (Method 1). This method was identical with that used by Wallis and Chakravorty.[21] The cottonseed oil was supplied by Canada Packers Limited, Toronto, Ontario. From 2.7 kg. of the oil a total of 4.5 g. of presumably pure β-sitosterol, m.p. 139°, $[\alpha]_D$ −35.1° was obtained. The constants remained unchanged on further recrystallization or aluminum oxide chromatography. Wallis and Chakravorty report a melting point of 140° and $[\alpha]_D$ −36°.

β-Sitosterol (Method 2). The bottom 25 to 30% of the steryl acetate fractions from the γ-sitosteryl acetate recrystallizations (pooled from Methods 1 and 2) were combined and saponified. The free sterols (m.p. 135°, $[\alpha]_D$ −33°) were converted into the benzoates by standard methods. The benzoates were recrystallized from a mixture of benzene-ethanol as described by Wallis and Fernholz[63] to remove any α-sitosteryl benzoates present. The top fractions reached a constant melting point at 146°, $[\alpha]_D$ −14°, after about sixteen recrystallizations. The recrystallization was con-

(57) J. M. Wallace and J. E. Copehaver, *J. Am. Chem. Soc.*, **63**, 699 (1941).

(58) E. Schwenk and G. J. Alexander, *Arch. Biochem. Biophys.* **76**, 65 (1958).

(59) R. L. Shriner and F. W. Neumann, *Org. Syntheses*, **Coll. Vol. III**, 73 (1955).

(60) M. H. Thornton, H. R. Kraybill, and J. H. Mitchell, Jr., *J. Am. Chem. Soc.*, 62, 2006 (1940).

(61) R. S. Tipson, *Technique of Organic Chemistry*, A. Weissberger, ed., Academic Press, Inc., New York, N. Y., 1956, Vol. 3, Part 1, p. 395 ff.

(62) W. Dirscherl, *Z. physiol. Chem. Hoppe-Seyler's*, **257**, 242 (1939).

(63) E. S. Wallis and E. Fernholz, *J. Am. Chem. Soc.*, **58**, 2446 (1936).

tinued and when sufficient material was collected with the above constants, it was saponified and the sterol isolated. After about four recrystallizations from methanol-ether the β-sitosterol melted at 139°, $[\alpha]_D$ −35.2°. Further crystallization of the sterol or adsorbtion chromatography failed to change these constants. A mixture with the β-sitosterol preparation from Method 1 melted at 140°.

Stigmastanol. This compound was prepared by catalytic hydrogenation[64] of the β-sitosterol isolated from cottonseed oil. It melted at 140°, $[\alpha]_D$ +25°; lit.,[65] m.p. 140°; $[\alpha]_D$ +24.9° for stigmastanol prepared from β-sitosterol by similar methods.

γ-Sitostanol was prepared from γ-sitosterol by the method described for stigmastanol. Both γ-sitosterol preparations gave the same saturated derivative, m.p. 144° and $[\alpha]_D$ +18°; lit.,[31] m.p. 144°; $[\alpha]_D$ +19°. For poriferastanol, prepared synthetically, but supposedly identical with γ-sitostanol, Lyon and Bergmann[66] report m.p. 143°; $[\alpha]_D$ +25°. The material gave a faint Liebermann-Burchard test[67] after 10 to 15 min. at room temperature.

Preparation of steryl esters (Method 1). The sterols (0.1–0.2 g.) were dissolved in benzene, toluene, or xylene (50 cc.), depending on the ester to be made, and the solution dried by distilling a small portion of the solvent. To the dried solution were added 1.2–1.5 molecular equivalents of the acid anhydride and a few drops (1 ml. max.) of dry pyridine. After refluxing for 1 hr. or standing overnight at room temperature, the solutions were suitably diluted with light petroleum (b.p. 30–60°) and chromatographed on silicic acid as previously described.[20] The steryl esters were eluted with 20% benzene in light petroleum (b.p. 30–60°), free fatty acids with 5% diethyl ether in light petroleum (b.p. 30–60°), and free sterols with 25% diethyl ether in petroleum (b.p. 30–60°). Any undecomposed fatty acid anhydrides were recovered with the free fatty acids or free sterols. Yields of chromatographically purified esters varied from quantitative for the acetates to as low as 10% for the stearates and other long chain fatty acid anhydrides. In the latter cases the yields could be improved by extending the time of heating.

Preparation of steryl esters (Method 2). The sterols (0.1–0.2 g.) were dissolved in benzene (10 cc.) and petroleum ether (b.p. 60–80°) or benzene (90 cc.) added. The solution was refluxed for a few minutes, and dried by distilling a portion (25 cc.) of the solvent. To the dried solution was added about 1.2–1.5 molecular equivalents of acid chloride dissolved in the appropriate solvent and just enough pyridine to combine with the hydrogen chloride formed. After refluxing for 30 min., the solution was cooled to room temperature, diluted with petroleum ether, if necessary, and poured on a silicic acid column. The chromatography was performed as described above. The pyridine hydrochloride formed and any undecomposed acylpyridinium chloride remained at the top of the column. Yields ranged from 60 to 95% of chromatographically purified material. For the determination of the physical constants the esters were recrystallized from methanol-ether mixtures.

Purity of products. Following the preparation of the steryl esters, reversed phase paper partition chromatographic techniques were developed for their separation and identification.[68] By this means it was demonstrated that the steryl esters synthesized and purified by the methods described above were free of readily detectable amounts of homologs and isomers and were not contaminated with unesterified sterol. Digitonide precipitation,[41] customarily employed for ascertaining the absence of free sterol from steryl ester preparations, was observed not to be sufficiently sensitive.

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