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THE STEROLS OF PINE BARK*

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Abstract—The sterols of several pine barks have been shown to consist predominantly of β-sitosterol with about one-tenth as much campesterol. The presence of traces of various dihydrosterols, cholesterol, α_1-sitosterol, triterpenoids, $\Delta^{3,5}$-stigmastadien-7-one, Δ^4-stigmasten-3-one, and 7-keto-β-sitosterol was indicated. Possible biosynthetic interrelationships are discussed.

DURING a survey being made at the U.S. Forest Products Laboratory on the benzene extractives of pine barks, we had occasion to examine the sterols from the barks of jack pine (*Pinus banksiana* Lamb.), lodgepole pine (*P. contorta* Dougl.),[1] sugar pine (*P. lambertiana* Dougl.), and loblolly pine (*P. taeda* L.). The sterols were precipitated from the unsaponifiable fraction of the benzene extract with digitonin and then carefully chromatographed. The results are shown in Table 1. As expected, the major sterol is β-sitosterol (Δ^5-stigmasten-3β-ol, 24α-ethylcholesterol).[2-4] This sterol has been often reported in this genus and in tall oil,[5] and indeed, appears to be ubiquitous in higher plants.[6] It occurs free, as esters of various fatty and aromatic acids, and as glycosides.

Pure β-sitosterol has been obtained only recently.[7] It is now recognized that all previous

* Previous paper in this series: J. W. ROWE and J. H. SCROGGINS, "Benzene extractives of Lodgepole Pine bark. Isolation of new diterpones," *J. Org. Chem.* **29**, 1554 (1964).

† Maintained at Madison, Wis., in co-operation with the University of Wisconsin.

[1] J. W. ROWE and J. H. SCROGGINS, *J. Org. Chem.* **29**, 1554 (1964).

[2] Representations of the stigmastane skeleton which assign a 24β orientation to the ethyl group have been shown to be incorrect. Y. KISHIDA, *Chem. Pharm. Bull. (Tokyo)* **8**, 357 (1960); K. TSUDA, R. HAYATSU and Y. KISHIDA, *Chem. & Ind. (London)* 1411 (1959); Y. KISHIDA, *Chem. & Ind. (London)*, 465 (1960); K. TSUDA, Y. KISHIDA and R. HAYATSU, *J. Am. Chem. Soc.* **82**, 3396 (1960). Thus campesterol and β- sitosterol have the same α orientation at C(24), and this is opposite to that of ergosterol and brassicasterol.[3] See also Ref. 9*c* in which the stereochemistry at 24 is proven for spinasterol ($\Delta^{7,22}$-stigmastadien-3β-ol).

[3] It should be noted that although W. BERGMANN, *Comparative Biochemistry*, Vol. IIIA (Edited by M. FLORKIN and H. S. MASON), pp. 114–115, Academic Press, New York (1962), correctly states that β-sitosterol is 24α-ethylcholesterol, campesterol, in contrast to most authorities, is listed as 24β-methylcholesterol. However, the illustrations show just the opposite stereochemistry due to Bergmann's incorrect assignment of the stereochemistry of the 20-methyl group.[4a] See also W. BERGMANN, "The Plant Sterols," in *Annual Review of Plant Physiology*, Vol. 4, (Edited by D. I. ARNON), p. 383, Annual Reviews, Inc., Stanford, Calif. (1953).

[4] L. F. FIESER and M. FIESER, *Steroids*, Reinhold Publishing Co., New York (1959), (*a*) pp. 337–340, (*b*) chap. 11–12.

[5] See index listings under "Sterols" in J. WEINER, *Tall Oil*, Bibliographic Series No. 133–135, 3rd Ed., Institute of Paper Chemistry, Appleton, Wis. (1959).

[6] For general references on plant steroids, and especially β-sitosterol, see W. KARRER, *Konstitution und Vorkommen der organischen Pflanzenstoffe*, pp. 844–862, Birkhauser Verlag, Basel (1958); A. STOLL and E. JUCKER, *Modern Methods of Plant Analysis*, (Edited by K. PAECH and M. V. TRACEY), Vol. III, pp. 141–176, Springer-Verlag, Berlin (1955); E. HEFTMANN, "Biochemistry of Plant Steroids", in *Annual Review of Plant Physiology*, (Edited by L. MACHLIS and W. R. BRIGGS), Vol. 14, Annual Reviews, Inc., Palo Alto, 1963; and references 3, 4*b*, 12*a*, 15, 23.

[7] J. A. STEELE and E. MOSETTIG, *J. Org. Chem.* **28**, 571 (1963).

β-sitosterol preparations contained 5–31 per cent of campesterol (24α-methylcholesterol) which cannot be removed by the usual procedures, but is readily separated on gas chromatography.[8] We have observed that all of our β-sitosterol preparations from pine barks likewise show the presence of a major impurity on gas chromatography whose retention time is identical to that of authentic campesterol. The recent suggestion[9a] that the impurity might instead be 22-dihydrobrassicasterol was based upon the wrong stereochemistry for the stigmastane skeleton.

TABLE 1. PINE BARK STEROLS

Bark (% benzene extract)	% sterols in benzene extract	Percent composition						
		β-Sitosterol	Campesterol	α_1-Sitosterol	Oxidized β-sitosterol*			Other
					A	B	C	
Lodgepole pine[1] (28·7)	3·9	73	5	4	2	0·4	-	—
Loblolly pine (4·6)	2·8	68	7	4	3	—	1·8	12 pinusenediol 1 cholesterol
Sugar pine (2·1)†	5·0	63	9	—	—	0·2	—	—
Jack pine (4·2)	2·0	60	6	4	2	0·1	—	5 pinusenediol 1 cholesterol 5 dihydrosterols 0·5 methoxy-triterpene[1]

* A = 7-keto-β-sitosterol, B = $\Delta^{3,5}$-stigmastadien-7-one, C = Δ^{4}-stigmasten-3-one
† Chloroform extract.

As Bergmann[3] has observed, primitive living systems, such as sponges and coelenterates, are characterized by the presence of a large variety of different sterols, including cholesterol. At higher levels of animal evolution, other sterols disappear and cholesterol rises to an increasingly dominant position until it has become the principal sterol of the vertebrate. However, the vast bulk of plant sterols carry substituents at C(24) in the side chain. Primitive plants, such as fungi, lichens, and certain algae, produce preferentially the 24-methyl or methylene derivatives of cholesterol. Among higher plants, the 24-ethyl or ethylidene derivatives are most conspicuous, especially β-sitosterol. The role of β-sitosterol is largely unknown; it may have a function in cell wall permeability.

That lanosterol (4,4,14-trimethyl-$\Delta^{8,24}$-cholestadien-3β-ol) is the biogenetic precursor of β-sitosterol is supported by tracer studies using labeled mevalonic acid.[10,11] It has been shown that the 24α-ethyl group is introduced via a double methylation,[9] which probably converts the unsubstituted Δ^{24} side chain first to the 24-methylene derivative and then to the

[8] M. J. THOMPSON, S. J. LOULOUDES, W. E. ROBBINS, J. A. WATERS, J. A. STEELE and E. MOSETTIG, *Biochem. Biophys. Res. Commun.* **9**, 113 (1962)

[9] (*a*) M. CASTLE, G. BLONDIN and W. R. NES, *J. Am. Chem. Soc.* **85**, 3306 (1963); (*b*) H. J. NICHOLAS and S. MORIARTY, *Fed. Proc.* **22**, Part I, 529 (1963); (*c*) S. BADER, L. GUGLIELMETTI and D. ARIGONI, *Proc. Chem. Soc.* 16 (1964); (*d*) W. R. NES and G. A. BLONDIN, IUPAC Symposium *The Chemistry of Natural Products*, Kyoto, Japan, April, 1964.

[10] E. J. HERBERT and G. W. KIRBY, *Tetrahedron Letters* No. 23, 1505 (1963). A. R. BATTERSBY and G. V. PARRY, *Tetrahedron Letters* No. 14, 787 (1964); H. J. NICHOLAS, *Nature* **189**, 143 (1961)

[11] D. F. JOHNSON, F. HEFTMANN and G. V. C. HOUGHLAND, *Arch. Biochem. Biophys.* **104**, 102 (1964)

24-ethylidene derivative, which upon reduction gives the saturated side chain as in β-sitosterol, or its isomer, clionasterol (24β-ethylcholesterol, Δ^5-poriferasten-3β-ol).[12k,13] This probably involves a methylating agent, such as S-adenosylmethionine.[14] These methylations may well occur before removal of all of the three extra angular methyl groups in lanosterol. A number of tetracyclic 24-methylene and 24-ethylidene steroids and triterpenoids which still contain one or more of these angular methyl groups are found in nature.[3,4b,5,12,15,16] In the column chromatograms of the pine barks sterols, small amounts of high-melting fractions, believed to be triterpenoids, were observed in all cases. Gas chromatograms indicated the presence of a compound with the same retention time as authentic α_1-sitosterol (4α-methyl-$\Delta^{7,24(28)}$-stigmastadien-3β-ol)[16] in the sterols from lodgepole, jack, and loblolly pine barks. This compound, which is also reported in *P. densiflora* F. and Z.,[17] is probably either a biogenetic intermediate between lanosterol and β-sitosterol, or closely related to such an intermediate. These biosynthetic interrelationships postulated are illustrated in Fig. 1.

The presence of a minor peak, with the retention time of cholesterol, in the gas chromatograms of the crude loblolly and jack pine sterols, is interesting. Cholesterol has been reported only occasionally in higher plants.[11,18,19] That cholesterol may be a direct precursor of β-sitosterol is not considered biosynthetically probable. Biological alkylation of an unactivated, saturated carbon atom is unlikely, whereas alkylation of a double bond is quite acceptable. Thus cholesterol and β-sitosterol both undoubtedly arise from lanosterol as a common intermediate. Reduction of the unsubstituted side chain, perhaps by the same enzyme that reduces the 24-ethylidene side chain, as in fucosterol,[12l] would thus prevent alkylation and lead to cholesterol as the end product. Likewise, reduction of the 24-methylene side chain before the second alkylation would lead to campesterol as the end product. The presence of small amounts of cholesterol and campesterol in pine bark sterols may perhaps be due to a lack of specificity of the hydrogenase. This agrees with the suggestion that in older genera, such as *Pinus*, the enzymes seem to be less specific and families of closely related compounds occur; e.g., the various monoterpenes and diterpene resin acids in pine oleoresin, and the polyphenols in pine heartwood extractives.[20]

Since stigmastan-3β-ol is normally present in small amounts in most β-sitosterol preparations,[12a] including those from *P. sylvestris* L.,[21] *P. densiflora*,[17] and tall oil,[22] the purified sterols from jack pine bark were oxidized and chromatographed to yield 5 per cent of stigmastan-3-one,[12l] in addition to the major products, Δ^4-stigmasten-3-one,[12d] and Δ^4-stig-

[12] F. RADT, *Elsevier's Encyclopaedia of Organic Chemistry*, Vol. 14, Elsevier Publishing Co., New York (1954 & 1959); pp. (*a*) 1780–1828s, (*b*) 2682s, (*c*) 1595s, (*d*) 2467s, (*e*) 2424s, (*f*) 2696s, (*g*) 2454s, (*h*) 2470s, (*i*) 2469s, (*j*) 2913s, (*k*) 1839s, (*l*) 1793s, (*m*) 2420s.

[13] γ-Sitosterol has been shown to be a mixture of campesterol and β-sitosterol rather than the 24-epimer of β-sitosterol as formerly believed. M. J. THOMPSON, W. E. ROBBINS and G. L. BAKER, *Steroids* **2**, 505 (1963).

[14] L. W. PARKS, *J. Am. Chem. Soc.* **80**, 2023 (1958).

[15] C. DJERASSI, *Biochemistry of Steroids*, (Edited by E. MOSETTIG), Vol. IV, *Proc. 4th Int. Congr. Biochem.*, pp. 1–20, Pergamon Press, New York (1959).

[16] K. SCHREIBER and G. OSSKE, *Experientia* **19**, 69 (1963).

[17] S. ITO, *Nippon Daigaku Kogaku Kenkyusho Iho*, No. 13, 114 (1956).

[18] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, *Science* **140**, 198 (1963); M. VON ARDENNE, K. STEINFELDER, R. TÜMMLER and K. SCHREIBER, *Experientia* **19**, 178 (1963).

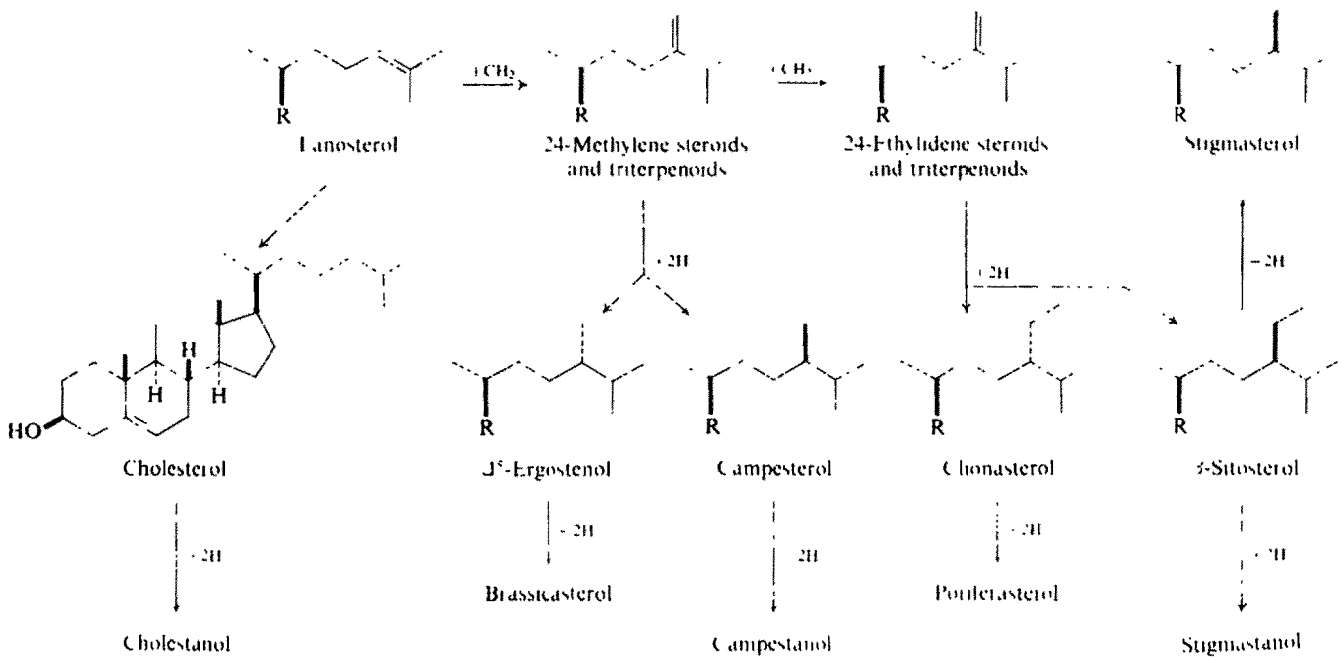
[19] Charles H. Schaefer, Shell Development Co., Modesto, Calif., informed me that he has identified about 1 per cent cholesterol in the sterols from Virginia pine foliage via retention time of the sterol, trimethylsilyl ether, acetate, and dihydrosterol on gas chromatography on NGS, SE-30, and QF-1.

[20] H. ERDTMAN, *Pure Appl. Chem.* **6**, 679 (1963).

[21] K. PAJARI, *Ann. Acad. Sci. Fennicae*, **59A**, 7 (1942); *Fette u. Seifen*, **50**, 506 (1943).

[22] D. H. R. BARTON and E. R. H. JONES, *J. Chem. Soc.* 599 (1943); T. ENKVIST, *Svensk Papperstidn.* **50**, 351 (1947); T. HASSELSTROEM, *Paper Trade J.* **128**, No. 7, 17 (1949).

BIOGENESIS OF PHYTOSTEROLS



R = 17 (Δ5-androstenyl) or related skeleton

mastene-3,6-dione.[12j,23] In a parallel determination, the crude sterol acetates from jack pine bark were oxidized with osmium tetroxide to yield 3β-acetoxy-5α,6α-dihydroxy-stigmastane as the major component, together with a fraction enriched in stigmastan-3β-yl acetate. This was then further oxidized with chromic acid to yield a small amount of the saturated acetates. Gas chromatography indicated that these also contained 8 per cent of campestan-3β-yl acetate and 0·9 per cent of cholestan-3β-yl acetate. The fact that these three saturated sterols are present in only trace amounts, and occur in approximately the same ratio as the parent Δ^5 sterols, may perhaps be again due to poor specificity of the hydrogenase which reduces the side chain. If so, these saturated sterols would then be metabolic errors.

The new triterpene, pinusenediol, which had been previously isolated from the hexane-insoluble terpenoids of jack pine bark,[24] was found in appreciable quantities in the sterols of loblolly and jack pine bark.* Another high-melting compound isolated from the sterols of jack pine bark appeared to be a methoxytriterpenol, perhaps related to a monomethyl ether of pinusenediol.[25]

No trace of stigmasterol (24α-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol; $\Delta^{5,22}$-stigmastadien-3β-ol) could be detected in any of the pine barks, and indeed, it has not been reported in the genus *Pinus*. None of the gas chromatograms of the crude sterols showed any indication of a peak between campesterol and β-sitosterol where stigmasterol is observed to appear, and none of the i.r. spectra showed any indication of a band at 975 cm^{-1} as expected for a *trans* disubstituted double bond.[26] The absence of stigmasterol is not surprising, however, since it has recently been shown that its biosynthesis is probably from β-sitosterol by dehydrogenation (see Fig. 1).[11,27] Apparently, this older genus has not yet acquired this dehydrogenase.

Several fractions isolated from the chromatograms of the sterols gave indications of containing small amounts of oxidation products of β-sitosterol. Although these were not obtained pure, their positions in the chromatogram, together with i.r. and u.v. spectra, were very suggestive. Thus Δ^4-stigmasten-3-one[12d] may well be present in loblolly pine bark. This compound was recently reported in the sterol fraction from two trees.[28] Its occurrence in β-sitosterol preparations may be widespread in analogy with the common occurrence of traces of Δ^4-cholesten-3-one in cholesterol.[12e] This could arise via autoxidation at the allylic C(4) position, followed by dehydration. Allylic oxidation at C(7) would be expected to give 7-keto-β-sitosterol[12f] analogous to the formation of 7-ketocholesterol[12b,c] on autoxidation of cholesterol; and indeed, 7-keto-β-sitosterol was indicated in lodgepole, jack, and loblolly pine barks. This should readily lose water to form $\Delta^{3,5}$-stigmastadien-7-one[12h]

* In an interesting sidelight, Dr. H. L. Hergert, Rayonier, Inc., informed me that he had isolated a minute amount of a triterpenediol from longleaf pine (*P. palustris* Mill.) bark whose m.p., 295°, and infrared spectrum were identical to those of pinusenediol. A small sample of his mother liquors gave us only two spots corresponding to β-sitosterol and pinusenediol on thin-layer chromatography. The sample was therefore subjected to gas chromatography, which showed it to consist of a trace of cholesterol, 3 per cent campesterol, 42 per cent β-sitosterol, 3 per cent α_1-sitosterol, and 38 per cent pinusenediol. Insufficient material was available for further study.

[23] P. Crabbe, E. A. Azpeitia and C. Djerassi, *Bull. Soc. Chim. Belges* **70**, 168 (1961). (Report on many derivatives of β-sitosterol.)
[24] J. W. Rowe, *Abstr. Div. Org. Chem.*, p. 83Q, 140th Meeting, American Chemical Society, Chicago, Ill., 1961.
[25] A similar compound has been isolated from the hexane-insoluble terpenoids of sugar pine bark. J. W. Rowe, unpublished results.
[26] J. L. Johnson, M. F. Grostic and A. O. Jensen, *Analyt. Chem.* **29**, 468 (1957).
[27] R. D. Bennett, E. Heftmann, W. H. Preston, Jr., and J. R. Haun, *Arch. Biochem. Biophys.* **103**, 74 (1963).
[28] D. Lavie and I. A. Kaye, *J. Chem. Soc.* 5001 (1963).

(tremulone[29]) analogous to the formation of $\Delta^{3,5}$-cholestadien-7-one.[12c, g] This has been found together with β-sitosterol in aspen,[29] and was indicated in sugar, lodgepole, and jack pine barks. These oxidation products are probably formed by autoxidative rather than by enzymatic processes, and are thus unlikely to be actively involved in the physiology of pine bark. Although autoxidation is to be expected in tree barks, it is also possible that this may have occurred during isolation of the bark sterols. Certainly those products lacking a 3β-hydroxyl are more likely to have been formed after precipitation with digitonin.

EXPERIMENTAL

Unless otherwise stated, melting points were determined in evacuated capillaries in a copper block and are corrected; optical rotations in chloroform with a Rudolph model 80 polarimeter; u.v. spectra in ethanol with a Beckman DK-2 recording ratio spectrometer; i.r. spectra in KBr with a Baird-Atomic spectrophotometer; and NMR spectra in deuterochloroform at 60 mc with tetramethylsilane as an internal standard. Nuclear magnetic resonance spectra were determined by Dr. Donald P. Hollis, Varian Associates, Palo Alto, Calif. Column chromatography was carried out using Woelm alumina, neutral, activity II. Gas chromatography was carried out on low-loaded columns containing G. E. SE-30 silicone gum rubber at 200° or above, in part by Mr. Robert F. Sweeny, Applied Science Laboratories, State College, Pa. Composition was determined by normalization of peak areas. Retention values relative to β-sitosterol for authentic standards are as follows. Cholesterol, 0·6; campesterol, 0·8; stigmasterol, 0·9; β-sitosterol, 1·0; lanosterol, 1·0; α_1-sitosterol, 1·3; pinusenediol, 2·6.

Digitonin Precipitation of Sterols

The sterols were all separated by the same general procedure. The unsaponifiable fraction of the benzene extract (50 g), which had sometimes already been treated with urea to separate *n*-aliphatics, was refluxed briefly with a solution of 17 g of digitonin in 1 l. of 95 per cent ethanol. The solution was taken to dryness under vacuum and 1 l. of benzene added to the residue. After refluxing for 1 hr, the fine suspension of digitonin and digitonides was filtered and washed thoroughly with benzene. The filtrate was treated a second time with digitonin (recovered from the first precipitation) in the same way.

Solutions of the digitonin precipitates[30] in 200 ml of pyridine were each refluxed briefly, cooled, and 2 l. of ether–benzene added with thorough shaking. After settling, the supernate was decanted and the gelatinous precipitate of digitonin filtered and thoroughly washed with benzene. The ether–benzene solutions of the sterols were each washed with dilute acid, dilute alkali, and water, and the solvents removed. The first precipitation generally yielded about 90 per cent of the sterols.

Characterization of Sugar Pine (P. lambertiana) *Bark Sterols*

The crude sterols (5·79 g) were chromatographed on 290 g of alumina, 70 fractions being collected. Petroleum ether, benzene, and mixtures of the two eluted 140 mg of a complex mixture, which appeared to contain, according to u.v. spectra, a total of 9 mg of $\Delta^{3,5}$-stigmastadien-7-one. λ_{max} 276 mμ, ν_{max} 1658 and 1608 cm^{-1}. Reported: λ_{max} 278 mμ, ϵ 20,000;

[29] R. A. ABRAMOVITCH and R. G. MICETICH, *Can. J. Chem.* **40**, 2017 (1962).

[30] A superior method of cleaving steroidal digitonides was recently reported by C. H. ISSIDORIDES, I. KITAGAWA and E. MOSETTIG, *J. Org. Chem.* **27**, 4693 (1962).

ν_{max} 1660, 1603 cm^{-1}.[29] Benzene:ether 50:1 then eluted 45 mg of a mixture of alcohols with melting points up to 276°, ν_{max} 3425 cm^{-1}, which had only a strong end absorption in the u.v. Fractions 20–32 (4·14 g), subsequently eluted with benzene–ether mixtures, were essentially β-sitosterol. The more polar fractions eluted after β-sitosterol were complex mixtures with no characteristic spectral properties, but with melting points as high as 257°. No indication of a peak at λ_{max} 237 mμ for 7-keto-β-sitosterol[12b,f] was observed.

The center cut of the β-sitosterol fractions was recrystallized from ethanol, hexane, and methanol for analysis without altering the melting point. A mixed m.p. with authentic β-sitosterol was undepressed, and their u.v. and i.r. spectra were identical. Gas chromatography showed 13 per cent campesterol and 87 per cent β-sitosterol. M.p. 140–141°; $[\alpha]_D^{23}$ −32° ($c = 1{\cdot}45$). Reported for campesterol-free β-sitosterol: m.p. 139–140°, $[\alpha]_D$ −33°.[7] (Found: C, 84·01; H, 12·36. Calc. for $C_{29}H_{50}O$: C, 83·99; H, 12·15%.)

Characterization of Loblolly Pine (P. taeda) *Bark Sterols*

The crude sterols on gas chromatography showed peaks for 1 per cent cholesterol, 7 per cent campesterol, 70 per cent β-sitosterol, 4 per cent α_1-sitosterol, and 12 per cent pinusenediol.

The crude sterols (1·73 g) were chromatographed on 80 g of alumina. Petroleum ether, benzene, mixtures of these two, and benzene–ether mixtures eluted 175 mg of a complex mixture, with melting points up to 151°, which appeared to contain a total of 31 mg of Δ^4-stigmasten-3-one, λ_{max} 242 mμ, ν_{max} 1661 and 1618 cm–[1]. Reported: λ_{max} 241 mμ, ε 20,000; ν_{max} 1667 and 1621 cm–[1].[12d,23] There was no indication of a peak at λ_{max} 278 mμ indicative of $\Delta^{3,5}$-stigmastadien-7-one.[29] Fractions 25–30 (1·06 g), eluted with benzene:ether 4:1, were essentially β-sitosterol. Fractions 31–38 (250 mg), eluted with benzene–ether and benzene–methanol mixtures, were white crystals, m.p. 288–292°. These showed only a high end absorption in the u.v., and an i.r. spectrum and gas chromatogram identical to those of pinusenediol.[24] They were crystallized from chloroform–ethanol and benzene to constant melting point for analysis, m.p. 299–301°, $[\alpha]_D^{23}$ −20° ($c = 1{\cdot}0$), undepressed mixed m.p. with pinusenediol, m.p, 300–301°, $[\alpha]_D^{22}$ −20½° ($c = 0{\cdot}9$). (Found: C, 81·40; H, 11·44. Calc. for $C_{30}H_{50}O_2$: C, 81·39; H, 11·38%.)

Fractions 39–43 (220 mg) were a complex mixture eluted with more polar solvents, which appeared to contain a total of 53 mg of 7-keto-β-sitosterol, λ_{max} 238 mμ, ν_{max} 1661 cm^{-1}. Expected: λ_{max} 237 mμ, ε 13,000.[12b,f]

The β-sitosterol was recrystallized several times from hexane and methanol to constant melting point for analysis, m.p. 139·5–140°, $[\alpha]_D^{22}$ −30° ($c = 1{\cdot}3$). A mixed m.p. with authentic β-sitosterol was undepressed, and their i.r. and u.v. spectra were identical. Gas chromatography showed it to consist of 9 per cent campesterol and 91 per cent β-sitosterol. Found: C, 84·14; H, 12·15%.)

Characterization of Jack Pine (P. banksiana) *Bark Sterols*

The crude sterols were purified by reprecipitation of the digitonides. A gas chromatogram showed 1 per cent cholesterol, 6 per cent campesterol, 83 per cent β-sitosterol, 4 per cent α_1-sitosterol, and some pinusenediol. The sterols (6·28 g) were chromatographed on 210 g of alumina. Petroleum ether and mixtures of petroleum ether–benzene eluted 112 mg of a complex mixture, which appeared to contain $\Delta^{3,5}$-stigmastadien-7-one, λ_{max} 276 mμ, ν_{max} 1666 and 1597 cm^{-1}, as in sugar pine bark.

Benzene eluted 360 mg from which 30 mg of white crystals, m.p. 245–249°, were obtained with difficulty. The u.v. spectrum showed only an end absorption. The NMR spectrum showed a broad singlet for a single vinylic proton at τ 4·72; a triplet at τ 6·8, $J = 9$ c.p.s., for a proton geminal to an equatorial hydroxyl; a triplet at τ 7·19, $J = \sim 2$ c.p.s., for a proton geminal to an axial methoxyl; a sharp singlet for a methoxyl at τ 6·71; and seven tertiary methyl groups (identical at 60 and 100 mc.) at τ 9·06, 9·11, 9·15, 9·19, 9·21, 9·25 and 9·34. The i.r. spectrum showed ν_{max} 3480 cm^{-1} (OH), 1650 and 794 cm^{-1} (trisubstituted olefin), and 1090 cm^{-1} (MeO). Gas chromatography gave a relative retention value of 2·3 and indicated it was not completely pure.

Fractions 16–22 (4·17 g), eluted with benzene:ether 50:1, were recrystallized several times from methanol and hexane for analysis, m.p. 140·5–141°, $[\alpha]_D^{22}$ −33·5° ($c = 1$). A mixed m.p. with authentic β-sitosterol was undepressed, and their u.v. and i.r. spectra were identical. Gas chromatography showed 11 per cent campesterol and 89 per cent β-sitosterol. (Found: C, 84·15; H, 12·04%.)

The acetate was prepared as usual from acetic anhydride–pyridine, m.p. 123·5–124·5°, $[\alpha]_D^{22}$ −38·5° ($c = 1$). Reported for authentic campesterol-free β-sitosteryl acetate: m.p. 121–122°, $[\alpha]_D$ −37°.[7] A mixed m.p. with β-sitosteryl acetate, m.p. 121·5–122°, prepared from soya bean β-sitosterol was undepressed, and the i.r. spectra were superimposable.

Fractions 26–27 (657 mg) were eluted with methylene chloride methanol 100:1. The u.v. spectrum showed λ_{max} 238 mμ, ε 2020, corresponding to 102 mg of 7-keto-β-sitosterol. However, this combined fraction when recrystallized from methylene chloride–methanol and methylene chloride–hexane several times yielded 343 mg of pinusenediol, m.p. 285–292°. A mixed m.p. was undepressed, and the NMR, u.v., and i.r. spectra, and retention time on gas chromatography were identical to those for pinusenediol originally isolated from the hexane-insoluble terpenoids of the same benzene extract of jack pine bark.[3,4]

Oxidation of Jack Pine β-Sitosterol

β-Sitosterol (935 mg) from the chromatogram of the jack pine bark sterols was dissolved in 70 ml of acetone and oxidized with 0·65 ml of 8 N chromic acid.[5] After 5 min at room temperature, the excess reagent was destroyed with methanol, the reaction mixture poured into water, and the product extracted with benzene in the usual way. The residue was dissolved in alcoholic sodium hydroxide and allowed to stand 72 hr in the refrigerator. Working it up in the usual way yielded 656 mg of neutral product, which was chromatographed on 33 g of alumina.

Fractions 10–11 (26 mg), eluted with petroleum ether:benzene 4:1, were recrystallized several times from methylene chloride–methanol for analysis, m.p. 156·5–159°, $[\alpha]_D^{22}$ +36 ($c = 0·6$), ν_{max} 1719 cm^{-1}. Reported for stigmastan-3-one: m.p. 156·5–161°, $[\alpha]_D^{20}$ +40·5°.[12] The u.v. spectrum exhibited no high intensity absorption, and the i.r. spectrum was identical to that of an authentic sample of stigmastan-3-one prepared by analogous oxidation of hydrogenated soya bean β-sitosterol. A mixed m.p. with the authentic sample, m.p. 158–159°, was undepressed. (Found: C, 84·18; H, 12·04. Calc. for $C_{29}H_{50}O$ C, 83·99; H, 12·15%.)

Examination of the u.v. spectra of fractions 10–13 indicated that these contained a total of approximately 46 mg of stigmastan-3-one.

Fractions 13–16 (183 mg), eluted with petroleum ether:benzene 1:1, were recrystallized several times from methylene chloride–methanol for analysis, m.p. 85–86°, $[\alpha]_D^{22}$ +71

[12] R. G. CURTIS, I. HEILBRON, E. R. H. JONES and G. E. WOODS, *J. Chem. Soc.* 457, 461 (1953)

($c = 1{\cdot}0$), ν_{max} 1678, 1623 cm^{-1}, λ_{max} 240 mμ, ϵ 17,900. Reported for Δ^4-stigmasten-3-one: m.p. 83–88°. $[\alpha]_D^{20}$ +83–86°, ν_{max} 1667, 1621 cm^{-1}, λ_{max} 241 mμ, ϵ 17,000–20,000.[12d,23] The u.v. and i.r. spectra were identical with those of an authentic sample of Δ^4-stigmasten-3-one prepared by analogous oxidation of soya bean β-sitosterol. A mixed m.p. with the authentic sample, m.p. 84–85°, was undepressed. (Found: C, 84·56; H, 11·52. Calc. for $C_{29}H_{48}O$: C, 84·40; H, 11·72%.)

The mother liquors from this crystallization showed an additional weak peak, λ_{max} 284 mμ. Expected for $\Delta^{4,6}$-stigmastadien-3-one: λ_{max} 285 mμ.[12m]

Fractions 19–22 (99 mg), eluted with benzene, were recrystallized several times from methylene chloride–methanol for analysis to yield yellow crystals, m.p. 172·5–173°, $[\alpha]_D^{22}$ −33·5° ($c = 0{\cdot}8$), ν_{max} 1681, 1605 cm^{-1}, λ_{max} 250·5 mμ, ϵ 11,500, λ_{max} 315 mμ, ϵ 996. Reported for Δ^4-stigmastene-3,6-dione: m.p. 170–172°, $[\alpha]_D$ −42°, ν_{max} 1686, 1610 cm^{-1}, λ_{max} 252 mμ, ϵ 12,600, λ_{max} 320 mμ, ϵ 1320.[12j,23] (Found: C, 81·67; H, 10·77. Calc. for $C_{29}H_{44}O_2$: C, 81·63; H, 10·87%.)

Losses due to formation of acids and polar neutral compounds were parallel in an analogous oxidation of soya bean β-sitosterol, and are probably due to the rapid air oxidation, especially in alkaline medium, of saturated and unsaturated steroid ketones.[32]

Oxidation of Jack Pine β-Sitosteryl Acetate

The sterols from jack pine bark (4·33 g) were acetylated with 100 ml of acetic anhydride: pyridine 1:1, and the product then thoroughly dried in a vacuum. The residue was dissolved in 200 ml of ether, and 20 ml of pyridine and 3 g of osmium tetroxide were then added. After 26 days in the dark at room temperature, the solution was saturated with hydrogen sulfide gas. After 15 min the solvents were removed in a vacuum, and the residue dissolved in benzene:chloroform then decolorized with active carbon. The dried residue (4·98 g) was chromatographed on 100 g alumina which contained a band of powdered silver to remove sulfur-containing impurities.

Fraction 15–22 (1·97 g), eluted with ether and ether-methanol, consisted of white crystals of 3β-acetoxy-5α,6α-dihydroxystigmastane, which were recrystallized alternately from methanol and hexane to constant melting point for analysis, m.p. 168–169·5°, $[\alpha]_D^{23}$ +10° ($c = 1{\cdot}15$). The u.v. spectrum was empty except for a very weak end absorption. No color was produced with tetranitromethane. (Found: C, 75·93; H, 11·31. Calc. for $C_{31}H_{54}O_4$: C, 75·87; H, 11·09%.)

Fractions 1–8 (1·41 g), eluted with petroleum ether, benzene, mixtures of the two, and benzene:ether 50:1, consisted of a mixture of white crystalline β-sitosteryl acetate and stigmastan-3β-yl acetate. This was dissolved in 75 ml of acetic acid, and a solution of 6·2 g of chromic acid in 4 ml of water plus 25 ml of glacial acetic acid then slowly added dropwise with stirring. After the addition, the solution was heated at 60° for 4 hr, cooled, and the excess chromic acid destroyed with methanol. The solution was poured into 1 l. of 1 N hydrochloric acid and extracted with benzene as usual to yield 156 mg of neutral product, which was chromatographed on 5 g of alumina. Fractions 3–4 of this chromatogram, eluted with hexane and petroleum ether: benzene 10:1, contained a small amount of white crystalline material whose i.r. spectrum was identical to that of authentic stigmastan-3β-yl acetate[12a] prepared from soya bean β-sitosterol. It showed only a very weak end absorption in the u.v. Gas chromatography (1 per cent SE-30 on Gas Chrom P at 210°) showed three peaks whose

[32] B. Camerino, B. Patelli and R. Sciaky, *Tetrahedron Letters* No. 16, 554 (1961).

areas in order of elution were 0·9, 8·4, and 91 per cent. The elution times were identical with authentic samples of cholestanyl acetate (16 min), campestanyl acetate (23 min), and stigmastanyl acetate (32 min), respectively.

Note added in proof

Pinusenediol has been shown to be identical to the unusual new triterpene, serratenediol.[33]

The double methylation biosynthesis of the ethyl side chain of the phytosterols has been further confirmed by V. R. Villanueva, *et al.*[34]

Dr. K. Schreiber has kindly run a negative ion molecular mass spectrum of the partly purified jack pine bark sterols. The major peak at $p-1=413$ corresponds to β-sitosterol. The major secondary peak at 399 corresponds to campesterol. Significant minor peaks were present at 427, 441, and 457 which could correspond to C_{30}, C_{31}, and C_{32} sterols, respectively.

A reviewer raised the question of the possible presence of lanosterol. The presence of appreciable amounts in the sterols of jack pine bark can be excluded since no significant mass peak is present at $p-1=425$. The peak at 427 cannot be dihydrolanosterol (Δ^8-4,4,14α-trimethylcholesten-3β-ol) because of the lack of a peak at 25·7 min in a gas chromatogram of the methyl ethers[35] of the crude sterols. This showed 1% cholesterol at 23 min, 1% unknown at 27·8 min, 10% campesterol at 37·7 min (which would have been unresolved from traces of lanosterol at 38·6 min), 86% β-sitosterol at 45·8 min, and 2% unknown at 52·9 min. However, thin-layer chromatography of the jack pine sterols on Kieselgel G (Merck), developed with ligroin (70–80°):benzene:ethyl acetate 75:10:15 yielded a major spot for β-sitosterol-campesterol together with four minor spots at 0·36, 0·50, 1·25, and 1·41 relative to β-sitosterol, and the spot at 1·41 is at the position observed for 4,4-dimethyl-3β-hydroxy steroids. This could include a 24α-ethyllanostanol corresponding to the mass peak at $p-1=457$.

Although the spot at 1·25 is at the position observed for 4α-methyl-3β-hydroxy steroids such as α_1-sitosterol, this compound is now eliminated by the lack of a mass peak at $p-1=425$. However, a 24(28)-dihydro-α_1-sitosterol ($p-1=427$) would be expected to have the same retention time on gas chromatography on SE-30 as α_1-sitosterol, and would be more logical biogenetically. The minor spots at 0·36 and 0·50 are probably autoxidation products.

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[33] J. W. ROWE, *Tetrahedron Letters*, No. 34, 2347 (1964).
[34] V. R. VILLANUEVA, M. BARBIER and E. LEDERER, *Bull. Soc. Chim. France* 1423 (1964).
[35] R. B. CLAYTON, *Biochemistry* **1**, 357 (1962).