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# Spinastanol and its Identity with Fucostanol and Stigmastanol

BY C. DONALD LARSEN

α-Spinasterol, first isolated from the unsaponifiable fraction of spinach fat by Clenshaw and Smedley-MacLean,[1] by Heyl, Wise and Speer,[2] and later by Simpson,[3] on the basis of the latest experimental evidence, is an isomer of stigmasterol and fucosterol. α-Spinasterol, like stigmasterol[4] and fucosterol,[5] was formerly shown,[6] by quantitative hydrogenation and perbenzoic acid titration experiments, to be doubly unsaturated. Whereas stigmasterol has been proved to be unsaturated in the 22,23-position[7] and, like fucosterol,[8] unsaturated in the 5,6-position,[9] α-spinasterol, although its points of unsaturation are unknown, apparently exhibits saturation in the more common sites of sterol unsaturation, the 5,6- and 22,23-positions. Exclusion of the 22,23-position as a site of one of the ethylenic linkages in α-spinasterol was demonstrated by the negative results of attempted ozonization.[6] Simpson[3] has drawn attention to the anomalous reactions of α-spinasterol, as compared to sterols with known 5,6-unsaturation, from which he has concluded that "this sterol differs from other sterols in that it does not contain the 5:6-double bond characteristic of the latter."

α-Spinasterol further differs from stigmasterol and fucosterol in that the former possesses a double bond that is unresponsive to hydrogenation[6] or has been caused to migrate, as has been postulated for the 7,8 double bond of ergosterol,[10] to a non-reducible position. In contrast to the nuclear double bond conjugation in ergosterol, Simpson has excluded, by the absence of a maleic anhydride reaction, the existence of conjugated unsaturations in the α-spinasterol nucleus.

Recent investigation of a series of derivatives of the completely saturated spinastanol and comparison of them with the constants of those similarly prepared from fucostanol by Heilbron and co-workers[8] and from stigmastanol[11-13] has established the identity of these three saturated sterols, and corroborates the isomerity of the unsaturated sterols from which they were derived.

Originally[2,14,15] $C_{27}H_{46}O$ was thought to be the correct formula for α-spinasterol. Later,[6,16] quantitative hydrolysis of numerous esters of α- and γ-spinasterol, and of the α- and β-spinastenols, gave molecular weight values for a $C_{28}$ or $C_{29}$ sterol, but preponderantly in favor of the former. Hydrolysis of the acetyl and benzoyl esters of entirely pure spinastanol and titrational determination of the equivalent weight of spinastanedicarboxylic acid now indicate for spinastanol the formula $C_{29}H_{52}O$, identical with the accepted formula for stigmastanol and fucostanol.

Critical examination of the data in Table II on the derivatives of spinastanol and comparison of their physical constants with those of the more carefully investigated $C_{29}$ saturated sterols in Table I establishes the identity of spinastanol, fucostanol and stigmastanol. On the other hand, the melting points and specific rotations of spinastanol, spinastanyl acetate and hydrocarbon, spinastane, are significantly lower than the corresponding values of Bengtsson's sitostanol and sitostane and Bergmann's sitostanyl (ostreastanyl) acetate (Table I). Therefore, it is concluded that spinastanol, fucostanol and stigmastanol are one and the same compound, and that they are different from sitostanol and ostreastanol, in accordance with the views previously set forth by Coffey, Heilbron and Spring[8] and Bergmann.[17] Comparative and mixed melting points of fucostanyl acetate and fucostanyl 3,5-dinitrobenzoate, for samples of which the writer is indebted to Dr. Heilbron, with the respective spinastanyl esters bear out the above conclusion of identity. Likewise, samples of ostreastanol and its phenylurethan, kindly furnished by Dr. Bergmann,

(1) Clenshaw and Smedley-MacLean, *Biochem. J.*, **23**, 107 (1929).
(2) Heyl, Wise and Speer, *J. Biol. Chem.*, **82**, 111 (1929).
(3) Simpson, *J. Chem. Soc.*, 730 (1937).
(4) Windaus and Hauth, *Ber.*, **39**, 4378 (1906).
(5) Heilbron, Phipers and Wright, *J. Chem. Soc.*, 1572 (1934).
(6) Larsen and Heyl, THIS JOURNAL, **56**, 2663 (1934).
(7) Guiteras, Nakamiya and Imhoffen, *Ann.*, **494**, 116 (1932).
(8) Coffey, Heilbron and Spring, *J. Chem. Soc.*, 738 (1936).
(9) Fernholz, *Ann.*, **508**, 215 (1934).
(10) Windaus and Langer, *ibid.*, **508**, 105 (1934).
(11) Bengtsson, *Z. physiol. Chem.*, **237**, 46 (1935).
(12) Windaus and Brunken, *ibid.*, **140**, 47 (1924).
(13) Dalmer, v. Werder, Honigmann and Heyns, *Ber.*, **68**, 1814 (1935).
(14) Collison and Smedley-MacLean, *Biochem. J.*, **25**, 606 (1931).
(15) Hart and Heyl, *J. Biol. Chem.*, **95**, 311 (1932).
(16) Heyl and Larsen, THIS JOURNAL, **56**, 942 (1934).
(17) Bergmann, *J. Biol. Chem.*, **104**, 317, 553 (1934).

TABLE I

COMPARISON OF SATURATED PHYTOSTEROLS

| Derivative | Fucostanol Heilbron M. p. | Fucostanol Heilbron $[\alpha]^{20}D$ | Stigmastanol Windaus M. p. | Stigmastanol Dalmer M. p. | Stigmastanol Dalmer $[\alpha]^{20}D$ | Stigmastanol Bengtsson M. p. | Stigmastanol Bengtsson $[\alpha]^{20}D$ | Sitostanol Bengtsson M. p. | Sitostanol Bengtsson $[\alpha]^{20}D$ | Ostreastanol Bergmann M. p. | Ostreastanol Bergmann $[\alpha]^{20}D$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| -stanol | 136 | +24.7 | 135 | | | 137 | +24.8 | 140 | +25.6 | 141 | +23.7 |
| Acetate | 130 | +15.1 | 128 | | | 131 | +15.3 | | | 137 | +14.6 |
| 3,5-Dinitrobenzoate | 215 | +13.8[a] | | | | 215 | +13.1[a] | 215 | +14.0 | | |
| Benzoate | 136 | | | | | 137 | | 139 | | | |
| Phenylurethan | | | | | | | | | | 175 | |
| -stanone | 157 | +40.2 | 156 | 157 | +42.0 | 157 | +40.6 | 158 | +40.8 | 157 | +41.9 |
| -stanone oxime | 218 | +30.1 | 216 | 219 | +30.0 | | | | | | |
| -stanedicarboxylic acid | 229 | | 230 | | | | | | | | |
| Dimethyl ester | 90 | | | | | | | | | | |
| -stane | | | 84.5 | | | | | | | | |

[a] Rotations in benzene; all others in chloroform.

TABLE II

PREPARED DERIVATIVES OF SPINASTANOL

| Compound | Formula | M. p., °C. | $[\alpha]^{20}D$ | Mol. wt. Calcd. | Mol. wt. Found | Analyses, % C Calcd. | C Found | H Calcd. | H Found | N Calcd. | N Found |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spinastanol | $C_{29}H_{52}O$ | 136 | +24.4 | 416.4 | | 83.6 | 81.7[a] | 12.6 | 12.6 | | |
| | | | | | | | 82.2[a] | | 12.4 | | |
| Acetate | $C_{31}H_{54}O_2$ | 132 | +14.9 | 458.4 | 457.4 | 81.2 | 81.1 | 11.9 | 11.8 | | |
| 3,5-Dinitrobenzoate | $C_{36}H_{54}O_6N_2$ | 215 | +13.1[b] | 610.4 | | 70.8 | 70.7 | 8.9 | 9.0 | 4.6 | 4.7 |
| Benzoate | $C_{36}H_{56}O_2$ | 136 | +19.6 | 520.4 | 516.6 | 83.0 | 83.1 | 10.8 | 11.0 | | |
| Phenylurethan | $C_{36}H_{57}O_2N$ | 172.5 | | 535.5 | | 80.7 | 80.6 | 10.7 | 10.8 | 2.7 | 2.7 |
| Spinastanone | $C_{29}H_{50}O$ | 157 | +40.5 | 414.4 | | 83.9 | 83.7 | 12.1 | 12.0 | | |
| Oxime | $C_{29}H_{51}ON$ | 217 | +29.2 | 429.4 | | 81.0 | 81.0 | 12.0 | 11.8 | 3.3 | 3.1 |
| Dicarboxylic acid | $C_{29}H_{50}O_4$ | 230 | +33.1 | 462.4 | 458.6 | 75.3 | 75.2 | 10.9 | 11.0 | | |
| Dimethyl ester | $C_{31}H_{54}O_4$ | 90 | +23.4 | 490.4 | | 75.8 | 75.8 | 11.1 | 11.0 | | |
| Spinastane | $C_{29}H_{52}$ | 84.5 | +25.5 | 400.4 | | 86.9 | 86.7 | 13.1 | 12.9 | | |

[a] Deviation from theory due to unremovable solvent of crystallization. [b] Rotation in benzene; all others in chloroform.

when compared with spinastanol and its phenylurethan, corroborate the non-identity of these saturated sterols.

It will be noted that spinastanedicarboxylic acid melts at 236–237° (corr.), 7–8° higher than the reported values for the acid from fucostanol and stigmastanol. Thanks to Dr. Heilbron, a sample of fucostanedicarboxylic acid has been available for comparison, for which the reported m. p. of 227–229° (corr.) was verified; when mixed with the acid from spinastanol a m. p. of 234–235° (corr.) was obtained. Due to the very close agreement in m. p. of the several derivatives of spinastanol with available data for derivatives of fucostanol and stigmastanol, such variation in m. p. of the respective acids must be not without reason. The sample of fucostanyl acetate furnished by Dr. Heilbron melted, as reported in the literature,[8] at 129–130°, and when mixed with spinastanyl acetate did not cause any m. p. depression. Nevertheless, subjection of fucostanyl acetate to the Liebermann–Burchard reaction was met with a comparatively faint but distinct development of green color. This fact seems to indicate that fucostanyl acetate contains a small amount of unsaturated, difficultly removable sterol. Analogously, it has been found that spinastanyl acetate is obtained pure only with difficulty; unless one recrystallizes the hydrogenation product of β-spinastenyl acetate from one of the better sterol solvents, such as ether or ethyl acetate, a pure, non-chromogenic spinastanyl acetate is not obtained. The pure, saturated ester is best obtained by treatment of the recrystallized hydrogenation product in chloroform with acetic anhydride and sulfuric acid according to the technique of Anderson and Nabenhauer.[18] Therefore, it seems possible that fucostanol, from which fucostanedicarboxylic acid was obtained by oxidation with chromic acid, might very well have contained a small amount of unsaturated sterol, oxidation of which may have led to a fucostanedicarboxylic acid contaminated with very small amounts of other, difficultly removable, acidic products. Similarly, the melting

(18) Anderson and Nabenhauer, THIS JOURNAL, 46, 1957 (1924).

point[12] of stigmastanedicarboxylic acid, reported as 229–230°, may be due to a small amount of contaminating oxidation product. It should be noted that the only recorded stigmastanedicarboxylic acid was prepared by Windaus and Brunken from a stigmastanol which melted at 134°; this alcohol, when pure, has since been found to melt at 136–7°.[11] It is possible that the low-melting stigmastanol of Windaus and Brunken contained a small quantity of contaminant which, through oxidation, led to a slightly contaminated dicarboxylic acid product of stigmastanol. A study of the dicarboxylic acids from fucostanol and stigmastanol of unquestioned homogeneity appears to be warranted.

## Experimental

**Spinastanyl Acetate.**—The preparation of spinastanyl acetate was accomplished by hydrogenation of β-spinastenyl acetate in the manner previously described.[6] The product was recrystallized repeatedly from ethyl acetate and from ethyl ether. Ten grams of the acetate, m. p. 130–131°, was dissolved in 50 cc. of chloroform, to which was added slowly 10 cc. of acetic anhydride and 1 cc. of sulfuric acid. The mixture was shaken and allowed to stand for a half hour, the small amount of colored material removed by washing with water, and the solution dried and evaporated to dryness. The residue, after crystallization from ethyl acetate or acetone, melted at 131.5–132°; $[\alpha]^{20}D$ +14.9°.

**Spinastanol.**—The above acetyl ester was hydrolyzed by boiling for one hour in 3% alcoholic potassium hydroxide. The recovered sterol, after crystallization from alcohol and from acetone, melted at 136.5–137.0°; $[\alpha]^{20}D$ +24.4°.

**Spinastanyl 3,5-Dinitrobenzoate.**—This ester was prepared in the usual manner from the sterol and the acid chloride in pyridine. The ester was crystallized from acetone and from ether; m. p. 214–215°; $[\alpha]^{20}D$ +13.1° (benzene).

**Spinastanyl Benzoate.**—The preparation of this ester was analogous to that of the 3,5-dinitrobenzoate. It was crystallized from acetone and ethyl acetate; m. p. 135–136°; $[\alpha]^{20}D$ +19.6°.

**Spinastanone.**—One gram of spinastanol was dissolved in 360 cc. of 96% acetic acid, to which was added 250 mg. of chromic anhydride in 60 cc. of acetic acid and 2 cc. of water with constant stirring during one-half hour. After standing for twenty hours the acetic acid was removed under reduced pressure, the residue digested with 100 cc. of 6 *N* sulfuric acid, and the oxidation products dissolved in ether. The acidic fraction was removed by extraction with 7% aqueous potassium hydroxide, the ether was evaporated from the neutral fraction, and the residue recrystallized from methyl and ethyl alcohol; m. p. 156.5–157°; $[\alpha]^{20}D$ +40.5°; yield, 50%.

**Spinastanone Oxime.**—The oxime of spinastanone was prepared in the usual manner with hydroxylamine hydrochloride and sodium acetate in alcoholic solution. The recovered oxime was crystallized from methyl alcohol and from acetone; m. p. 218–219°; $[\alpha]^{20}D$ +29.2°.

**Spinastanedicarboxylic Acid.**—Two grams of spinastanol was dissolved in 100 cc. of warm 96% acetic acid, to which was added 2.6 g. of chromic anhydride dissolved in a few drops of water. The mixture was heated on the steam-bath for two hours with occasional stirring. The solution was cooled, after which the oxidation products were precipitated with excess water and extracted with ether. Separation of the acidic fraction was made with 7% aqueous potassium hydroxide; about 0.2 g. of spinastanone was isolated from the neutral fraction. The acidic fraction was precipitated from the alkaline extract by acidification, extracted with ether, the ether evaporated and the residue recrystallized from methyl alcohol and from acetone. The nearly pure dicarboxylic acid, m. p. 234–235°, was dissolved in ether, again extracted as the potassium salt, precipitated with dilute hydrochloric acid and extracted with fresh ether. The residue from the evaporated ether solution was recrystallized from an alcohol–chloroform mixture (3:1); m. p. 236–237; $[\alpha]^{20}D$ +33.1°; yield 30%.

**Dimethyl Ester.**—The methylation of spinastanedicarboxylic acid was carried out in the cold with diazomethane. The recovered dimethyl ester was crystallized from methyl and ethyl alcohol; m. p. 89.5–90°; $[\alpha]^{20}D$ +23.4°.

**Spinastane.**—The reduction of 0.5 g. of spinastanone to the hydrocarbon was accomplished according to the directions of Windaus and Brunken.[12] The recovered hydrocarbon was crystallized from alcohol and acetone; m. p. 84.0–84.5°; $[\alpha]^{20}D$ +25.5°.

**Spinastanyl Phenylurethan.**—Preparation of the phenylurethan of spinastanol was carried out in benzene with phenyl isocyanate, in the usual manner. The derivative was crystallized from methyl and ethyl alcohol; m. p. 172.0–172.5°; $[\alpha]^{20}D$ +14.3.

**Molecular Weight Determinations.**—Samples of spinastanyl acetate were boiled for one hour in 0.1 *N* alcoholic potassium hydroxide, and the excess alkali titrated with accurately standardized 0.1 *N* hydrochloric acid. Blank samples of alkali were boiled and titrated simultaneously. Hydrolysis of 0.3141, 0.2770, 0.2919, 0.1980, 0.1657, 0.3222 and 0.2054 g. of spinastanyl acetate consumed 6.91, 6.02, 6.43, 4.32, 3.56, 7.31 and 4.47 cc. of 0.1000 *N* alkali, respectively, corresponding to molecular weights of 455.7, 460.1, 452.7, 458.3, 464.8, 454.4 and 459.4; average molecular weight found, 457.9; calculated on the basis of $C_{31}H_{54}O_2$, 458.4.

Samples of spinastanyl benzoate were hydrolyzed in a manner analogous to that for the acetyl ester. Hydrolysis of 0.2810 and 0.1413 g. of spinastanyl benzoate consumed 5.45 and 2.73 cc. of 0.1000 *N* alkali, respectively, corresponding to molecular weights of 515.6 and 517.6; average, 516.6; calculated on the basis of $C_{36}H_{56}O_2$, 520.4.

Samples of spinastanedicarboxylic acid were titrated with dilute potassium hydroxide for equivalent weight determination. Samples of 0.1020 and 0.0761 g. of the acid were dissolved in neutral alcohol, to which was added 7.49 and 6.50 cc. 0.1000 *N* hydrochloric acid, respectively. Total 0.1000 *N* alkali required for neutralization was 11.95 and 9.81 cc., respectively; net, 4.46 and 3.31 cc. Equiva-

lent weights found, 228.7 and 229.9; average, 229.3; calculated on the basis of $C_{29}H_{50}O_4$, 231.2.

## Summary

1. A series of derivatives of spinastanol have been prepared, the physical constants of which warrant the conclusion that this saturated sterol is identical with fucostanol and stigmastanol.

2. The identity of spinastanol, fucostanol and stigmastanol points out the isomerity of the naturally occurring sterols from which these saturated derivatives are obtained.

3. Spinastanol appears to differ from ostreastanol and sitostanol, probably in the arrangement of the side chain rather than in the carbon skeleton of the nucleus.

4. Hydrolysis of the acetyl and benzoyl esters of spinastanol, and titration of the equivalent weight of spinastanedicarboxylic acid, establish for the spinach sterols a skeleton of twenty-nine carbon atoms, in common with other well investigated phytosterols.

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# Studies in Organic Peroxides. V. *t*-Butyl Hydroperoxide[1]

BY NICHOLAS A. MILAS AND S. ARTHUR HARRIS[2]

The extraordinary stability of *t*-butyl hydrogen peroxide solution[3] and its therapeutic efficacy in the treatment of various fungus diseases[4] have stimulated our interest in the investigation of its chemical nature. In view of the lability of the hydroxyl group in tertiary alcohols, it was early suspected that pure hydrogen peroxide in non-aqueous solution would react with *t*-butyl alcohol reversibly to form *t*-butyl hydroperoxide. Although such a reaction between alcohols and hydrogen peroxide is unknown, an analogous reaction between organic acids and hydrogen peroxide in non-aqueous solvents has been known for some time.[5]

$$(CH_3)_3COH + HOOH \rightleftarrows (CH_3)_3COOH + H_2O$$

All of the alkyl hydroperoxides known, methyl,[6] ethyl,[6] isopropyl,[7] are much less stable than *t*-butyl hydroperoxide, which, besides being stable at room temperature, decomposes very slowly in alkalies or in the presence of pure liver catalase or horse-radish peroxidase. A distinct advantage, however, lies in the simplicity of its preparation. While the other hydroperoxides have been made by the alkylation of hydrogen peroxide with the corresponding dialkyl sulfates, *t*-butyl hydroperoxide can be prepared very easily from the anhydrous *t*-butyl alcoholic solution of hydrogen peroxide[3] by subjecting the latter to fractionation under reduced pressure in the presence of dehydrating agents such as anhydrous magnesium sulfate or preferably glacial metaphosphoric acid.

## Experimental Part

**Preparation of *t*-Butyl Hydroperoxide.**—To 600 cc. of 30% aqueous hydrogen peroxide "Albone C" was added with frequent shaking 2340 cc. of *t*-butyl alcohol (b. p. 81.7–81.8°) and the solution allowed to stand for fifteen minutes, when 225 g. of anhydrous sodium sulfate was added slowly with vigorous shaking. The mixture separated into two layers and the non-aqueous layer containing most of the peroxide was removed and shaken first with 225 g. more of anhydrous sodium sulfate, then with two 225-g. portions of anhydrous magnesium sulfate. The final mixture which was essentially free from water was filtered and the filtrate allowed to stand several days over 400 g. of glacial metaphosphoric acid. This treatment produced a peroxide solution having an active oxygen content equivalent to about 17% *t*-butyl hydroperoxide.

To obtain the pure hydroperoxide, samples of the above were fractionated several times under diminished pressure over glacial metaphosphoric acid or anhydrous magnesium sulfate using an all-glass apparatus and the fraction boiling at 38–38.5° (18 mm.) collected and analyzed. Both the metaphosphoric acid and the magnesium sulfate, when used as dehydrating agents, brought about the production of the same peroxide. That this peroxide is not a constant boiling mixture between hydrogen peroxide and *t*-butyl alcohol, is shown by its physical and chemical properties.

*Anal.* Calcd. for $C_4H_{10}O_2$: C, 53.33; H, 11.11; active (O), 17.78. Found: C, 53.68, 53.63; H, 11.10, 11.17; active (O), 17.84, 18.10, 18.21.

(1) For other papers in this series see THIS JOURNAL, **55**, 349, 352 (1933); **56**, 1219, 1221 (1934).

(2) Fellow of the Massachusetts Pharmaceutical Corporation.

(3) Milas and Sussman, THIS JOURNAL, **58**, 1302 (1936).

(4) Combes, *N. Y. State J. Med.*, **37**, No. 22 (1937).

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(6) Baeyer and Villiger, *Ber.*, **33**, 3387 (1900); **34**, 738 (1901); Rieche and Hitz, *ibid.*, **61**, 951 (1928); **62**, 218, 2458 (1929); Rieche, "Alkylperoxide und Ozonide," Theodor Steinkopff, Dresden, 1931, and "Die Bedeutung der organische Peroxyde für die chemische Wissenschaft und Technik," Ahrens Sammlung, **34**, 1 (1936), Ferdinand Enke, Stuttgart.

(7) Medwedew and Alexejewa, *Ber.*, **65**, 133 (1932).