Summary

1) The reaction of isonicotinylhydrazine with glucuronic acid or its sodium salt gave isonicotinylhydrazino-N-glucuronide and not isonicotinylhydrazone derivative.

2) The evidence that the N-glucuronide is in β-N-(glucopyranosid)uronic acid form was established by preparation of the pentaacetyl derivative of the glucuronide and its identification with the compound synthesized from isonicotinylhydrazine and methyl 2,3,4-tri-O-acetyl-α-D-glucopyranuronate as well as by comparison of their infrared spectra with that of the related compounds.

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85. Ken'ichi Takeda, Tokuo Kubota, and Yoshiki Matsui: Bile Acids and Steroids. X. On β-Spinasterol.

(*Research Laboratory, Shionogi & Co., Ltd.**)

β-Spinasterol was first isolated together with α-spinasterol by Heyl and Larsen[1] in 1933 from spinach fat, and later by King and Ball[2] from alfalfa seed oil. The sterol had been presumed as a double-bond isomer of α-spinasterol. Barton[3] suggested location of its two double bonds at C-7 and at C-24(25) on the basis of the molecular rotatory power, but this has not been confirmed yet. The present paper describes some results of experiments on β-spinasterol.

Previously, α-spinasterol was isolated from the root of *Bupleurum falcatum* L.[4] From a more soluble fraction of this α-spinasterol, another sterol corresponding to β-spinasterol (m.p. 147~149°, $[\alpha]_D = +6.8°$) was obtained by fractional crystallization of its dinitrobenzoate. The mixed melting point determination of the free sterol and Ball's β-spinasterol (m.p. 148~150°, $[\alpha]_D = +5.9°$) from alfalfa seed oil showed no depression, and the infrared spectra[5] of these two samples were almost identical only differing slightly in the relative intensity of the bands at 9.51 and 9.59 μ, and in the fine structures of the band at around 10.32 μ (Fig. 1). Also an absorption band at 12.01 μ in Ball's sample probably corresponds to the band at 12.04 μ in the present sample.

When the samples of the two β-spinasterols were dried over P_2O_5 at 80° *in vacuo* for one week, the infrared spectra of both samples changed and differences between the two spectra appeared at the following points:

1) An absorption maximum corresponding to hydroxyl group absorption appeared at 2.92 μ in Ball's sample, but at 2.83 μ in the present sample (Fig. 2).

2) An absorption band at 6.26 μ appeared only in Ball's sample.

3) A very weak absorption appeared at 8.41 μ in the present sample, but not in Ball's sample.

4) The intensity of the band at 10.32 μ was higher in Ball's sample than in the present sample (Fig. 2).

* Imafuku, Amagasaki, Hyogo-ken (武田健一, 久保田徳夫, 松井芳樹).

1) F.W. Heyl, D.J. Larsen: J. Am. Pharm. Assoc., **22**, 510(1933).
2) L.C. King, C.D. Ball: J. Am. Chem. Soc., **64**, 2488(1942).
3) D.H.R. Barton: J. Chem. Soc., **1945**, 813; **1946**, 512.
4) K. Takeda, K. Hamamoto, T. Kubota: Yakugaku Zasshi, **73**, 272(1953).
5) Infrared spectra discussed in this paper were determined in Nujol using a Parkin-Elmer Single-beam Infrared Spectrophotometer, Model 12 C.

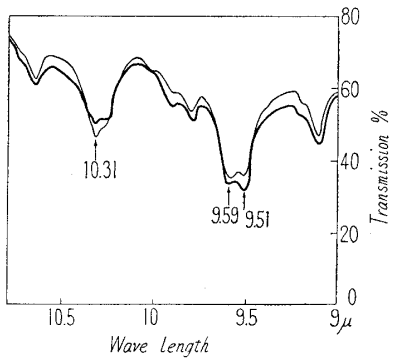

Fig. 1. Infrared Absorption Spectra (in Nujol)
——— β-Spinasterol from bupleurum
——— β-Spinasterol from alfalfa (Ball's)

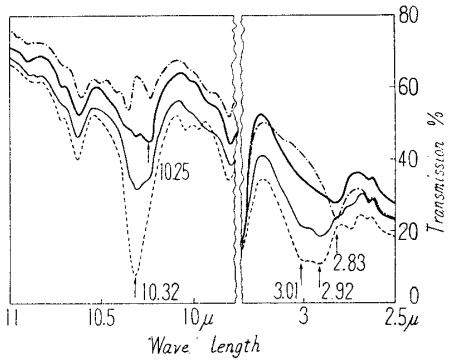

Fig. 2. Infrared Absorption Spectra (in Nujol)
——— Dried β-Spinasterol (authors')
——— Dried β-Spinasterol (Balls')
—·—·— Δ^7-Stigmastenol
---------- α-Spinasterol

5) The absorption band at 12.01 μ in the original Ball's sample shifted to 12.03 μ in the dried sample.

The infrared spectrum of this dried β-spinasterol rather resembles that of Δ^7-stigmastenol than that of Ball's sample, while the spectrum of the latter is more similar to that of α-spinasterol aside from intensity of the band at 10.32 μ, characteristic of the 22—23 double bond. Furthermore, not only the physical constants (Tables I and II) of this β-spinasteryl acetate but the infrared spectrum of each derivative also agrees well with those of Δ^7-stigmastenyl acetate.

From these findings the purity of β-spinasterol became doubtful. Recrystallization of the free sterol or its acetate did not effect any change but chromatography of its acetate on an alumina column afforded, from the first fraction, pure Δ^7-stigmastenol after hydrolysis. Identity was established by comparison of the infrared spectrum and by a mixed melting point determination with that of an authentic specimen of synthetic Δ^7-stigmastenol.[6] The physical constants of its acetate and benzoate were also in good agreement with those previously given in the literature[6~8] (Table II).

Another sterol having a melting point of 150~155° (acetate, m.p. 159~164°) was obtained by rechromatography of the crude material which was separated from the last fraction of the above-mentioned acetate. The infrared spectrum of this sterol is similar to that of α-spinasterol in many respects, but the material was insufficient for further purification.

Table I. β-Spinasterol

Sources	Spinach[1]		Alfalfa[2]		Bupleurum	
	m.p. (°C)	$[\alpha]_D$	m.p. (°C)	$[\alpha]_D$	m.p. (°C)	$[\alpha]_D$
Sterol	145~148	+7.7°	148~150	+5.9°	147~149	+6.8°
Acetate	150~154	+7.2°	153~155	+5.1°	155~157	+7.2°
Benzoate	—	—	181~183	+7.5°	181~183	+9.5°

Table II. Δ^7-Stigmastenol

Sources	Synthesized[7]		Wheat germ[6]		Rye germ[8]		Bupleurum	
	m.p. (°C)	$[\alpha]_D$	m.p. (°C)	$[\alpha]_D$	m.p. (°C)	$[\alpha]_D$	m.p. (°C)	$[\alpha]_D$
Sterol	144~145	+11°	146	+ 9.1°	144~145	+ 7.9°	145~147	+8.4°
Acetate	156~157	+ 8°	159	+ 6.6°	156~157	+ 6.7°	157~158	+6.9°
Benzoate	180.5	+13°	181	+12.0°	180~181	+12.0°	180~182	+9.9°

6) R. Idler, A.A. Kandutsh, C.A. Baumann: J. Am. Chem. Soc., **75**, 4325(1953).
7) D.H.R. Barton, J.D. Cox: J. Chem. Soc., **1948**, 1354.
8) H.A. Schuette, W.E. Link: J. Am. Chem. Soc., **76**, 4192(1954).

A synthetic mixture having a 10 : 1 ratio of Δ^7-stigmastenol to α-spinasterol showed the same melting point as the dried sample of β-spinasterol obtained in the present experiment and the infrared spectra of these substances were identical. Another sample having a 4 : 1 ratio of Δ^7-stigmastenol to α-spinasterol was identical in all respects with the dried sample of Ball's β-spinasterol.

As a result of the foregoing experiments, it is almost certain that the so-called "β-spinasterol" is an impure Δ^7-stigmastenol contaminated with a small amount of α-spinasterol.

The authors are indebted to Dr. C. D. Ball for his kind donation of the valuable sample of β-spinasterol. Thanks are also due to the members of the analysis room of this Laboratory for the microanalyses. This work was supported partly by the Grant in Aid for Scientific Research from the Ministry of Education.

Experimental[9]

Preparation of Crude Sterols—The dried root (30 kg.) of *Bupleurum falcatum* L. was percolated with 95% EtOH. After concentration of EtOH solution, the residue was extracted with hot petr. ether (b.p. 50~70°) to give 1.5 kg. of the extract which was saponified with EtOH-KOH. The solution was concentrated under a reduced pressure, poured into water, and extracted thoroughly with Et_2O. The combined Et_2O solution was washed with water and concentrated. The semi-crystalline residue (210 g.) was refluxed with 500 cc. of MeOH and after cooling, 65 g. of crystalline crude sterols was obtained.

Separation of the Fraction corresponding to β-Spinasterol—A mixture of the crude sterols and 1.2 L. of Ac_2O was refluxed for 2 hrs. After allowing to stand overnight, the precipitated acetate, m.p. 130~165°, was collected and hydrolyzed by boiling with EtOH-KOH for 2 hrs. Benzoylation of the sterols thus obtained gave 45 g. of crude benzoate, m.p. 177~193°, which was extracted 4 times with boiling 99% EtOH. Recrystallization of the remaining crystals from AcOEt yielded 15.8 g. of α-spinasteryl benzoate as described in the previous paper.[4]

The above EtOH extract was combined and concentrated to dryness. The residue (26 g.) was hydrolyzed with EtOH-KOH and then converted to the dinitrobenzoate with *m*-dinitrobenzoyl chloride in pyridine. Digestion of the crude dinitrobenzoate with boiling EtOH removed 12.7 g. of soluble, amorphous red substances. The insoluble crystals were repeatedly recrystallized from AcOEt and afforded 3.6 g. of yellow plates, m.p. 225~227°, $[\alpha]_D^{24}$ −3.7°(c=1.04). Details on this fraction will be reported in the following paper.

After evaporation of the above AcOEt mother liquor, the residue was purified by mechanical removal of the yellow plates and recrystallization from AcOEt to 1.6 g. of needles, m.p. 212~214°, $[\alpha]_D^{30}$ +7.4°(c=1.02).

Alkaline hydrolysis of this dinitrobenzoate and recrystallization from EtOH yielded a sterol, m.p. 147~149°, $[\alpha]_D^{20}$ +6.8°(c=1.24), which showed no depression by admixture with a sample of Ball's β-spinasterol. The infrared spectra of these two samples were almost identical.

This sterol on acetylation gave an acetate, m.p. 155~157°; $[\alpha]_D^{20}$ +7.2°(c=1.16).

The above sterol on benzoylation gave a benzoate, m.p. 181~183°; $[\alpha]_D^{18}$ +9.5°(c=1.10).

Chromatography of the β-Spinasterol Fraction—Above β-spinasteryl acetate (400 mg.) was chromatographed over 40 g. of Brockmann's alumina and gave the chromatogram as shown in Table III.

Table III.

Fract. No.	(cc.)	Eluant	Eluate m.p. (°C)	Eluate Yield (mg.)
1	160	petr. ether (b.p. 48~66°)	Nil	
2	80	petr. ether (b.p. 48~66°)	154~157	10
3	80	petr. ether (b.p. 48~66°)	153~156.5	80
4	80	petr. ether (b.p. 48~66°)	154~156	60
5	80	petr. ether (b.p. 48~66°)	152~155.5	40
6	80	petr. ether (b.p. 48~66°)	152~156	40
7	80	petr. ether (b.p. 48~66°)	154~157	40
8	80	petr. ether (b.p. 48~66°)	155~158	30
9	80	petr. ether (b.p. 48~66°) : Et_2O (9:1)	156~160	95
10	80	petr. ether (b.p. 48~66°) : Et_2O (4:1 to 1:1)	Nil	

9) All melting points are uncorrected. Specific rotations were measured in $CHCl_3$.

Fractions 2 to 6 were combined, hydrolyzed with EtOH-KOH, and recrystallized from EtOH to give Δ^7-stigmastenol as needles, m.p. 145~147°; $[\alpha]_D^{19}$ +8.4°(c=1.20). Identity with an authentic specimen was established by a mixed melting point determination and by comparison of the infrared spectra. *Anal.* Calcd. for $C_{29}H_{50}O$: C, 83.99; H, 12.15. Found : C, 83.75; H, 12.11.

Acetylation of this sterol gave an acetate, which was recrystallized from EtOH to plates, m.p. 157~158°; $[\alpha]_D^{19}$ +6.9°(c=1.05). *Anal.* Calcd. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48. Found : C, 81.64; H, 11.60.

Benzoylation of the sterol and recrystallization of the product from EtOH-AcOEt gave a benzoate as plates, m.p. 180~182°; $[\alpha]_D^{21}$ +9.9°(c=1.05). *Anal.* Calcd. for $C_{36}H_{54}O_2$: C, 83.34; H, 10.49. Found: C, 83.54; H, 10.42.

Fractions 7 to 9 from the above chromatogram were combined and rechromatographed in the same way. Chromatographic column was eluted thoroughly with 40 cc. each of petr. ether (b.p. 47~53°) until the eluate became smaller and then elution with petr. ether (b.p. 47~53°)-Et_2O (9:1) afforded 12 mg. of crystals with m.p. 158~163.5°. After recrystallization from EtOH, the melting point was raised to 159~164°. Alkaline hydrolysis of this acetate and recrystallization of the product from EtOH gave the sterol, m.p. 150~154°. The infrared spectrum of this substance agreed very closely with that of α-spinasterol, excepting that the band at 10.32 μ was weaker to some degree.

Hydrogenation of Δ^7-Stigmastenyl Acetate—Δ^7-Stigmastenyl acetate (23 mg.) dissolved in 10 cc. of 1:1 mixture of AcOH-AcOEt was shaken with 30 mg. of Adams' catalyst in H_2 for 5 hrs. The product crystallized from $CHCl_3$-MeOH in plates, m.p. 116~117°, which was identified as $\Delta^{8(14)}$-stigmastenyl acetate by a mixed melting point and by comparison of the infrared spectra.

Preparation of a Mixture of Δ^7-Stigmastenol and α-Spinasterol—(a) In a ratio of 10:1 : Authentic Δ^7-stigmastenol (10.60 mg.) and 1.06 mg. of α-spinasterol were dissolved in EtOH with warming and the solution was evaporated to dryness. m.p. 147~149°. The infrared spectrum of this sample was identical with that of the above-mentioned, dried β-spinasterol of the authors'.

(b) In a ratio 4:1 : A sample was similarly prepared from 6.90 mg. of Δ^7-stigmastenol and 1.72 mg. of α-spinasterol. It showed m.p. 147.5~150° and was identical with a dried sample of Ball's β-spinasterol by comparison of the infrared spectra.

Summary

From the root of *Bupleurum falcatum* L., a fraction corresponding to β-spinasterol was obtained by fractional crystallization of the dinitrobenzoates. Further investigation on this sterol showed that this so-called β-spinasterol is impure, and is Δ^7-stigmastenol contaminated with a small amount of α-spinasterol.

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