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# Neutral Constituents of the Methanol Extract from Twigs of *Metasequoia glyptostroboides* Hu et Cheng

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Neutral constituents extracted from *Metasequoia glyptostroboides* Hu et Cheng were investigated and seven steroids, $\beta$-sitosterol, stigmasterol, campesterol, stigmast-4-en-3-one, stigmasta-4,22-dien-3-one, campest-4-en-3-one, and 5$\alpha$-stigmastan-3,6-dione, together with $\alpha$-cadinol, *m*-cresol, and methyl esters of fourteen fatty acids, were identified. This is the first case that 5$\alpha$-stigmastan-3,6-dione, stigmasta-4,22-dien-3-one, and campest-4-en-3-one were identified from natural products.

*Metasequoia glyptostroboides* Hu et Cheng which is generally known as a typical living fossil, is considered to be placed between Taxodiaceae and Cupressaceae in plant taxonomy. Regarding chemical constituents of the leaves, isolation of sequoitol,[2] hinokiflavone,[3] and quercitrin[4] have been reported to date, and saccharides,[2,5] waxes,[6] and carotenoids[7] have also been detected.

The present investigation was undertaken to clarify neutral constituents extracted from the twigs with methanol. The extract was divided into three parts which were examined separately; (a) a crystalline substance which precipitated during concentration of the methanol extract solution, and (b) steam–volatile and (c) steam–nonvolatile parts of the residual extract.

## $\beta$-Sitosterol, Stigmasterol, and Campesterol

The crystalline substance (a), mp 138—139°, which was positive to both Liebermann and Salkowski color tests and formed an acetate derivative, mp 124—125°, was identified as $\beta$-sitosterol on the basis of its infrared (IR) spectrum and the mixed melting point determination.

The gas chromatogram, however, showed that this crystal of $\beta$-sitosterol was contaminated by four minor constituents, as shown in Fig. 1–A, two of which were proved to be stigmasterol and campesterol by gas chromatographic comparison of the substance, its acetate, and its hydrogenated product with their authentic specimens.

## Stigmast-4-en-3-one, Stigmasta-4,22-dien-3-one, and Campest-4-en-3-one

From the steam–nonvolatile part (c), white leaflets, $C_{29}H_{48}O$, mp 89.5—90.5°, which formed red 2,4-dinitrophenylhydrazone, mp 246.0—247.5°, were isolated. This substance was characterized as stigmast-4-en-3-one on the basis of its IR, ultraviolet (UV), and nuclear magnetic resonance (NMR) spectra, and by the mixed melting point determination.

The gas chromatogram (Fig. 1–D), as in the case of the crystalline substance (a), indicated that the crystals of stigmast-4-en-3-one were contaminated by two minor constituents, which were identified as stigmasta-4,22-dien-3-one and campest-4-en-3-one from the gas

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[1] Location: *Higashisenda-machi, Hiroshima.*
[2] T. Kariyone, M. Takahashi, K. Isoi, and M. Yoshikura, *Yakugaku Zasshi*, **78**, 801 (1958).
[3] T. Sawada, *Yakugaku Zasshi*, **78**, 1023 (1958).
[4] M. Takahashi, T. Ito, and A. Mizutani, *Yakugaku Zasshi*, **80**, 1557 (1960).
[5] M. Hida, S. Ono, and E. Harada, *Botan. Mag.* (Tokyo), **75**, 153 (1962).
[6] K. Isoi, *Yakugaku Zasshi*, **78**, 814 (1958).
[7] M. Hida and K. Ida, *Botan. Mag.* (Tokyo), **74**, 369 (1961).

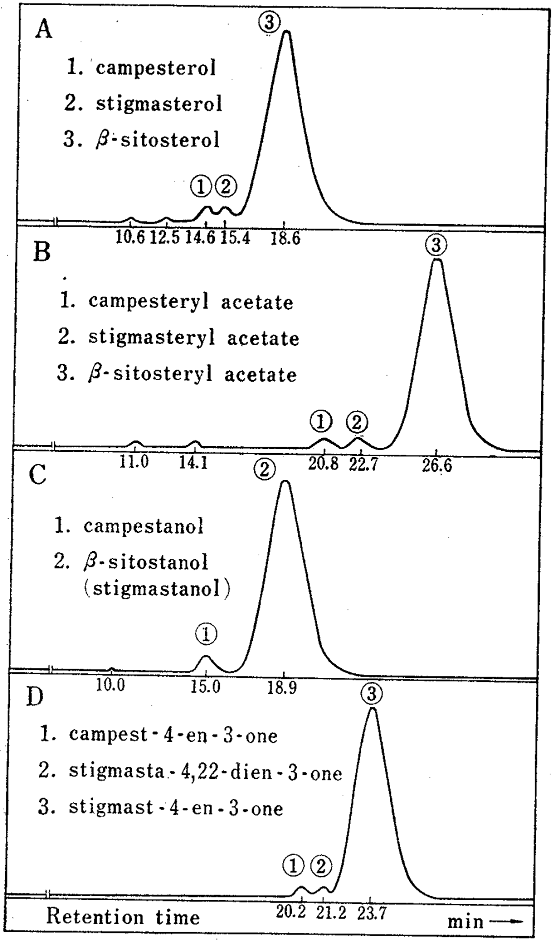

Fig. 1. Gas Chromatograms of β-Sitosterol (A), Its Acetate (B), and Its Hydrogenated Product (C), and Stigmast-4-en-3-one (D)

chromatographic comparison with the authentic specimens and from the appearance of detectable ions of $m/e$ 410 and 398, which correspond to their molecular weight, in the mass spectrum.

### 5α-Stigmastan-3,6-dione

Another white-powdered substance, $C_{29}H_{48}O_2$, mp 199.0—199.5°, which was negative to a color test by nitromethane, was also isolated from the nonvolatile part (c). It showed ketonic absorption bands in its IR (1705 cm$^{-1}$) and UV (284.9 m$\mu$, $\varepsilon$ 34) spectra, and formed mono-2,4-dinitrophenylhydrazone, mp 258.5—260.0°, whose IR spectrum still showed a ketone band at 1706 cm$^{-1}$. From these evidences it is deduced that this compound is a saturated diketone. Its mass spectrum, in which characteristic ions appeared at $m/e$ 428 ($M^+$), 287 ($M^+-C_{10}H_{21}$), and 245 ($M^+-C_{10}H_{21}-42$),[8] suggests $C_{29}$-dioxo steroid for the diketone.

The optical rotatory dispersion (ORD) curve showed a negative Cotton effect at 314 m$\mu$ and a positive specific rotation of about 9.5° at above *ca.* 400 m$\mu$. This pattern of the ORD curve is similar to that of 5α-cholestan-3,6-dione.[9] Besides, any isomerization was not observed in heating of the compound with mineral acid in acetic acid. By such a treatment 5β-cholestan-3,6-dione has been reported to be inverted to the α-form.[10] Thus, the compound was assumed to be 5α-stigmastan-3,6-dione.

This assumption was reasonably supported by the NMR spectrum of the substance. The signals appearing in the methyl region (0.76, 0.80, 0.91, and 0.98 ppm, total 18H) can be assigned to the six methyl groups of two angular and four side-chain methyls, and seven protons from 2.18 to 2.53 ppm are attributed to three active methylene groups and one active methine group adjacent to two carbonyl groups in stigmastan-3,6-dione. Moreover, the chemical shift of 0.76 and 0.98 ppm (each 3H, singlet) among the above methyl signals is in good agreement with the calculated values[11] for $C_{(18)}$- and $C_{(19)}$-protons ($C_{(18)}$: 0.751, $C_{(19)}$: 0.984 ppm) in 5α,14α-3,6-dioxo steroid.

This compound was finally confirmed as 5α-stigmastan-3,6-dione by its synthesis from β-sitosterol[12] through chromium trioxide oxidation followed by zinc dust reduction.

### Methyl Esters of Fatty Acids, α-Cadinol, and *m*-Cresol

In addition to the above steroids, methyl esters of many kinds of fatty acids from $C_{12}$ to $C_{20}$, shown in Table I, were identified from the steam-volatile (b) and steam-nonvolatile

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[8] H. Budzikiewicz and C. Djerassi, *J. Am. Chem. Soc.*, **84**, 1430 (1962).

[9] C. Djerassi and W. Closson, *J. Am. Chem. Soc.*, **78**, 3761 (1956); V. Prelog and E. Tagmann, *Helv. Chim. Acta*, **27**, 1880 (1944).

[10] T. Goto and Y. Kishi, *Nippon Kagaku Zasshi*, **83**, 1236 (1962).

[11] R.F. Zücher, *Helv. Chim. Acta*, **44**, 1380 (1961); *ibid.*, **46**, 2054 (1963).

[12] R.E. Marker, H.M. Crooks, Jr., E.M. Jones, and E.L. Wittbecker, *J. Am. Chem. Soc.*, **64**, 219 (1942).

(c) parts. Among them, linolate, oleate, and palmitate were predominant. In this respect, it might be considered that methyl esters were artifacts resulting from alcoholysis of fats and waxes during the methanol extraction procedure.

Further, α-cadinol and *m*-cresol were isolated from the steam–volatile part (b).

Table I. Methyl Esters of Fatty Acids obtained from *Metasequoia glyptostroboides* Extract

| Methyl ester of | Steam–volatile part (b) (%) | Steam–nonvloatile part (c) (%) | Relative content (%) |
|---|---|---|---|
| $C_{11}H_{23}COOH$ | trace | 1 | 0.3 |
| $C_{12}H_{25}COOH$ | trace | trace | 0.2 |
| $C_{13}H_{27}COOH$ | trace | 4 | 0.5 |
| $C_{14}H_{29}COOH$ | 1 | 6 | 1.2 |
| $C_{14}H_{27}COOH$ | 1 | 3 | 0.7 |
| $C_{15}H_{31}COOH$ | 16 | 45 | 16.8 |
| $C_{15}H_{29}COOH$ | 3 | 12 | 3.2 |
| $C_{15}H_{27}COOH$ | 2 | 1 | 1.8 |
| $C_{16}H_{33}COOH$ | 1 | 3 | 1.5 |
| $C_{17}H_{35}COOH$ | 3 | 1 | 2.5 |
| $C_{17}H_{33}COOH$ | 18 | 9 | 17.5 |
| $C_{17}H_{31}COOH$ | 34 | 14 | 33.0 |
| $C_{17}H_{29}COOH$ | 10 | trace | 9.5 |
| $C_{19}H_{39}COOH$ | trace | trace | 0.1 |

## Experimental

**1. Extraction**——Twigs of metasequoia that grew in Hiroshima city were pruned in August, and they were crushed to pieces with a chip machine after the fall of the leaves. The pieces (250 kg) were digested with MeOH at a room temperature. When the MeOH solution was concentrated, a white crystalline substance (a) precipitated and was collected (17.9 g) by filtration, when the solution was concentrated to one-third the original volume. The filtrate was evaporated and left a dark-brown resinous substance (345 g), which was extracted with petroleum ether. The extract, after removing acidic constituents by treatment with 5% $Na_2CO_3$ solution, was subjected to steam distillation and divided into volatile (b) (3.7 g) and nonvolatile (c) (116 g) parts.

**2. Constituents of the Crystalline Substance** (a)——β-Sitosterol: The crystalline substance, which precipitated from the MeOH extract solution, was recrystallized from MeOH to white leaflets, mp 138—139°, $[\alpha]^{25}_{D}$ −34.6° (*c*=1.0, $CHCl_3$). IR $\nu^{KBr}_{max}$ $cm^{-1}$: 3445, 3305, 1643, 1063, 955, 835.[13] This substance showed only one spot in thin-layer chromatography carried out with a mixed solvent of benzene and AcOEt (5:1, v/v) or hexane and AcOEt (4:1, v/v); the melting point did not depress on admixture with authentic β-sitosterol, mp 136.5—137.0°, which was isolated from cotton seed oil.

Acetate: mp 124—125°, $[\alpha]^{25}_{D}$ −49.9° (*c*=1.0, $CHCl_3$).[14] IR $\nu^{KBr}_{max}$ $cm^{-1}$: 1735, 1255.

β-Sitosterol (3.0 g) was also isolated from fraction F of the steam–nonvolatile part (c) (Table II).

Preparation of Saturated Product from the Crystalline Substance (a): The acetate (142 mg) was hydrogenated over Adams' $PtO_2$ catalyst (12 mg) in AcOH (16 ml) and absorbed about one molar equivalent of $H_2$. The hydrogenated product was freed from unreacted molecules by treatment with a small quantity of conc. $H_2SO_4$ in a mixture of $CCl_4$ and $Ac_2O$, according to the Anderson–Nabenhauer method,[15] and the saturated acetate, mp 132—133°, was saponified with 5% ethanolic solution of KOH. The resulting stanol, after recrystallization from EtOH, was obtained as white leaflets, mp 140—141°, which were negative to the Liebermann color test and showed no olefinic bands in its IR spectrum.

Gas Chromatography of the Crystalline Substance (a), Its Acetate, and Its Hydrogenated Product: For the gas chromatography of these compounds, Chromosorb–W impregnated with 1.5% SE–30 (column: 6 mm × 2 m) was used at a column temperature of 240°, a sample–injector temperature of 282°, and flow rates of $N_2$ and $H_2$ of 42.5 and 65 ml/min.

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[13] W.T. Beher, J. Parsons, and G.D. Baker, *Anal. Chem.*, **27**, 1148 (1957).

[14] I. Nishioka, N. Ikekawa, A. Yagi, T. Kawasaki, and T. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **13**, 379 (1965).

[15] R.J. Anderson and F.P. Nabenhauer, *J. Am. Chem. Soc.*, **46**, 1957 (1924).

Table II. Elution Chromatography of the Steam–Nonvolatile Part (c)

| Fraction | Solvent | Compound identified | Yield (g) |
|---|---|---|---|
| A | petroleum ether | hydrocarbons | 0.50 |
| B | petroleum ether | methyl esters | 25.30 |
| C | hexane–ethyl acetate (97:3) | stigmast-4-en-3-one | 9.35 |
| D | hexane–ethyl acetate (97:3) | stigmastan-3,6-dione | 3.40 |
| E | hexane–ethyl acetate (90:10) | $\beta$-sitosterol | 3.00 |
| F | ethyl acetate | unidentified | 32.05 |

3. **Constituents of the Steam-nonvolatile Part** (c)——The nonvolatile part was roughly eluted step by step through a silica gel–packed column with the solvents shown in Table II, and was separated into six fractions.

Stigmast-4-en-3-one: Fraction C (Table II) was purified by elution chromatography carried out with silica gel column and benzene–ether (85:15, v/v), and submitted to preparative thin–layer chromatography. A ketonic substance was isolated as white leaflets, mp 89.5—90.5°, $[\alpha]_D^{25}$ +89.4° ($c$=0.7, $CHCl_3$) after its recrystallization from EtOH. *Anal.* Calcd. for $C_{29}H_{48}O$: C, 84.36; H, 11.7. Found: C, 83.83; H, 11.5. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1678 (C=O), 1616 (C=C), 863 (C=C–H). UV $\lambda_{max}^{EtOH}$: 241 m$\mu$ (log $\varepsilon$ 4.19).[16] These data agreed with those of stigmast-4-en-3-one. The NMR spectrum[17] was similar to that of cholest-4-en-3-one[18]; 5.56 ppm (broad s, 1H, CO–CH=C), 2.13—2.38 (m, 2H, $-CH_2-CO$), 1.16 (s, 3H, $C_{10}-CH_3$), 0.68 (s, 3H, $C_{13}-CH_3$).

2,4-Dinitrophenylhydrazone, mp 246.0—247.5° (decomp.).[19] UV $\lambda_{max}^{EtOH}$: 389 m$\mu$ (log $\varepsilon$ 4.8). *Anal.* Calcd. for $C_{35}H_{52}O_4N_4$: C, 70.91; H, 8.84; N, 9.45. Found: C, 70.61; H, 8.53; N, 9.67.

The gas chromatogram, however, showed that the ketonic substance was contaminated with two minor constituents, which were identified as stigmasta-4,22-dien-3-one and campest-4-en-3-one.

5$\alpha$–Stigmastan–3,6–dione: Fraction D (Table II) was again eluted through a silica gel column with a mixed solvent of benzene–AcOEt (85:15, v/v), and subsequently recrystallized from MeOH. A diketonic substance, which exhibited only one peak in gas chromatography (3% SE–30 on Diasolid L, 240°) and one spot in thin–layer chromatography (benzene–AcOEt, 4:1, v/v), was isolated as a white powder, mp 199.0—199.5. *Anal.* Calcd. for $C_{29}H_{48}O_2$: C, 81.25; H, 11.37. Found: C, 81.78; H, 11.27. ORD ($c$=0.147, MeOH) $[\alpha]^{23}$ (m$\mu$): +9.5° (600), +9.5° (589), −333° (314), −257° (308) (shoulder), +200° (298) (inflection), +515° (289) (shoulder), +543° (284), +257° (254). CD ($c$=0.147, MeOH) $[\theta]^{23}$ (m$\mu$): −2346 (300).

Mono-2,4-dinitrophenylhydrazone, mp 258.5—260.0° (decomp.), IR $\nu_{max}^{KBr}$ 1706 $cm^{-1}$, UV $\lambda_{max}^{EtOH}$ 368 m$\mu$ (log $\varepsilon$ 4.18). *Anal.* Calcd. for $C_{35}H_{52}O_5N_4$: N, 9.42. Found: N, 9.21.

4. **Constituents of the Steam-volatile Part** (b)——The steam–volatile part of the neutral extract was separated into five fractions by elution chromatography using an alumina–packed column (Grade II) as shown in Table III.

The first fraction G was assumed to be hydrocarbons from its IR spectrum and thin–layer chromatography, but was not examined further because of its very small quantity.

Table III. Elution Chromatography of the Steam–Volatile Part (b)

| Fraction | Solvent | Compound identified | Yield (g) |
|---|---|---|---|
| G | petroleum ether | hydrocarbons | 0.1 |
| H | petroleum ether–ethyl acetate (97:3) | methyl esters | 1.1 |
| I | petroleum ether–ethyl acetate (85:15) | $\alpha$-cadinol | 0.8 |
| J | ethylacetate | *m*-cresol | 0.1 |
| K | methanol | unidentified | 0.4 |

Methyl Esters of Fatty Acids: The IR spectra of both fraction H (Table III) and fraction B of the nonvolatile part (Table II) agreed with those of methyl esters of fatty acids. Individual esters of these fractions were identified by a gas chromatographic comparison with the authentic specimens available in our laboratory and by employing the relationship between retention time and carbon chain length in fatty

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16) D. Lavie and I.A. Kaye, *J. Chem. Soc.*, **1963**, 5001.

17) The NMR spectrum in this experiment had been measured in 10% $CDCl_3$ solution.

18) G. Slomp and F.A. MacKellar, *J. Am. Chem. Soc.*, **84**, 204 (1962).

19) D.H.R. Barton and E.R.H. Jones, *J. Chem. Soc.*, **1943**, 559.

acids (separation column: 7.6% polyethylene glycol succinate on Gas-chrom. P and 5% SE-30 on Diasolid H).

α-Cadinol: White needles, mp 74.0—74.5°, were isolated by elution chromatography of fraction I (Table III). Its mass spectrum had abundant ions characteristic for α-cadinol: *m/e* 222 ($M^+$, 10%), 204 ($M^+$-18, 45), 161 (63), 121 (80), 95 (100); the IR spectrum[20] and the gas chromatogram agreed with those of α-cadinol.

The white crystalline substance, mp 76—77°, having a smaller retention time than that of α-cadinol was also isolated from the easy eluate of fraction I. IR $\nu_{\max}^{\text{Nujol}}$ $\text{cm}^{-1}$: 3290, 1370, 1361, 1290, 1227, 1153, 1125, 1105, 903. Further examination was not made because of the very small quantity available.

*m*-Cresol: From fraction J, a yellow oil was isolated which showed a positive color reaction to the $FeCl_3$ test. The IR spectrum was superimposable with that of *m*-cresol and further identification was carried out by a gas chromatographic comparison with the authentic *m*-cresol.

**Acknowledgement** The authors wish to express their thanks to Professor Shô Itô of Tôhoku University for his kind measurement of NMR spectra and to Professor Nobuo Ikekawa of Institute of Physical and Chemical Research for the donation of authentic campesterol.

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[20] O. Motl, V. Sykora, V. Herout, and F. Šorm, *Collection Czech. Chem. Commun.*, 23, 1279 (1958).