Notes

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Studies on the Constituents of Orchidaceous Plants. I. Constituents of *Nervilia purpurea* Schlechter and *Nervilia aragoana* Gaud. (1)

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Chemical constituents of *Nervilia purpurea* Schlechter and *Nervilia aragoana* Gaud. were examined. Phytol, a glycerin ester, cycloeucalenol, stigmasterol, linolic acid, linolenic acid, and L-norleucine were identified.

Keywords——*Nervilia purpurea*; *Nervilia aragoana*; Orchidaceae; triterpene; cycloeucalenol; sterol; norleucine; linolic acid; linolenic acid; GC–MS

Dried herbs of *Nervilia purpurea* Schlechter and *Nervilia aragoana* Gaud., Orchidaceous plants, are used for the treatment of visceral crisis, lung disease, and hypertension as a folk medicine "I-tiam-hong" in Taiwan.[1] We have examined the chemical constituents of these plants and the results are reported herewith.

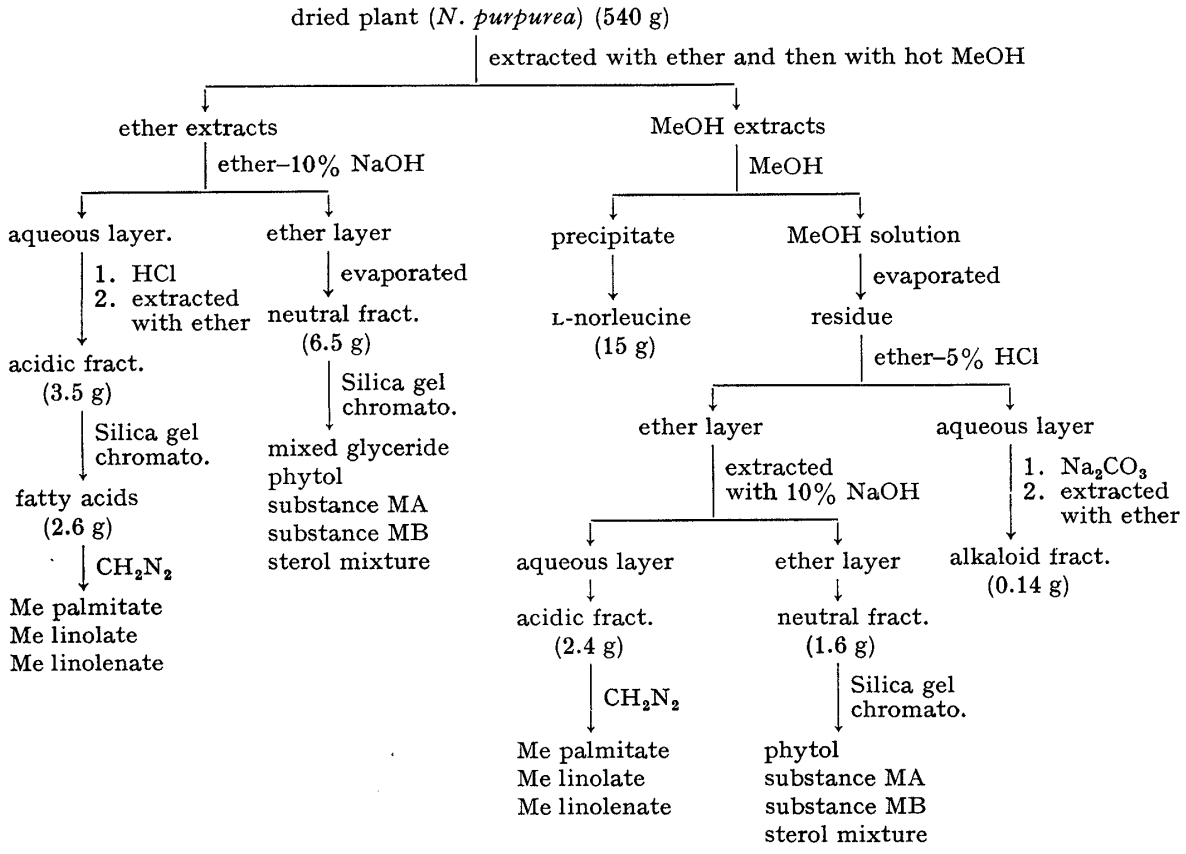

Chart 1

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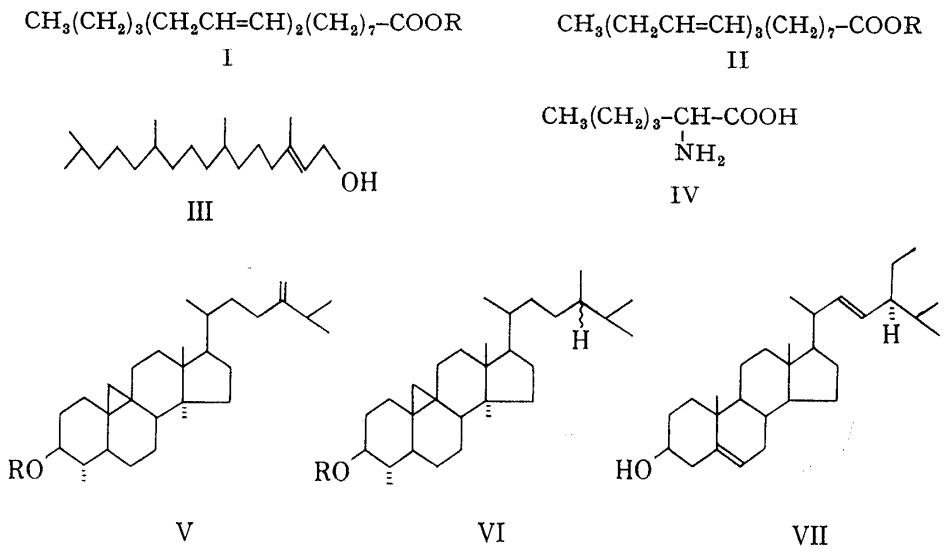

Chart 2

First, *N. purpurea* (dried whole plant) was extracted with ether at room temperature and then with hot methanol and the extracts were separated as shown in Chart 1.

The acidic fraction of the ether extracts was chromatographed on silica gel to give a mixture of fatty acids. Methylation of this mixture afforded a methyl ester, whose nuclear magnetic resonance (NMR) spectrum showed a pattern quite similar to that of methyl linolate (I, R=CH_3). In gas chromatography (GC), the methyl ester exhibited three major peaks with an approximate intensity ratio of 1:4:1 (retention times: 3.80, 8.96, and 10.88 min, respectively, on a 10% PEG-20M column). Eventually these peaks were assigned to methyl palmitate, methyl linolate (I, R=CH_3), and methyl linolenate (II, R=CH_3), respectively, by direct GC comparisons with authentic samples.

The neutral fraction of the ethereal extract was also chromatographed on silica gel to give phytol (III), an ester, two crystalline substances which were tentatively called MA and MB, and a sterol mixture.

The above ester exhibited a strong band at 1730 cm^{-1} in the infrared (IR) spectrum and its NMR spectrum was suggestive of the structural features of a glycerin ester. On alkaline hydrolysis, it furnished palmitic acid and linolic acid (I, R=H) in a ratio of approximately 1:2 as determined by GC examination. Therefore, this compound is considered to be a mixed glyceride.

Substances MA and MB gave a reddish-brown color upon Liebermann-Burchard reaction, suggesting that both are triterpene alcohols. Substance MA was shown to be a complex mixture by GC (Fig. 1) and GC–MS analyses. The mass chromatogram obtained by GC–MS is reproduced in Fig. 2, which shows the substance to consist of five components corresponding to the molecular formulae $C_{30}H_{50}O$ (A_{1a}: M^+ m/z 426), $C_{30}H_{52}O$ (A_{1b}: M^+ m/z 428), $C_{31}H_{52}O$ (A_2: M^+ m/z 440), $C_{31}H_{54}O$ (A_3: M^+ m/z 442), and $C_{32}H_{54}O$ (A_4: M^+ m/z 454).

Then, substance MA was acetylated as usual and the acetate was chromatographed on 20% $AgNO_3$-silica gel to give four fractions: a mixture of A_{1b} acetate and A_3 acetate, A_2 acetate (mp 129—131 °C), A_4 acetate (mp 149—151 °C), and A_{1a} acetate (mp 87—90 °C). Among these, the last was identified as cycloeucalenol acetate (V, R=Ac) by GC, IR (KBr), and MS comparisons with an authentic sample.

Compounds A_2 and A_4 were found to be new triterpenes, for which we propose the names cyclonervilol and cyclohomonervilol, respectively. Structure elucidation of these compounds will be reported elsewhere.[2] Incidentally, A_{1b} acetate was identified as dihydrocycloeucalenol acetate (VI, R=Ac) by GC and GC–MS analyses, while A_3 acetate was assigned as dihydro-

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cyclonervilol acetate.

The sterol mixture was also examined by GC (Fig. 1) and GC–MS, and the most prominent peak was assigned to stigmasterol (VII). Separation and structure elucidation of other sterols are currently under investigation.

On the other hand, the methanolic extract yielded a fairly large quantity of a crystalline compound, $C_6H_{13}O_2N$, $[\alpha]_D$ +32 °C (6 N HCl), which gave a positive Ninhydrin test. This compound was identified as L-norleucine (IV) by direct comparison with an authentic sample (IV) by means of amino acid analysis.

The methanol-soluble fraction of the methanolic extract was separated into neutral, acidic, and basic fractions in the usual manner. The basic fraction showed positive reactions with several alkaloidal reagents, but detailed examination could not be performed because of the small amount available.

The above acidic and neutral fractions were examined in the manner described for the ethereal extract, and additional crops of fatty acids, substances MA and MB, and sterols were obtained.

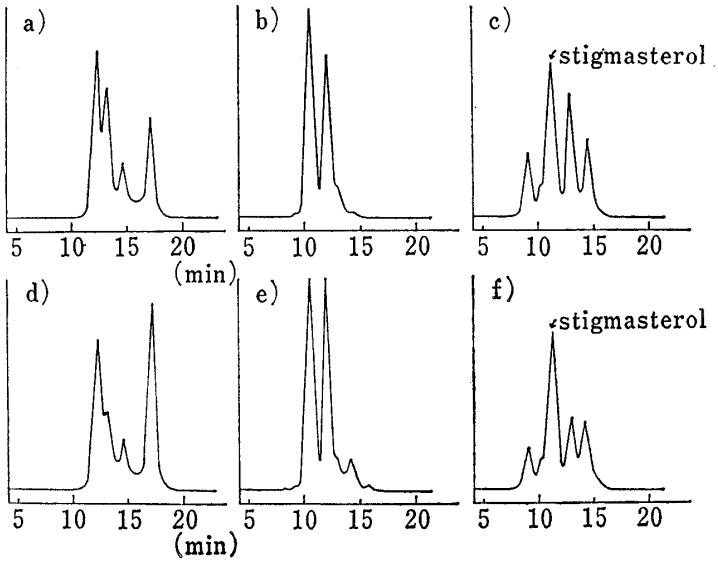

Fig. 1. Gas Chromatograms of the Constituents of *Nervilia* Species (2% OV-17 column)

a) substance MA, b) substance MB, c) sterol mixture from *N. purpurea*, d) substance MC, e) substance MD, f) sterol mixture from *N. aragoana*.

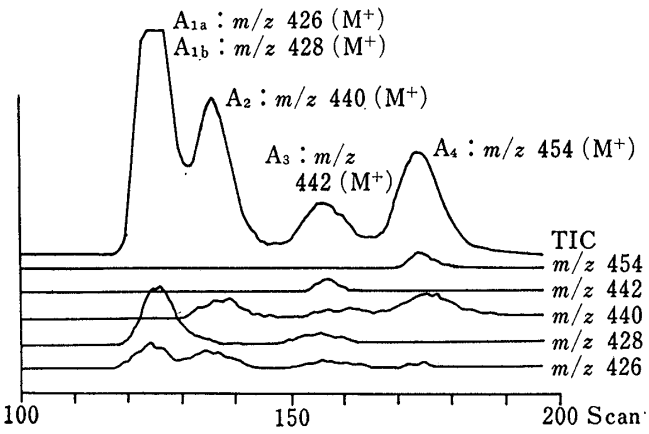

Fig. 2. Mass Chromatogram of Substance MA

Table I. Compounds obtained from *Nervilia* species

		N. purpurea	*N. aragoana*
L-Norleucine	Yield	2.7(%)	2.3(%)
Fatty acids			
Palmitic acid		0.12	0.2
Linolic acid		0.5	0.8
Linolenic acid		0.12	0.2
Ester (Mixed glyceride)		0.04	0.01
Phytol		0.03	0.01
Substance MA		0.04	
Substance MB		0.06	
Substance MC			0.09
Substance MD			0.05
Sterol mixture		0.03	0.03

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Next, we investigated the constituents of *N. aragoana* in the same manner. The results are summarized in Table I in comparison with those for *N. purpurea*.

It should be noted that substances MC and MD were indicated by GC (Fig. 1) and GC–MS analyses to be triterpene mixtures of the same components as those in substances MA and MB, respectively, differing only in compositions. Also, the two sterol mixtures were shown to be practically identical.

It is of interest that these two plants contain a large amount of free L-norleucine, but its biological significance remains unclear.

Experimental

Melting points were determined with a Kofler-type apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were measured for solutions in $CHCl_3$ unless otherwise noted and NMR spectra were recorded on a JEOL PMR-60 or a Varian Associates EM-390 spectrometer in $CDCl_3$ solutions using tetramethylsilane as an internal standard. GC analyses were done on a Shimadzu GC-6A gas chromatograph using 2 m glass columns (3 mm i.d.) packed with either 2% OV-17 on Gas-Chrom Q or 10% PEG-20M on Chromosorb WNAW at a column temperature of 280°C (OV-17) or 170°C (PEG-20M). Nitrogen was employed as a carrier at a flow rate of 40 ml/min. MS measurements were done on a Hitachi M-70 or a JEOL D-300 mass spectrometer using a direct inlet system or a GC injection system. GC-MS operating conditions were as follows: column, 2% OV-1 on Chromosorb W-AW DMCS (1 m × 3 mm i.d. glass tube) or 2% OV-17 on Gas-Chrom Q (2 m × 2 mm i.d. glass tube); column temperature, 240°C (OV-1) or 280°C (OV-17); injection temperature, 280°C (OV-1) or 300°C (OV-17); carrier gas, helium (40 ml/min); ionization energy, 20—23 eV; accelerating voltage, 3 kV. Amino acid analyses were performed on a Hitachi KLA-5 amino acid analyzer. Preparative thin-layer chromatography (TLC) was carried out on Merck Kieselgel GF_{254} with $CHCl_3$, and plates were examined under UV light (for UV-absorbing materials). Mallinckrodt silica gel was used for ordinary column chromatography, and 20% $AgNO_3$–silica gel was prepared according to Ghosh's description.[3] For drying organic solutions, anhydrous $MgSO_4$ was used.

A) Extraction and Separation of Constituents of *N. purpurea*

Dried herbs of *N. purpurea* (540 g), collected in Pingtung Hsen, Taiwan, were extracted with ether for 10 days at room temperature. This operation was repeated three times and the combined ether solutions were concentrated to give a green mass (*ca.* 16 g). The plant material was further extracted with boiling MeOH four times. Concentration of the MeOH extracts *in vacuo* afforded a dark green residue (*ca.* 30 g).

Treatment of the Ethereal Extract——The ethereal extract was dissolved in ether and extracted with 10% NaOH. The organic layer was dried and concentrated to give a neutral substance (*ca.* 6.5 g). The aqueous layer was acidified with 10% HCl and extracted with ether. After washing with H_2O, the ether solution was dried and concentrated to afford an oily dark brown residue (*ca.* 3.5 g), which was chromatographed on silica gel (30 g). Elution with CH_2Cl_2 and acetone–CH_2Cl_2 (1: 9) afforded a fatty acid mixture (2.6 g), which was treated with diazomethane to give a methyl ester. GC analysis of this methyl ester revealed the presence of methyl palmitate, methyl linolate (I, R=CH_3), and methyl linolenate (II, R=CH_3) (10% PEG-20M column, intensity ratio 1: 4: 1).

On the other hand, the neutral fraction was also chromatographed on silica gel (180 g). After development with hexane, elution with CH_2Cl_2–hexane (3: 7) gave a mixed glyceride as an oil (0.22 g), IR ν_{max} 1730 cm^{-1}. A portion (22 mg) of this oil was boiled with 5% KOH–MeOH (1 ml), then diluted with H_2O, and extracted with ether. Concentration of the ethereal extract gave only a trace of residue. The aqueous solution was then acidified with 10% HCl and the product was taken up in ether. Concentration of the ether solution gave a fatty acid mixture (20 mg) which was methylated with diazomethane in ether. The resulting material was proved to be a mixture of methyl palmitate and methyl linolate (ratio 1: 2) by GC analysis (10% PEG-20M column).

Subsequent elution of the column with CH_2Cl_2–hexane (1: 1) furnished an oily compound (III) (0.15 g), MS *m/z*: 296 (M^+, $C_{20}H_{40}O$), NMR δ 5.40 (1H, t, J=7 Hz, C=C<u>H</u>), 4.15 (2H, d, J=6 Hz, C<u>H</u>$_2$–OH), 1.65 (3H, broad s, C<u>H</u>$_3$–C=C). This compound was identified as phytol (III) by IR and NMR comparisons with an authentic sample. Further elution of the column with CH_2Cl_2 and acetone–CH_2Cl_2 (1: 9) afforded a semi-crystalline material (1.8 g), which was chromatographed again on silica gel (40 g) using CH_2Cl_2. The less polar fraction, on recrystallization from ether–MeOH, gave substance MA (0.20 g). High resolution GC–MS *m/z* (M^+): A_{1a}, Found 426.3816 (Calcd for $C_{30}H_{50}O$ 426.3861); A_{1b}, Found 428.3999 (Calcd for $C_{30}H_{52}O$ 428.4018); A_2, Found 440.4027 (Calcd for $C_{31}H_{52}O$ 440.4018); A_3, Found 442.4152 (Calcd for $C_{31}H_{54}O$ 442.4174); A_4, Found 454.4161 (Calcd for $C_{32}H_{54}O$ 454.4174). The more polar fraction, on fractional recrystallization from ether–MeOH, gave substance MB (0.30 g) and a sterol mixture (0.15 g). The main component of this sterol mixture was identified as stigmasterol (VII) by GC–MS analysis.

Separation of Substance MA——A mixture of substance MA (180 mg), acetic anhydride (1 ml), and pyridine (1 ml) was left to stand overnight at room temperature. After usual work-up, the product was

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recrystallized from ether–MeOH to give an acetate mixture (170 mg), which was chromatographed on 20% $AgNO_3$-silica gel (300 g) eluting with benzene–hexane (1: 5). The first eluate (45 mg) was a mixture of dihydrocycloeucalenol (A_{1b}) acetate (VI, R=Ac) and dihydrocyclonervilol (A_3) acetate, whose identities were confirmed by GC and GC–MS comparisons with authentic VI (R=Ac) and the dihydro compound of A_2, respectively. The second eluate, upon recrystallization from MeOH, gave cyclonervilol (A_2) acetate (25 mg), mp 129—131°C, MS *m/z*: 482 (M^+, $C_{33}H_{54}O_2$). The third eluate, upon recrystallization from MeOH, gave cyclohomonervilol (A_4) acetate (45 mg), mp 149—151°C, MS *m/z*: 496 (M^+, $C_{34}H_{56}O_2$). The last eluate gave, after recrystallization from MeOH, cycloeucalenol acetate (V, R=Ac) (6 mg), mp 87—90°C, MS *m/z*: 468 (M^+, $C_{32}H_{52}O_2$), whose identity was established by GC, IR (KBr), and MS comparisons with an authentic sample.

Treatment of the Methanol Extract——The MeOH extract yielded a crystalline precipitate (*ca.* 19 g) which was separated by filtration and washed with MeOH–CH_2Cl_2 (3: 7). Recrystallization[4] of this substance from aq. MeOH afforded colorless needles (IV) (15 g). An analytical sample was prepared by further recrystallizations from H_2O, mp 256—268°C, $[\alpha]_D^{20}$ +32°C (*c*=2.0, 6 N HCl), *Rf* 0.650 (BuOH–AcOH–H_2O 12: 3: 5). *Anal.* Calcd for $C_6H_{13}O_2N$: C, 54.74; H, 9.77; N, 10.42. Found: C, 54.94; H, 9.99; N, 10.68. This compound was identified as L-norleucine (IV) by direct comparison with an authentic sample by means of amino acid analysis.

On the other hand, the MeOH-soluble part of the MeOH extract was concentrated *in vacuo* and the residue was partitioned between ether and 10% HCl. The acidic aqueous layer was then neutralized with Na_2CO_3, extracted with ether, dried, and concentrated to leave a brown syrup (alkaloid fraction, 0.14 g). Further examination of this fraction was not possible because of the small amount obtained.

The ether layer was then shaken with 5% NaOH and the organic phase was washed with H_2O, dried, and concentrated *in vacuo* to afford an oily residue (1.6 g), which was chromatographed on silica gel (30 g) using hexane, hexane–CH_2Cl_2 (1: 1), CH_2Cl_2, and acetone–CH_2Cl_2 (1: 9). The acetone–CH_2Cl_2 eluate was further purified by preparative TLC to give substances MA (20 mg) and MB (40 mg), and a sterol mixture (15 mg).

The above NaOH phase, in turn, was acidified by addition of 10% HCl and extracted with ether. The extracts were washed with H_2O, dried, and concentrated *in vacuo*. The residue (2.4 g) was also chromatographed on silica gel (20 g) and eluted with acetone–CH_2Cl_2 (1: 9) to give an oily substance (1.5 g), which was proved to be a mixture of palmitic, linolic, and linolenic acids (ratio 1: 4: 1) by GC analysis.

B) Extraction and Separation of Constituents of *N. aragoana*

Dried herbs of *N. aragoana* (84 g), collected in Pingtung Hsen, Taiwan, were extracted with ether and with MeOH in the same manner as described for *N. purpurea* to afford the ethereal extract (*ca.* 2.4 g) and the methanolic extract (*ca.* 9.4 g).

Treatment of the Ethereal Extract——The above ethereal extract was divided into an acidic fraction (0.8 g) and a neutral fraction (1.1 g) in the usual manner. The acidic fraction was chromatographed on silica gel (20 g) and eluted with CH_2Cl_2 and acetone–CH_2Cl_2 (1: 9) to afford a brown oil (0.6 g). A portion of the latter was then treated with diazomethane in ether to give a methyl ester, which was found to be a mixture of methyl palmitate, methyl linolate (I, R=CH_3), and methyl linolenate (II, R=CH_3) (ratio 1: 4: 1) by GC analysis (10% PEG-20M column).

The neutral fraction was also chromatographed on silica gel (20 g) and eluted with hexane to give an oily ester (mixed glyceride, 10 mg), IR ν_{max} 1730 cm^{-1}. Subsequent elution of the column with CH_2Cl_2–hexane (3: 7) afforded phytol (III) (10 mg), whose identity was confirmed by IR and NMR analyses. Next, the eluate (0.2 g) with acetone–CH_2Cl_2 (1: 9) was re-chromatographed on silica gel (4 g) using CH_2Cl_2, whereupon substances MC (70 mg) and MD (25 mg) and a sterol mixture (25 mg) were obtained.

Treatment of the Methanol Extract——On standing, the MeOH extract yielded a crystalline mass (*ca.* 2.4 g), which was recrystallized from aqueous MeOH to afford colorless needles (IV) (1.9 g), mp 255—268°C, $[\alpha]_D^{20}$ +20°C (*c*=4.0, 6 N HCl). This compound was identified as L-norleucine (IV) by means of amino acid analysis.

The methanolic mother liquor was concentrated *in vacuo* and the residue was separated into a basic, an acidic, and a neutral fraction in the same way as described for *N. purpurea*. The basic fraction (25 mg) could not be purified further. The acidic fraction (0.75 g) was purified by silica gel chromatography to give a mixture of palmitic, linolic, and linolenic acids in a ratio of approximately 1: 4: 1 (0.45 g), whose composition and identities were determined by GC analysis of the corresponding methyl ester (10% PEG-20M column).

The neutral fraction (0.57 g) was also chromatographed repeatedly on silica gel using CH_2Cl_2 to give additional crops of substances MC (10 mg) and MD (15 mg).

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References and Notes

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2) A preliminary report of the structure elucidation of these triterpenes appears in *Tetrahedron Lett.*, **22**, 465 (1981).
3) A. Ghosh, M. Hoque, and J. Dutta, *J. Chromatogr.*, **69**, 207 (1972).
4) In the mother liquor of this fraction, trace amounts of glycine and isoleucine were detected by amino acid analysis.

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Isolation of Phenolic Compounds and Spectroscopic Analysis of a New Lignan from *Trachelospermum asiaticum* var. *intermedium*

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A new lignan **1** and two phenolic compounds, scopoletin and vanillic acid, were isolated from the stems of *Trachelospermum asiaticum* NAKAI var. *intermedium* NAKAI (Apocynaceae).

The structure of **1** was elucidated as (2*R*,3*R*) 2-4″-hydroxy-3″-methoxybenzyl-3-3′,4′,5′-trimethoxybenzylbutyrolactone by analysis of the carbon-13 nuclear magnetic resonance, mass and circular dichroism spectra.

Keywords——*Trachelospermum asiaticum* var. *intermedium*; Apocynaceae; phenolic compounds; scopoletin; vanillic acid; new lignan; (2*R*,3*R*) 2-4″-hydroxy-3″-methoxybenzyl-3-3′,4′,5′-trimethoxybenzylbutyrolactone; ^{13}C-NMR spectra; mass spectra; CD curves

We have already reported the isolation of four lignans, arctigenin, matairesinol, trachelogenin, and nortrachelogenin, from the ether extract of the stems of *Trachelospermum asiaticum* Nakai var. *intermedium* NAKAI (Apocynaceae) and their structure determination.[1-3]

As a continuation of our investigation on the constituents in the ether extract, a new lignan **1** and two phenolic compounds, scopoletin and vanillic acid, were isolated. Scopoletin and vanillic acid were identified by comparison with authentic samples.

This paper deals with the spectroscopic analysis of the structure of **1**, based on carbon-13 nuclear magnetic resonance (^{13}C-NMR), mass (MS) and circular dichroism (CD) spectra.

The extraction was carried out as described in "Experimental." The lignan **1** was isolated as a colorless syrup, $C_{22}H_{26}O_7$, $[\alpha]_D^{18}$ −25.1° (ethanol). The infrared (IR) absorption of **1** at 1765 cm^{-1} (CO) and the appearance of signals at δ 2.33—2.70 (4H, br.s, $C_{5,6}$–H), 2.77—3.07 (2H, br, $C_{2,3}$–H) and 3.97—4.27 (2H, m, C_4–H) in the proton nuclear magnetic resonance (PMR) spectrum suggested that **1** is a 2,3-dibenzylbutyrolactone lignan.

Methylation of **1** with diazomethane gave **2** as colorless needles, $C_{23}H_{28}O_7$, mp 122—123 °C, $[\alpha]_D^{18}$ −16.1° (chloroform).

Acetylation of **1** with acetic anhydride-pyridine gave **3** as a colorless syrup, $C_{24}H_{28}O_8$, $[\alpha]_D^{20}$ −26.3° (ethanol).

The PMR spectrum of **3** showed the presence of one phenolic acetoxyl (δ 2.30), four aromatic methoxyls (δ 3.83) and five aromatic protons (δ 6.27, 6.57—7.13).

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