Table 2. ^{13}C-NMR Spectroscopic Data for Crotocascarins A–H (**1**–**8**), and α and β (**9**, **10**) (100 MHz, $CDCl_3$)

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	74.6	75.8	76.6	74.5	75.4	74.6	76.7	76.3	75.5	75.7
2	33.3	32.7	32.5	33.2	32.6	33.4	32.8	32.5	34.4	34.5
3	36.9	36.4	36.3	36.9	36.4	36.9	36.3	36.3	34.3	34.3
4	60.4	60.1	60.2	60.5	60.2	60.4	60.1	60.2	64.8	65.2
5	57.9	57.8	57.9	57.6	58.0	57.8	57.7	57.9	75.4	75.6
6	56.5	55.9	56.5	56.1	56.4	56.4	55.9	56.5	88.0	87.9
7	44.3	44.4	44.4	44.1	44.7	44.2	44.2	44.4	48.0	47.4
8	159.0	162.0	158.6	161.7	159.0	158.7	161.9	158.5	60.1	62.2
9	107.5	78.4	106.4	82.2	109.5	107.2	78.3	106.4	174.7	175.1
10	41.9	44.1	45.7	37.7	40.4	42.0	44.1	45.8	35.8	27.1
11	34.8	72.7	73.7	36.2	73.7	34.8	72.6	73.7	67.9	27.2
12	146.8	148.9	148.4	146.0	149.3	146.6	148.7	148.3	146.1	141.0
13	39.5	31.7	31.8	40.7	31.4	39.5	31.7	31.6	32.4	34.7
14	68.9	68.8	68.8	68.7	68.8	68.8	68.8	68.7	66.0	65.9
15	130.4	128.2	129.8	128.5	129.9	130.6	128.2	129.9	202.0	201.7
16	170.8	173.4	170.3	173.0	170.7	170.5	173.3	170.2		
17	9.6	9.7	9.6	9.6	9.7	9.7	9.7	9.6	26.0	25.4
18	115.1	115.2	116.3	115.2	115.9	115.1	115.2	116.3	113.8	110.4
19	12.7	12.3	12.2	12.7	12.3	12.5	12.1	12.1	12.6	12.7
20	20.2	19.3	19.7	19.5	20.1	20.3	19.3	19.7	22.0	22.1
1′	175.4	178.0	178.8	175.2	178.1	175.6	178.1	179.0	175.5	175.0
2′	41.2	41.2	41.4	41.2	41.5	34.2	34.3	34.4	41.2	41.6
3′	26.7	26.6	26.6	26.8	26.5	19.3	19.3	19.2	26.6	26.6
4′	11.7	11.4	11.5	11.7	11.5	19.4	18.7	18.9	11.8	11.7
5′	17.2	16.2	16.6	17.1	16.5				16.9	17.3
$-OCH_3$					52.8					

the acid moiety and the five-membered ring.

Crotocascarin E (**5**), $[\alpha]_D^{25}$ +95.2, was isolated as an amorphous powder and its elemental composition was determined to be $C_{26}H_{34}O_8$ by HR-ESI-MS. The NMR spectroscopic data were essentially the same as those of crotocascarin C (Tables 1, 2), except for the presence of a methoxy signal [δ_H 3.57 (3H, s)], which crossed the ketal carbon one (δ_C 109.5) in the HMBC spectrum. Therefore, the structure of **5** was assigned, as shown in Fig. 1. The methoxy derivative is probably an artifact formed during the extraction and isolation processes.

Crotocascarins F (**6**), $[\alpha]_D^{25}$ +16.8, and G (**7**), $[\alpha]_D^{24}$ +81.7, were isolated as amorphous powders, and crotocascarin H (**8**), $[\alpha]_D^{24}$ +94.2, as colorless needles, and their elemental compositions were determined to be $C_{24}H_{30}O_7$, $C_{24}H_{30}O_7$ and $C_{24}H_{30}O_8$, respectively, by HR-ESI-MS. The ^{13}C-NMR spectral data for their diterpeneoid regions were essentially superimposable on those of crotocascarins A (**1**), B (**2**), and C (**3**), respectively. The common acyl moiety of these diterpenoids comprised four carbons, *i.e.*, two doublet methyls, one methine, whose proton was coupled as a septet, and a carbonyl carbon. Therefore, the structure of the acyl moiety was expected to be isobutanoic acid and the gross structures of **6**–**8** were shown to be as in Fig. 1. The absolute configurations of **6**, **7** and **8** were expected to be the same as those of crotocascarins A (**1**), B (**2**), and C (**3**), respectively, from similar respective optical rotation values and similar positive Cotton effect, [**6**: +4.66 (251), **7**: +1.41 (248) and **8**: +8.73 (252), respectively].

Crotocascarin α (**9**), $[\alpha]_D^{26}$ +78.7, was isolated as colorless plates and its elemental composition was determined to be $C_{24}H_{32}O_8$ by HR-ESI-MS. In the IR spectrum, absorption bands for carbonyl groups (1761, 1721 cm^{-1}) and a double bond (1634 cm^{-1}) were observed. The NMR spectroscopic data indicated the presence of 2-methylbutanoic acid as an acyl substituent, thus the terpenoid region comprised 19 carbons, *i.e.*, three methyls, two methylenes, three oxymethines, three methines, three oxygenated tertiary carbons, one quaternary carbon, an exomethylene moiety and two carbonyl carbons. The presence of the 2-methylbutanoic acid moiety and other functionalities, observed in one-dimensional NMR spectra, implied that crotocascarin α (**9**) was a compound related to crotofolanes. Since extensive examination of two-dimensional NMR data unfortunately did not lead to a structure which

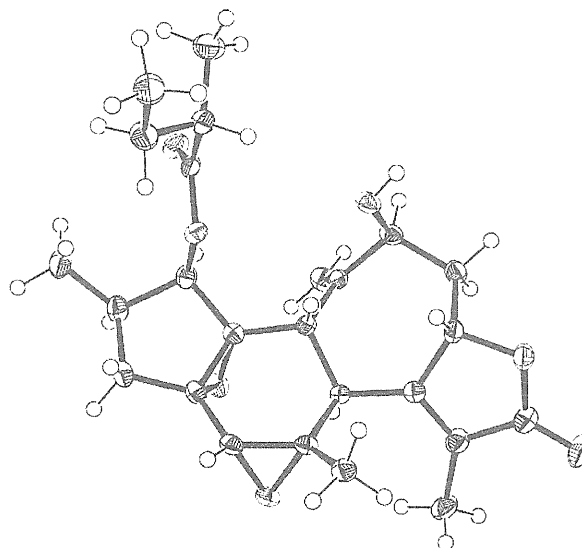

Fig. 5. ORTEP Drawing of **2**

The crystallization solvent is omitted from the structure.

satisfied all the spectroscopic data, an attempt was made to solve the structure by X-ray crystallographic analysis and an ORTEP drawing of **9** is presented in Fig. 6.[9] Crotocascarin α (**9**) has a new skeleton and was probably derived from some crotofolane, like crotocascarin B (**2**), through several steps, such as decarboxylation, C–C bond migration, oxidation, *etc.* (Fig. 7). The absolute configuration of the 2′-position was determined to be *S* by the same method used for **1** and **2**. Therefore, the structure of **9** is shown in Fig. 2, including the absolute one.

Crotocascarin β (**10**), $[\alpha]_D^{23}$ +35.0, was isolated as an amorphous powder and its elemental composition was determined to be $C_{24}H_{32}O_7$, which was one oxygen atom less than that of **9**. The NMR spectroscopic data indicated that crotocascarin β (**10**) was a congeneric compound to **9** with three methylene carbons. One of the oxymethine protons at δ_H 4.17, which was observed in the NMR spectrum of **9** was obviously replaced by methylene protons, with H-1 and H-5 remaining intact. Therefore, the structure of **10** was elucidated to be as shown in Fig. 2, namely 11-deoxycrotocascarin α. The absolute configuration of 2-methylbutanoic acid must also be the same as that in **1**, **2** and **8**, judging from the ^{13}C-NMR chemical shifts of the acid moiety and the five-membered ring.

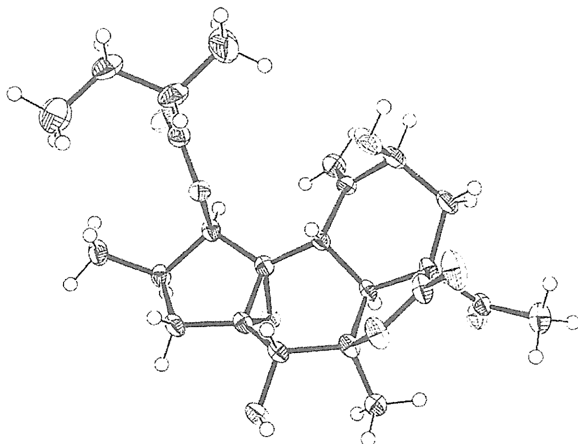

Fig. 6. ORTEP Drawing of **9**

Only seven crotofolane-type diterpenoids have been isolated so far, four from Jamaican *C. corylifolius*,[1,2] two from Kenyan *C. dichogamus*,[3] and one from Congolese *C. haumanianus*.[4] In these studies, without exception, the authors used an X-ray crystallographic method to come to a the final conclusion as to the relative structure. In this investigation on *C. cascarilloides*, a series of crotofolanes was isolated and two nor-diterpenes having a new skeleton probably derived from a crotofolane through the postulated biosynthetic scheme in Fig. 7. The structures of **1** and **2** were solved by X-ray crystallography using a direct method, and assignment of the absolute configuration of the acyl moiety, 2-methylbutanoic acid, obtained on chemical degradation of crotocascarins A (**1**) and B (**2**) as *S* provided information on the absolute structure of a crotofolane for the first time.

Experimental

General Melting points were measured on a Yanagimoto

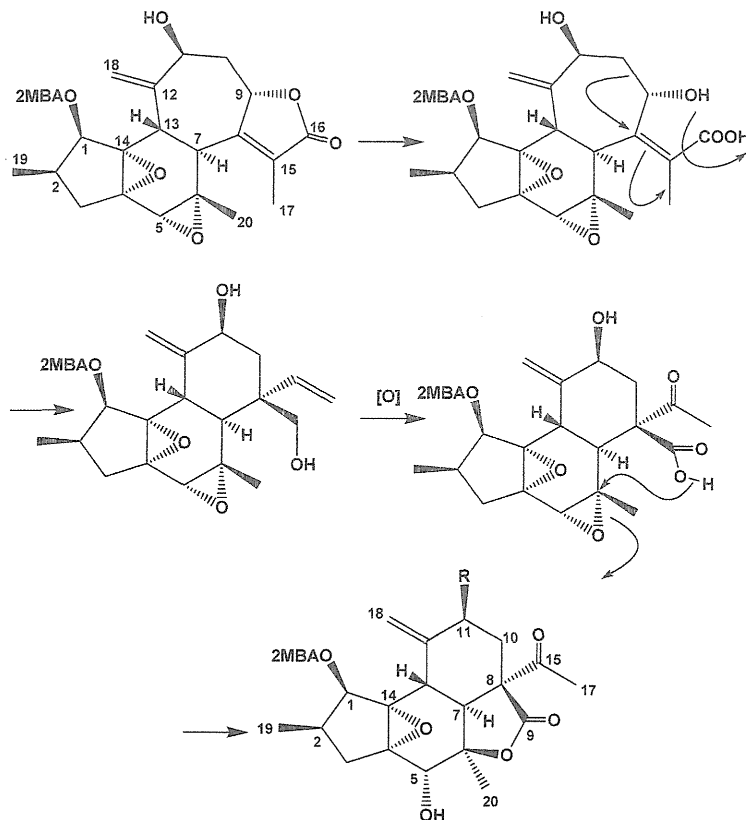

Fig. 7. Possible Biosynthetic Pathway from **2** to **9**

micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 digital polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/Vis spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM α-400 at 400 MHz and 100 MHz with tetramethylsilane as an internal standard. CD spectra were obtained with a JASCO J-720 spectropolarimeter. Positive-ion HR-ESI-MS was performed with an Applied Biosystems QSTAR XL NanoSpray™ System. Silica gel column chromatography (CC) was performed on Kiesel Gel (silica gel 60) (70–230 mesh) (E. Merck, Darmstadt, Germany) and reversed-phase octadecylsilanized (ODS) open CC on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) (Φ=50 mm, L=25 cm). HPLC was performed on an ODS column (Inertsil ODS-3; GL Science, Tokyo, Japan; Φ=6 mm, L=25 cm, 1.6 mL/min), and the eluate was monitored with UV (210 nm) and refractive index monitors. (*S*)-(+)-2-Methylbutanoic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Plant Material Stems of *C. cascarilloides* were collected at Okinawa in June 2004, and a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School of Biomedical and Health Sciences, Hiroshima University (04-CC-Okinawa-0628).

Extraction and Isolation Stems (14.5 kg) of *C. cascarilloides* were extracted with MeOH (15 L×3) for a week at 25°C. The combined extract was concentrated to 6 L and then partitioned with *n*-hexane (6 L, *n*-hexane extract: 92.1 g). The methanolic layer was concentrated and the resulting residue was suspended in 6 L of H_2O. The H_2O layer was partitioned with 6 L each of CH_2Cl_2, EtOAc and 1-BuOH to give 39.1 g, 10.5 g and 52.2 g of the respective residues.

The residue (39.1 g) of the CH_2Cl_2-soluble fraction was subjected to silica gel CC (400 g) (Φ=60 mm, L=30 cm) with $CHCl_3$ (5 L), $CHCl_3$–MeOH (15 : 1, 7 L and 12 : 1, 5 L), and MeOH (2 L). Fractions of 500 mL were collected. The residue (3.11 g) in fraction 14 was separated by two runs of ODS open CC [H_2O–MeOH (1 : 1, 1 L)→(1 : 9, 1 L) and then H_2O–MeOH (1 : 9, 250 mL)→MeOH (250 mL)], fractions of 10 g being collected. The residue (116 mg) in fractions 137–152 obtained on the first run of ODS open CC was again subjected to silica gel CC (Φ=10 mm, L=40 cm) with *n*-hexane–EtOAc [(9 : 1, 100 mL), (17 : 3, 100 mL), (4 : 1, 100 mL), (7 : 3, 100 mL), (3 : 2, 100 mL) and (1 : 1, 100 mL)], and EtOAc (100 mL). Fractions of 2 mL were collected, and 3.1 mg of **5** was obtained in fractions 135–144.

The residue (166 mg) in fractions 45–58 obtained on the second run of ODS open CC was again subjected to silica gel CC (Φ=10 mm, L=40 cm) with *n*-hexane (250 mL) → *n*-hexane–EtOAc (1 : 1, 250 mL), and then *n*-hexane–EtOAc (1 : 1, 250 mL). Fractions of 2 mL were collected. The residue (16.4 mg) in fractions 270–290 was finally purified by HPLC (H_2O–MeOH, 1 : 1) to give 3.5 mg of **9** from the peak at 35 min.

The residue (101 mg) in fractions 64–72 obtained on the second run of ODS open CC was again subjected to silica gel CC (Φ=10 mm, L=40 cm) with *n*-hexane (250 mL)→*n*-hexane–EtOAc (1 : 1, 250 mL), and then *n*-hexane–EtOAc (1 : 1, 250 mL). Fractions of 2 mL were collected. The residue (22.2 mg) in fractions 123–139 was purified by HPLC (H_2O–MeOH, 1 : 1) to give 12.3 mg of **7** from the peak at 76 min. From fractions 140–151, 10.5 mg of **8** was obtained in a crystalline state. The residue (12.2 mg) in fractions 152–151 was purified by HPLC [Inertsil (Ph-3), H_2O–MeOH, 1 : 1; 1.6 mL/min] to afford a further amount (5.1 mg) of **8** from the peak at 41 min.

The residue (155 mg) in fractions 73–85 obtained on the second run of ODS open CC was again subjected to silica gel CC (Φ=10 mm, L=40 cm) with *n*-hexane (250 mL)→*n*-hexane–EtOAc (1 : 1, 250 mL), and then *n*-hexane–EtOAc (1 : 1, 250 mL). Fractions of 2 mL were collected. The residue (2.3 mg) in fractions 106–113 was purified by HPLC (H_2O–MeOH, 2 : 3) to give 0.7 mg of **10** from the peak at 21 min. The residue (64.9 mg) in fractions 124–134 was purified by HPLC (H_2O–MeOH, 3 : 7) to yield 8.6 mg of **6** and 5.4 mg of **2** from the peaks at 10 min and 11 min. respectively. From fractions 135–147, 2.5 mg of **3** was obtained in a crystalline state.

The residue (126 mg) in fractions 86–95 obtained on the second run of ODS open CC was again subjected to silica gel CC (Φ=10 mm, L=40 cm) with *n*-hexane (250 mL)→*n*-hexane–EtOAc (1 : 1, 250 mL), and then *n*-hexane–EtOAc (1 : 1, 250 mL), fractions of 2 mL being collected. The residue (13.1 mg) in fractions 114–123 was purified by HPLC (H_2O–MeOH, 3 : 7) to give 6.5 mg of **1** and 2.9 mg of **4** from the peaks at 14 min and 15 min, respectively.

Crotocascarin A (**1**): Colorless rods (MeOH), mp 220–221°C, $[\alpha]_D^{26}$ +16.4 (c=0.95, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}: 3399, 2966, 2930, 1763, 1739, 1650, 1456, 1180, 1146, 1018, 802; UV λ_{max} (MeOH) nm (log ε): 223sh (3.87), 208 (4.21); ^{1}H-NMR (400 MHz, $CDCl_3$): Table 1; ^{13}C-NMR (100 MHz, $CDCl_3$): Table 1; CD $\Delta\varepsilon$ (nm): +3.41 (251), −8.39 (224) (c=2.02×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) *m/z*: 467.2046 [M+Na]$^{+}$ (Calcd for $C_{25}H_{32}O_7Na$: 467.2040).

Crotocascarin B (**2**): Colorless plates (2-PrOH), mp 152–153°C, $[\alpha]_D^{26}$ +81.8 (c=1.52, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3478, 2972, 2929, 1769, 1739, 1659, 1457, 1185, 1143, 1014, 804; UV (MeOH) λ_{max} nm (log ε): 218 (4.00); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +1.36 (249), −1.27 (210) (c 4.31×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) *m/z*: 467.2017 [M+Na]$^{+}$ (Calcd for $C_{25}H_{32}O_7Na$: 467.2040).

Crotocascarin C (**3**): Colorless plates (MeOH), mp 203–205°C, $[\alpha]_D^{24}$ +88.9 (c=0.82, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3448, 2974, 2938, 1759, 1734, 1649, 1140, 1081, 877; UV (MeOH) λ_{max} nm (log ε): 214 (3.85); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +4.47 (252), −10.61 (226) (c=1.78×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) *m/z*: 483.1985 [M+Na]$^{+}$ (Calcd for $C_{25}H_{32}O_8Na$: 483.1989).

Crotocascarin D (**4**): Amorphous powder, $[\alpha]_D^{24}$ +2.6 (c=0.19, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3463, 2972, 2932, 1794, 1748, 1651, 1457, 1161, 1112, 1062, 903; UV (MeOH) λ_{max} nm (log ε): 219 (4.00); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +1.17 (252), −3.21 (212) (c=2.26×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) *m/z*: 451.2085 [M+Na]$^{+}$ (Calcd for $C_{25}H_{32}O_6Na$: 451.2091).

Crotocascarin E (**5**): Amorphous powder, $[\alpha]_D^{25}$ +95.2 (c=0.15, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3480, 2970, 2934, 1767, 1739, 1457, 1190, 1139, 1085, 802; UV (MeOH) λ_{max} nm (log ε): 216 (3.92); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +4.23

(251), −12.68 (224) (c=3.06×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) m/z: 497.2129 $[M+Na]^+$ (Calcd for $C_{26}H_{34}O_8Na$: 497.2145).

Crotocascarin F (**6**): Amorphous powder, $[\alpha]_D^{25}$ +16.8 (c=0.22, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3466, 2973, 2932, 1765, 1739, 1651, 1459, 1337, 1191, 1154, 1066, 895; UV (MeOH) λ_{max} nm (log ε): 219 (3.88); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +4.66 (251), −10.18 (225) (c=2.56×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) m/z: 453.1897 $[M+Na]^+$ (Calcd for $C_{24}H_{30}O_7Na$: 453.1889).

Crotocascarin G (**7**): Amorphous powder, $[\alpha]_D^{24}$ +81.7 (c=0.82, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3436, 1741, 1634, 1459, 1195, 1157, 1072, 887; UV (MeOH) λ_{max} nm (log ε): 220 (4.08); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +1.41 (248), −4.22 (212) (c=1.91×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) m/z: 453.1888 $[M+Na]^+$ (Calcd for $C_{24}H_{30}O_7Na$: 453.1883).

Crotocascarin H (**8**): Colorless needles ($CHCl_3$), mp 242–244°C, $[\alpha]_D^{24}$ +94.2 (c=0.33, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3440, 2978, 2938, 1760, 1739, 1648, 1444, 1183, 1141, 1082, 880; UV (MeOH) λ_{max} nm (log ε): 214 (4.16); ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; CD $\Delta\varepsilon$ (nm): +8.73 (252), −23.36 (226) (c=1.47×10^{-5} M, MeOH); HR-ESI-MS (positive-ion mode) m/z: 469.1831 $[M+Na]^+$ (Calcd for $C_{24}H_{30}O_8Na$: 469.1832).

Crotocascarin α (**9**): Colorless plates ($CHCl_3$), mp 202–203°C, $[\alpha]_D^{26}$ +78.7 (c=0.13, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3479, 2968, 2926, 1761, 1721, 1634, 1461, 1193, 804; ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; HR-ESI-MS (positive-ion mode) m/z: 471.1973 $[M+Na]^+$ (Calcd for $C_{24}H_{32}O_8Na$: 471.1989).

Crotocascarin β (**10**): Amorphous powder, $[\alpha]_D^{23}$ +35.0 (c=0.04, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 3467, 2926, 1741, 1714, 1654, 1460, 1162, 889; ^{1}H-NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 2; HR-ESI-MS (positive-ion mode) m/z: 455.2044 $[M+Na]^+$ (Calcd for $C_{24}H_{32}O_7Na$: 455.2040).

X-Ray Ccrystallographic Analysis of Crotocascarin A (1) $C_{25}H_{32}O_7$, M=444.51, crystal size: 0.38×0.20×0.10 mm^3, space group: orthorhombic, $P2_12_12_1$, T=90 K, a=6.1970(15) Å, b=15.124(4) Å, c=25.145(6) Å, V=2356.6(10) $Å^3$, Z=4, D_c=1.253 Mg/m^3, F(000)=952. The data were measured using a Bruker SMART 1000 CCD diffractometer, using Mo$K\alpha$ graphite-monochromated radiation (λ=0.71073 Å) in the range of 3.04<2θ<56.7. Of the 14241 reflections collected, 5548 were unique (R_{int}=0.0421, data/restraints/parameters 5548/0/298. The structure was solved by a direct method using the program SHELXTL-97.[10] The refinement and all further calculations were carried out using SHELXTL-97.[11] The absorption correction was carried out utilizing the SADABS routine.[10] The H atoms were included at the calculated positions and treated as riding atoms using the SHELXTL default parameters. The non-H atoms were refined anisotropically using weighted full-matrix least-squares on F^2. Final goodness-of-fit on F^2=1.078, R_1=0.0408, wR_2=0.0997 based on I>2$\sigma(I)$, and R_1=0.0496, wR_2=0.1114 based on all data. The largest difference peak and hole were 0.645 and −0.402 $eÅ^{-3}$, respectively.

X-Ray Crystallographic Analysis of Crotocascarin B (2) $C_{28}H_{40}O_8$, M=504.60, crystal size: 0.50×0.30×0.15 mm^3, space group: orthorhombic, $P2_12_12_1$, T=120 K, a=10.1775(10) Å, b=10.4348(10) Å, c=25.908(3) Å, V=2751.5(5) $Å^3$, Z=4, D_c=1.218 Mg/m^3, F(000)=1088. Of the 13566 reflections collected in the range of 3.14<2θ<53.4, 3212 were unique (R_{int}=0.0224), data/restraints/parameters 3212/0/334. The structure was solved in a similar manner to as that for compound **1**. Final goodness-of-fit on F^2=1.048, R_1=0.0344, wR_2=0.0868 based on I>2$\sigma(I)$, and R_1=0.0378, wR_2=0.0891 based on all data. The largest difference peak and hole were 0.335 and −0.228 $eÅ^{-3}$, respectively.

Alkaline Hydrolysis of Crotocascarins A (1), B (2) and α (8) Crotocascarins A (**1**) (2.3 mg), B (**2**) (2.5 mg) and α (2.0 mg), and authentic (S)-(+)-2-methylbutyric acid were (500 μL) each dissolved in 1 mL of a 1 : 1 mixture of 10% KOH in H_2O and 50% aqueous dioxane, and then heated for 3 h at 100°C. The cooled reaction mixtures were neutralized with Amberlite IR-120B (H^+) and then the filtrates were evaporated. The four residues were analyzed by HPLC (column: Inertsil ODS-3, 6 mm×250 mm; solvent: 20% acetonitrile in H_2O containing 0.5% trifluoroacetic acid; flow rate: 1.6 mL/min) with a chiral detector (JASCO OR-2090*plus*) to give a peak of (S)-(+)-2-methylbutyric acid at 17.5 min with a positive optical rotation sign.

X-Ray Crystallographic Analysis of Crotocascarin α (10) $C_{24}H_{32}O_8$, M=448.50, crystal size: 0.30×0.15×0.15 mm^3, space group: monoclinic, $P2_1$, T=120 K, a=9.9294(12) Å, b=9.1267(11) Å, c=12.5443(15) Å, β=98.650(1)°, V=1123.9(2) $Å^3$, Z=2, D_c=1.325 Mg/m^3, F(000)=1088. Of the 5560 reflections collected in the range of 3.28<2θ<54.1, 2416 were unique (R_{int}=0.0154), data/restraints/parameters 2416/1/296. The structure was solved in a similar manner to as that for compound **1**. Final goodness-of-fit on F^2=1.056, R_1=0.0315, wR_2=0.0794 based on I>2$\sigma(I)$, and R_1=0.0335, wR_2=0.0809 based on all data. The largest difference peak and hole were 0.285 and −0.208 $eÅ^{-3}$, respectively.

Supplementary Data Supplementary X-ray crystallographic data for **1** (CCDC 894968), **2** (CCDC 761004), and **10** (CCDC 761005) can be obtained free of charge *via* www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, U.K.; fax: (+44) 1223–336–033; or deposit@ccdc.cam.ac.uk).

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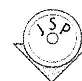

NOTE

Botanical origin of dietary supplements labeled as "Kwao Keur", a folk medicine from Thailand

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Abstract In the course of our study on the quality of dietary supplements in Japan, both the internal transcribed spacer (ITS) sequence of nrDNA and the *rps*16 intron sequence of cpDNA of products labeled as "Kwao Keur" were investigated. As a result, the DNA sequence of *Pueraria candollei* var. *mirifica*, which is the source plant of Kwao Keur, was observed in only about half of the products. Inferred from the determined sequences, source plants in the other products included *Medicago sativa*, *Glycyrrhiza uralensis*, *Pachyrhizus erosus*, and *Ipomoea batatas*, etc. These inferior products are estimated to lack the efficacy implied by their labeling. In order to guarantee the quality of dietary supplements, it is important to identify the source materials exactly; in addition, an infrastructure that can exclude these inferior products from the market is needed for the protection of consumers from potential damage to their health and finances. The DNA analysis performed in this study is useful for this purpose.

Keywords *Pueraria candollei* var. *mirifica* · Dietary supplement · DNA sequencing analysis · Regulatory science

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Introduction

The woody perennial climber *Pueraria candollei* var. *mirifica* (Syn: *P. mirifica*; Leguminosae) [1] is mainly found growing in deciduous forests in Chiengmai Province, Thailand. It is called "Kwao Keur" and its globular or pear-shaped tuberous roots have been used for their rejuvenating properties as a folk medicine [2]. The roots are rich in isoflavone derivatives, such as daidzein, puerarin, and mirificin, and potent estrogenic activity was found for miroestrol and deoxymiroestrol, which are characteristic compounds of the plant, together with kwakhurin [3, 4]. Recently, many dietary supplements labeled as "Kwao Keur" have been sold in Japan, owing to its rejuvenating and anti-aging effects, as well as its potential to improve skin appearance, infertility, and menopausal disorder. However, "Kwao Keur" is classified into three types, depending on color: white, red, and black [5, 6]. Among them, only white "Kwao Keur" is *Pueraria candollei* var. *mirifica* (PM). The others are *Butea superba* for red and *Mucuna collettii* for black; these two species do not have an estrogenic effect [5, 6]. They are often misidentified and, hence, the discrimination method based on PCR-RFLP utilizing *mat*K sequence differences between them was reported [6]. In addition to this confusing situation, congeners such as *P. montana* var. *lobata* and *P. montana* var. *thomsonii* are used as traditional medicine in Asian countries [7, 8]. Therefore, incorrect plants including such congeners as well as other Kwao Keur are probably used as the source material for dietary supplements claiming to be PM. The root of *P. lobata* is legally restricted to medicinal use in Japan [9]. Therefore, the accidental use of *P. montana* var. *lobata* as a source material of dietary supplements may pose a health risk to humans and is in violation of the Pharmaceutical Affairs Law in Japan.

In this study, we investigated whether dietary supplements labeled as "Kwao Keur" were definitely made from PM by using DNA analysis. Phylogenetic study of plants of the subtribe Glycininae using *rps*16 (ribosomal protein small subunit 16) intron sequences of cpDNA has already been reported [10]. Additionally, internal transcribed spacer (ITS) sequences of nrDNA of five *Pueraria* plants are registered in the international nucleotide sequence database (DDBJ/EMBL/GenBank; INSD). ITS sequence is advantageous for the analysis of mislabeled species because extremely large numbers of ITS sequences from various plants are registered in the INSD due to their usefulness for phylogenetic study. Therefore, we focused on the above two regions to analyze their sequences.

Materials and methods

Materials

"Kwao Keur" products were purchased online in 2005. The detailed information is summarized in Table 1, together with the results of DNA sequencing analysis. Authenticated plant specimens of PM were used as references (Ref-1 and Ref-2), which were kindly provided by Prof. T. Ishikawa of Chiba University, Japan [11] and "Wadayama-cho tokusanbutsu shijo kumiai" in Hyogo Prefecture, Japan, respectively. These samples are deposited in the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, Japan.

Methods

About 30 mg of each sample was crushed with a mixer mill, MM-300 (Qiagen, Germany), in liquid N_2. Genomic DNA was extracted from the powdered sample using the DNeasy Plant Mini Kit (Qiagen, Germany). The ITS region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) of nuclear rDNA and the *rps*16 intron region of cpDNA were separately amplified by two rounds of polymerase chain reaction (nested PCR) using the obtained genomic DNA as the template. Each primer for ITS and *rps*16 intron was designed on the basis of the conserved sequence in the plant kingdom and among *Pueraria* plants, respectively (Table 2). PCR was performed on DNA engine PTC-200 (MJ Research, USA; currently Bio-Rad, USA) using TaqNT DNA polymerase (Nippon Gene, Japan) with the following program: 94 °C, 4 min; 40 cycles of 94 °C, 30 s, 50 °C, 30 s, and 72 °C, 45 s; 72 °C, 4 min. After the removal of excess primers and dNTPs from the reaction mixture by Microcon-PCR (Millipore, USA), the amplicon was directly sequenced on an ABI

Table 1 Details of the dietary supplements used in this study and their plant species identified by DNA analysis

Sample no.	Product form	Indicated ingredients	DNA sequencing result	
			ITS[a]	*rps*16 intron[a]
Pu-1	Capsule	PM 100 %	*Medicago sativa* (99 %) + unknown[b]	No amplicon
Pu-2	Capsule	PM, sucrose fatty acid esters	*Pueraria candollei* var. *mirifica*	*Pueraria candollei* var. *mirifica*
Pu-3	Capsule	PM, fish collagen peptide, pearl powder	Unknown	No amplicon
Pu-4	Capsule	PM 100 %	No amplicon	No amplicon
Pu-8	Capsule	PM 252 mg/product 260 mg	*Glycyrrhiza uralensis* (100 %)	No amplicon
Pu-10	Powder	PM 100 %	*Pueraria candollei* var. *mirifica*	*Pueraria candollei* var. *mirifica*
Pu-11	Capsule	PM 100 %	*Ipomoea batatas* (99 %) + *Triticum aestivum* (99 %)	No amplicon
Pu-13	Capsule	PM 100 %	*Nelumbo nucifera* (99 %) + *Angelica* spp. (94.99 %)	No amplicon
Pu-14	Capsule	Kwao Keur mixture	*Pueraria candollei* var. *mirifica* + unknown[b]	*Pueraria candollei* var. *mirifica*
Pu-15	Capsule	Concentrated Kwao Keur 100 %	*Pueraria candollei* var. *mirifica*	*Pueraria candollei* var. *mirifica*
Pu-16	Powder	PM 100 %(natural)	*Pueraria candollei* var. *mirifica*	*Pueraria candollei* var. *mirifica*
Pu-17	Capsule	PM	*Pachyrhizus erosus* (99 %)	*Pachyrhizus erosus* (100 %)
Pu-18	Powder	PM 100 %	*Pueraria candollei* var. *mirifica*	*Pueraria candollei* var. *mirifica*

PM Pueraria candollei var. *mirifica*

[a] The values in parentheses indicate the similarity with the corresponding sequence in the international nucleotide sequence database (INSD)

[b] The minor sequence was found on direct sequencing, but the sequence could not be followed in subcloning

Table 2 Primer sequences used in this study

	Sense primer	Sequence (5′–3′)	Antisense primer	Sequence (5′–3′)
ITS first	ITS-S1	CTTTATCATTTAGAGGAAGGAG	ITS-AS1	TTTTCCTCCGCTTATTGATATGC
ITS second	ITS-S2	GGAAGTAAAAGTCGTAACAAGG	ITS-AS2	GTAGTCCCGCCTGACCTG
*rps*16 first	*rps*16-S1	CTAAACCCAATGATTCAAAG	*rps*16-AS1	AAACGATGTGGTAGAAAGCA
*rps*16 second	*rps*16-S2	AAAAGCTAAAAGATCATGGAA	*rps*16-AS2	CCCTAGAAACGTATAAGAAGTT

Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA). Cycle sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). In the case that the PCR product was obtained as a mixture from some plants, an amplicon was introduced into the pCR2.1-TOPO vector (Invitrogen, USA) using the TOPO TA Cloning Kit (Invitrogen, USA), and then the distinct clones were applied to the sequencing analysis. The DNA sequences were aligned using the ClustalW program [12].

Results

Genomic DNA was extracted from each sample, and the regions of interest (ITS and *rps*16 intron) were separately amplified by PCR using this DNA as the template. The resulting amplicon was directly sequenced. Nucleotide sequence variations of ITS and *rps*16 intron regions of PM found in this study are summarized in Tables 3 and 4, respectively. The sequence alignments of both regions are shown in the supplemental data (Figs. S1 and S2). ITS sequences (ITS1-5.8S rRNA-ITS2) from two reference PM samples, Ref-1 and Ref-2, were 691 bp in length and were identical to each other, except for five variable sites (aligned positions 40, 82, 83, 150, and 224), where nucleotide additivity [13] was found (Table 3 and Fig. S1). Besides one polymorphic site at aligned position 9, the insertion/deletion of ten nucleotides at positions 50–59 was observed in the *rps*16 intron region (326/336 bp in length) from the same reference samples (Table 4 and Fig. S2). The sequence information of referential plants is registered in the INSD with their own accession numbers (KC617871 and KC617873 for Ref-1 and KC617872 and KC617874 for Ref-2). The result of DNA analysis in commercial samples is indicated in Table 1, where the species names other than PM are the most similar ones, as determined using the BLASTn search program. PCR products could be amplified in 12 samples for ITS and seven samples for the *rps*16 intron region. In six samples (Pu-2, Pu-10, Pu-14, Pu-15, Pu-16, and Pu-18), both ITS and *rps*16 intron sequences identical or highly similar to those of PM were detected, and the others (Pu-1, Pu-3, Pu-8, Pu-11, Pu-13, and Pu-17) were different from that of PM. The unexpected

Table 3 Internal transcribed spacer (ITS) sequence with nucleotide variable sites found in this study

Sample no.	Aligned position							Accession no.
	40	82	83	150	224	574	669	
Ref-1	Y	Y	C	T	T	G	Y	KC617871
Ref-2	T	C	M	Y	Y	.	.	KC617872
Pu-2	T	C	.	.	.	R	T	
Pu-10	T	C	.	.	.	.	.	
Pu-14	T	C	.	.	.	.	T	
Pu-15	T	C	.	.	.	.	T	
Pu-16	T	C	.	.	.	R	T	
Pu-18	T	C	M	Y	Y	.	.	

A dot '.' indicates the same nucleotide as the Ref-1 sequence

M, A/C; R, A/G; Y, C/Y

Table 4 Ribosomal protein small subunit 16 (rps16) intron sequences with variable sites found in this study

Sample no.	Aligned position		Accession no.
	9	50–59	
Ref-1	A	–	KC617873
Ref-2	T	TCTAAAAAAT	KC617874
Pu-2	T	–	
Pu-10	.	–	
Pu-14	T	–	
Pu-15	T	–	
Pu-16	T	–	
Pu-18	.	–	

A dot '.' indicates the same nucleotide as the Ref-1 sequence, and a dash '–' shows aligned gap

materials from these samples were deduced as *Medicago sativa*, *Glycyrrhiza uralensis*, *Pachyrhizus erosus* (each Leguminosae), *Ipomoea batatas* (Convolvulaceae), *Nelumbo nucifera* (Nymphaeaceae), etc. on the basis of BLASTn search. All *rps*16 intron sequences of PM from commercial samples were completely identical or only one base different from that of Ref-2, and the deletion of 10 bp found in Ref-1 was not observed in commercial samples. ITS sequences of PM from commercial samples were also the same as those of reference samples, except for the five variable sites described above and two additional polymorphic sites (aligned positions 574 for Pu-2 and Pu-16

and 669 for Pu-2, Pu-14, Pu-15, and Pu-16 in Table 2 and Fig. S1).

Discussion

Two reference samples of PM have almost the same sequence in the two regions analyzed in this study. Although these sequences have high levels of similarity (94–97 %) with those of the congeners, such as *P. montana* var. *lobata* (acc. no.: AF338241 for ITS; acc. no.: AF311835 for *rps*16) and *P. montana* var. *thomsonii* (acc. no.: AF338217 for ITS) in the INSD, we could clearly discriminate PM from the above two species on the basis of sequence differences and insertion/deletion sites. The roots of *P. montana* var. *lobata* and *P. montana* var. *thomsonii* are prescribed as the traditional medicine, "Puerariae (lobatae/thomsonii) Radix", in Japanese and Chinese pharmacopoeia. Therefore, the distinguishability of these species and PM is very important from the viewpoint of regulatory science.

Of all the commercial samples tested, only about half of the products had DNA sequences identical or highly similar to those of PM (Table 3). The ITS and *rps*16 intron sequences with high similarity to PM observed in six commercial samples had slight differences from each other. However, they show only 1–6 base differences and all four differences of ITS were due to the nucleotide additivity. The *rps*16 intron region could be amplified in only about half of the tested samples. This may be attributable to the inadaptability of the primers, which were designed on the basis of the conserved sequence of *Pueraria* plants, but not to the poor efficiency of DNA extraction. The fact that the ITS region of most samples and the *rps*16 intron region of *Pachyrhizus erosus* (Pu-17), which is closely related to *Pueraria* plants, were successfully amplified supports this assumption. In the amplification of the ITS region, PCR product is sometimes obtained as a mixture of some sequences and, thus, subcloning to the vector is required for the sequencing. Although these processes are expensive and time-consuming, ITS sequence analysis has the advantage for the identification of unexpected species due to the extremely large number of known sequences in the database.

There were *Ipomoea batatas* (Pu-11) and *Pachyrhizus erosus* (Pu-17) within the unexpected species found in the tested samples. These plants are a vine and have a huge root (tubercle) similar to PM. Therefore, their existence in the commercial products might have resulted from the misidentification of PM. However, the other plants such as *Medicago sativa* (Pu-1) and *Glycyrrhiza uralensis* (Pu-8) are very likely to be adulterants added to the products on purpose. Additionally, not only *Ipomoea batatas* but also *Triticum aestivum* was detected in Pu-11. This fact suggests that the starch was contained in the product in spite of the description of "100 % PM" on its packaging.

It was found that about half of the commercial products tested in this study did not contain PM. These inferior products probably lack the efficacy implied on their packaging. The DNA analysis described in this study is useful for the identification of source materials of products and enable improvements in the quality of products via the exclusion of inferior products from the dietary supplement market. From the viewpoint of regulatory science, infrastructure that can exclude inferior material from the dietary supplement market is needed for the protection of consumers from potential damage to health and financial loss.

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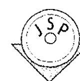

NOTE

Isoheleproline: a new amino acid-sesquiterpene adduct from *Inula helenium*

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Abstract A new amino acid–sesquiterpene adduct, isoheleproline (**1**), was isolated from the roots of *Inula helenium* (elecampane), together with four known sesquiterpene lactones (**2**–**5**). The planar configuration of **1** was elucidated on the basis of spectroscopic data analysis, and the relative configuration of **1** was determined by performing a detailed analysis of NOESY correlations and comparing its physicochemical data with the D- and L-proline adducts of **2** obtained by Michael addition. This is the first report of a new amino acid–sesquiterpene adduct from *Inula* plants.

Keywords *Inula helenium* · Asteraceae · Amino acid–sesquiterpene adduct · Sesquiterpene lactone

Introduction

The genus *Inula* is a member of the Asteraceae family, and *Inula* spp. are widely occurring perennial herbs in East Asia, North America, and Europe [1, 2]. The roots of *I. helenium* (elecampane) are used as a versatile medicinal herb against fever, lung disorders, bronchitis, indigestion, chronic enterogastritis, and infectious diseases [1]. It has been reported that *Inula* plants contain many sesquiterpene lactones and a few alkaloids such as royline and anthraniloyllycoctonine, which were isolated from *I. royleana* [3, 4]. However, as far as we know, there has been no report describing the isolation of an alkaloid from *I. helenium*. The presence/absence of an alkaloid is one of the main factors that influence whether a herbal material should be classified as a raw material that is exclusively used as pharmaceutical in Japan [5]. In our previous paper, we confirmed that shatavari (*Asparagus racemosus*) does not contain asparagamine A or any other alkaloids [6].

In this paper, we deal with the isolation and structure elucidation of a new amino acid–sesquiterpene adduct, isoheleproline (**1**). Its structure was elucidated by NMR spectral analysis using a 2D technique and a semisynthetic approach.

Materials and methods

General experimental procedures

Optical rotations were measured on a DIP-370 digital polarimeter (Jasco, Tokyo, Japan). UV spectra were obtained on a UV-2550 UV–visible spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were recorded on an FT/IR 6100 Fourier transform infrared spectrometer (Jasco). Direct analysis in real time (DART) time-of-flight (TOF) mass spectrometry (MS) was performed on an AccuTOF JMS-100 equipped with a DART-100 (both Jeol, Tokyo, Japan). HPLC was carried out using an LC-10ATVP pump equipped with an SPD-M10AVP detector (both Shimadzu) and an Inertsil ODS-3 column (for analytical HPLC; 250 × 4.6 mm i.d., 5 μm particle size; GL Sciences, Tokyo, Japan) as well as a Mightysil

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column (for preparative HPLC; 250 × 10 mm i.d., 5 μm particle size; Kanto Chemical, Tokyo, Japan). ^1H, ^{13}C, and 2D NMR spectra were recorded on an ECA-800 or ECA-600 spectrometer (JEOL), and the chemical shifts were referenced to TMS as an internal standard.

Plant material

The roots of *Inula helenium* were purchased from Amazon (Seattle, WA, USA). The source plant was identified based on the internal transcribed spacer sequence of the nrDNA. Briefly, the ITS region was amplified by PCR with the universal primer pair (ITS-S1, 5′- GGA AGT AAA AGT CGT AAC AAG G-3′; ITS-AS1, 5′- TCC TCC GCT TAT TGA TAT GC-3′) [7] using genomic DNA from plant material as the template. The amplicon was sequenced directly. The ITS sequence was completely identical with those of *I. helenium* on DDBJ (Acc. nos., EU239682; EU239683; FN870378).

Extraction and isolation

The roots of *I. helenium* were extracted with MeOH, and the extract (700 g) was treated with 3 % tartaric acid (pH 2) and then partitioned with EtOAc. The aqueous layer was treated with saturated aqueous Na_2CO_3 to obtain a pH of 9 and then extracted with $CHCl_3$ to give a basic fraction (2.6 g). The fraction was subjected to silica gel column chromatography (hexane/EtOAc 1/0 → 0/1 and then $CHCl_3$/MeOH 1/0 → 0/1) to give ten fractions. Fraction 4 was subjected to silica gel column chromatography (petroleum ether/EtOAc 1/0 → 6/4) to give isohelenin (**2**, 131.0 mg, 0.003 %) and alantolactone (**3**, 209.5 mg, 0.005 %). Fraction 6 was subjected to silica gel column chromatography ($CHCl_3$/acetone 1/0 → 0/1) followed by an additional silica gel column chromatography (hexane/acetone 1/0 → 0/1) to give 9 fractions (A–I). Fraction B was purified by ODS HPLC (50 % MeCN) to give 5a-epoxyalantolactone (**4**, 0.4 mg, 0.000009 %). Fraction E was subjected to ODS HPLC (40 % MeCN) to obtain 3-oxodiplophyllin (**5**, 1.2 mg, 0.00003 %). Fraction 8 was subjected to ODS column chromatography (MeOH/H_2O 40/60 → 0/100) followed by silica gel column chromatography (NH_3-saturated $CHCl_3$/MeOH 1/0 → 0/1) to give 9 fractions (I–IX). Fraction VI was subjected to ODS column chromatography (MeCN/H_2O 30/70 → 60/30) followed by ODS HPLC (67 % MeOH) to give isoheleproline (**1**, 1.8 mg, 0.00004 %).

Isoheleproline (1): Colorless solid; $[\alpha]_D^{24}$ +43 (*c* 1.0, MeOH); UV (MeOH) λ_{max} 300 (ε 130), 280 (180), 215 (3400), 200 (7500) nm; IR (KBr) ν_{max} 3440, 2930, 1760, 1640 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; DART-TOF-MS *m/z* 348.21983 (calcd. for $C_{20}H_{30}NO_4$ $[M+H]^+$, 348.21748).

Table 1 ^1H [δ_H (*J*, Hz)] and ^{13}C NMR data (δ_C) of isoheleproline (**1**) in $CDCl_3$

Position	δ_H	δ_C	HMBC
1a	1.24 (td, 6.6, 13.3)	42.1	C-2, C-3, C-5, C-9, C-10, C-14
1b	1.55 (t, 13.3)		C-2, C-3, C-5, C-9, C-10, C-14
2a	1.60 (m)	22.6	C-1, C-4, C-10
2b	1.60 (m)		C-1, C-4, C-10
3a	2.00 (td, 5.4, 13.3)	36.7	C-1, C-2, C-4, C-5, C-15
3b	2.32 (t, 13.3)		C-1, C-2, C-4, C-5, C-15
4		149.0	
5	1.82 (d, 12.7)	46.3	C-14, C-6, C-10, C-3, C-7, C-9, C-1, C-15, C-4
6a	1.12 (q, 12.7)	21.4	C-4, C-5, C-7, C-10
6b	1.60 (m)		C-4, C-5, C-7, C-8, C-10
7	2.67 (brs)	39.2	C-8
8	4.60 (brs)	78.4	C-6, C-10
9a	1.50 (dd, 3.6, 15.6)	41.1	C-1, C-5, C-10, C-14
9b	2.17 (d, 15.6)		C-1, C-4, C-5, C-7, C-8, C-10, C-14
10		34.7	
11	3.30 (brd, 5.4)	45.2	C-6, C-7, C-12
12		176.9	
13a	3.11 (dd, 5.4, 12.3)	51.3	C-7, C-11, C-12, C-2′, C-5′
13b	3.44 (dd, 5.4, 12.3)		C-7, C-11, C-12, C-2′, C-5′
14	0.78 (s)	17.7	C-1, C-5, C-9, C-10
15a	4.46 (s)	106.6	C-3, C-4, C-5
15b	4.79 (s)		C-3, C-4, C-5
1′		173.1	
2′	3.72 (brs)	68.4	C-4′
3′a	2.24 (brs)	29.7	C-5′
3′b	2.32 (brs)		C-1′, C-2′, C-4′,
4′a	1.98 (m)	24.1	C-2′, C-5′, C-3′
4′b	1.98 (m)		C-2′, C-5′, C-3′
5′a	2.74 (q, 8.8)	55.1	C-13, C-3′, C-4′
5′b	3.74 (brs)		C-2′, C-3′

Synthesis of the L- and D-proline adducts of 2

Compound **2** (20 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (0.2 mL) and EtOH (1.5 mL) with Et_3N (50 mL), and then L- or D-proline (35 mg, 0.3 mmol) was added. After the reaction mixture was stirred at room temperature for 15 h, the solvent was evaporated and excess L- or D-proline was

removed using ODS HPLC (67 % MeOH) to obtain the L- and D-proline adducts of **2**.

L-Proline adduct of 2: $[\alpha]_D$, ^{1}H and ^{13}C NMR data were identical with **1**.

D-Proline adduct of 2: $[\alpha]_D^{24}$ +84 (*c* 1.0, MeOH); ^{1}H NMR [$CDCl_3$, δ_H (*J*, Hz)] 0.77 (s, H_3-14), [1.05 (q, 12.6), 1.72 (ddd, 2.2, 5.8, 12.6), H_2-6], [1.25 (m), 1.56 (t, 13.6), H_2-1], [1.50 (dd, 4.8, 14.8), 2.15 (d, 14.8), H_2-9], 1.59 (m, H_2-2), 1.83 (d, 12.6, H-5), [2.00 (m), 2.32 (dd, 2.4, 16.0), H_2-3], 2.03 (m, H_2-4′), [2.26 (m), 2.35 (m), H_2-3′], 2.88 (m, H-7), [3.04 (q, 9.1), 3.79 (m), H_2-5′], [3.24 (dd, 6.0, 12.8), 3.45 (dd, 6.0 12.8), H_2-13], 3.50 (q, 6.0, H-11), 3.68 (m, H-2′), [4.48 (s), 4.77 (s), H_2-15], 4.64 (brs, H-8); ^{13}C NMR [$CDCl_3$, δ_C] 17.8 (C-14), 21.2 (C-6), 22.6 (C-2), 23.3 (C-4′), 29.1 (C-3′), 34.7 (C-10), 36.7 (C-3), 39.1 (C-7), 41.1 (C-9), 42.0 (C-1), 45.2 (C-11), 46.2 (C-5), 51.0 (C-13), 54.3 (C-5′), 70.1 (C-2′), 78.7 (C-8), 106.6 (C-15), 149.3 (C-4), 171.8 (C-1′), 176.8 (C-12).

Results and discussion

The chromatographic fractionation of a methanol extract of *I. helenium* afforded a new amino acid-sesquiterpene adduct, isoheleproline (**1**), together with four known sesquiterpene lactones: isohelenin (**2**) [8], alantolactone (**3**) [8], 5α-epoxyalantolactone (**4**) [8], and 3-oxodiplophylline (**5**) [9] (Fig. 1).

Isoheleproline **1:** {$[\alpha]_D^{24}$ +43 (*c* 1.0, MeOH)} showed a pseudomolecular ion peak at *m/z* 348.21983 $[M+H]^+$ in the DART-TOF-MS analysis, suggesting the molecular formula $C_{20}H_{29}NO_4$. The IR spectrum indicates the presence of carboxyl (3440 and 1640 cm^{-1}) and γ-lactone (1760 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Table 1) and the HSQC spectrum of **1** revealed the presence of two carbonyls, one sp^2 quaternary carbon, one sp^2 methylene, one sp^3 quaternary carbon, one methyl, five sp^3 methines, and nine sp^3 methylenes.

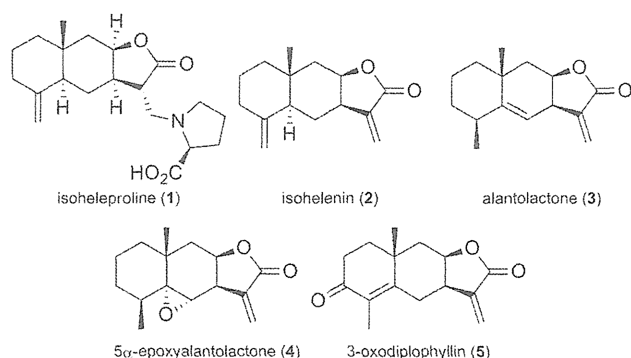

Fig. 1 A new amino acid–sesquiterpene adduct (**1**) and known sesquiterpene lactones (**2–5**) from *I. helenium*

The structure of **1** was deduced from detailed analysis of the two-dimensional NMR data, including the ^{1}H–^{1}H COSY, HSQC, and HMBC spectra in $CDCl_3$ (Fig. 2). The ^{1}H–^{1}H COSY and HSQC spectra revealed three partial structures (**a–c**), as shown in Fig. 2. The HMBC cross-peaks of H_2-2 (δ_H 1.60, 1.60) to both C-4 (δ_C 149.0) and C-10 (δ_C 34.7), H-5 (δ_H 1.82) to C-3 (δ_C 36.7), H_2-6 (δ_H 1.12, 1.60) to C-10, H-8 (δ_H 4.60) to C-10, and H_2-9 (δ_H 1.50, 2.17) to both C-5 (δ_C 46.3) and C-1 (δ_C 42.1) revealed the connection between partial structures **a** and **b**. The HMBC correlations of H_2-1 (δ_H 1.24, 1.55), H-5, and H-9 to C-14 (δ_C 17.7), H_2-15 (δ_H 4.46, 4.79) to both C-3 and C-5, and both H-11 (δ_H 3.30) and H_2-13 (δ_H 3.11, 3.44) to C-12 (δ_C 176.9) established that **1** possesses the eudesmane skeleton, while chemical shifts at C-13 (δ_C 51.3), C-2′ (δ_C 68.4), and C-5′ (δ_C 55.1) suggested that these carbon atoms bind to a nitrogen atom. In addition, the HMBC cross-peaks of H_2-13 to C-2′ and H_2-5′ (δ_H 2.74, 3.74) to C-13 suggested the connection between the eudesmane skeleton and partial structure **c**. Furthermore, the HMBC correlation of H_2-3′ (δ_H 2.24, 2.32) to C-1′ (δ_C 173.1) revealed that the proline moiety attaches to C-13 in the eudesmane skeleton (Fig. 2).

The relative configuration of **1** was elucidated by NOESY correlations, as shown in Fig. 3. The correlations H-1b/H_3-14, H-9b/H_3-14, and H-1b/H-9b indicated that the methyl group at C-14 is in a β configuration. The α configuration of H-5 was elucidated from the correlations H-1a/H-5 and H-9a/H-5. The observed correlations H-5/H-7, H-7/H-8, and H-7/H-9a suggest that H-7 and H-8 are α configurations. In addition, the correlation H-8/H-13a indicates that H-11 has a β configuration (Fig. 3).

Subsequently, the stereochemistry of proline conjugated at C-13 of **1** was determined by a semisynthetic approach. Treatment of **2** with L- and D-proline in EtOH–CH_2Cl_2 (15:2) in the presence of Et_3N at r.t. for 15 h furnished the L- and D-proline adducts of **2**, respectively. Their spectral data, $[\alpha]_D$ values, and retention times on HPLC analysis were compared with those of **1**. The above physicochemical data of **1** were identical to those of the L-proline adduct of **2**. Thus, **1** was found to have an L-proline moiety at

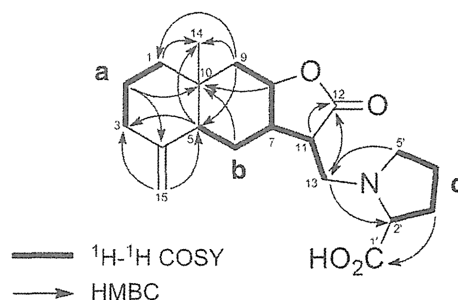

Fig. 2 Selected 2D NMR correlations for isoheleproline (**1**)

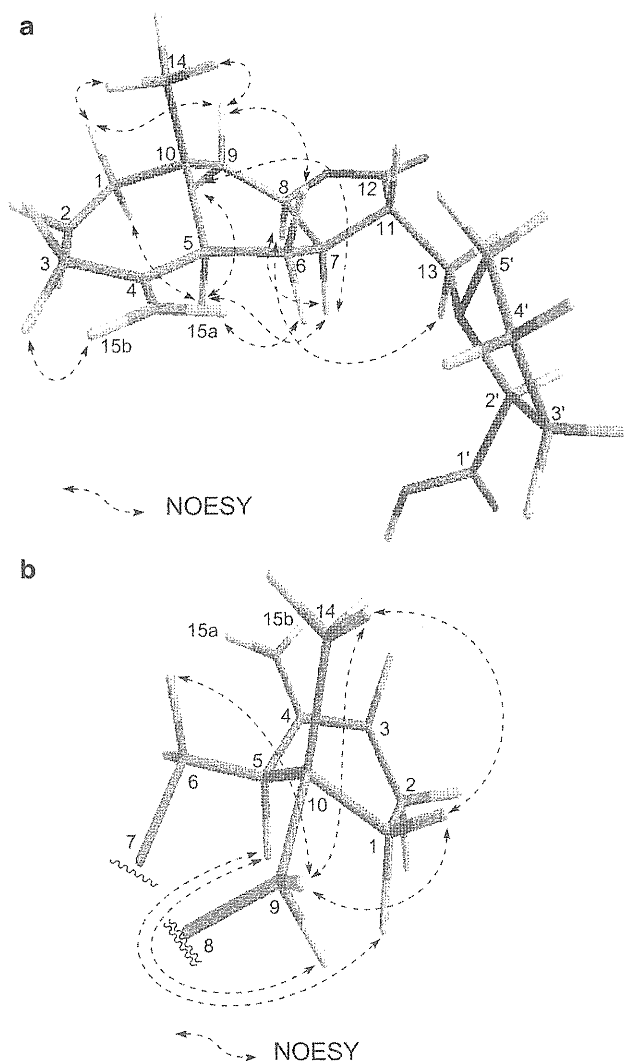

Fig. 3 **a** Selected NOESY correlations for isoheleproline (**1**). **b** Selected NOESY correlations for a partial structure of isoheleproline (**1**)

C-13. In conclusion, the structure of **1** was determined as (*S*)-1-(((3*S*,3a*R*,4a*S*,8a*R*,9a*R*)-8a-methyl-5-methylene-2-oxododecahydronaphtho[2,3-*b*]furan-3-yl)methyl)pyrrolidine-2-carboxylic acid. Although Yoshikawa et al. [10] reported a few amino acid–sesquiterpene adducts from *Saussurea lappa*, this is the first report of a new amino acid–sesquiterpene adduct isolated from *Inula* plants.

Amino acid–sesquiterpene adducts are regarded as alkaloids in the general sense. However, the biological activity of the constituent compounds is an important factor in judging whether a given material can be designated a raw material exclusively for pharmaceutical use. Therefore, further studies will be needed to revaluate whether or not *I. helenium* can be regarded a raw material exclusively for pharmaceutical use.

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—Symposium Review—

国立医薬品食品衛生研究所における痩身や強壮を標榜する健康食品中の医薬品成分の分析と同定

合田幸広

Analysis and Identification of Illegal Constituents in Health Food Products Implicitly Advertizing Tonic or Slimming Effect in the National Institute of Health Sciences in Japan

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With the prefectural governments' aid of the purchase, the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences (NIHS) successively has surveyed illegal constituents in health food products implicitly advertizing tonic or slimming effect since the fiscal year of 2002 (slimming type) or 2003 (tonic type). The average numbers of the analyzed products per year are about 100 (slimming type) and 150 (tonic type), respectively. We also continuously distribute standards of authentic samples of several illegal components such as *N*-nitrosofenfluramine (NFF) and sildenafil (SIL) to prefectural institutes and the average gross number per year is about 140. In the case of slimming type, the fact that the products containing NFF were widely sold in Japanese markets in 2002 is well known. In addition, phenolphthalein, fenfluramine, sibtramine, desdimethylsibtramine, orlistat, mazindol, Rhubarb, Senna Leaf, *etc.* have been found as illegal constituents. In the tonic type products, we have identified more than 20 synthetic compounds relating to the erectile dysfunction (ED) treatment drugs, SIL, vardenafil and tadalafil (TDF). Since 2005, their synthetic intermediates and the patented but non-approved PDE5 inhibitors also have been found. It should be noted that TDF was found in the shells of capsule in 2009 and that mutaprodenafil was found as pro-drug type illegal component in 2010. In this report identification method of these illegal constituents is briefly described and then analytical trend in this decade is reviewed.

Key words——health food product; illegal constituent; analytical trend; slimming type; tonic type

1. 緒言

国立医薬品食品衛生研究所（国立衛研，National Institute of Health Sciences; NIHS）生薬部では，平成14年度（2002年）より継続的に無承認無許可医薬品（Counterfeit medicines）についての実態調査を行っている．われわれが分析対象としている製品は，大きく分類して，痩身を暗に標榜する健康食品（Health food products intended for sliming: sliming type），強壮を暗に標榜する健康食品（Health food products intended for sexual enhancement: tonic type）及び，いわゆる違法（脱法）薬物（Non-listed compounds intended for psychotropic effects）があるが，本稿では，主に全国の都道府県の協力を得て買い上げられた（"買い上げ調査"で集められた）前2者について取り扱う．

2. 試験検体数と配布標品数

Table 1に，分析を行った試験検体数を年度別に示す．平成14年度より痩身を標榜する健康食品について，平成15年度より強壮を標榜する健康食品について分析を行っているが，前者の試験数は，年平均100件程度であり，後者は150件程度である．また，別に，都道府県，政令指定都市等で分析を行うための分析用標品（フェンフルラミン，*N*-ニトロソフェンフルラミン，シブトラミン，オリスタット，シルデナフィル，バルデナフィル，タダラフィ

The author declares no conflict of interest.

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本総説は，日本薬学会第133年会シンポジウムS30-105で発表した内容を中心に記述したものである．

Table 1. Number of Analyzed Products Obtained from Commercial Markets with Help of Province Governments from 2002 to 2011

Category	Fiscal year										Average
	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	
Slimming type	117	143	121	76	67	76	82	121	76	126	101
Tonic type	—	91	118	139	144	169	156	163	152	205	149

ル，ホンデナフィル，キサントアントラフィル，チオキナピペリフィル等）の配布も平成14年度より継続的に行っており，延べ数で年平均140物質を配布している．

3. 無承認無許可医薬品の分析方法

まず，表示等より含まれている可能性のある無承認無許可医薬品成分を推定する．次に，対象物が，天然物である場合と，化学的合成品である場合で分析方法は異なる．天然物由来である場合，痩身を標榜する製品からは，センナ (Senna) 葉，センナ実，大黄（Rhubarb)，甲状腺末（Thyroid gland powder)，マオウ（Ephedra）等が，強壮を標榜する製品からは，イカリソウ（*Epimedium grandiflorum*, Barrenwort)，ヨヒンベ（*Pausinystalia yohimbe*）等が検出される可能性がある．これらの分析同定方法は，主に，顕微鏡観察及びDNA抽出による遺伝子的同定による．センナ葉の場合，小葉中の葉軸は専ら医薬品成分である葉の一部とみなされるが，茎は専ら医薬品成分ではないので，その区別が重要となる．これには，細胞中の気孔を目安にした細胞の形状で区別可能である．[1] 甲状腺末については，酵素分解後，甲状腺末中の3,4,3′-トリヨード-L-チロニン（T3）及びL-チロキシン（T4）をLC-MS等で同定することで確認する．[2]

化学的合成品である場合には，化合物の精製，分離が重要である．既知の化合物であり，標品との比較が可能な場合には，あまり精製されていない段階でもLC-MSやGC-MSで分析することで同定可能である．一方，未知の化合物の場合には，単離して，NMR等を利用して最終的な構造決定を行い，そのものの構造情報等から，専ら医薬品成分であるかどうかを検討する必要がある．昨今，TOF-MSにより容易に高分解能MSが測定できるようになったため，表示情報に加えて，得られた推定分子式とデータ検索により，標品がなくても，既知化合物であれば，迅速に構造を予想できる場合がある．特に，DART-TOF-MSは，試料を未精製の段階で，かざすだけで，含有物の推定分子式情報が入手できるので，このような実態調査では，非常に強力な分析方法となるが，[3] 標品が入手できなければ，物質を0.5–1 mg程度は単離する必要があり，構造決定はNMRに頼らざるを得ない．

4. 無承認無許可医薬品より検出される承認薬成分

痩身を標榜する製品から検出される化学的合成品には，承認薬成分として，フェノールフタレイン (phenolphthalein，下剤)，フェンフルラミン（fenfluramine，食欲抑制剤)，シブトラミン（sibtramine，食欲抑制剤)，オリスタット（orlistat，抗肥満薬)，マジンドール（mazindol，向精神薬)，デヒドロエピアンドロステロン（dehydroepiandrosterone，ホルモン剤，体重増加抑制）等がある．また，アトピー性皮膚炎等に有効を標榜する外用製品からは，プロピオン酸クロベタゾール (clobetasol propionate)，デキサメタゾン (dexamethasone) 等，外用ホルモン剤が検出される可能性がある．また，高血圧等に有効を標榜する製品からは，グリベンクラミド（glibenclamid，血糖降下剤）が，コエンザイムQ10含有を謳う製品からは，イデベノン（idebenone）が検出された事例がある．一方，強壮を標榜する製品からは，シルデナフィル（sildenafil，勃起不全治療薬)，バルデナフィル（vardenafil，勃起不全治療薬)，タダラフィル（tadalafil，勃起不全治療薬）が検出される．

合田幸広

東京大学薬学部昭和55年卒業，東京大学大学院薬学系研究科博士課程昭和60年修了，国立衛生試験所（現国立医薬品食品衛生研究所）食品添加物研究員，同部主任研究官，食品部室長，生薬部長を経て平成25年4月より薬品部長．専門は医薬品及び違法薬物のレギュラトリーサイエンス，生薬学，食品衛生化学．

5. 痩身を標榜する製品から検出される承認薬関連成分

われわれは，2002年に，使用者に肝障害を引き起こした，痩身を標榜する中国産健康食品より，原因物質として*N*-ニトロソフェンフルラミン（*N*-nitrosofenfluramine）を世界で初めて単離，同定したが，筆者の知る限り，これが，承認薬成分の構造を若干変えた化合物の最初の出現事例である．承認薬成分の構造を一部改変すると，標品が入手できなくなるため，同定が格段に難しくなる．*N*-ニトロソフェンフルラミンの場合，特に，極性が低い方に変化しているため，通常の逆相HPLCでは，保持時間が長くなり，他の物質を分析目標としていると，検出できないことが予想される．また，*N*-ニトロソフェンフルラミンを含む製剤では，ニコチン酸，エピガロカテキン，ガロカテキン，カフェイン，エピカテキン，ビタミンB2，カテキン，ガロカテキンガレート，エピカテキンカレート（逆相HPLC溶出順に記載，これらのピークよりあとにフェンフルラミンが検出され，さらに，フェンフルラミンのほぼ2倍の保持時間で*N*-ニトロソフェンフルラミンが検出された）といった，他の天然物や抽出物（おそらく茶抽出物）に加えた形で製品化されているため，特に同定に時間がかかった．また，ニトロソ体は，通常の天然物では存在しないため，天然物に混ぜられると，予想外である合成物の構造推定には，天然物だけの事例より難易度が高かった記憶がある．

痩身を標榜する製品から検出される承認成分の構造改変体としては，*N*-ニトロソフェンフルラミンに加えて，脱ジメチルシブトラミン（desdimethylsibtramine），脱メチルシブトラミン（desmethylsibtramine）がある．なお，全国の都道府県の協力を得て買い上げられた痩身を標榜する製品からの無承認無許可医薬品成分の検出は，平成15年以降，センナ実の検出1件のみである．

6. 強壮を標榜する製品から検出される化学合成成分

2003年（平成15年度），強壮を標榜する製品から承認薬成分としてシルデナフィル，タダラフィル（当時は海外で承認薬）が検出されたが，その年，承認成分の構造改変体としてホモシルデナフィル（homosildenafil）が検出された．さらに，2004年（平成16年度）新規な構造改変体ヒドロキシホモシルデナフィル（hydroxyhomosildenafil）とホンデナフィル（hongdenafil，買い上げ調査外）を，2005年（平成17年度）プソイドバルデナフィル（pseudovardenafil），アミノタダラフィル（aminotadalafil），ノルネオシルデナフィル（norneosildenafil）と承認薬成分の構造改変体が毎年検出されたが，同年，承認薬シルデナフィルの合成中間体であるイミダゾサガトリアジノン（imidazosagatriazinone，買い上げ調査外）とゲンデナフィル（gendenafil，買い上げ調査外）も検出され，単に構造改変体だけでなく，合成中間体も検出される事例があらわれた．さらに，2006年（平成18年度）には，新規な承認薬成分であるバルデナフィル，ウデナフィル（udenafil，韓国での承認薬），構造改変体ヒドロキシホンデナフィル（hydroxyhongdenafil），カルボデナフィル（carbodenafil，買い上げ調査外），ニトロデナフィル（nitrodenafil）に加えて，新規なタダラフィルの合成中間体であるクロロプレタダラフィル（chloropretadalafil，買い上げ調査外）が検出された．[4]

フィル系承認薬は，ホスホジエステラーゼ5（PDE5）阻害により，erectile dysfunction（ED）治療作用を持つが，元々，シルデナフィルの薬効は，副作用から発見された経緯があることから，一部構造を改変しても，ある程度の活性が維持されるため，幅広い範囲で構造が改変された化合物が製造され，不法に健康食品中の成分として加えられるものと考えられる．ついで平成18年度後期には，ED治療薬を目指して開発されたが，開発中止され，藤沢製薬よりFR226807として論文報告が行われた化合物キサントアントラフィル（xanthoanthrafil，アステラス製薬の同意を得て命名）が検出され，[5] 違法な化合物を別に積極的に製造して，痩身を標榜する製品に加える事例が出現した．

2007年（平成19年度）には，構造改変体として，*N*-オクチルノルタダラフィル（*N*-octylnortadalafil，買い上げ調査外），[6] シクロペンチナフィル（cyclopentynafil，買い上げ調査外），[6] チオデナフィル（thiodenafil）が出現したが，協和発酵より特許報告された開発中止化合物であるKF31327（協和発酵の同意を得てthioquinapiperifilと命名）が新規に検出同定された．[7] これらの開発中止化合物は，なんらかの欠陥があって，医薬品にできなかった化

合物である可能性が考えられるため，当該欠陥が安全性の問題であった場合には，これらの化合物を含む製品を使用すると，重篤な副作用が出る可能性があり，承認化合物が入った無承認無許可医薬品製品より危険性が高いものと考えられる．

2008年，シンガポール当局から，トンカットアリ（Tongkat Ali，東南アジアの伝統薬）のカプセル基剤よりタダラフィルが検出されたとの報告が出された．2008年（平成20年度）のわれわれの実態調査で集められた製品には，カプセル型のものが32品目あった．本報告を受けて，それらについてカプセル内含有物だけでなく，カプセル基剤そのものについても分析を実施したところ，8製品について同様にタダラフィルが検出された．また，その含量は，0.4–0.8 mg程度であった．タダラフィルは，フィル系承認薬の中では，最も活性が強く，用量が5 mg程度であるため，この程度の微量でも，数カプセル使用すれば，ある程度の作用が予想されるため，このような形で含有させたものと推定される．また，2008年（平成20年度）には，ホモチオデナフィル（homothiodenafil）が，構造改変体として検出された．また，2009年（平成21年度）には，新たに構造改変体として，アセチルアシッド（acetil acid），ノルホンデナフィル（norhongdenafil），アイルデナフィル（aildenafil，買い上げ調査外）が検出された．

2010年（平成22年度）の調査では，プロドラッグタイプのムタプロデナフィル（mutaprodenafil）が新規化合物として単離同定された．[8] 本化合物は，酸性条件で，アイルデナフィルが加水分解されて生じる構造を持っている．多くのヘテロ原子を含む本化合物の構造決定は，難易度の高いものであった．当初，本化合物は，Venhuisらにより，thiazol環とnitroso基を持つnitrosoprodenafilとして報告されたが，N-HMBC及び2D-INADEQUATEを含むNMRのデータの詳細な解析より，同構造は明らかに間違いであることが判明し，最終的に，化合物を合成することで本物質の構造をimidazol環とニトロ基を持つmutaprodenafilであることを確定させた．なお，本物質は，ほぼ同時期に，都立健安センターのSakamotoらが，X線構造解析により，nitroprodenafilとして同じ構造を提出していることが判明したが，[9] 論文公開は，われわれのグループが明らかに早く，名称としてはmutaprodenafilが使用される．

強壮を標榜する製品から検出され，国立医薬品食品衛生研究所で構造を確認した化合物の構造式をFig. 1に示す．

7. 無承認無許可医薬品成分が検出された強壮を標榜する製品の経年変化

前述したように，2003年（平成15年度）から2011年（平成23年度）まで，毎年150平均の強壮を標榜する製品中の無承認無許可医薬品成分について分析を実施している．無承認無許可医薬品成分が検出された製品は，2003年，9検体で，以後，26, 28, 38, 30, 31検体と，ほぼ30検体前後なり，2009年より減少傾向に転じ，16, 13, 3検体となっている．本報告で取り上げた製品は，主に店頭買い上げの製品と考えられる．2009年以降，このように，検出数が減少傾向になったのは，店頭で流通する製品への，このような成分の添加率が下がったためと推定している．したがって，インターネット上の製品を買い上げた場合の結果とは異なるものと考えられる．また，2009年以降，日本で，向精神作用を持つ違法ドラッグ（いわゆる脱法ドラッグ）の流通が目立つようになったことを考慮すると，違法薬物の販売業者が，より利益率の高い，違法ドラッグへシフトした可能性も考えられる．

検出された化合物の経年変化をみると，検出数自体が少ない2011年を除き，毎年検出数のほぼ4–7割がシルデナフィルであり総数（193件）の49%を占める．ついで，タダラフィルが2008年まで継続的に検出され全体の13%を占めるが2009年以降は不検出である．バルデナフィルの検出は2006年の2件のみである．構造改変体では，ホモシルデナフィルが2008年まで，毎年検出され（全体の7%），ついでヒドロキシホモシルデナフィルが，2003年，2007年，2009年を除き毎年検出(5%)されている．2006年から2008年検出のアミノタダラフィル（4%），2007年から2009年に継続検出のチオキナピペリフィル（7%），2007年と2008年に複数検体検出されたチオデナフィル(4%)，2005年，2006年，2011年に検出されたプソイドバルデナフィル（2%）を除くと，検出例は3例以下，検出年も2年以下である．

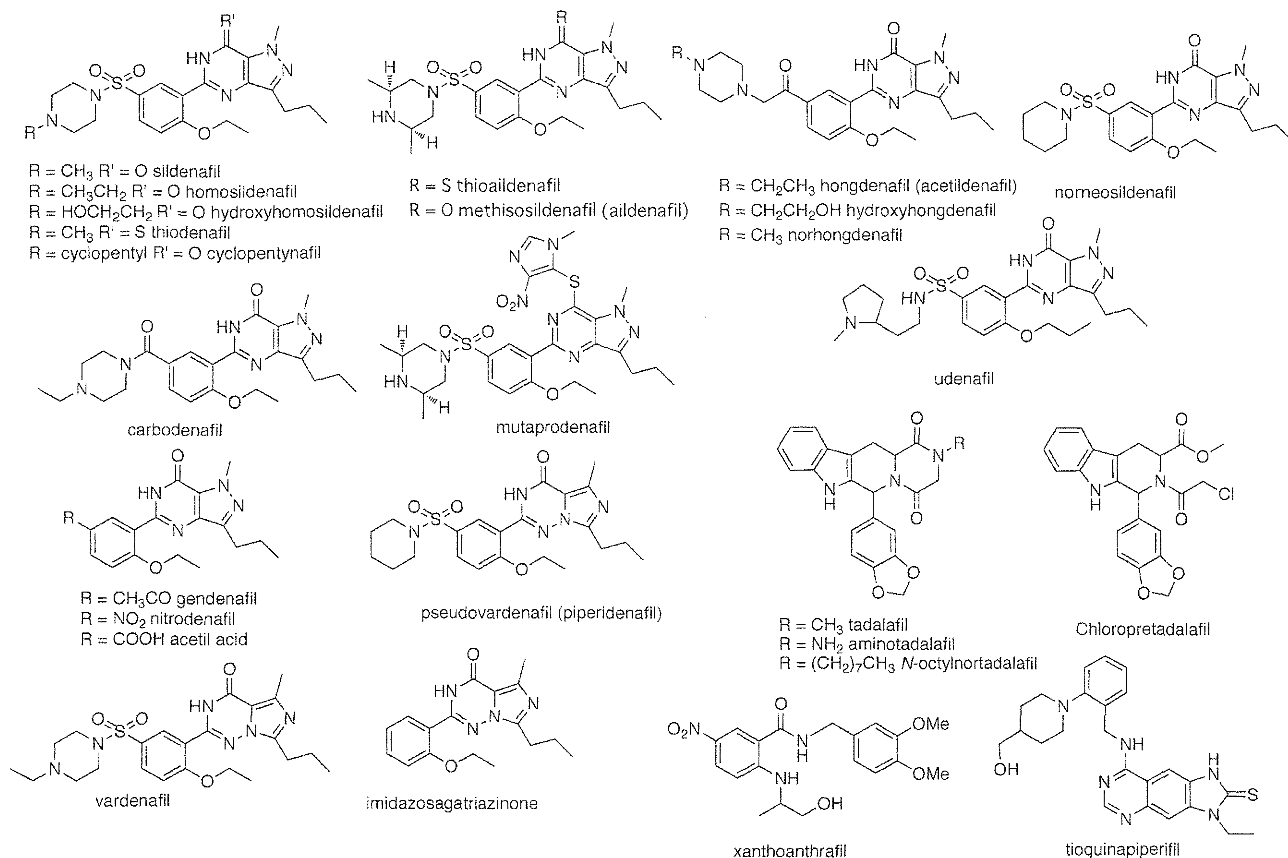

Fig. 1. Structures of Sildenafil, Tadalafil, Vardenafil and Determined Compounds in Dietary Supplements Promoting Sexual Enhancement

8. 結語

国立医薬品食品衛生研究所では，平成 14 年度（2002 年）より継続的に無承認無許可医薬品についての実態調査を行っている．検出される無承認無許可医薬品成分には，承認薬だけでなく，摘発を逃れるために，承認薬の構造改変体，開発中止化合物，プロドラッグタイプ化合物等があり，またカプセル基剤に成分を練り込んだ事例もある．今後も，実効ある取締りとするため，引き続き実態調査を実施するとともに，分析用標品の提供や，未知化合物の構造決定を行う予定である．

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