Further Investigation of Phenanthroindolizidine Alkaloids from *Tylophora tanakae*[1)]

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In addition to ten alkaloids previously described, we have isolated two alkaloid *N*-oxides from *Tylophora tanakae* MAXIM. (Asclepiadaceae). Further, the polar fraction of the leaves and caules of this plant cultivated in a farm afforded two new polar alkaloids. The structures of the four products were determined. The relation between the structure and cytotoxic activity of this group of alkaloids is discussed.

Key words *Tylophora tanakae*; phenanthroindolizidine alkaloid; cytotoxicity; Asclepiadaceae

Tylophora tanakae MAXIM. (Asclepiadaceae) is indigenous to the Ryukyu Islands of Japan. It is a feeding plant for caterpillars of *Ideopsis similis*. In the preceding paper of this series, we described the isolation and structure elucidation of ten phenanthroindolizidine alkaloids (**1**—**10**), including two known alkaloids, (+)-isotylocrebrine (**1**) and (−)-tylophorine (**2**), from the leaves and caules of this plant.[1)] Among these alkaloids, **1** and (−)-7-demethyltylophorine (**10**) showed remarkable oviposition-promoting activity towards *Ideopsis similis*.[2)] The cytotoxicity of phenanthroindolizidine alkaloids has been studied and **2** is known to inhibit protein biosynthesis in He La cells.[3)] During the further investigation of this plant, *N*-oxides of **2** and **10** (**11** and **12**, respectively) were isolated, as well as two new alkaloids (**13**, **14**). This paper deals with the isolation and characterization of these four products and with the cytotoxicity of some of the alkaloids.

From the chloroform-soluble fraction of a plant sample collected at Hateruma-jima, two alkaloids (**11**, **12**) were isolated in addition to **1**—**10**.[1)] Compounds **11** and **12** have *levo*-rotation values similar to those of **2** and **10**. Based on the FAB-MS, **11** was considered to have the same molecular formula, $C_{24}H_{27}NO_5$ as isotylocrebrine *N*-oxide (**5**), suggesting it to be an isomer of **5**. The ^{1}H-NMR spectrum of **11** showed similar signals and coupling patterns to those of **2**, except for the lowerfield shifts of H-9, 11 and 13a (Table 1). In the ^{13}C-NMR spectrum, lowerfield shifts were also observed for C-9, 11 and 13a, and upperfield shifts for C-13 and C-14 in comparison with those of **2** (Table 2). A similar shift pattern is seen between other alkaloids and *N*-oxides, such as **1** and **5**, or

Table 1. ^{1}H-NMR Spectral Data for the Four Alkaloids [δ (ppm) in CD_3OD–$CDCl_3$]

H	11	12	13	14[a)]
1	7.36 (s)[b,c)]	7.31 (s)[b,c)]	7.65 (d, 9)[b)]	7.97 (d, 9)[b)]
2			7.20 (d, 9)	7.18 (d, 9)
4	7.89 (s)[d)]	7.84 (s)[d)]		
5	7.89 (br s)[d)]	7.82 (s)[d)]	9.10 (s)[c)]	9.07 (s)[c)]
8	7.03 (br s)[e−g)]	7.09 (s)[e,f)]	7.09 (s)[d−f)]	6.87 (s)[d−f)]
9	5.17 (br d, 15)[e)]	5.09 (br d, 15)[e)]	4.52 (brd, 14)[d)]	4.11 (br d, 15)[d)]
	4.81 (br d, 15)[f)]	4.73 (br d, 15)[f)]	3.56 (br d, 14)[e)]	3.28 (br d, 15)[e)]
11	3.95 (td, 10, 2)	3.95 (br t, 9)	3.41 (t, 9)	3.18 (t, 9)
	3.71 (q, 10)	3.68 (br q, 9)	2.45 (q, 9)	2.29 (q, 9)
12	2.18 (m)	2.15 (m)	1.97 (2H) (m)	1.85—1.95
	2.46 (m)	2.44 (m)		
13	2.25—2.40	2.26 (m)	1.71 (m)	1.85—1.95
		2.35 (m)	2.22 (m)	2.25 (m)
13a	3.65 (m)	3.61 (m)	2.43 (m)	2.35 (m)
14	3.30—3.40[b)]	3.30—3.40[b)]	3.31 (dd, 16, 2)[b)]	4.97 (d, 2)[b)]
			2.87 (dd, 16, 13)	
-OMe	4.06 (2-)[c)]	4.04 (2-)[c)]	3.84 (4-)[c)]	3.83 (4-)[c)]
	4.13, 4.12	4.12, 4.11	4.03 (7-)[f)]	3.92 (7-)[f)]
	(3, 6-)[d)]	(3, 6-)[d)]		
	4.02 (7-)[g)]			

a) Dissolved in CD_3OD. *b*—*f*) or *g*) A cross-peak was observed between these signals in the NOESY spectrum.

Table 2. ^{13}C-NMR Spectral Data for the Four Alkaloids [δ (ppm) in CD_3OD–$CDCl_3$]

C	11	12	13	H[a)]	14[b)]
1	104.8	103.6	120.5		122.2
2	149.9[c)]	148.6[c)]	116.6		117.7
3	149.8[c)]	148.2[c)]	148.7[c)]		150.3[c)]
4	104.6[d)]	103.0[d)]	144.7	H-2, 4-OMe	145.7
5	104.3[d)]	102.9[d)]	113.2		114.1
6	149.8[c)]	147.6	145.6	H-8	145.7
7	149.7[c)]	145.9	148.4[c)]	H-8, 7-OMe	149.7[c)]
8	103.5	105.6	103.1		104.0
9	66.0	64.6	54.4		55.0
11	69.8	68.4	55.4		55.9
12	20.3	19.0	21.8	H-11a	22.5
13	27.7	26.4	31.3		25.2
13a	70.5	69.2	61.0	H-11b	66.7
14	28.1	26.7	33.8		65.7
ring C	125.4	123.9	126.9		129.1
	125.2	123.8	126.8		127.3
	124.9	123.6	126.3		126.9
	124.8	123.4	125.3		125.6
	124.3	122.9	124.0		125.6
	120.6	118.9	123.8		125.3
-OMe	56.5(×2)	55.2	59.9		60.0
	56.4(×2)	55.0(×2)	56.0		56.2

a) Proton signals coupled *via* 2 or 3-bonds in long-range ^{1}H–^{13}C COSY. *b*) Dissolved in CD_3OD. *c*, *d*) Assignments may be interchangeable.

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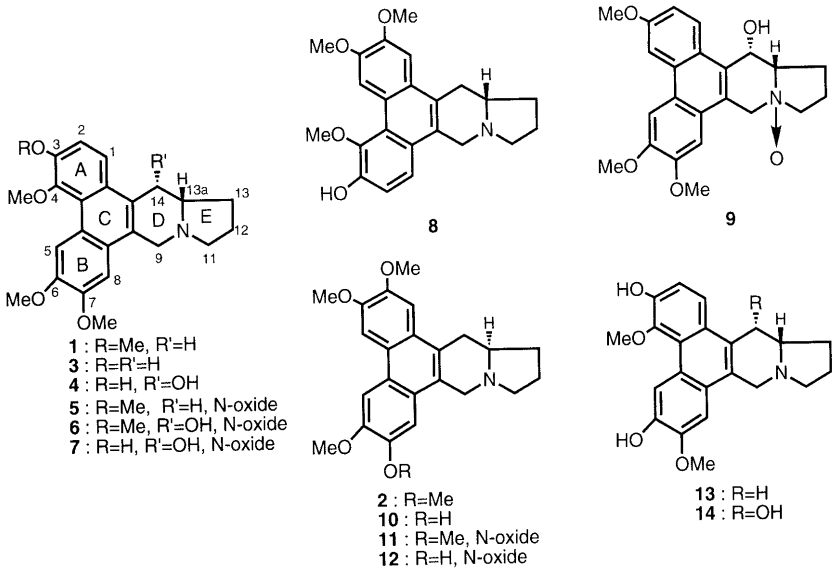

Chart 1

4 and **7**. Therefore, **11** was concluded to be the *N*-oxide of **2**.

Compound **12** was suggested to have the molecular formula $C_{23}H_{25}NO_5$, based on the $[M+H]^+$ peak at *m/z* 396.1803. The coupling pattern of the ^{1}H-signals in the phenanthrene moiety is similar to that of **10**. A similar chemical shift relation was observed between **10** and **12**; lowerfield shifts of H-9, 11, 13a and C-9, 11, 13a, and upperfield shifts of C-13 and 14. Compound **12** was thus considered to be the *N*-oxide of **10**.

Compounds **13** and **14**, which are dextro-rotatory, were isolated from the eluate from the reversed-phase column, after the removal of $CHCl_3$-soluble substances. In the ^{1}H-NMR spectrum of **13**, almost the same signals as those of **3** were observed, *e.g.*, one set each of *o*-coupling and *p*-coupling protons in the phenanthrene moiety, except for two methoxyl signals in **13**, instead of three in **3**, thus suggesting the presence of two hydroxyl and two methoxyl groups. In order to determine the locations of the hydroxyl and methoxyl groups, including the selection of either **1** or **8** type, 2-dimensional nuclear Overhauser effect (NOE) spectroscopy (NOESY) measurement was carried out. The signal at δ 3.31, assignable to H-14a, showed a cross peak to the H-1 signal at δ 7.65 (d, J=9 Hz), which was then linked to the signal at δ 7.20 in an *o*-coupling mode, indicating it to be due to H-2. On the other hand, the singlet signal at δ 7.09 (H-8) showed cross peaks to H-9 at δ 4.52 and also a methoxyl signal at δ 4.03, indicating that one of the methoxyl groups was attached to C-7. The H-5 (δ 9.10, s) signal showed NOE with the remaining methoxyl signal (δ 3.84), suggesting it to be located at C-4 or C-6. The location of the methoxyl group at C-4 was confirmed based on the cross peaks of C-4 to H-2 and the methoxyl signal in the long-range ^{1}H–^{13}C correlation spectroscopy (COSY) spectrum.

In the ^{1}H-NMR spectrum of **14**, two methoxyl and four aromatic proton signals were observed with almost the same multiplicity as those of **13**. The phenanthrene moiety was therefore considered to retain the same structure as that of **13**. One hydroxyl proton signal was observed at δ 4.97, showing a cross peak to H-1 in the NOESY experiment. In the ^{1}H–^{1}H COSY spectrum, the hydroxyl proton showed a cross peak to H-13a with a small coupling constant (d, J=2 Hz), similar to that of **4** (H-14β, δ 5.05, d, J=2 Hz). Therefore, the location and orientation of the hydroxyl group were assigned to be 14α. The hydroxyl-bearing carbon (C-14) signal at δ 65.7 also showed good coincidence with that of **4** (δ 64.3).[1] Based on the molecular formula of $C_{22}H_{23}NO_5$ (FAB-MS) and the ^{1}H- and ^{13}C-NMR considerations, **14** was characterized as 14α-hydroxy-3,6-didemethylisotylocrebrine.

Table 3. Cytotoxic Activity of Alkaloids toward Cultured Cancer Cells[a] (GI_{50} : ng/ml)

Compound	PC-6	MCF-7	SW620	NUGC-3	P388
4	0.0416	0.0625	0.0848	0.126	<0.038
3	0.0767	0.297	0.24	0.337	<0.038
8	0.068	0.181	0.245	0.337	<0.038
13	0.273	1.03	0.778	0.869	0.17
11	23.2	38.3	74.5	44.7	5.76
5-FU	298	1170	2930	2200	62
CDDP	256	2760	1380	83.4	12.9
10	0.41	0.517	0.581	0.701	<0.191
7	4.22	5.77	7.18	8.02	1.56
1	6.83	10.1	13.0	19.4	3.42
2	43.5	55.9	158	104	13.5
12	166	170	341	351	53.9
5-FU	447	515	1790	1240	56.2
CDDP	315	1430	786	63.5	16.2

a) PC-6, human lung; MCF-7, breast; SW-620, colon; NUGC-3, gastric; P388, murine leukemia. 5-FU = 5-Fluorouracil. CDDP = Cisplatin.

Although cytotoxicity of phenanthroindolizidine alkaloids, including **2** and several homologues, has already been reported,[3] growth inhibition assay[4] was carried out on some of the alkaloids obtained in this study. The results are listed in Table 3.

A remarkable decrease of the activity was observed upon transformation of **4** and **10** into their *N*-oxides, **7** and **12**, respectively, although **2** and its *N*-oxide, **11** afforded similar values in the 13a-αH (*R*) series. The compounds with one free hydroxyl group in the phenanthrene frame-

work, such as **3**, **4** and **8**, showed higher potency than those having two (**13**) or no hydroxyl groups (**1**), while no marked difference was observed between 3-hydroxyl (**3**) and 6-hydroxyl (**8**) structures. A similar relation was observed in the alkaloids of the 13a-αH (*R*) series, with **10** showing higher potency than **2**. A comparison of **3** and **4** indicates that the introduction of the additional 14α-hydroxyl group in the indolizidine moiety is effective, though further study is needed on this point. Direct comparison of 13a-H isomers was not possible but the 13a-βH (*S*) isomers seem to be more potent than the 13a-αH (*R*) group.

Experimental

Melting points were taken on a hot stage and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were measured on JEOL GX-400 and JNM A500 spectrometers in $CDCl_3+CD_3OD$ unless otherwise mentioned. Chemical shifts are given in δ values referred to internal tetramethylsilane, and the following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, dd=doublet of doublets, br=broad. FAB-MS were recorded on a JEOL HX-110 spectrometer. Optical rotations were measured on a JASCO-DIP 360 polarimeter. The UV spectra were taken in MeOH on a Shimadzu 200S double-beam spectrometer. The following solvent systems were used, 1: $CHCl_3$–MeOH–H_2O (10:1:2—7:3:1.2, lower layer), 2: EtOAc–MeOH–H_2O (5:1:4—4:1:3, upper layer), 3: H_2O–CH_3CN. Spray reagent for TLC: Dragendorff's reagent.

Extraction and Isolation. Procedure for 11 and 12 Fresh leaves and caules of *Tylophora tanakae* (3.0 kg), collected in June, 1994, at Hateruma-jima, were percolated with MeOH. The MeOH percolate was concentrated *in vacuo* and extracted with benzene (ext. 1.39 g) and then $CHCl_3$ (0.53 g). The benzene and $CHCl_3$ fractions were separately subjected to successive silica gel column chromatography and preparative TLC with solvent systems 1 and 2. The following alkaloids were isolated: **1** (17 mg), **2** (10 mg), **3** (15 mg), **4** (18 mg), **5** (8 mg), **6** (7 mg), **7** (13 mg), **8** (19 mg), **9** (9 mg), **10** (63 mg).

After separation of **1**—**10**, the remaining fractions containing alkaloids were further purified and crystallized from MeOH to give **11** (16 mg) and **12** (12 mg).

Procedure for 13 and 14 Fresh leaves and caules of *T. tanakae* (7.2 kg), cultivated in the medicinal plant garden of Fukuoka University and harvested in September, 1995, were treated with the same procedure described above. After extraction with $CHCl_3$ (ext. 4.95 g), the aqueous layer was passed through an MCI-gel column. The column was eluted with H_2O, 25%, 50%, 75% and 100% MeOH, successively. From the 50 and 75% MeOH eluates (ext. 70 g), **4**, **13** and **14** were obtained after repeated chromatography with silica gel (solvents 1 and 2) and YMC-gel (solvent 3) columns: **4** (11 mg), **13** (117 mg), **14** (48 mg).

Tylophorine *N*-Oxide (**11**): Yellowish fine prisms, mp 225—235 °C (dec.), $[\alpha]_D^{29}$ −8.6° (*c*=0.22, $CHCl_3$–MeOH (1:1)), FAB-MS *m/z*: 410.1970 $[M+H]^+$. Calcd for $C_{24}H_{28}NO_5$: 410.1968. UV λ_{max} nm (log ε): 220 (4.39), 238 (4.49), 250 (sh, 4.65), 257 (4.85), 282 (sh, 4.44), 287 (4.48), 302 (4.17).

7-Demethyltylophorine *N*-oxide (**12**): Brownish fine prisms, mp 194—204 °C (dec.), $[\alpha]_D^{28}$ −30.9° (*c*=0.11, $CHCl_3$–MeOH (1:1)), FAB-MS *m/z*: 396.1803 $[M+H]^+$. Calcd for $C_{23}H_{26}NO_5$: 396.1811. UV λ_{max} nm (log ε): 220 (4.51), 237 (4.57), 250 (sh, 4.72), 257 (4.90), 282 (sh, 4.50), 288 (4.46), 302 (4.26).

3,6-Didemethylisotylocrebrine (**13**): Brownish needles, mp 233—238 °C (dec.), $[\alpha]_D^{32}$ +102.7° (*c*=0.17, $CHCl_3$–MeOH (1:2)), FAB-MS *m/z*: 367.1781 $[M+2H]^+$. Calcd for $C_{22}H_{25}NO_4$: 367.1764. UV λ_{max} nm (log ε): 261 (4.44), 280 (4.15), 286 (4.14), 305 (sh, 3.75), 316 (3.75).

14α-Hydroxy-3,6-didemethylisotylocrebrine (**14**): An amorphous powder, $[\alpha]_D^{29}$ +17.4° (*c*=0.27, MeOH), FAB-MS (NG) *m/z*: 380.1493 $[M-H]^-$. Calcd for $C_{22}H_{22}NO_5$: 380.1498. UV λ_{max} nm (log ε): 241 (4.30), 260 (4.38), 280 (4.21), 285 (sh, 4.18), 304 (3.89), 316 (3.87).

Cytotoxicity Assay of the Alkaloids Cellular growth of PC-9, MCF-7, SW620, NUGC-3 and P388 in the presence or absence of the samples was determined according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay procedure.[4] The activity of each sample was represented as GI_{50} (ng/ml) (Table 3).

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