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Investigation of Uña De Gato I. 7-Deoxyloganic acid and ^{15}N NMR spectroscopic studies on pentacyclic oxindole alkaloids from *Uncaria tomentosa*☆

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Abstract

The C-8-(*S*) isomer of deoxyloganic acid (7-deoxyloganic acid), together with β-sitosteryl glucoside, five known stereoisomeric pentacyclic oxindole alkaloids and the tetracyclic oxindole isorhyncophylline, were isolated from the inner bark of *Uncaria tomentosa*. Structures of the isolated compounds were based on ^{1}H and ^{13}C NMR data, mainly 2D NMR experiments, including ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY correlation. Furthermore, the hitherto unreported ^{15}N chemical shifts of the isomeric oxindole alkaloids, using ^{1}H–^{15}N HMBC experiments, were utilized to facilitate their characterization. Uncarine D showed weak cytotoxic activity against SK-MEL, KB, BT-549 and SK-OV-3 cell lines with IC_{50} values between 30 and 40 μg/ml, while uncarine C exhibited weak cytotoxicity only against ovarian carcinoma (IC_{50} at 37 μg/ml).

Keywords: *Uncaria tomentosa*; Rubiaceae; Cat's claw; Alkaloids; Oxindoles; ^{15}N NMR; Iridoid glucoside; 7-Deoxyloganic acid; β-Sitosteryl glucoside; Cytotoxic activity

1. Introduction

Uncaria tomentosa (Wild.) DC. (Uña de Gato; Cat's Claw) is considered one of the most valuable botanicals in the rainforest and is widely used in traditional medicine by native people of Peru for numerous ailments (Jones, 1995). This plant displays a diverse range of bioactive secondary metabolites, including tetracyclic and pentacyclic oxindole alkaloids, triterpenes, glycosides, flavonoids and procyanidins (Aquino et al., 1990, 1991, 1997; Jones, 1995; Van Ginkel, 1996; Wirth and Wagner, 1997). The pentacyclic oxindole alkaloids are considered to be biochemical markers of cat's claw and are responsible, in part, for immunomodulatory, cytotoxic, anti-AIDS and anti-amyloidosis (Alzheimer's disease) activities (Wagner et al., 1985; Keplinger et al., 1986; Keplinger and Keplinger, 1994; Jones, 1995; Lemaire et al., 1999; Castillo and Snow, 2000). To date, nine natural heteroyohimbine-type pentacyclic oxindole isomers have been isolated from *Uncaria* species and six of them are restricted to *U. tomentosa* inner bark (Shamma et al., 1967; Seki et al., 1993).

As part of our continuing program to isolate marker compounds from traditional medicines and evaluate their biological activity, the present study deals with the isolation and characterization of a new constituent, 7-deoxyloganic acid (**1**) from the inner bark of *U. tomentosa*. In addition, the ^{15}N NMR spectral assignments of the stereoisomeric oxindoles (uncarines C, D and E, mitraphylline and isomitraphylline) is described and cytotoxicity of the isolated compounds evaluated.

2. Results and discussion

Column chromatography of the ethanolic extract, using 1–5% MeOH in $CHCl_3$ mixture as eluent (see

☆ For part II, see Ganzera et al. (2001).

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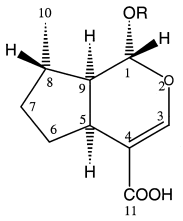
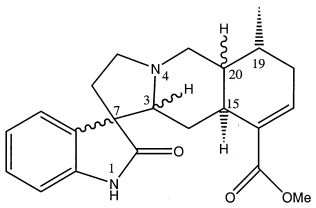


1 R=Glc

2 R=Glc(Ac)$_4$



Uncarine C (**3**) 3*S*, 7*R*, 15*S*, 19*S*, 20*S*
Uncarine D (**4**) 3*R*, 7*S*, 15*S*, 19*S*, 20*S*
Uncarine E (**5**) 3*S*, 7*S*, 15*S*, 19*S*, 20*S*
Mitraphylline (**6**) 3*S*, 7*R*, 15*S*, 19*S*, 20*R*
Isomitraphylline (**7**) 3*S*, 7*S*, 15*S*, 19*S*, 20*R*

Experimental), resulted in the isolation of five known stereoisomeric oxindoles [namely, uncarines C, D and E, mitraphylline and isomitraphylline (**3**–**7**) (Shamma et al., 1967; Seki et al., 1993)], the tetracyclic oxindole isorhyncophylline (**8**) (Phillipson and Hemingway, 1973; Yu et al., 1989), as well as 7-deoxyloganic acid (**1**).

Compound **1**, $C_{16}H_{24}O_9$, was obtained as colorless amorphous granules: mp. 94–96°; $[\alpha]_D^{27}$ −81.9°; which exhibited the presence of carboxylic acid (ν_{max} 1680 cm^{-1}; δ_C 170.1) and β-D-glucose [$\delta_{C\text{-}1'}$ 99.1, $\delta_{C\text{-}2'}$ 73.8, $\delta_{C\text{-}3'}$ 77.0, $\delta_{C\text{-}4'}$ 70.6, $\delta_{C\text{-}5'}$ 77.3, $\delta_{C\text{-}6'}$ 61.9; δ 4.65, 1H, *d* (J=7.9 Hz, H-1′) (Murai et al., 1984)] substituents. The ^{13}C NMR spectrum of **1** (Table 1) was found to be similar to those reported for 8-(*S*) isomer of 7-deoxyloganic acid tetraacetate (**2**) (Tagawa and Murai, 1983), save for the differences associated with the presence of the β-D-glucose moiety at C-1. Furthermore, a close comparison of the ^{13}C NMR spectrum of **1** with those of other deoxyloganic acid isomers, such as 8-*epi*deoxyloganic acid [8-(*R*) isomer of 7-deoxyloganic acid], 1,5,9-*epi*deoxyloganic acid and 5-*epi*deoxyloganic acid, isolated from *Nepeta cataria* (Tagawa and Murai, 1983; Murai et al., 1984), led to the conclusion that indeed **1** was the C-8-(*S*)-isomer of deoxyloganic acid, which has not been reported previously as a natural product. Thus, the structure and stereochemistry of **1** was unambiguously established by detailed 2D NMR spectroscopic studies, including ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY experiments. The ^{1}H–^{13}C HMBC experiment on **1** showed the key three-bond correlation between $\delta_{C\text{-}1'}$ 99.2 and δ 5.17 (H-1); $\delta_{C\text{-}11}$ 170.1 and δ 7.40 (H-3); $\delta_{C\text{-}9}$ 48.2 and δ 1.05 (H-10), confirming the placement of COOH, β-D-glucose and methyl substituents at C-1, C-4 and C-8, respectively.

The stereochemical assignment at the carbon centers C-1, C-5, C-8 and C-9 was inferred from 2D NMR ^{1}H–^{1}H NOESY experiments, which showed correlations between H-5 (δ 2.85), H-9 (δ 1.70) and H-10 (δ 1.05), indicating that they are *cis* (α-face) to each other. As a result, fusion between the five and six-member rings was also *cis* in **1**. Furthermore, the NOESY showed a *cis* (β-face) relationship between the protons H-1 (δ 5.17) and H-8 (δ 1.95), thereby confirming the assignment of β-D-glucose moiety at the α-face of the molecule. Compound **2** was previously reported as a catalytic hydrogenation product of asperuloside tetraacetate and its absolute configuration at C-8 was established as (*S*) (Inouye and Arai, 1964; Murai and Tagawa, 1980). Based on foregoing data compound **1** was established as the C-8 (*S*)-isomer of deoxyloganic acid.

The initial identity and purity of the isolated five stereoisomeric pentacyclic oxindoles (**3**–**7**) and the tetracyclic isorhyncophylline (**8**) were evaluated by HPLC (Ganzera et al., 2001), as well as by their optical purity ($[\alpha]_D$ values). All the oxindoles were found to be 99-99.5% pure by HPLC, except for isomitraphylline, which could only be purified up to 84.5%. The structures and relative stereochemistries of **3**–**7** were determined rigorously using high field 2D NMR spectroscopic studies, including ^{1}H–^{1}H NOESY and ^{1}H–^{13}C HMBC experiments and were found to be in agreement with those reported by Seki et al. (1993). In addition, the absolute stereochemistry of uncarine C (**3**) and uncarine E (**5**) were determined by single crystal X-ray crystallography,[1] which will be reported elsewhere. The pentacyclic oxindole isomers (**3**–**7**) are difficult and tedious to distinguish by simply comparing the ^{1}H or ^{13}C NMR spectral data. Therefore, the 2D ^{1}H–^{15}N NMR HMBC spectra were acquired to obtain ^{15}N chemical shifts that help to distinguish between most of the isomers (Table 2). The chemical shift of N-1 ranges from δ 136.9 to δ 135.1 and N-4 ranges from δ 54.6 to δ 64.8. The *allo*-type isomers (3*S*, 20*S*) uncarine C (**3**) and E (**5**) exhibited a drastic chemical shift differences at N-4 nitrogen (8-10 ppm) compared to those observed for *epiallo*-type (3*R*, 20*S*) uncarine D (**4**) and *normal*-type (3*S*, 20*R*) mitraphylline (**6**) and isomitraphylline (**7**). Although, the ^{15}N chemical shift differences between **4** and **6** are negligible, they can be very conveniently distinguished by their $[\alpha]_D^{27}$ values (+91° for **4** vs −8.6° for **6**). The ^{1}H–^{15}N HMBC experiment on uncarine C (**3**) demonstrated the correlation between the signals at δ_N 136.85 (N-1), $\delta_{N\text{–}H}$ 9.07 and $\delta_{H\text{-}12}$ 6.85; and δ_N 56.9 (N-4) and $\delta_{H\text{-}5}$ 1.95, $\delta_{H\text{-}6,14}$ 1.69, $\delta_{H\text{-}20}$ 1.53 and $\delta_{H\text{-}21}$ 3.29. All the correlated protons from ^{1}H–^{15}N HMBC data of five isomers studied (Table 2) were in good agreement with their original ^{1}H NMR spectroscopic assignments. Therefore, the ^{15}N chemical shifts and specific rotation values can be used as a valuable tool to distinguish quickly and conveniently all the five stereoisomers **3**–**7**. To our knowledge, this is the first report of ^{15}N NMR chemical shifts for *Uncaria* oxindole alkaloids.

All six oxindole alkaloids, as well as compound **1** and β-sitosteryl glucoside, were evaluated for cytotoxic activ-

[1] Fronczek, F.R., Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803-1804, USA, pers. commun.

ity, using a microplate assay (Dou et al., 1996) against human cancer cell lines of malignant melanoma (SK-MEL), epidermoid carcinoma (KB), ductal carcinoma (BT-549) and ovarian carcinoma (SK-OV-3). The *epiallo*-type C-3(*R*)-isomer uncarine D (**3**) showed weak cytotoxic activity against all the cell lines with IC_{50} values at 30, 35, 34 and 30 μg/ml, respectively, while *allo*-type C-3(*S*-)-epimer uncarine C demonstrated weak cytotoxicitiy only towards SK-OV-3 at 37 μg/ml (Table 3). The other three isomers, *allo*-type uncarine E, *normal*-type mitraphylline and isomitraphylline as well as isorhyncophylline, compound **1** and β-sitosteryl glucoside were found to be inactive. Interestingly, uncarine C and uncarine E had previously been reported from *U. guianensis* to possess weak but selective DNA damaging activities (Lee et al., 1999).

Table 1
^1H and ^{13}C NMR chemical shift values for compound **1**[a]

Proton/carbon	^1H	NOESY	^{13}C
1	5.17 *d* (5.7)[b]	H-8 β	96.9 *d*[c]
2	–	–	–
3	7.40 *d* (1.0)	–	151.8 *d*
4	–	–	112.0 *s*
5	2.85 *dd br* (7.6, 7.3)	H-9 α H-10 α	34.3 *d*
6	2.16 *m* ; 1.38 *m*	–	32.4 *t*
7	1.84 *m*; 1.16 *m*	–	33.2 *t*
8	1.95 *m*	H-1 β	35.6 *d*
9	1.70 *dd br* (6.2, 12.5)	H-5 α H-10 α	48.2 *d*
10	1.05 *d* (6.7)	H-5 α H-9 α	19.9 *q*
11	–	–	170.1 *s*
1′	4.65 *d* (7.9)	–	99.2 *d*
2′	3.29 *m*	–	73.8 *d*
3′	3.37 *t* (8.8), 3.28 *m*	–	77.0 *d*
4′	3.28 *m*	–	70.6 *d*
5′	3.29 *m*	–	77.3 *d*
6′	3.64 *m* 3.86 *dd* (1.6, 13.2)	–	61.9 *t*

[a] Spectra recorded in CD_3OD at 500 MHz (^1H) and 125 MHz (^{13}C). Assignments were aided by 2D NMR COSY, HMQC and HMBC experiments.
[b] Values in parentheses are coupling constants, *J* values in Hz.
[c] Multiplicities of carbon signals were determined by DEPT experiments.

3. Experimental

3.1. General

Mp uncorr.; NMR: were acquired on a Brüker Avance DRX-500 instrument at 500 MHz (^1H), 125 MHz (^{13}C) and 50 MHz (^{15}N) in $CDCl_3$, unless otherwise stated, using the residual solvent as int. standard; Multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC and NOESY) were obtained using standard Bruker pulse programs; ^1H–^{15}N HMBC spectra were calibrated using nitromethane as an external standard and setting the chemical shift to 380.2 resulting in ^{15}N chemical shifts are reported relative to liquid ammonia; HRMS: were obtained by direct injection using a Bruker Bioapex-FTMS with Electro-

Table 2
^1H–^{15}N NMR HMBC correlations and optical rotations of oxindoles **3**–**7**

Oxindoles	^{15}N (δ_N)		HMBC correlations with ^1H NMR (δ_H)								$[\alpha]_D^{27}$[a]
			N_1H[b]	H-3	H-5	H-6	H-12	H-14	H-20	H-21	
Uncarine C (**3**)	N-1	136.9	9.07	–	–	–	6.85	–	–	–	−106° (*c*, 0.2)
	N-4	56.9	–	–	1.95[d]	1.69[c]	–	1.69[c]	1.53[c]	3.29[d]	
Uncarine D (**4**)	N-1	135.1	9.31	–	–	–	6.85	–	–	–	+91° (*c*, 0.024)
	N-4	64.8	–	2.15[d]	–	1.99[d]	–	1.58[c]	–	–	
Uncarine E (**5**)	N-1	136.7	9.04	–	–	–	–	–	–	–	−80.5° (*c*, 0.554)
	N-4	54.6	–	–	3.18[d]	2.35[d]	–	–	1.60[c]	3.25[d]	
Mitraphylline (**6**)	N-1	135.1	8.41	–	–	–	6.85	–	–	–	−8.6° (*c*, 0.554)
	N-4	64.7	–	2.36[c]	3.36[d]	2.04[c]	–	2.36[c]	–	–	
Isomitraphylline (**7**)	N-1	135.8	9.02	–	–	–	6.85	–	–	–	+20° (*c*, 0.012)
	N-4	62.8	–	–	3.28[d]	1.97[c]	–	–	–	1.90[c]	

[a] All optical rotation values were measured in $CHCl_3$ at 27°C.
[b] One-bond N–H correlation.
[c] Two or three bond; α-protons.
[d] Two or three bond; β-protons.

Table 3
Cytotoxic activity of oxindole alkaloids[a]

Compounds	IC_{50} (μg/ml)				
	SK-MEL	KB	BT-549	SK-OV-3	VERO
Uncarine C (**3**)	> 50	> 50	> 50	37	> 50
Uncarine D (**4**)	30	35	34	30	39
Uncarine E (**5**)	> 50	–	–	> 50	–
DOX	< 1.1	1.7	2.0	1.9	> 10
5-FU	6.3	> 10	–	> 10	> 10

[a] SK-MEL, human malignant melanoma; KB, human epidermoid carcinoma; BT-549, human ductal carcinoma; SK-OV-3, human ovary carcinoma; VERO, (kidney, African green monkey) used as normal cell line; – inactive; DOX, doxorubicin; 5-FU, 5-fluorouracil.

Spray Ionization (ESI); Optical rotation measurements were taken on a JASCO DIP-370 digital polarimeter at 27°C; HPLC: was performed using a Waters 2690 alliance separation module with 996 PDA photodiode detector and a 3 μm Luna C-18 (Phenomenex) column; TLC: silica gel 60 F254 plates; solvent: hexane–EtOAc (4:6) and MeOH–$CHCl_3$(5:95); CC: flash-silica gel G (J.T. Baker, 40 μm); centrifugal preparative TLC (CPTLC, using Chromatotron®, Harrison Research Inc. Model 8924): 2 mm silica gel GF Chromatotron™ rotors (Analtech, Inc.), at a N_2 flow rate of 4 ml/min. The isolated compounds were visualized by observing under UV at 254 nm, followed by development in an iodine chamber or spraying with Dragendorff's reagent (Sigma Chemical Co.) and spraying with anisaldehyde–H_2SO_4 spray reagent.

3.2. Plant material

The inner stem bark of *Uncaria tomentosa* (Fam. Rubiaceae) was collected in April 2000 near Lima, Peru. A voucher specimen was deposited at the herbarium of University of Mississippi.

3.3. Extraction and isolation

The powdered inner stem bark of *U. tomentosa* (2.5 kg) was extracted by percolation with EtOH (3 × 2L). The combined extract was evaporated under reduced pressure and then freeze dried (yield 223 g). A portion of the crude extract (150g) was subjected to flash-CC over silica gel G (40 μm, 4.5 kg). Using 1% MeOH–$CHCl_3$ as solvent yielded uncarine E (**5**; needles, 500 mg), followed by mixture A, that was separated by a short flash-CC, using 25% EtOAc in *n*-hexane to afford uncarine C (**3**, plates, 225 mg) and isomitraphylline (**7**, amorphous powder, 180 mg). Further elution of the CC with 3–5% MeOH–$CHCl_3$ yielded mitraphylline (**6**, granules, 500 mg), followed by mixture B, which was purified by CPTLC (2 mm disc; solvent: 3% MeOH–$CHCl_3$) to give uncarine D (**4**, needles, 80 mg), followed by isorhyncophylline (**8**, needles, 100 mg; mp 138°, $[\alpha]_D^{27}$ +15°, lit.[2] mp 144°, $[\alpha]_D^{27}$ +8.3 ↔ +13°). Finally, elution with 6% MeOH in $CHCl_3$ yielded mixture C, which was separated and purified by CPTLC using 5% MeOH–$CHCl_3$ as eluent in a 2 mm disc to give **1** (80mg, R_f 0.30, solvent: 5% MeOH–$CHCl_3$) and β-sitosteryl glucoside (150 mg, R_f 0.25). The identity of β-sitosteryl glucoside was confirmed by the direct comparison with an authentic sample. The purity of the oxindoles was evaluated by an in-house HPLC method. The physical[2] and spectral data (Phillipson and Hemingway, 1975; Seki et al., 1993) of the oxindoles **3**–**7** were in agreement with those reported in literature.

3.4. 7-Deoxyloganic acid (*1*)

Obtained as colorless amorphous granules from $CHCl_3$, mp 94–96°; $[\alpha]_D^{27}$ −81.9° (MeOH, *c* 1.0); UV λ_{max}^{EtoH} nm (log ε): 220 sh (3.60), 238 (3.85) and 262 (3.88); IR ν_{max}^{KBr} cm^{-1}: 3357 (OH), 2952, 2870, 1680(COOH), 1630, 1548, 1071; ^1H and ^{13}C NMR: Table 1; HRMS (*m*/*z*): 361.1485 ($[MH]^+$, $C_{16}H_{24}O_9$.H); calculated for 361.1493.

3.5. Cytotoxicity assay

The in vitro cytotoxic activity was determined against four human cancer cell lines, SK-MEL, KB, BT-549, and SK-OV-3 (Table 3), obtained from American Type Culture Collection (ATCC, Rockville, MD). A primary assay for the oxindoles was conducted at a single concentration (100 μg/ml), followed by a secondary assay at three to six concentrations (between 50 and 1.56 μg/ml), using a culture-treated 96-well microplate (Dou et al., 1996). The level of toxicity of each sample was also determined by measuring their effect on fibroblast cell line from African green monkey kidney (VERO; non-transformed). The assay is based on the accumulation of neutral red dye in the lysosomes of viable cells. A subsequent addition of 2-propanol will lyse the cells releasing the dye into solution and the absorbance is measured at 490 and 630 nm. Corresponding growth inhibition was calculated and graphed. For secondary assays, IC_{50}s were determined from logarithmic graphs of growth inhibition values. The cytotoxic agents doxorubicin and 5-fluorouracil were used as positive controls, while the DMSO vehicle was used as negative control.

[2] The literature physical data (melting point and optical rotation values) were obtained from the Dictionary of Natural Products on CD ROM. 1999. Chapman and Hall, CR Cnet Base, Version 8:1, Chapman and Hall Numbers: CCG99-V, CCH03-T, CCH06-W, CGL89-T, CGL93-Q and CHF57-L.

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