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The Alkaloids of an *Uncaria rhynchophylla* (*Rubiaceae-Coptosapelteae*)

By

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With 8 Figures

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Key words: *Rubiaceae*, *Cinchonoideae*, *Coptosapelteae*, *Uncaria rhynchophylla*. – Indole alkaloids, oxindole alkaloids, phytochemistry. – Seedling morphology.

Summary

LAUS G. & TEPPNER H. 1996. The alkaloids of an *Uncaria rhynchophylla* (*Rubiaceae-Coptosapelteae*). – *Phyton* (Horn, Austria) 36(2): 185–196, 8 figures. – English with German summary.

Indole and oxindole alkaloids in the different parts of an *Uncaria rhynchophylla* plant were determined quantitatively by HPLC. They were identified spectroscopically and by TLC. The stereochemical relations were established by chemical transformations. Leaves, lateral branches and upper part of the stem show presence of the oxindole alkaloids isorhynchophylline and rhynchophylline as well as the corresponding unsaturated oxindole alkaloids isocorynoxine and corynoxine. On the other hand, in the roots almost only indole alkaloids (hirsutine, hirsutine and others) were present. The bark of the stem contains both, oxindole and indole alkaloids. The cotyledons are epigeal, the interpetiolar stipules of the lowermost nodes of the seedlings are entire.

Zusammenfassung

LAUS G. & TEPPNER H. 1996 Die Alkaloide einer *Uncaria rhynchophylla* (*Rubiaceae-Coptosapelteae*). – *Phyton* (Horn, Austria) 36(2): 185–196, 8 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Indol- und Oxindol-Alkaloide in den verschiedenen Teilen einer *Uncaria rhynchophylla*-Pflanze wurden mit HPLC quantifiziert. Die Identifizierung erfolgte

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spektroskopisch und mit DC. Die stereochemischen Zusammenhänge wurden durch chemische Umwandlungen geklärt. Blätter, Seitenzweige und oberer Teil des Stammes enthalten die Oxindolalkaloide Isorhynchophyllin und Rhynchophyllin sowie die entsprechenden ungesättigten Oxindolalkaloide Isocorynoxein und Corynoxein. Die Wurzeln enthalten fast ausschließlich Indolalkaloide (Hirsutein, Hirsutin und andere). Die Stamm-Rinde enthält Oxindol- und Indolalkaloide. Die Keimblätter sind epigäisch, die Interpetiolarstipeln an den untersten Knoten der Sämlinge sind ungeteilt.

Introduction

In the course of our studies on American *Uncaria* species (*U. guianensis*, *U. tomentosa*, TEPPNER & al. 1984, LAUS & KEPLINGER 1994) we decided to investigate also other species for comparison. In spite of a recent monograph about this genus (RIDSDALE 1978) the infrageneric classification does not seem to be completely satisfactory. Thus, *U. tomentosa*, *U. rhynchophylla* and *U. macrophylla* exhibit several common botanical features (RIDSDALE 1972 and own observations), although they are assigned to three different groups (PHILLIPSON & al. 1978: 565). While there are many reports on the alkaloid contents of *Uncaria* species including *U. rhynchophylla* (especially YAMANAKA & al. 1983), some contradictory results have been also published (PHILLIPSON & al. 1974; SAKAI 1976). As a few individuals of *U. rhynchophylla* were successfully grown in our greenhouse we attempted to solve these questions. It seemed to be of interest to investigate the alkaloid profile quantitatively in the different parts of a whole plant.

Until recently *Uncaria* has been treated within the tribe *Cinchoneae* of *Rubiaceae-Cinchonoideae*, but according to the revision of *Cinchoneae* proposed by ANDERSSON & PERSSON 1991 which was accepted by ROBBRECHT 1993: 174–175 today the genus is assigned to the *Coptosapel-teae*.

The distribution area of *U. rhynchophylla* includes SE.China and S.Japan (RIDSDALE 1978:94, OHWI 1965:823).

Material and Methods

Origin of seeds: *Uncaria rhynchophylla* (MIQ.) HAVIL., Hiroshima Botanical Garden, Kurashige, Itsukaichi, Hiroshima, Japan. Seed list 1984 No 129.

Seeds sown in soil March 17, 1986; begin of germination March 29, 1986 (Fig. 1); only one plant survived up to 1989 reaching a height of 46 cm. For further experiments cuttings from one in-vitro plant were used. Some of them were transferred to pots (soil) in March 1991 and 1992 and they reached heights of c. 2 m up to November 1993. One plant was used for analysis, but with its 'sisters' we had many problems in cultivation: the branches died or the plants died altogether. At last in spring 1996 one plant rejuvenated very quickly (Fig. 4) and attained a height of 5.4 m in August 1996.

In vitro cultivation: MURASHIGE & SKOOG minimal organics medium. Sown March 17, 1986, germination April 1986; transferred in test-tubes May 2, 1986; five plants were grown up to 1987 (Fig. 5). Because of the unexpected long time of inaccessibility of the laboratory all plants died except one (No 81/10). This one plant was propagated in-vitro by cuttings and they were then planted in pots with soil and transferred to the greenhouse of the Botanical Garden in Graz. The plant used for the chemical investigations was planted as an in-vitro cutting on November 11, 1991 (sixth passage of cuttings), it was potted in soil on January 14, 1992 and put in the greenhouse on March 3, 1992 for further growth.

Material used: The whole plant was harvested on September 9, 1993. Two lateral branches were deposited as herbarium specimens. The length of the stem was 188 cm. The plant was divided as follows and air-dried:

	Dry weight (g)
A) lower 60 cm of stem, bark	9.6
B) lower 60 cm of stem, wood	29.5
C) upper part of stem, bark + wood	34.1
D) 17 lateral branches incl. hooks, 4.5–57 cm long	18.9
E) leaves from stem and lateral branches	46.8
F) main root, ca. 27 cm, 6.5–5.5 mm diameter, bark	2.1
G) main root, ca. 27 cm, 6.5–5.5 mm diameter, central part	0.7
H) whole root system (without F and G)	21.5

Extraction: The weighed and coarsely powdered plant material (1.1–1.5 g) was macerated five times for 30 minutes using 10 ml portions of a mixture of methanol, water and concentrated hydrochloric acid (500:500:1) at room temperature. The combined extracts were neutralized with 2 M sodium hydroxide solution, and phosphate buffer pH 7 was added to a final volume of 100 ml. Wood samples (0.4 g) were extracted in an analogous manner using 3 ml portions and filled up to 20 ml. The solutions were filtered through a 0.45 µm hydrophilic filter (Sartorius) and used directly for TLC and HPLC analyses.

For the isolation of the indole alkaloids on a preparative scale the root (sample H, 15.47 g) was finely milled and macerated five times for 30 minutes using 200 ml portions of the above solvent mixture. After distilling off the methanol the resulting aqueous solution was adjusted to pH 2 by the addition of hydrochloric acid and defatted with *t*-butyl methylether. The aqueous phase was neutralized with sodium hydroxide solution and extracted three times with chloroform. The combined organic layers were filtered through hydrophobic filters (Macherey & Nagel), dried over anhydrous sodium sulfate and evaporated. The residue (70 mg) was subjected to silica gel column chromatography. Ethyl acetate eluted 2 fractions (corynantheine and dihydrocorynantheine), then ethyl acetate – methanol (9:1) eluted another 2 fractions (hirsuteine and hirsutine). The pure fractions were combined as indicated by HPLC, evaporated, redissolved in dichloromethane, filtered and extracted with 0.1 M hydrochloric acid. These extracts were neutralized and extracted again with dichloromethane, the organic layers dried and evaporated. Yields: 3 mg corynantheine, 2 mg dihydrocorynantheine, 21 mg hirsuteine and 26 mg hirsutine.

From the stem bark (Sample A, 7.38 g) akuammigine was isolated together with isocorynoxine and isorhynchophylline in an analogous manner. Akuammigine was

identified by comparison with an authentic sample in several TLC and HPLC systems. Owing to the low yield (<1 mg) no further purification was attempted.

Likewise, the oxindole alkaloids were isolated from the leaves (Sample E, 20 g). Again, the column was eluted by ethyl acetate (isocorynoxine and isorhynchophylline) followed by ethyl acetate – methanol (corynoxine and rhynchophylline). Yields: 4 mg isocorynoxine, 5 mg isorhynchophylline, 10 mg corynoxine and 15 mg rhynchophylline.

Thin Layer Chromatography (TLC): 5 μ l samples were dispensed as spots on TLC sheets (silica gel 60 F₂₅₄, thickness of layer 0.2 mm, 20 \times 20 cm, Merck). A mixture of ethyl acetate, 2-propanol and concentrated ammonia (100:2:1) was used as the mobile phase. The spots were detected (a) under ultraviolet light (254 and 366 nm), (b) by spraying with Ehrlich's reagent (1% 4-dimethylaminobenzaldehyde in ethanol) and hydrogen chloride vapors, and (c) by painting with 0.2 M ferric chloride solution in 35% perchloric acid and subsequent heating. The hR_F values of the pure alkaloids were correlated with retention times t_R obtained by HPLC. A summary of hR_F values of indole and oxindole alkaloids was published by PHILLIPSON & HEMINGWAY 1975.

High Performance Liquid Chromatography (HPLC): Merck-Hitachi pump L-6200 equipped with a UV-VIS-detector L-4250 and a column thermostat. Column Merck LiChrospher RP-18 (5 μ m) 125 \times 4 mm (I.D.), injected volume 200 μ l, wavelength 247 nm, flow 1.3 ml/min. Method A: acetonitril – 0.01 M phosphate buffer pH 7 (40:60) as eluent at 52 $^{\circ}$ C. Method B: acetonitril – 0.01 M phosphate buffer pH 7 (45:55) as eluent at 80 $^{\circ}$ C. The alkaloids were numbered according to the sequence of elution. Calibration was performed using ajmalicine (Fluka) for indole alkaloids and crystallized pteropodine (from *U. tomentosa*) for oxindole alkaloids, applying corrections for the actual molecular weights of the isolated compounds.

Mass Spectrometry (MS): Varian CH7. Electron impact ionization (70 eV). Ion source temperature 200–250 $^{\circ}$ C. MS data of the alkaloids were summarized by PHILLIPSON & HEMINGWAY 1975.

Nuclear Magnetic Resonance Spectroscopy (NMR): Varian 200 MHz (CDCl₃). Especially the signal of H-3 was of significant diagnostic value, since its chemical shift and coupling pattern allowed the assignment of the 3-epimers hirsuteine **5** (H-3_{equatorial}, δ = 4.54 ppm, J = 4 and 2 Hz) and corynantheine **8** (H-3_{axial}, δ = 3.32 ppm, J = 10 and 2 Hz). These values are in accordance with those given in the literature for the pentacyclic analogues 3-isoajmalicine and ajmalicine (LOUNASMAA & KAN 1980).

Isomerization of Oxindole Alkaloids: Equilibrating aqueous solutions of isocorynoxine **1** and isorhynchophylline **3** gave pH dependent mixtures with corynoxine **2** and rhynchophylline **4** (Table 1). Since the course of this isomerization is known (SEATON & al. 1960), the assignment of the C-7 epimers is possible.

Isomerization of Indole Alkaloids: Oxidation of corynantheine **8** and dihydrocorynantheine **9** by mercury(II)acetate and subsequent reduction by zinc yielded the C-3 epimers (WENKERT & ROYCHAUDHURI 1958). They were identical with the alkaloids **5** and **7**, possessing the same C-15 and C-20 configuration as corynantheine and dihydrocorynantheine. Hence, it was concluded that compounds **5** and **7** had to be hirsuteine and hirsutine, respectively.

Conversion of Indole to Oxindole Alkaloids: Oxidation by t-butyl hypochlorite and subsequent acid hydrolysis (ZINNES & SHAVEL 1966) converted

Table 1

Dependance of Isomerization Equilibrium of the Oxindole Alkaloids on pH in Water at 50 °C.

pH	4	5	6	7	8
Isocorynoxetine 1	25%	25%	31%	61%	68%
Corynoxetine 2	75%	75%	69%	39%	32%
Isorhynchophylline 3	23%	23%	27%	48%	66%
Rhynchophylline 4	77%	77%	73%	52%	34%

hirsuteine **5** to the corynoxetine isomers **1,2** and hirsutine **7** to the rhynchophylline isomers **3,4**. Thus, the stereochemical relationship was established.

Hydrogenation of Vinyl Substituted Alkaloids: The vinyl substituted alkaloids **1, 2, 5** and **8** were converted to the corresponding ethyl analogues by catalytic hydrogenation (AN Cu & al. 1957). The products were identical by HPLC with the alkaloids **3, 4, 7** and **9**, respectively, which showed that the corresponding pairs had the same configuration. Hydrogenation was performed in methanol – phosphate buffer pH 7 (30:70) solution in the presence of palladium coal at room temperature and atmospheric pressure. The starting material disappeared after 5–10 minutes to yield the products, which were further hydrogenated on prolonged reaction time.

Results and Discussion

Seedling Morphology

Germination of seeds took place between c. 12 and 30 days after sowing, at c. 25 °C day temperature. The cotyledons are epigeal. Fully grown cotyledons (Fig. 1) are late ovate and measure c. 1.5 × 1.3 mm (without petioles). After about half a year the plants in soil had ± seven nodes with leaves (the cotyledonar node included) below the apical bud (Fig. 2). At the first nodes with leaves the interpetiolar stipules are entire, but from the sixth node on, the stipules are bipartite (Fig. 3). The seedlings in the test-tubes reached heights of 1–8 cm and 5–10 nodes within c. 10 months (Fig. 5).

Phytochemistry

Since the elution behaviour of the indole and oxindole alkaloid groups was too different, two methods had to be developed in order to achieve separation of all alkaloids. The assignment to one of these groups was made on the basis of fluorescence and colour reactions in TLC (Table 2) and the ultraviolet spectra (λ_{\max} = 330 nm for indoles; 310 and 350 nm for oxindoles) recorded during HPLC. Retention times are summarized in Table 3. The isolated compounds were characterized by MS (Table 4) and,

Table 2

hR_F Values and Detection of the Alkaloids of *Uncaria rhynchophylla* on Silica Gel. *)

Alkaloid	hR _F	366 nm fluorescence	FeCl ₃ / HClO ₄	Ehrlich's reagent
Isocorynoxetine 1	64	–	pink	–
Corynoxetine 2	33	–	pink	–
Isorhynchophylline 3	63	–	pink	–
Rhynchophylline 4	29	–	pink	–
Hirsuteine 5	24	yellow	grey	purple
Akuammigine 6	52	yellow	grey	pink
Hirsutine 7	18	yellow	grey	purple
Corynantheine 8	71	yellow	grey	purple
Dihydrocorynantheine 9	70	yellow	grey	purple

*) Mobile phase: ethyl acetate, 2-propanol and conc. ammonia (100:2:1). All alkaloids show fluorescence quenching at 254 nm.

Table 3

Retention Times t_R of the Alkaloids on Reversed Phase HPLC.

Alkaloid	t _R – Method A (min)	t _R – Method B (min)
Isocorynoxetine 1	5.9	3.3
Corynoxetine 2	6.2	3.3
Isorhynchophylline 3	7.2	4.0
Rhynchophylline 4	8.0	4.1
Hirsuteine 5	10.8	4.7
Akuammigine 6	ca. 10.9	5.0
Hirsutine 7	13.6	5.8
Corynantheine 8	15.9	6.3
Dihydrocorynantheine 9	19.5	7.8

Table 4

Mass Spectra of the Alkaloids of *Uncaria rhynchophylla*.

Alkaloid	M ⁺ (%)	m/e (%)
Isocorynoxetine 1	382 (100)	367 (12), 351 (18), 222 (46), 108 (70)
Corynoxetine 2	382 (100)	367 (14), 351 (16), 222 (32), 108 (58)
Isorhynchophylline 3	384 (100)	369 (5), 367 (4), 355 (5), 353 (1), 224 (14)
Rhynchophylline 4	384 (100)	369 (3), 367 (2), 355 (3), 353 (4), 224 (19)
Hirsuteine 5	366 (100)	365 (43), 351 (71), 237 (13), 223 (15), 184 (63), 170 (45), 169 (34), 156 (58)
Akuammigine 6	368 *)	
Hirsutine 7	368 (82)	367 (47), 353 (100), 239 (8), 225 (17), 184 (47), 170 (21), 169 (48), 156 (53)
Corynantheine 8	366 (100)	365 (50), 351 (39), 237 (30), 223 (42), 184 (69), 170 (51), 169 (64), 156 (42)
Dihydrocorynantheine 9	368 (85)	367 (55), 353 (100), 239 (44), 225 (65), 184 (94), 170 (74), 169 (98), 156 (66)

*) In a mixture with 1 and 3.

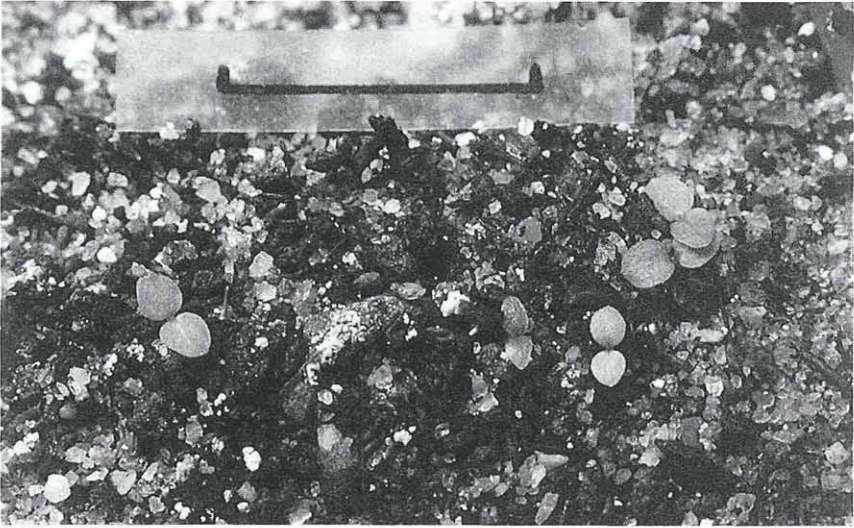


Fig. 1. *Uncaria rhynchophylla*, seedlings. 35 days after sowing and 23 days after the begin of germination. – Scale bar 1 cm.

in part, by $^1\text{H-NMR}$ spectroscopy. Stereochemical relationships were established by using chemical transformations. The structures of the alkaloids found are given in Fig. 6 and 7.

In the plant under investigation four tetracyclic indole and four tetracyclic oxindole alkaloids were identified. They occur as pairs with a

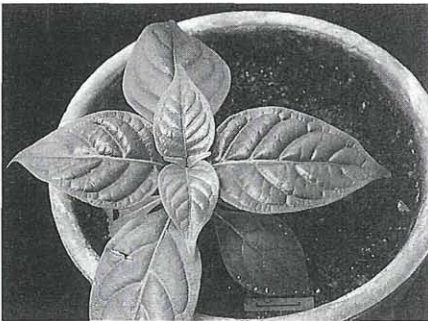


Fig. 2.

Fig. 2. *Uncaria rhynchophylla*, seedling c. half a year old. Uppermost half-developed leaf pair at the seventh node. – Scale bar 1 cm.

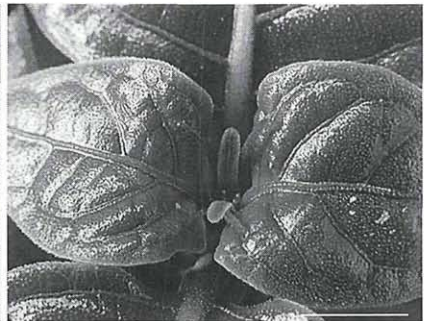


Fig. 3.

Fig. 3. *Uncaria rhynchophylla*, seedling c. half a year old. Uppermost leaf pair with its bipartited stipules and apical bud. In the bud the drip tips of the next leaf pair are visible. – Scale bar 3 mm.

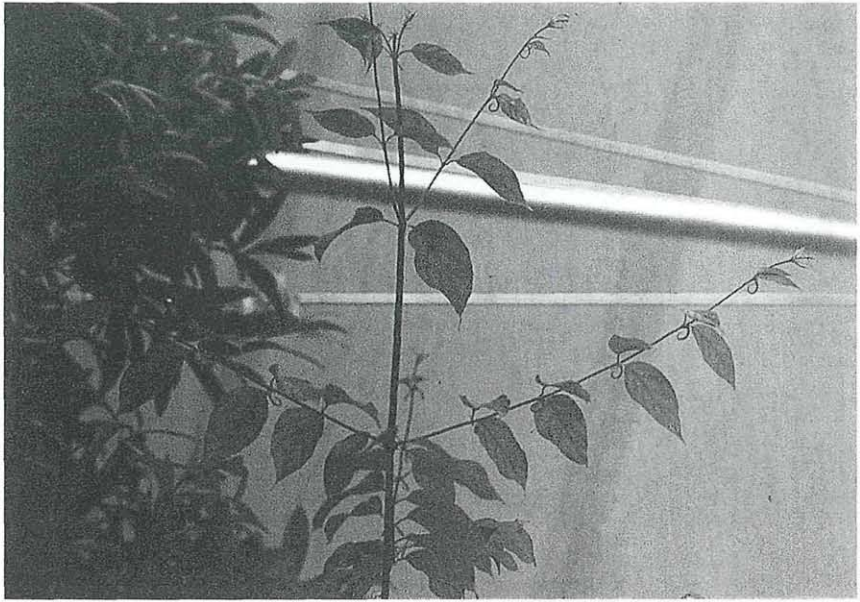


Fig. 4. *Uncaria rhynchophylla*. Upper part of a c. five years old plant. Length of the lower lateral branches c. 35 cm each.

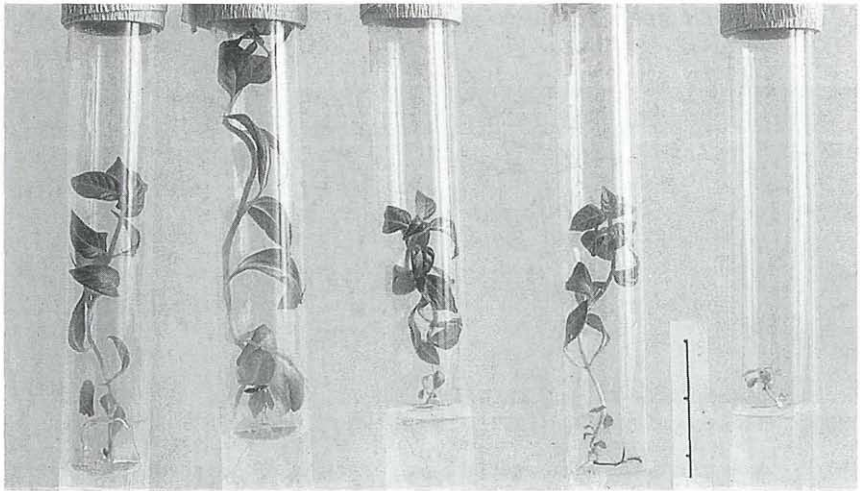


Fig. 5. *Uncaria rhynchophylla* seedlings in test-tubes, ca. 10 months after germination. The lowermost pair of leaves are the cotyledons, withered in some plants, still green in others. – Scale unit 1 cm.

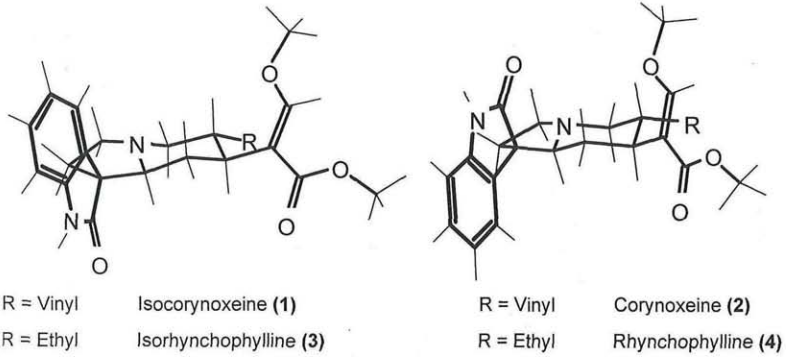


Fig. 6. Structures of the oxindole alkaloids of *Uncaria rhynchophylla*.

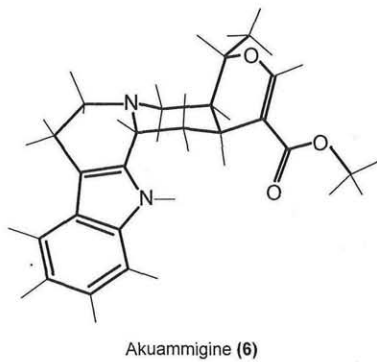
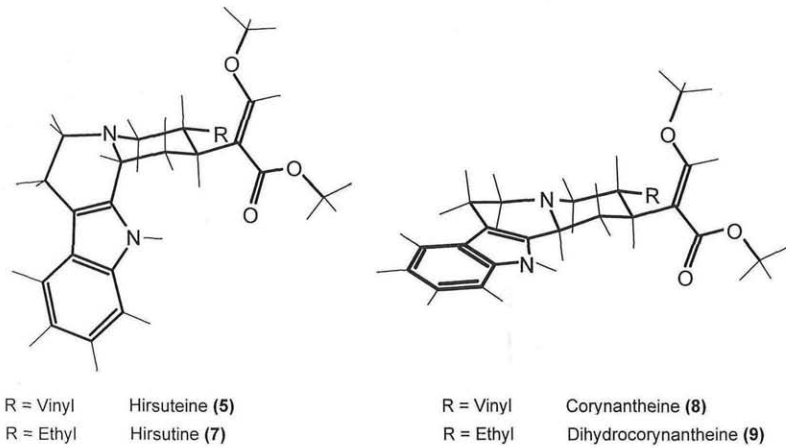


Fig. 7. Structures of the indole alkaloids of *Uncaria rhynchophylla*.

saturated and an unsaturated side chain. In the leaves, the lateral branches and the upper stem the oxindole alkaloids isorhynchophylline **3** and rhynchophylline **4** were found, which we had also isolated from *U. tomentosa* in a previous investigation (LAUS & KEPLINGER 1994). In addition the corresponding unsaturated oxindole alkaloids isocorynoxine **1** and corynoxine **2** were detected. These four alkaloids were reported to occur also in *U. longiflora* var. *longiflora* and, beside other alkaloids, in *U. borneensis* (KAM & al. 1992).

The root contained almost exclusively indole alkaloids, which were mainly located in the bark of the root. By comparison of their hR_F values with literature data and from the results of 1H -NMR and MS they were identified as predominantly hirsuteine **5** and hirsutine **7**. The isomers corynantheine **8** and dihydrocorynantheine **9** were present at lower concentrations. Surprisingly, the pentacyclic indole akuammigine **6** was also isolated, which in contrast to the other eight alkaloids exhibits the opposite configuration at C-20. It remains unclear how the presence of this alkaloid should be explained in terms of biosynthesis.

The stem bark contained both oxindoles and indoles, while the wood of the lower stem had almost no alkaloids. The results of the quantitative determinations are summarized in Table 5. Examples of chromatograms of the extracts from samples D and F are shown in Fig. 8.

We did not detect the pyridino-indolo-quinolizidinone alkaloids angustine, angustidine and angustoline, which were reported to be present

Table 5
Alkaloids in *Uncaria rhynchophylla*.

Alkaloid	mg Alkaloids / g Plant Material							
	Lower Stem Bark	Lower Stem Wood	Upper Stem	Lateral Branches	Leaves *)	Main Root Bark	Main Root Central	Other Roots
	A	B	C	D	E	F	G	H
Isocorynoxine 1	1.63	0.06	0.89	1.35	1.34	—	0.04	0.02
Corynoxine 2	0.67	0.05	0.41	0.60	0.47	—	0.01	0.01
Isorhynchophylline 3	1.60	0.10	0.76	0.98	0.96	0.01	0.04	0.04
Rhynchophylline 4	0.79	0.05	0.29	0.40	0.34	0.01	0.01	0.03
Hirsuteine 5	2.01	0.06	0.07	0.02	0.06	4.07	0.26	3.45
Akuammigine 6	0.89	0.01	—	—	—	2.44	0.14	2.09
Hirsutine 7	2.34	0.04	0.04	0.01	0.01	5.04	0.34	5.42
Corynantheine 8	0.09	0.05	0.02	0.02	0.01	0.55	0.17	1.31
Dihydrocorynantheine 9	0.21	0.08	0.04	0.03	0.02	0.71	0.20	2.11
Total	10.23	0.50	2.52	3.41	3.21	12.83	1.21	14.48

*) Mean value of 6 leaves.

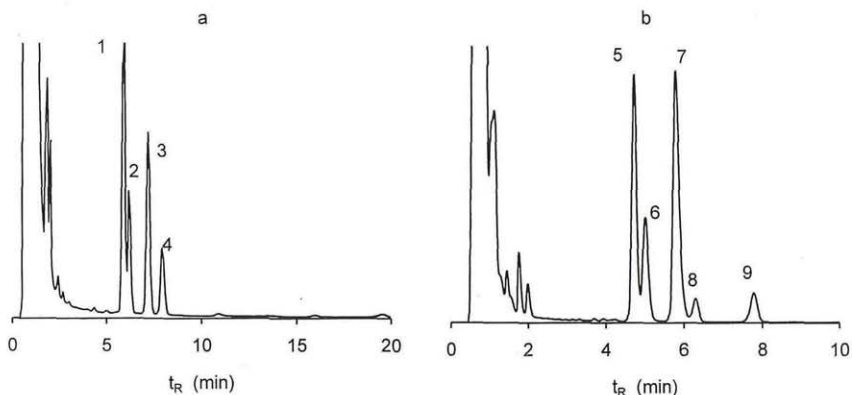


Fig. 8. Chromatograms of *Uncaria rhynchophylla* extracts. – a lateral branches (Sample D; Method A). – b Root bark (Sample F; Method B).

in the leaves (PHILLIPSON & al. 1974). However, our results are in accordance with those obtained by YAMANAKA & al. 1983. The observed alkaloid pattern puts *U. rhynchophylla* together with *U. sessilifractus* in an exceptional position within group V of the genus as proposed by RIDSDALE 1972 and PHILLIPSON & al. 1978. In contrast, chemical similarities with some species from group I, with *U. longiflora* from group III and *U. guianensis* from group VI are evident (according to data from PHILLIPSON & al. 1978 and KAM & al. 1992)

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