241. Structure Revision of the *Rauwolfia* Alkaloid Raucaffricine

by **Helmut Schübel, Arno Treiber** and **Joachim Stöckigt***

Institut für Pharmazeutische Biologie der Universität München, Karlstr. 29, D–8000 München 2

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Summary

The previous structure of the *Rauwolfia* alkaloid raucaffricine (vomilenine-α-D-galactoside) has been revised to be vomilenine-β-D-glucoside by enzymatic and NMR studies.

Introduction. – Raucaffricine, one of the rare glycoalkaloids of the monoterpenoid indole series was isolated 20 years ago from *Rauwolfia caffra* SONDER plants [1]. Its constitution was revised in 1970 [2] and the structure determined as being vomilenine-α-D-galactoside [2] [3]. A more recent, detailed ^{13}C-NMR analysis [4] confirmed the proposed structure by comparison with the ^{13}C-NMR data of rather distinct indole alkaloids and by only a limited number of sugar derivatives [4]. Recently, we found raucaffricine to be the major constituent of cell suspension cultures of *Rauwolfia serpentina* (L.) BENTH. ex KURZ and obtained this alkaloid in a higher gram range [5]. All physical data (m.p. IR, UV, $[\alpha]_D^{20}$, EI-MS, DCI-MS, ^{1}H- and ^{13}C-NMR) of the isolated compound were superimposable with those described in the literature or those measured of commercially available raucaffricine. After the isolation of a new co-factor-independent *Rauwolfia* enzyme, preliminarily named 'Raucaffricine glycosidase', which splits raucaffricine into vomilenine and glucose, we doubt the correctness of the currently accepted structure of the alkaloid. We describe here the structural revision of raucaffricine which turned out to be vomilenine-β-D-glucoside and not the earlier proposed vomilenine-α-D-galactoside.

Results and Discussion. – During a search for enzymes involved in the biosynthesis of ajmaline-type alkaloids, we have so far failed to give evidence for the enzyme raucaffricine synthase in a crude protein mixture of *R. serpentina* cell suspension cultures. We observed, however, that raucaffricine is efficiently hydrolyzed by this enzyme extract (0.3 nkat/mg protein) forming vomilenine and a sugar which co-migrated on TLC with glucose and not, as we expected, with galactose. To prove the identity of the sugar to be glucose, we applied two specific enzyme reactions catalyzed by glucose oxidase and glucose-6-phosphate-dehydrogenase [6] [7]. Both test systems clearly indicated glucose as the glycosidic component of raucaffricine if the glucoalkaloid isolated from cultured cells or obtained commercially (*Roth*, Karlsruhe) was hydrolyzed enzymatically or chemically. In full agreement, the specific galactose dehydrogenase showed no oxidation of the liberated sugar. These results suggested a glucoalkaloid rather than a galactoside.

To differentiate between an α- or β-glucoside, we attempted to split the glucoside using eight commercially available glycosidases (α- and β-glucosidases and galactosidases, respectively). Of these enzymes only β-galactosidase from *Aspergillus niger* showed a slight hydrolytic activity (0.1% of the above mentioned enzyme extract from *R. serpentina* cells). This contradictory result and the fact that in the previous ^{13}C-NMR analyses [4] considerable misinterpretations were made, prompted us to perform a complete NMR-analysis of raucaffricine.

^{1}H-COSY, DEPT and ^{1}H/^{13}C-correlated spectra measured in (D_5)pyridine allowed a clear assignment of ^{1}H- and ^{13}C-resonances and indicated an ajmalan skeleton [8]. Whereas the ^{13}C-signals for both CH_3-groups at 13 ppm (C(18)) and 21 ppm (C(23)) were earlier correctly assigned, C(14), C(15) and C(6) resonate at 25.3, 28.1 and 37.6 ppm, respectively. Of these resonances the high-field signal is correlated with H_β–C(14) (1.84 ppm) and H_α–C(14) (2.16 ppm). C(15) corresponds to a proton multiplet at 3.24 ppm (two unseparated protons, H–C(5) and H–C(15)). C(6) is correlated to the high-field H_α–C(6) (1.69 ppm, J = 11.5 Hz) and to the signal at 2.74 ppm (Hβ–C(6)). That the glycosidic component in raucaffricine is glucose instead of galactose is best supported by the fact that only large ^{1}H-coupling constants were observed for the penta-acetate of the glucoalkaloid (*trans*-diaxial orientation of H–C(1′), H–C(2′), H–C(3′), H–C(4′), H–C(5′)). In addition, this result also indicates, that the glucose moiety is β-glucosidic-bound. Previously, the resonance value of C(1′) (98.8 ppm in (D_6)DMSO) was taken as evidence for an α-glycosidic bond. However, using (D_5)pyridine as solvent we measured 102 ppm for C(1′) which suggests instead a β-orientated bond.

To resolve this point, J(C(1′), H–C(1′)) was determined and found to be 162 Hz. This value is in complete agreement with a β-junction [9]. Moreover, in the HETCOR spectrum, C(1′) allows the assignment of H–C(1′) at 5.29 ppm showing a J = 8 Hz, which is the third piece of spectroscopic evidence in favour of a β-glucosidic bond. Unfortunately, in the earlier structural determination, the broad signal of H–C(21) was claimed to be H–C(1′) and led to misassignment of the ^{1}H-NMR data. The line-broadening of the H–C(21)-signal is, however, due to a small coupling with H–C(18) as clearly shown from a ^{1}H-COSY experiment. In this context, it is noteworthy that H–C(21) is not correlated with H–C(19) indicating the absence of an allylic coupling (dihedral angle ~ 30°). This observation and the finding that vomilenine, which is enzymatically regenerated from raucaffricine, is cell-free transformed into ajmaline

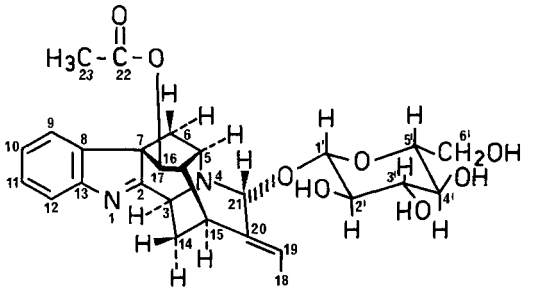

Figure. *Revised structure of raucaffricine*

with known C(21)-configuration points to the β-stereochemistry of H–C(21) in raucaffricine. From the data presented here, by NOE experiments, and by the comparison with the ^{13}C-values of ajmaline-type compounds [8] [10], we conclude that the correct structure of raucaffricine is as depicted in the *Fig.*

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Experimental Part

General. Optical rotation as determined using a *Perkin-Elmer 241* polarimeter. UV data were measured on a *Perkin-Elmer 551 S* instrument with MeOH as solvent, absorption in nm (log ε). ^{1}H- and ^{13}C-NMR spectra were determined at 300 MHz with a *Bruker AM 300* and at 400 MHz with a *Varian XL 400* instrument. Chemical shifts (σ) are reported in ppm and coupling constants (J) in Hz. Signal multiplicities: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br. = broad. Mass spectra (electron impact, EI-MS 70 eV; direct chemical ionization, DCI-MS 190 eV, with i-C_4H_{10}) were obtained on a *Finnigan MAT 44 S* instrument.

Enzymatic Analysis of the Sugar Moiety in Raucaffricine. – Enzymatic Raucaffricine Hydrolysis. Raucaffricine (2 mg, 3.9 µmol) was dissolved in 2 ml phosphate buffer (50 mM, pH 7.0) and 1 mg protein of a crude enzyme mixture (0.3 nkat/mg) which was filtrated on *Sephadex G 25* after isolation from *R. serpentina* cell cultures, were incubated for 1 h at 30°.

Chemical Hydrolysis of Raucaffricine. Raucaffricine (10 mg, 19.5 µmol) were refluxed in 1.5 ml TFA for 2 h. The solution was brought to pH 7 by adding 2N NaOH and $NaHCO_3$. In both mixtures glucose was determined; Glucose-oxidase *(EC 1.1.3.4)*/peroxidase *(EC 1.11.1.7)*-test: to 200 µl of each of the above hydrolysis mixtures were added 25 µl glucose oxidase (33 nkat), 25 µl peroxidase (166 nkat), 10 µl guaiacol solution (0.1M) and 0.5 ml phosphate buffer (50 mM, pH 7.0), this mixture was incubated for 1 h at 30° and the oxidation of guaiacol by the formed H_2O_2 was followed at 585 nm.

Hexokinase (EC 2.7.1.1)/*Glucose-6-phosphate-dehydrogenase* (EC 1.1.1.49)-*test.* To 200 µl of each of the hydrolysis mixtures were added 2.5 ml triethanolamine buffer (0.3M, pH 7.5, 4 mM $MgSO_4$), 100 µl $NADP^+$ (12 mM), 100 µl ATP (15 mM) and the absorption observed at 340 nm. Then, a mixture of both enzymes (20 nkat each) was added and the transformation of $NADP^+$ into NADPH was measured. Both test systems showed the presence of glucose to be the hydrolytic liberated sugar of raucaffricine. Control experiments with galactose-dehydrogenase *(EC 1.1.1.48)* were negative: 200 µl of the hydrolysis mixtures were incubated with 200 µl NAD^+ (7.5 mM containing 4 mM $MgSO_4$), 2 ml phosphate buffer (50 mM, pH 8.6). Addition of 8 nkat galactose-dehydrogenase resulted in an unchanged absorption at 340 nm.

Hydrolysis of Raucaffricine by Different Commercially Available Glycosidases. α-Galactosidase *(EC 3.2.1.22)* from *Aspergillus niger* and *Green Coffee Beans (Sigma)*, β-galactosidase *(EC 3.2.1.23)* from *Aspergillus niger (Sigma), Escherichia coli (Sigma)* and from *E. coli (Boehringer)*, α-glucosidase *(EC 3.2.1.20)* from *Brewers Yeast (Sigma)* and β-glucosidase *(EC 3.2.1.21)* from *Almonds (Sigma, Roth)* were tested: 2 mg (3.9 µmol) of the glucoalkaloid were incubated at 30° in 5 ml phosphate buffer (50 mM, pH 6.0) up to 8 days in presence of 16.7 nkat of the above glycosidases. Enzyme reactions were terminated by addition of MeOH. The denatured protein was separated and the supernatant was analyzed by HPLC for decreasing raucaffricine concentration and liberated aglycone (vomilenine). For the separation of both compounds a *RP 18* column *(Hibar LiChrocart®, Merck)* and a gradient of CH_3CN/10 mM $(NH_4)_2CO_3$ (20:80 to 60:40% within 30 min) was used (flow rate 1 ml/min). Raucaffricine t_R = 13.0 min, vomilenine t_R = 17.7 min. Vomilenine formation was only observed with β-galactosidase showing a specific activity of 0.3 pkat/mg for raucaffricine.

Raucaffricine from Cell Cultures. White plates from MeOH/AcOEt 1:8, m.p. 180–220° ([4]: 186–220°). $[\alpha]_D^{20}$ = + 14.5° (c = 0,4, EtOH) ([4]: + 14.5°). UV (MeOH): max. 258 (3.72), 220 (4.34); min. 240 (3.61). EI/MS: 351 (42, $M^+ - C_6H_{11}O_6$), 291 (10), 273 (16), 183 (30) 170 (21), 169 (100), 168 (51), 167 (25), 156 (29), 107

(18), 106 (19). DCI/MS: 569 (12, $[M + C_4H_9]^+$), 513 (100, $[M + H]^+$). ^{1}H-NMR (C_6D_5N): 7.81, 7.66 (*d*, *J* = 8, 2H–C(9, 12)); 7.42, 7.28 (*t*, *J* = 8, 2H–C(10, 11)); 5.86 (*q*, *J* = 6.5, H–C(19)); 5.43 (br. *s*, H–C(21)); 5.29 (*d*, *J* = 8, H–C(1′)); 5.23 (*s*, H–C(17)); 5.14 (*d*, *J* = 9.5, H–C(3)); 4.52, 4.33 (*dd*, *J* = 12 and 2, *J* = 12 and 5, 2H–C(6′)); 4.2–4.35 (*m*, 2H–C(3′, 4′)); 4.1 (*t*, *J* = 8.0, H–C(2′)); 3.94 (*m*, H–C(5′)); 3.24 (*m*, 2H–C(5, 15)); after addition of C_6D_6: 3.35 (*t*, *J* = 5, H–C(15)) and 3.30 (*t*, *J* = 6, H–C(5)); 2.74 (*dd*, *J* = 11.5 and 4.5, H–C(6)); 2.4 (*t*, *J* = 6, H–C(16)); 2.16 (s, $OCOCH_3$); 2.16 signal overlapped with $OCOCH_3$, after addition of C_6D_6: 2.26 (*dd*, *J* = 9.5 and 13.5, H–C(14)); 1.84 (*dd*, *J* = 5 and 13.5, H–C(14)); 1.69 (*d*, *J* = 11.5, H–C(6)); 1.50 (*d*, *J* = 6.5, H–C(18)). ^{13}C-NMR (C_6D_5N;*: exchangable): 184.6 (C(2)); 170.1 (C(22)); 157.8 (C(13)); 139 (C(20)); 137.6 (C(8)); 129* (C(11)); 125.9* (C(10)); 124.5 (C(9)); 123 (C(19)); 121.5 (C(12)); 102.2 (C(1′)); 90.6 (C(21)); 78.7 (C(3′, 5′)); 78.1 (C(17)); 75.6 (C(2′)); 71.6 (C(4′)); 65.7 (C(7)); 62.8 (C(6′)); 56.1 (C(5)); 51.5 (C(3)); 48.9 (C(16)); 37.6 (C(6)); 28.1 (C(15)); 25.5 (C(14)); 21 (C(23)); 13 (C(18)).

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