Novel Glucoalkaloids from *Rauwolfia* Cell Cultures – Acetylrauglucine and Related Glucosides

Carl Michael Ruyter, Helmut Schübel, and Joachim Stöckigt

Institut für Pharmazeutische Biologie, Karlstraße 29, Universität München, D-8000 München 2, Bundesrepublik Deutschland

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From cell suspension cultures of *Rauwolfia serpentina* grown in an optimized production medium for the glucoalkaloid raucaffricine, a novel glucoalkaloid was isolated and identified as 17-O-acetyl-21-O-β-D-glucopyranosyl-ajmaline (acetylrauglucine). This alkaloid is formed in very small amounts ($< 5 \times 10^{-4}$%). The biogenetically related N_a-demethylated base (acetyl-nor-rauglucine) and the deacetyl product rauglucine have also been detected in culture extracts. In addition 21(*R*)-(β-D-glucopyranosyl)-hydroxy-sarpagan-17-al has been isolated and identified as an artifact which originates from raucaffricine.

Introduction

The traditional medicinal plant *Rauwolfia serpentina* Benth. has been phytochemically investigated in great detail during the past 50 years [1].

Nevertheless recently several novel monoterpenoid indole alkaloids of the ajmalan/yohimban group have been detected in this plant [2–4] and many other species of the genus *Rauwolfia* have also been broadly characterized for alkaloid constituents [5].

Recently cell suspension cultures of *R. serpentina* have been successfully employed for the elucidation of the biosynthetic pathway leading to alkaloids of the ajmalan and sarpagan class [6]. During these investigations it was noted that the cultivated cells are an excellent source of the enzymes involved in the biosynthesis of these alkaloids.

Moreover, we have optimized this cell culture technique for the production of alkaloids during recent years and determination of the alkaloid profile resulted in the identification of a total of 23 different indole bases, among them the glucoalkaloid raucaffricine [7]. Under certain culturing conditions this alkaloid can be produced in concentrations of up to 1.6 g per 1 medium [8]. Because of both the impressive production rate and the high number of formed alkaloids, the Rauwolfia suspension is the most efficient cell culture system described for the indole alkaloid bearing families Apocynaceae, Rubiaceae or Loganiaceae. The glucoalkaloid raucaffricine can easily be isolated by rotation locular counter-current chromatography (RLCC) in the higher gram range [9].

When we investigated the remaining alkaloidal fractions obtained during the raucaffricine isolation we observed a great number of so far unidentified trace alkaloids with less polar properties together with a few rather polar components. The polar fraction comprised a series of structurally related ajmalan glycosides, the major alkaloid being 17-O-acetyl-21-O-β-D-glucopyranosyl-ajmaline, commonly named acetylrauglucine. Here we report the isolation and structure determination of these novel glucoalkaloids from cultured cells of *Rauwolfia serpentina*.

Materials and Methods

Cell suspension cultures

Cell suspensions of *Rauwolfia serpentina* were grown for 20 days in 1,5 l Fernbach flasks on a gyratory shaker at 100 rpm, 25 °C and 600 lux in an alkaloid production medium [10] which was modified by the sucrose and mineral salt content [8]. One l of this medium was inoculated with about 180 g cells grown for 10 days in a "growth medium" (LS medium [11]) under the above conditions. At the end of cultivation cells were harvested by suction filtration, frozen with liquid nitrogen and stored below −25 °C. Under these growth conditions ~400 g

Reprint requests to J. Stöckigt.

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fresh cells (corresponding to ~40 g dry cells) were obtained per l medium.

Chromatographic methods

Solvent systems:

- for XAD column chromatography
  (a) methanol (10%) (b) methanol
- for rotation locular countercurrent chromatography (RLCC); ascending mode (c) chloroform: methanol: water = 43:37:20 (organic phase = stationary phase; aqueous phase = mobile phase)
- for preparative and thin layer chromatography (TLC)
  (d) petroleum ether: acetone: diethylamine = 7:2:1
  (e) chloroform: methanol: ammonia = 8:2:0.1
  (f) ethylacetate: methanol: water = 7:2:1
  (g) chloroform: cyclohexane: diethylamine = 6:3:1
  (h) chloroform: methanol: water = 13:5:1.

A column (5 cm × 41 cm) containing XAD-2 (0.3–1 mm) from Merck (Darmstadt) was used to remove most of the extracted medium components (salts, sugars). RLCC instrument (Zinsser Analytik GmbH Frankfurt/Main) was employed. Prepurification of the crude alkaloid extract under the following instrumental conditions: The rotor slope was 35° and rotation was performed at 80 rpm. The flow rate of the mobile phase was 1 ml/min and fractions of 10 ml were collected. Preparative chromatography was carried out on 0.5 mm silica gel $60F_{254}$ plates (20 × 20 cm Merck Darmstadt) with solvent mixtures (e, f). TLC was performed on Sil gel plates G/UV_{254} (0.25 mm) obtained from Macherey-Nagel (Düren) with the solvent systems (d–g).

Alkaloids were detected by spraying the plates with ceric ammonium sulphate (CAS) reagent.

Alkaloid identification

Acetylrauglucine and related compounds were spectroscopically characterized by UV in methanol (Perkin Elmer 551 S spectrophotometer) and further analyzed by mass spectroscopy (MS) under electron impact (EI) mode using a Finnigan MAT 44S quadrupole instrument at 70 eV (major fragment ions are given). ^{1}H NMR analyses were carried out at 360 MHz using a Bruker AM 360 instrument. Pyridine-d_5 was used as solvent; coupling constants (J) are in Hz; br = broad, s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet.

The optical rotation values were recorded in ethanol using a Perkin Elmer 241 polarimeter. Due to small quantities of samples available the deviation of measurements were relatively high (15–20%).

Isolation of acetylrauglucine

Freeze-dried *R. serpentina* cells (0.4 kg) were extracted twice with 10 l methanol at room temperature for 24 h. After filtration the organic solvent was combined and evaporated under reduced pressure at 40 °C. The residue was dissolved in 0.1 l methanol, 0.9 l water was added and the solution after centrifugation subjected to XAD-2 chromatography. Substances not binding to the resin were removed from the column by washing with 1.5 l water. After elution of the column with the same volume of methanol the organic solvent was evaporated to dryness. Of this crude alkaloid extract 30% (7 g) were dissolved in 25 ml of the aqueous phase (d) for RLCC separation in ascending mode. Fractions (180) were collected and analyzed by TLC (systems d, e), CAS and UV. Fractions 100–130 containing a small part of raucaffricine and the glucoalkaloid under investigation were combined, evaporated under vacuum and then chromatographed on 0.5 mm gel plates in system (d). In this solvent mixture glycosides stay at the origin. The plates were then developed in system (e) and after drying in (f). The glucoalkaloids were scraped out and eluted from silica gel with methanol. These extracts were finally purified on pre-washed (methanol) plates in system (f).

After elution with methanol, centrifugation and evaporation approximately 2 mg of 17-O-acetyl-21-O-β-D-glucopyranosyl-ajmaline (acetylrauglucine) was recovered, which corresponds to 5×10^{-4}% of the dry Rauwolfia cells.

Physical data of acetylrauglucine

UV (λ_{max}): 284, 247 nm. $[\alpha]_D^{20} = +17°$ (c = 0.36, ethanol).

EI-MS (rel. int.): 530 (M^+, 12), 397 (26), 239 (12), 237 (17), 236 (18), 211 (14), 194 (19), 182 (100), 169 (25), 158 (25), 157 (23), 144 (62), 112 (54), 109 (33) m/z.

^{1}H NMR (pyridine-d_5): 7.57, 6.76 (2 × d, J = 7.5, H-C(9, 12)); 7.26, 6.94 (2 × dd, $J_1 = J_2 = 7.5$, H-C(10, 11)); 5.45 (br s, H-C(17)); 5.23 (d, J = 8.5, H-C(1′)); 4.63 (br s, H-C(21)); 4.52 (dd, J_1 = 11.4, J_2 = 4.8, H-C(6′)); 4.38 (dd, J_1 = 11.4, J_2 = 2.3, H-C(6′)); 4.29

(dd, $J_1 = J_2 = 8.8$, H-C(4′)); 4.23 (dd, $J_1 = J_2 = 8.8$, H-C(3′)); 4.07 (dd, $J_1 = 8.5$, $J_2 = 8.8$, H-C(2′)); 4.04 (d, $J = 9.7$, H-C(3)); 3.93 (m, H-C(5′)); 2.90 (t, $J = 5.4$, H-C(5)); 2.79 (s, $N\text{-}CH_3$); 2.56 (br s, H-C(2)); 2.42 (d, $J = 11.0$, H-C(6 α)); 2.34 (m, H-C(15)); 2.18 (s, $OCOCH_3$); 2.15 (m, H-C(16)); 1.98 (dd, $J_1 = 11.0$, $J_2 = 5.4$, H-C(6 β)); 1.77 (m, H-C (14 α)); 1.65 (m, H-C(20)); 1.47 (dd, $J_1 = 13.6$, $J_2 = 5.1$, H-C(14 β)); 1.4–1.2 (m, 2H-C(19)); 0.92 (t, $J = 7.4$, H-C(18)).

Derivatives of acetylrauglucine

Tetraacetate of the glucoalkaloid: Acetylation of acetylrauglucine was performed directly in the MS-crucible with dry pyridine/acetic anhydride (v/v) for 12 h. After evaporation of the reaction mixture the residue was subjected to EI-MS analysis (rel. int.): 698 (M^+, 4), 397 (1), 368 (4), 351 (2), 331 (8), 291 (2), 271 (3), 211 (5), 182 (9), 169 (100), 145 (10), 139 (5), 127 (13), 109 (66) *m/z*.

Enzymatic hydrolysis of the glucoalkaloid: 0.6 mg of the alkaloid were dissolved in 0.2 ml phosphate buffer (0.1 M, pH 6.0) and treated at 30 °C for 12 h with 0.5 mg of a crude sephadex G-25 purified protein extract from *R. serpentina* cell suspension culture.

The mixture (20 µl) was examined by TLC. Despite the starting material two major nonpolar compounds were observed: 17-O-acetylajmaline (purple CAS-reaction, at 360 nm bright fluorescence after 10 min) with $R_f = 0.65$ (f), $R_f = 0.20$ (d); and ajmaline (purple CAS-reaction, no fluorescence at 360 nm) with $R_f = 0.49$ (f), $R_f = 0.15$ (d). After extraction of the incubation mixture with CH_2Cl_2 and TLC purification both products exhibited the identical mass fragmentation as the authentic reference compounds.

A third product has been detected with a slightly higher polarity than raucaffricine; $R_f = 0.37$ (f). EI/MS: 488 (M^+, 1) *m/z*, 21-O-β-D-glucopyranosyl-ajmaline (rauglucine), acetylation resulted in a pentaacetate, 698 (M^+, 5) *m/z*. Rauglucine was also isolated from a cell extract UV (λ_{max}): 292, 249 nm; $[\alpha]_D^{20} = +34°$ (±5°, $c = 0.1$, ethanol) as well as identified in a solution of acetylrauglucine in methanol stored for 3 months at room temperature.

Isolation of acetyl-nor-rauglucine

An additional trace alkaloid ($R_f = 0.34$, (e)) with an orange CAS-reaction was isolated in amounts about 0.1 mg ($\sim 2 \times 10^{-5}$% of the cell dry weight).

EI/MS (rel. int.): 516 (M^+, 12), 383 (32), 368 (3), 354 (71), 339 (25), 325 (9), 311 (12), 293 (17), 277 (11), 265 (12), 249 (8), 239 (7), 224 (17), 208 (11), 196 (20), 180 (43), 168 (100), 156 (15), 143 (21), 130 (45), *m/z*.

Pentacetyl-acetyl-nor-rauglucine EI/MS (rel. int.): 726 (M^+, 0.3), 684 (0.5), 396 (4), 379 (3), 368 (1), 354 (2), 337 (1), 331 (10), 271 (6), 222 (3), 211 (7), 196 (5), 180 (1), 169 (100), 145 (6), 139 (4), 127 (10), 109 (50), *m/z*.

Isolation of 21(R)-(β-D-glucopyranosyl)-hydroxy-sarpagan-17-al

The RLCC-fractions 100–130 showed during chromatography in system (f) an alkaloid with grey CAS-reaction ($R_f = 0.48$). After elution of this compound 27 mg (5.4×10^{-3}% of the dried cells) were obtained and finally purified in system (h, $R_f = 0.43$). The following data refer to the major compound of an epimeric mixture.

UV (λ_{max}): 282 (4.14), 226 (4.70) nm. $[\alpha]_D^{20} = +51°$ ($c = 0.30$, ethanol).

EI/MS (rel. int.): 470 (M^+, 6), 441 (3), 337 (3), 331 (7), 307 (26), 291 (28), 279 (11), 270 (5), 264 (11), 246 (31), 231 (15), 183 (13), 169 (100), 156 (18), *m/z*.

^1H NMR (pyridine-d_5): 12.1 (s, NH); 9.7 (s, CHO); 7.7–7.2 (H-C(9, 10, 11, 12)); 6.0 (q, $J = 6.5$, H-C(19)); 1.6 (d, $J = 6.5$, H-C(18)). The presence of a formyl group was also confirmed by ^{13}C NMR analysis (signal at 201 ppm in pyridine-d_5).

Tetraacetate EI/MS (rel. int.): 638 (M^+, 5), 609 (3), 499 (3), 349 (2), 331 (9), 307 (22), 291 (27), 264 (11), 246 (32), 182 (6), 169 (100), 156 (13), *m/z*.

Results and Discussion

Rauwolfia serpentina plant cell culture systems are one of the richest sources of monoterpenoid indole alkaloids. We estimate that from 50 kg fresh cells more than 50 different alkaloids can be isolated and so far 23 indole bases have been identified in this laboratory from this particular cell culture.

Apart from the glucoalkaloids strictosidine and raucaffricine (vomilenine-β-D-glucoside) all other indolic components were *non*-glycosidic alkaloids.

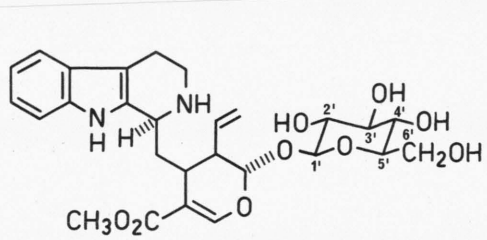
On the one hand it seems that glycoalkaloids of the indole class occur in plants in very low frequency. Only about 0.5% of all isolated indole alkaloids comprise glucoalkaloids. On the other hand, however,

the isolation, separation and identification of such glycosidic compounds is more tedious and difficult than those of the appropriate aglycones. Probably that is a further reason why little attention has been paid to the distribution of indolic glycosides in plants or cell cultures. The work of R. T. Brown's group on Adina alkaloids [12] might suggest that this group of alkaloids is not as rare as one would infer from the alkaloid patterns reported up to now. Particularly for the isolation of polar compounds RLCC has for several years proved to be an efficient method for the separation of different classes of natural products such as saponins [13], phenylpropanoids [14] and iridoid-glycosides [15] *e.g.* secologanin* [16]. The RLCC isolation of raucaffricine [9] proved this method to be excellent for the gram-scale and provided an alkaloid fractionation which should also be useful for the isolation of trace alkaloids from *R. serpentina* cell cultures.

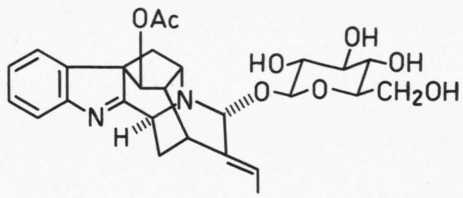
Indeed, when RLCC fractions of a slightly higher polarity than raucaffricine were analyzed by TLC and CAS reaction the presence of CAS-positive *Rauwolfia* alkaloids was indicated by reddish or orange colours. We expected such compounds to be of glycosidic nature. From 0.4 kg dried cells worked up as described in the experimental section ~ 2 mg of the major previously unknown compound were isolated.

This alkaloid exhibited the typical CAS-reaction of ajmaline (purple) which slowly turned to a yellow fluorescence on the TLC-plate (monitored at 360 nm). Such an effect is known for acetylated ajmaline alkaloids and this observation suggested a closely related structure for the unknown product. The TLC-properties of the compound (solvent system e, $R_f = 0.43$) were similar to vomilenine-β-D-glucosid (raucaffricine) (system e, $R_f = 0.40$) which suggested a glucosidic character. This assumption was supported by the fact that during incubation of this alkaloid in the presence of a crude Rauwolfia enzyme extract, which contained the enzyme raucaffricine-glucosidase, hydrolysis of the compound was observed. TLC examination of this reaction mixture clearly showed the formation of two less polar alkaloids which were the appropriate aglycones identi-

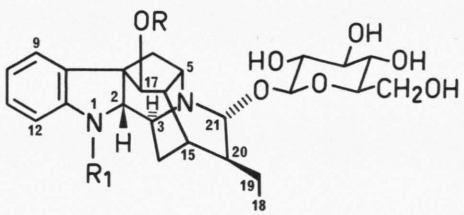
* Very recently Hermans-Lokkerbol and Verpoorte [17] have also described the isolation of secologanin by RLCC.



Strictosidine



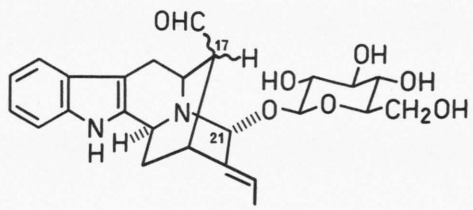
Raucaffricine



Acetylrauglucine R=Ac, R_1=CH_3

Acetyl-nor-rauglucine R=Ac, R_1=H

Rauglucine R=H, R_1=CH_3



21-Hydroxysarpagan-glucoside*)

* Is formed artificially from raucaffricine during the isolation procedure.

Fig. Glucoalkaloids from cell suspension cultures of *Rauwolfia serpentina* Benth.

cal (TLC, MS) to 17-O-acetylajmaline and ajmaline. The formation of the latter alkaloid was due to the presence in the crude enzyme mixture of 17-O-acetylajmalan-esterase which removed enzymatically the 17-O-acetylgroup from the ajmalan skeleton [18].

Our expectation that the isolated alkaloid was a 17-O-acetylajmaline glycoside was confirmed by MS and ^{1}H NMR data. In EI-mode we observed a molecular ion of M^+ 530 (12%, *m/z*) corresponding to an alkaloidal monoglycoside. The intensive fragment ion at 368 (98%) *m/z* results from the loss of this glycosidic part ($C_6H_{10}O_5^+$). According to this, the acetylation gave a more nonpolar alkaloid with M^+ 698 *m/z* which clearly accounts for the formation of a tetraacetate.

The ^{1}H NMR spectrum was in full agreement with the structure suggested above. The NMR-data were evident for a 17-O-acetylajmalan skeleton linked to a sugar moiety. The latter unit showed the same pattern of signals as previously found for the glucose unit in raucaffricine. A singlet at 2.18 ppm (3H) was consistent with an acetyl group at position 17 because of the down field shift of H-C(17) (5.45 ppm). Except for the proton at C(21) the remaining signals showed shift values very similar to those of 17-O-acetylajmaline as described by Lounasmaa [19]. The presence of a one proton doublet at 5.23 ppm with a coupling constant of 8.5 Hz was characteristic of the H-C(1′) of a β-glucosidic compound. The protons at C(2′−6′) of the sugar unit are located in the region between 3.9−4.5 ppm (H-C(2′) 4.07, H-C(3′) 4.23, H-C(4′) 4.29, H-C(5′) 3.93, 2H-C(6′) 4.38, 4.52) as shown by irradiation experiments. The fact, that the protons (1′−5′) exhibit only large coupling constants (8.0−8.8 Hz) proves the sugar moiety to be glucose.

In agreement with the ^{1}H NMR data of ajmaline [19], H-C(21) corresponds to a broad singlet at 4.63 ppm. The upfield shift of this signal in comparison to the corresponding proton in the glucoalkaloid raucaffricine (5.4 ppm) is due to the saturation of the ethylidene double bond (C-19, C-20).

All these data confirm that the isolated alkaloid is the novel compound 17-O-acetyl-21-O-β-D-glucopyranosyl-ajmaline (acetylrauglucine).

In principle there are two possibilities to explain the biosynthesis of these glucoalkaloids although no data are presently available concerning the appropriate enzymes. For instance the aglycones *e.g.* 17-O-acetylajmaline, which are involved in the ajmaline pathway could be enzymatically glucosylated by a glucosyl-transferase. In contrast, the enzymes converting the aglycone of raucaffricine (vomilenine) into 17-O-acetylajmaline by hydrogenation (N-1, C-2; C-19, 20) and $N_{(\alpha)}$-methylation might perform these reactions also at the raucaffricine stage; eventually with extremely low activities as compared to the main substrate vomilenine. Determination of the substrate specificities of these enzymes with the corresponding glucosylated substrates, which are now available, will indicate the detailed route of biosynthesis.

The additional occurrence of an appropriate N-demethylglucoalkaloid would nicely fit this idea. Because of the extremely low amounts isolated ($< 10^{-5}$%), this alkaloid has been only tentatively identified by CAS-reaction, its mass spectrum (M^+ 516 *m/z*) and the MS of the corresponding acetylated alkaloid (M^+ 726 *m/z*). Further work needs to be done to identify this compound (named acetyl-norrauglucine) in more detail. It also would be interesting to show whether 21-O-β-D-glucopyranosyl-ajmaline (rauglucine) which we obtained enzymatically from acetylrauglucine in a cell culture extract (or during storage in methanol over 3 months) is also a true constituent of the Rauwolfia cell system. For the isolated 21(*R*)-(β-D-glucopyranosyl)-hydroxy-sarpagan-17-al mixture it is evident that its formation takes place spontaneously from raucaffricine during the work up procedure of the alkaloids. When a solution of raucaffricine in methanol was allowed to stand at 18 °C for 3 months, the formed sarpagan-glucosides could be unequivocally identified by TLC and MS analysis. The mechanism of this reaction can easily be explained. After hydrolysis of the 17-O-acetylgroup of raucaffricine, the formed unstable 17-hydroxy-indolenine is transformed to the sarpagan structure. Under alkaline or acidic conditions this conversion can be easily performed [16] resulting in a mixture of C-16 epimers. Since we have not been able to demonstrate the occurrence of these sarpagan-glucosides in fresh *Rauwolfia serpentina* cells using fast and mild extraction conditions, its biogenetic formation is rather doubtful. We conclude from our data that these compounds are artifacts. Despite this fact, the currently investigated cell suspension culture of *R. serpentina* shows a remarkable capability to synthesize a whole variety of indole alkaloids including novel, water soluble glucoalkaloids (figure). The yields of these last-named compounds are at the present time extremely low.

It is, however, interesting to note that acetylated glycosides of ajmaline are of pharmacological value, exhibiting excellent antiarrhythmic properties [20].

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