18. Formation of the First Trimeric Monoterpenoid Indole Alkaloids

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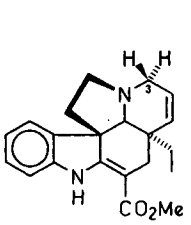
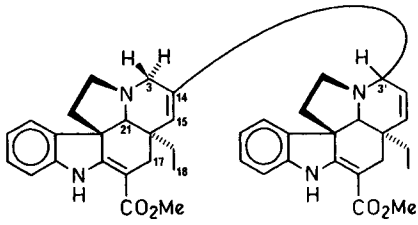
Under O_2, the *Aspidosperma* alkaloid tabersonine (**1**) was converted by a crude enzyme preparation from leaves of mature plants of *Catharanthus roseus* G. DON into the trimeric 3-hydroxy-14′-(3α″-tabersonyl)voafrine B (**4**) which was easily reduced by $NaBH_4$ to 14′-(3α″-tabersonyl)voafrine B (= tertabersonine; **5**). Compounds such as **4** and **5** are the first trimeric alkaloids in the series of monoterpenoid indole alkaloids.

Introduction. – During a broad screening of cultivated plant cells of different genera of the family Apocynaceae, we observed in *each* of our cultures the formation of monoterpenoid indole alkaloids ranging from the µg to the g scale per litre medium [1] [2]. This impressive capability for alkaloid accumulation in plant-cell suspensions is documented because of the remarkable number of alkaloids formed in some of these culture systems. For instance, cell suspensions of *Catharanthus roseus* G. DON synthesize 24 different alkaloids [3], and from *Rauwolfia serpentina* (L.) BENTH. cultures, 26 alkaloids have already been isolated [2].



1 tabersonine[1])
(= methyl 2,3,6,7-tetradehydro-5α,12β,19α-aspidospermidine-3-carboxylate)



2 voafrine A[1]), H_α–C(3′)
3 voafrine B[1]), H_β–C(3′)

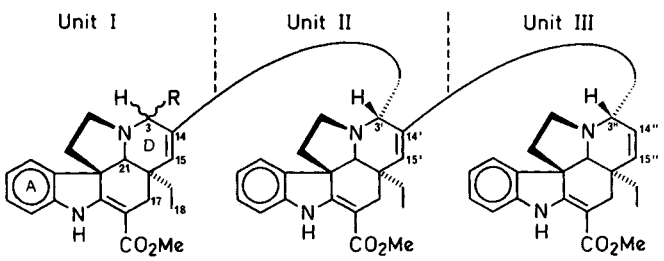
In several culture systems, we identified novel monoterpenoid indole alkaloids. An interesting example was the cell suspension of the tropical plant *Voacanga africana* STAPF. from which dimeric alkaloids **2** and **3** were isolated for the first time which we called voafrines[1]) [4]. These voafrines comprise a new structure originating by dimerization of the monomeric *Aspidosperma* alkaloid tabersonine[1]) (**1**) between positions C(3) and C(14) [4], although from other plants structurally related alkaloids have been isolated, *e.g.*

[1]) Trivial names and numbering; semisystematic names for **4** and **5** in the *Exper. Part.*

ditabersonine from *Crioceras dipladeniiflorus* (STAPF) K. SCHUM. [5] for which the complete structure is unknown and voacinol from *Voacanga grandifolia* (MIQ.) [6].

Recently, we investigated the biosynthesis of the voafrines and searched plants and cell cultures for enzymes capable of metabolizing tabersonine (**1**). In this respect, the so far most efficient enzyme was detected in leaves of *C. roseus*, dimerizing **1** only in presence of O_2. This coupling process resulted in the formation of 3-hydroxyvoafrines which could be simply reduced by $NaBH_4$ leading to the dimeric tabersonines voafrine A (**2**) and B (**3**) [7]. When we analyzed these incubations, a minor, rather polar alkaloid was identified comprising three tabersonine units. In the present paper, we describe the formation, isolation, and identification of this novel alkaloid which is the first recorded example of a trimeric monoterpenoid indole alkaloid and one of the largest alkaloids ever discovered.

Results and Discussion. – When crude enzyme preparations from a variety of plant cell suspension cultures and differentiated plants were incubated in the presence of the *Aspidosperma* alkaloid tabersonine (**1**), transformations of the alkaloid were observed to quite variable extents. TLC permitted simple testing for tabersonine derivatives because of their bright blue color after spraying with cerium(IV) ammonium sulfate. The enzyme mixture obtained from leaves of mature *C. roseus* plants proved to be highly effective in transforming **1**: under optimized conditions, a 70–80% conversion occurred. TLC analysis of this mixture exhibited, besides **1**–**3** [7], the formation of a more polar alkaloid **4**. The UV data of this compound demonstrated that the typical β-anilinoacrylate chromophore (λ_{max} 328 and 299 nm) of **1** was retained. Whereas in the MS the highest fragment ion was observed at only *m/z* 769, ^{1}H-NMR measurements at 360 MHz indicated a very complex structure revealing *ca.* 12 aromatic protons, more than 2 Et groups and 3 NH groups which were easily exchanged with D_2O. These data, on one hand, pointed to an alkaloid structure composed of at least 3 tabersonine units. On the other hand, the experiment with D_2O in which an OH signal at 5.49 ppm was exchanged and its original geminal coupling with H–C(3) lost, together with the observed instability of the compound, was in agreement with a hydroxylated trimer (OH group at C(3)) of **1**, *i.e.* with structure **4**.



4 R = OH, 3-hydroxy-14′-(3α″-tabersonyl)voafrine B[1])
5 R = H, 14′-(3α″-tabersonyl)voafrine B[1])

The aminoalcohol structure of **4** which has only sporadically been found in naturally occurring indole alkaloids (*e.g.* [8] [9]) as well as in the case of dimeric tabersonine derivatives (hydroxyvoafrines, [7]) could be efficiently reduced with $NaBH_4$, leading to a stable reduction product **5**. The latter exhibited identical UV data as **4** and **1**. In the IR spectrum of **5**, a strong absorption for a vinylogous amide unit could be detected at 1670

cm^{-1}, characteristic for **1**–**3**. Its EI-MS revealed a M^+ at 1004 of extremely small intensity, suggesting again the trimeric nature of the alkaloid. The successive loss of three fragment ions $C_{14}H_{15}NO_2$ (*m/z* 229) also agreed with this proposition. The obtained alkaloid could, therefore, be expected to be a 'tertabersonine' with a structure strongly related to the voafrines (= 14-(3′-tabersonyl)tabersonines) **3** and **4**. Taking advantage of the superb resolution of the 600-MHz ^{1}H-NMR spectra of **5**, the basic structure of the trimer could immediately be confirmed.

The 3 indole NH resonate at 9.66, 9.76, and 9.80 ppm. The *s* of 3 CO_2Me groups (3.64, 3.65, 3.69 ppm) and the *t* (each J = 7.4 Hz; 0,61, 0.65, 0.71 ppm) for 3 CH_3CH_2 side chains are nicely separated. Twelve aromatic protons exclude any attachment between the A rings of the three tabersonine moieties. The remaining NMR data suggest the same coupling of the three monomers as found for the voafrines. The geminal H–C(3) (J = 15.6 Hz) of unit I are located at 3.45 (H_α–C(3)) and 3.90 ppm (H_β–C(3)). In a long-range COSY experiment, H_α–C(3) shows small allylic coupling (J < 1 Hz) with H–C(15) which appears as a broad *s* at 5.45 ppm. No further large splitting of the H–C(15) signal also demonstrates that a neighbouring proton at C(14) is missing. In fact, C(14) is attached to the next tabersonine moiety (unit II) at its 3′α-position. H_β–C(3′) of unit II is found as a broad *s* at 4.34 ppm which shows long-range coupling with H_α–C(3), H_β–C(3), and H–C(15) of unit I. One additional fine splitting ($J \approx 0.5$ Hz) is observed for H_β–C(3′) due to coupling with H–C(15′). Moreover, careful inspection of the long-range COSY data demonstrates an extremely small but remarkable interaction of H_β–C(3′) of unit II and the corresponding H_β–C(3″) (3.82 ppm) of the third tabersonine moiety (unit III). Assignment of H_β–C(3′) is also in clear agreement with its further coupling (J = 4.5 Hz) to the vicinal olefinic H–C(14″). A small allylic splitting (J < 1 Hz) of H_β–C(3″) due to H–C(15″) is easily detected in the long-range COSY spectrum and by additional irradiation experiments. The remaining couplings of the olefinic protons of unit III are those of an *ABX* pattern as previously found for voafrine B (**3**) [4]. These results indicate that unit II is also bound to the more accessible 3″α-position of the final unit III.

Although tabersonine (**1**) is a constituent of *C. roseus* plants [10] and, additionally, *C. roseus* is one of the most intensively investigated alkaloid-bearing plants [11], the currently described alkaloids have never been isolated from these Apocynaceous species. Therefore, questions immediately arise about the formation of these trimeric tabersonines. Preliminary results concerning the formation of the dimeric tabersonines voafrine A (**2**) and voafrine B (**3**) [7] indicate that the iminium derivative of **1** (3,4-dehydrotabersonine) is involved as an intermediate. Whether the dimerization and trimerization following the dehydrogenation of tabersonine are spontaneous is presently under investigation as is the possibility of generating the trimers more efficiently by chemical synthesis. In this context, it is interesting to note that enzyme-catalyzed dimerization of the *Aspidosperma* base vindoline has already been achieved [12] [13]. The mechanism of such a reaction might be very similar to the here described formation of 14′-(3α″-tabersonyl)-voafrine B (**5**) which is the first example of a trimeric monoterpenoid indole alkaloid and one of the largest alkaloids so far known.

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Experimental Part

General. Anal. TLC: *Polygram, Sil G/UV$_{254}$* plates *(Macherey & Nagel)*. Prep. TLC: purification of the alkaloids in the mg range on 0.5-mm silica-gel plates *(Merck)* which were washed with MeOH before analyses; eluents: Et_2O/hexane/MeOH/Et_2NH 45:45:2:2 (*A*) and 40:40:5:0.1 (*B*). Optical rotation: *Perkin-Elmer-241* polarimeter. UV Spectra (MeOH): *Perkin-Elmer-*551S instrument; λ_{max} (log ε) in nm. IR spectra: spectrometer *Accu Lab 1* from *Beckman;* $\tilde{\nu}$ in cm^{-1}. NMR spectra ((D_6)DMSO): at 360 and 600 MHz on *Bruker* instruments *AM 360* and *AM 600*. MS: *Finnigan MAT 44S* quadrupol instrument, EI conditions at 70 eV; *m/z* (rel. int. %); recording up from *m/z* 100.

Isolation. Plants of *Catharanthus roseus* G. DON were grown under green-house conditions. Leaves of mature plants were the source for enzyme isolation by the following general procedure. Leaves (800 g) were frozen with liq. N_2 and ground. The obtained powder was stirred for 1 h with 800 ml of 0.1M phosphate buffer (pH 7.0) containing 14 mM of 2-mercaptoethanol. After filtration of the slurry through cheese-cloth, the enzyme soln. was centrifuged for 30 min at 16000 *g*. To the supernatant, $(NH_4)_2SO_4$ was added up to 70% and the soln. stirred for 30 min. After centrifugation, the precipitated protein was dissolved in 60 ml of phosphate buffer (0.1M, pH 7.5) and desalted on a *Sephadex-G-25* column; 100 ml of eluate represented the enzyme solution (1.2 g of protein). After adding 0.7 l of this buffer, a soln. of 1 g (2.7 mmol) of tabersonine hydrochloride (**1**·HCl) in 20 ml of MeOH was added under stirring. This mixture was shaken under O_2 at 100 rpm for 18 h. After extraction with CH_2Cl_2 (3 × 0.5 l) and evaporation of the solvent, 10% of the alkaloidal residue was chromatographed yielding 0.8 mg of *3-hydroxy-14′-(3α″-tabersonyl)voafrine B* (= *trimethyl 2,2′,2″,3,3′,3″,6,6′,6″,7,7′,7″-dodecadehydro-8-hydroxy[7,8′α:7′,8″α-ter(15α,12β,19α-aspidospermidine)]-3,3′,3″-tricarboxylate;* **4**). UV (MeOH): 328, 299. MS: 769 (7), 543 (6), 335 (9), 328 (11), 229 (56), 224 (18), 170 (100), 169 (74), 168 (93), 154 (59), 143 (30).

The remaining alkaloid mixture was dissolved in 10 ml of MeOH and reduced with an excess of $NaBH_4$ for 1.5 h. This mixture was chromatographed on 0.5-mm silica-gel plates with solvent *B*. The bands with R_f 0.5 were eluted with MeOH/CH_2Cl_2 1:1: 7.5 mg (0.9%) of *14′-(3α″-tabersonyl)voafrine B* (= *trimethyl 2,2′,2″,3,3′,3″,6,6′,6″,7,7′,7″-dodecadehydro[7,8′α:7′,8″α-ter(15α,12β,19α-aspidospermidine)]-3,3′,3″-tricarboxylate;* **5**). $[\alpha]_D^{20} = -169°$ (*c* = 0.34, MeOH). UV: 329 (4.33), 300 (4.19), 222 (4.29). IR (KBr): 2940, 1670, 1600, 1430, 1375, 1282, 1238, 1200, 1180, 1165, 735. ^{1}H-NMR: 9.8, 9.76, 9.66 (3 *s*, 3 NH); 7.4–6.8 (12 arom. H of units I–III); 6.0 (*d*, *J* = 10.4, H–C(15′)); 5.78 (br. *s*, H–C(15)); 5.77 (*dd*, *J* = 10.3, 4.6, H–C(14′)); 5.45 (br. *s*, H–C(15″)); 4.34 (br. *s*, H–C(3)); 3.90 (*d*, *J* = 15.7, H_β–C(3″)); 3.82 (*d*, *J* = 4.5, H–C(3′)); 3.69, 3.65, 3.64 (3 *s*, 3 CO_2Me); 3.45 (*d*, *J* = 15.6, H_α–C(3″)); 3.0–2.8 (3 br. *s*, H–C(21,21′,21″)); 2.6–2.5 (3 *dd*, *J* = 14.9, 1, H_α–C(17,17′,17″)); 2.2–1.9 (3 *d*, *J* = 14.9, H_β–C(17,17′,17″));1.1–0.8 (*m*, CH_2(19,19′,19″)); 0.71, 0.65, 0.61 (3 *t*, *J* = 7.4, CH_3(18,18′,18″)). MS: 1005 (< 0.2, $M^+ + 1$), 1004 (< 0.2, M^+), 775 (4, $M^+ - 229$), 546 (3), 440 (4), 335 (7), 318 (4), 317 (3), 230 (18), 229 (60), 228 (26), 214 (22), 198 (14), 197 (24), 196 (16), 182 (10), 171 (33), 170 (100), 169 (78), 168 (93), 167 (35), 156 (26), 155 (43), 154 (60), 133 (24), 128 (37), 115 (56); only major fragment ions.

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