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CATHARANTHUS ROSEUS ENZYME MEDIATED SYNTHESIS OF 3-HYDROXYVOAFRINE A AND B–A SIMPLE ROUTE TO THE VOAFRINES

WALTER FAHN, VERENA KAISER, HELMUT SCHUBEL, JOACHIM STÖCKIGT and BRUNO DANIELI†

Institut für Pharmazeutische Biologie der Universität München, Karlstraße 29, D-8000 München 2, F R G, †Dipartimento di Chimica Organica e Industriale, Facoltà di Scienze, Università degli Studi di Milano, Centro CNR di Studio per le Sostanze Organiche Naturali, Via G Venezian 21, I-20133 Milano, Italy

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Key Word Index—*Catharanthus roseus*, Apocynaceae, dimeric indole alkaloids, tabersonine, 3-hydroxyvoafrine, voafrine, enzyme mediated synthesis

Abstract—Dimerization of tabersonine by a crude enzyme preparation from *Catharanthus roseus* led to the novel 3-hydroxyvoafrines A and B which were reduced enzymatically or chemically to the voafrines A and B, recently isolated in trace amounts from cell cultures of *Voacanga africana*

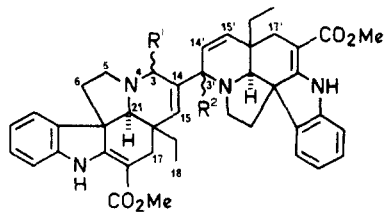
INTRODUCTION

Plant cell suspension cultures have been established as an excellent source for the isolation of enzymes involved in the biosynthesis of secondary metabolites Cultures of different genera of the indole alkaloid bearing plant family Apocynaceae have also been proved in our laboratory to synthesize a wide range of monoterpenoid indole bases indicating a large synthetic capability of cultivated cells. A broad phytochemical screening of such cell systems has led to the identification of *ca* 90 alkaloids of various structural types. More than 10% of these compounds were novel indole alkaloids which were not previously found in differentiated plants. Only three of the alkaloids, voafrine A, voafrine B [1] and vobtusine [2], belong to the group of dimeric or bisindole alkaloids The isolation of these metabolites represented the first example of cultivated plant cells capable of producing complex dimeric alkaloids, although the yields were rather low (under optimized conditions 30 mg for the voafrines per 1 medium) We also investigated the enzymatic formation of the voafrines by the enzyme-catalysed dimerization of the appropriate monomeric alkaloid tabersonine. Here we report that crude enzymes of *Catharanthus roseus* can oxidize tabersonine to the novel dimers 3-hydroxy-voafrine A and B and we describe an enzymatic and chemical procedure for their reduction to the voafrines, thus allowing for the first time the formation of the latter compounds in substantial amount

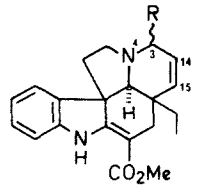
RESULTS AND DISCUSSION

The detection of the novel ditabersonines voafrine A and voafrine B in cell suspension cultures of *Voacanga africana* [1] immediately raised the question of their biosynthesis. There was no evidence of an artificial formation from the monomeric tabersonine under the cell growth conditions and, furthermore, the observation that different *Voacanga* strains synthesized different ratios of both alkaloids suggested an enzyme catalysed process for voafrine synthesis. We therefore searched for tabersonine metabolizing enzymes from plants and plant cell cultures by incubating the alkaloid (1 mg tabersonine hydrochloride) with crude enzyme preparations (1 5 mg protein) followed by TLC-analysis and alkaloid detection with ceric ammonium sulphate (CAS) Under these conditions protein from leaves of mature plants of *C. roseus* showed excellent conversion rates of *ca* 50% of the starting material. When tabersonine was incubated with the enzyme mixture in the presence of acetone (2.5%), TLC revealed the formation of several novel products with strong characteristic blue CAS-reaction In order to get a first impression of the structure of these products, the major compounds were analysed by UV and mass spectroscopy (EI-mode). The less polar product (system b, R_f = 0.4) displayed in the UV spectrum the three maxima of the β-anilino acrylate chromophore (223, 300, 329 nm) but showed a low intensity $[M]^+$ at m/z 392, i e. 56 mu higher than in tabersonine The spectrum was dominated by peaks at m/z 228, 168 and 167 as in tabersonine, indicating no substitution in ring A, B, C [3], and contained a selective intensive ion at m/z 335 (31%) corresponding to the loss of a fragment of 57 mu. Reduction with $NaBH_4$ afforded a pair of closely running more polar compounds with the same mass spectrum displaying a $[M]^+$ at m/z 394 and a fragmentation pattern very similar to that of the non-reduced compound

As a working hypothesis, a ring-D acetonyl-tabersonine was proposed and this could be verified by ^{1}H NMR analysis after scaling up the incubations 50-fold and rigorous chromatographic purification of the compound In the spectrum, recorded in acetone-d_6, a sharp singlet at δ2.22 was easily attributed to the methyl group of the acetone appendage and, in addition, the resonance of the two diastereotopic protons of the methylene group were observed at δ2.85 and 2.66, with a geminal coupling of 15.8 Hz and vicinal couplings of 6.0 and 6 2 Hz, respectively, to a signal at δ3 99. The last signal was further coupled to the two olefinic protons H-14 (J=4 5 Hz) and H-15 (J=1.3 Hz) and must therefore be due to one of the protons of position 3. Other signals of easy attribution were the AB system at δ2.18 and 2.58 (J = 15.8 Hz) featuring the methylene group at C-17, in which the downfield signal is additionally coupled in a



R¹=H , R²=H α , voafrine A (1)
R¹=H , R²=H β , voafrine B (2)
R=OH , R²=H α , 3-OH-voafrine A (3)
R¹=OH , R²=H β , 3-OH-voafrine B (4)



R = 3α-(CH₂-C(=O)-CH₃), 3α-acetonyl-tabersonine (5)
R = H , tabersonine (6)

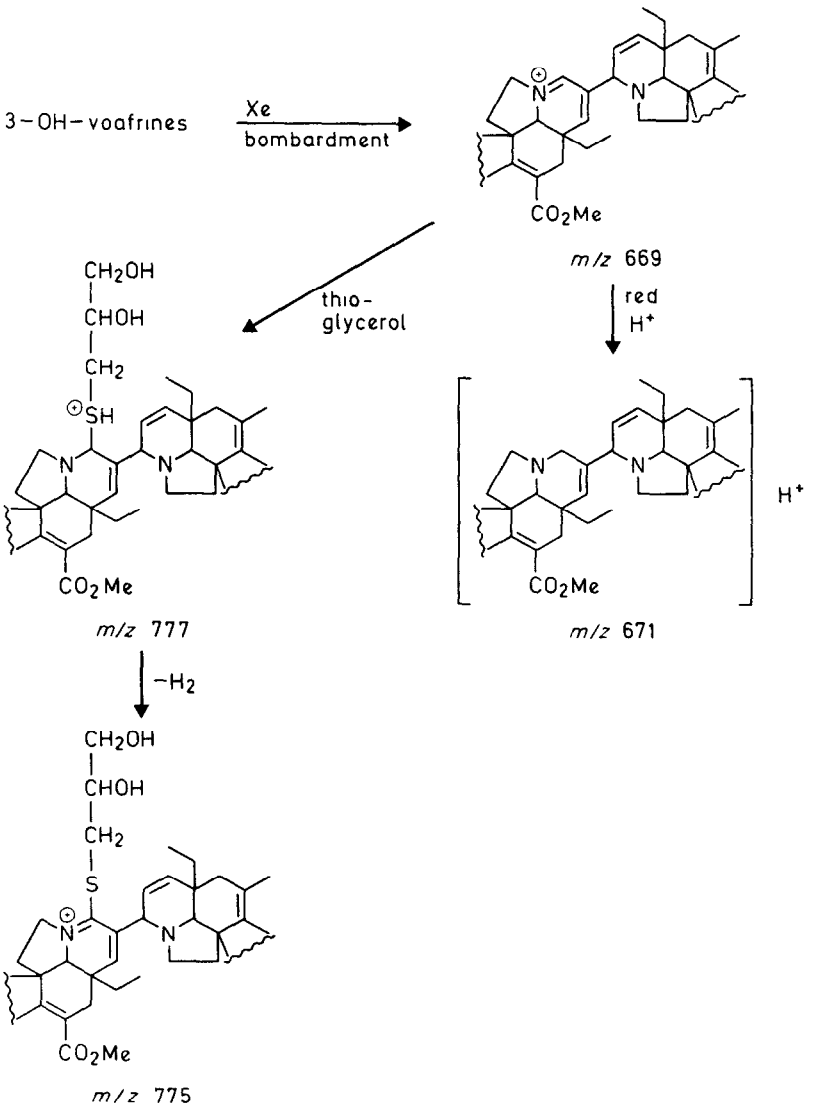
periplanar *W*-arrangement to the signal at δ 3 09 due to H-21 The multiplicity of the H-3 signal as well as the presence of H-14 and H-15 suggested a linkage of the acetone moiety at position 3 The value of coupling to H-14 clearly proved, in comparison with the data for tabersonine [4], that the stereochemistry was β for H-3

The new compound is thus 3α-acetonyl-tabersonine (**5**) formed by addition of acetone to an enzymatically produced reactive tabersonine derivative A plausible mechanistic reason for this addition involves the nucleophilic attack of the enolic form of acetone on a 3,4-dehydrotabersonine iminium ion under stereoselective control from the less hindered convex face of the molecule

The second alkaloid isolated (system b, R_f 0 1) gave the same UV spectrum as for the above mentioned compound The highest peak found in the EI- mass spectrum was at *m/z* 726 Considering also the occurrence of the fragment *m/z* 335 (tabersonyl unit), a dimeric tabersonine incorporating an acetone moiety could be suggested Following the structure determination of 3α-acetonyl-tabersonine, reduction gave a rather polar compound ($[M]^+$ *m/z* 728) The comparable fragmentation of both non-reduced and reduced alkaloids to voafrine A and B [1] indicated the structure of an acetonyl-voafrine, which again was confirmed by analysis of ^{1}H NMR spectra in acetone-d_6 performed with the aid of a 2D-COSY experiment In addition to two NH, eight aromatic protons, two carbomethoxy and two ethyl groups, the signals of H-15′, H-14′ and H-3′ were found at δ 6 07, 5 88 and 4 03 The H-3′ had a coupling constant of 4 8 Hz to H-14′ and a small coupling to H-15′ These values were identical to those found in voafrine B, thus indicating that the new compound had the same 3′(*S*) configuration The location of the acetonyl moiety at C-3 and its α-orientation (H_β-3 configuration) was easily deduced by comparison with the data for 3α-acetonyl-tabersonine (**5**) The H_β-3 is a *br dd* at δ 4 70 with 10 9 and 3 9 Hz couplings to the diastereotopic methylene protons of the acetone group A small additional coupling to H-21 at δ 3 15, according to the *W*-rule, is responsible for the broadening of H-3 signal, but no appreciable coupling could be detected to H-15 at δ 5 66 This implies a quasi coplanar orientation at H-3 with respect to the double bond and hence an α-orientation of the acetonyl group

As control experiments with denatured protein did not show any metabolism of tabersonine, the synthesis of the alkaloids described herein must be enzymatically catalysed From these results we also conclude, in analogy to the formation of acetonyl-tabersonine, that a corresponding reactive but dimeric intermediate should be involved in the production of this acetonyl-voafrine B Possibly this intermediate could also play an important role in the formation of voafrine B

We therefore decided to analyse the enzymatically formed alkaloid mixture in more detail and obtained three further β-anilino acrylic alkaloids by preparative TLC using solvent system (a), alkaloid A, R_f = 0 38, alkaloid B, R_f = 0 3, alkaloid C, R_f = 0 1 The mass spectra of alkaloids A and B exhibited a strongly related pattern of fragment ions, and also displayed marked similarities to the spectra of the voafrines The higher mass ions were measured at *m z* 670 668 which would correspond to those of voafrine and dehydrovoafrine However, the spectra could only be obtained under harsh conditions (heating at > 300) and the appearance was somewhat erratic, being strongly dependent upon experimental conditions No information could be gained from CI and DCI spectra, in contrast to the voafrines, whereas the FAB technique furnished structurally valuable suggestions For example, when a sample of voafrine B was dissolved in the matrix of choice, glycerol, with the aid of little methanol, a complex spectrum was obtained in which peaks due to the glycerol clusters predominated Low abundance peaks of the compound could be detected at *m z* 671 and 669, formally corresponding to a protonated voafrine and a protonated dehydrovoafrine or dehydrovoafrine iminium ion Many weak peaks were also found above 671 mu and some of these were at *m/z* 701/699 and 761 759, indicating addition of the matrix components methanol and glycerol to the cationic species at *m/z* 669 accompanying dehydrogenation In order to obtain better results, the matrix was changed to a 1 1 mixture of glycerol and thioglycerol Under these conditions, a clean spectrum appeared which still contained peaks at *m z* 671/669, however at higher mass an unusually abundant cluster at *m z* 777 775 due to the addition of thioglycerol appeared Very weak fragment ions were observed at *m z* 443 (335 + 108), 335 228 and 215/214 The addition of HBF_4 to the matrix, caused rapid disappearance of high-mass peaks and only *m/z* 669 was found as base peak Although desorption of some species from the matrix as a consequence of sputtering is not a completely understood phenomenon, the possibility that chemical reactions take place in the energized condensed phase is well documented A plausible explanation of the above complex behaviour could be found considering that a dehydrovoafrine iminium ion is present in the matrix or formed upon atomic bombardment and thereafter desorbed This electrophilic species can suffer a well-



Scheme 1 FAB-Mass spectral behaviour of 3-hydroxyvoafrine

known solvent mediated reduction to afford voafrine (desorbed as $[M+H]^+$ ion at m/z 671) or undergoes a nucleophilic attack by glycerol or, more probably, by thioglycerol to form ionic adducts. It will be shown later that alkaloid B has the basic skeleton of voafrine B The FAB mass spectral behaviour can thus be depicted in the following scheme in which the nature of the putative precursor ion of m/z 669 remains undefined. The FAB data suggested that the products were the assumed dimeric reactive intermediates

In order to get sufficient material for detailed NMR analyses the enzyme incubation procedure was optimized From 0 8 1 incubation mixtures containing 1 g tabersonine hydrochloride dissolved in methanol instead of acetone and 1.2 g *Catharanthus* enzyme, 0 26 g of alkaloids A and B (ratio 1 1) were isolated after shaking for 20 hr under oxygen. The yield of alkaloid C was 8 mg The ^{1}H NMR spectrum of alkaloid B displayed in sharp contrast to that of A a relatively good separation of signals and marked similarities to the known spectrum of voafrine B Surprisingly, the aromatic region of this product displayed in the NMR spectrum more than the eight signals which we expected for a voafrine-like dimer Moreover, two signals were found for H-3', 4 N–H groups (9.8–9.6 ppm) were observed and many of the other signals occurred also as pairs. In agreement with the ^{1}H NMR, the ^{13}C spectrum showed the same situation, for example several pairs of signals in the aromatic region, more than two ethyl functions, etc. Since the FAB spectra ruled out higher M_r than for a dimeric alkaloid, the NMR results suggested a mixture of two compounds for alkaloid B, which also became evident by 2D COSY spectra. However, all our attempts to separate this mixture chromatographically failed. The ratio of both compounds was not always constant and was obviously dependent on the purification procedure used and on the water content of

the sample Indeed the ^1H and ^{13}C NMR spectra changed dramatically after addition of D_2O and a 'clean' pattern of signals appeared which was consistent with the structure of one dimeric alkaloid. Analysis of the proton spectrum of alkaloid B was carried out with the aid of a 2D-COSY experiment Additionally, the assignments were corroborated by extensive decoupling experiments That alkaloid B was a 3-substituted voafrine B, was soon apparent by the finding of an ABX system due to H-3', H-14' and H-15' at δ 4 10, 5 74 and 5 91 respectively, with a vicinal coupling between the first and second signals of 4 6 Hz, ensuring the β-orientation of H-3' The other half of the molecule contained H-15 as a singlet at δ 5 46 and only one of the aminomethylene protons at C-3 could be found as a broad singlet at δ 5 22. The broadening was caused by a long-range coupling to H-21 at δ 3 29 in a *W*-shaped arrangement In the position of the missing H-3, an oxygen should be present because the proton and carbon (δ 76 7) shifts of H-3 are those expected for a carbinolamine moiety, more precisely, that the oxygen function is an hydroxy is clearly proved by the spectrum in DMSO-d_6, in which the δ 5 24 signal is a doublet (J = 5 7 Hz), coupled to a doublet at δ 5 34, which disappeared on deuteration The assignment of the signals to one of the two possible 3-hydroxy-voafrine B stereoisomers facilitated the interpretation of the spectrum in DMSO-d_6 The resonances of another ABX system for H-3', H-14' and H-15' were found at almost the same values (δ 4 04, 5 76 and 5 97, respectively) and with identical couplings H-15 was at δ 5.53 and one H-3 was found at δ 4 94, both as slightly broadened singlets The carbon shift of C-3 of this stereoisomer was at δ 85 5, almost 9 ppm downfield with respect to the previous compound These data fit well with the structure of the other 3-hydroxyvoafrine stereoisomer and a comparison allows one to conclude that in DMSO-d_6/D_2O the 3α-hydroxy compound exists as the unique stereoisomer (proton at C-3 downfield because in a quasi-equatorial orientation, C-3 upfield because OH is quasi-axial), whereas in DMSO it is accompanied by the 3β-hydroxy stereoisomer (proton at C-3 upfield because in axial orientation, C-3 downfield because OH is quasi equatorial) The different patterns of H_α-3 and H_β-3 signals in DMSO-d_6 require some comments Examination of the molecular models indicates that the two halves of 3α-hydroxyvoafrine B have almost the same conformation and relative disposition as voafrine B On the other hand, in the case of 3β-hydroxy-voafrine B there is the possibility of the formation of a hydrogen bridge between the OH group and N-4' through a quasi six-membered ring This bridge modifies the relative orientation of the two tabersonine units resulting in different shifts of corresponding protons and, more interestingly, causes the dihedral angle H_α-3-O-H to assume a fixed value, near to 90 As $J_{H\text{-}C\text{-}O\text{-}H}$ shows the same dependence from the dihedral angle as $J_{H\text{-}C\text{-}C\text{-}H}$, this could be the reason why H_α-3 has no coupling to OH Alkaloid B displayed similar NMR behaviour in acetone-d_6 and acetone-d_6/D_2O In the first solvent, the 3α- and 3β-hydroxy-voafrines gave a complex spectrum in which H-3 resonated at δ 5 43 and 5 08 and H-15 at δ 5 60 and 5 65, respectively The addition of D_2O caused the appearance of signals of the 3α-hydroxy stereoisomer only When trifluoroacetic acid was added to the 3α- and 3β-hydroxy-voafrine B mixture in acetone-d_6, a dramatic change occurred in the spectrum All signals showed a downfield shift which was very pronounced for the protons close to N-4 The usual ABX system for H-3', H-14' and H-15' was located at δ5.01, 6 12 and 6 42, whereas for the ring D protons, a broad quartet with J = 1 Hz appeared at δ9 47 and a broad singlet at δ 7 51 A doublet at 4 9 (J = 1 Hz) was attributed to H-21 coupled to H_α-17 at 2 81 but not to H_β-17 at 2 31 Surprisingly the aminomethylene protons at C-5 resonated at unusually low field, one proton at δ 4 68 as a broad *ddd* ($J_1 = J_2 = 13\ 0$, $J_3 = 5\ 8$), the other at 4 79 as a broad *dd* ($J_1 = 13\ 0$, $J_2 = 6\ 9$) Careful inspection of the COSY spectrum and signal shape after decoupling established that the small quartet at 9 47 was due to H-3, because it coupled with both H-5 and H-21 but not with H-15 In agreement with the behaviour of carbinolamine upon treatment with non-nucleophilic acids, our data can be accounted for by the formation of a conjugated stabilized 3,4-dehydrovoafrine B iminium ion

All the above NMR data leave no doubt that alkaloid B is 3-hydroxyvoafrine B This compound exists in solution in neutral dry solvents in the two possible diastereomeric forms 3α-hydroxyvoafrine B and 3β-hydroxyvoafrine B These species should have different energies and must be in fast equilibrium through the intermediacy of a reactive and not detectable iminium form In fact, in the presence of excess of water or by heating, the equilibrium is rapidly shifted against the 3α-hydroxy species which must be the more thermodynamically stable The iminium form is of course the unique product in the presence of acids

The 3-hydroxyvoafrines A and B are reduced by borohydride in high yields leading to voafrine A and B Both alkaloids, which were previously only available in extremely small quantities from cultured *Voacanga africana* cells, can now be easily synthesized All these dimeric alkaloids are therefore available in gram quantities and are now being checked for their pharmacological properties The reduction of hydroxyvoafrines can also be achieved enzymatically in the presence of NADPH and *Catharanthus* enzyme and might be therefore indicative for the biosynthesis of voafrine A and B

The spectrum of compound C was even more complex. It turned out, that there was one more tabersonine moiety linked to hydroxyvoafrine B The structure elucidation of this novel alkaloid will be discussed elsewhere

From the enzymatic point of view, we could demonstrate that the formation of the hydroxyvoafrines is completely dependent on oxygen In fact, in the presence of oxygen the highest conversion rate of tabersonine (**6**) to **3** and **4** was observed (78%) In contrast, under a nitrogen atmosphere tabersonine was not metabolized As only 8 or 30% of the starting material was converted in presence of O_2/KCN or O_2/NaN_3, respectively, both CN^- and N_3^- act as inhibitors of the dimerization process Carbon monoxide pretreatment also produced complete inhibition Analysis of the incubation mixtures containing KCN revealed one product besides tabersonine, which was identified as 3α-cyanotabersonine The mass spectrum showed a $[M]^+$ at m/z 361 and the ^1H NMR spectra the presence of a single H-3 at δ 4 82 coupled to H-14 at 5 86 and to H-15 at δ 5 95 with coupling constants of 4 8 and 1 5 Hz, respectively These data agree with those reported earlier for 3α-cyanotabersonine [5] Because oxidative conditions are required for the *in vitro* metabolization of tabersonine and additionally the cyano or acetonyl derivatives are easily formed, an iminium species of tabersonine seems to be

very likely as an intermediate Cyanide trapping has been several times used for indirect evidence of iminium compounds [6–8].

Extensive investigations performed by Rosazza's group on the metabolism of vindoline, which is structurally most closely related to tabersonine, gave comparable results Vindoline was transformed into the enamine dimer and a monomeric precursor to the dimer by the biochemical systems of the bacterium *Streptomyces griseus* [9], by copper oxidases including human ceruloplasmin and by plant and fungal laccases [10] or by horseradish peroxidase [11]. It was postulated that an appropriate iminium derivative is involved in these transformations. Evidence for the existence of this intermediate [9] was obtained when 16-*O*-acetylvindoline was selected as a substrate, because the protected 16-hydroxyl group could not undergo internal etherification and the resulted iminium intermediate could be accumulated [6].

The dimeric hydroxy-voafrines may have been formed through a similar mechanism of dimerization because they exhibit the same coupling positions as found for the vindoline dimer The different structural outcome of the dimerization of vindoline and tabersonine depends on the intramolecular ether formation by the 16-hydroxy in vindoline.

Further investigations implied that the dimerization of the enzymatically produced iminium tabersonine does not necessarily depend on protein catalysis Photochemical experiments with tabersonine demonstrated the formation of the intermediate iminium species by cyanide trapping [5] When omitting potassium cyanide in the reaction mixture only ditabersonines other than the voafrine-typical C-3′, C-14 linkage could be detected as major components In contrast, the generation of iminium tabersonine by oxidation of tabersonine with mercuric acetate or by eliminating the cyanide group of 3-α-cyanotabersonine with silver carbonate yielded the hydroxy-voafrines or their 3-cyano-derivatives, respectively

In conclusion, the above results strongly suggest, that the first step of 3-hydroxyvoafrine production by a crude enzyme preparation of *C. roseus* is the enzyme catalysed formation of iminium-tabersonine No indication could be gained on the mechanism of the subsequent dimerization process Due to the structure of 3-hydroxyvoafrines, other enzyme reactions might be involved in the above process At least for the *Catharanthus* system, the hydroxylated voafrines are the precursors of the corresponding voafrines (**1**) and (**2**). A more facile access to these rare compounds in large amount can now be reached by a combination of enzyme oxidation of tabersonine followed by *in situ* reduction with sodium borohydride

EXPERIMENTAL

General Plant cell suspension cultures were cultivated in 1 l Erlenmeyer flasks on a gyratory shaker at 100 rpm and 25° *Catharanthus roseus* G Don plants were grown under standard greenhouse conditions For TLC Polygram Sil G/UV_{254} plates (0 25 mm, Macherey & Nagel) or 0 5 mm silica gel 60 F_{254} plates (Merck) were used Alkaloids were visualized with ceric ammonium sulphate/H_3PO_4 (CAS) All described alkaloids gave a strong blue colour with CAS EIMS were recorded at 70 eV, FAB-MS were measured using Xe with a beam energy and intensity of 8 keV and 25 μA, respectively ^{1}H NMR analyses were carried out at 360 MHz ^{13}C NMR data were obtained at 90 56 MHz Two dimensional COSY ^{1}H NMR analyses were carried out in DMSO-d_6 (containing 5% D_2O) Chemical shifts (δ) are in ppm, coupling constants (J) in Hz Protein concns were determined by the method of ref [12]

Chromatographic systems The following solvent systems were used for TLC analysis and alkaloid purification, (a) Et_2O–*n*-hexane–MeOH–Et_2NH, 45 45 2 2, (b) Et_2O–*n*-hexane, 1 1, (c) Et_2O–*n*-hexane-MeOH, 25 25 1

Enzyme isolation In a typical procedure, 850 g of mature *Catharanthus roseus* leaves were frozen with liquid N_2 and ground in a mill (Type IKA M 20) The resultant powder was stirred in 1 l K Pi buffer (50 ml, pH 7) with 12 mM β-mercaptoethanol for 1 hr The obtained slurry was filtered through cheesecloth and the filtrate centrifuged at 20 000 × *g* for 30 min To the supernatant $(NH_4)_2SO_4$ was added over 30 min to a final concn of 70% After centrifugation as above, the pptd protein was dissolved in 80 ml K-Pi buffer (0 1 M, pH 7 5) and low M_r contaminations were removed by Sephadex G-25 filtration On average 150 ml crude enzyme soln (protein content, 5 mg/ml) were obtained and used for the synthesis of the dimeric alkaloids

Preparation of 3-hydroxyvoafrines A and B Usually 1 g (2.69 mmol) tabersonine HCl was dissolved in 20 ml MeOH This soln was added under shaking to 800 ml of a dil enzyme soln (0 1 M K-Pi, pH 7 5) containing *ca* 1 2 g of the above isolated protein The mixt was shaken at 25° (100 rpm) under O_2 for 20 hr Alkaloids were then extracted × 4 by adding successive 500 ml portions of CH_2Cl_2 After continued shaking for 10 hr the organic phases were combined and evapd yielding the crude alkaloid mixt This mixt was chromatographed on 0 5 mm silica gel plates in solvent system (a) Major alkaloid containing bands were scraped off and eluted with MeOH After centrifugation the organic solvent was evapd and the residue dried yielding 131 mg (0.191 mmol) 3-hydroxyvoafrine A (14%) and 133 mg (0 194 mmol) 3-hydroxyvoafrine B (15%).

Preparation of voafrine A and voafrine B This was carried out by reducing the appropriate 3-hydroxyvoafrines individually, or as a mixt, in MeOH with excess $NaBH_4$ for 20 min. The reaction mixt was directly subjected to TLC (0 5 mm layer) in solvent system (a) Voafrines A (R_f 0 45) and B (R_f 0 42) were usually obtained in yields between 80 and 90% The synthesis of voafrines also can be carried out in a 'one-pot' reaction, 200 mg (0 54 mmol) tabersonine HCl were transformed with 0 24 g *Catharanthus* protein in 200 ml Pi buffer (0 1 M, pH 7) at 25° under O_2 for 12 hr The incubation mixt was acidified (pH 4) with HOAc and reduced with 0 5 g $NaBH_4$ After neutralization with K_2HPO_4 the voafrines were extracted with CH_2Cl_2 (2 × 200 ml) and chromatographically purified yielding voafrine A 42 mg (23%) and voafrine B 44 mg (24%) The spectroscopic data (MS, ^{1}H NMR), chromatographic properties and optical rotation values were identical with those described for voafrines isolated from *V. africana* cell suspension cultures [2]

Reduction of 3-hydroxy-voafrines is furthermore performed by *Catharanthus* enzyme solns. To 20 ml K-Pi buffer (0 1 M, pH 7, 50 mg protein), a NADPH regenerating system was added (30 mg glucose-6-phosphate (5 mM), 10 mg $NADP^+$ (0 5 mM), 2 5 U glucose-6-phosphate-dehydrogenase). 3-Hydroxyvoafrine A (15 mg) was incubated for 12 hr at 25° From the resulting mixt, 1 2 mg voafrine A (8%) were isolated and identified by ^{1}H NMR. A control incubation without $NADP^+$ did not convert 3-hydroxyvoafrine A

HPLC-Assay for enzymatic conversion of tabersonine (inhibition studies) K-Pi buffer (0 5 ml 0 1 M, pH 7) containing 2 mg prepurified *Catharanthus* protein were preincubated with and

without inhibitor for 10 min, at 30°. Then tabersonine HCl (1 mM) was added and after 5 min the incubation was stopped by extn with EtOAc (0·5 ml). Aliquots of the organic solvent were evapd. The residue was dissolved in MeOH and subjected to HPLC-analysis under the following conditions: column Select B RP 18 (Merck), solvent system MeCN–MeOH–0·1% H_3PO_4, 17 : 10 : 73, gradient to 19 : 5·26 in 15 min, in 5 min to 8 : 1·1 at a flow rate of 1 ml/min. Tabersonine R_t 12 min, voafrines and hydroxyvoafrines R_t 24 min (not sepd), detection at 328 nm. Conversion rates (%) of tabersonine were in presence of O_2 78%, O_2 + 40 mM NaN_3 30%, O_2 + 10 mM KCN 8%, CO before O_2 treatment 0% and N_2 0%.

In vitro formation of derivatives of tabersonine and voafrine. 3α-*Cyanotabersonine*. Tabersonine HCl (100 mg, 0·27 mmol) in 3 ml MeOH were incubated at 30° with 0·3 g *Catharanthus* protein in 100 ml K Pi buffer (0·1 mM, pH 7) in the presence of 16·3 mg (0·25 mmol) KCN for 12 hr under O_2. Extn of the mixt with EtOAc and TLC analysis revealed one major component (R_f 0·44, system b), which was chromatographically purified (26 mg, yield 29%).

3α-Acetonyl tabersonine and 3α-acetonylvoafrine B. Tabersonine HCl (130 mg, 0·35 mmol) was converted under the conditions as described for the cyanocompound but KCN was omitted and replaced by 2·5 ml Me_2CO in the incubation mixt. Sepn and purification was performed on 0·5 mm plates, 3α-acetonyltabersonine, yield 4 mg (R_f 0·4, system b), 3α-acetonylvoafrine B) yield 1 mg (R_f = 0·13, system b).

Physical data of alkaloids

3-*Hydroxyvoafrine A*. Slightly yellow, amorphous $[\alpha]_D^{20}$ −21° ± 5° (MeOH, *c* 0·4). UV λ_{max} 328 (4·48), 300 (4·35), 226 (4·35). EIMS (rel int.) *m/z* 669 $[M-OH]^+$, (15), 668 (18), 442 (9), 347 (31), 335 (19), 228 (58), 213 (15), 194 (42), 182 (21), 168 (100), 167 (83), 154 (19). After reduction with $NaBH_4$ in MeOH the resulting compound showed identical spectroscopic behaviour to a reference sample of voafrine A [1].

3-*Hydroxyvoafrine B*. Slightly yellow, amorphous $[\alpha]_D^{20}$ −68° ± 5° (MeOH, *c* 0·45). UV λ_{max} 328 (4·15), 300 (4·0), 226 (3·97). EIMS (rel int.) *m/z* 669 $[M-OH]^+$, (6), 668 (7), 439 (5), 410 (2), 347 (21), 335 (17), 229 (42), 228 (41), 213 (57), 168 (100), 167 (79), 154 (41). ^{1}H NMR [DMSO-d_6 containing 5% (v/v) D_2O] 7·29, 7·22 (H-9′, 9), 7·10 (H-10′, 10), 5·91 (*d*, *J* = 10·2, H-15′), 5·74 (*dd*, J_1 = 10·2, J_2 = 4·6, H-14′), 5·46 (*s*, H-15), 5·22 (*s*, H_β-3), 4·10 (*d*, *J* = 4·6, H-3′), 3·67, 3·62 (2 × *s*, 2 × COOMe), 3·29 (*br s*, H-21), 3·1–2·9 (*m*, 2H-5, 2H-5′), 2·91 (*br s*, H-21′), 2·57 (*dd*, J_1 = 14·7, J_2 = 1·0, H_α-17′), 2·25 (*dd*, J_1 = 14·7, J_2 = 1·0, H_α-17), 2·14 (*d*, *J* = 14·7, H_β-17), 2·07 (*d*, *J* = 14·7, H_β-17′), 1·94–1·5 (*m*, 2H-6, 2H-6′), 1·0–0·7 (*m*, 2H-19, 2H-19′), 0·65 (*t*, *J* = 7·5, 3H-18′), 0·53 (*t*, *J* = 7·5, 3H-18). ^{1}H NMR (DMSO-d_6): 5·53, 5·48 (2 × *s*, H-15), 5·40 (*d*, *J* = 5·7, OH_α-3), 5·24 (*d*, *J* = 5·7, H_β-3), 4·94 (*s*, H_α-3).

^{1}H NMR (acetone-d_6, containing 5% (v/v) D_2O): 7·4–7·3 (2 × *d*, *J* = 7·4, H-9,9′), 7·15–7·08 (2 × *dd*, J_1 = J_2 = 7·4, H-11, 11′), 7·05–7·0 (2 × *d*, *J* = 7·4, H-12,12′), 6·9–6·8 (2 × *dd*, *J* = 7·4, H-10,10′), 5·95 (*dd*, J_1 = 10·2, J_2 = 1·3, H-15′), 5·77 (*dd*, J_1 = 10·2, J_2 = 4·7, H-14′), 5·60 (*br s*, H-15), 5·42 (*br s*, H_β-3), 4·23 (*d*, *J* = 4·7, H-3′), 3·7–3·6 (2 × *s*, 2 × CO_2Me), 3·47 (*d*, *J* = 1·4, H-21), 3·4–3·0 (*m*, H-5,5′), 3·1 (*d*, *J* = 1·4, H-21′), 2·66 (*dd*, J_1 = 15·1, J_2 = 1·7, H_α-17′), 2·39 (*dd*, J_1 = 14·8, J_2 = 1·7, H_α-17), 2·30 (*d*, *J* = 14·8, H_β-17), 2·15 (*d*, *J* = 15·1, H_β-17′), 2·0–1·7 (*m*, H-6,6′), 1·1–0·8 (*m*, H-19,19′), 0·7–0·6 (2 × *t*, *J* = 7·4, H-18,18′). ^{1}H NMR (acetone-d_6): 5·65, 5·60 (2 × *s*, H-15), 5·43 (*br s*, H_β-3), 5·08 (*s*, H_α-3), 4·3–4·1 (2 × *d*, *J* = 4·7, H-3′). ^{1}H NMR (acetone-d_6 + CF_3CO_2H): 9·7–9·6 (2 × *s*, 2 × NH), 9·47 (*br q*, *J* = 1, H-3), 7·6–6·9 (H-9-12, 9′-12′), 7·51 (*br s*, H-15), 7·34 (*s*, H-15), 6·42 (*dd*, J_1 = 10·2, J_2 = 2·0, H-15′), 6·12 (*dd*, J_1 = 10·2, J_2 = 3·7, H-14′), 5·01 (*br dd*, J_1 = 3·7, J_2 = 2·0, H-3′), 4.90 (*br s*, H-21), 4·79 (*br dd*, J_1 = 13·0, J_2 = 6·9, H-5), 4·68 (*br ddd*, J_1 = J_2 = 13·0, J_3 = 5·8, H-5), 3·74 (*d*, *J* = 1·4, H-21′), 3·7 (2 × *s*, 2 × CO_2Me), 3·7–3·6 (*m*, 2H-5′), 2·81 (*dd*, J_1 = 15·9, J_2 = 1·2, H_α-17), 2·78 (*dd*, J_1 = 16·1, J_2 = 1·4, H_α-17′), 2·50 (*br ddd*, J_1 = J_2 = 13·0, J_3 = 6·9, H-6), 2·40 (*br dd*, J_1 = 13·0, J_2 = 5·8, H-6, overlapped with H_α-6′), 2·31 (*d*, *J* = 15·9, H_β-17), 2·28 (*d*, *J* = 16·1, H_β-17′), 2·15 (*ddd*, J_1 = 12·0, J_2 = 6·4, J_3 = 4·3, H_β-6′), 1·2–1·0 (*m*, H-19,19′), 0·8–0·7 (2 × *t*, *J* = 7·3, H-18,18′).

3α-*Cyanotabersonine*. UV λ_{max} 328 (4·18), 295 (4·12), 247 (4·52) nm. $[\alpha]_D^{20}$ = −354° (MeOH, *c* 0·6). EIMS (rel int.) *m/z* 361 $[M]^+$, (32), 360 (22), 334 (7), 302 (5) 229 (52), 228 (32), 214 (37), 201 (42), 168 (75), 167 (68), 160 (100), 159 (47), 144 (73). ^{1}H NMR (DMSO-d_6): 9·7 (*s*, NH), 7·2–6·9 (H-9,10,11,12), 5·95 (*dd*, J_1 = 9·7, J_2 = 1·5, H-15), 5·86 (*dd*, J_1 = 9·7, J_2 = 4·8, H-14), 4·82 (*dd*, J_1 = 4·8, J_2 = 1·5, H-3), 3·7 (*s*, CO_2Me), 3·06 (*m*, H_α-5), 2·85 (*d*, *J* = 1·4, H-21), 2·79 (*m*, H_β-5), 2·49 (*dd*, J_1 = 14·9, J_2 = 1·4, H_α-17), 2·25 (*d*, *J* = 14·9, H_β-17), 1·91 (*m*, H_β-6), 1·68 (*m*, H_α-6), 0·92 (*m*, H_α-19), 0·78 (*m*, H_β-19), 0·59 (*t*, *J* = 7·3, H-18).

3α-*Acetonyltabersonine*. UV λ_{max} 329, 300, 223 nm. EIMS (rel int.) *m/z* 392 $([M]^+$, 2), 361 (1), 349 (1), 335 (31), 279 (23), 228 (42), 196 (8), 168 (63), 167 (100), 166 (21). ^{1}H NMR (acetone-d_6): 9·4 (*s*, NH), 7·24 (*d*, *J* = 7·5, H-9), 7·13 (*dd*, J_1 = J_2 = 7·5, H-11), 7·03 (*d*, *J* = 7·5, H-12), 6·86 (*dd*, J_1 = J_2 = 7·5, H-10), 5·88 (*dd*, J_1 = 10·2, J_2 = 4·5, H-14), 5·76 (*dd*, J_1 = 10·2, J_2 = 1·3, H-15), 3·99 (*m*, J_1 = 6·0, J_2 = 6·2, J_3 = 4·5, J_4 = 1·3, H_β-3), 3·70 (*s*, CO_2Me), 3·1 (*m*, H-5), 3·09 (*br s*, H-21, 2·98 (*m*, H-5), 2·85 (*m*, CH_2CO), 2·66 (*dd*, J_1 = 15·8, J_2 = 6·2, CH_2CO), 2·58 (*dd*, J_1 = 15·4, J_2 = 1·6, H_α-17), 2·22 (*s*, Ac), 2·18 (*d*, *J* = 15·4, H_β-17), 2·0 (*m*, H_β-6), 1·89 (*m*, H_α-6), 1·05–0·85 (*m*, 2H-19), 0.67 (*t*, *J* = 7·5, H-18).

3α-*Acetonylvoafrine B*. UV λ_{max} 328, 300, 222 nm. EIMS (rel int.) *m/z* 726 $([M]^+$, 3), 670 (3), 669 (3), 440 (9), 336 (10), 355 (28), 229 (25), 228 (72), 213 (13), 196 (25), 169 (31), 168 (100), 167 (33), 154 (16), 121 (9). *m/z* ^{1}H NMR (acetone-d_6): 9·5–9·4 (2 × *s*, 2 × NH), 7·39 (2 × *d*, *J* = 7·4, H-9,9′), 7·13 (2 × *dd*, J_1 = J_2 = 7·4, H-11,11′), 7·05–7·0 (2 × *d*, *J* = 7·4, H-12,12′), 6·96–6·85 (2 × *dd*, J_1 = J_2 = 7·4, H-10,10′) 6·07 (*dd*, J_1 = 10·4, J_2 = 1·1, H-15′), 5·88 (*dd*, J_1 = 10·4, J_2 = 4·8, H-14), 5·66 (*br s*, H-15), 4.70 (*dd*, J_1 = 10·9, J_2 = 3·9, H-3), 4·03 (*br d*, *J* = 4·8, H-3′), 3·7, 3·65 (2 × *s*, 2 × CO_2Me), 3·15 (*br d*, *J* = 1·2, H-21), 3·09 (*br d*, *J* = 1·2, H-21′), 2·9–2·8 (*m*, CH_2CO), 2·35 (*s*, Ac), 1·1–0·8 (*m*, H-19,19′), 0·76, 0·69 (2 × *t*, *J* = 7·4, H-18,18′).

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