3-Oxo-rhazinilam: A New Indole Alkaloid from *Rauvolfia serpentina* × *Rhazya stricta* Hybrid Plant Cell Cultures

Irina Gerasimenko,[†,‡] Yuri Sheludko,[†,‡] and Joachim Stöckigt*[,†]

Department of Pharmaceutical Biology, Institute of Pharmacy, Johannes Gutenberg-University, Staudinger Weg 5, D-55099 Mainz, Germany, and Institute of Cell Biology and Genetic Engineering, National Academy of Science of Ukraine, Zabolotnogo 148, 252143 Kiev, Ukraine

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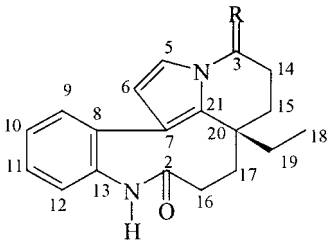
A new monoterpenoid indole alkaloid, 3-oxo-rhazinilam (**1**), was isolated from intergeneric somatic hybrid cell cultures of *Rauvolfia serpentina* and *Rhazya stricta*, and the structure was determined by detailed 1D and 2D NMR analysis. It was also proved that 3-oxo-rhazinilam (**1**) is a natural constituent of the hybrid cells.

In the course of our investigation of new *in vitro* plant systems, such as intergeneric somatic hybrid cell cultures, for the biosynthesis of new and known monoterpenoid indole alkaloids, we have phytochemically analyzed the unique cell culture *RxR*17 of two pharmaceutically valuable species *Rauvolfia serpentina* Benth. ex Kurz and *Rhazya stricta* Decaisne (Apocynaceae),[1−4] which was previously obtained using somatic hybridization techniques.[5] In a recent paper we discussed the results of the phytochemical analysis of *RxR*17K hybrid cell line and reported the identification of 10 monoterpenoid indole alkaloids from this cell culture.[4] Compounds characteristic of *R. stricta* as well as alkaloids typical of *R. serpentina* were detected,[4] suggesting that the biosynthetic pathways of both parental species are expressed in *RxR*17K hybrid cells even after a long period of cultivation (about 10 years) as callus cultures. In addition to known compounds, several other substances were detected in *RxR*17K cells. In this paper we report the isolation and structure elucidation of 3-oxo-rhazinilam, a new monoterpenoid indole alkaloid of the rare rhazinilam type (**1**).

Ring-opened indole alkaloids such as the rhazinilam-type alkaloids have been isolated from several species of the Apocynaceae family (e.g., *R. stricta*, *Aspidosperma quebracho blanco*, *Leuconotis griffithii*, etc.) and are considered to be artifacts formed from an unidentified aspidosperma precursor.[6] The alkaloid rhazinilam (**2**) was cytotoxic.[7] Recent work described the isolation of rhazinilam (**2**) from *RxR*17K hybrid cell suspension cultures.[4] The new compound 3-oxo-rhazinilam (**1**), $C_{19}H_{20}N_2O_2$, exhibited a characteristic UV spectrum with a strong absorption only at 241 nm (log ϵ 3.63). The mass spectrum of **1** displayed close similarity to that of rhazinilam[8] (**2**), but the molecular ion peak and the following intense fragments were shifted upfield by 14 units, indicating a carbonyl group instead of a methylene group. The DI−MS of **1** revealed the molecular ion at *m*/*z* 308 with another significant fragment ion at *m*/*z* 279 [$M^+ - 29$], formed probably by the loss of the ethyl side chain and corresponding to the fragment ion at *m*/*z* 265 of rhazinilam (**2**).[9]

The ^{1}H NMR spectrum in pyridine-d_5 indicated the presence of N−H as a broad singlet at δ 10.00, four aromatic protons (multiplet, H-9−H-12, δ 7.33−7.51), and an ethyl side chain with a methyl triplet at δ 0.62 (3H-18, J = 7.3 Hz). Two adjacent protons, H-5 and H-6 were observed as doublets at δ 7.55 (J = 3.2) and δ 5.94 (J = 3.2), respectively. Although in pyridine-d_5 the signal of H-5 proton interfered with one of the solvent signals, the corresponding doublet was easily recognizable at δ 7.39 (J = 3.2) when CD_2Cl_2 was used as solvent. The low field shift of the H-5 signal, in comparison with that of rhazinilam (**2**) (δ 6. 46 in $CDCl_3$),[9] was caused by deshielding due to the carbonyl group at C-3. This was supported by the ^{13}C NMR spectrum where the C-3 amide carbonyl carbon was observed at δ 168.3. The data described above are in accordance with the ^{1}H NMR data of the other structurally related alkaloid, 3-oxo-14,15-dehydro-rhazinilam (**3**), isolated from cell suspension cultures of *A. quebracho blanco*.[10] H-5 of this alkaloid (**3**) was detected at δ 7.53.[10] The signals of the 10 aliphatic protons of 3-oxo-rhazinilam (**1**) were observed in the area between δ 1.25 and 2.93. In contrast to rhazinilam (**2**),[9] no signals were detected in the low field area from δ 3.0 to 4.0, suggesting that both protons of C-3 amide carbon were substituted by oxygen. Repetition of ^{1}H NMR measurement in CD_2Cl_2 aided in the resolution of H-17 (dd, δ 1.56, J = 12.3, 7.9), H-15β (ddd, δ 1.72, J = 14.1, 5.3, 3.2 Hz), and two multiplets of H-19 protons (δ 1.31 and 1.46).



1 R=O
2 R=H_2
3 R=O, Δ^{14}

The ^{13}C NMR spectra (in CD_2Cl_2) revealed 19 distinct carbon resonances, including two amide carbonyl carbons (C-2 at δ 176.4 and C-3 at δ 168.3), the quaternary carbon (C-20 at δ 38.8), and the high-field resonance at δ 8.1 ascribed to the methyl carbon. The signal at δ 30.1 was assigned to the methylene carbon of the ethyl moiety. The aromatic region displayed 10 resonances, indicating six protonated carbons and four quaternary carbons. The two

* To whom correspondence should be addressed. Tel.: +49-6131-39-25751. Fax: +49-6131-39-23752. E-mail: stoeckig@mail.uni-mainz.de.
† Johannes Gutenberg-University.
‡ Institute of Cell Biology.

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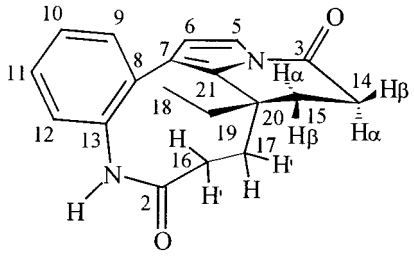

Figure 1. Relative stereochemistry of **1**.

strong signals at δ 116.5 and 115.1 are due to the unsubstituted pyrrole carbons C-5 and C-6, respectively. The four strong resonances between δ 128.1 and 130.8 belong to the unsubstituted benzene carbons C-9−C12. The remaining aromatic signals were assigned to quaternary carbons C-7, C-8, C-13, and C-21. Four signals (δ 28.7, 29.5, 32.2, and 34.0) remained to be designated. The two highest field resonances were attributed to the methylene carbons C-16 and C-14, respectively, adjacent to both carbonyl groups; the other two signals were assigned to C-15 and C-17.

These spectral data suggested a ring-opened indole alkaloid similar in structure to rhazinilam (**2**) but bearing an additional carbonyl group at C-3 (**1**). Confirmation of the proposed structure and of the ^{1}H and ^{13}C NMR chemical shift assignments was accomplished via ^{1}H−^{1}H COSY and HMQC measurements in which one-bond proton−proton and proton−carbon chemical shift correlations, respectively, were established.

To obtain definite evidence for the relative stereochemistry of **1**, measurements of difference NOE were performed. As a result, NOEs were found between 3H-18 and H-9, 2H-19, H-15α, and H-15β and between H-15α and H-15β, 3H-18, H-14α, and H-14β, besides other well-anticipated pairs. The presence of the NOE signal between 3H-18 and H-9 indicated a β-position of the ethyl chain. All these spectral data together with the data concerning the stereochemistry of the known structurally similar alkaloid **2**[11] indicated the relative stereochemistry of **1** to be as shown in Figure 1. As calculated by PCMODEL,[12] the distance between protons of the methyl group and H-9 was close enough (2.69 Å) to exhibit a significant NOE signal after irradiation of 3H-18.

Although the rhazinilam-type alkaloids were assumed to be artifacts produced from an aspidosperma precursor either during isolation of the alkaloids or by prolonged exposure of the plant material to air,[8,9,13] there was an example in which the ring-opened alkaloid leuconolam was proved to be a natural product.[8] In our study, fresh cells (30 g) were harvested, filtered, and immediately extracted with cold MeOH (50 mL) for 15 min. Another portion of the cells was freeze-dried and extracted in the same manner. After HPLC investigation, a peak with retention time (33.2 min) and UV characteristics identical to those of a standard 3-oxo-rhazinilam (**1**) sample was detected in both extracts. The appropriate fractions were collected, and, after freeze-drying and TLC purification, MS-analysis revealed the mass spectrum of 3-oxo-rhazinilam (**1**). Re-investigation of the extracts after storage in MeOH for 1 month at 4 °C showed no change in the alkaloid pattern. Such experiments indicated unequivocally that 3-oxo-rhazinilam (**1**) is a natural constituent of the hybrid cells.

The isolation of the novel monoterpenoid indole alkaloid 3-oxo-rhazinilam (**1**) from *RxR*17K somatic hybrid cell cultures indicates their high potential in the production of new putatively pharmacologically active substances. In addition, the described cell cultures might be an important source for rare alkaloids that could be used as substrates for the detection of new enzymes involved in the biosynthesis of particular alkaloid types such as ring-opened alkaloids. We can also conclude that *RxR*17K cell suspensions retain apparent features of alkaloid biosynthesis reconstruction when compared with the original parental species even after a 10-year period of cultivation.

Experimental Section

General Experimental Procedures. Melting points were determined using a Büchi apparatus and are uncorrected. Optical rotations in $CHCl_3$ were recorded on a Perkin-Elmer 241 MC polarimeter. UV spectra were measured in MeOH using a Perkin-Elmer Lambda 2 spectrophotometer. EIMS measurements were carried out with a quadrupole instrument (Finnigan MAT 44S) at 70 eV. HREIMS was recorded on VG ZAB-2F mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AM 400 instrument with pyridine-d_5 and CD_2Cl_2 as solvents (400 MHz for ^{1}H and 100.6 MHz for ^{13}C). The COSY and HMQC experiments were performed on a Bruker DRX 400 instrument. HPLC was performed with a Merck/Hitachi system connected to a CC 250/4 Nucleosil 100−5 C_{18} column (Macherey-Nagel, Düren, Germany) with the solvent system MeCN−39mM NaH_2PO_4−2.5mM hexanesulfonic acid buffer (pH = 2.5), gradient 15:85 → 20:80 within 5 min, → 60:40 within 40 min, → 80:20 within 15 min, 1 mL/min flow rate and detection at 243 nm. Flash chromatography was performed with Si gel 60 (230−400 mesh) (Merck, Darmstadt, Germany). For TLC precoated 0.5-, 0.25-, and 0.2-mm Si gel 60 F_{254} plates, 20 × 20 cm (Merck, Darmstadt, Germany) were used. Alkaloids were detected by their absorption at 254 nm; the plates were sprayed with ceric ammonium sulfate (CAS) reagent.

Plant Material. The *RxR*17K hybrid cell suspensions were cultivated in 1-L Erlenmeyer flasks containing 350 mL of liquid LS-medium[14] at 25 °C and 100 rpm (gyratory shaker) in diffuse light (600 lux) for a 14-day cultivation period.

Extraction and Isolation. For alkaloid extraction we used the protocol of Smith et al.[15] with minor modifications: freeze-dried cells (125 g) were extracted with 12.5 L of MeOH under sonication for 30 min at 40 °C. After filtering, the extract was evaporated to dryness under vacuum at 40 °C. The residue was taken up in 500 mL of 50 mM bicarbonate buffer (pH 10.0) and partitioned three times into equal volumes of EtOAc. Organic fractions were bulked and evaporated under vacuum at 40 °C yielding 2.4 g of crude extract. The extract was then fractionated by flash chromatography on 140 g of Si gel using solvent system Me_2CO−petroleum ether−diethylamine (7:2:1). After TLC testing, fractions revealing appropriate CAS reactions were combined and evaporated. The residue was chromatographed over Si gel plates with the solvent systems EtOAc−C_6H_6 (2:1, R_f = 0.21) and $CHCl_3$−cyclohexane−diethylamine (6:3:1, R_f = 0.49) sequentially, and this yielded in 4.5 mg of pure 3-oxo-rhazinilam (**1**).

3-Oxo-rhazinilam (1): white powder; mp 201−204 °C; $[\alpha]^{25}_D$ −247.2° (*c* 0.3; $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 241 (3.63); ^{1}H NMR (pyridine-d_5, 400 MHz) δ 10.00 (1H, s, N−H), 7.55 (1H, d, J = 3.2 Hz, H-5), 7.50 (1H, d, J = 7.3 Hz, H-9), 7.33−7.47 (3H, m, H-10−H-12), 5.94 (1H, d, J = 3.2 Hz, H-6), 2.88 (1H, ddd, J = 18.2, 13.5, 5.3 Hz, H-14α), 2.65 (1H, dd, J = 13.2, 12.3 Hz, H-17′), 2.60 (1H, ddd, J = 18.2, 4.7, 3.2 Hz, H-14β), 2.51 (1H, dd, J = 14.1, 12.3 Hz, H-16′), 2.19 (1H, dd, J = 14.1, 7.9 Hz, H-16), 1.91 (1H, ddd, J = 14.1, 13.5, 4.7 Hz, H-15α), 1.50 (1H, ddd, J = 14.1, 5.3, 3.2 Hz, H-15β), 1.47 (1H, m, H-19′), 1.46 (1H, dd, J = 13.2, 7.9 Hz, H-17), 1.29 (1H, m, H-19), 0.62 (3H, t, J = 7.3 Hz, H-18); ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 7.27−7.45 (4H, m, H-9−H-12), 7.39 (1H, d, J = 3.2 Hz, H-5), 6.68 (1H, s, N−H), 5.95 (1H, d, J = 3.2 Hz, H-6), 2.91 (1H, ddd, J = 17.9, 13.5, 5.3 Hz, H-14α), 2.66 (1H, ddd, J = 17.9, 4.7, 3.2 Hz, H-14β), 2.38 (2H, m, H-16′, H-17′), 2.11 (1H, ddd, J = 14.1, 13.5, 4.7 Hz, H-15α), 2.03 (1H, dd, J =

12.3, 7.9 Hz, H-16), 1.72 (1H, ddd, $J = 14.1, 5.3, 3.2$ Hz, H-15β), 1.56 (1H, dd, $J = 12.3, 7.9$ Hz, H-17), 1.46 (1H, m, H-19′), 1.31 (1H, m, H-19), 0.72 (3H, t, $J = 7.3$ Hz, H-18); ^{13}C NMR (CD_2Cl_2, 100 MHz)[16] δ 176.4 (s, C-2), 168.3 (s, C-3) 138.0 (s, C-13),[17] 137.7 (s, C-21),[17] 134.1 (s, C-8), 130.8 (d, C-11), 129.3 (d, C-9),[17] 128,1 (d, C-10),[17] 128,1 (d, C-12), 122.5 (s, C-7), 116.5 (d, C-5), 115.1 (d, C-6), 38.8 (s, C-20), 34.0 (t, C-17), 32.2 (t, C-15), 30.1 (t, C-19), 29.5 (t, C-14), 28.7 (t, C-16), 8.1 (q, C-18); EIMS *m*/*z* (rel int %) 308 $[M]^+$ (17), 279 $[M - CH_2CH_3]^+$ (100), 251 (45), 237 (15), 223 (25), 195 (15); HREIMS *m*/*z* 308.1535 (calcd for $C_{19}H_{20}N_2O_2$, 308.1524); 279.1141 (calcd for $C_{17}H_{15}N_2O_2$, 279.1133), $[M - CH_2CH_3]^+$.

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(17) These values may be interchanged.

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