ISOLATION OF TWO NEW NITROGENOUS METABOLITES FROM THE CULTURED CELLS OF ASPIDOSPERMA QUEBRACHO-BLANCO

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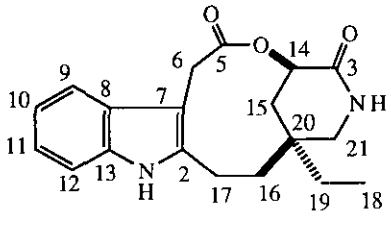
Abstract Two novel nitrogen containing metabolites were isolated from cultured cells of *Aspidosperma quebracho-blanco* Their structures were elucidated as 11-hydroxytubotaiwine and 1,6-propano-3-ethylideno-1,4-piperazine-2,5-dione.

Aspidosperma quebracho-blanco (Apocynaceae) attracts the continuing interest of natural product chemists because of its importance as the folk medicine employed in South American countries and its production of a rich variety of monoterpenoid indole alkaloids.[1] The importance of this plant species stimulated us to develop tissue and cell suspension cultures. We succeeded in establishing a cell strain which shows steady growth as a fine cell suspension in liquid media.[2]

In our previous paper[2] we described the isolation and structure determination of two new monoterpenoid indole alkaloids from these cultured cells. Aspidochibine (**1**) was found to be a new type of Aspidosperma alkaloid which is considered to be biosynthesized from a member of quebrachamine class of alkaloids through oxidation at C-5, hydrolytic cleavage of the resulting amide linkage between C-5 and N_b, and macrocyclic lactone formation between the C-5 carboxylic acid and the hyroxyl group on C-14 The stereochemistry and the conformation of this molecule were also studied. Another new



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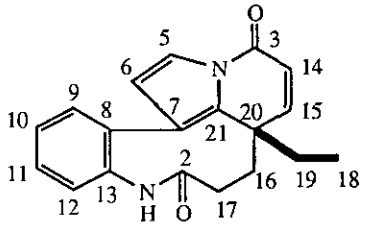

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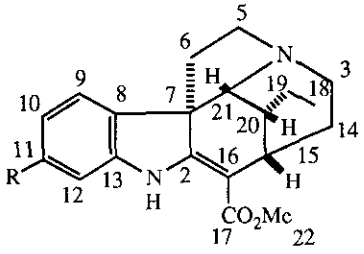
compound was 14,15-dehydro-3-oxo-rhazinilam (**2**), an alkaloid with a highly advanced oxidation level and belonging to a very small group of alkaloids (rhazinilams)

In our further studies on the metabolites of the cultured cells of *Aspidosperma quebracho-blanco* we have now isolated two new nitrogen containing metabolites and determined their structures

The compound AQC-20 (**3**) (2.2 mg) was isolated from a basic fraction of the methanol extract obtained from the lyophilized cultured cells of the named plant. The production yield of **3** was almost comparative to those of **1** and **2** in the cultured cells The high resolution ms revealed the molecular formula of **3** to be $C_{20}H_{24}N_2O_3$ The uv spectrum showed the absorption maxima at 255 and 328 nm which clearly indicated the presence of the β-anilinoacrylic ester chromophore The fragmentation pattern of EI-ms of **3** strongly suggested that the molecule is a member of tubotaiwine group of compounds[3] having one additional oxygen to the molecule of tubotaiwine (**5**).

The comparison of the ^{13}C-nmr spectra of AQC-20 with that of tubotaiwine[4] (**5**) revealed their close similarity The signals of the carbons of the non-aromatic part of **3** were almost superimposable on those of tubotaiwine This observation clearly indicated that the additional oxygen is located on the aromatic ring as a phenolic hydroxyl group Next the location of the hydroxyl group was clarified In the ^{1}H-nmr spectrum an aromatic proton was observed at δ 6.46 as a singlet The corresponding carbon showed the chemical shift of δ 95.9 ppm, a shift position which indicates a strongly shielded aromatic ring carbon. The location of the oxygen at C-11 is in excellent agreement with such a shielding effect to the neighboring position. Moreover, close similarity of the aromatic carbon shifts of **3** with the described carbon shifts for 11-methoxytubotaiwine[5] (**6**) strongly supported this structural assignment.



R = OH (**3**)

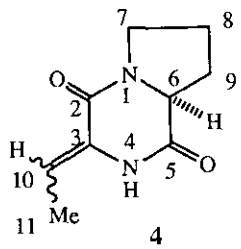
R = H (**5**)

R = OMe (**6**)

Tubotaiwine has been found to be a constituent of many Apocynaceous plants and has also been isolated from some cell suspension cultures. It was found in suspension cultures of *Stemmadenia tomentosa* Grenman var. *plmeri*,[6] *Tabernaemontana divaricata* (L) R. Br *ex* Roem *et* Schult , and *T. iboga* Baill.[7] The stereochemistry of the molecule, in particular the configuration of the chiral center at C20 was unsettled for many years From a broad survey of described isolations of tubotaiwine and by means of NOE, ^{13}C-H coupling constants and protonation shifts it was concluded that all natural tubotaiwines possess *S* configuration at C-20.[4] Close similarity of the shift values and coupling constants of the carbons and protons around C-20 together with the big dextrorotatory $[\alpha]_D$ value strongly indicated that AQC-20 (**3**) belongs to the same stereochemical group as the known natural tubotaiwines. All nmr-data were in complete agreement with the structure (**3**) as illustrated

The $CHCl_3$ eluant from SiO_2 column purification of the basic fraction and further purification by mplc (medium pressure liquid chromatography) gave the second cellular metabolite, AQC-14 (**4**). High resolution ms measurement indicated that the molecular formula was $C_9H_{12}N_2O_2$. The ^{1}H-nmr disclosed the presence of an ethylidene group by the doublet for the methyl

group at δ 1.75 (J= 7 4 Hz) and a quartet for the vinyl H at δ 6.13 (J= 7.4 Hz). Furthermore a series of seven protons on four sequential carbons was observed. The last proton, which was found at δ 7.45 as a broad singlet, was exchangeable with D_2O This proton was reasonably ascribable to an amide NH These observations are consistent with the dioxopiperazine structure (**4**) as shown.



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The uv spectrum possessing an absorption maximum at 224 nm also strongly supports the depicted structure which carries a conjugated amide chromophore [8] All the nmr-data, including the ^{13}C-values, clearly support the deduced structure. It is most likely that this molecule has been formed through condensation of L-proline and L-threonine followed by elimination of one molecule of water In accord to this view the absolute configuration at C-6 is safely posturated as shown The configuration of the double bond remains to be determined At this moment, however, we can not exclude the possibility that 4 is a secondary product resulting from condensation of the corresponding amino acids under the isolation conditions

EXPERIMENTAL

The nmr spectra were run on a JEOL JNMA500 or a JNM-GSX400 instrument in $CDCl_3$ with tetramethylsilane as an internal standard. The mass spectrometer used for the measurements of EI- and FAB-ms was a JEOL JMS HX-110. For measurment of FAB-ms *m*-nitrobenzyl alcohol was used as the matrix. Adsorbent for open column chromatography was silica gel G, Merck. For flash column chromatography silica gel 60, Merck, 230-400 mesh, was used. A prepacked column Si-5, Kusano Kagaku Kikai Co. was used for medium pressure liquid chromatography Plant cell suspension culturers were performed in the LS and 4X- medium as previously described [2]

Isolation of AQC-20 (3) and AQC-14 (4)

Extraction of the cultured cells (98.2 g) with hot methanol (2 l, 1.5 l, 1.5 l) for each 24 h gave the crude extract (25.4 g), which was then dissolved in 1N-HCl After being washed with AcOEt the aqueous layer was basified with conc NH_4OH. Extraction with $CHCl_3$ gave a crude residue (129 mg) Flash column chromatography separation of the residue gave a syrup on elution with $CHCl_3$. Repeated separation of the syrupy residue gave AQC-14 (**4**) as a homogeneous material (3 mg) Fractions eluted from the above open column with 20-40 % MeOH in $CHCl_3$ were combined. Separation of the residue with repeated medium pressure liquid chromatography afforded AQC-20 (**3**) (2 2 mg) as a homogeneous syrup.

AQC-20 (11-Hydroxytubotaiwine) (3): An amorphous powder, $C_{20}H_{24}N_2O_3$ High resolution FAB-ms, Found m/z, 341.1872; Calcd for $C_{20}H_{25}N_2O_3$ $(M + H)^+$; m/z 341 1865 Uv λmax (EtOH), 255 (log ε 3 78) and 328 (log ε 3.87) nm. λmax (EtOH + NaOH), 285 nm. $[\alpha]_D$ +207° (c=0 04, EtOH) EI-ms m/z (%); 340 (M^+, 11), 283 (5), 245 (11), 196 (11), 183 (8), 167 (5), 154 (6), 115 (15), 95 (33), 83 (39), 71 (100). ^1H-Nmr (400MHz, $CDCl_3$) δ, 2 94 (ddd, J= 12.1, 12 1, 5.0

Hz, H-3), 3 60 (m, H-3), 3.16 (ddd, *J*= 11.5, 7 3, 2.4 Hz, H-5), 3 60 (m, H-5), 2.19 (m, H-6), 2.76 (ddd, *J*= 14.1, 11.5, 7.5 Hz, H-6), 7.02 (d, *J*= 8.4 Hz, H-9), 6 46 (d, *J*= 8 6 Hz, H-10), 6 46 (s, H-12), 1 97 (m, H-14), 2.15 (m, H-14), 3 23 (br s, H-15), 0.69 (3H, dd, *J*= 7.2, 7 2 Hz, 18-Me), 0 85 (2H, m, H-19), 2.19 (m, H-20), 4.43 (br s, H-21), 3.80 (3H, s, COOMe), 8.75 (s, NH), 12.4 (br s, OH). ^{13}C-Nmr (100 MHz, $CDCl_3$) δ, 160.0 (C-2), 43 9 (C-3), 53 7 (C-5), 45.2 (C-6), 54.4 (C-7), 128 7 (C-8), 120 2 (C-9), 107.5 (C-10), 156 2 (C-11), 98.4 (C-12), 144 9 (C-13), 28.4 (C-14), 30.8 (C-15), 95.9 (C-16), 170.9 (C-17), 11.6 (C-18), 23 8 (C-19), 41 2 (C-20), 65.3 (C-21), and 51 1 (C-22).

AQC-14 (1,6-Propano-3-ethylideno-1,4-piperazine-2,5-dione) (4) · An amorphous powder. $C_9H_{12}N_2O_2$. High resolution EI-ms; Found 180.090, Calcd for $C_9H_{12}N_2O_2$ (M^+); 180 090 Uv λmax (EtOH); 224 nm. ^{1}H-Nmr (500 MHz, $CDCl_3$) δ; 1.75 (3H, d, *J*= 7.4 Hz, 11-Me), 6.13 (q, *J*= 7.4 Hz, 10-H), 4.19 (dd, *J*= 10.2, 6.6 Hz, 6-H), 3.58 (ddd, *J*=12 4, 9 3, 3.0 Hz, 7a-H), 3.7 (ddd, *J*= 12 5, 8.3, 8.3 Hz, 7b-H), 1 9 - 2 1 (3H, m, 8a, 8b, 9b-H), 2 44 (dd, *J*= 10.4, 6.4 Hz, 9a-H), 7 45 (br s, NH). ^{13}C-Nmr (100MHz, $CDCl_3$) δ; 158.0 (C-2), 128.8 (C-3), 166.1 (C-5), 59.0 (C-6), 45.3 (C-7), 21.8 (C-8), 28.9 (C-9), 113 3 (C-10), 11 0 (C-11).

ACKNOWLEDGEMENTS

We like to thank Prof W E Court (Mold) for linguistic advice. Our thanks are also due to the Ministry of Education, Science and Culture, Japan (International Scientific Research Program, Joint Research, No. 04044035 and a Grant-in-Aids for Scientific Researches; No 06453187), the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg (SFB 145) and to the Fonds der Chemischen Industrie (Frandfurt/Main) for financial support

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Received, 4th July, 1994