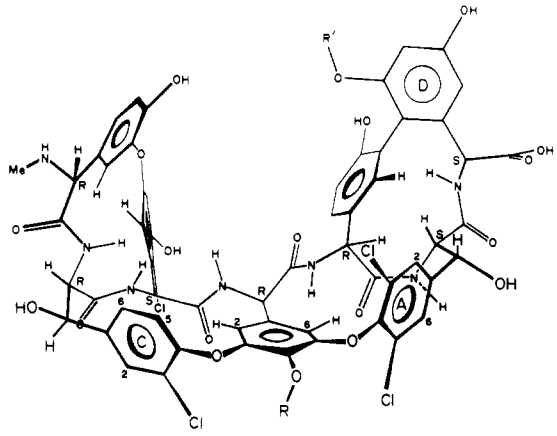

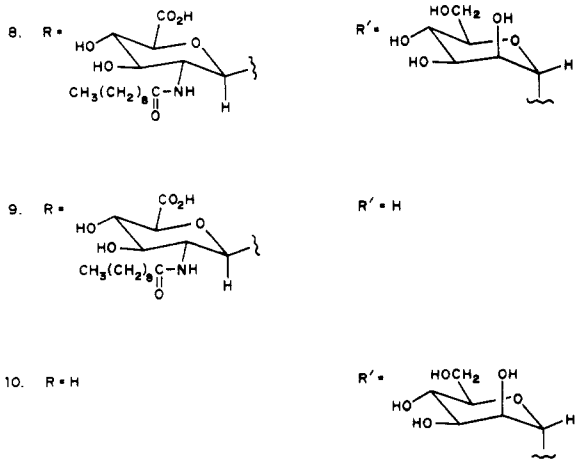

spectrum containing strongly coupled, overlapping resonances of the sugar ring protons (see Figure 4A). Also, the latter occur in the same region as the strong H_2O signal from the solvent, which is largely suppressed under the conditions of the double quantum excitation.[47] The resulting spectrum obtained at pH 5.6 contained seven amide NH signals that could be identified and assigned from double quantum cross peaks to neighboring α-CH protons. Most interestingly, a clear coupling pattern was evident for each of the protons in the two sugar rings in the contour plot of the 2D double quantum spectrum of **8**, a segment of which is shown in Figure 4B.

In double quantum transitions, pairs of spins i and j resonate at the sum of their corresponding chemical shift frequencies $\sigma_i + \sigma_j$. These transitions are indirectly observed via the allowed single quantum transitions of both participating nuclear spins. Hence, in double quantum spectra, pairs of peaks appear in F_1, which are centered around the axis $(2F_1,F_2)$, with coordinates of $\sigma_i + \sigma_j$ in F_1 and σ_i and σ_j in F_2.

In the upper part of the depicted portion of the double quantum spectrum, these pairs of peaks are clearly visible for the double quantum transitions of (Sm2, Sm3), (Sm3, Sm4), and (Sg3, Sg4). In other cases, particularly in the lower part of the figure, only one of the observed double quantum peaks is visible, and the complementary peaks appear at chemical shift positions in F_2 outside the displayed range, e.g., σ_{SgNH} is at 7.83 ppm.

Conclusion

The structures of aridicin A and its aglycon are derived as **8** and **2**, respectively, from studies based upon a variety of two-dimensional NMR methods. The NMR results, particularly those obtained from 2D NOE spectra, provide approximate intramolecular hydrogen distances that, when used as distance constraints, provide the expeerimental data for interactive computer-assisted modeling to derive the three-dimensional structure of the aglycon **2**. Elaboration of the remaining details of the parent antibiotic is accomplished by further NMR studies in which delayed COSY, NOESY, and double quantum methods are employed.

The methods described here not only should prove especially useful in providing detailed structural information on new members of this class of molecules but also should be readily applicable to other classes of complex structures.

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Stereochemistry and Total Synthesis of Amavadin, the Naturally Occurring Vanadium Compound of *Amanita muscaria*

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Abstract: The structure and stereochemistry of the vanadium compound of *Amanita muscaria*, amavadin, are elucidated, and a total synthesis of amavadin is reported. It is shown to be the monohydrated complex of VO^{2+} with two molecules of *N*-(L-1-carboxyethyl)-*N*-hydroxy-L-alanine. The ligand belongs to a new type of complexing agents. Amavadin is the first naturally occurring vanadium compound of which the structure of an organic ligand has been established and synthesized.

Vanadium recently has attracted increasing interest in biochemistry.[1,2] Of the numerous effects of vanadium on organisms reported, its stimulatory effect on the growth of algae and plants[3] and the inhibitory action of vanadate(V) on Na,K-ATPase[4] are especially worth mentioning. However, there is still little knowledge about the structure and biological function of naturally

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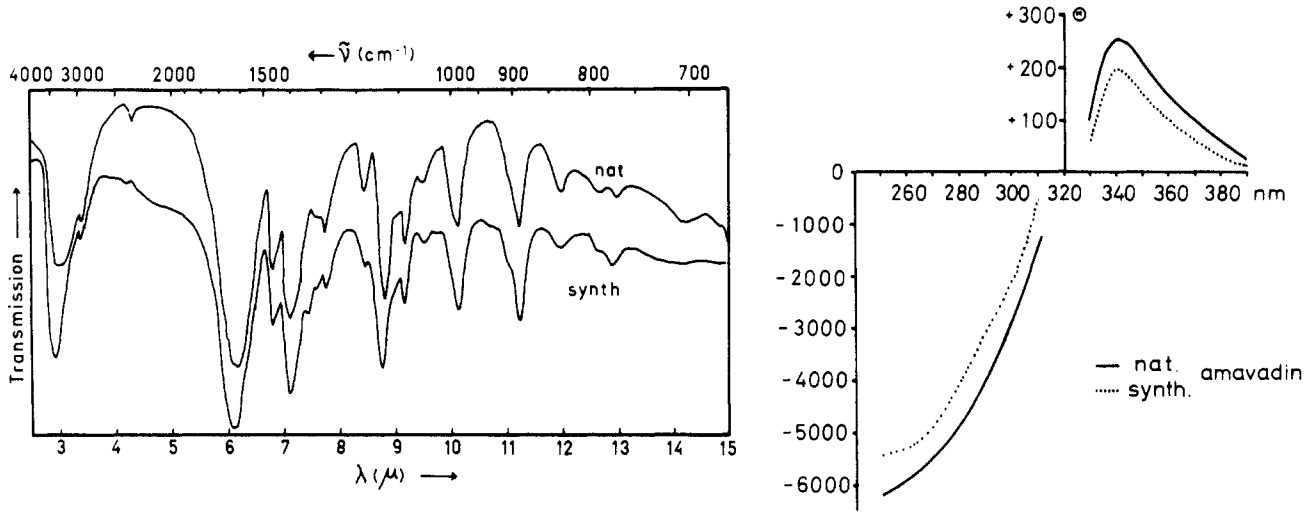


Figure 1. Infrared (left) and circular dichroism spectra (right) of natural and synthetic amavadin.

occurring vanadium compounds. Whereas the occurrence of vanadium in living organisms usually does not exceed 5–10 ppm, accumulation of vanadium is known in tunicates (up to 10 000 ppm)[5] and in the mushroom *Amanita muscaria* (up to 200 ppm).[6,7] No defined organic ligand has been isolated from the vanadium compound of tunicates.

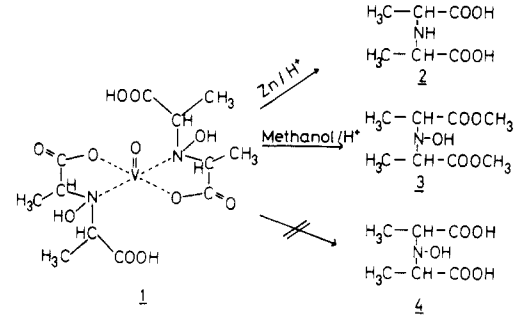
We have previously reported on the isolation of the vanadium compound from *Amanita muscaria*, which we called "amavadin"[8] (**1**). On the basis of preliminary data it had been proposed that **1** is the complex of oxovanadium(IV) with two molecules of *N*-(1-carboxyethyl)-*N*-hydroxyalanine (**4**).[9,10] However, the question concerning the configuration of the two chiral carbon atoms remained to be confirmed. Finally, proof of this structure by total synthesis of the naturally occurring amavadin with the correct stereochemistry had to be achieved, because all attempts to crystallize amavadin for X-ray crystallography so far have failed.

We now report on the determination of the stereochemistry of the ligand and synthesis of optically active amavadin.

Results

For the assignment of the stereochemistry of the ligand **4** of amavadin two compounds are available (Scheme I). The reduction of amavadin by zinc dust resulted in the formation of *N*-(1-carboxyethyl)alanine (**2**). After methanolysis of amavadin, the methyl ester **3** of the ligand can be obtained. Both compounds isolated from natural amavadin contain the two asymmetric carbon atoms and can exist in the form of the L, D, and meso isomers. The imino compound **2** has been already described in the literature.[11] All three isomers were synthesized and compared with the product obtained from amavadin. The methyl esters of the D and L enantiomers could not be separated by gas chromatography on an optically active phase (Chirasil-Val[12]). The separation of the enantiomers was, however, achieved by the use of

Scheme I



isopropyl isocyanate as derivatizing agent. By comparison of the retention times, the degradation product of amavadin was shown to be the L enantiomer. This indicated that also in amavadin the asymmetric carbon atoms have an L configuration.

The stereoisomers of the ligand **4** had not been described in the literature prior to this work. Our earlier report[9] on the synthesis of **4** as well as the recent report by Felcman[13] did not discuss the occurrence of isomers.

Total Synthesis of Amavadin. For the synthesis of **4** either of two ways seemed feasible: the direct oxidation of the imino compound **2** or a synthesis by starting from hydroxylamine and D-2-bromopropanoic acid. In the latter case a stereospecific synthesis of the L enantiomer could be expected, avoiding racemate separation.

First attempts to obtain **4** by oxidation of **2** were not successful and, therefore, the condensation reaction of 2-bromopropanoic acid with hydroxylamine in the presence of sodium carbonate was chosen. The separation of **4** from the reaction mixture was easily achieved by precipitation after addition of zinc acetate and the zinc ion removed by cation exchange, yielding pure **4**. By starting from D-2-bromopropanoic acid the product contained mostly the L enantiomer of **4** but also considerable amounts of the meso form. Purification could be achieved by preparative ion-pair HPLC. The structure was confirmed by NMR spectra. The purity of the synthesized L enantiomer was analyzed by capillary gas chromatography of its dimethyl ester on Chirasil-Val.[12] The recrystallized product contained less than 10% of the D enantiomer and about 2% of the meso isomer. Retention times in gas chromatography of the dimethyl ester of the synthesized L enantiomer of **4** and of the corresponding compound obtained from amavadin were identical.

To prove that the synthesized *N*-(1-carboxyethyl)-*N*-hydroxyalanine had indeed the L configuration in both asymmetric centers, it was reduced with retention of its configuration. The

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resulting *N*-(1-carboxyethyl)alanine, after derivatization with isopropyl isocyanate, gave GC retention times on Chirasil-Val identical with that of authentic *N*-(L-1-carboxyethyl)-L-alanine.

From **4** the synthesis of amavadin could be performed easily by adding the stoichiometric amount of vanadyl sulfate. The synthesized product and amavadin isolated from *Amanita muscaria* showed good agreement in all analytical and spectroscopic data like infrared, ESR, UV, and the circular dichroism spectra (Scheme I; Figure 1).

Discussion

Amavadin is the first naturally occurring vanadium compound isolated in pure form of which the structure has been elucidated. The ligand **4** forms a very stable complex with vanadium, which obviously is a main reason for the accumulation of vanadium in *Amanita muscaria*. *N*-(Carboxyalkyl)-*N*-hydroxy amino acids with **4** as its first representative could, therefore, be expected to form an interesting new class of chelating agents. A certain structural relationship with ethylenediaminetetraacetic acid (EDTA) is obvious. However, the complex stabilities of **4** for vanadium are several orders of magnitude higher for VO^{2+} as compared with EDTA–VO^{2+} chelates. The properties of amavadin show no similarity to these of the vanadium compounds of tunicates.

Until now, nothing is known about the biological function and the biosynthesis of amavadin. The deoxygenated organic ligand of amavadin **2** has recently been described as a natural product ("alanopine") from marine invertebrates.[14,45] Other opines are known from crown gall tumors where they are involved in the ecology of the plant pathogen *Agrobacterium tumefaciens*.[16] Opines are generally formed under the action of certain dehydrogenases and reduced nicotinamide adenine dinucleotide by the reaction of pyruvate with amino acids and other substrates. In the case of L-alanine, *meso*-alanopine is formed and, therefore, the alanopine from marine invertebrates is different from the L-alanopine obtainable from amavadin.

Experimental Section

Amavadin was isolated as described before.[8]

***N*-(L-1-Carboxyethyl)-L-alanine.** A sample of 43.7 mg of amavadin (0.1 mmol) was reacted under stirring with 75 mg of Zn dust in 2 mL of a 1:1 mixture of acetic acid and water for 12 h and then the resultant mixture subjected to ion-exchange chromatography [column size, 30 × 1.5 cm; Dowex 50W-X8, 200–400 mesh; equilibrium and elution with pyridine (0.1 M)/formic acid (0.4 M) in water]. *N*-(1-Carboxyethyl)-alanine eluted after 15 mL. After evaporation of the solvent, the product was converted into the trimethylsilyl derivative by heating it with 100 μL of bis(trimethylsilyl)trifluoroacetamide at 100 °C in a screw-cap vial. GC separation was achieved on a 25-m quartz capillary column (i.d. 0.32 mm) CpSil5 (Chrompack) at 120 °C with He as carrier gas. The product appeared after 14 min, and its mass spectrum was identical with that of the bis(trimethylsilyl) derivative of *N*-(L-1-carboxyethyl)-L-alanine synthesized according to literature.[11] Mass spectrum (from GC/MS, not corrected: *m/e* 305 (M^+, 2%), 290 ($-CH_3$, 6), 262 (3), 188 ($-COO{:}Me_3Si$, 100), 172 (15), 75 (12), 73 (17), 70 (55).

Determination of the Configuration of *N*-(1-Carboxyethyl)alanine (2). **2** was derivatized with isopropyl isocyanate as described by Koenig et al.[17] The reaction product was separated by gas chromatography on a glass capillary column (20 m × 0.28 mm) coated with Chirasil-Val[12] [temperature program, 80 °C (3 min), 4 °C/min to 200 °C; carrier gas, hydrogen; inlet pressure, 40 kPa]. The meso form of **2** appeared after 23.31 min, the L form after 25.15 min, and the D form after 25.31 min. CI-mass spectra (methane as ionizing agent) showed a molecular peak at *m/e* 270, indicating that a cyclization product had been formed.

Methanolysis of Amavadin. One milligram of amavadin was dissolved in a solution of 0.02 mL of concentrated H_2SO_4 in 1 mL of methanol in a vial with a Teflon-lined screw cap. The reaction mixture was kept for 1 h at 65 °C. After addition of 1 mL of H_2O, the solution was made alkaline by the addition of $NaHCO_3$ and extracted with ether. The ether extract was dried over sodium sulfate, concentrated by a stream of N_2, and subjected to GC/MS (20-m quartz capillary with Chirasil-Val[12] [temperature program, 70 °C (2 min), 4 °C/min to 120 °C; carrier gas, H_2]. The main product eluted after 11.5 min. Mass spectrum for the dimethyl ester of **4**: *m/e* 205 (M^+, 2%), 186 (3), 146 ($M - COOCH_3$, 77), 130 (8), 128 (5), 114 (17), 100 (6), 87 (10), 86 (14), 70 (28), 60 (18), 59 ($-COOCH_3$, 100), 55 (27), 42 (38).

Synthesis of *N*-(L-1-Carboxyethyl)-*N*-hydroxy-L-alanine. A sample of 1.4 g of $NH_2OH{\cdot}HCl$ (0.02 mol) and 9.2 g of D-bromopropionic acid[18] (0.06 mol) were dissolved in 50 mL of water. Na_2CO_3 (13.5 g) was slowly added. In the beginning, CO_2 evolution caused foaming. The mixture was heated at 80 °C for 2 h under N_2. After cooling, the precipitate was removed by filtration and the filtrate brought to pH 4 by the addition of 6 M HCl and reacted with 40 mL of an aqueous solution of zinc acetate [10 g of $Zn(CH_3COO)_2{\cdot}2H_2O$ in 100 mL]. Yield of the crude zinc complex was 1.75 g (36.5%). (The complex contained 1 mol of **4**/1 mol of Zn.) The precipitate was dissolved in about 50 mL of 0.2 M HCl, and the solution was applied onto a column (30 × 1 cm) filled with Dowex 50W-X8 (H^+) that was previously equilibrated with 0.1 M HCl. Elution was achieved with 0.05 M NaOH. The product appeared after elution of about 30 mL, and its presence was detected by the pH value (about 1.8) and by isotachophoresis. After lyophilization, 720 mg (20.2%) of a white crystalline powder was obtained. This product contained about 40% of the meso form as a byproduct and had to be purified by HPLC.

HPLC Separation of L- and *meso*-*N*-(1-Carboxyethyl)-*N*-hydroxyalanine. The HPLC system consisted of a Waters M 6000 A pump and U6K injector, a preparative HPLC column (Knauer, RP 18, 7 μm, 25 × 1.6 cm), and a LKB UV detector with a 8-μL flow cell and an interference filter to measure the light absorption at 206 nm. Eluant (0.25 mL) (25 mmol of NaH_2PO_4, 5 mmol of *n*-heptanesulfonic acid, 2.5 mL of methanol, and H_3PO_4—to obtain pH 2.2—in 1 L of water) containing 50 mg of raw product was injected and separated at room temperature with a flow rate of 6 mL/min. The meso form was collected from 16.5 to 18.5 min (peak at 17.5 min) and the L form from 20 to 25 min (peak at 21 min). The collected fractions of several runs were purified by application onto an ion-exchange column and elution with 0.05 M NaOH as described above. A recrystallization of the lyophilizate was possible by solution in a small volume of 90% ethanol and cooling to −30 °C: yield 105 mg (2.9%); mp 145–146 °C dec; ^1H NMR (D_2O) δ 1.43 (d, 6 H), 4.05 (q, 2 H); IR (KBr) 705 (s), 755 (w), 793 (m), 873 (m), 890 (m), 952 (m), 969 (m), 1059 (m), 1080 (s), 1105 (s), 1164 (w), 1120 (s, br), 1260 (m), 1315 (m), 1370 (s), 1400–1410 (s, br), 1580–1610 (m, br), 1745 (s), 2500 (br), 2940 (w), 2980 (w), 3420 (br) cm^{-1}. Anal. Calcd for $C_6H_{11}NO_5$: C, 40.68; H, 6.26; N, 7.91. Found: C, 40.70; H, 6.72; N, 7.94.

Synthesis of Amavadin. A sample of 35.4 mg (0.2 mmol) of **4** (L-isomer) and 50.6 mg (0.2 mmol) of $VOSO_4{\cdot}5H_2O$ were dissolved in 1 mL of water. $BaCO_3$ (60 mg, 0.3 mmol) was added. After shaking, was centrifuged and the supernatant subjected to ion-exchange chromatography on a small column (10 × 0.5 cm) was Dowex 50W-X8 (H^+) and with water as eluant. The blue fraction of amavadin, which was eluted at the front, was finally lyophilized; yield 33 mg (79%). Anal. Calcd for $C_{12}H_{22}N_2O_{12}V$: C, 32.96; H, 5.07; N, 6.41, V, 11.65. Found: C, 32.77; H, 4.69; N, 6.06; V, 11.8. (Vanadium was determined by atomic absorption spectroscopy.)

Acknowledgment. We thank E. Koch for valuable discussions and G. Nicholson for performing the gas chromatographic and mass spectrometric analyses.

Registry No. 1, 12705-99-6; **2**, 92283-80-2; **2** bis(trimethylsilyl) derivative, 101756-84-7; **3**, 101670-93-3; **4**, 50825-12-2; $NH_2OH{\cdot}HCl$, 5470-11-1; D-bromopropionic acid, 10009-70-8.

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