SEQUENCE DETERMINATION OF ACTAGARDINE, A NOVEL LANTIBIOTIC, BY HOMONUCLEAR 2D NMR SPECTROSCOPY

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By combination of several ^{1}H NMR techniques, the sequence of actagardine has been elucidated. It has been shown that it is a tetracyclic 19-residue peptide antibiotic. It differs from the previously described lanthionine-containing peptide antibiotics belonging to the lantibiotic class.

Actagardine† (**1**), an antibiotic produced by fermentation of *Actinoplanes* strains ATCC 31048 and ATCC 31049,[1~3] belongs to the group of lanthionine-containing antibiotics to which the common name "lantibiotics" has been proposed.[4] So far only two members of this class of antibiotics have been fully characterized by NMR spectroscopy.[5,6]

Actagardine is a heterodetic polypeptide formed by 19 amino acids, 8 of them forming 1 lanthionine and 3 β-methyllanthionine units through intramolecular thioether bridges. Some physico-chemical properties were presented previously.[3] The sequence elucidation described in this paper was carried out by ^{1}H NMR experiments on actagardine (**1**)[3] and its 3,3-dimethylamino-1-propylamide on the terminal carboxyl (as monohydrochloride) (**2**), whose preparation is reported in the accompanying paper.[7]

The following 2D NMR techniques were used: Double quantum filter (DQF)-COSY,[8] relayed-COSY,[9] total correlation spectroscopy (TOCSY),[10] NOESY[11] and DQF-relayed-NOESY.[5]

Several solvents and solvent mixtures as DMSO, DMSO-benzene, water-DMSO were used to record NMR spectra of actagardine and its derivative **2** in the temperature range of 37~50°C. Unfortunately, under all these measurement conditions the resonance lines were quite broad and the amide proton resonances hardly resolved. However, the NMR spectra of 25 mg actagardine or **2** dissolved in a mixture of 0.4 ml DMSO, 0.1 ml phosphate buffer pH 7.38, 0.05 ml hexafluoro-2-propanol and some drops of CF_3COOH recorded at 39°C showed improved resolution and most of the amide protons were resolved in the 2D spectra.

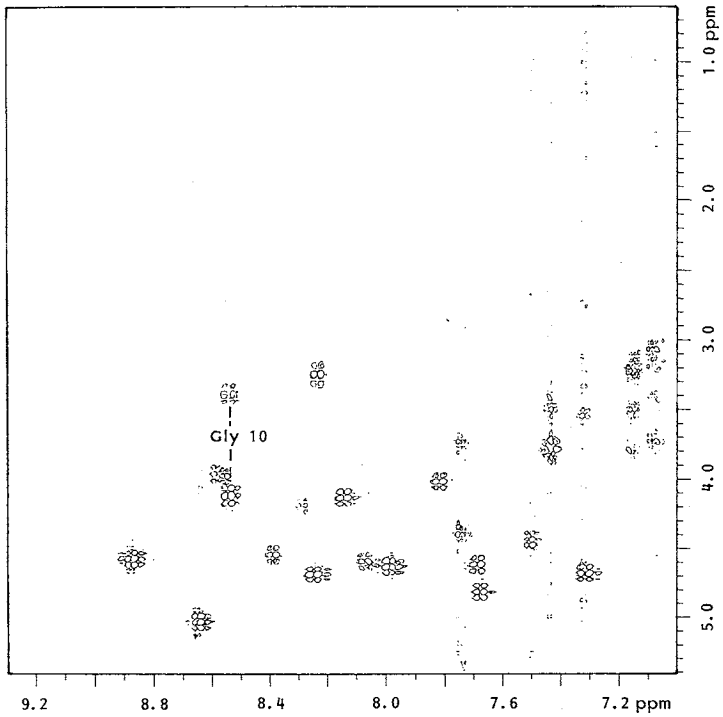
Assignment of the Individual Amino Acid Spin Systems

The amino acid composition of actagardine determined by hydrolysis and physico-chemical characterization[1,3] is reported in Table 1. Three amino acid spin systems occur only once, Ala, Glu and Leu. Three spin systems Ile, Val and Gly appear twice, while the α-aminobutyric acid (Abu) part of the β-methyllanthionine units appears three times. Finally, the amino acids lanthionine, the Ala part of the β-methyllanthionine units, Ser and Trp give rise to 7 ABMX spin systems.

Fig. 1 shows the NH-HC$_\alpha$ region of the DQF-COSY spectrum, showing only 18 NH-HC$_\alpha$ connectivities out of the 22 which are expected according to Table 1 if the spin systems of the Gly units are not overlapped.

† Actagardine is the recommended International Nonproprietary Name (WHO) of the antibiotic previously designated as gardimycin.[1]

Fig. 1. NH, HC_α region of 500 MHz DQF-COSY spectrum.



This might be due to the fact that the amide resonances are still quite broad and that the peptide is not cyclic.

Severe overlapping of the HC_α resonance in the DQF-COSY spectra did not allow the assignment of the amino acid spin systems unambiguously except for the Gly-10.

Relayed-COSY and TOCSY experiments (Fig. 2), relaying magnetization further from the NH proton *via* HC_α to HC_β and HC_γ protons allowed the unambiguous assignment of the spin systems of the two Val and the three Abu moieties.

Table 1. Amino acids and chiralities.

Amino acids	Symbol	Number of units	Chirality
Alanine	Ala	1	L
Glutamic acid	Glu	1	nd
Glycine	Gly	2	
Isoleucine	Ile	2	L
Lanthionine	Ala, Ala	1	nd
Leucine	Leu	1	L
β-Methyllanthionine	Abu, Ala	3	nd
Serine	Ser	1	nd
Tryptophan	Trp	1	nd
Valine	Val	2	L

nd: Not determined.

For Leu and Ile none of these connectivities were detected, but an assignment of their spin systems could be partially done by reference to the NOE data.

Major difficulties were encountered in attributing the 7 ABMX systems to their individual amino acids and to determine the site of the sulfur bridges in the β-methyllanthionine and lanthionine moieties.

The spin system of the Trp unit was easily determined by the long range crosspeaks from the HC_{indole} residue to the HC_β protons.

The DQF-relayed-NOESY spectra allowed the determination of the site of the 4 sulfur bridges and so to discriminate between the Ala parts of the β-methyllanthionine units (see Fig. 3). After the NOE between the HC protons of the Abu and Ala part of the β-methyllanthionine units the magnetization is transferred

Fig. 2. Relayed-COSY spectrum: Some NH-HC_α-HC_β connectivities are shown.

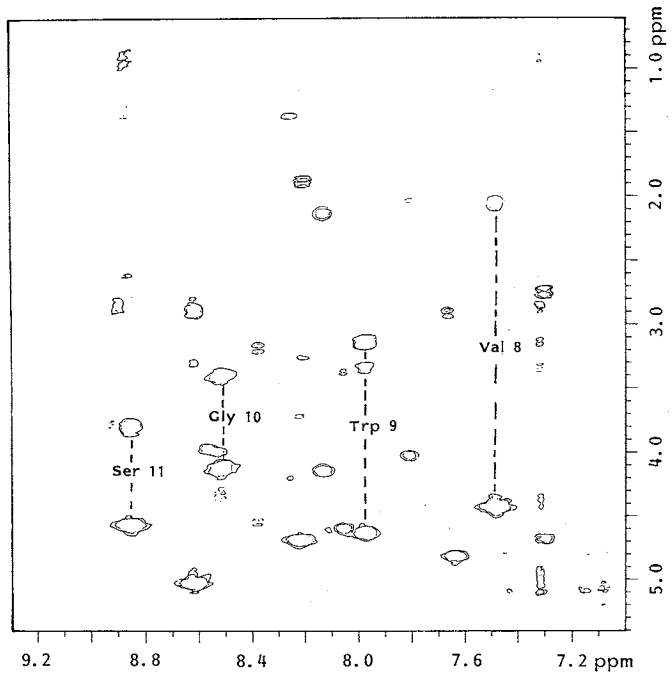
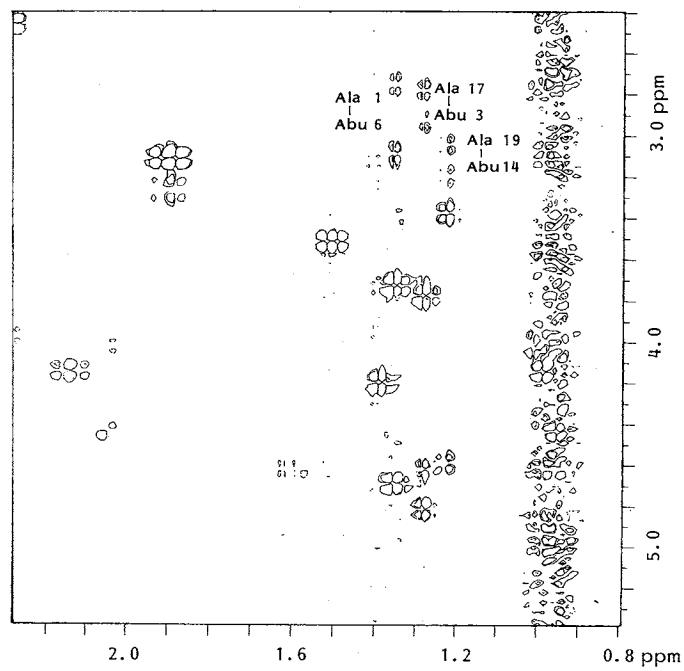


Fig. 3. High-field part of the DQF-relayed-NOESY spectrum.
Identification of the sulfur bridges of the three β-methyllanthionine units.



in the relay step *via* scalar coupling to the H_3C_γ groups of the Abu residue.

These H_3C_γ groups show now two crosspeaks to the H_2C_β protons of the Ala part, leading unambiguously to their side chain connectivities, as the original NOE is transferred into a less crowded spectro-

Table 2. ^{1}H NMR chemical shifts (δ ppm, DSS=0) in DMSO, phosphate buffer pH 7.38, hexafluoro-2-propanol, T=39°C.

Amino acid	HN	HC_α	HC_β	Others
1 Ala	7.30	4.74	3.19, 2.79	—
2 Leu[a]	8.06	4.17	1.54	nd
3 Abu	7.73	4.81	3.76	HC_γ 1.26
4 Ile[a]	8.58	4.12	1.82	H_3C_γ 0.92, H_2C_γ 1.78, H_3C_δ 0.89
5 Glu	8.60	4.03	2.31, 2.12	HC_γ 2.50
6 Abu	8.24	4.71	3.73	HC_γ 1.33
7 Ala	8.67	5.05	3.33, 2.96	
8 Val	7.52	4.44	2.11	HC_γ 0.97
9 Trp	7.99	4.70	3.38, 3.19	HN_{indole} 11.12, HC_{indole} 7.38, 7.80, 7.50, 7.19, 7.11 H-arom
10 Gly	8.57	4.16, 3.40		
11 Ser	9.82	4.53	4.73	
12 Ala	n.d.	4.38	3.30, 2.88	
13 Gly	n.d.	4.04, 3.80		
14 Abu	8.08	4.58	3.33	HC_γ 1.23
15 Val	8.14	4.17	2.13	HC_γ 0.95
16 Ile[a]	7.92	3.97	2.01	H_2C_γ 1.24, H_3C_γ 0.98
17 Ala	7.71	4.68	2.93, 2.76	
18 Ala	8.32	4.14	1.37	
19 Ala	8.39	4.53	3.21, 3.03	

[a] Partly assigned by NOE.
nd: Not determined.
Signals of the 3,3-dimethylamino-1-propylamido group of compound **2**: HN 8.16; H_2C_α 3.93, 3.76; H_2C_γ 3.64; H_2C_β 2.01; 2 H_3C 2.83 ppm.

scopical region. The same connectivities are found for the ABMX spin system at NH_δ 8.5 ppm with the H_2C group of Ala-12 connecting the two Ala parts of the lanthionine moiety, leaving only one ABMX system without any side chain connectivity which has to be attributed to Ser.

The above assignments of the individual amino acid spin system are collected in Table 2.

Sequence Assignments by NOESY

Three types of through space interactions or dipolar couplings are generally used for the sequence determination of polypeptides and proteins: HN_i-$HC_{\alpha i+1}$; HN_i-$HC_{\beta i+1}$ and HN_i-HN_{i+1}.

A part of the NOESY spectrum is shown in Fig. 4 and the results of the NOE experiments and the obtained amino acid sequence are presented in Fig. 5. One critical point in the sequence analysis is the lack of any information about the connectivity Ala-12→Gly-13. The only indirect proof of the existence of this peptide bond is the exclusion of all other possibilities.

Except for the middle chain amino acid sequence Ala-7—Ala-12 no regular secondary structure elements could be detected by inspection of the NOE's and all data show that in all the solvent mixtures used actagardine has a random coil conformation.

FAB-MS Studies

Previous data and the present detailed NMR analysis of actagardine suggest the molecular formula $C_{81}H_{124}N_{20}O_{23}S_4$. FAB-MS analysis (Fig. 5) to confirm the molecular mass of this antibiotic gave, however, a surprising result: The found mass was 16.0 dalton higher than expected, and a similar difference was observed for amide **2**. These seem to be the real molecular masses and not artifacts in FAB-MS, based on various points:

Fig. 4. NOESY spectrum: As example the sequence Val-8→Trp→Gly→Ser-11 is shown.

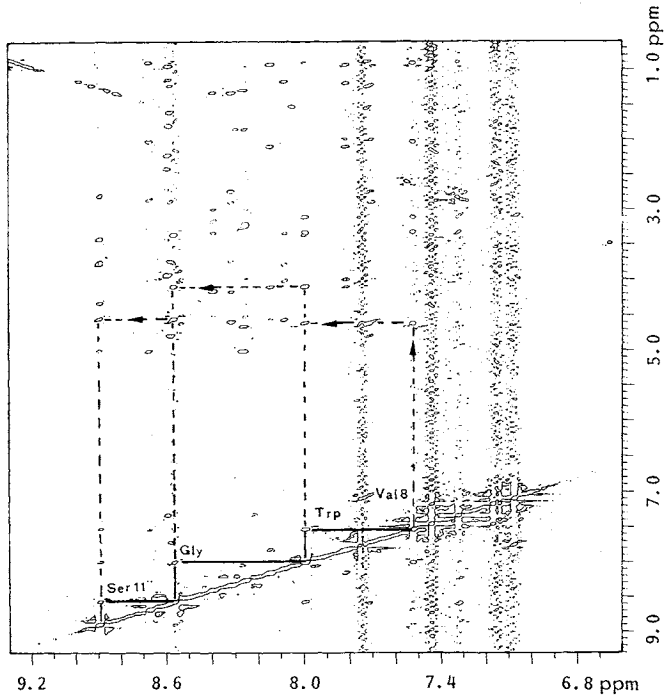
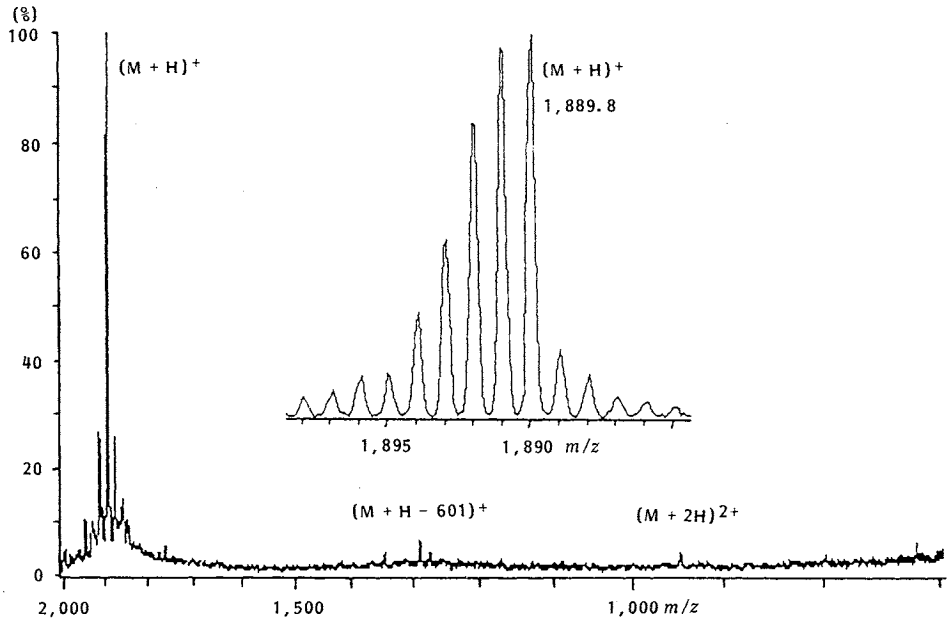


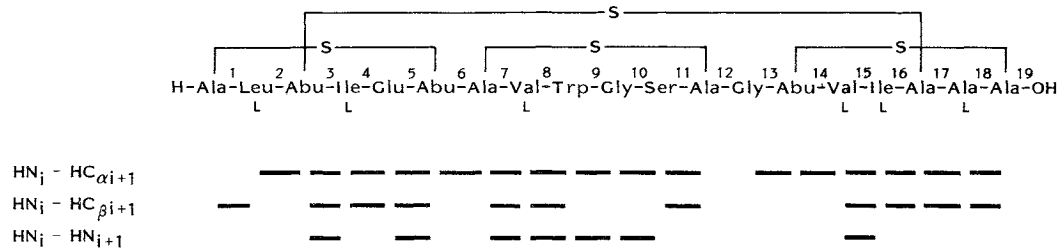
Fig. 5. FAB-MS spectrum of actagardine in thioglycerol matrix.
The molecular ion part of the spectrum is also shown on an expanded scale.



i) There is no example in the literature of an interference peak 16 dalton higher than the expected $(M+H)^+$ in FAB.

ii) Under various experimental conditions the spectra did not show a significant time dependence.

Fig. 6. Sequence of actagardine and correlations for the determination of sequential connectivities by NOESY experiments.



Positive ion FAB-MS spectra in glycerol, thioglycerol, nitrobenzyl alcohol matrices all gave an $(M+H)^+$ ion at *m/z* 1,889.8 (mass for the lowest isotope), 16 dalton higher than expected, together with a smaller $(M+Na)^+$ ion at *m/z* 1,911.8.

iii) Negative ion FAB-MS was also obtained showing an $(M-H)^-$ ion at 1,887.8.

iv) FAB-MS results on other lanthionine antibiotics, taken from the literature,[4,12,13] indicated the correct molecular weight. As a further control, nisin, whose mass spectra have been recently investigated,[14] showed on our spectrometer the expected $(M+H)^+$ ion.

These results demonstrate that one more oxygen is present in the actagardine, and that the elemental formulae of actagardine and its amide **2** are $C_{81}H_{124}N_{20}O_{24}S_4$ and $C_{86}H_{136}N_{22}O_{23}S_4 \cdot HCl$, respectively.

In the FAB-MS spectrum of actagardine there are very few fragment ions. One small but characteristic fragment formed by loss of 601 dalton from both actagardine and **2** showed the loss of the amino acid sequence between Ala-7 and Ala-12 containing one sulfur bridge.

Conclusions

Modern highfield NMR-techniques proved to be a powerful tool for the structure elucidation of actagardine, which is characterized by quite broad NMR lines in every solvent mixture used so far, thus avoiding amino acid sequencing which showed to be particularly troublesome due to the resistance to enzymatic cleavages and to the bad reactivity toward the desulfurization treatment required for the Edman degradation.

Actagardine (Fig. 6) differs from the lantibiotics listed by KELLNER *et al.*,[4] and in particular from the 19-membered compounds cinnamycin, Ro 09-0198, duramycin, ancovenin[4] and the recently described lanthiopeptin[13] and mersacidin.[15]

Experimental

The NMR spectra were recorded in the temperature range from 37 to 50°C on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer. Prior to FT the time domain data were multiplied with phase-shifted sine-bell windows and extended with zero-filling.

DQF-COSY Spectrum

Sequence: D1-90°-t_1-90°-D2-90°-t_2; D1 = 1.5 s; D2 = 3 μs; 90°-pulse = 11.5 μs; size = 2K; 512 increments with 32 transitions each; acquisition time = 170 ms.

Relayed-COSY Spectrum

Sequence: D1-90°-t_1-D2-180°-D2-90°-t_2; D1 = 1.5 s; D2 = 18 ms; 90°-pulse = 11.5 μs; size = 2K; 512 increments with 48 transitions each; acquisition time = 170 ms.

TOCSY Spectrum

With MLEV-17 cycle: D1=2.1 s; 90°-pulse=31 μs; 180°-pulse=62 μs; D2=70~100 ms; size=2K; 512 increments with 48 transitions each; acquisition time=170 ms.

NOESY Spectrum

Sequence: D1-90°-t_1-90°-D9-90°-t_2; D1=2.1 s; D9=180±20 ms; 90°-pulse=11.5 μs; size=2K; 512 increments with 32 transitions each; acquisition time=170 ms.

DQF-relayed-NOESY Spectrum

Sequence: D1-90°-t_1-90°-D9-90°-D2-180°-D2-90°-D3-90°-t_2; D1=2.4 s; D9=300±20 ms; D2=18 ms; D3=0.9 μs; 90°-pulse=11.5 μs; size=2K; 512 increments with 64 transitions each; acquisition time=170 ms.

Mass Spectrometry

FAB spectra were recorded on a Kratos MS-50 double focusing mass spectrometer using fast Xe atoms (at 6 kV, 1 mA). Data were acquired using a DS-90 software package in raw data acquisition mode. Calibration was performed with a CsI-NaI salt mixture; mass accuracy is better than ±0.2 dalton.

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