RAMOPLANIN (A-16686), A NEW GLYCOLIPODEPSIPEPTIDE ANTIBIOTIC

IV. COMPLETE SEQUENCE DETERMINATION BY HOMONUCLEAR 2D NMR SPECTROSCOPY†

J. K. KETTENRING, R. CIABATTI, G. WINTERS, G. TAMBORINI and B. CAVALLERI

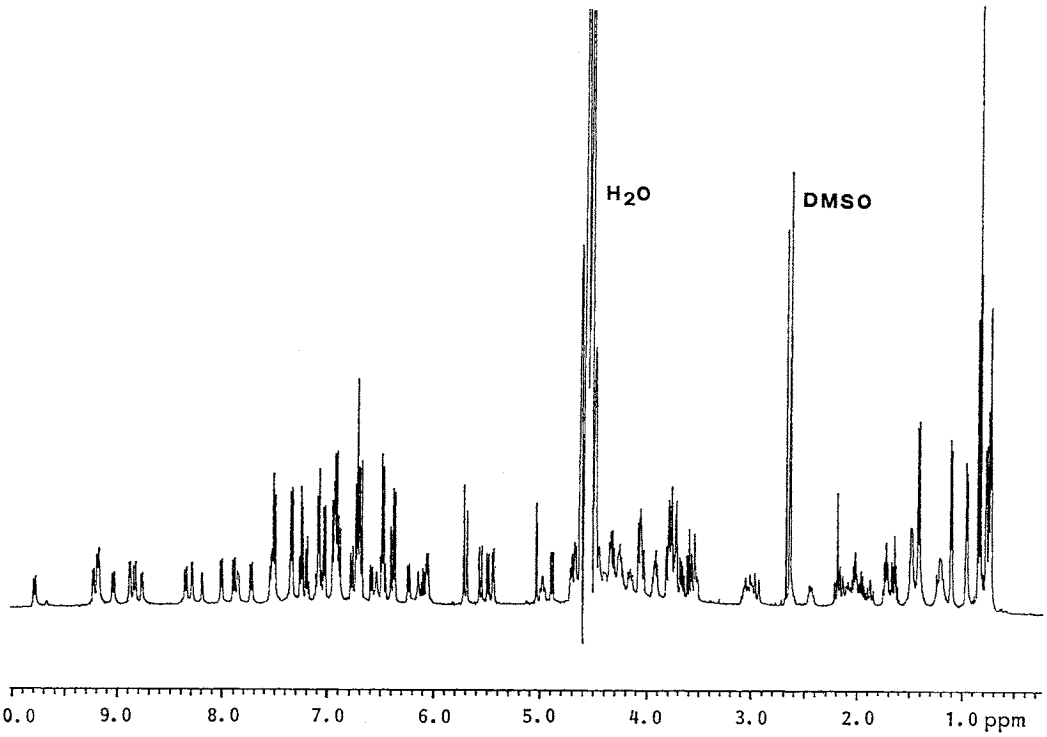
Merrell Dow Research Institute-Lepetit Research Center, 21040 Gerenzano (VA), Italy

(Received for publication August 9, 1988)

Homonuclear 2D NMR spectroscopy double quantum filter correlation spectroscopy (DQF-COSY), relayed-COSY, nuclear Overhauser enhancement spectroscopy (NOESY), and DQF-relayed-NOESY) allowed the complete determination of the core depsipeptide of antibiotic ramoplanin (A-16686). In particular, the DQF-relayed-NOESY experiments were essential in assigning the single signals close to the diagonal.

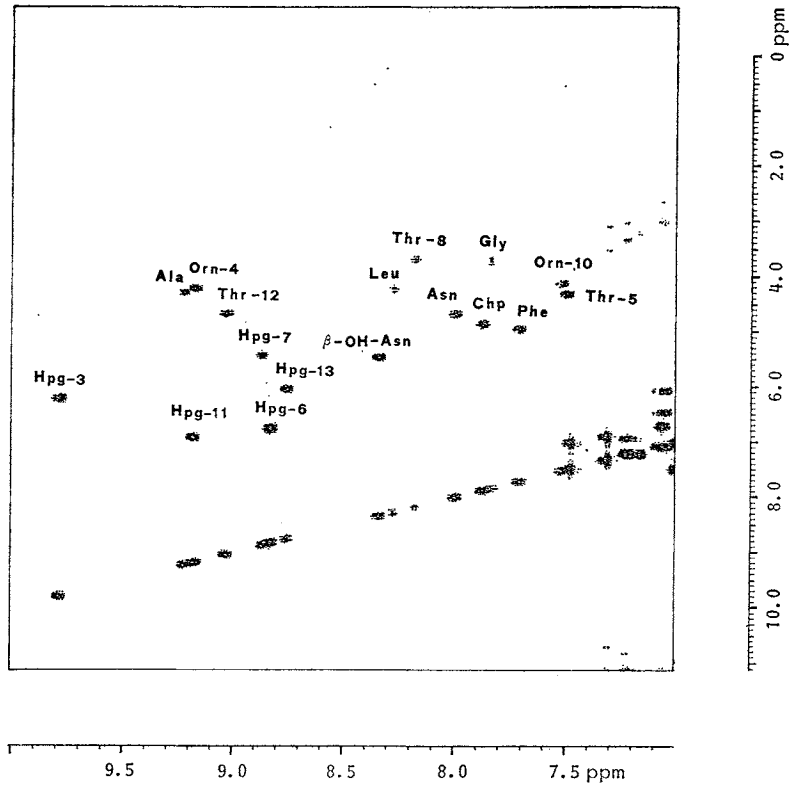
Ramoplanin (A-16686) is a new antibiotic produced by *Actinoplanes* sp. ATCC 33076. The taxonomy of the producing organism, the production, isolation and properties of the antibiotic were previously described.[1,2] The antibiotic was shown to consist of three components designated A1,

Fig. 1. ^1H NMR spectrum (500 MHz) of A-16686 factor A2 in H_2O - DMSO (4 : 1) at pH 4.6, 40°C.



† Some of these data were presented at the 2nd International Symposium on "New Bioactive Metabolites from Microorganisms", Gera (GDR), May 2, 1988.

Fig. 2. NH, HC_α region of 500 MHz DQF-COSY spectrum of A-16686 factor A2.



A2 and A3 by reversed-phase high pressure liquid chromatography (HPLC).[1]

Chemical and spectroscopic studies revealed that the three factors are closely related. They possess the same cyclic depsipeptide skeleton formed by seventeen amino acids, carrying a dimannosyl unit linked by a semiacetalic bond to a 4-hydroxyphenylglycine (Hpg), and differ only in the presence of a diunsaturated fatty acid residue acylating the free NH_2 group of a terminal asparagine unit.[3]

Factor A2 was obtained[3] as a dihydrochloride freely soluble in water. The molecular formula $C_{119}H_{154}ClN_{21}O_{40}\cdot 2HCl$ (MW 2,627.02) was assigned by fast atom bombardment (FAB)-MS. It is characterized by a 7-methylocta-2,4-dienoic acid chain.

The ^1H NMR experiments presented in this paper were carried out on factor A2 using the following 2D NMR techniques: Double quantum filter correlation spectroscopy (DQF-COSY),[4] relayed-COSY,[5] COSY with enhancement of long range couplings,[6] nuclear Overhauser enhancement spectroscopy (NESYO)[7] and DQF-relayed-NOESY.[8]

The ^1H NMR spectrum of factor A2 can be divided in several characteristic spectral regions. Thus, the aromatic and the NH-protons resonate between 6.5 and 10 ppm, the amino acid protons as well as the olefinic protons between 4 and 6 ppm and the aliphatic amino acid protons in the range 0.7~3.5 ppm (Fig. 1).

The individual amino acid spin systems are:

a) Aliphatic amino acids: The scalarly coupled spin systems were identified in a DQF-COSY experiment. In cases where severe overlapping of several resonances occurred and prevented the

Fig. 3. Relayed-COSY spectrum of A-16686 factor A2 (40°C).
Some NH-HC_α-HC_β connectivities are shown.

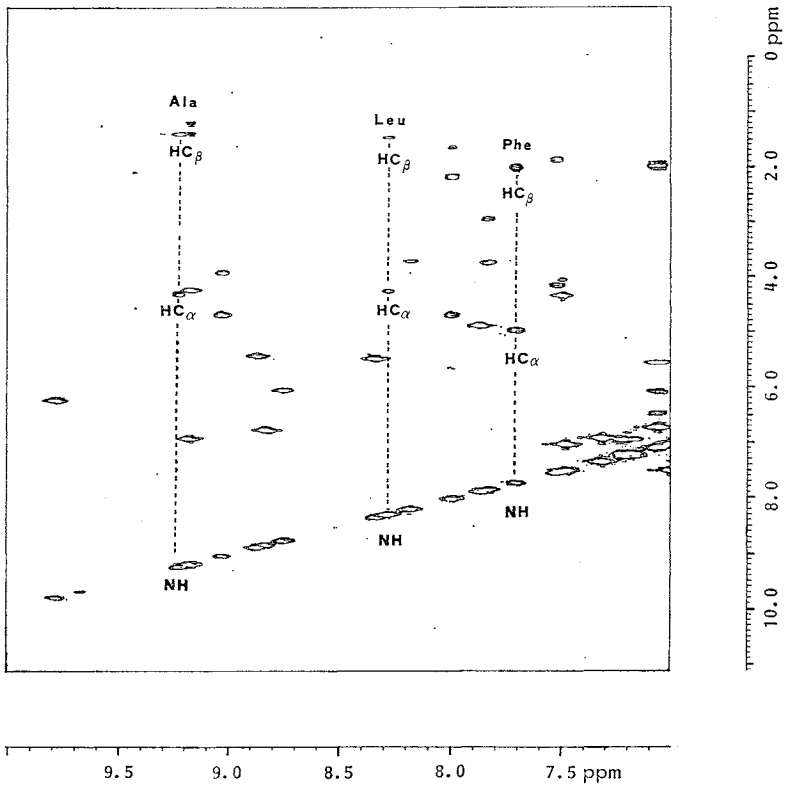


Table 1. ^1H NMR chemical shifts (δ, TMS=0) of A-16686 factor A2 in H_2O - DMSO (4:1) at pH 4.6, 40°C (see Fig. 6).

Amino acid	HN	HC_α	HC_β	Others
1 Asn	8.02	4.76	2.17, 1.62	—
2 β-OH-Asn	8.35	5.49	5.69	—
3 Hpg	9.78	6.21	—	Phenyl 7.46 (b, f), 7.02 (c, e)
4 Orn	9.18	4.21	1.42, 1.28	HC_γ 1.18 HC_δ 2.68, 2.43
5 Thr	7.50	4.36	4.03	HC_γ 1.04
6 Hpg	8.83	6.78	—	Phenyl 6.82 (b, f), 6.36 (c, e)
7 Hpg	8.88	5.44	—	Phenyl 6.67 (b, f), 6.45 (c, e)
8 Thr	8.18	3.71	3.92	HC_γ 0.82
9 Phe	7.73	4.97	2.04	Phenyl 7.22 (b, f), 6.92 (c, d, e)
10 Orn	7.53	4.20	2.18, 1.89	HC_γ 1.67, HC_δ 3.02
11 Hpg	9.18	6.94	—	Phenyl 7.34 (b, f), 6.95 (c, e)
12 Thr	9.03	4.72	3.94	HC_γ 0.95
13 Hpg	8.78	6.09	—	Phenyl 7.08 (b, f), 6.70 (c, e)
14 Gly	7.85	3.76, 2.99	—	—
15 Leu	8.28	4.26	1.47	HC_γ 1.47, HC_δ 0.74
16 Ala	9.22	4.32	1.41	—
17 Chp	7.87	4.91	—	Phenyl 6.83 (f), 6.51 (b), 6.36 (e)

Dimannosyl moiety: 5.70, 5.02 anomeric protons, 4.01, 3.91~3.71, 3.60~3.51
7-Methylocta-2,4-dienoyl residue: 5.52 (HC_α), 6.12 (HC_δ), 6.25 (HC_β), 7.26 (HC_γ), 2.00 (CH_2), 1.67 (HC_ζ), 0.84 (two CH_3)

Fig. 4. Correlations for the determinations of sequential connectivities in A-16686 factor A2 by NOESY and DQF-relayed-NOESY experiments.

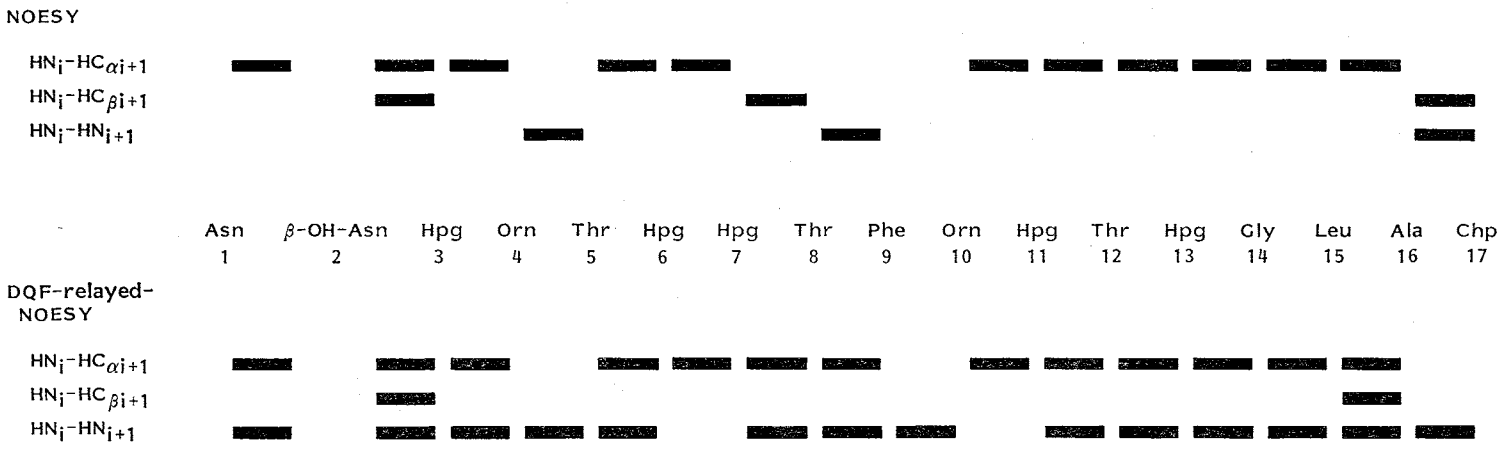
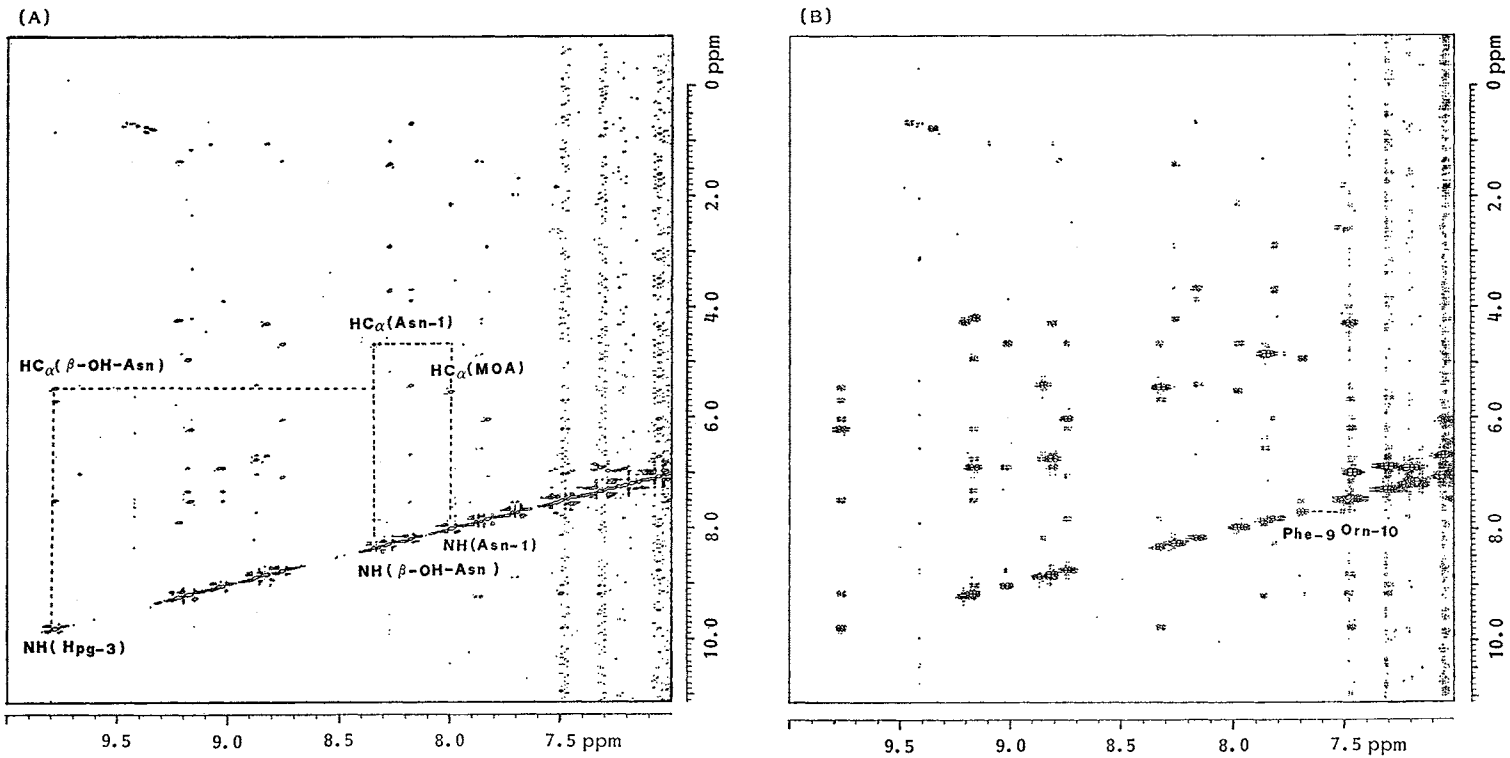


Fig. 5.

(A) NH, HC_α region of NOESY spectrum (40°C). The sequence Hpg-3 → β-OH-Asn → Asn-1 → 7-methylocta-2,4-dienoic amide is shown.

(B) NH, HC_α region of DQF-relayed-NOESY spectrum (40°C). The connectivity Phe-9 → Orn-10 is shown.

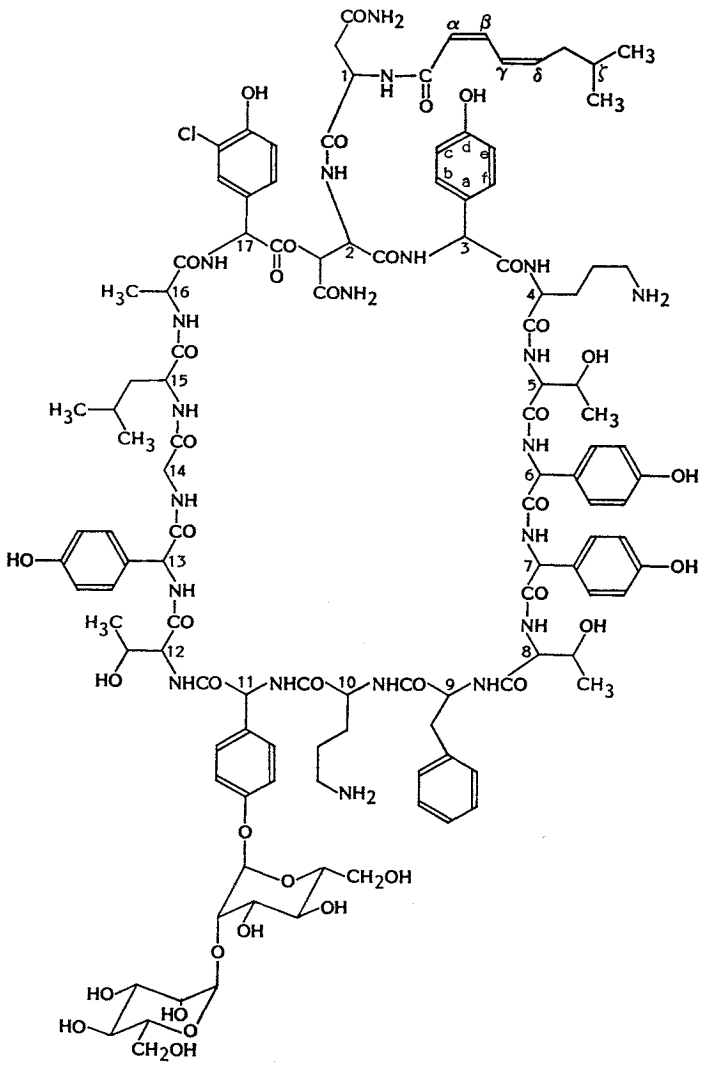


straightforward identification, the aliphatic amino acid spin systems could be unambiguously assigned by the use of a relayed-COSY experiment. In Figs. 2 and 3 the identification of the amino acids through the NH-HC_α and NH-HC_α-HC_β connectivities, respectively, is shown.

b) Aromatic amino acids: The NMR methods described above failed to distinguish among the five molecules of Hpg and 3-chloro-4-hydroxyphenylglycine (Chp) and to assign their aromatic protons. The reason is due to the lack of a mutual coupling between their HC_α and the aromatic protons. A carefully tuned COSY experiment with enhancement of long-range coupling helped to overcome this problem. Due to the symmetrical substitution pattern of Hpg only two cross peaks from HC_α to the *ortho* and *meta* aromatic protons were observed, whereas for Chp connectivities from HC_α to the three different aromatic protons were found.

The HC_α of two Hpg units are buried under the aromatic protons and so are their weak cross peaks in this COSY experiment. The assignment of their aromatic protons was achieved by NOESY meas-

Fig. 6. Structure of A-16686 factor A2.



urements *via* the dipolar connectivities between their NH protons to the *ortho* and *meta* protons. All the chemical shift assignment are summarized in Table 1.

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}.

The results of the NOE-experiments of A-16686 are presented in Fig. 4. The sequence could be established by the use of the above mentioned connectivity determinations. Only for the link between Phe-9 and Ornithine (Orn)-10 none of the sequential connectivities could be found in a conventional NOESY experiment. Their connectivity could however be established by a DQF-relayed-NOESY experiment.[8] This experiment, compared with a normal NOESY experiment (Fig. 5), has several advantages:

a) Due to the DQF the diagonal is cleaner, therefore cross peaks close to the diagonal can be distinguished easily;

b) The NOE is transferred from crowded spectroscopic regions to less crowded ones.

As it is shown in Fig. 5 only three NH_i-NH_{i+1} cross peaks can be detected in the NOESY experiment, whereas in the DQF-relayed-NOESY experiment fourteen of them can be found, including the missing link between Phe-9 and Orn-10.

Conclusions

The NMR experiments described above, combined with the information obtained by chemical degradation studies, led to the elucidation of the complete structure of A-16686 factor A2 (Fig. 6) and of the other components of the complex.

Experimental

Approximately a 15-mmol solution of factor A2 in H_2O - DMSO (4:1), carefully deoxygenated with an argon stream, was used (pH 4.6). NMR spectra were recorded in the temperature range from 25 to 60°C on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer. Prior to Fourier transformation 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°-D_2-90°-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.

COSY Spectrum with Delay

Sequence: D1-90°-D2-t_1-90°-D2-t_2; D1=1.5 s; D2=180 ms; 90°-pulse=13.5 μs; size=2K; 512 increments with 16 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; D2=18

ms; D3=0.9 μs; 90°-pulse=11.5 μs; size=2K; 512 increments with 64 transitions each; acquisition time=170 ms.

Acknowledgments

The authors thank Prof. G. G. GALLO for helpful discussions.

References

1) CAVALLERI, B.; H. PAGANI, G. VOLPE, E. SELVA & F. PARENTI: A-16686, a new antibiotic from *Actinoplanes*. I. Fermentation, isolation and preliminary physico-chemical characteristics. J. Antibiotics 37: 309~317, 1984
2) PALLANZA, R.; M. BERTI, R. SCOTTI, E. RANDISI & V. ARIOLI: A-16686, a new antibiotic from *Actinoplanes*. II. Biological properties. J. Antibiotics 37: 318~324, 1984
3) CIABATTI, R.; J. K. KETTENRING, G. WINTERS, G. TUAN, L. ZERILLI & B. CAVALLERI: Ramoplanin (A-16686), a new glycolipodepsipeptide antibiotic. III. Structure elucidation. J. Antibiotics 42: 254~267, 1989
4) MARION, D. & K. WUETHRICH: Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of ^{1}H-^{1}H spin-spin coupling constants in proteins. Biochem. Biophys. Res. Commun. 113: 967~974, 1983
5) CHAZIN, W. J.; D. P. GOLDENBERG, T. E. CREIGHTON & K. WUETHRICH: Comparative studies of conformation and internal mobility in native and circular basic pancreatic trypsin inhibitor by ^{1}H nuclear magnetic resonance in solution. Eur. J. Biochem. 152: 429~437, 1985
6) BAX, A. & R. FREEMAN: Investigation of complex networks of spin-spin coupling by two-dimensional NMR. J. Magn. Reson. 44: 542~561, 1981
7) MACURA, S.; K. WUETHRICH & R. R. ERNST: The relevance of *J* cross-peaks in two-dimensional NOE experiments of macromolecules. J. Magn. Reson. 47: 351~357, 1982
8) KESSLER, H.; S. STEUERNAGEL, D. GILLESSEN & T. KAMIYAMA: Complete sequence determination and localisation of one imino and three sulfide bridges of the Nonadecapeptide RO 09-0198 by homonuclear 2D-NMR spectroscopy. The DQF-RELAYED-NOESY-Experiment. Helv. Chim. Acta 70: 726~741, 1987