Volume 291, number 1, 109–112 FEBS 10231 October 1991

ADONIS 0014579391009416

Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*[a]

A. Ballio[1], D. Barra[1], F. Bossa[1], A. Collina[2], I. Grgurina[1], G. Marino[3], G. Moneti[4], M. Paci[5], P. Pucci[3], A. Segre[2] and M. Simmaco[1]

[1]*Dipartimento di Scienze Biochimiche 'A. Rossi-Fanelli' and Centro di Biologia Molecolare del CNR, Università La Sapienza, 00185 Roma, Italy,* [2]*Istituto di Strutturistica Chimica 'G. Giacomello' del CNR, Casella Postale 10, 00016 Monterotondo Stazione, Italy,* [3]*Dipartimento di Chimica Organica e Biologica, Università Federico II, 80134 Napoli, Italy,* [4]*Centro di Spettrometria di Massa, Dipartimento di Farmacologia dell'Università, 50134 Firenze, Italy* and [5]*Dipartimento di Scienze e Tecnologie Chimiche, Università Tor Vergata, 00173 Roma, Italy*

Received 16 June 1991; revised version received 2 August 1991

The primary structure of some new lipodepsipeptides named syringopeptins, produced by plant pathogenic strains of *Pseudomonas syringae* pv. *syringae* has been determined by a combination of chemical methods, ^{1}H and ^{13}C NMR spectroscopy and FAB mass spectrometry. Two syringomycin-producing strains afforded 3-hydroxydecanoyl-Dhb-Pro-Val-Val-Ala-Ala-Val-Val-Dhb-Ala-Val-Ala-Ala-Dhb-aThr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr, with Tyr acylating aThr to form a macrolactone ring, and smaller amounts of the 3-hydroxydodecanoyl homologue. Evidence was obtained that a third syringomycin-producing strain and a syringotoxin-producing strain synthesize 3-hydroxydecanoyl-Dhb-Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Dhb-Val-Dhb-Ala-Val-Ala-Ala-Dhb-aThr-Ser-Ala-Val-Ala-Dab-Dab-Tyr, with Tyr and aThr forming again the macrolactone ring, and smaller amounts of the 3-hydroxydodecanoyl homologue.

Phytotoxin; Lipodepsipeptide; Syringopeptin; *Pseudomonas syringae* pv. *syringae*

1. INTRODUCTION

In recent years it has been shown that different ecotypes of *Pseudomonas syringae* pv. *syringae* produce closely related bioactive lipodepsipeptides. Syringomycins [1–3], syringostatins [4,5] and syringotoxin [6,7] have been isolated respectively from stone fruits, pear, grass and sugar cane isolates, from lilac isolates, and from citrus isolates, and all of them have been shown to be composed of a nonapeptide moiety with the C-terminal sequence Dhb-Asp(3-OH)-Thr(4-Cl) and an N-terminal Ser *N*-acylated by a long-chain unbranched 3-hydroxy fatty acid and *O*-acylated by the C-terminal carboxyl to form a macrolactone ring. The 5 amino acid residues located between the N-terminal Ser and the C-terminal tripeptide, mostly of an uncommon structure, form the variable region of the peptide moiety. During our previous work on syringomycin [1,2] and syringotoxin [6] we had noticed that partially purified preparations of these toxins gave rise in HPLC (beside peaks due to the toxins themselves and to related compounds) to peaks due to more hydrophobic substances with a larger molecular size and a different amino acid composition. Our attention turned back to these substances when it was noticed that the phytotoxicity of the unfractionated mixture was only in part accounted for by their content of syringomycin or syringotoxin [8]. As reported in the present paper, we have isolated some of these substances and demonstrated that they are new phytotoxic metabolites, here called syringopeptins (SPs). They are lipodepsipeptides that share a limited number of structural features with the previously investigated bioactive substances produced by the same strains.

[a]Dedicated to Professor Doriano Cavallini on the occasion of his 75th birthday.

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; TBDMS, *t*-butyldimethylsilyl; Asp(3-OH), 3-hydroxyaspartic acid; Thr(4-Cl), 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid; aThr, allothreonine; NOESY, nuclear Overhauser effect correlated spectroscopy; TOCSY, total correlated spectroscopy.

Correspondence address: A. Ballio, Dipartimento Scienze Biochimiche, Università La Sapienza, P.le Aldo Moro, 5, 00185 Roma, Italy. Fax: (39)(6) 4440062.

2. MATERIALS AND METHODS

2.1. *Preparation of syringopeptins*

Syringopeptins were isolated by freeze-drying appropriate HPLC fractions originating from the partially purified preparations of syringomycin [1,2] or syringotoxin [6] used in our previous investigations.

2.2. *Analytical methods*

Amino acid analyses were carried out with a Pharmacia Alpha Plus 4151 analyzer after hydrolysis in vapor phase with 6 N HCl at 110°C

for 24 h in vacuo, or by GC-MS after transformation into TBDMS derivatives [9]. N-terminal analyses were performed by the dansyl chloride method [10,11]. FAB-mass spectra were mostly obtained as reported in [1]. NMR spectra were run on a Bruker AM 400 instrument operating at 400.13 MHz. Homonuclear 2D NMR experiments were performed in phase-sensitive mode using TPPI phase cycle [12] with 2K of memory typically for 512 increments. Spectral width and the number of scans were optimized to obtain the best resolution and a satisfactory signal-to-noise ratio. Correlation experiments (COSY) were performed in the Double Quantum Filtered mode (DQF) [13,14]. Total Correlation experiments (TOCSY) were performed using the MLEV-17 spinlock composite pulse sequence [15,16]. NOE dipolar correlated 2D spectra (NOESY) were obtained using the normal pulse sequence [17,18] or by using the rotating frame dipolar correlated 2D spectroscopy [19–21]. The mixing time for the magnetization exchange is reported for every experiment. Data were weighted by a sinebell apodization function shifted typically of $\pi/3$ in both dimensions and processed on a MicroVax II with the 2D NMR software written in FORTRAN 77. The program was kindly provided by Prof. R. Kaptein, Department of Organic Chemistry, Afd. NMR, Utrecht, The Netherlands. A matrix of 1024 × 1024 phase-sensitive absorption spectra was thus obtained with a digital resolution of ~4 Hz/point. An accurate baseline correction was carried out in both dimensions by using a polynomial fit provided by the same program. ^{13}C chemical shift values in D_2O were obtained from proton bearing Carbon atoms with a reverse detection probe using heteronuclear multiple quantum correlation spectroscopy [22]. In the F1 domain 1K experiments were performed corresponding to 15 Hz resolution, followed by zero filling to 2K (spectrum obtained by courtesy of Dr G. Gatti, Bruker, Milan).

2.3. *Chemical methods*

Partial acid hydrolyses were performed in 70% formic acid for 72 h at 45°C. After lyophilization the hydrolysates were fractionated by HPLC on a macroporous reverse phase column (Aquapore RP-300, 7.0 × 250 mm purchased from Applied Biosystems, San Jose, CA, USA). The addition of mercaptoethanol to SPs was carried out by reacting about 2 nmol of peptide with an excess of reagent, under conditions very similar to those described for the detection of phosphoseryl residues [23]. After reaction the sample was loaded onto the glass fiber filter of an Applied Biosystems model 475A gas phase protein sequencer. The opening of the lactone ring was obtained by incubation with 6.5% aqueous triethylamine (RP reagent from Carlo Erba, Milan) at room temperature for 2 h.

2.4. *Enzymatic hydrolyses*

Digestion with carboxypeptidase A (Type I-DFP from Sigma, St.Louis, MO, USA) was performed in 0.1 M triethylamine pH 8.5, at 37°C for 30 min.

3. RESULTS AND DISCUSSION

The partially purified preparations obtained from three syringomycin-producing strains of *P. syringae* pv. *syringae* according to Surico and DeVay [24] and used in our previous investigations gave in HPLC an elution pattern that consistently showed, beside syringomycin peaks, one or more frequently two other hydrophobic peaks (Fig. 1). Fractions corresponding to each of the latter peaks were pooled and their amino acid composition and molecular size determined. The results demonstrated that these substances were quite different from those of previously studied bacterial metabolites and were therefore named syringopeptins (SPs). In particular, SP_{22}-A and SP_{22}-B were obtained from strains B3A and B301, and SP_{25}-A and SP_{25}-B from strain

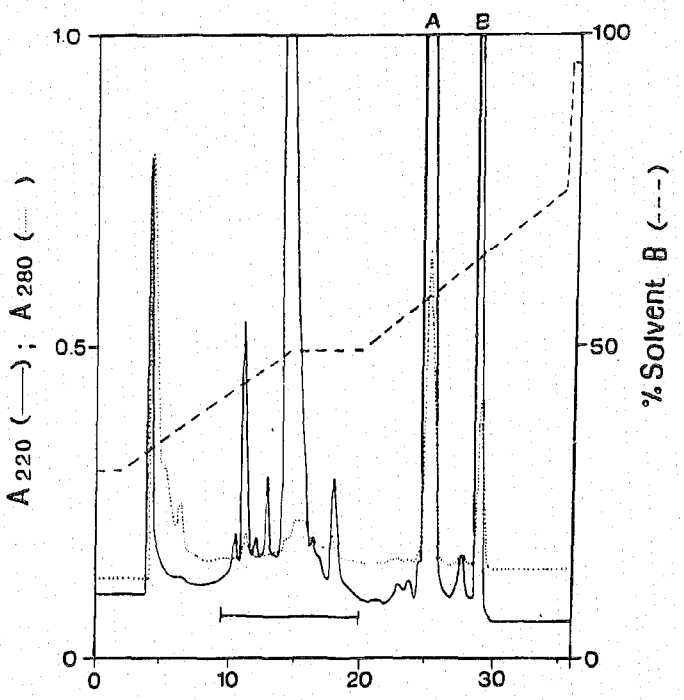

Fig. 1. Reverse-phase HPLC of a syringomycin preparation from *Pseudomonas syringae* pv. *syringae* strain B-359. Horizontal bar indicates the elution of the syringomycins. The letters indicate individual syringopeptins: A= SP_{25}-A, B= SP_{25}-B.

B359. Similarly, the syringotoxin-producing strain B427 also afforded SP_{25}- A and SP_{25}-B.

Unless otherwise stated, the experiments reported in this paper were carried out with SP_{25}-A. Quantitative amino acid analysis indicated the composition: Pro(1), Val(5), Ala(9), Leu(1), Thr(1), Ser(1), Dab(2), Tyr(1). GC-MS analysis of the TBDMS amino acids demonstrated the *allo* configuration of Thr. The combined use of DQF COSY, TOCSY and HMQC techniques (in D_2O) not only confirmed this composition, but furthermore extended it with the identification of four Dhb residues, all with the *Z* stereochemistry, and of a 3-hydroxydecanoyl residue. At least some of the residues must be in a cyclic arrangement since the sum of their masses in a linear arrangement does not correspond to the molecular weight (2397) found by FAB-MS; this consideration, together with the occurrence of a 3-hydroxyacid moiety, a weak IR signal at 1745 cm^{-1} (lactone) and a strong IR band at 1672 cm^{-1} (amide), suggested that SP_{25}-A was a new lipodepsipeptide. This hypothesis was corroborated by the observed addition of one molecule of water to SP_{25}-A on treatment with 6.5% aqueous triethylamine (MH^+ 2416), and by the blocked N-terminus in the same metabolite. On partial acid hydrolysis SP_{25}-A yielded a major product that emerged from the HPLC column before the starting compound. The quantitative amino acid analysis of the partial hydrolysis product gave results identical to those obtained with SP_{25}-A, but at variance with SP_{25}-A, the peptide had an unblocked N-terminus (Pro) and showed

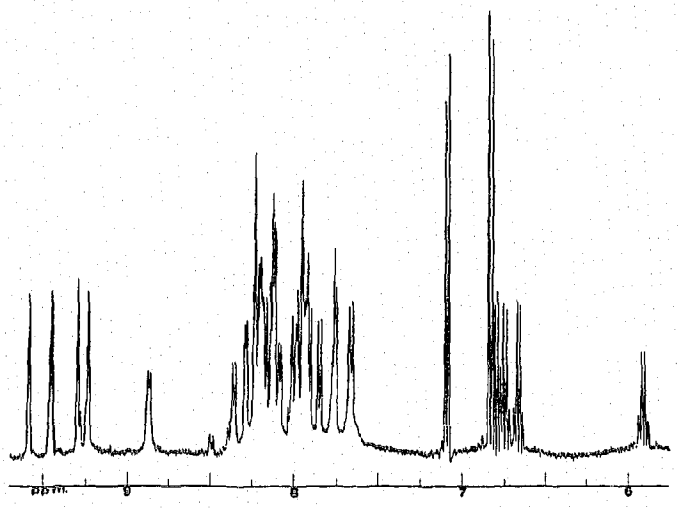

Fig. 2. ^{1}H 400.13 MHz NMR spectrum of SP_{25}-A, 1 mg/0.5 ml in H_2O, pH = 5.5. Only the low field portion of the spectrum is shown, corresponding to the amidic protons (7.7–9.7 ppm range), to the aromatic Tyr protons, and showing the 4 quartets due to β protons of 4 different Dhb. All resonances, given in ppm, have been assigned as follows: Dhb(1) 9.60; Dhb(12) 9.50; Dhb(17) 9.35; Dhb(10) 9.27; Ala(22) 8.91; Ala(15) 8.53; Leu(7) 8.40; Ala(20) 8.32; Ala(16) 8.27; Ala(8) 8.25; Val(21) 8.25; Dab(23) 8.22; Tyr(25) 8.18; Ala(9) 8.16; Val(14) 8.15; Ala(4) 8.12; Ala(5) 8.05; aThr(18) 7.99; Val(3) and Val(6) 7.96; Dab(24) 7.88; Ser(19) 7.80; Ala(13) 7.79; Val(11) 7.70; Tyr(*O*) 7.11; Tyr(*m*) 6.86; β Dhb(17) 6.83; β Dhb(10) 6.78; β Dhb(12) 6.70; β Dhb(1) 5.94.

a protonated molecular ion at MH^+ 1891. In order to make this material suitable for sequencing by the automated Edman degradation, it was reacted with mercaptoethanol to form a stable adduct with the Dhb residues (MH^+ 2710). The sequence analysis established the following primary structure: Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Xaa-Val-Xaa-Ala-Val-Ala-Ala-Xaa-Thr-Ser-Ala-Val-Ala-Dab-Dab-Tyr, where Xaa corresponds to a pair of PTH-derivatives, originating from the addition of mercaptoethanol to the double bond of Dhb residues.

Tyr is also the C-terminal amino acid of SP_{25}-A, as shown by the loss of 163 mass units from this metabolite after treatment first with triethylamine, to open the lactone ring (MH^+ 2416), and then with carboxypeptidase A. The partial primary structure was confirmed and integrated (Fig. 3) to yield the complete sequence of SP_{25}-A by TOCSY and NOESY experiments with different mixing times carried out in a H_2O solution. The ^{1}H-NMR spectrum relative to the aromatic region and to the amidic protons showed the resonances due to all backbone amides (Fig. 2). All amino acid spin systems were detected and the through-space connectivities in the sequential steps were unambiguously identified. These are reported in Fig. 3 where, for the sake of clarity, only the observed sequential NOE contacts are reported. Furthermore, on the basis of the combined

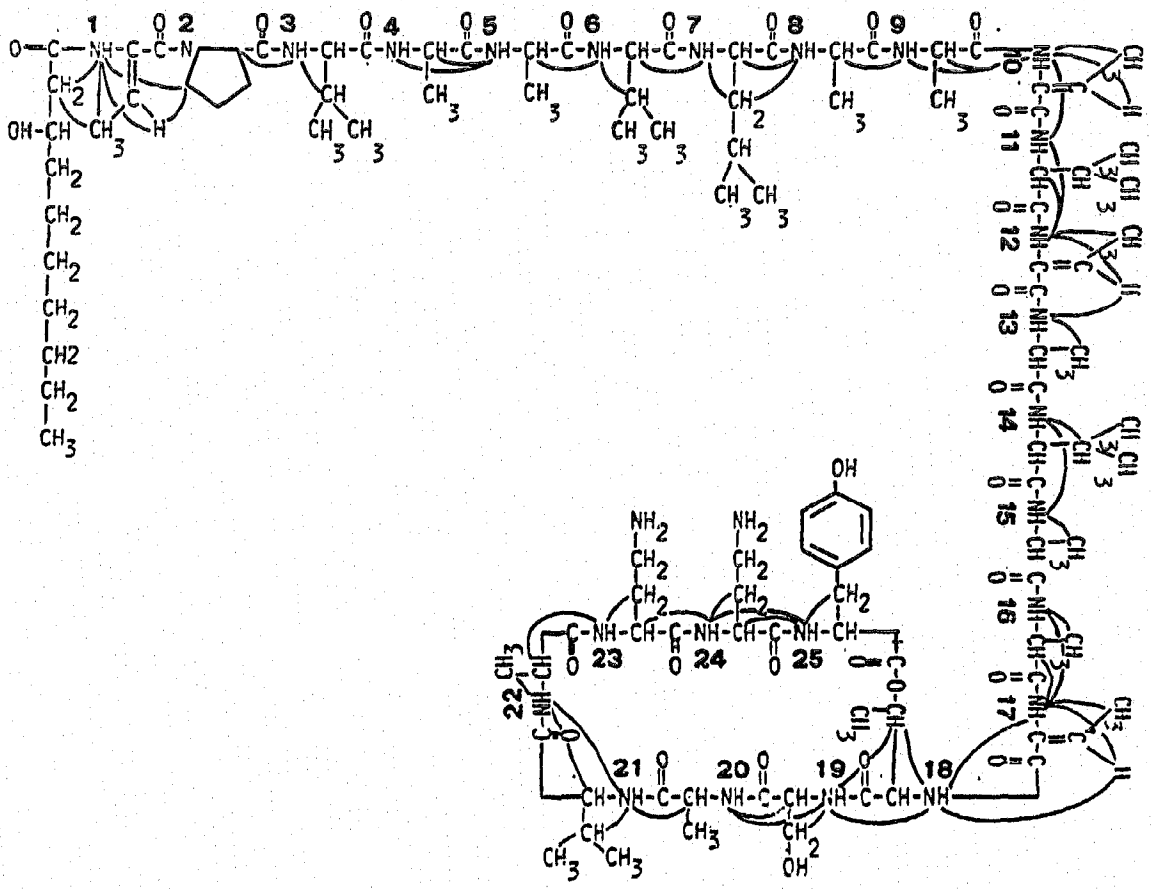

Fig. 3. Chemical structure of SP_{25}-A. Only the observed sequential NOE contacts have been reported.

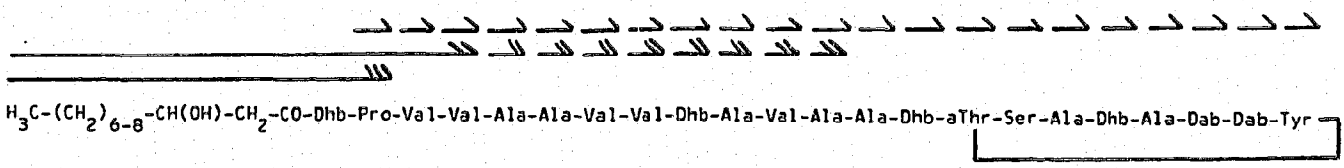

Fig. 4. Chemical structure of SP_{22}–A and SP_{22}–B. Sequence information was obtained by Edman degradation (→), FAB-MS (⇝) and NMR (⇶).

use of $J_{\alpha\text{-}\beta}$ (~9 Hz) and the NOE effect, the presence of aThr was confirmed. The closure of the lactone ring between the carboxyl of Tyr and the β-OH of aThr residue was clearly indicated by diagnostic ^1H and ^{13}C chemical shift values. Many other NOE contacts have been observed and identified both from close proximity protons and from sequentially remote parts of the molecule, indicating the presence of a rather structured conformation. Further elaboration of these data is in progress. The structure of SP_{25}-B (MH^+ 2426), a higher homologue of SP_{25}-A, derives from the complete homogeneity of PTH-amino acids formed in the Edman degradation of a mixture with SP_{25}-A.

The FAB-MS data, the amino acid analyses and the results of an Edman degradation after modification of the Dhb residues with mercaptoethanol have suggested that strains B-3A and B-301 produce two lipodepsipeptides similar to SP_{25}-A and B. They were named SP_{22}-A (MH^+ 2143) and SP_{22}–B (MH^+ 2171), differed from each other only for the 3-hydroxyacyl moiety (3-hydroxydecanoyl and 3-hydroxydodecanoyl, respectively), and appeared to have the structure shown in Fig. 4. The nature of the hydroxyacyl group, its linkage with Dhb-Pro, and the involvement of Tyr and aThr in the formation of the lactone ring were demonstrated by NMR spectroscopy, which however raised doubts about the homogeneity of the products.

Some structural features of the above metabolites are similar to those found in tolaasin, a biologically active lipodepsi octadecapeptide produced by *P. tolaasi* [25].

The isolation of SPs from syringomycin- and syringotoxin-producing strains and the disclosure of their high phytotoxicity [8] stresses once more the recommendation [1] that future investigations on the mode of action of *P. syringae* pv. *syringae* metabolites should be conducted with single purified components and encourages the re-examination, with pure substances, of some of the results obtained by previous investigators with unfractionated mixtures.

Acknowledgements: This work was supported in part by grants of the Italian National Research Council (CNR), special ad hoc programme 'Chimica fine II' subproject 3, and of the Italian Ministry of University and Scientific and Technological Research (MURST), and by NATO grant 86/0659 to A.B. Mass spectral data were obtained at Servizio di Spettrometria di Massa del CNR, Università di Napoli. We thank Miss Alessandra Franco for excellent technical assistance.

REFERENCES

[1] Ballio, A., Barra, D., Bossa, F., DeVay, J.E., Grgurina, I., Iacobellis, N.S., Marino, G., Pucci, P., Simmaco, M. and Surico, G. (1988) Mol. Physiol. Plant Pathol. 33, 493–496.
[2] Segre, A., Bachman, A., Ballio, A., Rossa, F., Grgurina, I., Iacobellis, N.S., Marino, G., Pucci, P., Simmaco, M. and Takemoto, J.Y. (1989) FEBS Lett. 255, 27–31.
[3] Fukuchi, N., Isogai, A., Yamashita, S., Suyama, K., Takemoto, J.V. and Suzuki, A. (1990) Tetrahedron Lett. 31, 1589–1592.
[4] Isogai, A., Fukuchi, N., Yamashita, S., Suyama, K. and Suzuki, A. (1989) Agric. Biol. Chem. 53, 3117–3119.
[5] Isogai, A., Fukuchi, N., Yamashita, S., Suyama, K. and Suzuki, A. (1990) Tetrahedron Lett. 31, 695–698.
[6] Ballio, A., Bossa, F., Collina, A., Gallo, M., Iacobellis, N.S., Paci, M., Pucci, P., Scaloni, A., Segre, A. and Simmaco, M. (1990) FEBS Lett. 269, 377–380.
[7] Fukuchi, N., Isogai, A., Nakayama, J. and Suzuki, A. (1990) Agric. Biol. Chem. 54, 3377–3379.
[8] Iacobellis, N.S., Lavermicocca, P., Grgurina, I., Simmaco, M. and Ballio, A., Mol. Physiol. Plant Pathol. (submitted).
[9] Chaves Das Neves, H.J. and Vasconcelos, A.M.P. (1987) J. Chromatogr. 392, 249–258.
[10] Gray, W.R. (1972) Methods Enzymol. 25, 121–138.
[11] Simmaco, M., De Biase, D., Barra, D. and Bossa, F. (1990) J. Chromatogr. 504, 129–138.
[12] Marion, D. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974.
[13] Piantini, U., Sørensen, O.W. and Ernst, R.R. (1982) J. Am. Chem. Soc. 104, 6800–6801.
[14] Rance, M., Sørensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[15] Braunschweiler, L. and Ernst, R.R. (1983) J. Magn. Reson. 53, 521–528.
[16] Levitt, M.H., Freeman, R. and Frenkiel, T.A. (1982) J. Magn. Reson. 47, 328–338.
[17] Jeener, J., Meier, B.H., Bachman, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[18] States, D.J, Haberkorn, R.A. and Ruben, D.J. (1982) J. Magn. Reson. 48, 286–297.
[19] Bother-By, A.A., Stephens, R.L., Lee, J., Warren, C.D. and Jeanloz, R.W. (1984) J. Am. Chem. Soc. 106, 811–813.
[20] Davis, D.G. and Bax, A. (1985) J. Am. Chem. Soc. 107, 2820–2821.
[21] Neuhaus, D. and Williamson, M. (1989) The Nuclear Overhauser Effect in Structural and Conformational Analysis, VCH Publishers, New York.
[22] Bax, A., Griffley, R.H. and Hawkins, B.L. (1983) J. Magn. Reson. 55, 301–315.
[23] Meyer, H.E., Hoffman-Posorske, E., Korte, H. and Heilmeyer Jr, L.M.G. (1986) FEBS Lett. 204, 61–66.
[24] Surico, G. and DeVay, J.E. (1982) Physiol. Plant Pathol. 21, 39–53.
[25] Nutkins, J.C., Mortishire-Smith, R.J., Packman, L.C., Brodey, C.L., Rainey, P.B., Johnstone, K. and Williams, D.H. (1991) J. Am. Chem. Soc. 113, 2621–2627.