Biosynthetic origin of syringomycin and syringopeptin 22, toxic secondary metabolites of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*[1]

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Abstract The biosynthesis of syringomycin (SR) and syringopeptin 22 (SP22), bioactive lipodepsipeptides of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*, was studied by feeding ^{14}C-labeled precursors to chloramphenicol-containing bacterial suspensions. The preferential sites of incorporation were determined by comparing the specific activities of the intact radiolabeled metabolites and their single structural elements, obtained by hydrolytic degradation followed by derivatization and isolation by high performance liquid chromatography. The results show that, upon feeding L-[^{14}C(U)]-Thr, 35.0 and 31.0% of the SR radioactivity is retained in 2,3-dehydro-2-aminobutyric acid (Dhb) and 4-chlorothreonine (Thr(4-Cl)), respectively. L-[^{14}C(U)]-Asp labels the same sites, though less efficiently, and is also incorporated in 2,4-diaminobutyric acid (Dab) and 3-hydroxyaspartic acid (Asp(3-OH)). Dhb is also labeled by Thr and Asp in SP22. These are the first data on the biosynthetic origin of the modified residues in *P. syringae* lipopeptides.

Key words: Lipodepsipeptide; Biosynthetic precursor; 3-Hydroxyaspartic acid; 4-Chlorothreonine; 2,4-Diaminobutyric acid; 2,3-Dehydro-2-aminobutyric acid; *Pseudomonas syringae* pv. *syringae*

1. Introduction

Syringomycin (SR) [1,2] and syringopeptin 22 (SP22) [3] are extracellular secondary metabolites produced in vitro by several strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*. As shown in Fig. 1, these peptides share some structural features, like a lactone macrocycle and a medium length chain hydroxylated lipid moiety. Both are produced as mixtures of homologous forms differing in the length of the acyl chain. Following their isolation and structural elucidation, analogous investigations, extended to other strains of the bacterium, led to the discovery of a number of new metabolites. *P. syringae* lipopeptides known so far can be classified into two groups on the basis of their structure and biological activities. The first comprises, besides SR, other nine amino acid residues-containing lipopeptide lactones characterized by strong anti-fungal activity [4], like syringotoxin [5], syringostatin [2] and pseudomycin [6], each produced by a different strain. The second group comprises phytotoxic and anti-bacterial compounds [4] with a larger peptide moiety of 22 or 25 residues. These include SP22 [3] and its isoforms [7,8] as well as two SP25 isoforms [3,9]. Like most bacterial peptide metabolites, they contain several uncommon amino acids. For example, 2,4-diaminobutyric acid (Dab) and the C-terminal tripeptide 2,3-dehydroamino-butyroyl-3-hydroxyaspartyl-4-chlorothreonine (Dhb-Asp(3-OH)-Thr(4-Cl)) are conserved in all the lipodepsinonapeptides. Dhb and Dab are also contained in SPs.

Intensive investigations into the biological activities of these metabolites, recently reviewed [10], are aimed at understanding their role in plant pathogenesis and the development of medical and biotechnological applications. Studies of their mechanism of action in vitro demonstrated that the disruption of cell membrane integrity lies at the basis of their biocidal action on plant, animal and microbial cells. However, the different toxicity of the two types of lipopeptides towards various biological systems suggests that the interaction with lipid bilayers could be only part of a more complex process and that specific events, in which the role of the modified amino acids should be considered, could take place in the mechanism of action of each particular system. Besides Thr(4-Cl), whose relevance, but not function, in the anti-fungal activity of lipodepsinonapeptides has been demonstrated [11], *P. syringae* metabolites contain various other amino acids which have been implicated in the biological activity in different systems. For example, Asp(3-OH) and Dab were shown to possess antibiotic and neurotoxic activity, respectively [12,13]. Moreover, 2,3-unsaturated residues were shown to contribute to the anti-microbial property of the compounds that contain them [14,15].

The interesting chemical structures and the vast array of biological activities of *P. syringae* pv. *syringae* lipodepsipeptides, as well as the significant agricultural damage caused by this phytopathogenic organism, motivated us to study the biosynthesis of this group of peptide secondary metabolites. We have shown previously [16] that the thiotemplate mechanism of peptide synthesis [17] operates in the formation of SR and SP22. In previous studies, the entire SR biosynthetic gene cluster was sequenced and the role of some domains was demonstrated biochemically [18]. Here, we present the evi-

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Abbreviations: Asp(3-OH), 3-hydroxyaspartic acid; Thr(4-Cl), 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid; RP-HPLC, reversed phase-high performance liquid chromatography; SR, syringomycin; SP22, syringopeptin 22; DNPH, 2,4-dinitrophenylhydrazone; DNFB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl

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dence on the biosynthetic precursors of four modified amino acids in *P. syringae* lipopeptides.

2. Materials and methods

2.1. Preparation of SR and SP

P. syringae pv. *syringae* strain B301D-R was grown in 1l Roux bottles as previously described [11]. The mixtures of the secondary metabolites were extracted from the culture broth and isolated by reversed phase-high performance liquid chromatography (RP-HPLC) on an Aquapore RP 300 column (4.6×250 mm, 7 μm, Applied Biosystems), using a Beckman System Gold 126 instrument under conditions described in [11].

SR and SP22 obtained from the appropriate HPLC fractions were lyophilized, redissolved in water and quantified by measuring the absorbance at 220 nm ($\varepsilon = 30\,000$ and $\varepsilon = 140\,000$ M^{-1} cm^{-1}, respectively). A calibration curve was constructed for each metabolite using samples quantified by amino acid analysis after hydrolysis. Radioactivity was determined by scintillation counting on a LKB Wallac 1211 instrument.

2.2. Incubation experiments

Chloramphenicol was added to 24 h old bacterial cultures to a final concentration of 0.1 mg/ml. The radioactive precursors, L-[^{14}C(U)]-Val or L-[^{14}C(U)]-Thr (244 mCi/mmol), or L-[^{14}C(U)]-Asp (224.8 mCi/mmol), all purchased from Du Pont de Nemour and previously diluted with unlabeled carrier material, were subsequently added. After 120 h of incubation at 25°C, the metabolites were extracted and purified to constant specific radioactivity as described in [11].

2.3. Chemical methods

The mixtures of the amino acids obtained by hydrolysis with HCl 6 N (24 h, 110°C) of radiolabeled lipodepsipeptides were derivatized with 2,4-dinitrofluorobenzene (DNFB) [19] and with 2,4-dinitrophenylhydrazine [20]. The dinitrophenyl (DNP) amino acids were fractionated by RP-HPLC on a C_{18} column, 4.6×250 mm, 5 μm (Beckman, Ultrasphere), and eluted with a gradient of solvent A (KH_2PO_4, 25 mM, pH 6.8) and solvent B (acetonitrile:2-propanol, 3:1) at a flow rate of 1.0 ml/min. The gradient program was as follows: the initial concentration, 7% solvent B, constant for 1 min, changed linearly to 23% in 30 min. After 1 min of isocratic elution, the concentration of B reached 60% in 12 min and after 1 min, the initial conditions were restored. The 2,4-dinitrophenylhydrazone (DNPH) of 2-oxobutyric acid was isolated by RP-HPLC under conditions described in [11]. The derivatives were quantified by measuring the absorbance at 360 nm ($\varepsilon = 12\,235$ and $\varepsilon = 21\,033$ M^{-1} cm^{-1} for mono- and diamino acids, respectively; $\varepsilon = 25\,600$ M^{-1} cm^{-1} for DNPH of 2-oxobutyric acid) and their radioactivity was measured. Thr(4-Cl) was obtained by hydrolyzing its derivative *N*-Cbz-Thr(4-Cl) methyl ester, prepared from the corresponding vinylglycine derivative as described in [21]. The latter was synthesized starting from glutamic acid according to Hanessian's procedure [22].

3. Results

3.1. Production of [^{14}C]SR and [^{14}C]SP22

To establish the most suitable timing for the addition of the radiolabeled precursors, the production of SR and SP22 in *P. syringae* pv. *syringae* cultures was monitored during the bacterial growth. The data shown in Fig. 2 indicate that the secretion of the two metabolites started during the logarithmic growth phase and reached a maximum in the early stationary phase. The peak of SP22 production was delayed, compared to SR.

Twenty-four hours after the inoculation was subsequently chosen as the most suitable time for the addition of a protein synthesis inhibitor and a radiolabeled precursor. This represents a compromise between the amount of cells, sufficient to ensure further observable production of the metabolites, and the amount of lipodepsipeptides produced at the time of feeding (3.0 nmol/ml and 0.9 nmol/ml for SR and SP22, respectively), in order to minimize the dilution of the labeled compounds. In a typical feeding experiment, a precursor with a specific activity of 4.3×10^6 cpm/μmol was administered to the chloramphenicol-containing bacterial suspension. Initially, the experiment with L-[^{14}C(U)]-Val was run as control. As expected, this amino acid was incorporated into SP22 which contains several valine residues (specific molar incorporation 4.45%) but did not label significantly SR (specific molar incorporation 0.83%), thus showing that the dispersion of the label via degradative processes was negligible. Threonine and aspartic acid were chosen as possible precursors because, on the basis of the data presented in the literature [13,23], they could be expected to be incorporated into Dhb and Dab. In fact, by feeding L-[^{14}C(U)]-Thr or L-[^{14}C(U)]-Asp, lipodepsipeptides with specific radioactivity in the range of 105 cpm/μmol were obtained. The specific molar incorporations ranged between 2 and 6%. The radioactive peptide metabolites were diluted with unlabeled material prior to degradation.

3.2. Degradation of [^{14}C]SR and [^{14}C]SP22 and determination of the labeling pattern

To determine the preferential sites of label incorporation, the specific radioactivity of the intact metabolite was compared to that of the single amino acid residues. To achieve this, a procedure suitable for the isolation of the latter was deviced. Typically, the mixture obtained after hydrolysis of

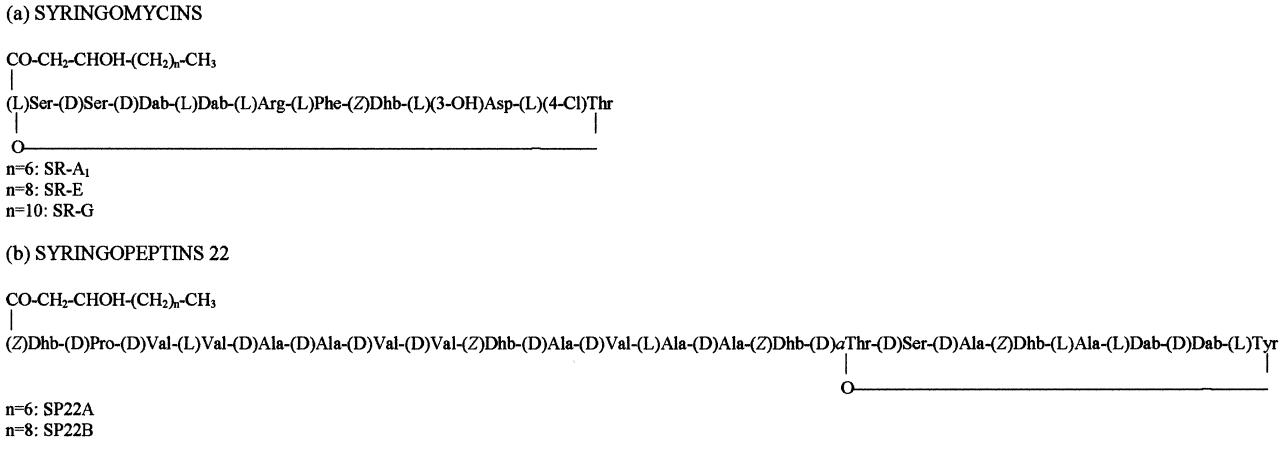

Fig. 1. Structures of SR (a) and SP22 (b).

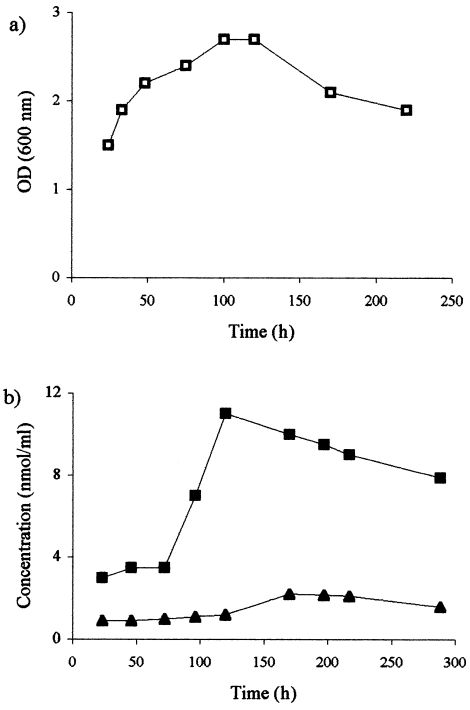

Fig. 2. Growth curve of *P. syringae* pv. *syringae*, strain B301D-R (a), time course of the production of SR (■) and SP22 (▲) (b).

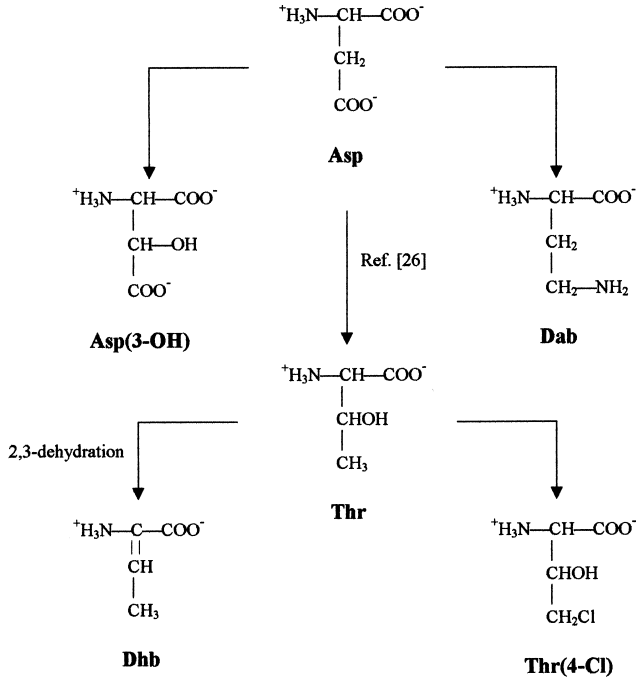

Fig. 3. Schematic overview of the origin of Asp(3-OH), Dab, Dhb and Thr(4-Cl).

[^{14}C]SR was divided into two portions: one was treated with 2,4-dinitrophenylhydrazine, which allows us to derivatize 2-oxobutyric acid produced by deamination of Dhb [24], and the other with DNFB, which is adequate suitable for the derivatization of all the other residues. Following fractionation of the DNP derivatives, all the amino acid residues and their by-products formed during the course of the acid hydrolysis were separated and assigned by analyzing the elution profiles of the derivatized hydrolysis mixtures of each amino acid present in SR. The results of the degradation studies of samples of [^{14}C]SR deriving from incubations with L-[^{14}C(U)]-Thr and L-[^{14}C(U)]-Asp are summarized in Table 1. They represent the average of the values obtained in three different experiments. The specific radioactivity of the Ser residue could not be quantified because its DNP derivative co-eluted with DNFB.

Radioactive threonine was incorporated essentially into Dhb and Thr(4-Cl), which contained 35.0 and 31.0% of the radioactivity of the intact SR, respectively, while the distribution of the label deriving from aspartic acid was more scattered. It labeled Asp(3-OH) and Dab to a similar extent (13.2 and 15.9% of the SR radioactivity, respectively) and, in accordance with its role as a precursor of threonine, it was incorporated, albeit less efficiently, into the same residues labeled by Thr. For experiments with SP22, only the radioactivity retained in Dhb was determined and shown to be 21.8 and 14.6% feeding labeled Thr and Asp, respectively.

4. Discussion

The degradation studies carried out on SR and SP22, produced upon feeding radiolabeled precursors, shed light on the origin of some of the modified residues in these molecules. The overview of the biosynthetic pathways is shown in Fig. 3. The level of radioactivity found in Dhb, in the experiments with L-[^{14}C(U)]-Thr, demonstrates that this modified amino acid is formed by 2,3-dehydration of the corresponding 3-hydroxy acid. This confirms that the most frequently encountered route for the production of α,β-unsaturated amino acids [25] also operates in *P. syringae*. The lower level of the label retention in Dhb observed by feeding [^{14}C]-Asp was expected on the basis of the biochemical pathway that leads to threonine from aspartate [26]. Conversely, the efficient incorpora-

Table 1
Distribution of ^{14}C label in the single amino acid residues of [^{14}C]SR, produced upon feeding L-[^{14}C(U)]-Asp (a) and L-[^{14}C(U)]-Thr (b) to *P. syringae* pv. *syringae* (strain B301D-R) cell suspensions

	Specific activity (cpm/μmol)		% of SR radioactivity retained in the residues	
	(a)	(b)	(a)	(b)
[^{14}C]SR	7.00×10^4	4.50×10^4	100	100
[^{14}C]DNP-Asp(3-OH)	0.92×10^4	0.14×10^4	13.2	3.1
[^{14}C]DNP-Dab	1.11×10^4	0.06×10^4	15.9	1.4
[^{14}C]DNP-Phe	0.10×10^4	0.06×10^4	1.4	1.4
[^{14}C]DNP-Thr(4-Cl)	0.58×10^4	1.40×10^4	8.3	31.0
[^{14}C]DNP-Arg	0.21×10^4	0.09×10^4	3.0	2.0
[^{14}C]DNPH of 2-oxobutyric acid	1.36×10^4	1.58×10^4	19.5	35.0

tion of radiolabeled threonine into its chlorinated analogue is the first report on the biosynthetic origin of this uncommon amino acid. The level of incorporation, similar to that observed for Dhb, whose formation from Thr implies only one reaction, suggests that threonine is a very close, if not the direct, precursor of its chlorinated analogue. However, the direct halogenation of this amino acid appears improbable in the light of the present knowledge on the mechanism of action of chloroperoxidases, enzymes involved in biological halogenations. All the known haloperoxidases, classified into three groups on the basis of the type of cofactor used, catalyze the formation of an electrophilic form of chlorine which requires an electron rich substrate [27–29]. Recently, an elegant study on the formation of trichloroleucine in the marine cyanobacterial metabolite barbamide suggested a novel mechanism of chlorination, possibly involving radicals [30]. Our data point towards the role of threonine as direct precursor of Thr(4-Cl) but do not allow us to rule out the 3,4-dehydration leading to vinylglycine, which would be a more suitable substrate of the known chloroperoxidases, as reaction intermediate. The identification of the biosynthetic precursors of the modified residues is essential for the complete understanding of a biosynthetic pathway. For example, our knowledge on the role of Thr as precursor of Thr(4-Cl) was helpful in the determination of the catalytic activity of the synthetase module encoded by *syrB1* [18].

The radioactivity derived from [^{14}C]-Asp besides being incorporated, as expected, into residues whose nearer precursor is threonine was also retained in Dab and Asp(3-OH). The biosynthesis of Dab by transamination of aspartic semihaldehyde with glutamate has recently been demonstrated in *Halomonas elongata* [31]. This three step pathway is compatible with the level of incorporation observed in SR. The Asp(3-OH) was shown to originate by direct hydroxylation of Asp in the blood clotting cascade enzymes [32] and by the aldol type condensation, between glycine and glyoxylate, in *Micrococcus denitrificans* [33]. The results of our feeding experiments do not clearly point towards a direct hydroxylation of Asp. Nevertheless, they allow us to rule out the second route in the biosynthesis of SR. Firstly, threonine, which is an intermediate on the pathway from aspartate to glycine, should be a better precursor than aspartate and the contrary is observed. Moreover, the multistep sequence required to produce glycine and glyoxylate from aspartic acid is not compatible with the Asp(3-OH) radioactivity level measured (13.2% of the intact metabolite). In fact, the scrambling of the label is reflected in approximately 3% of the radioactivity, as exemplified by the label retention level in Phe and Arg.

In conclusion, we determined the biosynthetic precursors of four modified amino acids in SR, which are constituents of all *P. syringae* lipodepsinonapeptides. Two of these residues, Dhb and Dab, are also contained in all SPs. It is reasonable to assume that the conclusions of this investigation can be extended to other structurally related *Pseudomonas* metabolites.

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References

[1] Segre, A., Bachman, R.C., Ballio, A., Bossa, F., Grgurina, I., Iacobellis, N.S., Marino, G., Pucci, P., Simmaco, M. and Takemoto, J.Y. (1989) FEBS Lett. 255, 27–31.
[2] Fukuchi, N., Isogai, A., Nakayama, J., Takayama, S., Yamashita, S., Suyama, K., Takemoto, J.Y. and Suzuki, A. (1992) J. Chem. Soc. Perkin. Trans. 1, 1149–1157.
[3] Ballio, A., Barra, D., Bossa, F., Collina, A., Grgurina, I., Marino, G., Paci, M., Pucci, P., Segre, A. and Simmaco, M. (1991) FEBS Lett. 291, 109–112.
[4] Lavermicocca, P., Iacobellis, N.S., Simmaco, M. and Graniti, A. (1997) Physiol. Mol. Plant Pathol. 50, 129–140.
[5] 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.
[6] Ballio, A., Bossa, F., Di Giorgio, D., Ferranti, P., Paci, M., Pucci, P., Scaloni, A., Segre, A. and Strobel, G.A. (1994) FEBS Lett. 355, 96–100.
[7] Isogai, A., Iguchi, H., Nakayama, J., Kusai, A., Takemoto, J.Y. and Suzuki, A. (1995) Biosci. Biotech. Biochem. 59, 1374–1376.
[8] Grgurina, I., Scaloni, A. and Iacobellis, N.S. (1997) Ital. Biochem. Soc. Trans. 9, 397.
[9] Scaloni, A., Camoni, L., Di Giorgio, D., Scortichini, M., Cozzolino, R. and Ballio, A. (1997) Physiol. Mol. Plant Pathol. 51, 259–264.
[10] Bender, C.L., Alarcón-Chaidez, F. and Gross, D.C. (1999) Microbiol. Mol. Biol. Rev., 266–292.
[11] Grgurina, I., Barca, A., Cervigni, S., Gallo, M., Scaloni, A. and Pucci, P. (1994) Experientia 50, 130–133.
[12] Ishiyama, T., Furuta, T., Takai, M. and Okimoto, Y. (1975) J. Antibiot. 10, 821–823.
[13] Nigam, S.N. and Ressler, C. (1966) Biochemistry 11, 3426–3429.
[14] Morris, S.L., Walsh, C. and Hansen, J.N. (1984) J. Biol. Chem. 259, 13590–13594.
[15] Mackintosh, R.B., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P. and Mackintosh, C. (1995) FEBS Lett. 371, 236–240.
[16] Grgurina, I. and Benincasa, M. (1994) Ital. Biochem. Soc. Trans. 5, 143.
[17] Kleinkauf, H. and von Dfhren, H. (1996) Eur. J. Biochem. 236, 335–351.
[18] Guenzi, E., Galli, G., Grgurina, I., Gross, D.C. and Grandi, G. (1998) J. Biol. Chem. 273, 32857–32863.
[19] Sanger, F. (1945) J. Biol. Chem. 39, 507–515.
[20] Hemming, B.C. and Gubler, C.J. (1979) Anal. Biochem. 92, 31–40.
[21] Shaw, K.J., Luly, J.R., Rapoport, H. and Rapoport, H. (1985) J. Org. Chem. 50, 4515–4523.
[22] Hanessian, S. and Sahoo, S.P. (1984) Tetrahedron Lett. 25, 1425–1428.
[23] Mocek, U., Zanopie, Z., O'Hagan, D., Zhou, P., Fan, L.D., Beale, J.M. and Floss, H.G. (1993) J. Am. Chem. Soc. 115, 7992–8001.
[24] Liesch, M.J., Millington, D.S., Pandey, R.C. and Rinehart, K.L. (1976) J. Am. Chem. Soc. 98, 8237–8249.
[25] Schmidt, U., Hdusler, J., Fhler, E. and Poisel, H. (1979) Prog. Chem. Nat. Prod. 37, 251–327.
[26] Lehninger, L.A. (1970) Biochemistry, Worth Publishers, New York.
[27] Libby, R.D., Thomas, J.A., Kaiser, L.W. and Hager, L.P. (1982) J. Biol. Chem. 25, 5030–5037.
[28] de Boer, E., Tromp, M.G., Plat, H., Krenn, G.E. and Weaver, R. (1986) Biochim. Biophys. Acta 872, 104–115.
[29] Hofmann, B., Tflzer, S., Pelletier, I., Altenbuchner, J., van Pée, K.H. and Hecht, H.J. (1998) J. Mol. Biol. 279, 889–900.
[30] Sitachitta, N., Rossi, J., Roberts, M.A., Gerwick, W.H., Fletcher, M.D. and Willis, C.L. (1998) J. Am. Chem. Soc. 120, 7131–7132.
[31] Ono, H., Sawada, K., Khunajakr, N., Tao, T., Yamamoto, M., Hiramoto, M., Shinmyo, A., Takano, M. and Murooka, Y. (1999) J. Bacteriol. 181, 91–99.
[32] Stenflo, J., Holme, E., Lindstedt, S., Chandramouli, N., Tsai Huang, L.H., Tam, J.P. and Marrifield, R.B. (1989) Proc. Natl. Acad. Sci. USA 86, 444–447.
[33] Gibbs, R.G. and Morris, J.G. (1964) Biochim. Biophys. Acta 85, 501–503.