Biosynthesis of PF1022A and Related Cyclooctadepsipeptides

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PF1022A belongs to a recently identified class of N-methylated cyclooctadepsipeptides (CODPs) with strong anthelmintic properties. Described here is the cell-free synthesis of this CODP and related structures, as well as the purification and enzymatic characterization of the responsible synthetase. For PF1022A synthesis extracts of Mycelia sterilia were incubated with the precursors L-leucine, D-lactate, D-phenyllactate, and S-adenosyl-L-methionine in the presence of ATP and MgCl2. A 350-kDa depsipeptide synthetase, PFSYN, responsible for PF1022A synthesis was purified to electrophoretic homogeneity. Like other peptide synthetases, PFSYN follows a thiotemplate mechanism in which the substrates are activated as thioesters via adenylation. N-Methylation of the substrate L-leucine takes place after covalent binding prior to peptide bond formation. The enzyme is capable of synthesizing all known natural cyclooctadepsipeptides of the PF1022 type (A, B, C, and D) differing in the content of D-lactate and D-phenyllactate. In addition to PF1022 types A, B, C, and D, the in vitro incubations produced PF1022F (a CODP consisting of D-lactate and N-methyl-L-leucine), as well as di-, tetra-, and hexa-PF1022 homologs. PFSYN strongly resembles the well documented enniatin synthetase in size and mechanism. Our results suggest that PFSYN, like enniatin synthetase, is an enzyme with two peptide synthetase domains and forms CODP by repeated condensation of dipeptide building blocks. Due to the low specificity of the D-hydroxy acid binding site, D-lactate or D-phenyllactate can be incorporated into the dipeptides depending on the concentration of these substrates in the reaction mixture.

In the course of screening for new anthelmintic compounds, Sasaki et al. (1) isolated a new N-methylated cyclodepsipeptide, PF1022A, from the fungus Mycelia sterilia. PF1022A exhibits strong anthelmintic properties in combination with low toxicity and is, therefore, one of the most outstanding anthelmintics to emerge since the discovery of the avermectins and milbemycins.

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Porption of [14C]L-methionine carried out as described by Zocher was used as an inoculum for the main culture. A 5-ml aliquot of the cultivation for 72 h at 26 °C on a rotary shaker (200 rpm), the mycelium and bassianolide from product in M. sterilia onine, or [14C]D-lactate in a final volume of 100 mCi/ml were purchased from Amersham Pharmacia Biotech. PF1022A, -B, -C, -D, and -F (a new derivative extracted from mycelium of M. sterilia) and diketotidol building blocks (10).

No information on the biosynthesis of cyclooctadepsipeptides like PF1022A and bassianolide has been available so far, whereas the biosynthesis of enniatins is well established (8, 9, 13, 14). Here we report the cell-free synthesis of PF1022A and related cyclooctadepsipeptides and the purification and characteristic of the responsible N-methyl-cyclooctadepsipeptide synthetase PFSYN.

**EXPERIMENTAL PROCEDURES**

**Radioisotopes and Chemicals—**L-[14C]Methionine (60 mCi/mmol), L-[14C]leucine (89 mCi/mmol), D-[14C]lactate (60 mCi/mmol), and S-adenosyl-L-[14C-methyl]methionine (60 mCi/mmol) were purchased from Amersham Pharmacia Biotech. PF1022A, -B, -C, -D, and -F were generously donated by Miyoi Kaisha Seika Ltd. (Kayama, Japan). All other chemicals were of the highest purity commercially available.

**Growth of Organism—**M. sterilia PF1022, donated by Miyoi Seika Kaisha Ltd., was maintained on agar slants (1.95% potato dextrose). Pieces from agar slants were transferred into 250-ml baffled flasks and glass beads containing 50 ml of preculture medium (3% glucose, 1% peptone, 1% yeast extract, 1% malt extract, 0.05% KH₂PO₄, pH 7). After cultivation for 72 h at 26 °C on a rotary shaker (200 rpm), the mycelium was used as an inoculum for the main culture. A 5-ml aliquot of the preculture was transferred into 500-ml baffled flasks containing 100 ml of production medium (1% Pharmamedia, 2% wheat germ, 0.2% MgSO₄·7H₂O, 0.2% NaCl, 0.2% CaCl₂, pH 7). After incubation for 90 h at 26 °C on a rotary shaker (200 rpm), the mycelium reached its maximum capacity for PF1022A formation, measured by the in vivo incorporation of [14C]-methionine carried out as described by Zocher et al. (13).

Cells were harvested by suction filtration on a Büchner funnel, washed three times with 0.1 M KCl solution, and stored at -80 °C.

**ATP/PP Exchange Reaction—**Amino or hydroxy acid-dependent ATP/PP exchange was carried out as described by Lee and Lippman (15).

**Measurement of Covalently Bound Substrates—**Covalently bound substrates were measured according to Gevers et al. (16). One picomolar of purified PFSYN was incubated in the presence of 5 mM ATP, 10 mM MgCl₂, and 0.5 µCi of [14C]-leucine, S-adenosyl-L-[14C-methyl]methionine, or [14C]-lactate in a final volume of 100 µl. To show the ATP-dependent covalent binding of the substrates, the control was incubated without ATP. The reaction was stopped after 1 h by addition of 1 ml of 7% trichloroacetic acid. The reaction mixture was centrifuged for 3 min at 3000 rpm. The protein precipitate was then washed twice with 1 ml of ethanol and dried at 60 °C for 1 h.

C-terminal to domain EB is a third phosphopantetheine that is C-terminal to domain EB.

**Kinetic Measurements—**For kinetic measurements, [14C]methyl-AdoMet was added in total volume of 100 µl for 1 h at 26 °C. After addition of 2 ml of water, the mixture was extracted with 2 ml of ethyl acetate. The organic phase was evaporated, and the residue was subjected to TLC. Autoradiography was performed with Konica medical A2 x-ray film. For synthesis of other cyclooctadepsipeptides, the ratio of ω-lactate-ω-phényllactate in the assay was changed to ω-lactate-ω-benzoyllactate.

**Protein Determination—**Protein concentrations were determined by a modified Bradford procedure (20) with bovine serum albumin as a standard.

**Western Blot Analysis—**Purified PFSYN was subjected to 5% SDS-PAGE. The protein was electrotransferred to nitrocellulose in a semidry blotting procedure (BioMetra). After the transfer, immunoblot analysis used was using rabbit anti-ESYN polyclonal antibodies according to Haese et al. (9).

**Detection of 4'-Phosphopantetheine—**Purified PFSYN was hydrolyzed with 1 N NaOH for 2 h at 80 °C and incubated after adjusting the pH to 8 with or without (control) 15 units of bovine alkaline phosphatase for 2 h at 37 °C. The column was calibrated with alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa), and cyclosporine synthetase (1700 kDa). Vₑ was determined using blue dextran (2000 kDa).

**Enzymic Formation of PF1022A—**For in vitro synthesis of PF1022A, enzyme fractions were incubated in the presence of 5 mM ATP, 10 mM MgCl₂, 8 mM ω-lactate, 2 mM ω-phényllactate, 5 mM L-leucine, and 0.05 mM [14C]methyl-AdoMet in a total volume of 100 µl for 1 h at 26 °C. After addition of 2 ml of water, the mixture was extracted with 2 ml of ethyl acetate. The organic phase was evaporated, and the residue was subjected to TLC. Autoradiography was performed with Konica medical A2 x-ray film. For synthesis of other cyclooctadepsipeptides, the ratio of ω-lactate-ω-phényllactate in the assay was changed (see Fig. 4).

**Enzymic Formation of PF1022F—**For in vitro synthesis of PF1022F, enzyme fractions were incubated in the presence of 5 mM ATP, 10 mM MgCl₂, 5 mM L-leucine, and varying concentrations of ATP, 10 mM MgCl₂, 5 mM L-leucine, and varying concentrations of ω-phényllactate in a final volume of 100 µl. Each concentration ranging from 0.1 to 10 mM was tested in triplicate. After incubation for 30 min at 26 °C, the reaction was stopped by the addition of 2 ml of water, and the product was extracted with 2 ml of ethyl acetate. Quantitative measurements of the radioactive product in the organic phase were done with a Wallac 1409 liquid scintillation counter.
Purification and Characterization of PFSYN

Two grams of lyophilized cells per extraction were used.

| Total amount of protein | Activity* | Specific activity | Purification |
|-------------------------|-----------|------------------|--------------|
| mg                      | pkat      | pkat/mg          | -fold        |
| Crude extract           | 140       | 1                | 0.007        | 1             |
| Polymethyleneimine precipitate | 128       | 1.2              | 0.01         | 1.4           |
| Hydrophobic chromatography | 0.5       | 2.5              | 5            | 228           |

* One picokatal is the amount of enzyme catalyzing the formation of 1 pmol of PF1022A.

The molecular mass of the native enzyme determined by SDS-PAGE was found to be 350 kDa. As can be seen in Fig. 2, the enzyme comigrates with ESYN, which has a molecular mass of 350 kDa. Lane 1, ESYN (6); lane 2, purified PFSYN.

**RESULTS**

**Purification of PFSYN**—PFSYN from *M. sterilia* was purified in two steps (Table I). The presence of 60% glycerol was essential for the isolation of active enzyme preparations. In the butyl-agarose step, the bulk of the protein was found to be in the run-through fraction. PFSYN could be eluted with a decreasing gradient of ammonium sulfate (30–0%) at about 15% saturation. PFSYN activity was monitored by measuring the \( \Delta \)-phenyllactate-dependent ATP/PP, exchange. After the butyl-agarose step, the PFSYN peak fraction reached about 95% homogeneity, as judged by SDS-PAGE (Fig. 2). The purified enzyme could be preserved for several months by storage at \(-80^\circ\)C in the presence of 20% glycerol without significant loss of activity.

**Molecular Properties of PFSYN**—The molecular mass of the denatured enzyme determined by SDS-PAGE was found to be 350 kDa. As can be seen in Fig. 2, the enzyme comigrates with ESYN. The molecular mass of the native enzyme determined by gel filtration chromatography was found to be 1400–1600 kDa, suggesting an oligomeric structure.

PFSYN showed strong cross-reaction with polyclonal antibodies directed against ESYN in a Western blot analysis. No reaction with purified PFSYN (Coomassie Blue stain). PFSYN comigrates with ESYN, which has a molecular mass of 350 kDa. Lane 1, ESYN (6); lane 2, purified PFSYN.

**In vitro synthesis of radiolabeled PF-compounds.** Reaction products were separated by TLC and detected by autoradiography. Lane 1 reveals the products of a complete assay for PF1022A synthesis (5 mM ATP, 10 mM MgCl\(_2\), 8 mM \( \Delta \)-phenyllactate, 2 mM \( \Delta \)-lactate, 5 mM \( \Delta \)-leucine, and 2 mM AdoMet at 26 °C for 2 h). The products were extracted with 2 ml of ethyl acetate. The organic phase was evaporated, and the residue was dissolved in 200 \( \mu \)l of 5% acetonitrile/water. Sample volumes of 50 \( \mu \)l were injected. Separation of products was performed on a Nova-Pak column (Waters, Milford, MA). The following step gradient was used: 0 min/5% acetonitrile, 10 min/5% acetonitrile, 15 min/10% acetonitrile, 25 min/50% acetonitrile, 30 min/80% acetonitrile, 40 min/80% acetonitrile, 45 min/100% acetonitrile, 60 min/100% acetonitrile (flow rate 1 ml/min). The LC effluent was split by means of an adjustable flow splitter (Upchurch; GAT, Bremerhaven, Germany). The mass spectrometer was operated in the electrospray mode with positive ionization. Identification of the products was done by retention time, UV spectra, and the protonated and sodiated quasimolecular ions in the mass spectra.

**Chromatographic Procedures**—Products were separated on silica gel plates (Merck, Darmstadt) with ethyl acetate/chloroform (1:2) as the solvent system. After TLC separation, radioactive products were extracted from the silica gel plates with ethanol and applied to HPLC. A step gradient was used as described above. Identification of the products was done by co-chromatography with DKM and CODP references. These references were synthesized by Meiji Seika Kaisha Ltd. Structural documentation was done by NMR and MS analysis. Radiotracers \( \left[^{14}\text{C}\right]\text{methyl}-\Delta\text{-leucine and }\left[^{14}\text{C}\right]\text{-leucine were separated on silica gel plates in butanol/acetate/water (4:1:1).}

**Fig. 2.** SDS-PAGE of purified PFSYN (Coomassie Blue stain). PFSYN comigrates with ESYN, which has a molecular mass of 350 kDa. Lane 1, ESYN (6); lane 2, purified PFSYN.

**Fig. 3.** In vitro synthesis of radiolabeled PF-compounds. Reaction products were separated by TLC and detected by autoradiography. Lane 1 reveals the products of a complete assay for PF1022A synthesis (5 mM ATP, 10 mM MgCl\(_2\), 10 mM \( \Delta \)-Lac, 1 mM \( \delta \)-Phelac, 5 mM \( \Delta \)-Leu, 0.05 mM \( \left[^{14}\text{C}\right]\text{-methyl-AdoMet). Lane 2, as for lane 1, but ATP was omitted. Lane 3 confirms that no product is formed in the absence of AdoMet in the complete assay (\( \left[^{14}\text{C}\right]\text{-leucine served as a radiolabel).}

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2 K. Iiuma, personal communication.
PFSYN from the butyl-agarose step was used to detect the presence of enzyme-bound 4'-phosphopantetheine. After alkaline hydrolysis and subsequent alkaline phosphatase treatment, bacterial growth could be stimulated. Without phosphatase treatment, the growth rate of the test organism was strongly reduced due to the limited uptake of phosphopantetheine (results not shown). From these results we suggest that 4'-phosphopantetheine was released from the enzyme.

Cell-free Synthesis of PF1022A and Related Cyclooctadepsipeptides—we were able to establish the cell-free synthesis of PF1022A and related compounds when the enzyme was incubated with D-lactate, D-phenyllactate, L-leucine, AdoMet, ATP, and MgCl₂. Product formation is dependent on the presence of ATP as shown in Fig. 3 (lanes 1 and 2). In these experiments [¹⁴C-methyl]AdoMet was used. The same product pattern was observed when [¹⁴C]Leu (and unlabeled AdoMet) was used (data not shown). Besides PF1022A, the synthesis yielded some additional cyclooctadepsipeptides of the PF1022 type, as detected by TLC analysis using authentic PF1022-related compounds as standards. Using different molar ratios of D-phenyllactate/D-lactate, the product formation in vitro could be shifted from PF1022A to PF1022B (four D-phenyllactate residues) (Fig. 4). Thus, PFSYN is responsible for the synthesis of all known natural occurring cyclooctadepsipeptides in M. sterilia. N-Methylation is necessary for product formation, as shown in Fig. 3 (lane 3). As can be seen, in the absence of the methyl donor, no product formation occurs. This behavior is obviously different from ESYN, where the formation of unmethylated enniatins takes place in the absence of AdoMet (8).

Substrate Activation—Like other peptide and depsipeptide synthetases, PFSYN activates its substrates as thioesters via adenyllylation. The first activation step (acyl adenyllylation) can be followed by monitoring the ATP/[³²P]pyrophosphate exchange reaction, which is a specific indicator for adenylation reactions (21). When the enzyme was incubated with the appropriate substrates (L-Leu, D-Lac, D-Phelac) in the presence of ATP and [³²P]pyrophosphate, the formation of radioactive ATP could be monitored. All primary precursors of PF1022A were found to be activated as adenylates. The second activation step (ATP-dependent thioacylation) could be demonstrated using the method of Gevers et al. (16). This is shown in Fig. 5. Bound substrates could be released by oxidation with performic acid. When the enzyme was charged with [¹⁴C]leucine in the presence of the methyl donor AdoMet, N-methyl-leucine was found after oxidation (see Fig. 5, lanes 4 and 7). From the fact that formic acid did not release radioactive products, we conclude that the substrates are indeed bound as thioesters.

The band at the origin in Fig. 5 (lanes 7 and 8) is most probably labeled protein that was not oxidized by performic acid treatment and does not migrate in TLC.

Substrate Specificity of the Hydroxy Acid Binding Site—M. sterilia produces high levels of PF1022A as well as low levels of PF1022B, -C, and -D. Because PFSYN is able to synthesize all naturally occurring cyclooctadepsipeptides in vitro, the question arose as to whether product formation depends mainly on the substrate specificity of the enzyme (concerning the hydroxy acid binding site) or on the substrate pool in the fungal cell. Therefore, $K_m$ values of D-lactate and D-phenyllactate were determined from initial rates of PF1022F formation (contains only D-lactate as the hydroxy acid constituent) and PF1022B formation (contains only D-phenyllactate as the hydroxy acid constituent), respectively. $K_m$ values for these substrates were found to lie in the micromolar range (0.77 ± 0.15 µM for D-lactate, 0.45 ± 0.12 µM for D-phenyllactate), indicating a high affinity of the enzyme for both hydroxy acids compared with
Fig. 6. A, formation of the DKM derivatives during PF1022A synthesis in vitro as revealed by autoradiography of TLC. Lane 1, complete assay for CODP in vitro synthesis (5 mM ATP, 10 mM MgCl₂, 10 mM D-Lac, 1 mM D-Phelac, 5 mM L-Leu, 0.05 mM [³⁵Cl]methyl-AdoMet). Lane 2, as for lane 1, but without D-lactate. Lane 3, as for lane 1, but without D-phenyllactate. Lane 4, as for lane 1, but without D-phenyllactate and D-phenyllactate. B, autoradiography of TLC separation of the D-phenyllactate-dependent DKM derivative after acid hydrolysis. Identification of the radioactive product was done by co-chromatography with NMeLeu (for further details, see “Results”).

leucine ($K_m = 20 \pm 3 \mu M$). $k_{cat}/K_m$ values showed the same order of magnitude ($k_{cat}/K_m$ for D-lactate is $0.0022 \text{s}^{-1} \mu M^{-1}$, $k_{cat}/K_m$ for D-phenyllactate is $0.0037 \text{s}^{-1} \mu M^{-1}$), indicating that there is no distinct preference for one substrate. These results suggest that the formation of different PF-related compounds is mainly controlled by the molar ratio of the hydroxy acids and not by the specificity of the hydroxy acid binding site of the synthetase. This might be due to the elongation and reactions on the enzyme (see “Discussion”), which are probably limited by the different hydrophobic residues of the incorporated substrates D-Lac and D-Phelac. Thus, a strong excess of D-Lac compared with D-Phelac in the reaction mixture of the cell-free synthesis is necessary for the formation of PF1022-related compounds containing D-Lac as can be seen in Fig. 4. Interestingly, several homologous hydroxy acids like D-2-hydroxyisocaproate, D-2-hydroxyvalerate, D-2-hydroxybutyrate, D-2-hydroxyisocaproate, D-2-hydroxy-3-methyl-valerate, and p-nitro- and p-amino-D-phenyllactate could be incorporated into new products, indicating a rather low specificity of the hydroxy acid binding site (22, 23).

**Formation of Di-, Tetra-, and Hexadepsipeptides**—A product of the reaction mixture from the in vitro synthesis could be identified as a diketomorpholine derivative whose formation depends either on the hydroxy acids D-phenyllactate or D-lactate, the amino acid L-leucine and the methyl donor AdoMet. This is shown in Fig. 6, where [³⁵Cl]methyl-AdoMet was used as a radiolabel. In the absence of D-hydroxy acids, no product formation was observed (Fig. 6A, lane 4). Acid hydrolysis of this radioactive product yielded radioactive N-methyl-l-leucine (Fig. 6B). HPLC analysis showed a similar retention time with the authentic DKMs c(D-LacNMeLeu) and c(D-Phelac NMeLeu) (see Fig. 7). The in vitro formation of N-methylated cyclodipeptides and diketopiperazines is well known from enniatin synthetase (13) and cyclopore synthetase, respectively (24, 25).

Further analysis of the in vitro PF-related products was carried out by HPLC/MS analysis (see Table II). When a molar ratio of 10:1 (10 mM/1 mM) D-lactate/D-phenyllactate in the in vitro reaction mixture was chosen, the formation of all natural cyclooctadepsipeptides, PF1022A, -B, -C, and -D, could be observed (Fig. 8). Interestingly, a hitherto postulated and chemically synthesized (26) cyclooctadepsipeptide called PF1022F consisting of D-lactate and N-methyl-l-leucine was found in the in vitro reaction mixture (Fig. 7). Besides the known PF1022A type structures, we could also detect two new cyclooctadepsipeptides and a new cyclohexadepsipeptide in the in vitro reaction mixture by HPLC/MS analysis (see Fig. 7). Thus, PFSYN is capable of synthesizing not only cyclooctadepsipeptides but also di-, tetra- and hexadepsipeptides.

**DISCUSSION**

PFSYN was purified from crude extracts of *M. sterilia PF 1022*. This is the first reported purification and characterization of a N-methyl-cyclooctadepsipeptide synthetase. This multifunctional enzyme catalyzes: 1) activation of the substrates via adenylation; 2) covalent binding of the substrates via thioesters; 3) N-methylation of thioesterified leucine with AdoMet; and 4) condensation reaction, elongation, and cyclization. Interestingly, the molecular size of the purified enzyme in SDS-PAGE is identical with that of the ESYN, although the latter enzyme is responsible for the synthesis of a cyclohexadepsipeptide and therefore assembles only six residues instead of eight to form the final product enniatin. It has been previously dem-
onstrated that peptide and depsipeptide synthetases consist of repeating 120-kDa domains responsible for the activation and condensation of the corresponding substrates to form the final peptide metabolite (for review, see Ref. 10). The molecular mass of 350 kDa estimated for PFSYN suggests that, like ESYN, PFSYN also comprises two peptide synthetase domains each of more than 100 kDa with an integrated methyltransferase portion of about 50 kDa (9, 12).

Interestingly, estimation of the native molecular weight by gel filtration chromatography yielded higher values (>1 Mda) than the denatured enzyme indicating a possible oligomeric structure. Work is in progress to analyze the exact oligomeric structure of PFSYN by means of analytical ultracentrifugation. Based on the assumption that PFSYN is a two-domain enzyme like ESYN, catalyzing the biosynthesis of PF1022-related compounds via an iterative mechanism, we suggest that one do-

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**TABLE II**

Cyclodepsipeptides found by HPLC/MS in the in vitro synthesis catalyzed by PFSYN

| Cyclodepsipeptides<sup>a</sup> | Molecular formula | Mass peak | Intensities relative to PF1022<sup>b</sup> | Retention time (t<sub>R</sub>) |
|-------------------------------|-------------------|-----------|---------------------------------|-----------------------------|
| Tetradepsipeptide             |                   |           |                                 |                             |
| (NMeLeu<sub>2</sub> d-Lac d-Phelac) | C<sub>20</sub>H<sub>35</sub>N<sub>2</sub>O<sub>6</sub> | [M + H]<sup>+</sup> 475.28 | 33 | 32.33 |
| (NMeLeu d-Phelac NMMeLeu d-Phelac) | C<sub>20</sub>H<sub>35</sub>N<sub>2</sub>O<sub>6</sub> | [M + H]<sup>+</sup> 551.114 | 25 | 34.67 |
| Hexadepsipeptide              |                   |           |                                 |                             |
| (NMeLeu<sub>3</sub> d-Lac<sub>2</sub> d-Phelac) | C<sub>30</sub>H<sub>55</sub>N<sub>3</sub>O<sub>6</sub> | [M + H]<sup>+</sup> 674.164 | 7 | 34.15 |
| Octadepsipeptide              |                   |           |                                 |                             |
| (NMeLeu<sub>4</sub> d-Lac<sub>4</sub>) PF1022F | C<sub>40</sub>H<sub>65</sub>N<sub>4</sub>O<sub>12</sub> | [M + Na]<sup>+</sup> 972.227 | 22 | 33.8  |
| (NMeLeu<sub>4</sub> d-Lac<sub>3</sub> d-Phelac) PF1022D | C<sub>40</sub>H<sub>65</sub>N<sub>4</sub>O<sub>12</sub> | [M + Na]<sup>+</sup> 1025.252 | 11 | 44.75 |
| (NMeLeu<sub>4</sub> d-Lac<sub>2</sub> d-Phelac<sub>2</sub>) PF1022B | C<sub>52</sub>H<sub>76</sub>N<sub>4</sub>O<sub>12</sub> | [M + Na]<sup>+</sup> 1101.379 | 2 | 46.29 |

<sup>a</sup> Cyclic structures are based on their calculated and observed molecular mass, as well as on their behavior in HPLC.

<sup>b</sup> As estimated by relative peak areas in the HPLC/MS analysis.

<sup>c</sup> Not found.

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**Fig. 8.** HPLC/MS analysis of products synthesized in vitro by PFSYN. A, total ion current (TIC); B–F, mass traces of the individual cyclodepsipeptides (for peak assignments, see Table II); G, diode array at 210 nm (for details, see “Experimental Procedures”).
main of PFSYN activates l-leucine and is responsible for N-methylation. The other domain activates d-lactate as well as d-phenyllactate. The low substrate specificity of the latter domain is also illustrated by the fact that the enzyme activates a wide range of different d-2-hydroxy acids. The occurrence of different natural PF1022A-related compounds varying in the d-lactate and d-phenyllactate position appears to be the consequence of a competition of the hydroxyacids for one binding site on the enzyme leading to the formation of different dipeptidols. Elongation of the growing depsipeptide chain occurs by linking dipeptidol units either of N-methyl-l-leucine and d-lactate or of N-methyl-l-leucine and d-phenyllactate. After four dipeptidol units have entered the process, cyclization terminates the reaction sequence of the enzyme. The elongation reaction links the different combinations of the two possible dipeptidol units consisting either of d-lactate/N-methyl-leucine or of d-phenyllactate/N-methyl-leucine and therefore leads to the complete natural product spectrum of M. sterilia including PF1022A, -B, -C, -D, and -F. The detection of DKM derivatives, tetra- and hexadepsipeptides, in the in vitro system provides strong evidence for this mechanism of stepwise dipeptidol condensations, whereas the odd-numbered tri-, penta-, and heptadepsipeptides were not detectable in the MS analysis of the in vitro products. We could demonstrate by in vitro studies that PF1022F, which consists of four residues of d-lactate and four residues of N-methyl-l-leucine, is also synthesized by PFSYN. From the six possible arrangements of d-lactate, d-phenyllactate, and N-methyl-l-leucine in the corresponding PF1022-related molecules, there should be one isomer of PF1022A in which the two d-phenyllactate residues are neighbors (Fig. 1, R1 = R2 = CH2C6H5, R3 = R4 = CH3). Because of their structural homology, the two PF1022A isomers have not been distinguished by mass spectrometry analysis. Work is in progress to detect the two isomers by MS/MS and NMR techniques.

DKM formation appears to be a general reaction in cyclodepsipeptide-producing fungi. Recently, a DKM derivative named lateritium (27), consisting of d-2-hydroxyisovalerate and N-methyl-l-phenylalanine, which are the alternating constituents of the cyclohexadepsipeptide beauvericin (28).

The ability to produce mixed-type structures is also known from enniatin biosynthesis (29). However, in this case variations in the amino acid moieties of the enniatin molecule occur, whereas the hydroxy acid component d-HIV is conserved. Similarly, it has been demonstrated by in vitro studies with beauvericin synthetase from B. bassiana that l-phenylalanine could be replaced by l-leucine and a variety of homologous amino acids (28).

In summary, these results show that cyclodepsipeptides with repetitive dipeptidole building blocks like PF1022A-type cyclooctadepsipeptides, enniatins, and beauvericins are synthesized by two-domain enzymes in an iterative process in contrast to peptides with irregular structure, e.g., cyclosporin, which is synthesized by a peptide synthetase with 11 domains.

REFERENCES
1. Sasaki, T., Takagi, M., Yaguchi, T., Miyadoh, S., Okada, T., and Koyama, M. (1992) J. Antibiot. (Tokyo) 45, 692–697
2. Terada, M., Ishih, A., Tungtrongchitr, A., Sano, M., and Shomura, T. (1993) Jpn. J. Parasitol. 42, 199–210
3. Kachi, S., Terada, M., and Hashimoto, H. (1998) Jpn. J. Parasitol. 47, 235–245
4. Scherenbeck, J., Harder, A., Plant, A., and Dyker, H. (1998) Bioorg. Med. Chem. Lett. 8, 1035–1040
5. Suzuki, A., Kanoaka, M., Isogai, A., Murakoshi, S., Ichinoe, M., and Tamura, S. (1977) Tetrahedron Lett. 25, 2167–2170
6. Bergendorff, O., Anke, H., Dekeermendjian, K., Nielsen, M., Rudong, S., Sterner, O., and Witt, R. (1994) J. Antibiot. (Tokyo) 47, 1560–1561
7. Tomoda, H., Nishida, H., Huang, X. H., Masuma, R., Kim, Y. K., and Omura, S. (1992) J. Antibiot. 45, 1207–1215
8. Zocher, R., Keller, U., and Kleinkauf, H. (1982) Biochemistry 21, 28–44
9. Haese, A., Schubert, M., Herrmann, M., and Zocher, R. (1995) Mol. Microbiol. 7, 905–914
10. Zocher, R., and Keller, U. (1997) Adv. Microb. Physiol. 38, 85–131
11. Lawen, A., and Zocher, R. (1990) J. Biol. Chem. 265, 11355–11360
12. Weber, G., Scho¨rgendorfer, K., Schneider-Scherzer, E., and Leitner, E. (1994) Curr. Genet. 26, 120–125
13. Zocher, R., Keller, U., and Kleinkauf, H. (1983) Biochem. Biophys. Res. Comm. 110, 292–299
14. Pieper, R., Haese, A., Schro¨der, W., and Zocher, R. (1995) Eur. J. Biochem. 230, 119–126
15. Lee, S., and Lipmann, F. (1975) Methods Enzymol. 53, 585–602
16. Gevers, W., Kleinkauf, H., and Lipmann, F. (1969) Proc. Natl. Acad. Sci. U. S. A. 63, 1335–1342
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Blum, H., Beiger, H., and Gross, H. J. (1987) Electrophoresis 8, 93–99
19. Quandt, N., Stindl, A., and Keller, U. (1993) Anal. Chem. 65, 490–494
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Eigner, E. A., and Lohfeld, R. B. (1974) Methods Enzymol. 29, 601–619
22. Weckwerth, W. (1998) Studies on the Biosynthesis of Cyclooctadepsipeptides from Mycelia sterilia. Ph.D. thesis, Technical University Berlin, Berlin
23. Jeschke, P., Bonse, G., Thielking, G., Etzel, W., Harder, A., Mencke, N., Kleinkauf, H., Zocher, R., Inuma, K., and Miyamoto, K. (September 23, 1983–1987 (September 1, 1994) International Patent WO9419334
24. Zocher, R., Niihara, T., Paul, E., Maday, Peeters, H., Kleinkauf, H., and Keller, U. (1986) Biochemistry 25, 550–553
25. Hoffmann, K., Schneider-Scherzer, E., Kleinkauf, H., and Zocher, R. (1994) J. Biol. Chem. 269, 12710–12714
26. Ohyama, M., Ohishi, M., Okada, Y., Koyama, M., Sumi, S., Murai, Y., Takagi, M., Okada, T., Sakakana, O., Yoneta, T., Inuma, K., and Shibahara, S. (September 1, 1994) International Patent WO9419334
27. Hasumi, K., Shinohara, C., Iwanaga, T., and Endo, A. (1993) J. Antibiot. (Tokyo) 46, 1782–1787
28. Peeters, H., Zocher, R., and Kleinkauf, H. (1988) J. Antibiot. (Tokyo) 12, 1135–1342
29. Pieper, R., Kleinkauf, H., and Zocher, R. (1992) J. Antibiot. (Tokyo) 45, 1273–1277