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In vitro Synthesis of New Cyclodepsipeptides of the PF1022-Type: Probing the α-D-Hydroxy Acid Tolerance of PF1022 Synthetase

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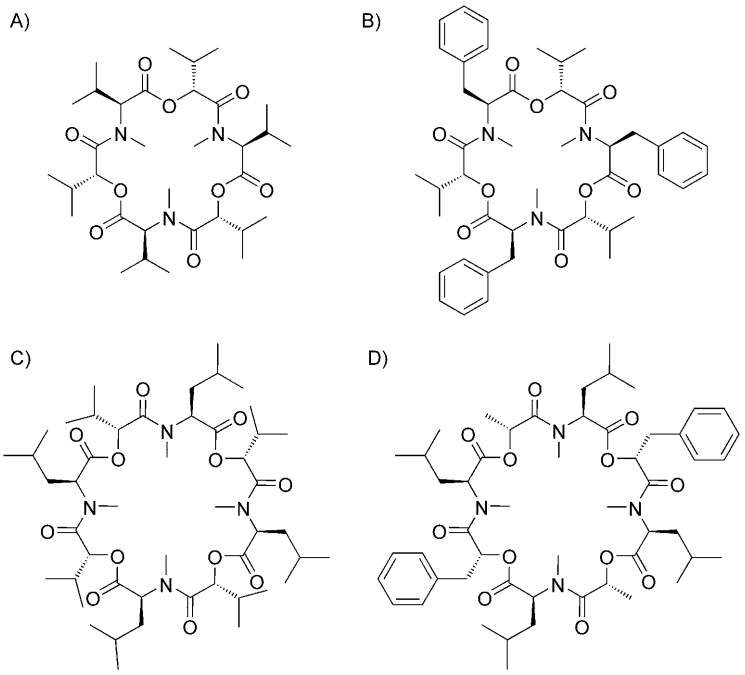
In memory of Rainer Zocher.

The nonribosomal peptide synthetase PF1022-synthetase (PFSYN) synthesises the cyclooctadepsipeptide PF1022 from the building blocks D-lactate, D-phenyllactate and N*-methylleucine. The substrate tolerance of PFSYN for hydroxy acids was probed by in vitro screening of a set of aliphatic and aromatic α-D-hydroxy acids with various structural modifications in the side chain. Thus, new PF1022 derivatives for example, propargyl-D-lactyl-PF1022 and β-thienyl-D-lactyl-PF1022 were generated. The promiscuous behaviour of PFSYN towards aliphatic and aromatic α-D-hydroxy acids is considerably larger than that of related enniatin synthetase (ESYN) and thus gives rise to the enzymatic generation of various new PF1022 derivatives.*

Introduction

Natural products are considered to be biologically validated lead structures. Compounds with novel or enhanced biological properties are expected from the generation of structural diversity in natural product libraries. Cyclodepsipeptides (CDPs) represent an important category of natural peptide products that are characterised by the occurrence of at least one ester linkage. The great interest that this class of natural products has elicited is explained by the broad range of biological activities, including antitumour (destruxins A, B and E),[1] antibacterial (ramoplanin), antifungal (W493A and B),[2] insecticidal (verticillide)[3] or anthelmintic activities (PF1022A).[4] Helminths, including parasitic nematodes, cause significant health problems in both humans and animals and thus are a cause of diseases such as epileptic seizure and allergic shock or even onchocerciasis.[5] In this context, development of anthelmintic drugs is an important matter because many nematodes have become resistant to traditional anthelmintics, including benzimidazole derivatives and macrocyclic lactones, for example, avermectin.[6] In the course of a directed screening for anthelmintic properties Sasaki et al. isolated the *N*-methylated cyclodepsipeptide PF1022A (Scheme 1), which is produced by the fungus *Mycelia sterilia*.[7,8] PF1022 belongs to a class of CDPs[9] that consist of an alternating arrangement of four *N*-methyl-L-leucines and a varying content of α-D-hydroxy acids, from four D-lactates (PF1022F) to four D-phenyllactates (PF1022B), respectively (Scheme 2). A semisynthetic derivative of PF1022, emodepside, is currently used as a highly effective drug against helminths.[10,11] Structurally, PF1022 is related to CDPs, like the cyclohexadepsipeptides enniatin (*Fusarium scirpi*)[12] and beauvericin (*Beauveria bassi-*



Scheme 1. Structures of the cyclohexadepsipeptides A) enniatin B, B) beauvericin and the cyclooctadepsipeptides C) bassianolide and D) PF1022A.

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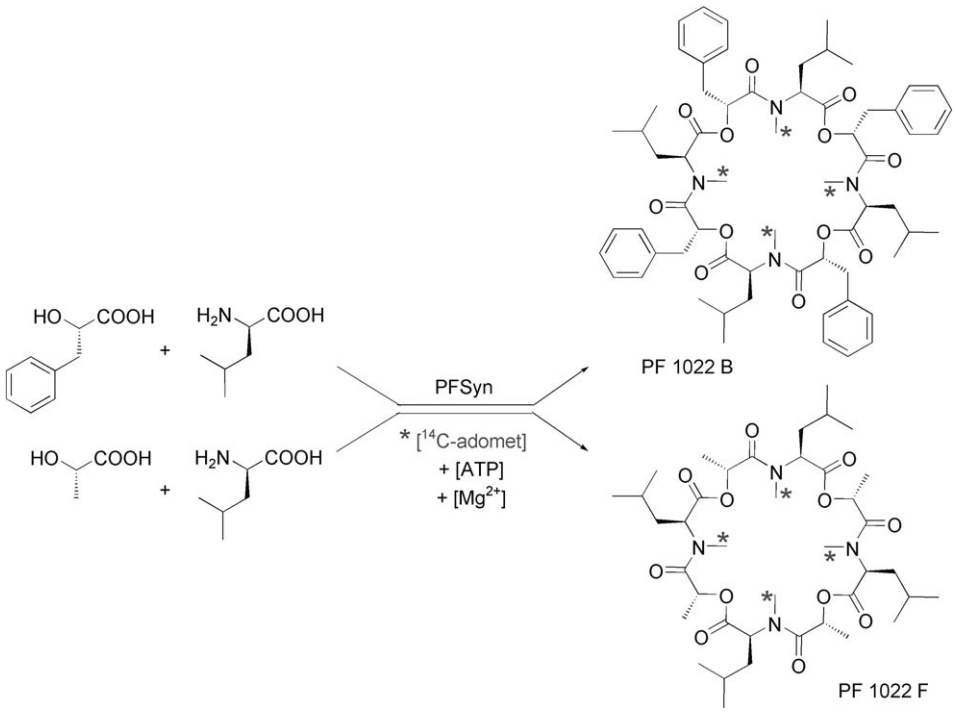


Scheme 2. Assay for the in vitro generation of new PF1022 derivatives (here PF1022B and F) from α-D-hydroxy acid (D-Lac/D-PheLac), L-Leu, ATP and AdoMet. Modular sketch of the domains architecture of PF1022 synthetase PFSYN: C (condensation), A (adenylation), T (thiolation), M (methylation).

ana) and the cyclooctadepsipeptide bassianolide (*Beauveria bassiana*),[13,14] with various hydrophobic α-hydroxy and α-amino acid side chains (Scheme 1). All of these CDPs are synthesised by a corresponding nonribosomal peptide synthetase. The nonribosomal peptide synthetase of PF1022 is PFSYN (350 kDa), which accepts L-leucine, D-lactate, D-phenyllactate, *S*-adenosyl-L-methionine (AdoMet), and ATP as substrates. The domain structure of the PFSYN is analogous to that of ESYN.[17] It consists of two modules (MA and MB); MA is responsible for α-D-hydroxy acid activation and MB for L-amino acid activation and *N*-methylation.[15] Both modules activate their substrates as pantetheine-derived thioesters via the corresponding acyl adenylates. The process of substrate activation starts with the recognition step by the adenylation domains of modules MA and MB. The specificity of these domains is responsible for the selection of the α-L-amino and α-D-hydroxy acids that are incorporated into the depsipeptide ring. PF1022 is synthesised in an iterative process by forming a linear depsipeptide from intermediate dipeptidol building blocks, which are finally condensed head-to-tail to form and thus finalise the cyclooctadepsipeptide. In this process a third protein-bound pantetheine group is involved by acting as a "waiting position" for the growing peptide chain.[16] N-Methylation is catalysed by a N-methyltransferase domain with *S*-adenosyl-L-methionine as a methyl donor. Omission of AdoMet yields the corresponding desmethyl PF1022 analogue, and at low concentrations of the methyl donor AdoMet, partially methylated PF1022A is produced. The occurrence of five natural derivatives, PF1022A–F, which vary in their content of D-lactate and D-phenyllactate implies a lowered α-hydroxy acid specificity of the adenylation domain of module MA.[17]

Recently, our group has established the in vitro synthesis of a number of enniatin derivatives that are catalysed by ESYN by probing the α-hydroxy acid binding pocket.[18] A considerable number of α-D-hydroxy acids has been accepted as substrates, thus enabling a remarkable structural diversity in newly generated enniatins to be generated. Herein we report an extension of this in vitro approach to PFSYN (Scheme 2). In contrast to the α-hydroxy-acid-activating domain of enniatin synthetase ESYN, the corresponding domain of PFSYN accepts both D-lactate (D-Lac) and the sterically demanding aromatic D-phenyllactate (D-PheLac).[19] In order to evaluate the substrate specificity of the α-D-hydroxy acid activating domain of PFSYN, a set of thirty-three α-D-hydroxy acids was synthesised that bear various modifications in the side chain (see Supporting Information).

Results and Discussion

For in vitro synthesis, PFSYN was incubated with L-Leu, ATP, Mg^{2+} and the corresponding α-D-hydroxy acid in mM concentrations. [^{14}C-methyl]-AdoMet was used as a radiolabel. Under these conditions PFSYN was under substrate saturation, and this allowed us to estimate the corresponding relative k_{cat} values from the initial velocity measurements. The radioactive PF1022 analogues that were generated were extracted with EtOAc and subjected to scintillation counting. To proof the formation of PF1022 analogues, autoradiographs of TLC separation were performed for conversions with α-D-hydroxy acids. An additional characterisation was performed by means of HPLC–ESI-MS and MS/MS analyses. In this case unlabeled AdoMet was used (see Supporting Information). Figures 1 and 2 show incorporation of propargyl-D-lactic acid (**16**) and thienyl-D-lactic acid (**28**) into the cyclooctadepsipeptide ring to yield "propargyl-D-lactyl-PF1022" and "thienyl-D-lactyl-PF1022′", respectively.

Probing substrate tolerance of PFSYN toward α-D-lactate and analogues

In order to deduce substrate–enzyme activity relationships various α-D-hydroxy acids were used to probe the substrate tolerance of PFSYN. In the first test panel only aliphatic α-hydroxy

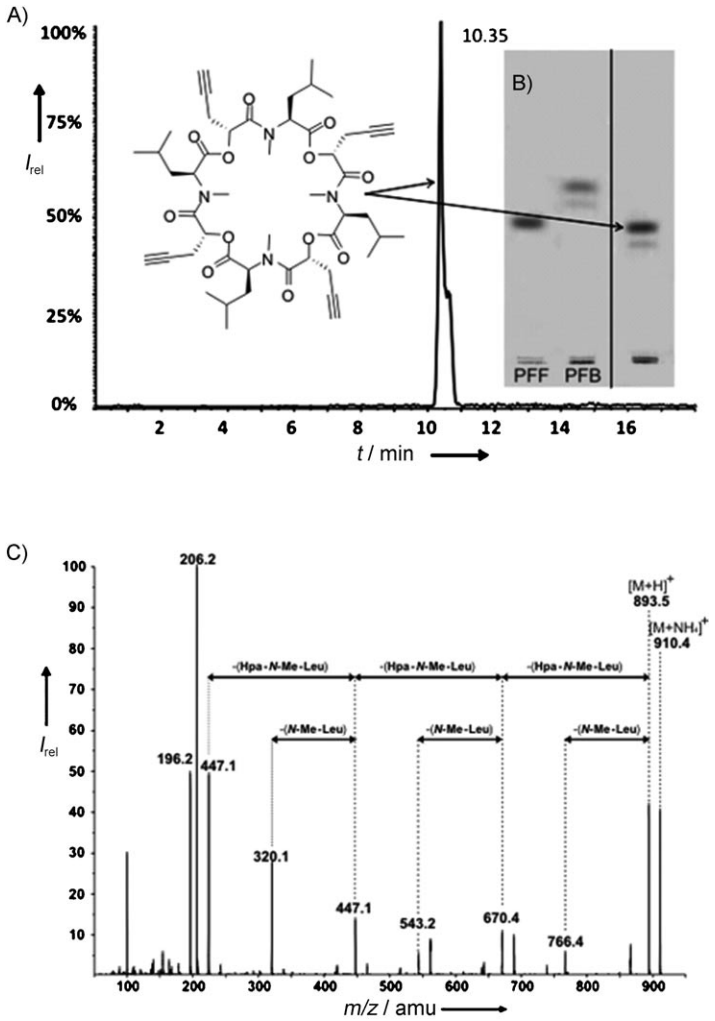


Figure 1. In vitro synthesis of propargyl-D-lactyl-PF1022 is catalysed by PFSYN: Incorporation of β-propargyl-D-lactate. A) Extracted ion chromatogram of the HPLC-ESI-MS, B) autoradiograph of the TLC separation showing enzymatically synthesised PF1022F (PFF), PF1022B (PFB) and propargyl-D-lactyl–PF1022. C) MS–MS spectrum of propargyl-D-lactyl-PF1022; calcd: 893.48 $[M+H]^+$; found: 893.5.

acids were tested. Scheme 3 shows the relative k_{cat} of aliphatic α-D-hydroxy acids incorporated into the cyclooctadepsipeptide ring compared to the natural substrate D-lactate (**1**; defined as 100%). As can be seen, PFSYN has a relaxed substrate tolerance towards substrates other than lactate (**1**). Tolerated substitutions of the methyl side chain of lactate include the chloromethyl derivative **2**, which shows the highest activity (174%), fluoromethyl derivative **3** (166%) and bromomethyl derivative **4** (121%). Interestingly, the propargyl residue of **16**, which has an activity of 156% is a significantly better substrate than the corresponding saturated derivative **6.** Elongation of the aliphatic chain to linear analogues with ethyl (analogue **5**), *n*-propyl (analogue **6**), and *n*-butyl (analogue **7**) side chains do not affect PFSYN activity (93%, 110% and 100%, respectively). Substitution of the terminal methylene group in the side chain of **7** by sulfur, which gives analogue **8**, even raises the activity to 112%; however, branched aliphatic side chains in the 2-position such as isopropyl analogue **12** (60%), *tert*-butyl compound **15** (54%), or in the 3-position, as in isobutyl compound **9** (59%) and neopentyl analogue **10** (68%), show significantly reduced activities.

A branched side chain of opposite configuration in 2-position, as exemplified by compound **13**, which has a *sec*-butyl group with a *R,R* configuration is moderately accepted by the enzyme (51%), but a complete loss of activity (0%) was observed for the corresponding analogue **14**, which is derived from *allo*-Ile and has a *R,S* configuration. Side chains with H-bonding capability, which are exemplified by compound **11** significantly reduce PFSYN activity (73%).

Probing substrate tolerance of PFSYN toward α-D-phenyl lactate and analogues

In the second test panel mono- and multinuclear aromatic as well as heterocyclic α-D-hydroxy acids were tested. In addition α-D-hydroxy acids with cyclohexane side chains were included in the assay in order to evaluate conformational influences of six-membered rings. Scheme 4 shows the relative k_{cat} values of the α-D-hydroxy acids that are incorporated into the cyclooctadepsipeptide ring compared to the natural substrate, D-phenyl lactate **17** (defined as 100%). As can be seen, PFSYN allows extensive modifications of the phenyllactate core, for instance the 4-bromo analogue **19** (101%), the 4-fluoro analogue **20** (85%) and the 4-chloro compound **21** (82%) are well tolerated. Interestingly, the pentafluoro derivative **18** shows the highest activity of all the phenyllactates (209%). Substitution of the *para* position by a nitro group (**30**) raises the activity to 120%, whereas *para* substitution by a cyano group (**31**) reduces the activity to 65%. Chain elongation by one methylene group (e.g., **24**) or truncation by one methylene group, as in **25**, reduces PFSYN activity slightly to 87 and 74%, respectively. Introduction of sterically demanding biphenyl group in compound **26** (47%), naphthyl in **27** (42%) and even diphenylmethyl groups in **32** (49%) are tolerated, but the activity is significantly reduced. Most remarkably, the substitution of phenyl with thienyl in compound **28** gave a considerable activity of 120%. In contrast, imidazole substitution in **29** caused a dramatic loss of activity (42%); this is probably due to the slightly basic character of the imidazole side chain. Finally, aliphatic cyclohexyl substituents in the 2-position (as in compound **23**) or 3-position (**22**) reduce the reactivity (88 and 67%, respectively).

Conclusions

Properties of the α-D-hydroxy acid binding pocket

The data on the relative enzymatic activities of PFSYN that are presented in Schemes 3 and 4 suggest that the α-D-hydroxy-acid-recognizing module MA of PFSYN has an enormous sub-

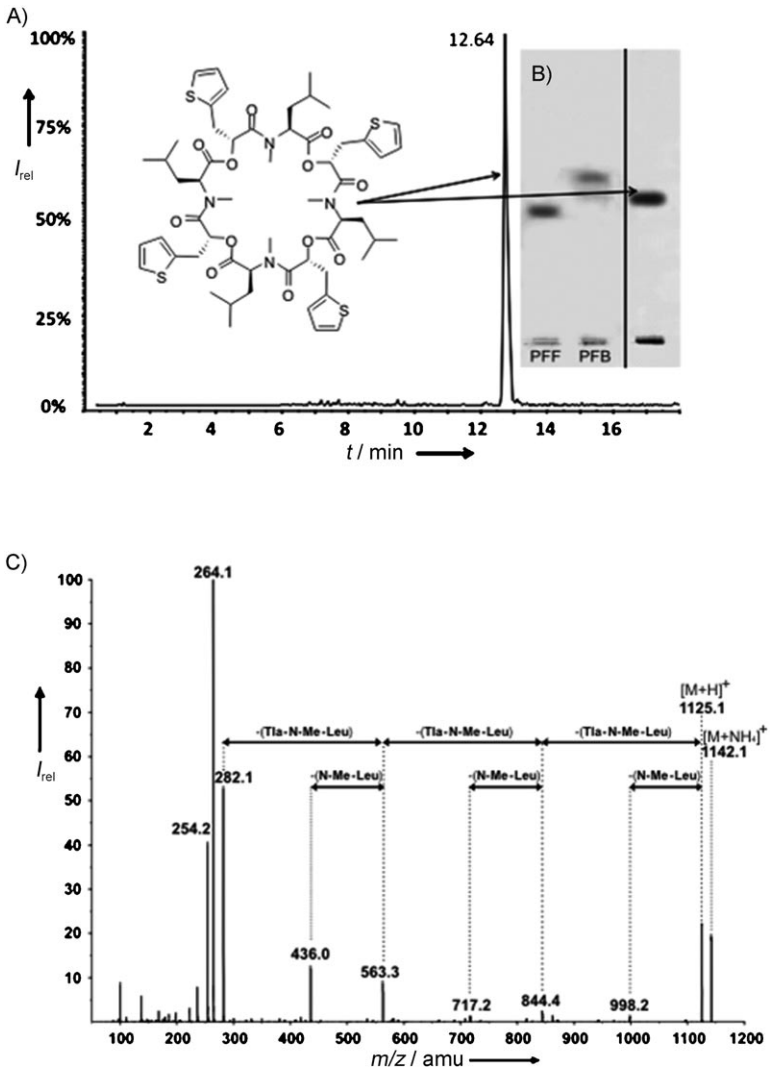


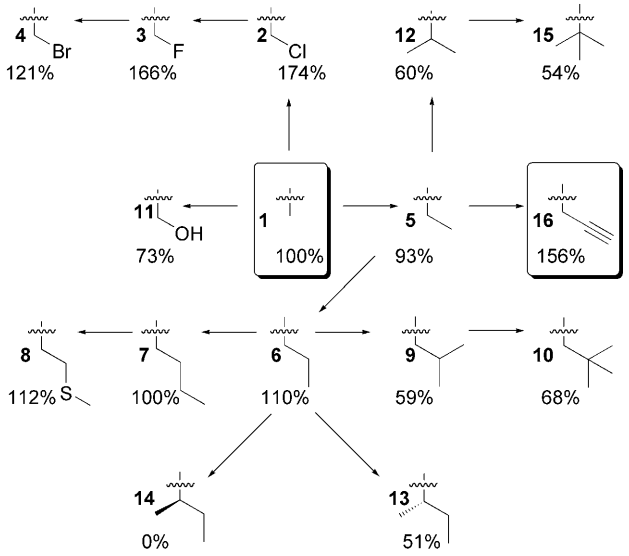
Figure 2. In vitro synthesis of thienyl-D-lactyl–PF1022 is catalysed by PFSYN: Incorporation of β-thienyl-D-lactate. A) Extracted ion chromatogram of the HPLC–ESI-MS, B) autoradiograph of a TLC separation showing enzymatically synthesised PF1022F (PFF), PF1022B (PFB) and thienyl-D-lactyl–PF1022. C) MS–MS spectrum of thienyl-D-lactyl–PF1022; calcd: 1125.43 $[M+H]^+$; found: 1125.1.

strate tolerance towards both aliphatic and aromatic amino acids. A qualitative comparison to enniatin synthetase ESYN shows that PFSYN is significantly less restrictive in its acceptance of structurally diverse α-D-hydroxy acids.[13,18]

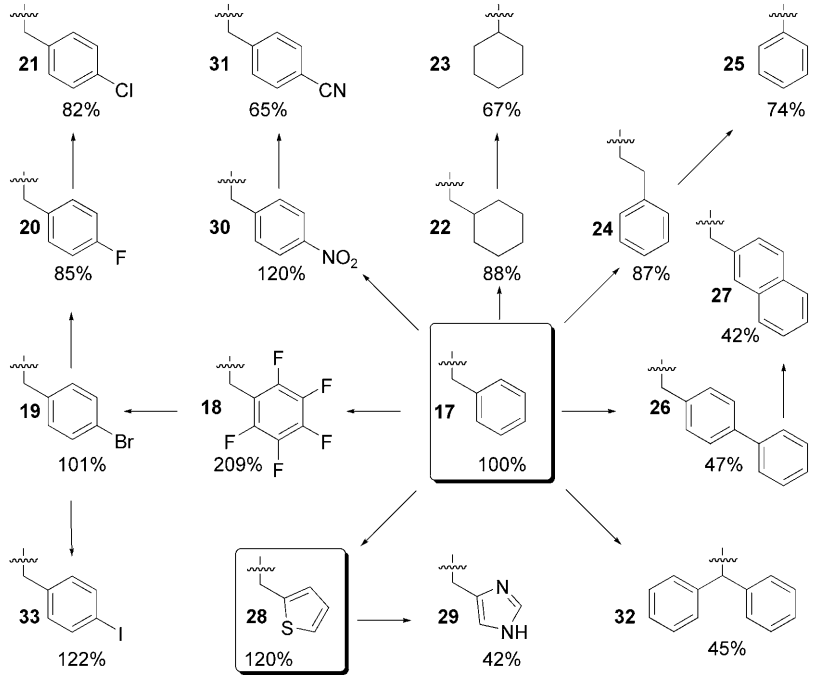
A closer look at activity differences gives the following picture: the α-D-hydroxy acid recognition site is relatively insensitive towards substitutions at the 2-position, albeit linear non-branched aliphatic chains (e.g. compounds **6**–**8**) are clearly preferred over sterically demanding methyl substituents (e.g. compounds **12** and **15**). Interestingly, the *R* enantiomer of **13** with the *sec*-butyl group is a moderate substrate whereas the *S* enantiomer **14** is inactive. This enantioselectivity is paralleled by ESYN,[18] and as a consequence with regard to enantioselectivity, homologous α-hydroxy acid recognition sites can be assumed for this structural motif. Halogenated lactates, which are represented by 3-chloro-, 3-fluoro- and 3-bromolactate (compounds **2**, **3** and **4**, respectively) all show an increased activity (174, 166 and 121%, respectively) compared to lactate (**1**).

An increasing steric demand in the 3-position, which is reflected by methyl group branching of derivatives **9** and **10** again decreases PFSYN activity. Substitution of the terminal methylene group of *n*-butyl derivative **7** with sulfur (e.g. compound **8**) does not affect the enzymatic activity negatively. Remarkably, the best activity among non-aromatic α-D-hydroxy acids is achieved by the propargyl analogue **16** (156%).

Among aromatic α-hydroxy acids, various substitutions in the *para* position are accepted as substrates. Substitution with halogens, 4-bromobenzyl compound **19** (101%), 4-fluorobenzyl analogue **20** (85%), 4-chlorobenzyl analogue **21** (82%) and 4-iodobenzyl analogue **33** (122%) are tolerated. Remarkably, the pentafluorobenzyl substrate **18** (209%) shows the best reactivity of all substrates. With regard to inductive and mesomeric effects, no clear rules could be deduced. Electron-withdrawing substituents in the *para* position such as 4-cyanobenzyl (**31**) and 4-nitrobenzyl (**30**) are similarly accepted as the natural substrate (**17**). An increasing steric demand exemplified by multinuclear aromatics (e.g. **26**, **27** and **32**) is less tolerated, as well as changing the number of methylene groups that link the phenyl group by α (e.g. **25**) or γ substitution (e.g. **24**). Interestingly, heterocycles such as **28** and **29** are also substrates, although the tendency to form hydrogen bonds appears to have a negative effect on enzyme activity. Finally, comparing activities of PFSYN for the sp^2-hybridised aromatic systems **17** and **25** (100 and 74%, respectively) to the sp^3-hybridised cyclohexane systems **22** and **23** (88 and 67%, respectively), seems to slightly favour sterically less-demanding planar aromatic substituents. Nevertheless, concomitant acceptance of aliphatic and aromatic α-D-hydroxy acids is characteristic for PFSYN, and thus, it is not surprising, that PFSYN has the capability to synthesise bassianolide from the α-hydroxy acid D-hydroxyisoveleric acid **12**. In return, it remains to be seen, whether bassianolide synthetase can synthesise PF1022.



Scheme 3. Reactivity of synthetic aliphatic D-hydroxy acids **1**–**16** in the assembly line of PFSYN expressed as a percentage of $v_{max.app}$; incorporation of D-lactate (**1**) as 100%



Scheme 4. Reactivity of cyclic-D-hydroxy acids **18**–**33** in the assembly line of PFSYN expressed as a percentage of $v_{max.app}$; the incorporation of D-phenyllactate (**1**) as 100%.

The characterisation of the analogue system,[18] that is, α-hydroxy acid recognition site of ESYN clearly shows that beauvericin cannot be synthesised by ESYN.

In summary, we have shown that PFSYN has a hitherto-unknown substrate tolerance towards an extremely broad spectrum of aliphatic and aromatic α-D-hydroxy acids in comparison to other NRPS enzymes. The present results suggest a relative independence of the α-D-hydroxy acid binding pocket from electronic effects, as well as a large steric tolerance for numerous substitutions, except for substituents with hydrogen-donating properties (e.g., **11** and **29**). The substrate tolerance is more pronounced than for ESYN and thus permits the synthesis of an enormous diversity of new PF1022 derivatives with versatile functional groups. The present characterisation of PF1022 substrate tolerance sets the stage for experiments proving whether this approach of generating new cyclodepsipeptides is suited for mutasynthesis experiments.[20] Future efforts will be directed to the generation of preparative amounts of PF1022 derivatives and testing for their bioactivities.

Experimental Section

Chemicals were purchased from Sigma (Deisenhofen, Germany), and radio chemicals were purchased from Amersham (Brunswick, Germany). Details for the synthesis of the α-D-hydroxy acids compounds **1**–**33** are given in the Supporting Information.

PFSYN was purified as described previously.[17] Protein concentrations were determined by the dye-binding method of Bradford by using bovine serum albumin as a standard.[21] For the in vitro synthesis of PF1022 analogues that contained different α-D-hydroxy acids (see Supporting Information) purified PFSYN was incubated in the presence of the corresponding α-D-hydroxy acids (3 mM), $MgCl_2$ (10 mM), ATP (5 mM), AdoMet 0.5 μCi (60 Ci mol^{-1}) and L-val (2 mM) in a final volume of 123 μL. The enzymatic reaction was stopped by the addition H_2O (1 mL) and extracted with EtOAc (2 mL). An aliquot of the organic phase (100 μL) was tested in the Scintillation counter. The residual amount of the organic phase was evaporated, dissolved in of EtOAc (50 μL) and applied to thin layer plates (silica gel plates, Merck Darmstadt, Germany). The plates were developed in $CHCl_3$/EtOAc (2:1, *v/v*). Radioactive substances were visualised by autoradiography by using Konica X-ray films and detected by radio scanning (Bertold Linear TLC analyzer, Bad Wildbad, Germany).

To determine the incorporation rates of the different substrates, $k_{cat.rel.}$ were calculated from initial rate plots. At time intervals 0, 2, 4, 6, 8, 10 min the amount of labelled PF1022 derivatives from the above-described assay was measured under saturation conditions ($v_{max.app.}$) by liquid scintillation counts (Wallac 1409, Turku, Finland). From the linear plots $k_{cat.rel.}$ were calculated from the best-fit straight lines (Origin 7.0, Frankfurt am Main, Germany). All experimental series were performed along with an assay that contained D-lac as a $k_{cat\ rel}$ standard.

For mass spectrometric analysis, unlabelled PF derivatives were synthesised by using a concentration of the methyl donor of

1 mM. After incubation for 2 h at 25 °C, the reaction was stopped by the addition of H_2O (1 mL) and the products were extracted with EtOAc (2 mL). The organic phase was evaporated, dissolved in MeOH (50 μL) and measured by HPLC–ESI-MS(/MS). The experiments were recorded on a capillary-LC system (1100 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany) that was coupled to a QTrap2000 with a TurbolonSpray source (Applied Biosystems, Darmstadt, Germany). HPLC was performed by using a 50×1 mm Luna 3u C18(2) 100 Å column (Phenomenex, Aschaffenburg, Germany) with a flow rate of 50 μL min^{-1} in micro mode by using a gradient from 30% to 100% MeCN (0.1% HCOOH) in H_2O (0.1% HCO_2H) in 10 min.

The TurbolonSpray source-dependent parameters were optimised for the used flow rate of 50 μL min^{-1} to: CUR 30, IS 4200, nebulizer gas 70, turbo gas 70, TEM 300. The used compound-dependent parameters were: DP 30, EP 10, CE 10, Q3 entry barrier 8. MS and MS–MS spectra were recorded in a mass range from *m/z* 50–1700, by using the mass analyser in linear ion-trap mode. The EMS scans were carried out in positive mode, with a LIT scan rate of 1000 amu s^{-1} and dynamic fill time. The EPI scans had the following parameters: Q1 resolution unit, LIT scan rate: 1000 amu s^{-1}, fixed LIT fill time: 400 ms, CE 50, CES 20, CAD gas high.

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Keywords: biosynthesis · cyclodepsipeptides · enzymes · hydroxy acids · nonribosomal peptide synthetase

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