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Cytotoxic Tetramic Acid Derivative Produced by a Plant Type-III Polyketide Synthase

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Supporting Information

ABSTRACT: The tetramic acid (2,4-pyrrolidinedione) scaffold has been recognized as an important structural feature because of its mycotoxic, antibacterial, antiviral, and antioxidant activities. This important class of natural products is reportedly produced by the type-I polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS) hybrid megaenzyme systems. In contrast, the benzalacetone synthase (BAS) from *Rheum palmatum* is a structurally simple, plant-specific type-III PKS that catalyzes the one-step decarboxylative condensation of malonyl-CoA with 4-coumaroyl-CoA. The type-III PKS exhibits unusually broad substrate specificity and notable catalytic versatility. Here we report that *R. palmatum* BAS efficiently produces a series of unnatural, novel tetramic acid derivatives by the condensation of malonyl-CoA with aminoacyl-CoA thioesters chemically synthesized from L- and D-amino acids. Remarkably, the novel tetramic acid dimer D-**5** formed from D-phenylalanoyl-CoA showed moderate antiproliferative activity against murine leukemia P388 cells.

Pyrrolidin(on)e alkaloids, including tetramic acids, are quite important because of their wide distribution in terrestrial and marine organisms.[1,2] They are common structural scaffolds found in biologically active natural products such as cylindramide,[3] aurantosides,[4] reutericyclin,[5] and discodermide[6] (Figure 1). In addition, the substituted rigid and complex ring system containing hydrogen-bond donors and acceptors would also be favored in synthetic materials to generate a variety of medicinal activities.[7,8] Although numerous synthetic efforts directed toward the development of pyrrolidin(on)e alkaloids have been performed, an efficient and novel methodology for the construction of the tetramic acid scaffolds would contribute to the development of versatile chemical libraries with promising biological activities.

The members of the chalcone synthase (CHS) superfamily of type-III polyketide synthases (PKSs) are involved in the biogenesis of pharmaceutically and biologically important polyphenols, including chalcone, stilbene, and benzalacetone.[9,10] The type-III PKSs are structurally and mechanistically simple enzymes that accept free CoA thioesters as substrates without the involvement of an acyl carrier protein and perform iterative condensation and cyclization reactions to produce an array of chemically and structurally divergent polyphenol scaffolds. Remarkably, they exhibit unusually broad and promiscuous substrate specificities

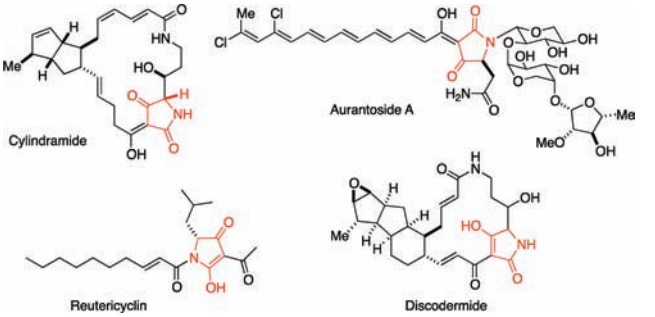

Figure 1. Representative tetramic acid-containing natural products.

and accept a variety of nonphysiological substrates.[9,10] The catalytic versatility is extremely useful for the production of unnatural novel polyketides by precursor-directed biosynthesis.[10,11]

We previously reported that the benzalacetone synthase (BAS) from rhubarb (*Rheum palmatum*) is a unique plant-specific type-III PKS that catalyzes the one-step decarboxylative condensation of malonyl-CoA with 4-coumaroyl-CoA as a starter substrate, producing the C_6-C_4 skeleton of a diketide benzalacetone (Scheme 1A).[12] The diketide-forming activity is attributed to the characteristic substitution of the CHS's conserved active-site residue Phe215 with Leu in *R. palmatum* BAS (numbering according to *Medicago sativa* CHS2).[13] Indeed, our X-ray crystallographic analysis of *R. palmatum* BAS at 1.8 Å resolution revealed that the F215L substitution causes not only conformational changes at the active-site cavity but also a 2-fold increase in the surface area of the active-site entrance of BAS relative to that of *M. sativa* CHS.[14] This widening of the active-site entrance contributes to the unique substrate and product specificities of *R. palmatum* BAS and allows it to accept a wide range of exogenous substrates, including bulky *N*-methylanthraniloyl-CoA as a starter molecule.[15,16]

The tetramic acid (2,4-pyrrolidinedione) scaffold is reportedly produced by the modular and iterative type-I polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS) hybrid megaenzyme systems. This process involves initial amide bond formation by coupling of an activated amino acid with an polyketide moiety and subsequent cyclization by the Dieckmann condensation.[17−23] In contrast, it was anticipated that if the structurally simple, promiscuous type-III PKS enzyme were to accept aminoacyl-CoA as a starter substrate and catalyze the condensation with malonyl-CoA to produce an aminoacyl−polyketide intermediate, it would then be possible to generate

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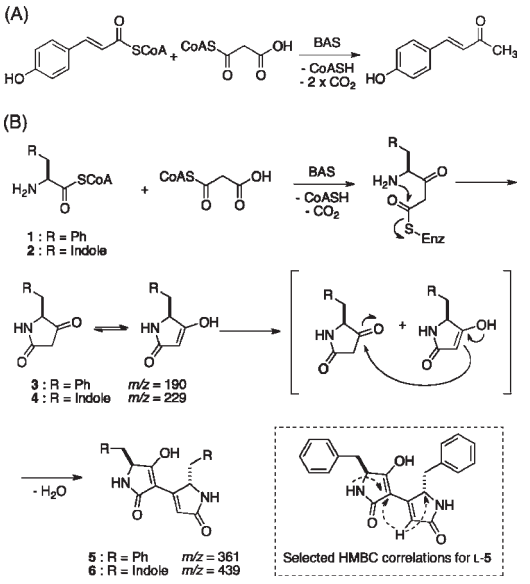
Scheme 1. Proposed Mechanism for the Formation of (A) Benzalacetone and (B) Tetramic Acid Derivatives by *R. palmatum* BAS (the HMBC Spectrum Was Recorded in DMSO-d_6)



the tetramic acid scaffold by intramolecular lactamization (Scheme 1B). To test this hypothesis, we investigated the enzymatic synthesis of tetramic acid derivatives by utilizing *R. palmatum* BAS and chiral aminoacyl-CoA thioesters that were chemically synthesized from L- and D-amino acids.

According to the published method,[24] the chiral aminoacyl-CoAs derived from L- and D-phenylalanine or tryptophan were chemically synthesized by the succinimide-based condensation of the corresponding *N*-Boc amino acids with CoASH. Final deprotection of the Boc group afforded the desired aminoacyl-CoAs. When L-phenylalanoyl-CoA (**1**) and malonyl-CoA were incubated with the purified recombinant *R. palmatum* BAS,[12] the enzyme reaction generated a 1:10 mixture of two products having different parent ion peaks $[M + H]^+$ at m/z 190 (minor) and 361 (major) in the liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS) spectrum, with a combined yield of 11% (Figure 2A). These products were not detected in control experiments performed with the boiled enzyme. The spectroscopic data (LC−ESI-MS, UV, ^{1}H and ^{13}C NMR) of the minor compound were characteristic of the tetramic acid **3** and identical to those of a chemically synthesized authentic compound, as described below. The minor product **3** is thus thought to be produced by the one-step decarboxylative condensation of L-phenylalanoyl-CoA with malonyl-CoA followed by lactam formation by the enzyme-bound diketide intermediate (Scheme 1B).

On the other hand, the spectroscopic data (LC−ESI-MS, UV, ^{1}H and ^{13}C NMR) of the major compound indicated that the structure was the dehydrated dimer **5** of the minor product **3**. Thus, in the ^{1}H NMR spectrum of **5** in DMSO-d_6, two sets of signals corresponding to phenylalanine were observed. Furthermore, a sharp singlet proton at δ_H 5.87, which was not apparently related to phenylalanine, was exchangeable with CD_3OD and showed correlation to the olefinic carbon at δ_C 118.3 in the HMQC spectrum. Finally, the HMBC correlations with the carbons at δ_C 59.7 and δ_C 100.2 indicated the connectivity with

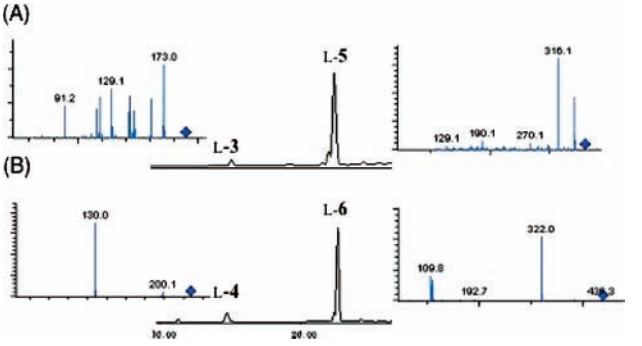


Figure 2. Reversed-phase HPLC and ESI-MS data for the products derived from (A) L-phenylalanoyl-CoA and (B) L-tryptophanoyl-CoA. The chromatograms were detected using (A) 250 and (B) 280 nm UV light.

the α-carbon and enol carbon, respectively. The unnatural, novel tetramic acid dimer **5** is likely to be produced by the non-enzymatic, spontaneous dimerization of **3** between each tautomer via an aldol-type condensation (Scheme 1B).

The structures of these enzyme reaction products were confirmed by comparison with chemically synthesized authentic compounds. Thus, according to the literature,[25] condensation of *N*-Boc-phenylalanine with Meldrum's acid and subsequent cyclization by refluxing in AcOEt gave the N-protected tetramic acid derivative. During the final deprotection with trifluoroacetic acid, **5** was produced by spontaneous dimerization with the concomitant removal of the Boc group. The chromatographic and spectral data of synthetic **5** were identical to those of the enzymatic product. It should be noted that the previous synthetic work described by Courcambeck et al.[7] did not lead to the direct deprotection of the N-protected tetramic acid derivative. Therefore, the spontaneous dimerization was not reported during their synthetic process.

Interestingly, *R. palmatum* BAS efficiently accepted both L- and D-phenylalanoyl-CoAs to produce the corresponding tetramic acid derivatives in nearly equal yields. A steady-state kinetic analysis gave K_M = 11.7 μM and k_{cat} = 27.8 min^{-1} for L-phenylalanoyl-CoA and K_M = 3.3 μM and k_{cat} = 8.1 min^{-1} for D-phenylalanoyl-CoA.

Next, we sought to extend this reaction to the bulkier aminoacyl-CoA thioesters from L- and D-tryptophan. As in the case of phenylalanoyl-CoA, *R. palmatum* BAS accepted both L- and D-tryptophanoyl-CoAs as starter molecules with nearly equal efficiencies and carried out a single condensation with malonyl-CoA to produce a 1:10 mixture of monomeric and dimeric tetramic acid derivatives (**4** and **6**) with a combined yield of ∼10% (Figure 2B). The structures of these products were also determined on the basis of the spectroscopic data for synthetic materials. A steady-state kinetic analysis gave K_M = 9.8 μM and k_{cat} = 14.7 min^{-1} for L-tryptophanoyl-CoA and K_M = 8.4 μM and k_{cat} = 11.0 min^{-1} for D-tryptophanoyl-CoA, which are comparable to the corresponding values for phenylalanoyl-CoA. These results indicate that the substrate-binding pocket of *R. palmatum* BAS is large enough to accommodate the bulky L- and D-aminoacyl-CoAs as starter molecules, and they are supported by the results of docking studies based on the crystal structure of *R. palmatum* BAS[14] (Figure 3).

Tetramic acids (2,4-pyrrolidinediones) have been widely used in the design of pseudopeptides as aspartyl, serine, and cysteine protease inhibitors and as nonpeptidic neuraminidase inhibitors.

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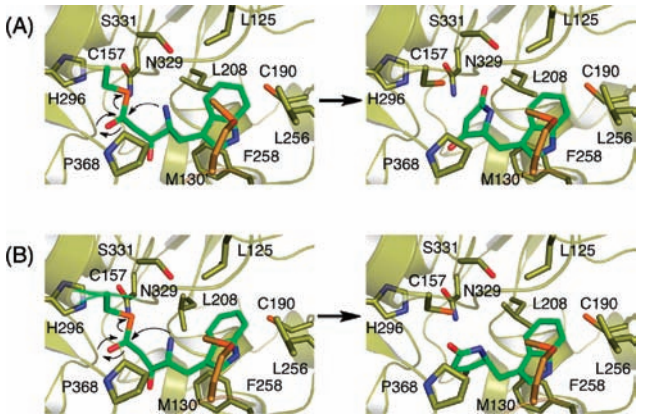

Figure 3. Active-site structure of BAS. The diketide intermediate and the tetramic acid product **6** from (A) L-tryptophanoyl-CoA and (B) D-tryptophanoyl-CoA are shown as green stick models.

Furthermore, the cytotoxic and antibacterial activities of tetramic acid-containing natural products have also been reported. Therefore, the above-obtained tetramic acid derivatives were tested for their antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus* and for their cytotoxic activity against murine leukemia P388 cells. None of the products was active against any of the bacteria, but the tetramic acid dimer D-**5** derived from D-phenylalanine showed moderate cytotoxicity (IC_{50} = 1 μg/mL) against murine leukemia P388 cells. It is quite remarkable that the differences in the stereochemistry and aromatic ring substituent significantly affected the cytotoxic activity. Further investigations to elucidate the detailed mechanism of the cytotoxicity are now in progress.

To the best of our knowledge, there have been no reports of the generation of the tetramic acid (2,4-pyrrolidinedione) scaffold by structurally simple type-III PKS. As mentioned above, previous reports have shown that the tetramic acid scaffold is produced by the modular and iterative type-I PKS/NRPS hybrid megaenzyme systems.[17−23] Thus, after polyketide chain elongation, NRPS appends an additional amino acid to the thioester terminus, thereby generating a β-ketoamide aminoacetyl thioester. It has been suggested that during the final cyclization step, the NRPS reductive (R) domain catalyzes Dieckmann condensation rather than reductive cleavage of the thioester to generate tetramic acids.[19−22] In contrast, the type-III PKS BAS initially accepts the aminoacyl-CoA as a starter substrate and then recruits malonyl-CoA for a Claisen condensation to generate the γ-amino-β-ketothioester (Scheme 1B). The free γ-amino group of the enzyme-bound intermediate could cleave the thioester bond with concomitant intramolecular lactamization. Since the cavity volume of the active site is apparently large enough for the monomer (**3** or **4**), but not for the dimer (**5** or **6**) (Figure 3), it is likely that the dimerization proceeds nonenzymatically.[26] The ability of the type-III PKS to accept the bulky aminoacyl-CoA as a starter molecule suggests that further structural variants of tetramic acids can be readily prepared from various amino acids.[27] The catalytic versatility of BAS is extremely useful for precursor-directed biosynthesis.

In conclusion, we have demonstrated for the first time that the structurally simple type-III PKS catalyzes the formation of a series of unnatural, novel tetramic acid derivatives. Remarkably, the novel tetramic acid dimer D-**5** from D-phenylalanoyl-CoA showed moderate cytotoxicity against a tumor cell line. This efficient and novel methodology for the construction of the tetramic acid scaffold should thus contribute to the development of versatile chemical libraries with promising biological activities.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) Spontaneous dimerization of the chemically synthesized monomers **3** and **4** also proceeded immediately.
(27) The lack of reports that type-III PKS produce tetramic acids in nature would reflect the fact that aminoacyl-CoAs are very rare in nature.