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Peniphenone and penilactone formation in *Penicillium crustosum via* 1,4-Michael additions of *ortho*-quinone methide from hydroxyclavatol to γ-butyrolactones from crustosic acid

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Supporting Information Placeholder

ABSTRACT: Penilactones A and B consist of a γ-butyrolactone and two clavatol moieties. We identified two separate gene clusters for the biosynthesis of these key building blocks in *Penicillium crustosum*. Gene deletion, feeding experiments, and biochemical investigations proved that a non-reducing PKS ClaF is responsible for the formation of clavatol and the PKS-NRPS hybrid TraA is involved in the formation of crustosic acid, which undergoes decarboxylation and isomerization to the predominant terrestric acid. Both acids are proposed to be converted to γ-butyrolactones with involvement of a cytochrome P_{450} ClaJ. Oxidation of clavatol to hydroxyclavatol by a non-heme Fe^{II}/2-oxoglutarate-dependent oxygenase ClaD and its spontaneous dehydration to an *ortho*-quinone methide initiate the two non-enzymatic 1,4-Michael addition steps. Spontaneous addition of the methide to the γ-butyrolactones led to peniphenone D and penilactone D, which undergo again stereospecific attacking by methide to give penilactones A/B.

Penilactones A (**1**) and B (**2**) (Figure 1A) are rare fungal metabolites and were firstly isolated from *Penicillium crustosum* PRB-2.[1] Together with their putative precursors peniphenone D (**3**) and penilactone D (**4**) (Figure 1A), they were also identified in other *Penicillium* species.[2-4] Feeding experiments suggested that **1** and **2** are derived from acetyl-CoA and L-malic acid (Figure 1B).[1] It was proposed that **1** and **2** are formed by 1,4-Michael additions of two clavatol (**5**) molecules in its active form *ortho*-quinone methide (**6**) with a γ-butyrolactone (tetronic acid), *i.e.* (*R*)-5-methyl (**7**) or (*S*)-5-carboxylmethyltetronic acid (**8**).[1,2] This hypothesis was confirmed by a biomimetic synthesis.[5-7] Acetate of hydroxyclavatol (**9**) instead of **5** was used at 110 °C for the synthesis. Hydroxyclavatol methyl ether (**10**) was also isolated from *P. crustosum*.[3]

5 can be considered as a polyketide synthase (PKS) product. However, the responsible enzyme is unknown before. Neither the direct precursor nor the biosynthesis of **7** and **8** has been reported. Michael addition as a thermodynamically controlled 1,4-addition of active methylenes to activated olefins such as α,β-unsaturated carbonyl derivatives[8] are widely used in the chemical synthesis[9-12] and also involved in the biosynthesis of natural products.[13] However, the substrates, enzymes and conditions for Michael addition involved in the formation of **1**–**4** in nature have not been reported yet.

For secondary metabolite (SM) production in PRB-2, several culture conditions were tested and the extracts were analyzed on HPLC (Figure S1 in the Supplement Information (SI)). Three dominant peaks were detected in a 7 days-old PD culture (Figure S1), which were identified as **9**, **10**,[14] and terrestric acid (**11**)[15] after isolation and structure elucidation (See SI for details, NMR data and spectra are given in Tables S6–S10 and Figures S28–S45). The stereochemistry of **11** was confirmed by determination of its optical rotation and comparison with the published data.[15] **9** has not been described before and therefore was confirmed by X-ray analysis (Table S11). Two additional minor peaks were proven to be **5** and hydroxyclavatol ethyl ether (**12**) (Figures 1A and S1).[14]

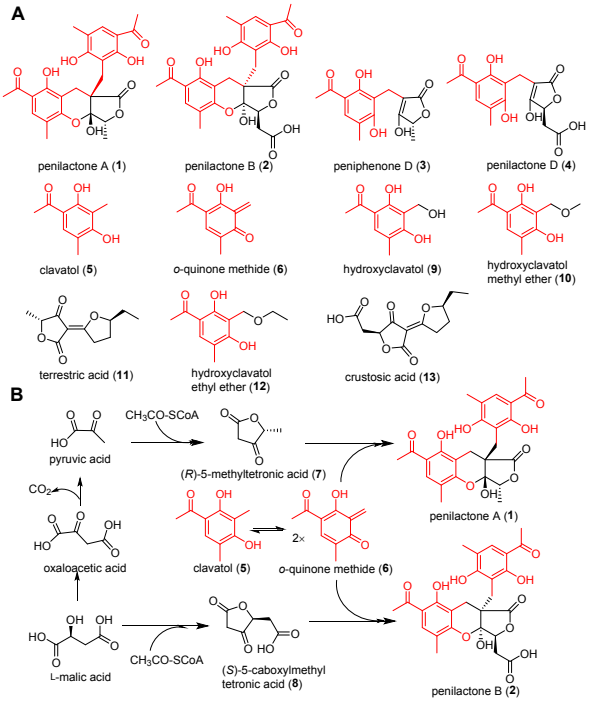


Figure 1. Metabolites from PRB-2 (A) and proposed biosynthetic routes to **1** and **2** (B).[1]

However, peniphenones and penilactones could only be detected in extracted ion chromatograms (EICs, data not shown). To increase their productivity, PRB-2 was cultivated in PD surface culture for 14 days. LC-MS analysis revealed clear accumulation of **1**–**4** (Figures 2, S1, and S2). A 30 days-old rice

culture also accumulated **1**–**4** and was therefore used for isolation and structure elucidation by MS, NMR (Tables S6–S7 and Figures S28–S31), optical rotation, and CD spectra (Figures S46–S49).[1,3] The CD spectra of **1** and **2** (Figures S46 and S47) correspond very well to those reported previously.[1] The stereochemistry of **3** and **4** was determined by chemical synthesis from **7** and **8** with known configuration at C-5 (Figure S21, see below for the formation of **3** from **7** and **4** from **8**). Under these conditions, the production of **9** and **10** was strongly reduced. In comparison, **11** was detected as the predominant peak. Furthermore, a new peak was identified as carboxylated derivative of **11**, termed crustosic acid (**13**) hereafter (Figures 1A, S44, and S45). **13** has an [α]20 D value of -164.1, while that of **11** at +37.1. The configuration of **13** was assigned by comparison with the optical rotation data of 5-methyl- and 5-carboxylmethyltetronic acids.[16]

For biosynthetic studies on **1** and **2**, the genome of PRB-2 was sequenced and the draft genome sequence was used for prediction of putative gene clusters by using AntiSMASH.[17] For gene inactivation, we established a gene replacement protocol using the split marker strategy and hygromycin B as selection marker, which significantly enhances the homologous recombination events at the target gene (Figure S3).[18]

Based on its aromatic character, **5** is expected to be assembled by a non-reducing PKS (NR-PKS).[19] One of the six NR-PKS genes *pcr3094* within a 36.2 kbp large cluster (Figure 2A and Table S4) has a SAT-KS-AT-PT-ACP-MeT-TE domain structure (Abbreviations for PKS and NRPS domains as given before[19,20]). It shares a sequence identity of 57.7 % with CitS from *Monascus ruber*[21] and 64.4 % with EAW12049.1 from *Aspergillus clavatus* (Table S4). Deletion of *pcr3094*, termed *claF* (from the clavatol cluster) hereafter, completely abolished the production of **1**−**5**, **9**, and **10** (see SI for manipulation). The two tetronic acids **11** and **13** accumulated with much higher yields in the *ΔclaF* mutant than in PRB-2 (Figures 2B, S4, and S6). Feeding **5** to the mutant restored the production of **1**–**4** and **9** (Figure S15).

To provide more evidence for the function of ClaF as a clavatol synthase, *pcr3094* was cloned into pYH-wA-pyrG and expressed in *A. nidulans*.[22-24] The formation of **5** in the transformant JF11 was confirmed by LC-MS (Figure S20) and ^{1}H NMR analyses after isolation. These results proved that ClaF is responsible for **5** formation in the biosynthesis of **1**–**4** (Scheme 1).

To identify the genetic potential for **7** and **8**, we focused on PKS-NRPS hybrid enzymes, because tetronic acids like carlosic acid are usually assembled by such enzymes.[25] Analysis of the draft sequence revealed the presence of a candidate gene *pcr11009*, termed *traA* (from the terrestric acid cluster), within a 33.6 kbp large cluster. TraA with a domain structure KS-AT-DH-MeT-KR-ACP-C-A-PCP (Figure 2A) shares a sequence identity of 69.6 % with CaaA in the carlosic acid biosynthesis (Table S5).[25] Deletion of *traA* completely abolished the production of **1–4**, indicating its involvement in the biosynthesis. As expected, **5**, **9**, and **10** were accumulated in the Δ*traA* mutant (Figures 2C and S8). Surprisingly, the production of **11** and **13** were also totally blocked. To restore the production of **1–4**, we chemically synthesized **7** and **8** (Figure S21)[5,7,26,27] and fed them to the Δ*traA* mutant. LC-MS analysis revealed that feeding **7** restored the production of **1** and **3**, but not **2** and **4**. In contrast, **2** and **4**, but not **1** and **3** were detected in the culture of Δ*traA* mutant fed with **8** (Figures 2C, S16 and S17). This proved that TraA is involved in the formation of **7** and **8**, which cannot be converted to each other (Scheme 1).

For understanding the role of **11** and **13** for **1–4**, they were isolated from Δ*claF* mutant and fed into Δ*traA* mutant. Feeding **11** only restored **1** and **3** production, while **1–4** were detected after feeding with **13** (Figures 2C, S18 and S19). More interestingly, **11** was also restored after feeding with **13**, but not *vice versa* (Figure 2D). This proved that **13** is the precursor of both **8** and **11**. **11** serves then as a precursor of **7** (Scheme 1).

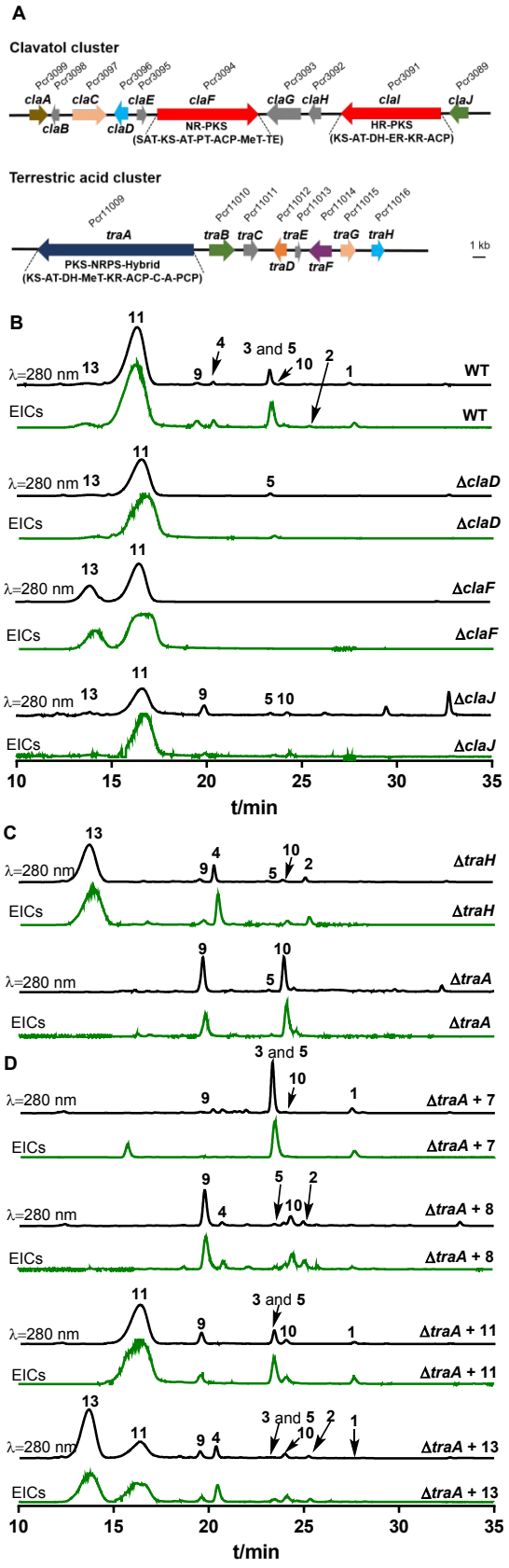


Figure 2. Schematic representation of clavatol and terrestric acid clusters in PRB-2 (A) and LC-MS results of deletion mutants (B and C) as well as of *ΔtraA* mutant fed with putative precursors (D). EICs refer $[M+H]^+$ ions of **1**–**6** and **11**, **13** or $[M+Na]^+$ of **9** and **10** with tolerance ranges of ± 0.005.

It can be concluded that **13** is the product of TraA with or without other enzymes and mainly converted to the predominant product **11** in PRB-2. Only small amounts of **11** and **13** undergo degradation to **7** and **8** for the formation of **1**–**4** (Figure 2D and Scheme 1).

Having the both backbone genes/enzymes identified, we intended to investigate the conversion of **13** to **8** and **11**, **11** to **7**, and the metabolism of **5**. Inactivation of the oxygenase gene *traH* abolished the production of **1**, **3**, and **11**, confirming its involvement in the decarboxylation and isomerization of **13** to **11** (Figures 2C and S13). In the deletion mutants of the cytochrome P_{450} *traB* and the dehydrogenase *traD,* no accumulation of **11**, **13**, or **1–4** was detected (Figures S9 and S10), proving their roles in the **13** formation (Scheme 1). Deletion of *traE* and *traF* did not result in significant changes in SM production (Figures S11 and S12).

Regarding Michael addition, we presumed a more active intermediate than **5** for the formation of **6**. Detailed inspection of the *cla* cluster (Figure 2A, Table S4) revealed the presence of genes coding for an oxygenase (*claD*) and a cytochrome P_{450} (*claJ)*. ClaD comprises 338 amino acids and shares a sequence identity of 53.8 % with CitB in the citrinin biosynthesis.[21] It also contains the typical conserved 2-His-1-Asp ion-binding triad (His_{184}, His_{202} and Asp_{187}) of non-heme Fe^{II}/2-oxoglutarate-dependent oxygenases (Figure S22). Deletion of *claD* abolished the production of **1–4** and **9**, whereas **5** was clearly accumulated (Figures 2B and S5). Feeding **9** in the *ΔclaD* mutant restored the production of **1**–**4** (Figure S14), proving its role in the conversion of **5** to **9**.

For biochemical characterization, *claD* was amplified and cloned into pET28a (+). The purified ClaD (Figure S23) was used for incubation with **5** in the presence of ascorbate (AA), $Fe[(NH_4)_2(SO_4)_2]$, and 2-oxoglutarate (2OG).[28,29] HPLC analysis confirmed the oxidation of **5** to **9** with a conversion yield of 22.5 % after incubation with 2 μg protein at 37°C for 30 min (Figure 3A). Nearly no consumption of **5** was detected in the assays without ascorbate or 2-oxoglutarate. Replacing ascorbate by dithiothreitol (DTT) or without additional Fe^{II} reduced the activity significantly. These results proved that ClaD acts as a non-heme Fe^{II}/2-oxoglutarate-dependent oxygenase and oxidizes **5** to yield **9**. Determination of kinetic parameters gave a K_M of 0.30 mM toward **5** and a turnover number (*kcat)* of 0.26 s^{-1} (Figure 3B).

To prove the conversion between **9** and **6**, **9** was incubated in H_2O and $H_2{}^{18}O$ at 25°C for 16 h. MS data in positive and negative modes confirmed the incorporation of ^{18}O into **9** and therefore the equilibration (Figures 3C and S24).

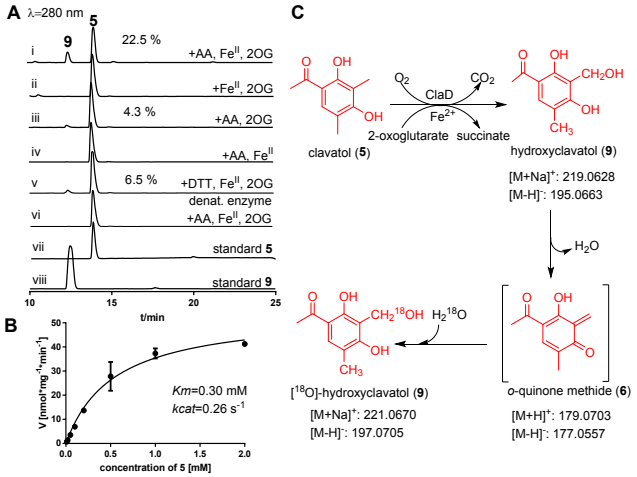


Figure 3. Functional proof of ClaD as a non-heme Fe^{II}/2-oxoglutarate-dependent clavatol oxidase (A and B) and determination of the equilibration between **9** and **6** (C).

ClaJ shares clear sequence homology with fungal cytochrome P_{450} enzymes, *e.g.* 42.0 % identity with BAJ04372.1 from *Aspergillus oryzae*.[30] Deletion of *claJ* resulted in the abolishment of **1**–**4** (Figures 2B and S7), but still retained the production of **5**, **9**, **11**, and **13**. This indicates its role in the C-C double bond cleavage of **11** and **13** (Scheme 1). However, ClaJ could also catalyzes the connection of the two building blocks *via* Michael addition.

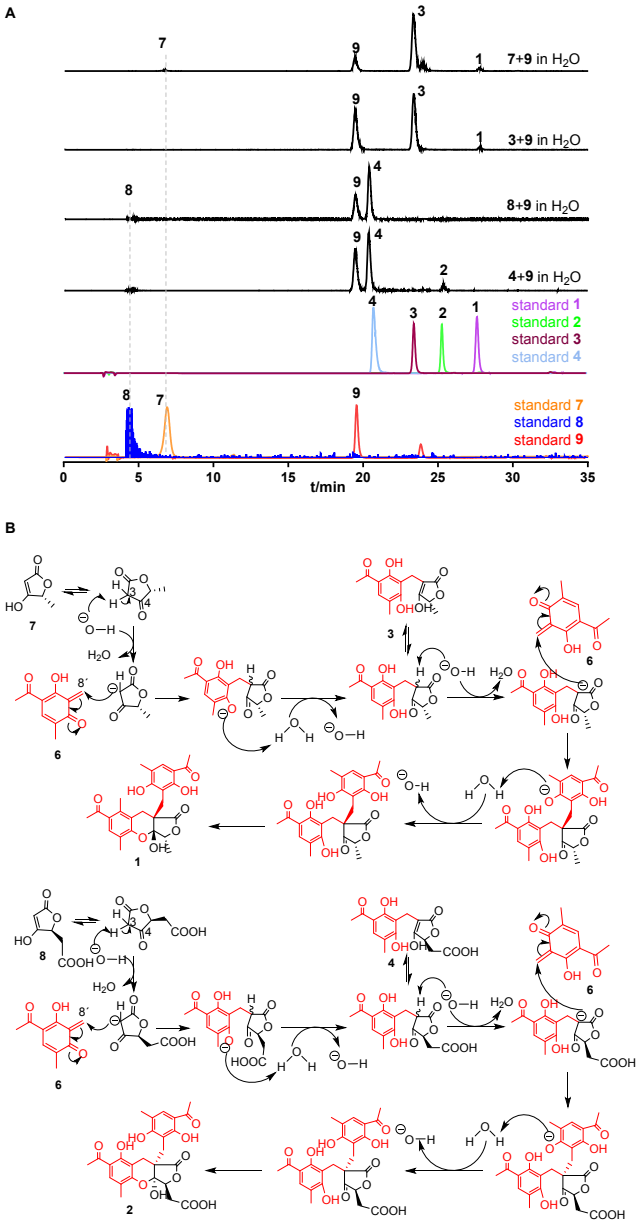
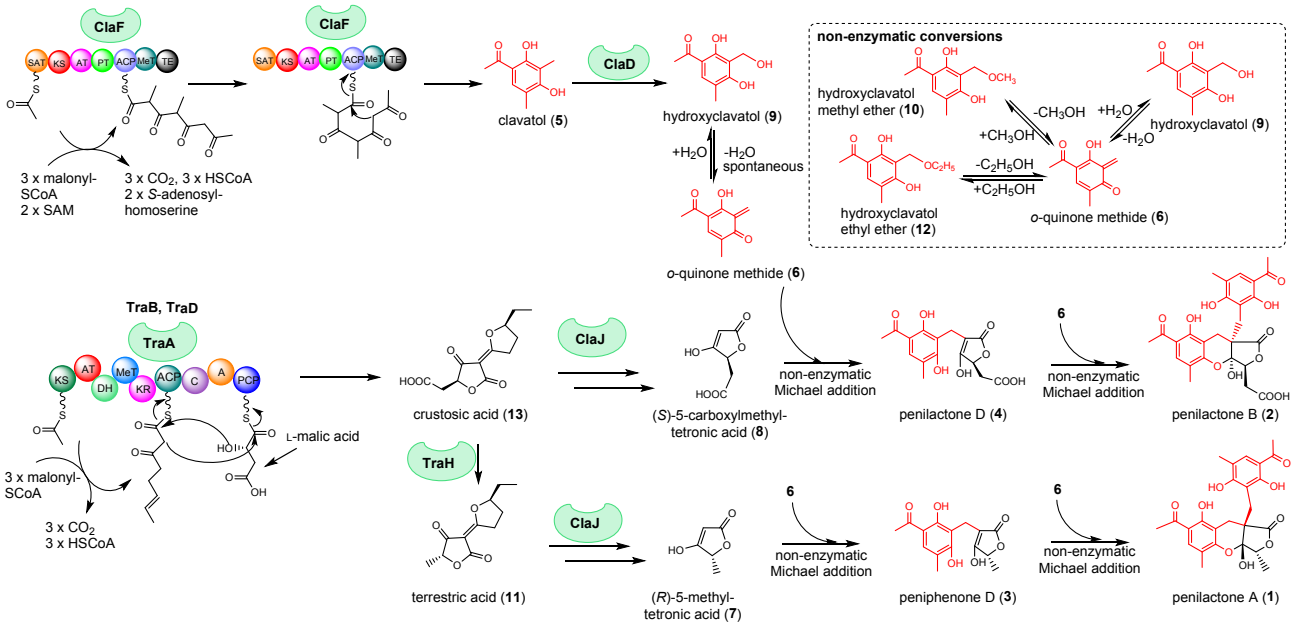


Figure 4. Non-enzymatic formation of penilactones and peniphenone. (A) LC-MS analysis of 48 h-incubation mixtures. Absorptions at 254 nm (**1**–**4**, **9**) or EICs (**7** and **8**) are illustrated. (B) Proposed mechanism of non-enzymatic formation of **1**–**4** *via* Michael addition.



Scheme 1. Proposed biosynthetic pathways of penilactones and peniphenones in *P. crustosum*.

For preparing feeding experiments in *ΔclaJ*, we carried out control incubations of **7** with **9** and **8** with **9** in water at 25°C, which delivered surprising results, i.e. the non-enzymatic Michael addition under these mild conditions (Figure 4A). In the first combination, **3** was detected as the major and **1** as a minor product, while **4** as the major and **2** as a minor product in the case of **8** with **9**. When **3** and **4** were incubated with **9**, **1** and **2** were detected (Figure 4A). Formation of **3** and **4** is time- and pH-dependent (Figures S25 and S26). **3** and **4** are formed under neutral or acidic conditions. When pH values were higher than 5.0, diclavatol[3] was also detected (Figure S26). It is obvious that the active intermediate **6** can be easily formed from **9** in aqueous system and initiates the Michael additions (Figure 4B), which was confirmed by incubation of **9**, **10**, and **12** in different solvents. They are stable in acetonitrile. Alcohols determined the end products of **6** (Figure S27, Scheme 1). All these results indicate that ClaJ is likely not involved in the Michael addition, probably in the conversion of **11** to **7** and **13** to **8** (Scheme 1).

Taken together, **1**–**4** are formed by enzymes from independent pathways of two separate gene clusters (Scheme 1). The *tra* cluster assembles **13**, which is converted to **11**. Both acids deliver the two γ-butyrolactones **7** and **8**. The *cla* cluster provides the highly active **6** by a spontaneous dehydration of **9**. This initiates the two step non-enzymatic Michael additions by the intermolecular nucleophile attacking of **6** to **7** or **8** and subsequent reaction with **3** and **4**. Thus, this study provides an excellent example for SM with complex structures that are formed by enzymes from different pathways and by combination of enzymatic and non-enzymatic reactions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Materials, experimental procedures, physiochemical properties and spectroscopic data (PDF).

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Notes

The authors declare no competing financial interests.

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