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Note

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Friedel–Crafts Alkylation of Acylphloroglucinols Catalyzed by a Fungal Indole Prenyltransferase

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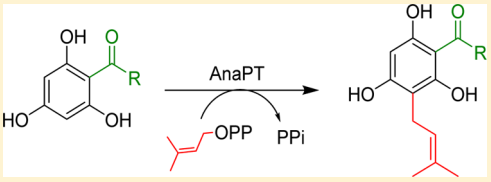
Supporting Information

ABSTRACT: Naturally occurring prenylated acylphloroglucinol derivatives are plant metabolites with diverse biological and pharmacological activities. Prenylation of acylphloroglucinols plays an important role in the formation of these intriguing natural products and is catalyzed in plants by membrane-bound enzymes. In this study, we demonstrate the prenylation of such compounds by a soluble fungal prenyltransferase AnaPT involved in the biosynthesis of prenylated indole alkaloids. The observed activities of AnaPT toward these substrates are much higher than that of a microsomal fraction containing an overproduced prenyltransferase from the plant hop.



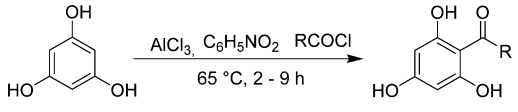
Polyprenylated acylphloroglucinols are found in a limited number of plant families including Clusiaceae, Hypericaceae, and Cannabaceae.[1−5] Their fascinating chemical structures and intriguing biological activities have attracted increasing attention. Hyperforin, a polyprenylated acylphloroglucinol (Scheme S1, Supporting Information), and the naphthodianthrone hypericin are considered as active constituents in extracts of *Hypericum perforatum* (St John's wort, Hypericaceae) used for treatment of depression in Europe and the USA.[2] Humulone (α-acid) and lupulone (β-acid, Scheme S1, Supporting Information) are prenylated acylphloroglucinols in hop cones, the female flowers of *Humulus lupulus* (Cannabaceae), and are responsible for the bitter taste and pharmacological effects.[3] Hops are used primarily as a flavoring and stabilizing agent in beer. In traditional medicines, hops are also used as mild sedative drugs.[3]

Biogenetically, the acylphloroglucinol cores in hyperforin and lupulone are formed by condensation of three malonyl-CoA molecules with different start units, isobutyryl-CoA in the case of the hyperforin precursor phlorisobutyrophenone (**1a**) and isovaleryl-CoA in the case of the lupulone precursor phlorisovalerophenone (**2a**). This key step is catalyzed by a polyketide synthase (PKS) and followed by an alkylation on the benzene ring catalyzed by prenyltransferases (Scheme S1, Supporting Information).[1,6,7] Several plants from the families Clusiaceae and Hypericaceae also use benzoyl-CoA as start unit and produce phlorbenzophenone (**3a**) and derivatives thereof such as grandone (Scheme S1, Supporting Information).[4,5,8,9]

In sharp contrast to many new prenylated acylphloroglucinol derivatives reported in 2014,[1,5,8] little is known about the enzymes related to the biosynthesis of these compounds. Until now, only one recombinant enzyme involved in their biosynthesis, the prenyltransferase HIPT-1 from *Humulus lupulus*, was investigated biochemically.[6] The membrane-bound HIPT-1 was overproduced in baculovirus-infected insect cells[6] and the microsomal fraction of the protein extract was used for enzyme assays. It catalyzed the prenylation of phlorisovalerophenone (**2a**) in the presence of dimethylallyl diphosphate (DMAPP) and also accepted phlorisobutyrophenone (**1a**) as prenylation substrate. Its catalytic activities for both substrates were very low, being 11 pmol per mg of microsomal fraction per minute for phlorisovalerophenone (**2a**), and 29.5% of that for phlorisobutyrophenone (**1a**). Like most membrane-bound proteins, HIPT-1 is more difficult to overproduce and purify than soluble enzymes. These features strongly prohibit its potential use as a biocatalyst for chemoenzymatic synthesis for production of prenylated acyphloroglucinols. Therefore, there is a need to find alternative enzymes with better properties. In this study, we tested the acceptance of such acylphloroglucinols by prenyltransferases of the DMATS superfamily from fungi, which are soluble proteins and can be easily overproduced in *Escherichia coli* with significantly higher yields.[10]

Phlorisobutyrophenone (**1a**), phlorisovalerophenone (**2a**), and phlorbenzophenone (**3a**) were synthesized according to protocols described previously[11−13] (Scheme 1) and incubated with 13 purified soluble fungal prenyltransferases including four tryptophan, seven cyclic dipeptide, and two tyrosine prenyltransferases. HPLC analysis of the incubation mixtures revealed that AnaPT from *Neosartorya fischeri*, which catalyzes the *C*3-

Scheme 1. Synthesis of Acylphloroglucinols



R=$CH(CH_3)_2$ phlorisobutyrophenone (**1a**, 70.8 %)

R=$CH_2CH(CH_3)_2$ phlorisovalerophenone (**2a**, 67.1 %)

R=C_6H_5 phlorbenzophenone (**3a**, 65.8%)

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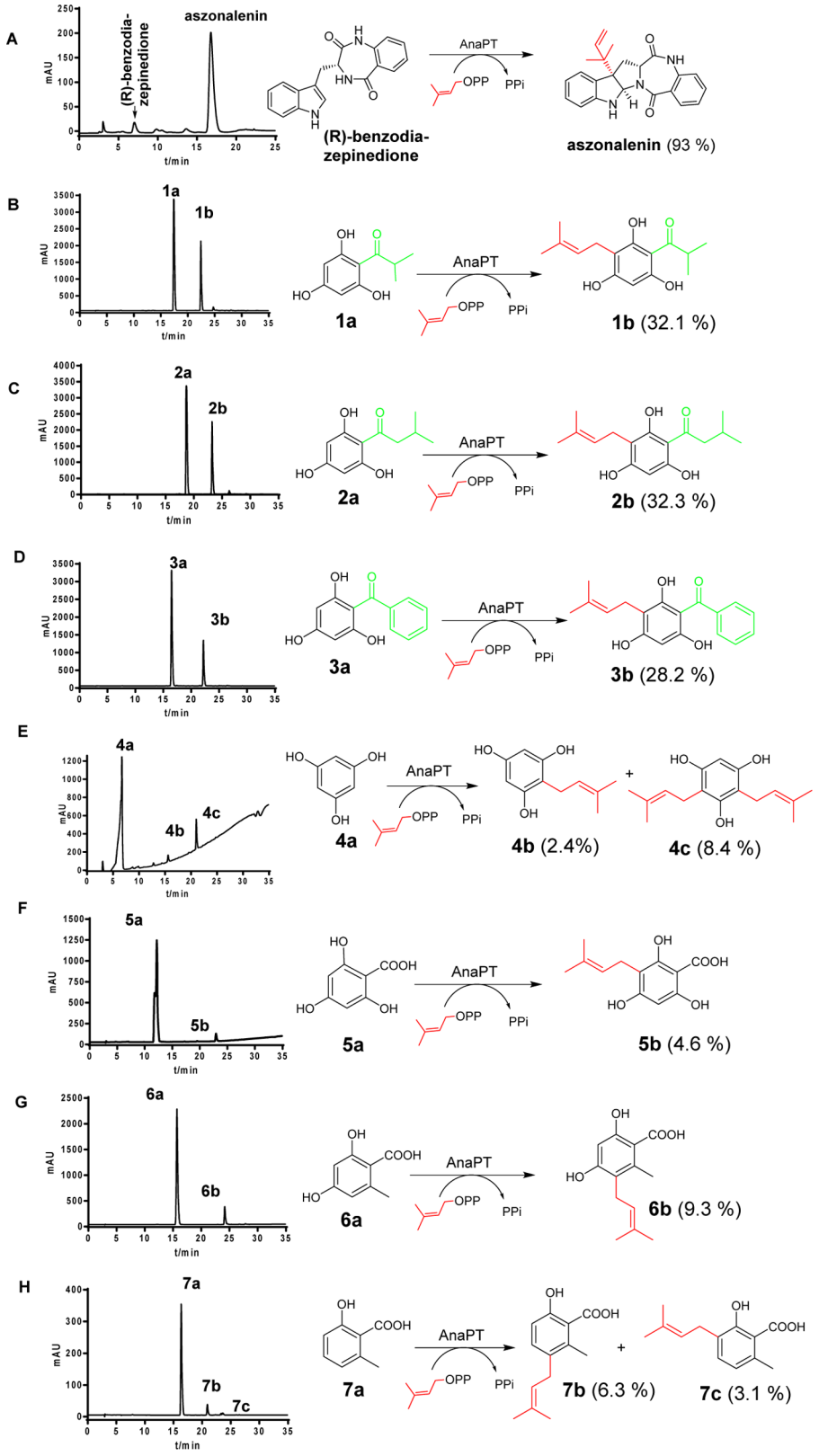

Figure 1. HPLC chromatograms and prenyl transfer reactions catalyzed by AnaPT. Different wavelengths were used for illustration of product formation: 230 nm (**4a**), 254 nm ((*R*)-benzodiazepinedione and **5a**), 266 nm (**6a**), 291 nm (**1a**, **2a**, and **7a**), and 306 nm (**3a**).

prenylation of (*R*)-benzodiazepinedione (Figure 1A) and is involved in the biosynthesis of acetylaszonalenine,[14] showed higher activities toward these compounds than other tested enzymes (Table S1, Supporting Information). Conversion yields between 12% and 14% were achieved for these substrates after incubation with 5 μg of AnaPT in 100 μL assay at 37 °C for 16 h. No product formation was detected with heat-inactivated AnaPT (data not shown). Using 50 μg of protein,

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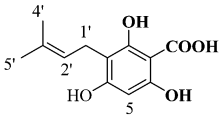
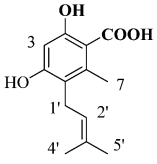
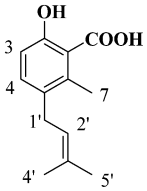
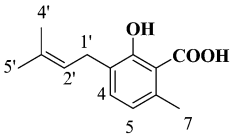
Table 1. ^{1}H NMR Data (500 MHz) of the Enzyme Products 5b, 6b, 7b, and 7c in Methanol-d_4[a]

Comp				
	5b	**6b**	**7b**	**7c**
Pos.	δ_H, (J in Hz)	δ_H, (J in Hz)	δ_H, (J in Hz)	δ_H, (J in Hz)
3	/	6.13, s	6.97, d (8.4)	/
4	/	/	6.57, d (8.4)	6.91, d (8.4)
5	5.73, s	/	/	6.50, d (8.4)
7	/	2.50, s	2.46, s	2.53, s
1′	3.16, d (7.0)	3.28, d (7.0)	3.24, d (7.0)	3.24, d (6.9)
2′	5.20, m	5.02, m	5.16, m	5.32, m
4′	1.63, s	1.66, s	1.71, s	1.70, s
5′	1.73, s	1.75, s	1.72, s	1.73, s

[a]Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz.

we found product formation to be nearly linear in the first 6 h (Figure S1, Supporting Information). Conversion yields of 32.1%, 32.3%, and 28.2% were calculated for **1a**, **2a**, and **3a**, respectively, after incubation with 50 μg of protein at 37 °C for 6 h (Figure 1). Under this condition, 93% of (*R*)-benzodiazepinedinone was converted to aszonalenin (Figure 1A). The tryptophan prenyltransferase 7-DMATS from *Aspergillus fumigatus*[15] showed slightly lower activities than AnaPT, with product yields of about 10% in the assays of **1a** and **2a** with 5 μg of protein (Table S1, Supporting Information). Compound **1a** was accepted by CdpC3PT from *Neosartorya fischeri*[16] with a conversion yield of 5.7% (Table S1, Supporting Information).

These results encouraged us to test the acceptance of phloroglucinol (**4a**) and its carboxylic acid **5a**, as well as orsellinic acid (**6a**) and 6-methylsalicylic acid (**7a**), as substrates. The latter two compounds were identified as PKS products in different microorganisms.[17−19] As shown in Figure 1, all of these substances were accepted by AnaPT. Conversion yields of 4.6−10.8% were observed using 50 μg of AnaPT after incubation at 37 °C for 6 h. It is obvious that acylphloroglucinols **1a**−**3a** are better substrates for AnaPT than **4a**−**7a**. Interestingly, **6a** with a methyl instead of a hydroxy group in **5a** was a better substrate for AnaPT (Figure 1F,G). Detailed inspection of the HPLC chromatograms B−H in Figure 1 revealed the presence of one predominant product each in the incubation mixtures of **1a**−**3a**, **5a**, and **6a**. Compounds **4a** and **7a** were converted to at least two products.

In a previous study,[20] we demonstrated that AnaPT also used geranyl diphosphate (GPP) as prenyl donor. Therefore, **1a**−**7a** were incubated with 50 μg of AnaPT in the presence of GPP. Indeed, product formation was observed in all incubation mixtures, but with relatively lower activities than with DMAPP for a given aromatic substrate. After incubation for 16 h, conversion yields of 6−10% were obtained for **1a**−**3a** and less than 5% for other substrates (Figure S2, Supporting Information).

To elucidate the structures, nine enzyme products, **1b**, **2b**, **3b**, **4b**, **4c**, **5b**, **6b**, **7b**, and **7c**, were isolated from the incubation mixtures of **1a**−**7a** with AnaPT and DMAPP and subjected to NMR and HR-EI-MS analysis (Table 1 and Tables S2 and S3 and Figures S3−S14, Supporting Information). In addition, **1b** was also isolated from the incubation mixtures of **1a** with CdpC3PT or 7-DMATS. The spectra of **1b** from the three different incubation mixtures are nearly identical, proving the same product of different enzymes. With the exception of **4c**, the M^+ ions of the isolated products are 68 Da larger than the respective substrates (Table S2, Supporting Information), proving the monoprenylation of these compounds. The M^+ ion of **4c** is 136 Da larger than that of **4a**, proving the diprenylation in its structure. This conclusion was confirmed by their molecular formula deduced from HR-MS analysis (Table S2, Supporting Information). The signals at δ3.16−3.29 (d, 2H $-CH_2-$), 5.02−5.32 (m, 1H, $-C{=}CH$), 1.61−1.71 (s, 3H, $-C{=}C-CH_3$), and 1.72−1.76 ppm (s, 3H, $-C{=}C-CH_3$) in the ^{1}H NMR spectra of the isolated products confirmed the presence of regular dimethylallyl moieties in their structures. The resonance of the methylene group in the range of 3.16−3.29 ppm proved their attachment to aromatic carbon atoms.[21] Comparison of the NMR data with those of previously reported compounds led to identification of the structures of **1b**,[22] **2b**,[22] **3b**,[23] **4b**,[24] and **4c**,[24] as shown in Figure 1. The structures of **5b**, **6b**, **7b**, and **7c** have not been reported prior to this study.

Only one singlet for an aromatic proton at δ5.73 ppm was found in the ^{1}H NMR spectrum of **5b**, which proved unequivocally the attachment of the prenyl moiety at C-3 of the benzene ring (Table 1, Figure 1F). One singlet for an aromatic proton (δ6.13 ppm) was also observed in the ^{1}H NMR spectrum of **6b**, confirming the prenylation at C-3 or C-5 of the benzene ring. Coupling between CH_3 at C-6 and H-5 was observed in the ^{1}H NMR spectrum of **6a** (data not shown). No coupling between the signal for this methyl group at δ2.50 ppm with the singlet at δ6.13 ppm was observed in the ^{1}H NMR spectrum of **6b**, indicating a prenylation at C-5. The NOESY correlation of the signal at δ2.50 ppm with that of H-

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1′of the prenyl moiety at δ3.28 ppm proved unequivocally the prenylation at C-5, as shown in Figure 1. The ^{1}H NMR spectrum of **7b** showed two doublets at δ6.97 (1H, d, J = 8.4 Hz, ArH-3) and δ6.57 ppm (1H, d, J = 8.4 Hz, ArH-4), and the NOESY correlation of the signal at δ2.46 ppm for CH_3 at C-6 with that of H-1′of the prenyl moiety at 3.24 (d, J = 7.8 Hz) confirmed the prenyl moiety at C-5 of the benzene ring. The spectrum of **7c** also showed signals of two aromatic protons at δ6.91 (1H, d, J = 7.5 Hz, ArH-3) and δ6.50 (1H, d, J = 7.5 Hz, ArH-4). NOESY correlation was observed for the signal of CH_3 at C-6 with that of H-5, suggesting the prenylation at C-3 in **7c** (Figure 1H).

In summary, our data provide evidence that the soluble fungal indole prenyltransferase AnaPT catalyzed the same prenylation reaction of acylphloroglucinols as the membrane-bound prenyltransferases involved in the biosynthesis of the prenylated acylphloroglucinols in plants like HIPT-1,[6] but with much higher conversion yields than HIPT-1. To the best of our knowledge, this is the first report on the prenylation of acylphloroglucinols by microbial enzymes. Furthermore, AnaPT also prenylated hydroxylated benzoic acids such as orsellinic acid (**6a**) and 6-methylsalicylic acid (**7a**), which are typical PKS products of microorganisms.[17−19] Therefore, prenylated and hydroxylated benzoic acids could be produced by introducing *anaPT* into the producers of **6a** and **7a** or by coexpression of the responsible PKS genes[17−19] with *anaPT* in suitable hosts. Thus, this work extends significantly the substrate and catalytic promiscuity of the prenyltransferases of the DMATS superfamily as well as their potential applications.

To get more insights into the catalytic efficiency of the tetrameric AnaPT[14,25] toward acylphloroglucinols and hydroxybenzoic acids, kinetic parameters including Michaelis−Menten constants (K_M) and turnover numbers (k_{cat}) were determined at pH 7.5 for (*R*)-benzodiazepinedinone, **1a**−**7a**, and DMAPP by Hanes−Woolf, Eadie−Hofstee, and Lineweaver−Burk plots and were compared with each other (Figures S15−24, Supporting Information). As given in Table 2, AnaPT displayed similar affinity to **1a**−**3a** as to its natural aromatic substrate (*R*)-benzodiazepinedinone,[14] while **4a**−**7a** showed lower affinity to AnaPT. With **1a** as aromatic substrate, a slightly higher K_M value was determined for DMAPP than in the presence of (*R*)-benzodiazepinedione,[14] while a significantly higher K_M value was obtained in the presence of **2a**. As expected, the turnover numbers of AnaPT for **1a**−**3a** are very low, only 0.7−1.0% of that of (*R*)-benzodiazepinedione determined in this study. Undoubtedly, catalytic efficiency of AnaPT toward acylphloroglucinols should be improved in the future by suitable approaches such as mutagenesis experiments. However, it should be mentioned that the turnover number of AnaPT toward its natural substrate is much higher (up to 10-fold) than most prenyltransferases of the DMATS superfamily to their natural substrates. The determined V_{max} values for **1a**−**3a** are in the range of 3.7−5.1 nmol mg protein^{-1} min^{-1}. Therefore, it can be concluded that AnaPT is already an interesting candidate as a biocatalyst for prenylation of phloroglucinol analogues, especially of acylphloroglucinols.

Table 2. Kinetic Parameters of AnaPT toward Selected Substrates

substrate	K_M [mM]	V_{max} (nmol mg protein^{-1} min^{-1})	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
(*R*)-benzodiazepinedione	0.22	526	1.72	7818
1a	0.22	3.71	0.012	54.5
2a	0.33	5.07	0.017	51.5
3a	0.24	4.54	0.015	62.5
4a	1.64	0.71	0.0023	1.4
5a	0.87	1.21	0.0040	4.8
6a	0.42	3.54	0.012	28.6
7a	0.52	1.11	0.0037	7.1
DMAPP with **1a**	0.21	2.56	0.0084	40.0
DMAPP with **2a**	0.38	3.29	0.011	28.9

EXPERIMENTAL SECTION

General Experimental Procedures. Phloroglucinol (**4a**) was obtained from Acros Organics, 2,4,6-trihydroxybenzoic acid (**5a**) and orsellinic acid (**6a**) from Alfa Aesar, and 6-methylsalicylic acid (**7a**) from Chempur. NMR spectra were recorded on a JEOL ECA-500 spectrometer, processed with MestReNova 5.2.2. Chemical shifts were referenced to the signal of acetone-d_6 at 2.05 ppm or methanol-d_4 at 3.31 ppm. The enzyme products were also analyzed by electron impact mass spectrometry (EI-MS) on an Auto SPEC (Micromass Co. UK Ltd.).

Synthesis of DMAPP, GPP, and Acylphloroglucinols 1a, 2a, and 3a. The triammonium salts of dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) were synthesized according to the method described for geranyl diphosphate by Woodside et al.[26] Compounds **1a**, **2a**, and **3a** in yields of 70.8%, 67.1%, and 65.8% were prepared by Friedel−Crafts acylations of phloroglucinol with isobutyryl chloride,[11] methylbutanal chloride,[13] and benzoyl chloride[12] in the presence of $AlCl_3$, respectively (Scheme 1).

Overproduction and Purification of the Recombinant AnaPT and Enzyme Assay. Protein overproduction and purification were carried out as described previously.[14] The enzyme assays (100 μL) contained **1a**−**7a** (1 mM), $CaCl_2$ (5 mM), DMAPP/GPP (2 mM), glycerol (1.0−6% v/v), dimethyl sulfoxide (DMSO, 5% v/v), 50 mM Tris-HCl (pH 7.5), and purified recombinant protein (5−50 μg). The reaction mixtures were incubated at 37 °C for different times and terminated by addition of 100 μL of methanol. The protein was removed by centrifugation at 13000 rpm for 20 min. Assays for isolation of the enzyme products were carried out in large scale (10−15 mL) containing aromatic substrates (1 mM), DMAPP (2 mM), $CaCl_2$ (5 mM), glycerol (1.0−9.9% v/v), DMSO (5% v/v), 50 mM Tris-HCl (pH 7.5), and 5 mg of recombinant protein per 10 mL assay. After incubation for 16 h at 37 °C, the reaction mixtures of **1a**−**3a** were extracted 3−4 times with a double volume of ethyl acetate. The organic phases were combined and evaporated. The residues were dissolved in methanol (0.5−1.0 mL) and purified by HPLC. The reaction mixtures of **4a**−**7a** were terminated by addition of a double volume of methanol. Protein was removed by centrifugation at 6000 rpm for 30 min. The supernatants were collected, concentrated, and dried on a rotary evaporator. The residues were dissolved in methanol (0.5−1.0 mL) and after centrifugation, the supernatants were purified on HPLC. Assays for determination of kinetic parameters (100 μL) contained $CaCl_2$ (5 mM), glycerol (1.0−9.9% v/v), DMSO (5% v/v), 50 mM Tris-HCl (pH 7.5), DMAPP (2 mM), 50 μg of AnaPT, and (*R*)-benzodiazepinedinone or **1a**−**7a** at final concentrations of up to 20.0 mM. For determination of the kinetic parameters of DMAPP, **1a** or **2a** at a final concentration of 1 mM and DMAPP of up to 5.0 mM were used. The reaction mixtures were incubated for 60 min ((*R*)-benzodiazepinedione), 120 min (**1a**, **2a**, **3a**, **5a**, DMAPP, and **6a**), or 240 min (**4a** and **7a**). Product formation was found to be linear within such incubation times (Figure S1, Supporting Information). The reactions were then terminated with 100 μL of methanol. Protein was removed by centrifugation at 13000 rpm for 20 min.

Analysis of Enzyme Products by HPLC and Structure Elucidation by NMR and MS Analysis. An Agilent HPLC series

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1200 was used for analysis and isolation of the enzyme products. A Multospher 120 RP-18 column (250 mm × 4 mm, 5 μm C+S Chromatographie Service, Langerwehe, Germany) was applied for analysis at a flow rate of 1 mL/min, and a Multospher 120 RP18 column (250 mm × 10 mm, 5 μm) was used for isolation at a flow rate of 2.5 mL/min. Water containing 0.5% trifluoroacetic acid (solvent A) and acetonitrile containing 0.5% trifluoroacetic acid (solvent B), were used as solvents. A linear gradient of 2−100% (v/v) solvent B in 30 min was used for analysis of the enzymatic products. The column was then washed with 100% solvent B for 5 min and equilibrated with 2% solvent B for another 5 min. Detection was carried out on a photodiode array detector. Solvents for isolation of the enzyme products are water (solvent C) and acetonitrile (solvent D) without acid. The enzyme products were isolated with a linear gradient of 10−100% D in C in 25 min. After each run, the column was equilibrated with 10% solvent D for 5 min. Analysis of product from (*R*)-benzodiazepinedione by HPLC was carried out as described previously.[14]

■ ASSOCIATED CONTENT

Supporting Information

Examples of polyprenylated acylphloroglucinols and their biosynthetic origins, results of different enzyme assays as tables or HPLC chromatograms, MS and ^{1}H NMR data, NMR spectra, and, kinetic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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