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Complementary Flavonoid Prenylations by Fungal Indole Prenyltransferases

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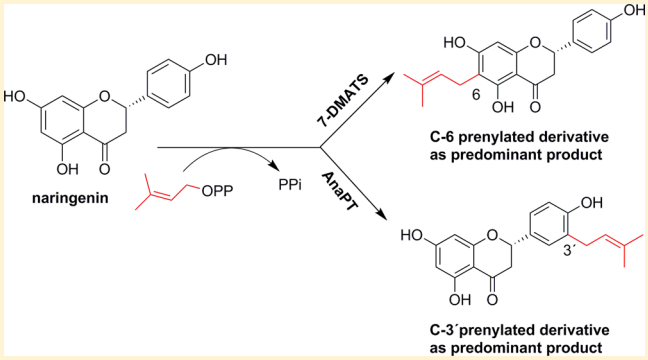
Supporting Information

ABSTRACT: Flavonoids are found mainly in plants and exhibit diverse biological and pharmacological activities, which can often be enhanced by prenylations. In plants, such reactions are catalyzed by membrane-bound prenyltransferases. In this study, the prenylation of nine flavonoids from different classes by a soluble fungal prenyltransferase (AnaPT) involved in the biosynthesis of the prenylated indole alkaloid acetylaszonalenin is demonstrated. The behavior of AnaPT toward flavonoids regarding substrate acceptance and prenylation positions clearly differs from that of the indole prenyltransferase 7-DMATS. The two enzymes are therefore complementary in flavonoid prenylations.



Flavonoids are valuable natural products widely distributed in the plant kingdom. On the basis of their structures, they are categorized into dihydrochalcones, chalcones, flavanones, dihydroflavonols, flavones, flavonols, isoflavones, isoflavonols, pterocarpans, coumestans, aurones, neoflavonoids, and anthocyanidins.[1,2] Flavonoids have been shown to have a wide range of biological and pharmacological activities in in vitro studies, including anti-inflammatory, antibacterial, antiviral, antiallergic, cytotoxic, and antitumor activities.[3] They are also considered potential candidates for the treatment of neurodegenerative and vasodilatory diseases.[2] Prenylations at the two benzene rings often increase the lipophilicity of the backbone compounds, leading to enhancement of their affinity to cell membranes and of their interaction with target proteins.[4−6] Owing to the impressive biological activities and their diverse chemical structures, prenylated flavonoids have been studied by scientists from different research disciplines including natural product chemistry,[7] plant physiology,[8] and chemical syntheses.[9,10]

In plants, the prenyl moieties are transferred from prenyl diphosphates onto the flavonoid skeleton by membrane-bound prenyltransferases.[11,12] For example, SfN8DT-1/SfPFT, SfG6DT, and SfiLDT from *Sophora flavescens* catalyze prenylations of flavanones, isoflavonoids, and chalcones, respectively.[11,13,14] SfN8DT-1, the first identified flavonoid-specific prenyltransferase, was shown to be responsible for the prenylation of a few select flavanones at C-8.[14] SfFPT displayed a high catalytic efficiency for different types of flavonoids with high regiospecificity at C-8.[13] SfG6DT was found to specifically prenylate the isoflavone genistein at C-6,[11] while SfiLDT functions as a chalcone-specific prenyltransferase.[11] LaPT1 from *Lupinus albus* acts as an isoflavonoid-specific B-ring prenyltransferase,[15] while G4DT from *Glycine max* is specific for pterocarpans.[16] Recently, two isoliquiritigenin 3,3-dimethylallyltransferases MaIDT and CtIDT have been identified in *Morus alba* and *Cudrania tricuspidata,* respectively.[17] With the exception of a few members, these enzymes usually showed high substrate specificities and accepted only their natural substrates or just a few substances with similar structures.

In addition to membrane-bound prenyltransferases from plants, soluble prenyltransferases from bacteria such as NphB and SCO7190 also accept flavonoids as substrates.[18,19] NphB from a *Streptomyces* sp. is a hydroxynaphthalene geranyltransferase.[18] Its homologue SCO7190 from *Streptomyces coelicolor* used dimethylallyl diphosphate (DMAPP) as a prenyl donor and also catalyzes the prenylation of naringenin at C-6.[19] We have demonstrated that the recombinant indole prenyltransferase 7-DMATS from the fungus *Aspergillus fumigatus* accepted chalcones, isoflavonoids, and flavanones much better than flavones and flavonols and mainly catalyzed prenylation at C-6.[20] These results encouraged us to find more prenyltransferases with different substrate specificities and prenylation positions on the flavonoid skeleton, in order to utilize these enzymes for the production of prenylated flavonoids. Preliminary results from the previous study[20] indicated that AnaPT, which catalyzes the C-3 prenylation of (*R*)-benzodiazepindinone in the biosynthesis of acetylaszonalenin[21] and uses diverse aromatic substances for prenylation,[22−25] could be a good candidate.

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RESULTS AND DISCUSSION

Twenty-one flavonoids were initially incubated with 75 μg of the recombinant AnaPT in the presence of DMAPP at 37 °C for 16 h. HPLC analysis revealed that naringenin (**1a**), 7-hydroxyflavanone (**2a**), eriodictyol (**3a**), hesperetin (**4a**), silibinin (**5a**), phloretin (**6a**), apigenin (**7a**), genistein (**8a**), and biochanin A (**9a**) were readily accepted by AnaPT under these conditions. Compounds **1a**−**3a** and **5a**−**9a** were incubated with 75 μg of AnaPT at 37 °C for different times (Figure S1, Supporting Information). With the exception of the preferred flavonoid **1a**, product formation was found to be nearly linear for up to 2 h for other substrates. With **1a** as substrate and 40 μg of protein, the AnaPT reaction was found to be linear for up to 2 h (Figure S2, Supporting Information). For better comparison of their acceptance, **1a**−**9a** were incubated with 40 μg of AnaPT at 37 °C for 2 h (Table 1, Figure S3, Supporting Information). Under these conditions, **1a** was accepted as the best substrate with a product yield of 48.2%, which was calculated by comparison of peak areas in the HPLC chromatogram and intensities of signals in the ^{1}H NMR spectrum of the reaction mixture. Compounds **1a**−**9a** were subsequently incubated with 40 μg of 7-DMATS at 37 °C for 2 h. Under these conditions, product formation with its best substrate **3a**[20] was found to be linear (Figure S2, Supporting Information). HPLC analysis confirmed **3a** as the best substrate, with a product yield of 36.0%, followed by **1a** and **6a**, with product yields of 26.9% and 23.1%, respectively.

Table 1. Product Yields of 1a−9a Catalyzed by 7-DMATS and AnaPT[a]

	product yield (%)	
substrate	7-DMATS	AnaPT
1a	26.9 ± 0.3	48.2 ± 1.0
2a	1.2 ± 0.0	3.2 ± 0.2
3a	36.0 ± 0.4	4.4 ± 0.3
4a	15.9 ± 2.7	1.5 ± 0.0
5a	0.2 ± 0.2	3.2 ± 0.1
6a	23.1 ± 0.5	12.0 ± 0.3
7a	0.9 ± 0.0	3.3 ± 0.2
8a	6.7 ± 0.2	4.8 ± 0.3
9a	4.5 ± 0.2	7.1 ± 0.0

[a]The enzyme assays (100 μL) contained one of the flavonoids **1a**−**9a** (1 mM), $CaCl_2$ (10 mM), DMAPP (2 mM), glycerol (1.0−6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), and the purified recombinant proteins (40 μg). The reaction mixtures were incubated at 37 °C for 2 h.

To enhance product formation, compounds **1a**−**9a** were incubated with 40 μg of AnaPT or 7-DMATS at 37 °C for 16 h (Figure 1). Under these conditions, product yields of more than 10% were calculated for seven AnaPT reactions (**1a**−**3a**, **5a**, **6a**, **8a**, and **9a**), with **1a** as the best substrate (58.6%) and six 7-DMATS reactions (**1a**, **3a**, **4a**, **6a**, **8a**, and **9a**) with **3a**, **4a**, and **6a** as the best substrates (56.0−77.8%). It is obvious that the flavanones **1a**, **3a**, and **4a**, chalcone **6a**, and isoflavones **8a** and **9a** were better substrates for one or both of the two enzymes than other subgroups. AnaPT accepted **1a**, **2a**, **5a**, **7a**, and **9a** much better than 7-DMATS, while **3a**, **4a**, **6a**, and **8a** were better substrates for 7-DMATS. Interestingly, silibinin (**5a**, also termed silybin), a hepatoprotective dihydroflavonol lignoid from the medicinal plant *Silybum marianum*,[26] was accepted by AnaPT with a product yield of 11.3%. Flavones were poor substrates for both enzymes. The product yield of the preferred flavone apigenin (**7a**) was determined for AnaPT at 6.7%.

Inspection of the HPLC chromatograms revealed that more than one product peaks were detected in a number of reaction mixtures. Interestingly, the major products of several AnaPT and 7-DMATS reactions differed from each other. For example, the dominant product **1b** in the 7-DMATS reaction with **1a** (product yield 29.6%) was detected only as a minor product, with a yield of 4.4% in its reaction mixture with AnaPT. Instead, product **1c**, with a yield of 54.2%, was found as the main product. Eriodictyol (**3a**) was much better accepted by 7-DMATS, and several products including **3b**, **3c**, and **3d** with comparable yields were observed. In contrast, **3c**, with a product yield of 16.4%, was detected as the main product of the AnaPT reaction (Figure 1). Phloretin (**6a**) was converted by 7-DMATS to one predominate product, **6b**, while a number of products including **6b**, **6c**, and **6d** were detected in its reaction mixture with AnaPT. These results indicate different prenyl transfer reactions catalyzed by these two enzymes.

Previously, we demonstrated that AnaPT also used geranyl diphosphate (GPP) as prenyl donor for prenylation of cyclic dipeptides.[23] Therefore, **1a**−**9a** were incubated with AnaPT in the presence of GPP. HPLC analysis revealed that these compounds were also accepted by AnaPT in the presence of GPP. However, for a given aromatic substrate, the activity was much lower than that with DMAPP. After incubation with 40 μg of AnaPT for 16 h, the highest product yield of 5.9% was found for **6a** (Figure S4, Supporting Information). Under the same conditions, 7-DMATS also used GPP as prenyl donor for its reaction with **1a**−**9a**, but with lower product yields than with AnaPT. The highest product yield of approximately 1% was found for **1a** with 7-DMATS after incubation at 37 °C for 16 h (data not shown).

For structure elucidation, 12 enzyme products **1b**, **1c**, **2b**, **3c**, **5b**, **6b**, **6c**, **6d**, **7b**, **8b**, **8c**, and **9b** were isolated via preparative HPLC from incubation mixtures of **1a**−**3a** and **5a**−**9a** with AnaPT and DMAPP, respectively. Compounds **8b** and **8c** were also isolated from the incubation mixture of **8a** with 7-DMATS and DMAPP. The isolated products were subjected to NMR and HREIMS analyses. With the exception of **6d** and **8c**, the M^+ ions of the isolated products are 68 Da larger than the respective substrates, proving the monoprenylation of these compounds. The M^+ ions of **6d** and **8c** are 136 Da larger than those of **6a** and **8a**, respectively, corresponding to those of diprenylated derivatives. This conclusion was also confirmed by their molecular formula deduced from HREIMS analysis. The signals at δ_H 3.24−3.55 (d, 2H, $-CH_2-$), 5.18−5.36 (tsept or m, 1H, $-C{=}CH$), 1.62−1.71 (d or s, 3H, $-C{=}C-CH_3$), and 1.67−1.78 (d or s, 3H, $-C{=}C-CH_3$) in the ^{1}H NMR spectra of the isolated products indicated the presence of dimethylallyl moieties in their structures (Tables 2 and 3, Experimental Section, and Figures S5−S8 and S12−S18, Supporting Information). The resonance of the methylene group in the range 3.24−3.55 ppm proved the attachment of the dimethylallyl moieties to aromatic carbon atoms.[22] Compounds **1b**,[27] **1c**,[20] **2b**,[28] **3c**,[20] **6b**,[20] **7b**,[29] **8b**,[20] **8c**,[30] and **9b**[20] were identified as known compounds by comparison of their ^{1}H NMR data with reported data. These compounds are 6- (**1b**, **2b**, **6b**, **7b**, **8b**, and **9b**) or 3′-prenylated (**1c** and **3c**) or 6,3′-diprenylated derivatives (**8c**).

The ^{1}H NMR spectrum of **5b** (Figure S9, Supporting Information) was similar to that of its substrate, silybin,[31] and

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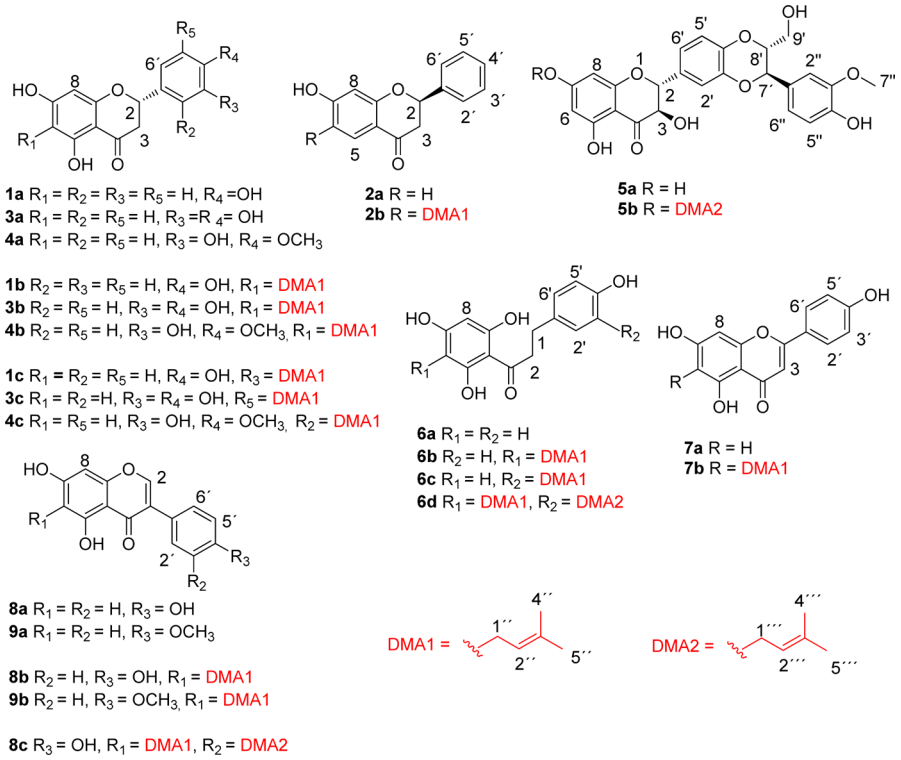
Chart 1



showed additional signals for a dimethylallyl moiety (δ_H 4.63, 2H, d, J = 8.0 Hz, H-1‴; 5.45, 1H, m, H-2‴; 1.77, 3H, s, H-5‴; and 1.74, 3H, s, H-4‴) (Table 2). The chemical shift of H-1‴ at 4.63 ppm indicated that the prenylation has taken place at an oxygen atom. This conclusion was supported by the same number and coupling pattern of the aromatic protons in the spectra of **5b** and its substrate. To prove the prenylation position in **5b**, HSQC and HMBC spectra (Figures S10 and S11, Supporting Information) were also taken into consideration. As shown in Figure S11 (Supporting Information), correlations of H-1‴ with C-7, C-2‴, and C-3‴ were evident, proving the prenylation of the 7-hydroxy group (Table 2).

Comparing the ^{1}H NMR spectra of **6c** (Figure S13, Supporting Information) and **6d** (Figure S14, Supporting Information) with that of **6a** revealed the disappearance of the AA′BB′ systems for B-ring protons in **6c** and **6d**. Instead, an ABX system was observed in their spectra. This indicates the prenylation of both substances at C-3′. Signals of two prenyl moieties (δ_H 3.24, 2H, d, J = 7.3 Hz; 5.22, 1H, tsept, J = 7.3, 0.9 Hz; 1,74, 3H, d, J = 0.7 Hz; 1.62, 3H, d, J = 1.1 Hz, and δ_H 3.28, 2H, d, J = 7.3 Hz; 5.32, 1H, tsept, J = 7.3, 1.1 Hz; 1.70, 3H, d, J = 1.1 Hz; 1.68, 3H, d, J = 1.3 Hz) were observed in the ^{1}H NMR spectrum of **6d**. Comparing the spectrum of **6d** with that of **6b** revealed the disappearance of the signal for H-6 in both cases. These results proved that **6c** is a 3′-monoprenylated derivative and **6d** bears the two prenyl moieties at C-6 and C-3′ (Table 3). A literature search indicated that the structures of **5b**, **6c**, and **6d** have not been reported prior to this work.

In summary, AnaPT displayed in several cases different behaviors regarding prenylation position from those of 7-DMATS reported previously.[20] For **1a**, **3a**, and **6a**, 7-DMATS is preferred for C-6 prenylation, and 6-prenylated derivatives **1b**, **3b**, and **6b** were detected as predominant or one of the main products. In contrast, 3′-prenylation was observed as the main reactions in the assays of these compounds with AnaPT.

Kinetic parameters including Michaelis–Menten constants (K_M) and turnover numbers (k_{cat}) were determined at the pH optimum of the AnaPT reactions (pH 7.5) in a Tris-HCl buffer system by Hanes–Woolf, Eadie–Hofstee, and Lineweaver–Burk plots. The data obtained for (*R*)-benzodiazepinedinone, **1a**–**3a**, and **5a**–**9a** were compared (Figures S19–S26, Supporting Information). As shown in Table 4, **1a**, **3a**, and **5a** were found to have comparable affinities to (*R*)-benzodiazepinedione toward AnaPT, with K_M values of 0.26, 0.29, and 0.18 mM, respectively, while **2a** and **6a** showed lower affinity to AnaPT. The K_M values of **7a** and **9a** at 0.11 mM are even lower than that of (*R*)-benzodiazepinedione. As reported previously,[20] 7-DMATS displayed affinity to **6a** and **8a** similar to its natural substrate L-tryptophan. The turnover numbers of AnaPT with **1a**–**3a** and **5a**–**9a** in the range 0.001–0.05 s^{-1} are much smaller than that with (*R*)-benzodiazepinedione, at 1.72 s^{-1}. It is evident that catalytic efficiency of AnaPT toward flavonoids should be improved in the future by suitable approaches such as mutagenesis experiments.

In conclusion, AnaPT used in this study was identified in the ascomycetous fungus *Neosartorya fischeri* and proved to be responsible for the 3-prenylation of (*R*)-benzodiazepindinone in the biosynthesis of acetylaszonalenin.[21] This enzyme was demonstrated to exhibit significant substrate and catalytic promiscuity in vitro. It has an unprecedented ability to recognize diverse aromatic substrates such as tryptophan-containing cyclic dipeptides, hydroxynaphthalenes, and acylphloroglucinols and catalyzes Friedel–Crafts alkylations.[22,24,25,32] In addition to its prenyl donor DMAPP, AnaPT also accepted GPP and unnatural alkyl donors as substrates.[23,33]

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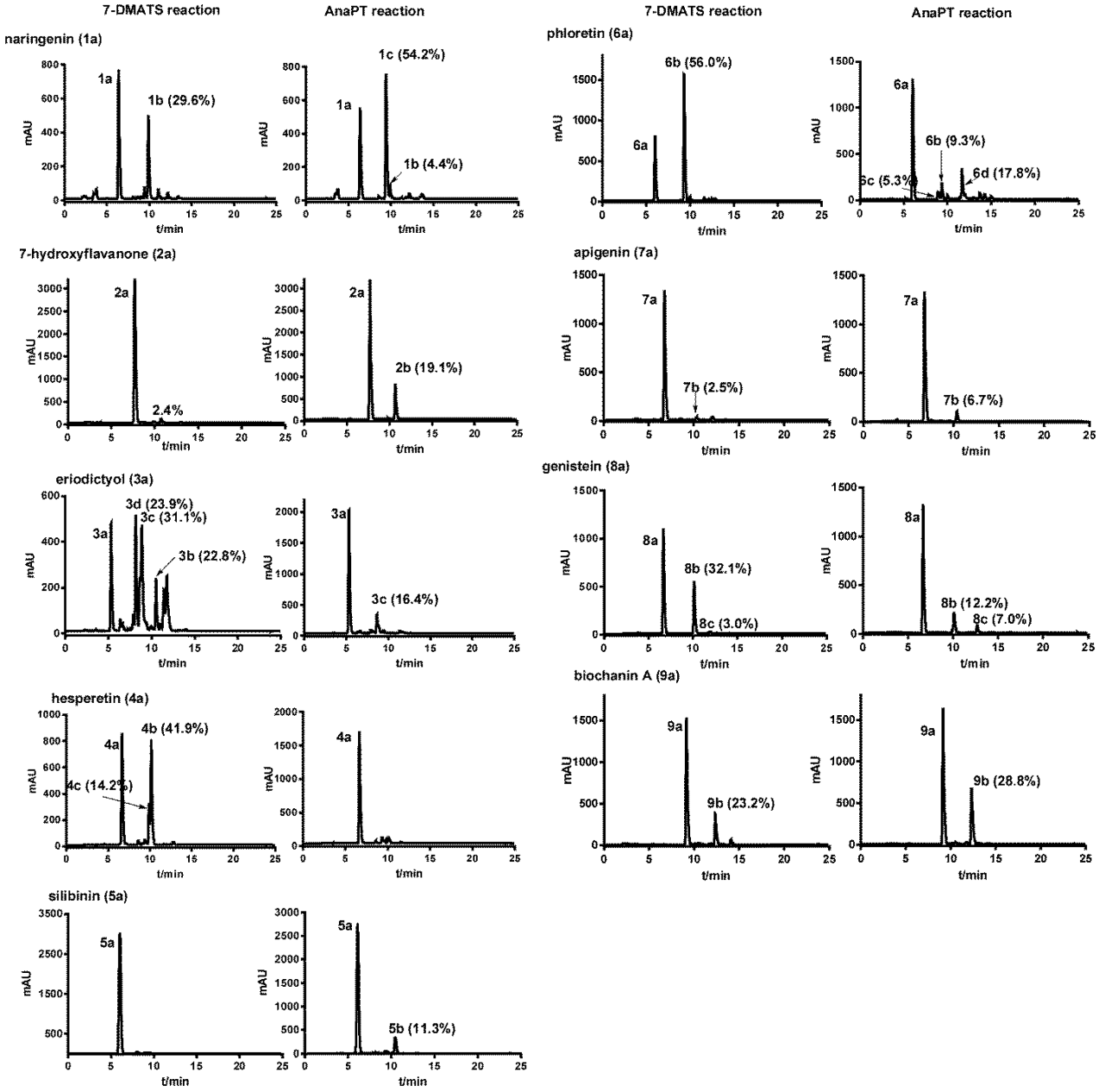

Figure 1. HPLC analysis of the reaction mixtures of 7-DMATS and AnaPT. The enzyme assays (100 μL) contained one of the flavonoids **1a**−**9a** (1 mM), $CaCl_2$ (10 mM), DMAPP (2 mM), glycerol (1.0−6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), and the purified recombinant proteins (40 μg). The reaction mixtures were incubated at 37 °C for 16 h and detected on a diode assay detector. The absorption at 277 nm was used for illustration of the reaction with **2a** and 296 nm for other substrates.

In this study, we demonstrated prenylations of different flavonoids such as flavanones and isoflavones by AnaPT at C-6 of the A ring or C-3′ of the B ring, which expands significantly its potential for modification of small molecules. More importantly, AnaPT and 7-DMATS displayed different substrate preferences and prenylation positions, so that these two fungal indole prenyltransferases could be used complementarily for prenylation of flavonoids. Prenylations of the flavonoid skeleton contribute significantly to structural diversity and biological activity of natural products and are usually crucial in the biosynthesis of these compounds. Therefore, the soluble indole prenyltransferases AnaPT and 7-DMATS could also be used for production of prenylated flavonoids in microorganisms by synthetic biological approaches.

EXPERIMENTAL SECTION

General Experimental Procedures. Flavonoids and solvents used in this study were purchased from Alfa Aesar (Karlsruhe, Germany), Acros Organics (Geel, Belgium), Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany), and TCI (Zwyndrecht, Belgium). The triammonium salts of DMAPP and GPP were synthesized according to the method described for GPP by Woodside and co-workers.[34] NMR spectra were recorded at room temperature on a JEOL ECA-400 or -500 or a Bruker Avance 600 MHz spectrometer and processed with MestReNova 5.2.2. Chemical shifts were referenced to the signal of acetone-d_6 at 2.05 ppm. The enzyme products were also analyzed by EIMS on an Auto SPEC (Micromass Co. UK Ltd.).

Overproduction and Purification of AnaPT and 7-DMATS as Well as Enzyme Assay. Overproduction and purification of AnaPT and 7-DMATS were carried out as described previously.[21,35] The

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Table 2. NMR Spectroscopic Data (600 Hz, Acetone-d_6) for 5b

pos	δ_C, type	δ_H, mult (J in Hz)	HMBC	pos	δ_C, type	δ_H, mult (J in Hz)	HMBC
2	83.5, CH	5.13, d (13.8)	C-3, C-4, C-9, C-1′, C-2′, C-6′	1″	128.1, C		
3	72.5, CH	4.38, d (13.8)		2″	111.0, CH	7.14, d (2.1)	C-1″, C-3″, C-6″
4	198.1, C			3″	147.7, C		
5	165.2, C			4″	147.4, C		
6	94.5, CH	6.06, d (3.3)	C-5, C-7, C-8, C-10	5″	114.8, CH	6.88, d (8.4)	C-1″, C-2″, C-4″, C-6″
7	168.4, C			6″	120.6, CH	6.98, d (8.0, 2.1)	C-7′, C-1″, C-2″, C-4″, C-5″
8	95.5, CH	6.09, d (3.3)	C-6, C-7,C-9, C-10	7″	55.5, CH_3	3.87, s	C-3″
9	162.2, C			1‴	65.3, CH_2	4.63, d (8.0)	C-7, C-2‴, C-3‴
10	101.1, C			2‴	119.0, CH	5.45, m	C-4‴, C-5‴
1′	130.2, C			3‴	138.2, C		
2′	116.5, CH	7.14, d (2.1)	C-2, C-6′, C-7′	4‴	17.3, CH_3	1.74, s	C-2‴, C-3‴, C-5‴
3′	143.9, C			5‴	24.8, CH_3	1.77, s	C-2‴, C-3‴, C-4‴
4′	144.2, C						
5′	116.4, CH	6.98, d (8.4)	C-1′, C-3′, C-6′, C-8′				
6′	121.0, CH	7.10 dd, (8.4, 2.1)	C-2, C-2′, C-3′, C-4′				
7′	76.5, CH	5.00, d (8.0)	C-8′, C-9′, C-1″, C-2″, C-6″				
8′	78.7, CH	4.16, m					
9′	60.9, CH_2	3.52, dd (14.5, 4.9)	C-7′, C-8′				
		3.75, dd (14.5, 2.6)					

Table 3. ^{1}H NMR Spectroscopic Data (500 Hz, Acetone-d_6) for 6c and 6d

	6c	6d
pos	δ_H, mult (J in Hz)	δ_H, mult (J in Hz)
1	2.84, t (7.7)	2.87, t (7.7)
2	3.29, t (7.7)	3.32, t (7.7)
6	5.90, s	
8	5.90, s	6.07, s
2′	6.96, d (2.2)	6.99, d (2.1)
5′	6.70, d (8.1)	6.72, d (8.2)
6′	6.88, dd (8.1, 2.2)	6.90, dd (8.2, 2.1)
1″	3.26, d (7.2)	3.24, d (7.3)
2″	5.30, tsept (7.2, 1.1)	5.22, tsept (7.3, 0.9)
4″	1.66, d (1.3)	1.62, d (1.1)
5″	1.68, d (1.1)	1.74, d (0.7)
1‴		3.28, d (7.3)
2‴		5.32, tsept (7.3, 1.1)
4‴		1.68, d (1.3)
5‴		1.70, d (1.1)

enzyme assay mixtures (100 μL) contained **1a**–**9a** (1 mM), $CaCl_2$ (10 mM), DMAPP or GPP (2 mM), glycerol (1.0–6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), and purified recombinant protein (40 μg). The reaction mixtures were incubated at 37 °C for different times and terminated by addition of 100 μL of MeOH. The proteins were removed by centrifugation at 13 000 rpm for 20 min. Assays for isolation of the enzyme products were carried out in large scales (10–15 mL) containing aromatic substrates (1 mM), DMAPP (2 mM), $CaCl_2$ (10 mM), glycerol (1.0–6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), and 7.5 mg of recombinant protein per 10 mL assay. After incubation for 16 h at 37 °C, the reaction mixtures of **1a**–**3a** and **5a**–**9a** were extracted three or four times with double the volume of EtOAc. The organic phases were combined and evaporated. The residues were dissolved in acetone-d_6 for recording ^{1}H NMR spectra. After measurement, the NMR samples were evaporated, dissolved in MeOH (0.5–1.0 mL), and purified by HPLC. Assays for determination of kinetic parameters (100 μL) contained $CaCl_2$ (10 mM), glycerol (1.0–6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), DMAPP (2 mM), (*R*)-benzodiazepinedinone, **1a**–**3a**, or **5a**–**9a** at final concentrations of up to 5.0 mM and different amounts of AnaPT, i.e., 1 μg for (*R*)-benzodiazepinedinone, 40 μg for **1a**, or 75 μg for **2a**, **3a**, and **5a**–**9a**. To keep product formation in the linear region (Figures S1 and S2, Supporting Information), the reaction mixtures were incubated for different times: 15 min for **1a**, 60 min for (*R*)-benzodiazepinedione, 120 min for **3a** and **9a**, 180 min for **2a**, **5a**, and **6a**, or 240 min for **7a** and **8a**. The reactions were terminated with 100 μL of MeOH. Protein was removed by centrifugation at 13 000 rpm for 20 min.

Table 4. Kinetic Parameters of AnaPT and 7-DMATS Reactions

	AnaPT			7-DMATS[a]		
substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
L-tryptophan				0.14	0.23	1643
(*R*)-benzodiazepinedione	0.22	1.72	7818			
naringenin (**1a**)	0.26	0.042	161.5	0.99	0.023	23
7-hydroxyflavanone (**2a**)	0.48	0.0042	8.8			
eriodictyol (**3a**)	0.29	0.0039	13.4	1.26	0.39	312
hesperetin (**4a**)				1.10	0.026	24
silibinin (**5a**)	0.18	0.0017	9.4			
phloretin (**6a**)	0.81	0.0025	3.1	0.13	0.036	286
apigenin (**7a**)	0.11	0.0018	16.4			
genistein (**8a**)	0.51	0.0042	8.2	0.16	0.027	171
biochanin A (**9a**)	0.11	0.011	100.0	0.07	0.019	261

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Analysis of Enzyme Products by HPLC, NMR, and MS. An Agilent HPLC series 1200 was used for analysis and isolation of the enzyme products. A Multospher 120 RP-18 column (250 × 4 mm, 5 um C+S Chromatographie Service, Langerwehe, Germany) was used for analysis at a flow rate of 1 mL/min, and a Multospher 120 RP18 column (250 × 10 mm, 5 μm) for isolation at a flow rate of 2.5 mL/min. H_2O (solvent A) and MeCN (solvent B), both containing 0.5% TFA, were used as solvents. A linear gradient of 40−100% (v/v) solvent B in 15 min was used for analysis of the enzymatic products. The column was then washed with 100% solvent B for 5 min and equilibrated with 40% solvent B for another 5 min. Detection was carried out using a photodiode array detector. Solvents for isolation of the enzyme products were H_2O (solvent C) and MeCN (solvent D) without acid. The enzyme products were isolated with a linear gradient of 50−100% D in C in 25 min. After each run, the column was equilibrated with 50% solvent D for 10 min. HPLC analysis of the (*R*)-benzodiazepinedione reaction was carried out as described previously.[21]

Compound ***1b:*** t_R = 10.02 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 288 nm; MS *m/z* 340.1293 (calculated for $C_{20}H_{20}O_5$, 340.1311); ^{1}H NMR (500 Hz, acetone-d_6) δ 7.39 (2H, d, *J* = 8.7 Hz, H-2′/H-6′), 6.89 (2H, d, *J* = 8.7 Hz, H-3′/H-5′), 6.03 (1H, s, H-8), 5.43 (1H, dd, *J* = 12.9, 3.0 Hz, H-2), 5.23 (1H, tsept, *J* = 7.3, 1.5 Hz, H-2″), 3.17 (1H, dd, *J* = 17.1, 12.9 Hz, H-3), 3.24 (2H, d, *J* = 7.3 Hz, H-1″), 2.72 (1H, dd, *J* = 17.1, 3.0 Hz, H-3), 1.75 (3H, d, *J* = 0.8 Hz, H-5″), 1.64 (3H, d, *J* = 1.1 Hz, H-4″).

Compound ***1c:*** t_R = 9.52 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 296 nm; MS *m/z* 340.1309 (calculated for $C_{20}H_{20}O_5$, 340.1311); ^{1}H NMR (500 Hz, acetone-d_6) δ 7.29 (1H, d, *J* = 2.2 Hz, H-2′), 7.21 (1H, dd, *J* = 8.3, 2.2 Hz, H-6′), 6.89 (1H, d, *J* = 8.3 Hz, H-5′), 5.95 (1H, d, *J* = 2.2 Hz, H-6), 5.94 (1H, d, *J* = 2.2 Hz, H-8), 5.43 (1H, dd, *J* = 12.9, 3.0 Hz, H-2), 5.35 (1H, tsept, *J* = 7.4, 1.5 Hz, H-2″), 3.34 (2H, d, *J* = 7.4 Hz, H-1″), 3.18 (1H, dd, *J* = 17.1, 12.9 Hz, H-3), 2.71 (1H, dd, *J* = 17.1, 3.0 Hz, H-3), 1.71 (3H, d, *J* = 0.7 Hz, H-5″), 1.70 (3H, d, *J* = 1.4 Hz, H-4″).

Compound ***2b:*** t_R = 12.43 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 240, 277, 320 nm; MS *m/z* 308.1389 (calculated for $C_{20}H_{20}O_3$, 308.1412); ^{1}H NMR (500 Hz, acetone-d_6) δ 7.59 (1H, s, H-5), 7.57 (2H, br d, *J* = 8.2 Hz, H-2′/H-6′), 7.44 (2H, t, *J* = 8.2 Hz, H-3′/H-5′), 7.38 (1H, tt, *J* = 8.2, 1.4 Hz, H-4′), 6.49 (1H, s, H-8), 5.54 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 5.33 (1H, tsept, *J* = 7.4, 1.5 Hz, H-2″), 3.28 (2H, d, *J* = 7.4 Hz, H-1″), 3.01 (1H, dd, *J* = 16.7, 13.0 Hz, H-3), 2.72 (1H, dd, *J* = 16.7, 3.0 Hz, H-3), 1.73 (3H, d, *J* = 0.9 Hz, H-5″), 1.71 (3H, d, *J* = 1.4 Hz, H-4″).

Compound ***3c:*** t_R = 8.99 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 290 nm; MS *m/z* 356.1234 (calculated for $C_{20}H_{20}O_6$, 356.1260); ^{1}H NMR (500 Hz, acetone-d_6) δ 6.90 (1H, d, *J* = 2.0 Hz, H-2′), 6.80 (1H, d, *J* = 2.0 Hz, H-6′), 5.95 (1H, d, *J* = 1.2 Hz, H-6), 5.94 (1H, d, *J* = 1.2 Hz, H-8), 5.37 (1H, dd, *J* = 12.9, 3.0 Hz, H-2), 5.35 (1H, tsept, *J* = 7.3, 1.5 Hz, H-2″), 3.35 (2H, d, *J* = 7.3 Hz, H-1″), 3.12 (1H, dd, *J* = 17.1, 12.9 Hz, H-3), 2.70 (1H, dd, *J* = 17.1, 3.0 Hz, H-3), 1.71 (3H, d, *J* = 0.6 Hz, H-5″), 1.70 (3H, d, *J* = 1.1 Hz, H-4″).

Compound ***5b:*** t_R = 11.08 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 290 nm; MS *m/z* 550.1839 (calculated for $C_{30}H_{30}O_{10}$, 550.1900); ^{1}H NMR (600 Hz, acetone-d_6) Table 1.

Compound ***6b:*** t_R = 9.09 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 290 nm; MS *m/z* 342.1438 (calculated for $C_{20}H_{22}O_5$, 342.1467); ^{1}H NMR (500 Hz, acetone-d_6) δ 7.09 (2H, d, *J* = 8.5 Hz, H-2′/H-6′), 6.74 (2H, d, *J* = 8.5 Hz, H-3′/H-5′), 6.07 (1H, s, H-8), 3.24 (2H, d, *J* = 7.2 Hz, H-1″), 5.22 (1H, tsept, *J* = 7.2, 1.1 Hz, H-2″), 3.33 (2H, t, *J* = 7.7 Hz, H-2), 2.88 (2H, t, *J* = 7.7 Hz, H-1), 1.74 (3H, d, *J* = 0.7 Hz, H-5″), 1.62 (3H, d, *J* = 1.1 Hz, H-4″).

Compound ***6c:*** t_R = 8.70 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 285 nm; MS *m/z* 342.1505 (calculated for $C_{20}H_{22}O_5$, 342.1467); ^{1}H NMR (500 Hz, acetone-d_6) Table 2.

Compound ***6d:*** t_R = 11.59 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 230, 290 nm; MS *m/z* 410.2071 (calculated for $C_{25}H_{30}O_5$, 410.2093); ^{1}H NMR (500 Hz, acetone-d_6) Table 2.

Compound ***7b:*** t_R = 11.55 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 220, 277, 330 nm; MS *m/z* 338.1149 (calculated for $C_{20}H_{18}O_5$, 338.1154); ^{1}H NMR (500 Hz, acetone-d_6) δ 7.93 (2H, d, *J* = 9.0 Hz, H-2′/H-6′), 7.02 (2H, d, *J* = 9.0 Hz, H-3′/H-5′), 6.62 (1H, s, H-3), 6.64 (1H, s, H-8), 5.27 (1H, tsept, *J* = 7.3, 1.4 Hz, H-2″), 3.35 (2H, d, *J* = 7.3 Hz, H-1″), 1.78 (3H, d, *J* = 1.0 Hz, H-5″), 1.65 (3H, d, *J* = 1.1 Hz, H-4″).

Compound ***8b:*** t_R = 10.52 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 215, 265 nm; MS *m/z* 338.1175 (calculated for $C_{20}H_{18}O_5$, 338.1154); ^{1}H NMR (500 Hz, acetone-d_6) δ 8.15 (1H, s, H-2), 7.45 (2H, d, *J* = 6.5 Hz, H-2′/H-6′), 6.90 (2H, d, *J* = 8.7 Hz, H-3′/H-5′), 6.51 (1H, s, H-8), 5.27 (1H, tsept, *J* = 7.2, 1.2 Hz, H-2″), 3.36 (2H, d, *J* = 7.2 Hz, H-1″), 1.78 (3H, d, *J* = 0.7 Hz, H-5″), 1.65 (3H, d, *J* = 1.2 Hz, H-4″).

Compound ***8c:*** t_R = 12.73 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 215, 266 nm; MS *m/z* 406.1768 (calculated for $C_{25}H_{26}O_5$, 406.1780); ^{1}H NMR (500 Hz, acetone-d_6) δ 8.10 (1H, s, H-2), 7.32 (1H, d, *J* = 2.3 Hz, H-2′), 7.25 (1H, d, *J* = 8.3, 2.3 Hz, H-6′), 6.88 (1H, d, *J* = 8.3 Hz, H-2′), 6.50 (1H, s, H-8), 5.36 (1H, m, H-2‴), 5.26 (1H, m, H-2″), 3.35 (2H, d, *J* = 7.5 Hz, H-1‴), 3.33 (2H, d, *J* = 6.0 Hz, H-1″), 1.76 (3H, d, *J* = 0.8 Hz, H-4‴), 1.71 (3H, d, *J* = 1.2 Hz, H-5″), 1.70 (3H, d, *J* = 1.2 Hz, H-5‴), 1.63 (3H, d, *J* = 1.1 Hz, H-4″).

Compound ***9b:*** t_R = 13.55 min; UV (extracted from PDA) (MeCN/H_2O) λ_{max} 215, 265 nm; MS *m/z* 352.1310 (calculated for $C_{21}H_{20}O_5$, 352.1311); ^{1}H NMR (500 Hz, acetone-d_6) δ 8.18 (1H, s, H-2), 7.54 (2H, d, *J* = 8.8 Hz, H-2′/H-6′), 7.00 (2H, d, *J* = 8.8 Hz, H-3′/H-5′), 6.51 (1H, s, H-8), 5.27 (1H, tsept, *J* = 7.2, 1.5 Hz, H-2″), 3.84 (3H, s, OCH_3), 3.36 (2H, d, *J* = 7.2 Hz, H-1″), 1.78 (3H, d, *J* = 0.6 Hz, H-5″), 1.65 (3H, d, *J* = 1.1 Hz, H-4″).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00422.

NMR spectra; HPLC chromatograms of enzyme assays with GPP; kinetic parameters (PDF)

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Notes

The authors declare no competing financial interest.

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