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Rational Engineered C-Acyltransferase Transforms Sterically Demanding Acyl Donors

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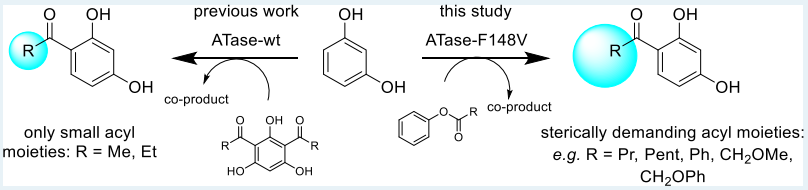
Supporting Information



ABSTRACT: The biocatalytic Friedel−Crafts acylation has been identified recently for the acetylation of resorcinol using activated acetic acid esters for the synthesis of acetophenone derivatives catalyzed by an acyltransferase. Because the wild-type enzyme is limited to acetic and propionic derivatives as the substrate, variants were designed to extend the substrate scope of this enzyme. By rational protein engineering, the key residue in the active site was identified which can be replaced to allow binding of bulkier acyl moieties. The single-point variant F148V enabled the transformation of previously inaccessible medium chain length alkyl and alkoxyalkyl carboxylic esters as donor substrates with up to 99% conversion and up to >99% isolated yield.

KEYWORDS: *Friedel−Crafts reaction, acylation, biocatalysis, biotransformation, enzyme engineering, side-directed mutagenesis*

INTRODUCTION

Carbon−carbon bond formation by the Friedel−Crafts reaction[1] is state-of-the-art in organic synthetic chemistry.[2] Various catalysts and different types of activated molecules have been employed.[3] As an extension to established protocols and because of environmental awareness and increasingly legal regulations, alternative concepts are investigated including biocatalytic approaches.[4] Research on Friedel−Crafts alkylation using enzymes for methyl-, prenyl-, and glycosylation has recently been published.[5] For instance, *S*-adenosyl-L-methionine-dependent *C*-methyltransferases were identified to modify flavonoid and alkaloid scaffolds to yield products with a specific methylation pattern.[6] Prenyltransferases (PTases) were reported to catalyze cofactor-independent transfer reactions of prenyl moieties from isoprenoid donors to the aromatic ring of prenyl acceptors, leading to a wide range of terpenoids.[7,8] Wild-type PTases typically exhibited a strict specificity for prenyl donors. Extended engineering studies on a recently discovered PTase (AtaPT, from *Aspergillus terreus*) allowed tuning of both the substrate scope and the prenylation selectivity.[9]

Sequence and structure-guided mutagenesis has been successfully applied to *O*-glycosyltransferases, which resulted in replacing the O-glycosylation to C-glycosylation activity.[10,11]

All the above mentioned biocatalytic reactions represent alkylations; in contrast, a biocatalytic equivalent to the classical Friedel−Crafts acylation based on a natural disproportionation[12] has been published recently for the C-acylation of phloroglucinol and resorcinol derivatives bearing alkyl, alkoxy, and halo-functionalities by a recombinant acyltransferase from *Pseudomonas protegens* (*Pp*ATaseCH).[13] The biocatalytic reaction proceeded with exquisite regioselectivity at the C6 position under ambient reaction conditions using various non-natural donor substrates,[13,14] to form dihydroxyphenyl ketones which are key motifs found in many therapeutic targets, fine chemicals, and natural products.[3,15] Furthermore, ATase exhibited reaction promiscuity and accepted aniline derivatives as substrates for amide bond formation.[16] Elucidation of the crystal structure of the biocatalyst and soaking experiments indicated the involvement of a cysteine (C88) in the subunit PhlC in the mechanism reacting with acetyl donors to give a thioester as an intermediate.[17] The carbonyl oxygen of the C88-bound acetyl group is positioned in the oxyanion hole, while the methyl group of acetyl is directed toward a small hydrophobic cavity formed by F148, L209, and L300.

The scope of acyl donors was previously restricted to acetyl and propionyl groups. Here, structure-based molecular modeling allowed us to expand significantly the scope of acyl

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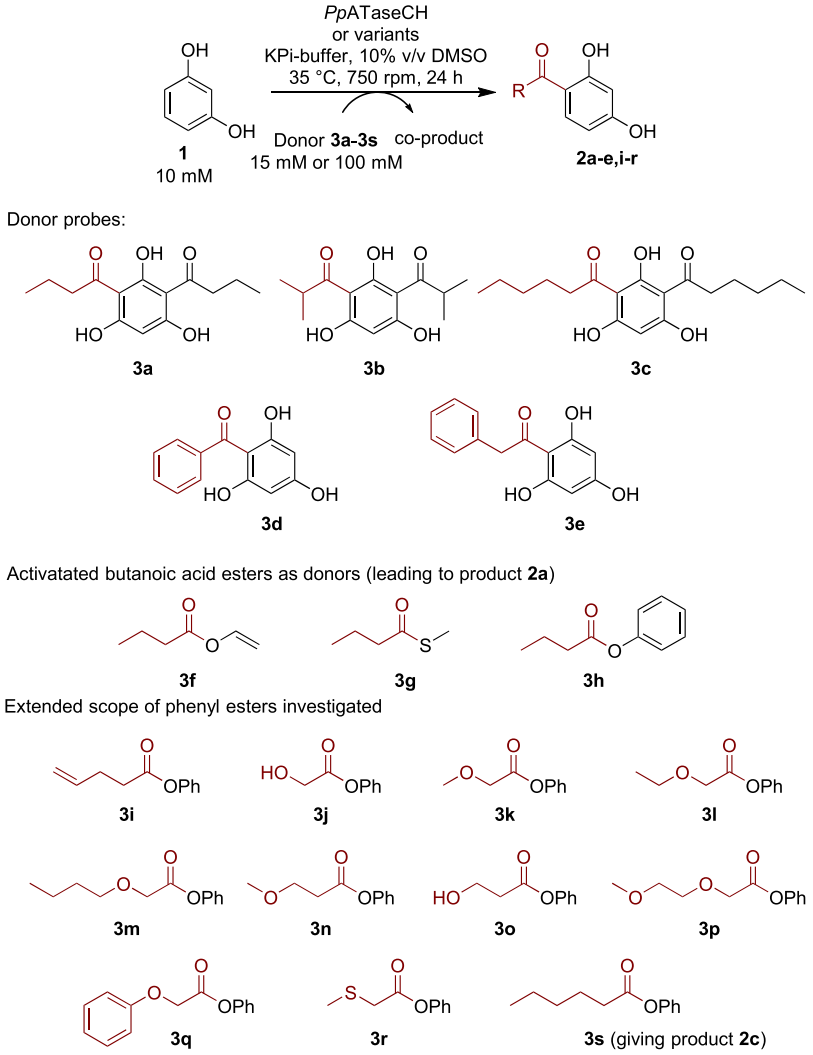
Scheme 1. Biocatalytic Friedel–Crafts Acylation Investigated[a]



[a]Acyl donors **3a–3e** were selected as initial probes to investigate acyltransferase variants to accept sterically more demanding substrates. Donors **3f–3s** were investigated subsequently.

donors to transform medium-chain length acyl and alkoxyacetyl moieties.

RESULTS AND DISCUSSION

The acyltransferase from *P. protegens* transfers an acetyl group or a propionyl group to resorcinol (**1**); however, no activity was detected toward acyl substrates bearing longer side-chains, such as butanoyl.[13] To investigate the transfer of sterically more demanding acyl groups, 2,6-diacylated phloroglucinol derivatives mimicking the native donor substrate diacetylphloroglucinol were synthesized. Thus, butanoyl (**3a**), isobutanoyl (**3b**), hexanoyl (**3c**), benzoyl (**3d**), or phenylacetyl (**3e**) were attached to the phloroglucinol scaffold (Scheme 1).

To identify residues in the enzyme which might be responsible for the limited substrate scope, a rational approach was chosen. From the crystal structure of *Pp*ATaseCH (PDB codes 5M3K, 5MG5) (Figure S2, Supporting Information), it was deduced that residues F148, L300, L383, Y386, and Y298 located in the environment of the acetyl group may limit the size of the cavity for substrate binding. The acetyl group covalently attached to C88 in the crystal structure of the enzyme was manually modeled to a butanoyl moiety in order to identify residues which might sterically interfere with the binding of longer acyl chains. Based on this analysis, F148 was subsequently replaced by valine and alanine (Figure 1). Also,

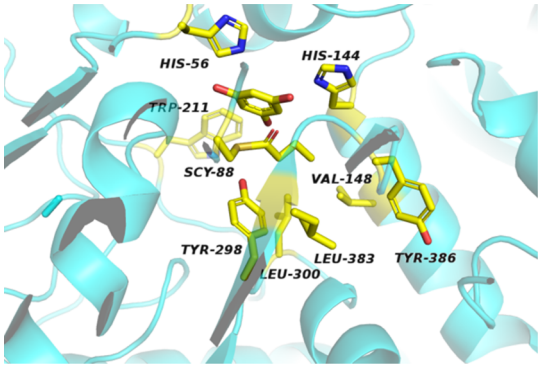


Figure 1. Crystal structure with the butanoyl residue modeled into the active site by extending the acetyl moiety manually and introducing mutation F148V by PyMOL mutation wizard. Figures were prepared using PyMOL.

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Table 1. Conversions of ATase Variants with Acyl Donor Substrates 3a−3e

		conv. [%][a]					
entry	*Pp*ATaseCH variant	**3a**	**3b**	**3c**	**3d**	**3e**	specific activity [mU/mg][b]
1	wild type	<0.1	<0.1	<0.1	<0.1	<0.1	55.4 ± 4.1
2	F148A	52.5 ± 2.0[c]	6.1 ± 0.1	54.8 ± 0.9	1.0 ± 1.7	<0.1	8.6 ± 1.1
3	F148V	66.8 ± 1.5[c]	8.8 ± 3.6	24.0 ± 0.9	1.0 ± 1.7	<0.1	10.2 ± 0.8
4	F148A/L383A	2.3 ± 0.6	<0.1	19.1 ± 2.0	0.6 ± 0.5	<0.1	7.4 ± 1.7
5	F148A/L383V	35.4 ± 2.7	2.8 ± 0.8	45.7 ± 5.1	0.8 ± 0.2	<0.1	9.1 ± 0.8
6	F148V/L383A	2.3 ± 0.6	<0.1	14.6 ± 0.4	0.9 ± 0.8	<0.1	8.5 ± 3.5
7	F148V/L383V	37.5 ± 2.0	4.3 ± 1.2	16.9 ± 1.2	2.3 ± 0.3	<0.1	4.0 ± 0.8
8	F148V/Y386A	21.3 ± 1.3	2.9 ± 0.8	10.4 ± 1.4	0.7 ± 0.7	<0.1	4.9 ± 1.1
9	F148V/Y386V	29.2 ± 0.7	2.2 ± 1.0	17.7 ± 3.7	0.6 ± 0.6	<0.1	9.3 ± 0.2
10	F148V/L300A	30.8 ± 0.5	<0.1	21.2 ± 1.9	2.8 ± 0.2	<0.1	12.5 ± 1.4
11	F148V/L300V	22.5 ± 2.2	<0.1	19.2 ± 0.9	1.2 ± 1.0	<0.1	19.1 ± 1.8
12	F148V/W211A	8.5 ± 1.3	2.0 ± 0.9	6.6 ± 0.5	1.0 ± 0.9	<0.1	10.7 ± 1.5
13	F148V/W211A/Y386A	2.2 ± 0.2	1.1 ± 0.1	1.8 ± 1.2	<0.1	<0.1	8.7 ± 0.9

[a]Conditions: cell-free *Escherichia coli* extract containing ATase variant (1.5 mg total protein) in K-phosphate-buffer (100 mM, pH 7.5, total volume 1 mL), resorcinol (**1**, 0.01 mmol), donor **3a−3e** (0.015 mmol), imidazole (100 mM added from a 1 M stock solution prepared in the reaction buffer), 35 °C, 750 rpm, 24 h. Conversions were determined based on three experiments by high performance liquid chromatography (HPLC) using calibration curves with authentic samples. [b]Specific activity refers to the activity of the enzyme for the disproportionation of monoacetylphloroglucinol into diacetylphloroglucinol and phloroglucinol. [c]Cell-free *E. coli* extract containing ATase variant (3 mg total protein).

Table 2. Acyltransferase F148V-Catalyzed Acylation of 1 with Butanoyl Donors (3f−3h) under Varied Conditions[a]

entry	donor	donor conc. [mM]	acceptor conc. [mM]	additives	enzyme loading [mg]	conv. [%]
1	**3f**	100	10	Im 100 mM	1.5	18.2 ± 5.4
2	**3g**	100	10	Im 100 mM	1.5	13.7 ± 2.1
3	**3h**	100	10	Im 100 mM	1.5	30.4 ± 2.8
4	**3h**	100	10	Im 100 mM	3	34.5 ± 2.8
5	**3h**	100	10	Im 100 mM	6	53.7 ± 0.2
6	**3h**	100	10	DMSO (10% v/v)	1.5	32.1 ± 3.0
7	**3h**	100	10		1.5	27.3 ± 2.3
8	**3h**	15	10	Im 100 mM	1.5	13.3 ± 1.2
9	**3h**	50	10	Im 100 mM	1.5	28.5 ± 1.0
10	**3h**	100	10	Im 100 mM; Triton X-100	1.5	31.4 ± 3.1
11	**3h**	100	10	Im 100 mM; Tween 40	1.5	34.5 ± 0.6

[a]Reaction conditions: cell-free *E. coli* extract containing ATase F148A (1.5−6 mg total protein) in KPi-buffer (100 mM, pH 7.5, total volume 1 mL), resorcinol (**1**, 0.01 mmol), donor **3d−3h** (0.1 mmol) with or without imidazole (Im, 100 mM added from a 1 M stock solution prepared in the reaction buffer), or with DMSO (10% v/v), or Triton X-100 (0.25 mM), or Tween 40 (0.03 mM), 35 °C, 750 rpm, 24 h.

the region of the lid, besides the substrate binding region, is involved in modulating activity, selectivity, and stability in selected enzymes (e.g., lipases and kinases).[18] Studies have demonstrated that an access to the enzyme active site is controlled by the position of the lid, that undergoes a transition between an open and a closed conformation as the enzyme binds the substrate in the catalytic cycle. It was shown that protein engineering of lid structures might provide either enzymes with new properties or reduction in the enzyme activity.[19] To investigate if residue W211, possessing the lid function, impedes the entrance of bulky substrates to the active site, it was replaced by smaller residues.

The selected amino acids were replaced by side-directed mutagenesis for sterically less demanding amino acids such as alanine or valine. Consequently, a focused library consisting of 11 single variants (F148A, F148V, W211A, W211F, Y298A, Y298V, L300A, L300V, L383A, L383V, and Y386V) was prepared and examined to catalyze the acylation of **1** using **3a−3e** as acyl donor substrates.

All of the tested variants were functional proteins and displayed reduced specific activity toward the native substrate MAPG (Supporting Information, Table S1). Importantly, only the exchange of F148 to either alanine or valine (F148A/V) enabled to transfer sterically more demanding acyl moieties (Table 1). For instance, using **3a** as the substrate, the butanoyl moiety was transferred to resorcinol with 53 and 67% conversion using F148A and F148V, respectively (Table 1, entry 2−3). Side-chain elongation of the acyl group to six carbon atoms (**3c**) resulted still in 55 and 24% conversion, and for a branched side-chain (**3b**), 6 and 9% conversion, respectively, was obtained. Even the benzoyl moiety (**3d**) was accepted, although just 1% conversion was reached under the conditions employed. In contrast, substrate **3e** bearing the phenylacetyl group was not transformed. Residue F148 was clearly identified as the key position for acyl donor selectivity, and the mutation of this residue to either alanine or valine enables the acceptance of enlarged acyl donors.

Consequently, variants F148V and F148A were chosen as the base for further variants, in which the variants F148A/V were combined with the other residues mentioned before (W211, L300, L383, and Y386), leading to the preparation of nine double variants (Table 1) and one triple variant (F148V/W211A/Y386A).

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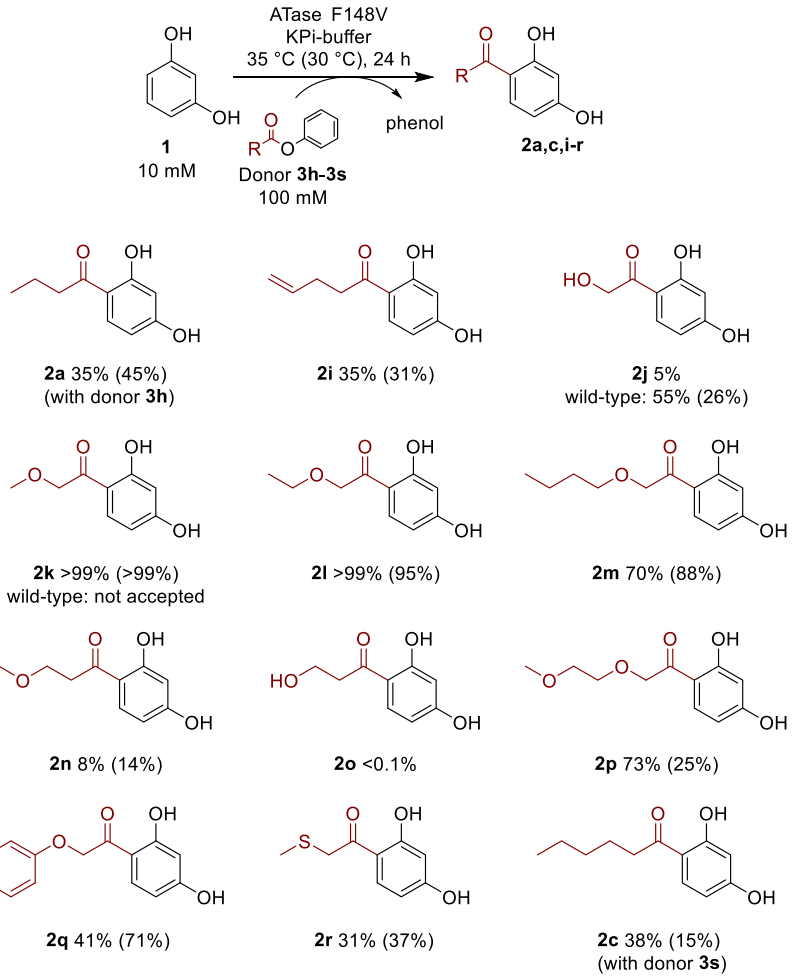
Scheme 2. Biocatalytic Friedel–Crafts Acylation of Resorcinol Using Phenyl Esters as Donors[a]



[a]Values before brackets represent conversions performed on an analytical scale. Values in brackets represent isolated yield from reaction performed on 0.25 mmol scale for **1**. Reaction conditions for semi-preparative scale: ATase F148V, 30 °C, 120 rpm, 24 h, horizontal tube position.

All double variants and the triple variant were able to catalyze the acyl transfer from donor substrates **3a** and **3c**; however, only a few of them accepted **3b** and **3d** and again none of them converted **3e** (Table 1, entries 4–13). Actually, all double/triple variants investigated displayed lower conversions than the single variant F148V. Although we expected initially that an enlarged active site would even more easily accommodate bulky acyl donors, the results may indicate that probably besides steric restraints, also positive interactions are responsible to ensure the transformation of a substrate. Concluding, for the probes **3a–d** investigated, F148V led to the highest conversion.

Thermal stability experiments (Supporting Information Figures S4–S7) were performed for the most interesting variants (F148A, F148V, and F148V/L383A) and the native enzyme. For all catalysts, immediate thermal denaturation was observed at 70 °C. The native enzyme displayed high stability in a temperature range of 30–50 °C, maintaining >85% activity after 240 min. Enzyme variants showed lower stability and may be used at 35 °C with half-life around 50 min for F148A, 140 min for F148V, and 240 min for F148V/L383A.

Based on previous studies on acetyl derivatives,[14] it was expected that other activated acyl donors may be suited as well. Consequently, the reactivity of the F148V variant was assayed using vinyl butyrate (**3f**), methyl thiobutyrate (**3g**), and phenyl butyrate (**3h**). Because the transformation with these donors led to reduced conversion when using the same reaction conditions as for **3a**, the influence of the donor concentration and the influence of additives such as imidazole, detergents, or cosolvents was investigated to obtain improved conversion (Table 2). Imidazole was previously identified to improve the reaction with 2-propenyl acetate.[14b]

It turned out that an excess of acyl donor had a beneficial effect on the conversion (entry 3 vs 8–9). Moreover, the increase in enzyme loading to 6 mg improved product conversion from 30 to 54% (entry 3 vs 4). Subsequently, the influence of various additives was investigated. Compared to reaction performed without additives (27%, entry 7), the addition of imidazole (30%, entry 3) or dimethyl sulfoxide (DMSO) (32%, entry 6) led to a small improvement. Likewise, surfactants (31, 35%, entries 10–11) slightly promoted the reaction. Finally, phenyl esters were chosen as the most attractive type of acyl donors because not only did they lead to highest conversion (30%, Table 2, entry 3 vs entries 1 and 2) but also many are commercially or accessible via simple synthetic methods.

To provide further insight into the scope of donors, the variant F148V was tested with a broad range of phenyl esters with varied side chains as donor substrates (**3i–3s**, Scheme 2). Compared to the wild-type, the F148V variant clearly showed

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Table 3. Kinetic Parameters for Wild-Type *Pp*ATaseCH and Variant F148V in the Biocatalytic Friedel−Crafts-Type Acylation

substrate	enzyme variant	K_M [mM]	V_{max} [mM min^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1} s^{-1}]
MAPG[a]	*Pp*ATaseCH	0.29 ± 0.04	4.83 ± 0.57	72 ± 8	251 ± 15
MAPG[a]	F148V	0.24 ± 0.09	0.82 ± 0.15	7.2 ± 1.9	31 ± 4
MBPG[a]	*Pp*ATaseCH		<0.014[c]		
MBPG[a]	F148V	0.54 ± 0.07	0.62 ± 0.11	5.3 ± 0.6	9.9 ± 0.8
3h[b]	F148V	3.2 ± 0.1	0.04 ± 0.02	0.012 ± 0.002	0.0037 ± 0.0006
3k[b]	F148V	5.0 ± 1.0	0.58 ± 0.23	0.188 ± 0.016	0.0387 ± 0.0085

[a]Spectrophotometric assay: potassium phosphate buffer (100 mM, pH 7.5) and substrate 0.04−1.6 mM (added from a 40 mM stock solution prepared in DMSO) were added to a cuvette and preheated to 35 °C. The reaction (1 mL total volume) was started by the addition of purified enzyme (0.18 mg). The reaction was followed for over 1 min. An increase in absorption is recorded because of the formation of DAPG (λ = 370 nm) or DBPG (λ = 370 nm). All reactions were performed in triplicates. [b]HPLC assay: potassium phosphate buffer (100 mM, pH 7.5), donor **3h** or **3k** 0.5−25 mM (added from a 50 mM stock solution prepared in DMSO), resorcinol (**1**, 0.275 mg, 10 mM), imidazole (100 mM, added from a 1 M stock solution in phosphate buffer) were added to an Eppendorf tube. The reaction (0.25 mL total volume) was started by the addition of purified enzyme (0.75 mg). The reaction mixture was shaken (5 min for **3k** and 60 min for **3h**) at 35 °C and 750 rpm in an orbital shaker. Reactions were quenched by addition of acetonitrile (0.25 mL). The precipitated protein was removed by centrifugation (20 min, 14 000 rpm), and the supernatant was subjected to HPLC for determination of conversions. [c]Reaction rates were lower than the detection limit, V < 0.014 mmol min^{-1}.

a significant extended substrate scope. Donor scaffolds bearing functional groups, such as double bonds or hydroxyl groups, were investigated. For instance, phenyl pent-4-enoate (**3i**) led to conversion up to 35%, while neither phenyl (*E*)-but-2-enoate nor phenyl 3-methylbut-2-enoate was transformed (both possessing a conjugated double bond, Supporting Information, Scheme S2). Short-chain substrate **3j** bearing a hydroxyl group in position 2 was poorly accepted by F148V (5%), but significant activity was observed for the wild-type enzyme (55%). When the hydroxyl moiety was protected as methyl ether, wild-type ATase displayed no measurable conversion for the corresponding phenyl 2-methoxyacetate (**3k**), which is consistent with previous observations, that the native enzyme accepts only short acyl moieties. Interestingly, the same acyl donor **3k** was well accepted by the F148V variant, reaching completion at 10 mmol substrate concentration within 24 h. Thus, compared to the aliphatic donor (**3h**) with the same number of atoms, the 2-alkoxy compound (**3k**) led to the significantly higher conversion (35 vs >99%).

Since 1990s, alkyl methoxyacetates have been used as an efficient source of acyl moieties for lipase-catalyzed resolutions of chiral amines.[20] The presence of a methoxy substituent accelerated the reaction, so the initial acylation rate was 100 times faster than the corresponding reaction with ethyl butyrate.[21] Interaction between the β-oxygen atom in methoxyacetate and the amine nitrogen atom was found to be a key factor in the rate enhancement. High preference for the alkoxyacetyl moiety had also been identified for an acyltransferase in amide bond formation.[22]

Consequently, various alkoxyacetyl donor substrates were subjected to the Friedel−Crafts bioacylation. Elongation of the alkoxy side chain from methoxy to ethoxy (substrate **3l**) allowed again to achieve >99% conversion, and also for groups (**3m**, 70%), good conversion was reached. However, when shifting the oxygen atom in the acyl moiety from position 2 to position 3 resulted in reduced conversion, such as 8% for the butanoyl analogue (**3n**) and the unprotected hydroxyl groups (**3o**, <0.1%). On introduction of a second oxygen atom in the side chain of the well accepted donor **3m**, donor **3p** led to a comparable conversion of 73%.

Even the donor bearing a phenyl group linked to the oxygen atom in 2-position was accepted with reasonable conversion (**3q**, 41% conv.). This may be of importance because **2q** constitutes the core structure of various antivirulence agents and potential adjuvants for the treatment of *Staphylococcus aureus*-derived diseases.[23]

Because oxygen in the side chain showed a tremendous influence on the conversion, we wondered what might be the effect if the oxygen atom in, for example, donor **3k** gets exchanged with sulfur (donor **3r**). The side chains of the O- and S-donors differ only slightly in their sizes, but sulfur is a weaker hydrogen bond acceptor.[24] The corresponding product **2r** was obtained with 31% conversion, which represents a lower value compared to the analogue compound **2k** (>99% conv.). Obviously, the oxygen at position 2 has a significant effect; however, the exact binding could not be elucidated using docking experiments.

Interestingly, other acyl donors possessing an aromatic ring, such as benzoyl phenylacetyl or phenylpropionyl were not transformed (see Supporting Information, Scheme S2). Medium-chain length donor **3s** led to similar conversion as for native-substrate mimicking analogue **3c**.

To demonstrate the applicability of the biotransformation, semipreparative transformations were performed starting with 0.25 mmol of substrate **1**. Reactions were run for 24 h, and products were isolated with high yields with up to 99% (Scheme 2, values in brackets). Generally, isolated product yields were comparable to the conversions obtained in analytical-scale experiments. In few cases, isolated product yield values were even higher because of the slightly altered reaction conditions including lower reaction temperature (30 °C instead of 35 °C), shaking speed (120 rpm instead of 750 rpm), and horizontal tube positioning while shaking (instead of vertical). In selected cases, hydrolysis of O-acetylated coproduct was required. As previously shown,[13] reaction with carboxylic acid esters may give the Friedel−Crafts product or lead to spontaneous O-acylation, whereby the O-acylated products may serve again as the acyl donor; this is in contrast to phloroglucinol derivatives for which no O-acetylation was detected.

Finally, to evaluate the effect of the mutation F148V, the kinetic parameters of the variant and the wild-type enzyme *Pp*ATaseCH were compared for the disproportionation of monoacetylphlorogucinol (MAPG) and monobutanoylphlorogucinol (MBPG) into the corresponding products, diacetylphloroglucinol (DAPG) and **3a**, respectively. Initial velocities were measured at substrate concentrations of 0.04−1.6 mM. Variant F148V showed 10 times lower catalytic efficiency

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toward the native substrate MAPG than the wild type as indicated by k_{cat}/K_M (Table 3). The lowered k_{cat}/K_M value results mainly of a 10-fold decrease in k_{cat}. The comparable K_M values indicate that binding is similar. In the case of the more bulky compound MBPG, the wild type showed no detectable activity, while F148V led to kinetic parameters similar as observed for the variant with MAPG.

Because a significant effect of an oxygen atom in position 2 of the acyl donor was noted, kinetic parameters for **3h** and **3k** were determined in the acylation of resorcinol **1**. The amount of resorcinol was kept constant (10 mM), while the initial velocities were measured at different concentrations of donors (0.5−25 mM). The transformation of each donor into the corresponding product was monitored by measuring the increase in product concentration.

The results show a significant difference in the k_{cat} values of the butanoyl and methoxyacetyl side chains; for the methoxyacetyl side chain, the k_{cat} was 15.7-fold higher than for butanoyl.

CONCLUSIONS

For extending the spectrum of acyl groups accepted by the wild-type ATase, rational enzyme engineering was used to identify potential amino acid exchanges, which may generate more space in the active site pocket. Enzyme variants generated by side-directed mutagenesis were screened first toward a set of diacylated phloroglucinols as donor substrates, being closest to the native substrate. The results showed that phenylalanine 148 is a key position for mutagenesis, whereby F148V turned out to be suited best. Additional mutations to increase the cavity of the active site even further did not lead to the desired effect. Investigation of different types of the activated butanoyl group indicated that phenyl esters are highly suitable substrates. A broad set of various functionalized phenyl esters was investigated as acyl donors. Interestingly, acyl donors possessing an oxygen atom in position 2 turned out to be well accepted substrates, leading to significant higher conversion than the corresponding methylene substrates; for instance, methoxyacetyl led to >99% conversion, while butanoyl led to 35% conversion under comparable conditions. This methodology allowed us to use ATase for the synthesis of 1-(2,4-dihydroxyphenyl)-2-phenoxyethan-1-one (**2q**), which constitutes the core structure of various antivirulence agents.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.9b04617.

Enzyme specific activity, SDS-PAGE analysis of the cell-free *E. coli* extracts, structure of the active site of the native enzyme, plasmids, primers, SDS-PAGE analysis, ATase-wt thermostability, UV-Vis spectrum, plot of initial rates against substrate concentration, ^{1}H-NMR, ^{13}C-NMR, MS, HPLC-chromatogram, ESI-HRMS spectrum, and detailed experimental procedures (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ATase, acyltransferase; *Pp*ATaseCH, acyltransferase produced by codon-harmonized construct from *P. protegens*; Im, imidazole; DMSO, dimethyl sulfoxide; MAPG, monoacetylphloroglucinol; DAPG, 2,4-diacetylphloroglucinol; MBPG, monobutyrylphloroglucinol; DBPG, 2,4-dibutyrylphloroglucinol; DPPG, diisobutyrylphloroglucinol; HPLC, high performance liquid chromatography; *E. coli*, *Escherichia coli*

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