

Biocatalysis

Efficient Enzymatic Synthesis of Carbamates in Water Using Promiscuous Esterases/Acyltransferases

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Abstract: Biocatalysis provides an attractive approach to facilitate synthetic reactions in aqueous media. Motivated by the discovery of promiscuous aminolysis activity of esterases, we exploited the esterase from *Pyrobaculum calidifontis* VA1 (PestE) for the synthesis of carbamates from different aliphatic, aromatic, and arylaliphatic amines and a set of carbonates such as dimethyl-, dibenzyl-, or diallyl carbonate. Thus, aniline and benzylamine derivatives, aliphatic and even secondary amines could be efficiently converted into the corresponding benzyloxycarbonyl (Cbz)- or allyloxycarbonyl (Alloc)-protected products in bulk water, with (isolated) yields of up to 99%.

Protecting groups are irreplaceable in organic synthesis to prevent undesired side-reactions in subsequent reaction steps. Even though protection and deprotection introduces uneconomical steps into synthetic routes, their complete avoidance remains the prospect of current research.^[1] In this regard, (nucleophilic) amine groups often require protection.^[2] Traditionally, acid chlorides are employed for the protection of amines, transforming them into amides or carbamates.^[3] However, handling risks and the formation of toxic by-products call for less harmful substitutes for acid

chlorides.^[4] On this matter, biocatalysis, which employs enzymes for the synthesis of (complex) organic molecules, offers a promising solution.^[5] Not only do biocatalysts exhibit excellent chemo-, regio-, and stereoselectivity; they usually operate under benign reaction conditions in aqueous media, highlighting their potential for the development of environmentally friendly and more sustainable processes.^[6] In the context of amine transformations, several enzymes have been investigated to catalyze the formation of amide bonds.^[7] For instance, carboxylic acid reductase adenylation domains (CAR-As) and amide bond synthetases (ABSs) are relevant as biocatalysts for the pharmaceutical industry.^[8] Although this enzyme-catalyzed coupling of free carboxylic acids and amines is appealing, a major challenge for broader application has been the dependency on stoichiometric amounts of co-factors like ATP, which requires additional recycling systems.^[9] In the past, the promiscuous aminolysis of esters and carbonates mediated by lipases was used to synthesize amides and carbamates, respectively.^[10] *Candida antarctica* lipase B (CAL-B), for example, catalyzed this biotransformation in pure organic solvents to achieve product formation and to avoid the undesired hydrolysis of the target products.^[11] Thus, industrially suitable enzymes for the transformation of amines are still searched for.^[12] Esterases have also been shown to possess promiscuous acyltransferase activity.^[13,14] Both enzyme families depend on a nucleophile-base-acid (often serine-histidine-aspartate) catalytic triad, to form the key acyl enzyme intermediate. This intermediate undergoes subsequent hydrolysis by an organic nucleophile, yielding the acyl transfer product (Scheme S1).^[15] Despite these similarities, a clear advantage of esterases is their ability to catalyze aminolysis in bulk water. Land et al. first described this promiscuous activity for *Mycobacterium smegmatis* acyltransferase (MsAcT), a SGNH-hydrolase.^[13] A remarkable discovery was made by Zeng et al., who identified *Sphingomonas* sp. HXN-200 esterase (Spl), an α/β -hydrolase fold enzyme, able to catalyze aminolysis with free carboxylic acids.^[14] Recently, we reported the promiscuous aminolysis activity of EstCE1, a metagenome-derived β -lactamase-like esterase, and illustrated its pharmaceutical potential by synthesizing moclobemide as a representative amide-containing active pharmaceutical ingredient (API).^[16] In a plethora of APIs, amide and carbamate bonds are important structural motifs.^[17] Especially, carbamates are of interest in current drug discovery and can be found in various small molecule drugs.^[17] While biocatalytic amide synthesis has been exten-

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sively studied with esterases, their potential for carbamate synthesis has been barely tapped.^[18]

Extending the previous study by Müller et al.,^[16] we aimed at exploring the biocatalytic synthesis of carbamates in aqueous solution. In this study, we focused on the transformation of amines that are frequently found in APIs, and the enzyme-catalyzed introduction of commonly used protecting groups (Scheme 1).

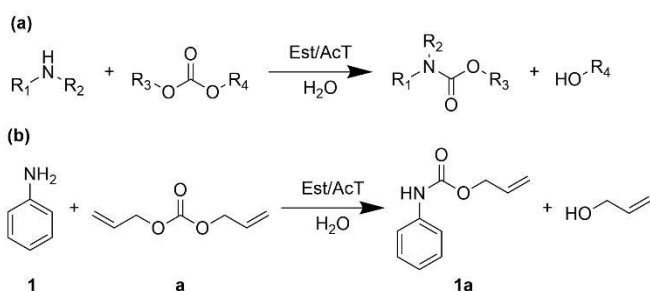
Prioritizing the identification of enzymes suitable for the desired biotransformation of amines into carbamates, a panel of 23 esterases was investigated (Table S1). The selected enzymes originate from three major superfamilies (SGNH-hydrolases, β -lactamase-like hydrolases, and α/β -hydrolases), which are also the only three esterase superfamilies known so far to feature biocatalysts with promiscuous aminolysis activity. Apart from wild-type enzymes, engineered variants were also included in the panel such as MsAcT_S11C, which was described to accept secondary amines.^[18] The corresponding mutation was also introduced into PestE to investigate whether the effect of this substitution can be transferred to another enzyme. Further, EstA_WGG, a variant of *Arthrobacter nitroguajacolicus* esterase in which two residues in the oxyanion hole were mutated,^[16] was included since this variant accepted polar substrates like sugars in promiscuous transesterification reactions in water.^[16,19] Lastly, the variants EstCE1_MAA and a triple mutant of PestE (PestE_trip) were included in this work. Both better accept the cheap ethyl acetate as acyl donor in transesterification reactions.^[19,20]

For the initial screening, crude *E. coli* cell lysates containing one of the recombinantly expressed panel enzymes were used. To account for undesirable background activity, lysate of untransformed *E. coli* cells was used as control. Three amines—**1** (aniline), pentylamine (**25**), and piperidine (**28**)—were chosen as representatives for primary aromatic, primary aliphatic, and secondary heterocyclic amines, respectively. Diallyl carbonate (**a**) was employed as donor substrate initially because it enables the introduction of the Alloc protecting group (Figures S2–4).

Initial results suggested that carbamate formation with the aromatic amine **1** was catalyzed by enzymes from the

α/β -hydrolase superfamily with high conversions (50–99%). Among them, Spl, PestE, and the variant PestE_trip achieved conversions >80%. Using the aliphatic amine **25**, high conversions of 70–91% were observed. However, also different enzymes were identified to accept **25** as substrate. While Spl and PestE still showed the highest conversions (88% and 91%, respectively), EstCE1 and its MAA-variant showed equally high conversions (~90%); EstM2 and Est2 yielded conversions above 70%. The secondary amine **28** was only accepted by a few enzymes with low conversions (10–18%). Surprisingly, the wild-type MsAcT better converted **28** into the carbamate than the S11C variant (11% and 6%, respectively), indicating that carbamate formation with secondary amines does not require this mutation. EstM2, EstY29, and PestE achieved even higher conversions (13%, 15%, and 18%, respectively). These findings support that the serine to cysteine exchange is not a prerequisite for Est/AcTs to accept secondary amines for this promiscuous carbamate synthesis and, more intriguingly, that the activity is prevalent among the three investigated enzyme superfamilies. However, **1** and **25** were more efficiently converted with various enzymes, the secondary amine **28** remains a more challenging substrate for the tested enzymes. Presumably, accessibility of the secondary amine by the enzymes is limited due to steric hindrance. Interestingly, the exchange of the catalytic serine to cysteine, while effective for MsAcT, did not improve the activity of PestE. In fact, carbamate formation using PestE_S157C was not observed for any of the three amine substrates (**1**, **25**, and **28**). Additionally, a discrepancy between amine consumption and product formation was found for all three reactions when employing crude cell lysates (Figures S2–S4). This can be due to the oxidation of amines by endogenous host enzymes, for example.^[21] To address this and potential differences in soluble expression levels in lysates, the four most promising enzymes—MsAcT, EstCE1, PestE, and Spl—were purified. Satisfyingly, results with purified biocatalysts are in good agreement with initial screenings and no background consumption of the amine substrate was found in blank reactions (Figure 1). While **25** seems to be the best substrate for all four selected Est/AcTs, **1** is best converted by PestE and Spl (74% and 97%), and **28** by MsAcT and PestE (15% and 17%). Hence, PestE was selected for an in-depth investigation of the accessible substrate scope regarding both amines and different carbonates.

For the latter, PestE was purified as before and aniline (**1**) converted with a broad panel of carbonate substrates (Table 1); **1** was chosen since aromatic amines are present in many APIs.^[22] At the optimal temperature of 33 °C, the highest conversion of **1** was achieved with 200 mm donor **a** (Figure S8). With dibenzyl carbonate (**b**), PestE facilitated the introduction of the Cbz-protecting group, yielding 42% isolated **1b** under experimental conditions. Additionally, aliphatic carbonates with chain lengths of one to five carbons (**c**, **d**, **e**, **f**, **g**, and **i**) were accepted by PestE. The enzyme showed a preference for carbonates with three to five carbon atoms (**e**, **g**, and **i**), while no conversion was observed with **j** (six carbon atoms).



Scheme 1. (a): Promiscuous esterase/acetyltransferase (Est/AcT)-catalyzed carbamate synthesis. Est/AcTs yield carbamates from an amine acceptor substrate and a carbonate donor. In the case of primary amines, R_2 is a hydrogen; $R_3 = R_4$ in case of symmetric carbonates. (b): The formation of allyl phenyl carbamate (**1a**) from aniline (**1**) and diallyl carbonate (**a**) was used as reference reaction in this work.

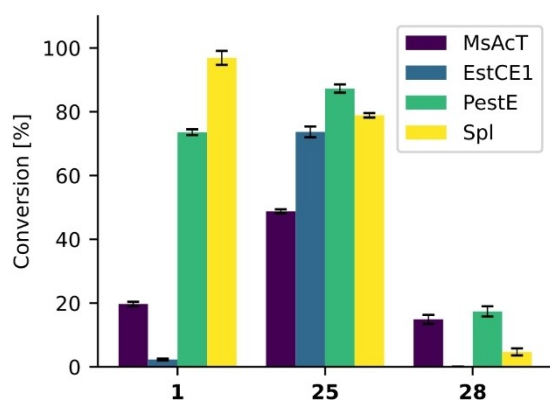


Figure 1. Activity of selected purified promiscuous Est/AcTs. Amines **1**, **25**, and **28** were converted to the carbamate products. Reactions employed the indicated purified enzyme (0.5 mg mL^{-1}), 50 mM amine substrate, and 200 mM carbonate **a** in 100 mM sodium phosphate buffer ($\text{pH}=8.0$). Conversions were calculated with respect to buffer controls not containing enzyme. Conversions shown as mean values \pm standard deviation (SD) of triplicates.

Table 1: Carbonate substrates studied in the conversion of **1** with PestE.

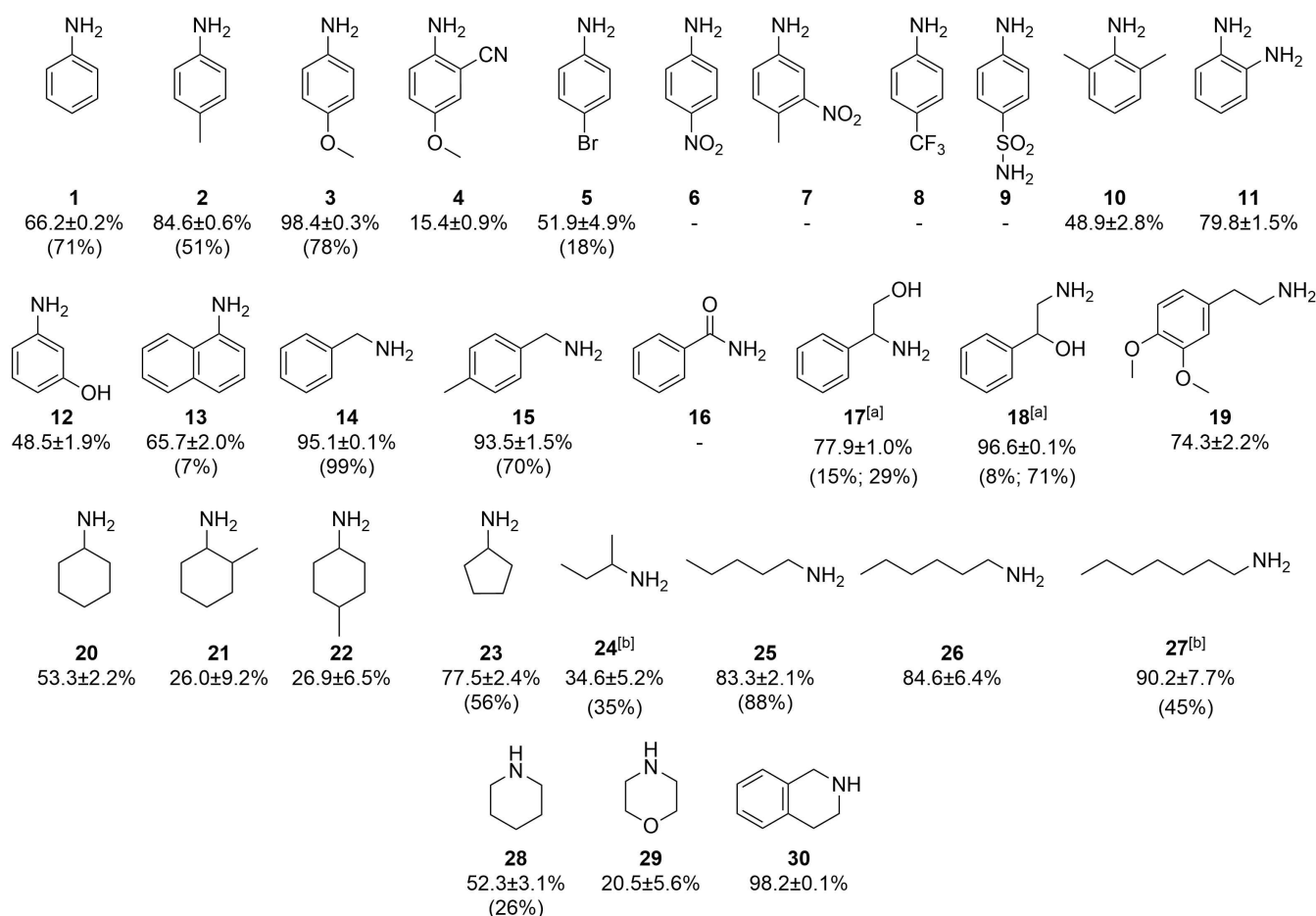
Carbonate	R ₁	R ₂	Amine Consumption (Yield) [%] ^[a]
a	$-\text{CH}_2-\text{CH}=\text{CH}_2$	$=\text{R}_1$	66.2 ± 0.2 (71)
b	$-\text{CH}_2-\text{Ph}$	$=\text{R}_1$	43.6 ± 0.3 (42)
c	Me	$=\text{R}_1$	20.4 ± 0.9
d	Et	$=\text{R}_1$	25.2 ± 2.8 (15)
e	Pr	$=\text{R}_1$	52.6 ± 2.2 (38)
f	<i>i</i> -Pr	$=\text{R}_1$	< 1
g	Bu	$=\text{R}_1$	59.7 ± 1.1 (85)
h	<i>t</i> -Bu	$=\text{R}_1$	–
i	Pe	$=\text{R}_1$	55.8 ± 2.0
j	Hex	$=\text{R}_1$	–
ad	$-\text{CH}_2-\text{CH}=\text{CH}_2$	Et	32.7 ± 1.9 ^[b]
hd	<i>t</i> -Bu	Et	–

[a] The carbonate substrates (200 mM) were reacted with **1** (50 mM), using purified PestE (0.05 mg mL^{-1}) for 24 h in 100 mM sodium phosphate buffer ($\text{pH}=8.0$). Reactions were performed in triplicates to determine the average amine consumption \pm SD with respect to a blank reaction. Formation of product carbamates (**1x**) was verified by GC-MS. Isolated yield of preparative scale reactions is given in brackets if applicable (see also Supporting Information). [b] Carbamate **1d** and **1a** were detected in a 6:1 ratio.

One of the most commonly used protecting group for amines is the *tert*-butyloxycarbonyl group.^[23] However, employment of the respective carbonate **h** showed no conversion of **1** to the corresponding carbamate. Notably, screening of the entire enzyme panel did not yield a positive hit (data not shown). We assume that this is due to the bulky *tert*-butyl group as only a few esterases are known to accept them.^[24] Thus, the sterically less demanding di-isopropyl carbonate (**f**) was investigated. The biotransformation catalyzed by PestE led to the identification of the carbamate product below the limit of quantification (< 1 % conversion). However, screening of the whole Est/AcT panel revealed that, besides EstCE1 and EstY29 (43 % and 22 %, respec-

tively), Spl yielded high conversions (95 %; Figure S6). The asymmetric carbonate **hd** was not accepted by PestE, likely again, due to the bulky *tert*-butyl group. The other investigated asymmetric carbonate (**ad**) was accepted by PestE. However, analysis of the formed product revealed that both the ethyl carbamate (**1d**) as well as the allyl carbamate (**1a**) were formed in a 6:1 molar ratio (**1d**:**1a**). This emphasizes the need for symmetric carbonates as donor substrates to prepare the desired carbamates.

Regarding the amine substrate scope of PestE, a selection of aromatic, aliphatic, and secondary amines was investigated (Scheme 2). As before, diallyl carbonate (**a**) was used as acyl donor, achieving good to high conversions. Aniline derivatives were readily accepted by PestE (49–98 % conversion, except for 2-amino-5-methoxy benzonitrile (**4**)). The difference in conversion may be explained by the influence of ring substituents on the electron density of the aromatic amine. Comparing *p*-anisidine (**3**) with **4**, which differ by an additional nitrile group in the *ortho*-aniline position, a five-fold lower conversion was measured, likely due to the deactivating effect of the nitrile substituent. In contrast to unsubstituted **1**, an increase in conversion by up to 30 % was measured for substrates **2**, **3**, and **11**, which contain an activating substituent in *para*- or *ortho*-position with respect to the amine group. A remarkable 98 % conversion of **3** was achieved. The conversion of *p*-bromoaniline (**5**) is reduced compared to **1**, which can be explained by the deactivating effect of the halogen-substituent in *para*-position. Similarly, substrates with deactivating substituents (**6**, **7**, **8**, and **9**) were not converted by PestE. Furthermore, low conversion of 2,6-dimethylaniline (**10**) was observed, even though the methyl groups should exert an activating effect in *ortho*-position. Presumably, the substituents might hinder the access of the amine substrate into the active site of PestE. The two arylamines *o*-phenylenediamine (**11**) and *m*-aminophenol (**12**) contain two nucleophilic groups, which could potentially both function as acceptors for the acyl transfer. However, for **11** and **12**, only a single acylated product, the corresponding carbamate, was detected. Interestingly, 30 % higher conversion of **11** compared to **12** was observed. Both the amine and hydroxyl group exhibit an activating effect. Likely because in **11**, the activating group is in *ortho*-position, an exceptional high conversion of 79 % was achieved. In **12**, the hydroxyl group is in *meta*-position to the amine functionality. This should have no influence on its nucleophilicity. The bulkier substrate 1-naphthylamine (**13**) was equally well converted as **1**, showing that the enzyme also accepts a bicyclic aromatic substrate. Benzylic amines (**14**, **15**, **17**, **18**, and **19**) were readily converted by PestE, yielding 74 % to 97 % of the corresponding carbamates. Benzamide (**16**), however, was not accepted. Presumably, the amide cannot function as substrate due to the delocalized electron pair of the nitrogen to the carbonyl oxygen. The constitutional isomers 2-amino-2-phenylethanol (**17**) and 1-amino-2-phenylethanol (**18**) were similarly well converted. A slight preference, by roughly 20 %, was found for **18**. Separate transformations of both enantiomers of **17** showed 87 % and 75 % conversion for the (*R*)- and (*S*)-enantiomer, respectively (Figure S9).



Scheme 2. Investigated amine substrates. Amine substrates (1–30: 50 mM) for purified PestE (0.05 mg mL⁻¹) were reacted with **a** (200 mM) as carbonate substrate. Reactions were performed for 24 h in triplicates and are reported as average amine consumption ± SD with respect to a control reaction in the absence of PestE. Product formation of the expected carbamate was verified by GC-MS. Isolated yields from preparative scale reactions are given in brackets if applicable (see also Supporting Information). [a] The formation of two products was found. The isolated yield is given for both products. [b] Product formation was calculated because amine consumption could not be quantified reliably.

We conclude from this that PestE exhibits no enantioselectivity in its promiscuous acyltransferase activity. A possible explanation for the observed difference in conversion of **17** and **18** might be that the amine in **17** is less nucleophilic than the amine in **18**.^[25] With dimethoxyphenylethylamine (**19**) high conversions were found with PestE. This moiety is present in natural compounds, suggesting broad application of PestE to also convert such amines.^[26] Linear and cyclic aliphatic amines connected in a molecule by a carbamate bond are also readily found in APIs.^[16] Ring sizes of cyclic aliphatic amines seem to have an influence on their conversion as shown for cyclopentylamine (**23**; 78%), which is better converted by about 25% than cyclohexylamine (**20**; 53%). PestE shows no preference for a particular carbon chain length of linear aliphatic amines (**25**, **26**, and **27**), converting them all at an average of 80%. The enzyme, however, showed a 20% reduced conversion of *sec*-butylamine (**24**), in which the amine is attached to a secondary carbon. This difference might be due to the higher nucleophilicity of primary amine groups. Another finding was that a methyl group, as in 2-methylcyclohexylamine (**21**)

or 4-methylcyclohexylamine (**22**), also influences the conversion. Compared to **20**, the conversion of **21** and **22** was reduced by half. As already found during the enzyme screening, the conversion of secondary amines into carbamates was generally low. With purified PestE conversions of roughly 50% were achieved with **28**. To date, only Contente et al. have investigated secondary amines as substrates for the Est/AcT MsAcT.^[18] The secondary amines morpholine (**29**) and 1,2,3,4-tetrahydroisoquinoline (**30**) were tested due to their importance as structural motifs in small molecule drugs and natural compounds.^[27] Only about 20% of **29** were converted, but the benzylic **30** was almost completely converted into the carbamate product. Conclusively, it is not possible to generally propose that secondary amines are only poorly accepted due to sterical hindrance of the amine group. The amino acids glycine and lysine were also investigated as substrates. However, no conversion could be detected (data not shown). This had been previously reported for MsAcT.^[13]

Lastly, preparative scale reactions were performed for which the concentration of the amine substrate was doubled

(100 mM) and the reaction volume was increased to 10 mL. For most reactions, the isolated yield agreed with analytical scale data (Table 1 and Scheme 2). Most interestingly, reactions with **17** and **18** yielded two products. A single acylated product (**18a-I** and **17a-I**) with only the carbamate formed and a double-acylated species (**18a-II** and **17a-II**) with an additional carbonate formed at the hydroxyl group (Figure S66 and S73). The major product was in both cases the carbamate product (**18a-I** and **17a-I**). Measuring a time course of the reaction showed that both products are simultaneously formed. However, the double acylated product is consumed again after 6 h (Figure S7). This highlights the property of the enzyme to catalyze reversible transesterification, while aminolysis was found to be irreversible. Allowing for a chemo-selective biocatalytic transformation of the amine group in molecules containing also hydroxyl functionalities as potential acceptor groups.

In summary, we have shown in this work that the enzyme PestE can be used for the synthesis of a broad panel of carbamates. A few other enzymes from our investigated panel were also active. However, PestE was by far the synthetically most useful enzyme. While known enzymes like the cofactor-dependent ABS and CAR-A catalyze the synthesis of amides from amines and free carboxylic acids, the herein presented enzymatic route gives access to organic carbamates in water. Not only is this a convenient tool for the introduction of commonly used protecting groups; the straight-forward scale-up of reactions highlights the synthetic potential of PestE for industrial applications. Currently, we regard the use of organic carbonates as acyl donor to be one major discrepancy in our catalytic route to comply with the principles of green chemistry.^[6] Organic carbonates are commonly synthesized from phosgene, for example.^[28] However, on-going research investigates the use of carbon dioxide as safer and less toxic alternative to make the synthesis of organic carbonates more sustainable in the future.^[28]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article. This also includes further references.^[29–46]

Keywords: Acyltransferase · Amine · Biocatalysis · Carbamate · Esterase

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