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Dehydration in water: solid-supported lipases as green catalysts for esterification†

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Catalytic esterification in water has been achieved with the aid of a commercially available polymer-supported lipase, Novozym-435. Remarkably, this well-known biocatalyst demonstrates significant activity towards esterification using just water as the reaction medium. This methodology features representative esterifications of complex acids and alcohols, in the presence of unprotected amines. The work disclosed leading to esters typically requires only two equivalents of alcohol, although conversion for water-soluble alcohols appears to require increased loadings. Recycling of both the aqueous medium and catalyst are documented, highlighting the potential of this new technology, especially using directed evolution on the enzyme involved. Pharmaceutically relevant compounds are efficiently esterified (*e.g.*, Ibuprofen, Tolmetin, and Ticagrelor) and multi-step, one-pot chemoenzymatic sequences can be performed to demonstrate the robustness of this catalytic aqueous system.

Introduction

Dehydration reactions are at the core of a wide variety of chemical industries, and yet, may be among the most waste-generating processes. While the pharmaceutical industry relies significantly on amide bond formation,[1] the $29 billion dollar flavor and fragrance industry[2] depends heavily on syntheses of a wide variety of esters.[1,3] Furthermore, ester functionality is pervasive in functional materials, natural products, and other bioactive molecules.[4,5] Although made less frequently than amides, pharma has also found significant uses for esters, particularly as pro-drugs of NSAIDs or antibiotics. These derivatives of carboxylic acids improve cell permeability and hence, remain an important area of research (Fig. 1).[6]

Linked by their shared loss of water, dehydration reactions rely on several stoichiometric reagents to activate a carboxylic acid towards coupling with the desired nucleophile.[7] Although these reagents, such as DCC, COMU, HATU, *etc*. can be very effective, they generate considerable amounts of organic waste, while some have been identified as dangerous to human health.[8–10] For every equivalent of acid undergoing coupling, a stoichiometric amount of by-product, by definition, is generated as the activating group is lost.[10–12] This issue is compounded by the generally high molecular weights of the activating agents, leading to reagent-based by-products that may exceed the molecular weights of the products.[10–12] Additionally, these by-products of selected coupling reagents can also be explosive; nonetheless, they are still commonly used by several industries.[8,9,13]

To minimize waste formation, as highlighted as the very first among the *12 Principles of Green Chemistry*, coupling reagents have been developed where some component is recoverable and recyclable in efforts to ameliorate the environmental impact over the life-cycle of the reagent.[10–12] While these represent a step forward in terms of greenness, such recycling processes require toxic materials. Other chemical methods *en route* to esters are well known, such as the traditional acid-catalyzed Fischer esterification, while generally efficient, is limited by the strong acid and high temperatures required leading to potential issues of substrate(s) functional group tolerance.[14] Acyl chlorides are also fundamental, but generation of HCl can be problematic.[15]

Additional concerns arise, especially at scale, from toxic chlorinated reagents such as oxalyl chloride or thionyl chloride.[16,17] Moreover, acid halides are notoriously moisture-sensitive, as are the more commonly used chlorinating reagents.

In light of these routinely faced aspects associated with esterification, a search for the greenest (and therefore, safest)

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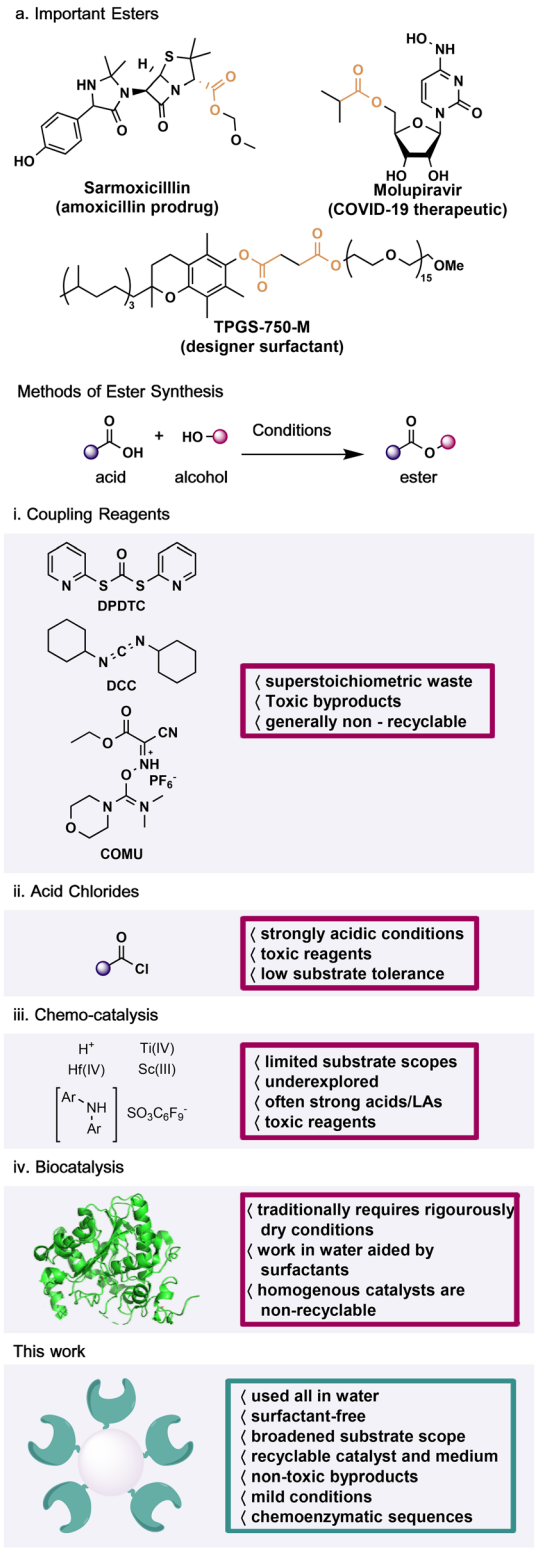

Fig. 1 Methods of making esters.

solution involving catalysis directly from partner acids and alcohols seems long overdue. While a variety of alternative catalysts have been developed, they typically rely on strong Lewis acids or solid Brønsted acids to activate the carboxylic acid.[18–20] Other modes of catalysis have recently been explored, including electro-catalytic esterification reported by Nacsa, *et al.*[3] Nonetheless, this space remains perhaps surprisingly under-explored, as no environmentally friendly catalytic system has emerged tackling this problem. A significant part of this challenge is that the solvents typically used for these couplings, *e.g.*, CH_2Cl_2, DMF, *etc.*, are labelled as hazardous, and hence, far from green.[5,21,22] Therefore, timely methodologies for effecting catalytic esterification under environmentally responsible conditions remain sparse, at best.

One alternative option is to consider use of biocatalysis. This is a rapidly growing field due to both the high activity and selectivity characteristic of enzymatic catalysts, done under mild and green conditions.[23–25] Lipases and esterases used in Nature's natural reaction medium, water, may comprise the most widespread and synthetically useful class of enzymes.[26–30] Due to their high thermostability, solvent tolerance, and promiscuous nature, they have found extensive use in the chemical industry,[31–33] where the market was projected to exceed USD $590 million in 2023.[34]

Lipases and esterases natively hydrolyze acyl triglycerides into glycerol and a fatty at the lipid–water interface.[35] These enzymes contain a conserved catalytic triad of serine, histidine, and aspartic or glutamic acid that catalyze this important chemistry.[36,37] The acid and histidine residues serve to increase the nucleophilicity of the catalytic serine. This allows for an efficient nucleophilic attack on the carbonyl substrate (either acid or ester) and subsequent collapse of the tetrahedral intermediate to form an enzyme-bound ester, the acyl-enzyme intermediate. This common intermediate can then be freed from the enzyme by either hydrolysis or alcoholysis to produce the carboxylic acid or ester product (Fig. 2).

Importantly, this cycle is fully reversible and hence, lipases have been used effectively in the reverse, ester-forming direction in organic solvents, where the water being generated must be removed. Typically, dehydrating reagents such as molecular sieves have been used, adding to the overall waste produced by these reactions[38–41]

Much early utility was found in the chiral resolution of alcohols by the hydrolysis of esters.[26–28,34,42,43] Furthermore, lipases grew in popularity due to their organic solvent tolerance, leading to the development of a wide variety of esterifications and amidations of alcohols and amines, respectively.[44] However, although the catalyst is biological in origin, the need for organic solvents renders esterifications, as currently practiced, environmentally egregious.[37–40] Thus, in principle, half of the synthetic value of enzymatic catalysis is lost due to the lack of a green technology for their use for esterification reactions.

Alternative enzymes that have been used to catalyze similar transformations include a variety of promiscuous acyltransferases such as that from *M. smegmatis*, or the Penicillin G-acylases.[45–48] These enzymes have been shown to affect a variety of acyl transfer reactions such as transesterifications and amidations and have been applied at industrial scales.[46] However, the acyl donor is typically some form of active ester,

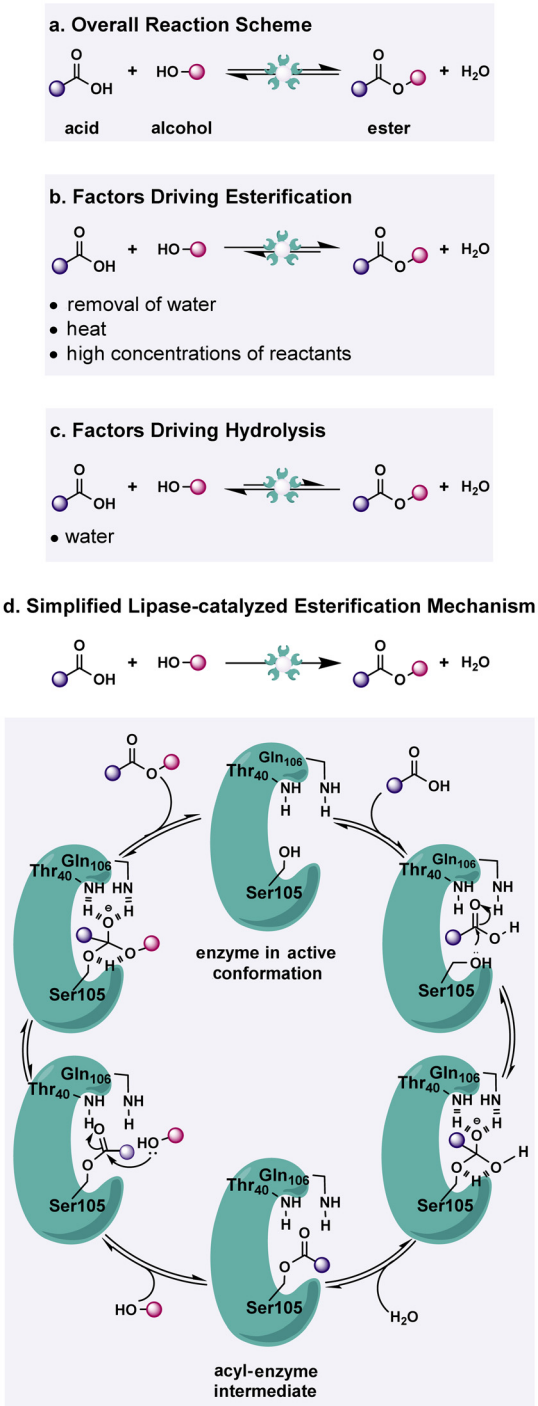

Fig. 2 Mechanistic considerations associated with lipase catalysis.

and rarely are carboxylic acids directly condensed into ester or amide products.

Prior studies from our group described the first such report, using Palatase 20000L (a lipase from *Rhizomucor Miehie*), where catalyzed esterifications could be carried out in water (only).[49] The buffered, aqueous micellar medium containing a designer surfactant (TPGS-750-M)[49,50] was enabling, with low yields observed in its absence. In stark contrast to the typical use of excess of one reactant in most esterification methodologies, these enzyme-catalyzed reactions require only equimolar amounts of acid and alcohol. Unfortunately, however, Palatase 20000L is limited in substrate scope, as two methylene residues as spacers are essential, being located between the carboxylic acid and other functionality further down the chain.

Given that lipases have tremendous value in so many capacities within the chemical enterprise, a study was initiated to develop a new, more general technology, thereby providing lipase-catalyzed esterifications to the fine chemicals industry.

Results and discussion

Initially, the focus was on expanding the substrate scope of the enzymatic esterification by screening commercially available lipases for activity with both phenylacetic and benzoic acids; neither are accepted by Palatase 20000L.[49] Included in the evaluation was a mixture of lyophilized lysates (lipases from *B. cepacia*, *C. Rugosa*, and *R. Niveus*), glycerol suspensions (lipase from *R. miehie*; Palatase 20000L), and *P. Antarctica* (aka *Candida Antartica* Lipase or CALB), along with one immobilized lipase, CALB (sold as Novozym 435). Remarkably, only the heterogeneous forms of CALB, both as a glycerol suspension and as Novozym 435, were catalytically active towards esterification in water of both phenylacetic acid and the significantly more challenging benzoic acid (Fig. 3).

Interestingly, immobilized CALB afforded significantly higher yields than the glycerol suspension at the same enzyme loading (normalized by catalytically active units). Immobilization was then conducted with other lipases using the same polymethylmethacrylate (PMMA) resin used in the production of Novozym 435. Subsequent screening confirmed the previous finding that only CALB was active toward esterification of both acids. These observations led to the choice of the ubiquitous, commercially available, solid-supported Novozym 435 as catalyst for further study.

The nature of the reaction medium was then determined. Based on previous studies, a noticeable surfactant effect had been observed, where nonionic amphiphiles such as TPGS-750-M in a buffered aqueous medium were found to enhance the extent of lipase-catalyzed esterification.[49] While this effect was consistent between mixtures of either solutions of Palatase 20000L or CALB, no surfactant effect was observed in any of these esterifications using the immobilized lipase.

These observations support an interfacial activation effect of surfactants with lipases and eliminates the reservoir effect observed with other classes of enzymes.[51–54] As previously discussed, lipases hydrolyze triglycerides into glycerol and fatty acids at the lipid–water interface.[26] This interface is typically a lipid that self-assembles into a micelle-like array with the fatty acid chains forming the inner cores. Lipases share the α/β-hydrolase fold motif with serine proteases, which have a defined α-helical "lid" structure over the active site.[54] It is the movement, or opening, of this "lid" that is commonly

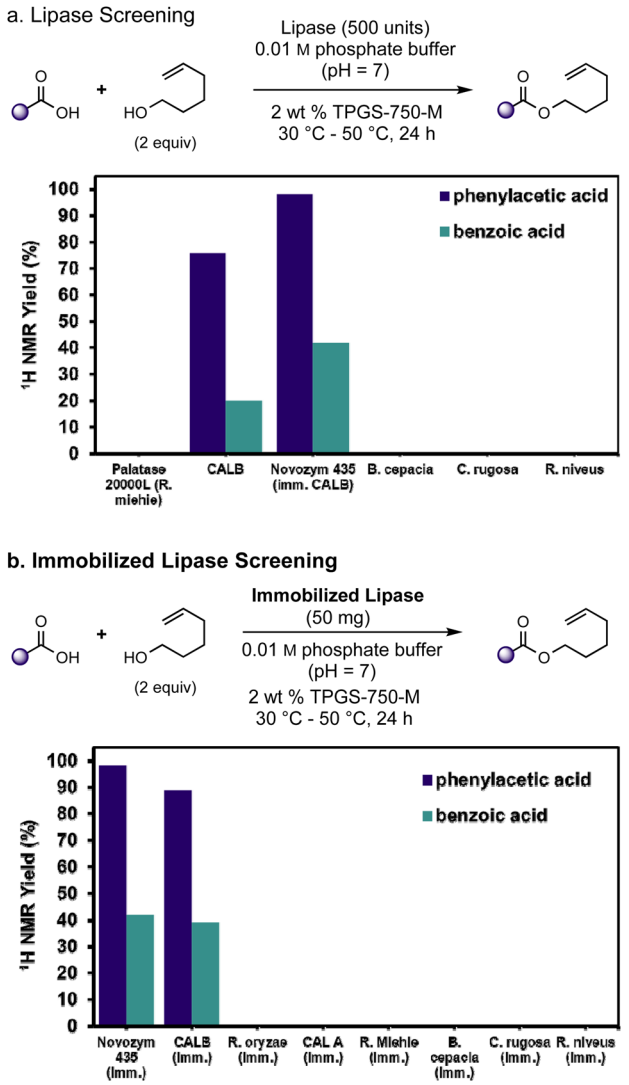

Fig. 3 Results from screening various lipases.

described as key to the mechanism of interfacial activation in lipases.[55] Even CALB, with its small lid structure, has been shown to benefit from immobilization, as demonstrated by the myriad industrial applications of Novozym 435.[53]

The lack of dependence on the surfactant in the esterification yield using Novozym 435 is consistent, where immobilization 'pre-activates' it on the surface of the polymer.[56] The supported enzyme, therefore, does not require the presence of a surfactant to become catalytically active. Equally surprising was the lack of a "reservoir effect" when the surfactant was removed. In our prior work, we hypothesized that the significantly more lipophilic ester products would be more sequestered in the surfactant micelles than their respective acid and alcohol starting materials, thus protecting them from hydrolysis by the enzyme. The absence of this effect disproves this hypothesis. Furthermore, the lack of buffering salts in the medium was also a welcomed finding, the implication being that the optimized reaction medium is just deionized water (Fig. 4).

Optimization of esterification conditions led to important mechanistic understandings of these reactions. Screening of yield *versus* equivalents of alcohol showed that for lipophilic alkanols, three equivalents led to complete consumption over *ca.* 16 hours (Fig. 5). In the case of water-soluble alcohols, including methanol and ethanol, a strong correlation between concentration dependence and yield was observed. The difference between use of one and ten equivalents of alcohol led to a dramatic increase in yield; on the order of 4 or 5 times than that obtained using a 1 : 1 ratio.

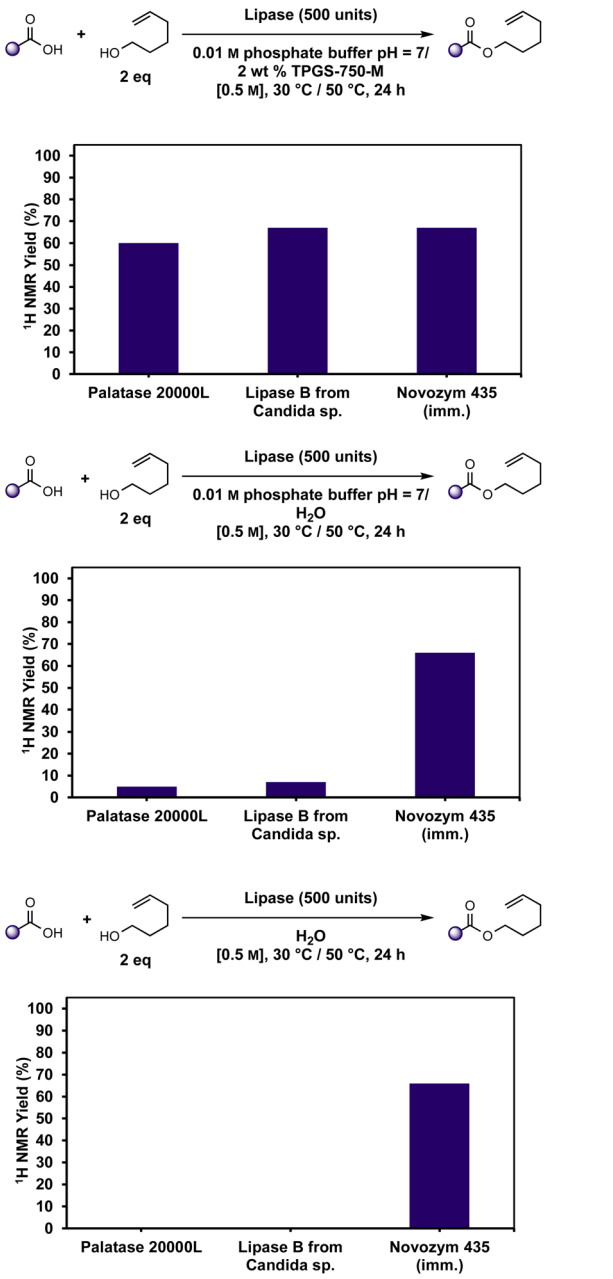

Fig. 4 Optimization of the reaction medium.

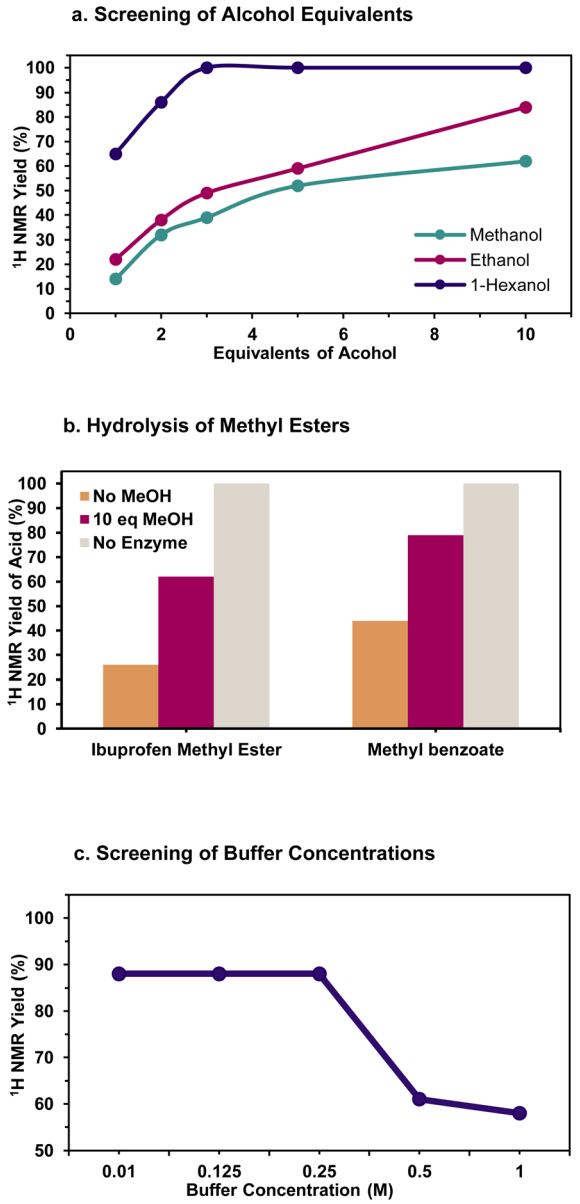

Fig. 5 Factors affecting product conversion.

A hydrolysis experiment further supported this conclusion where the degree of hydrolysis of Ibuprofen methyl ester and methyl benzoate was greatly inhibited by the presence of 10 equivalents of methanol. Importantly, control experiments showed that these hydrolyses were enzyme-mediated, as they did not proceed in the absence of immobilized CALB. This product hydrolysis is consistent with the current understanding of enzymatic "reverse hydrolysis" being under kinetic control. As the acyl-enzyme intermediate is common to both the synthesis and hydrolysis reactions, product hydrolysis is to be expected. At any given time, the ratio of product to starting material is determined by the kinetic synthesis/hydrolysis ratio, and eventually the system with return to thermodynamic equilibrium (even if it was not reached in the timescale of this experiment).[45]

Another key parameter investigated was the pH dependence of these reactions. A screening of buffer concentration was carried out, affording interesting results. Using the same potassium phosphate buffer (pH = 7) as had been used in our previous work,[49] increasing its concentration up to 0.5 M showed no effect on yield. However, at concentrations ≥0.5 M, the observed yield dropped significantly. As this concentration was equal to the acid concentration, much of the acid is deprotonated, with the resulting carboxylate being an insufficiently competent electrophile for the intended catalysis. When the buffer was excluded and the reaction pH measured at the start and end; the pH had increased from 3.88 to *ca.* 6. Running the model esterification in a 1 M phosphate/citrate buffer at pH = 3.88 led to no change in yield, confirming that the immobilized enzyme is more competent at lower pH. Ultimately, the observed consistency in yields suggested that the buffer be completely excluded from the reaction medium. This is unlike most enzymatic reactions, which require the presence of a buffer for correct folding of the protein. Here, the fixed conformation of the lipase due to immobilization likely obviates this. Use of plain water to the exclusion of any buffer in the medium represents a key strength of this methodology, as it could exclude otherwise expensive downstream wastewater processing.[57]

The final parameter of consequence in this optimization study was concentration. A drawback associated with several enzymatic processes is that they typically require low concentrations, as many synthetically relevant substrates have low solubility in aqueous media.[58] Based on the hydrolytic experiments, higher concentrations should favor esterification, as lowering the concentration of water present should favor alcohol access to the acyl-enzyme intermediate, and thus product formation. A simple study varying concentration supported this notion, and a 1 M reaction concentration was ultimately chosen. On larger scales, concentration can presumably be further increased, but due to the volume occupied by the beads in the 4 mL vials used, 1 M global concentration afforded enough solvent to enable uniform stirring of the reaction.

With general conditions optimized, a screening for substrates was undertaken (Fig. 6). Although linear, primary alcohols gave the highest yields, allylic and propargylic alcohols were also isolated in moderate-to-good yields.[59] Substituted benzyl alcohols were also generally well-tolerated, with 3-bromobenzyl alcohol providing ester **1** in high yield. 4-Chloro-substitution led to moderate yields (**11**), while *ortho*-substitution on the benzylic alcohol was deleterious to the reaction (*e.g.*, the 2-fluoro case giving only 17% ester **2**. An important improvement over our previously reported methodology with Palatase-20000L was that methyl and ethyl esters of a variety of substrates were both realizable.[49] although 10 equivalents of these water-soluble alcohols were required for good yield.

Alcohols containing other heteroatoms were also accepted by CALB without additives, unlike previous observations using Palatase 20000L.[49] 2-(3-Thiophenyl)ethanol was efficiently esterified to product **20**. Noteworthy are cases in which atypi-

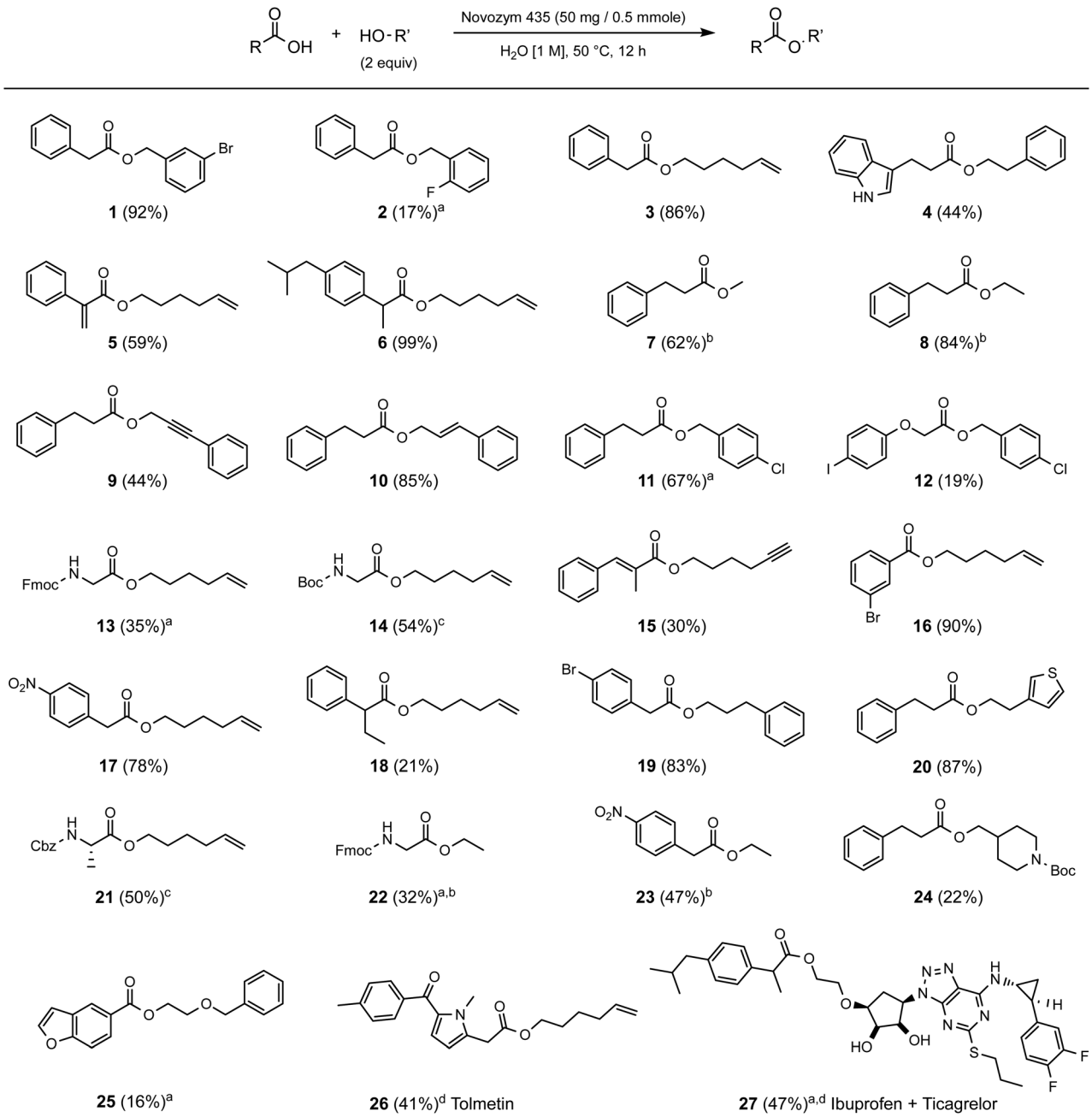

Fig. 6 Substrate scope. [a] 2 wt% TPGS-750-M/H_2O used as reaction medium, [b] 10 equiv. alcohol used. [c] 4 equiv. alcohol used. [d] 100 μL of DMSO used as co-solvent.

cal, nitrogen-containing alcohols were also tolerated (sometimes with decreased yields). Thus, esters bearing a *p*-nitro group (as in **17** and **23**), amino acid derivatives (see **13**, **14**, **21**, and **22**), and a protected piperidine-methanol (as in product **24**) were all accepted by the enzyme. 4-Aminobenzyl alcohol was accepted as a substrate by this immobilized enzyme using two equivalents of carboxylic acid, thereby maintaining an acidic pH. Although no amidation was observed, the product rapidly decomposed upon attempted purification. *N*-Protection of this alcohol would likely result in higher yields as the aniline is both less stable and significantly more water soluble under these acidic conditions, leading to an unfavorable reaction as the substrate is less accessible to the enzyme.

Compared to our previous report, major strides have been made in diversifying the scope of the participating carboxylic acid. Several phenylacetic acids can now be efficiently esterified with a variety of alcohols. Importantly, their α-substitution is well tolerated, with near quantitative yields of ibuprofen hexenyl ester (**6**) being obtained under these heterogeneous conditions. Additionally, α-ethylphenylacetic acid is also toler-

ated, although the isolated yield of product **18** is significantly decreased due to the steric bulk of the ethyl group. Likewise, rigid (unsaturated) substitution at the α-position proved to be a consistent steric factor for immobilized CALB, as both 1-phenylacrylic and cinnamic acids gave lowered yields of esters **5** and **15**, respectively. By contrast, α-methyl phenylacetic acids were easily accepted with Ibuprofen ester **6** giving near-quantitative yields. Furthermore, substitution at the 4-position of the phenyl ring is well accepted by this enzyme.

Surprisingly, 3-bromobenzoic acid proved to be a high yielding substrate leading to product **16**, although 4-methylbenzoic acid failed to yield the corresponding ester to any degree. This may be due to the orientation of the phenyl group in the acyl-binding pocket of the enzyme. A benzofuran carboxylic acid was also esterified to product **24** in low yield, indicative of the capacity of the enzyme to accept some more bulky functionality. Fmoc- and Cbz-protected glycine and alanine could also be esterified in moderate yields; however, the steric bulk of the α-substituent limits the utility of this particular enzyme, as no other amino acids were accepted.

Substrate mapping of this enzyme being used in its immobilized state in pure water was made all-the-more complex, since some highly functionalized drug molecules could also be used as substrates together with a lipophilic ester (*e.g.*, of the NSAID Tolmetin) leading to product (**26**) of esterification in 41% yield. In addition, an ester (**27**) between Ibuprofen and the blood thinner Ticagrelor was also formed in 47% isolated yield. Since both components are crystalline solids, a small amount of DMSO was used to initially break up the crystal lattice, while TPGS-750-M (2 wt%) was added to the medium to aid with stirring. Although the presence of surfactant was not shown to affect yield, it was found that it aided in homogenizing certain substrates, leading to higher yields.

Scalability of the reaction was tested utilizing 7.5 mmol (1.021 g) of phenylacetic acid together with 5-hexen-1-ol (2 equiv., 1 M, H_2O, 55 °C, 12 h) to afford the desired ester (**3**) in 90% isolated yield (Fig. 7).

Given the anticipated stability of this immobilized CALB, its recyclability was tested, as shown in Fig. 8. An extractive workup using the green solvent 2-methyl THF[21] allowed for recycling of both the catalyst and medium three times, without significant loss of yield.[60] Simple filtration of the crude material over silica gave pure product, which translated to an average process mass intensity (PMI) of just 40.8 over three recycles, with most of the waste coming from purification

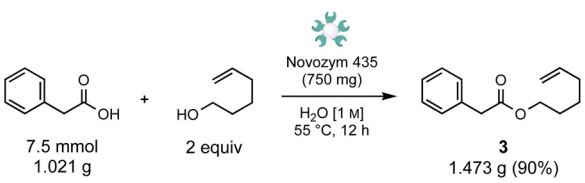

Fig. 7 Gram scale reaction.

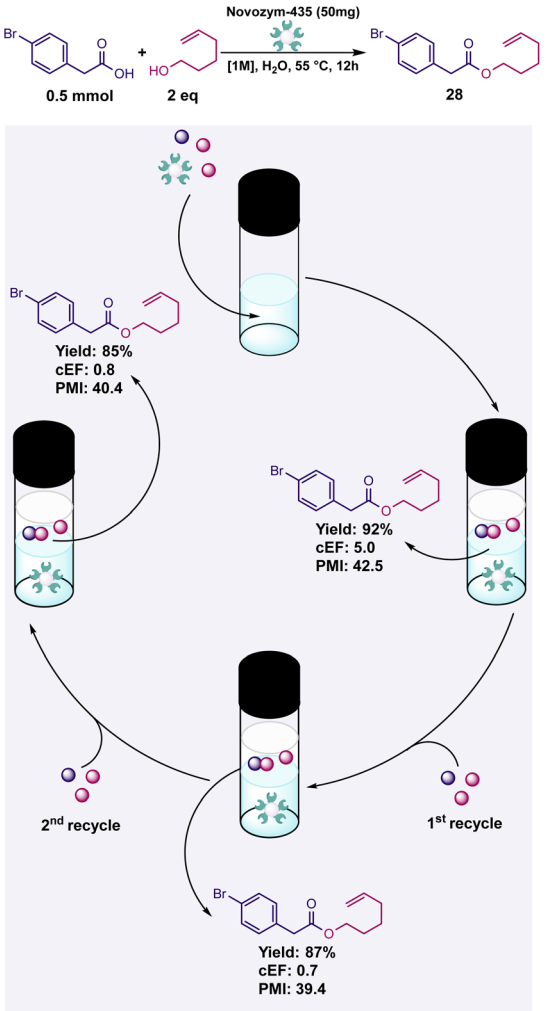

Fig. 8 Recycling study.

(which varies as a function of product). Another green metric, the complete E-factor (cEF),[61] describes the ratio of mass inputs to product output calculated for the reactions. For the initial reaction, this was calculated to be 5.03 while for each of the recycles this was just 0.7 and 0.8 each. By contrast, work by Sneddon, *et al.*[5] at GSK showed that for Steglich-type esterifications, PMIs were between 173 and 2043, which are orders of magnitude higher, likely due to the simple workup and high concentrations of our methodology.

The esters created were also amenable to telescoping into multi-step chemoenzymatic syntheses. In one three-step, two-pot sequence (Fig. 9A), 4-bromophenylacetic acid and 5-hexen-1-ol were esterified at 65 °C to form ester **30**. This increased temperature was critical as it allowed for the complete conversion of the carboxylic acid to prevent remaining acid from poisoning further palladium catalysts. This ester was then extracted three times with 2-methyl THF as in the recycling study and dried into another vial.

The catalyst beads were filtered off to be re-used and the aqueous layer was then added back to the crude ester. The

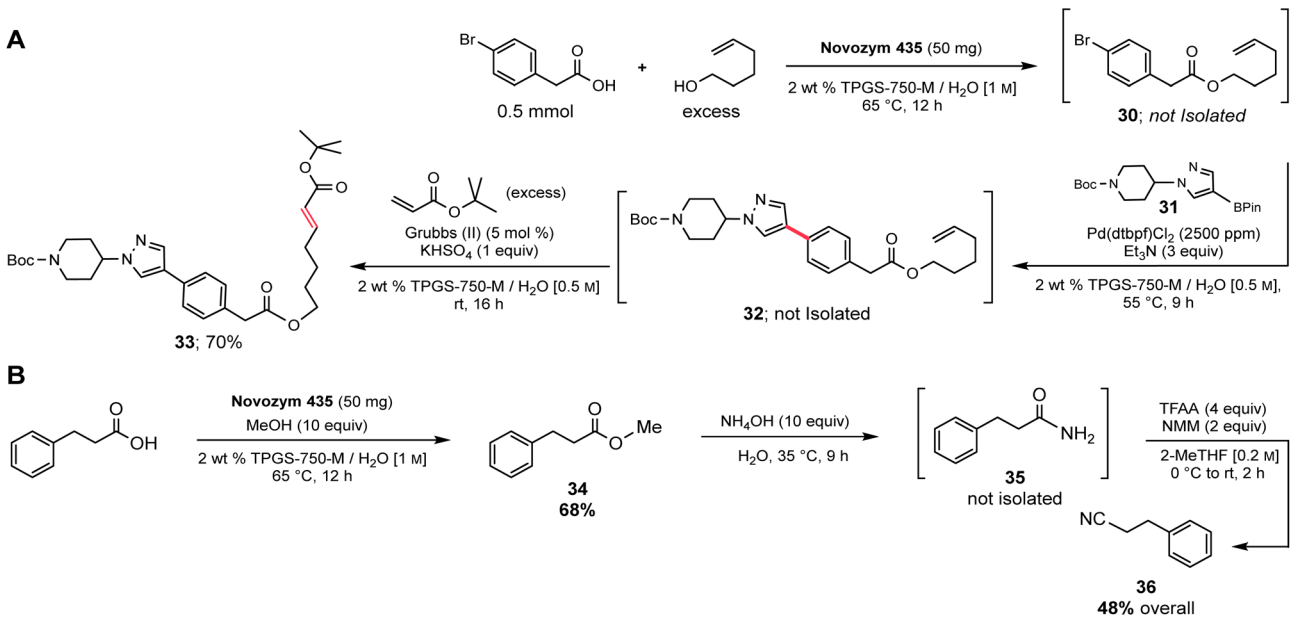

Fig. 9 Telescoped sequences using our esterification methodology.

volume was adjusted to give a global concentration of 0.5 M and the whole vial was sparged with argon for 15 minutes to remove dissolved oxygen. Boronic ester **31** (a precursor Pfizer's non-small cell lung cancer drug crizotinib) was added, followed by triethylamine and just 2500 ppm of $Pd(dtbpf)Cl_2$ as catalyst for the Suzuki–Miyaura coupling to yield product **32**. Upon completion, $KHSO_4$ was added followed by 4 mol% of Grubbs-2 catalyst and 10 equivalents of *t*-butyl acrylate, in sequence, and the reaction left to stir at room temperature overnight to give product **33** in 70% isolated yield.

In another sequence (Fig 9B), a methyl ester of phenylpropionic acid was synthesized using our enzymatic methodology. The isolated product ester was then stirred at room temperature with 10 equivalents of 40 wt% aqueous ammonium hydroxide to yield primary amide **35**. This crude primary amide could then be extracted and azeotropically dried with toluene before being dehydrated to form nitrile **36** in 48% overall yield.

Conclusions

A more general, scalable, and green technology has been developed involving a lipase-catalyzed esterification in water. For the first time, the polymer-supported, commercially available lipase, Novozym 435, was found to effectively esterify a variety of functionalized carboxylic acids and alcohols with no organic solvent or dehydrating agents present.

The catalyst also shows significant improvements over past methodologies with substituted phenylacetic and benzoic acids being effectively converted into their product esters. Furthermore, small, water soluble alcohols like methanol and ethanol were esterified for the first time in an aqueous medium. The catalyst also showed unprecedented reactivity allowing for the esterification of the API's Ticagrelor, Tolmetin and Ibuprofen.

Importantly, these esterifications are co-factor free, making both the catalyst and medium easily recyclable. This methodology provides justification for practical esterifications to be carried out on scale, especially after applying an element of directed evolution to broaden enzyme tolerance. The heterogenous nature of the catalyst, also allows, for the first time, a recycling of the enzymatic catalyst and aqueous medium without significant loss of yield.

Thus, the key advances in terms of both synthesis and environmentally responsible chemistry being reported include:

- use of a solid-supported, commercially available enzyme-based catalyst;
- recyclable catalyst and medium;
- reactions performed in just water; no surfactant or buffer required.
- a variety of alcohols, both simple and complex, as coupling partners;
- dramatic improvement, and extension, of substrate scope over prior art.

These reactions also document the prospects for potentially converting some of the more challenging esterifications to environmentally attractive processes. We anticipate expanding this platform in efforts to tackle a variety of related enzymatic dehydrations known to be of great value to the chemical industry.

Author contributions

R. M. T. conceptualized the project and performed optimizations and compound synthesis and characterization and

directed D. B. O., K. G. and M. L. L in their contributions. R. M. T also drafted the manuscript and compiled the ESI.† M. L. L., K. G., and D. B. O. performed experiments and assisted in compiling the SI. Y. Y. provided guidance and advice and manuscript edits. B. H. L. directed the research and assisted in manuscript preparation and editing.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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