Advanced Synthesis & Catalysis

Accepted Article

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To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.201901434

Link to VoR: http://dx.doi.org/10.1002/adsc.201901434

10.1002/adsc.201901434

FULL PAPER

DOI: 10.1002/adsc.201((will be filled in by the editorial staff))

Application of Transaminases in a Disperse System for the Bioamination of Hydrophobic Substrates

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Received: ((will be filled in by the editorial staff))

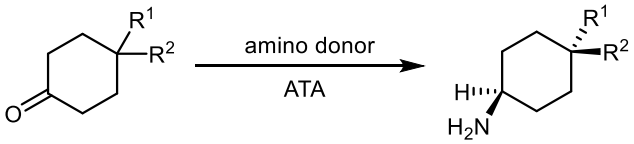
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. The challenging bioamination of hydrophobic substrates has been attained through the employment of a disperse system consisting of a combination of a low polarity solvent (e.g. isooctane or methyl-*tert*-butylether), a non-ionic surfactant and a minimal amount of water. In these conditions, amine transaminases (ATA) were shown to efficiently carry out the reductive amination of variously substituted cyclohexanones, providing good conversions often coupled with a superior stereoselectivity if compared with the corresponding chemical reductive amination. An array of synthetically useful 4-substituted aminocyclohexanes was consequentially synthesized through biocatalysis, analyzed and stereochemically characterized.

Keywords: biotransformations; amination; multiphase catalysis; enzyme catalysis; diastereoselectivity; chemoenzymatic synthesis

Introduction

4-Substituted cyclohexylamines are important building blocks in medicinal chemistry.[1] A highly selective and general synthesis methodology for this class of compounds is therefore desired. Here, a biocatalytic approach for the stereoselective conversion of 4,4-disubstituted cyclohexanones into the corresponding amines using amine transaminases (ATAs, EC 2.6.1.x) as biocatalysts (Scheme 1) is presented. The reactions are performed in a non-conventional disperse system in order to overcome the solubility issues associated with such hydrophobic substrates.



Scheme 1. Biocatalytic reductive amination of achiral cyclohexanone derivatives.

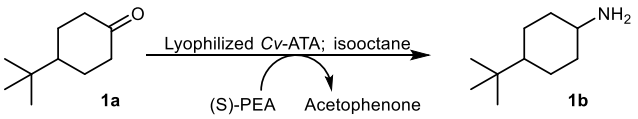
The reactions catalyzed by ATAs are commonly carried out in aqueous media, often in combination with a minor amount of hydrophilic solvents (e.g. DMSO) when an increase in solubility of a hydrophobic substrate is sought. In contrast, there are only a few reports covering the application of ATAs in pure organic solvents.[2] The first ATA successfully employed in a substantial amount of organic solvent (i.e. 50 % DMSO) was reported in 2010 for the synthesis of sitagliptin,[3] where the immobilization of the enzyme permitted even the employment of neat organic solvents.[4] Since then, a number of reports on immobilized transaminases have been published.[5] However, the employment of immobilized ATAs for synthetic purposes is far from being of wide use. The main drawbacks may be related to mass transfer problems or to the scarce solubility of the cofactor (pyridoxal 5'-phosphate, PLP) in organic solvents.[6]

Recently, the application of some lyophilized ATAs in ethyl acetate and methyl-*tert*-butyl ether (MTBE) was shown.[2a] Increased stability of the enzyme and the absence of substrate inhibition were underlined. The key factor of this work is the activity of water in the organic medium; this is in line with the generally accepted mechanism of transamination,[7] where a water molecule is involved in the catalytic cycle. The same authors also report on the use of this approach in the stereoselective synthesis of valinol,[8] and for 3-substituted cyclohexylamine derivatives.[9] The use of surfactants (i.e. Brij® C10 and octyl β-D-glucopyranoside) as an

additive prior to the lyophilization of the enzyme for employment in organic solvent was recently reported for the biocatalytic reductive amination of methoxyacetone. In that study, the ATA from *Chromobacterium violaceum* (*Cv*-ATA) was applied as a suspension of surfactant-stabilized lyophilized powder for the stereoselective conversion of methoxyacetone to the corresponding amine in dry isooctane employing (S)-phenylethylamine ((*S*)-PEA) as amino donor (Scheme 2).[10] Unfortunately, this approach led to unsatisfactory results when applied to 4-substituted-cyclohexanones, in particular in terms of reaction rate. In order to address this issue, a novel approach for the application of ATAs in organic solvents was developed. In this procedure, an aqueous solution of the enzyme, a non-ionic surfactant, the substrate, and an appropriate organic solvent are mixed to form a macroscopically homogeneous system. Such procedure was successfully applied to the reductive amination of a library of hydrophobic 4,4-disubstituted cyclohexanones using different ATAs, and led to interesting results also in terms of stereoselectivity.

Results and Discussion

At first, following the procedure described by Chen et al.,[10] *Cv*-ATA was employed as a surfactant-stabilized powder in dry isooctane at 37 °C using 4-*tert*-butyl-cyclohexanone (**1a**) as a model substrate (Scheme 2). This resulted in a very low reaction rate, and only 40 % conversion was observed after 8 days. On the other hand, the diastereomeric ratio (24/76 *cis/trans*) of the newly formed amine was the opposite with respect to the product obtained through metal-mediated reductive amination.[11]



Scheme 2. Model reaction: bioamination of 4-*tert*-butylcyclohexanone (**1a**).

A MS analysis of the crude reaction mixture showed that (*S*)-PEA spontaneously reacted with the substrate giving the corresponding Schiff base as a by-product. This reaction is reasonably favored by the low water activity in the reaction media. Despite the reversibility of the Schiff base formation, the depletion of the ketone from the mixture is probably responsible for the reduction of the reaction rate and, consequently, of the overall conversion. In order to increase the reaction efficiency, the reaction parameters were tuned, in particular focusing on the lyophilization time and the water activity in the solvent.[12] Indeed, an increase of the freeze-drying time brought some beneficial effects in terms of catalyst activity (see SI), even if the reaction rate still was too low to be of practical use. Since the water activity in the organic solvent generally plays a major role in the enzyme performance,[2a] the effect of small additions of water into the reaction mixture was explored and established to have, indeed, some beneficial effects on both the reaction rate and the final conversion, also by preventing the imine formation, as demonstrated by GC-MS (see SI). However, the high hydrophobicity of isooctane allows the addition of only a tiny amount of water before a new liquid phase appears (water activity reaches 1), preventing an efficient mixing of the components and complicating the downstream processing.

As an alternative approach stemming from the same components, a ternary reaction mixture containing a hydrophobic organic solvent, a small amount of water and an appropriate surfactant, with the aim to generate amphiphilic supramolecular assemblies was designed. Such systems have been extensively described in the 1980s by Luisi and coworkers,[13] and are based on the formation of reverse micelles encapsulating the enzyme. Thus, a water-in-oil disperse environment suitable for ATAs was designed and employed. According to literature, in this work, we refer to *micelles* (oil in water) and *reverse micelles* (water in oil) when the assemblies dimensions are around 5-10 nm, *microemulsions* when droplets dimensions are 10-100 nm and *emulsions* when the droplets size ranges 0.5-200 mm.[14] Briefly, a non-ionic surfactant (*e.g.* Polyethylene-glycolhexadecyl-ether, Brij® C10) is at first dissolved in a hydrophobic organic solvent (isooctane or MTBE) together with the substrates at 37 °C. After complete dissolution, a small amount of aqueous enzymatic preparation (1 % v/v) was added to the reaction mixture and then vigorously shaken. In order to find suitable reaction conditions for screening our library of ketones, a model transamination system was explored with the use of **1a** (25 mM), (*S*)-PEA, Brij® C10 (as surfactant) and *Cv*-ATA (0.95 U) as biocatalyst. Through a preliminary characterization of the system, the following parameters were evaluated: donor/acceptor ratio, nature of organic solvent and surfactant, reaction temperature, nature of amino donor.

When the reaction solvent is isooctane and the surfactant employed is Brij® C10 (50 mM) at 37 °C the reaction mixture is perfectly clear and transparent. The addition of 1.0 % v/v of the aqueous enzymatic preparation, upon vigorous shaking, turns the mixture into a macroscopically homogeneous milky emulsion. This mixture is stable as long as the necessary agitation is provided. Interruption of the shaking leads to phase separation and to a drastic slowdown

of the conversion rate. On the other hand, when employing no more than 0.2 % v/v of water, a stable, transparent and clear solution is obtained. In this case, we assume that such a configuration may be considered as a micellar system, where the enzyme solution is entrapped in thermodynamically stable reverse micelles. Such a hypothesis is confirmed by dynamic light scattering (DLS) experiments (Figure 1, green peaks), which show that the reaction system is composed of a homogeneous population of particles with a mean hydrodynamic radius of 16 nm.

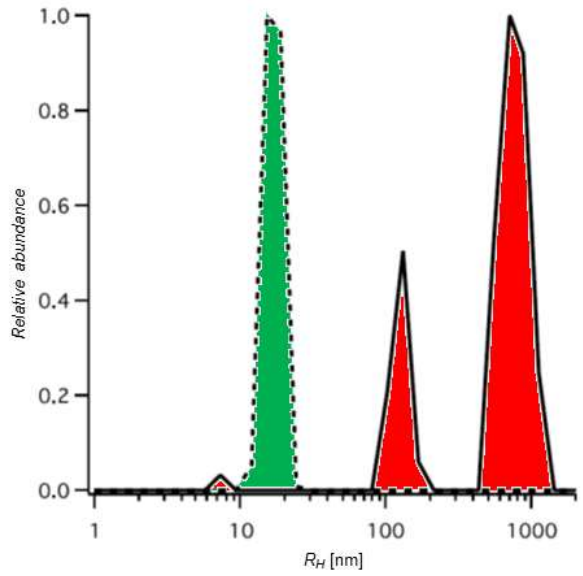


Figure 1. Dynamic light scattering showing the mean hydrodynamic radius distributions of the particles obtained using 50 mM of Brij® C10 in isooctane at 37 °C. Dashed line, green peaks: 0.2 % v/v of aqueous enzymatic preparation. Continuous lines, read peaks: 1.0 % of aqueous enzymatic preparation.

This is consistent with the hypothesis of a solution containing spherical inverse micelles with a confined aqueous phase encapsulating the enzyme, as illustrated in the literature.[13] An increase of the buffer concentration leads to a destabilization of the micelles, which coalesce in droplets and form an emulsion stabilized by the surfactant (Figure 1, red peaks). When isooctane is substituted with MTBE, DLS analysis indicates the presence of two supramolecular populations, the former on a nanometric scale and the latter in the range of hundreds of nm, even employing a small amount (0.2 % v/v) of aqueous solution (data shown in SI). According to the aforesaid definitions, the system should be in this case described as an emulsion, even though the mixture looks macroscopically like a transparent and clear solution. The different assemblies are summarized in Table 1

Table 1. Effect of water concentration on the system self-assembly. Micelles shape is only based on the measured hydrodynamic radius. Conditions: 25 mM ketone **1a**; 50 mM (*S*)-PEA; 50 mM decane; 50 mM Brij® C10. A proper amount of Hepes buffer pH 8.2, 50 mM, with or without 13 mg/mL *Cv*-ATA.

Water concentration *v/v* [%]	Enzyme	Solvent	
		isooctane	MTBE
0	No	Spheric micelles	Oblong micelles
0.2	No	Spheric micelles	Stable emulsion
0.2	Yes	Spheric micelles	Stable emulsion
1.0	Yes	Stable emulsion	Unstable emulsion

In order to evaluate the performance of this system, the conversion of **1a** to the corresponding amine was studied. Two different experiments were carried out employing the same amount of enzyme (0.13 mg, 0.82 U) diluted in order to obtain a final concentration of the aqueous solution of 1.0 and 0.2 % v/v, respectively. A third experiment was performed using 1.0 % v/v of the enzymatic preparation at a higher concentration (0.45 mg, 2.8 U).

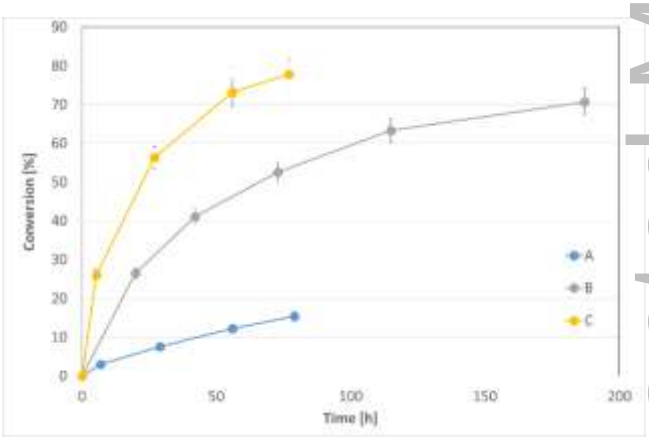


Figure 2. Effect of water content. A. 0.2 % v/v of buffer containing 0.82 U *Cv*-ATA. B. 1.0 % v/v of buffer containing 0.95 U *Cv*-ATA. C. 1.0 % v/v of buffer containing 2.8 U *Cv*-ATA. Reaction conditions: 25 mM ketone **1a**; 50 mM (S)-PEA; 50 mM decane; 50 mM Brij® C10; enzyme preparation in HEPES buffer (pH 8.2, 50 mM); isooctane to final volume 1 mL.

The results are reported in Figure 2. According to these data, the emulsion systems possess better performances when compared to the corresponding micellar systems. Moreover, as expected, employing about 3.5 times more enzyme leads to an approximately three-fold faster reaction.

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Another interesting aspect is that the reaction workup is quick and easy if compared to a standard extractive protocol. By just centrifuging the cooled (4 °C) reaction mixture it is possible to separate the organic phase, containing substrates and products, from the solids, composed of salts, enzyme, cofactor and surfactant.

The stability of *Cv*-ATA in the studied reaction medium is remarkably high: the enzyme retains its activity all along the explored reaction time (187 h, > 1 week). The addition of a different substrate, i.e. methoxyacetone, to the solution after reaching the conversion plateau immediately reactivates the reaction: a further aliquot of (*S*)-PEA is consumed and the corresponding amount of acetophenone is released (Figure 3). This also demonstrates that the conversion observed at the end of the reaction depends on the assessment of the thermodynamic equilibrium and is not due to enzyme inactivation.

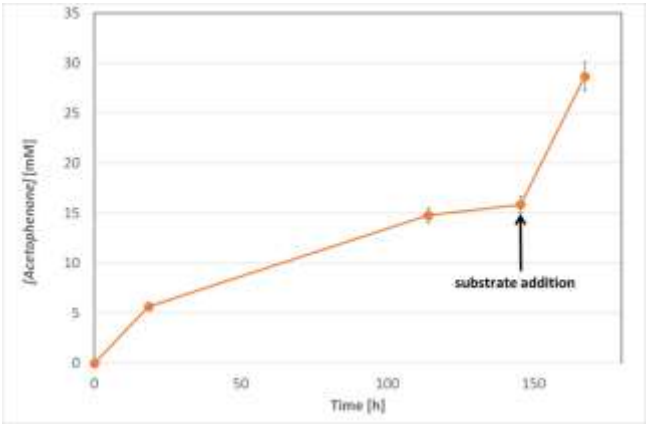


Figure 3. Stability of the enzymatic preparation: Addition of methoxyacetone (25 mM) after 144 h of reaction. Reaction conditions: 25 mM ketone **1a**; 50 mM *(S)*-PEA; 50 mM decane; 50 mM Brij® C10; 10 µL enzyme preparation (0.95 U) in HEPES buffer (pH 8.2, 50 mM); isooctane to a final volume of 1 mL.

Figure 4 reports the results of a set of experiments aimed at investigating the influence and proper ratio between amino donor and amino acceptor. These experiments were carried out with a fixed concentration of **1a**, while the concentration of (*S*)-PEA was varied. Apparently, while a slight excess of the amino donor has a positive effect, higher concentrations of (*S*)-PEA lead to significant inhibition. However, for the purpose of screening the ketones library (see later), 50 mM of (*S*)-PEA was applied to push the thermodynamic equilibrium towards the product.[15] At the same time, experiments were performed using different amino donors, in particular benzylamine and isopropylamine at different concentrations. Although both amines are known in the literature for being accepted as amino donors by *Cv*-ATA,[16] no conversion was observed in all cases.

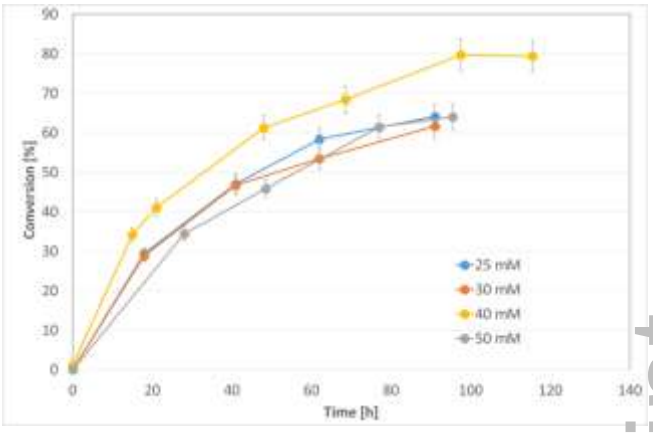


Figure 4. Influence on conversion by the ratio between ketone **1a** and amino donor. Reaction conditions: 50 mM *(S)*-PEA; 50 mM decane; 50 mM Brij® C10; 10 µL of *Cv*-ATA preparation (0.95 U) in HEPES buffer (pH 8.2, 50 mM); 37 °C; isooctane to a final volume of 1 mL.

Next, different solvents; MTBE, isooctane, ethyl acetate (AcOEt), cyclohexane, chloroform, toluene and dimethoxyethane (DME) were explored. Among them, only DME is miscible with water. In our experiments, isooctane allowed the highest conversions. Good results were also obtained using MTBE and cyclohexane, while ethyl acetate was less efficient (Figure 5).

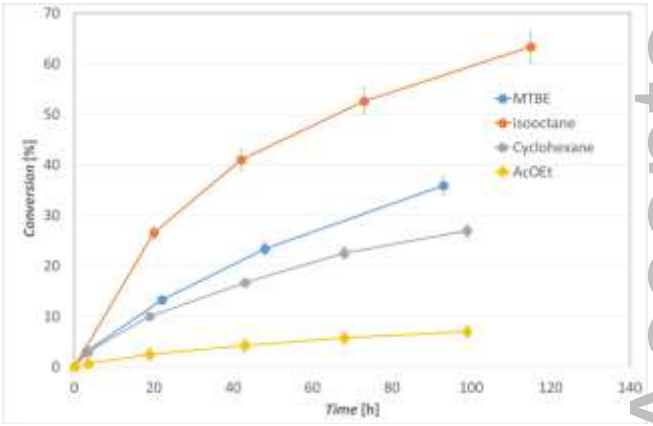


Figure 5. Influence of organic solvent. Reaction conditions: 25 mM ketone **1a**; 50 mM (S)-PEA; 50 mM decane; 50 mM Brij c10; 10 µL enzyme preparation (0.95 U) in HEPES buffer (pH 8.2, 50 mM); organic solvent to a final volume of 1 mL; 37°C.

No conversion was detected using either chloroform, toluene or DME. We point out that this solvent screening was focused on improving the

activity of *Cv*-ATA, and the solvent tolerability may differ for other ATAs. Moreover, the identity of each organic solvent might also affect the final position of the reaction equilibrium.

After selecting the best performing organic solvent, the model reaction was evaluated in isooctane at different temperatures (see SI). The highest conversions were obtained at 37 °C. Temperatures lower than 30 °C lead to the incomplete dissolution of Brij® C10 in the medium, while higher temperatures resulted in lower conversions. Albeit the enzyme in other conditions showed good thermostability,[17] it is nevertheless known that the presence of (*S*)-PEA significantly lowers its melting point.[10, 18] This fact, in combination with the particular environment we describe, might explain the thermal inactivation we observed.

Table 2. Employed surfactants. AOT stands for Aerosol OT, or dioctyl sodium sulfosuccinate.

Surfactant	Type	HLB
Brij® C10	Non-ionic	12.0
Triton™ X-100	Non ionic	13.5
Brij® 58	Non-ionic	15.7
Tween® 20	Non-ionic	16.7
AOT	Ionic	-

Once set up the reaction conditions, five different surfactants were investigated (Table 2, structures in SI). The surfactants were evaluated for the conversion of 25 mM of **1a** to the corresponding amine (Table 3).

Table 3. Effect of surfactant on final conversion [%] (n.c. = no conversion).

Surfactant	*Concentration* [mM]							
	1.0	2.5	5.0	10	25	50	75	100
Brij® C10	< 3	< 3	< 3	< 3	6.5	65	25	
Brij® 58	25	27	21	24	8.1	6.0	3.9	1.5
Triton™ X-100	27		20	22	10	8.2		
Tween 20	22		25	15	6.1	11		
AOT	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.

Reaction conditions: 25 mM ketone 1a; 50 mM (S)-PEA; 50 mM decane; 10 µL of enzyme preparation (0.95 U) in HEPES buffer (pH 8.2, 50 mM); 37 °C; isooctane to a final volume of 1 mL.

The use of Brij® C10, at a concentration of 25 mM or lower, did not result in any detectable conversion. However, a higher concentration (50 mM) of the same surfactant gave acceptable results (65 % conversion). A further increase in concentration is restricted by its solubility in isooctane at 37 °C. Conversely, all the other non-ionic surfactants (Brij® 58, Triton™-X100 and Tween 20) activated the reaction at very low concentrations (1-5 mM), but the achieved conversions were in all cases disappointing (3-27 %). Moreover, any increase in the surfactant concentration led to a decrease in the conversion.

The use of AOT resulted in no conversion in all the performed experiments. Such a result is somehow surprising since AOT is the most common surfactant reported in the literature for enzymatic micellar systems.[13]

In order to explore the versatility of the disperse system, a library of twelve 4-substituted and 4,4-disubstituted cyclohexanones (Figure 6) was selected and employed.

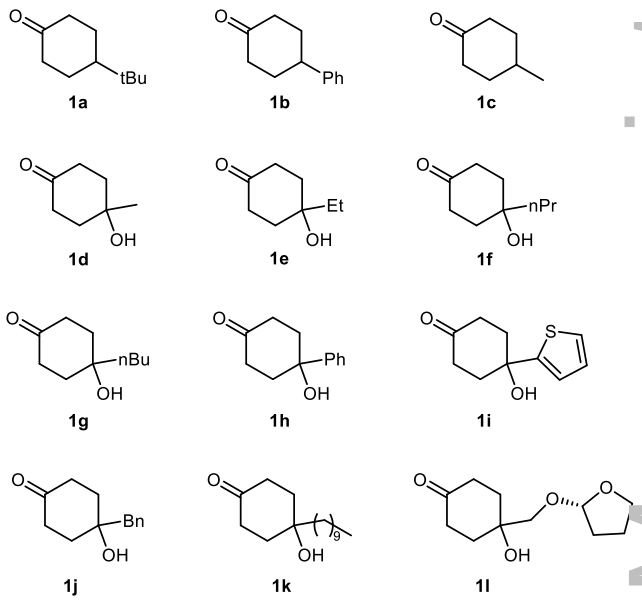
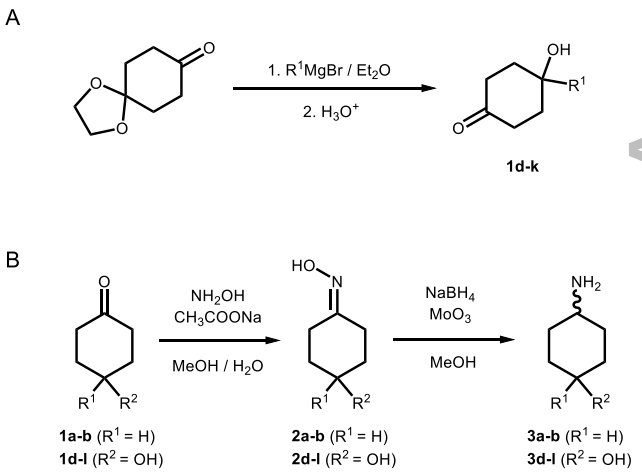


Figure 6. Ketones library.

Ketones **1d-k** were synthesized starting from the commercial precursor 1,4-cyclohexanedione monoethylene acetal (Scheme 3A). The latter is reacted with the appropriate Grignard reagent as the first step.



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Scheme 3. A. Synthesis of ketones **1d-k**. B. Metal-mediated synthesis of the reference amines **3a-k**.

Then, the protective group is easily removed by mild acid hydrolysis, and quantitative yields of the desired ketone (**1d-k**) are obtained after purification by flash chromatography or by distillation. Ketone **1l** is a key intermediate for the synthesis of the antiviral drug radalbuvir (coded as GS-9669),[19] and was kindly provided by Flamma SpA (Chignolo D'Isola, Italy).

All the corresponding amines (**3a-l**) were chemically synthesized through metal-mediated reductive amination in order to be employed as references for the enzymatic reaction (Scheme 3B), with the exception of amine **3c**, which is commercially available as a mixture of *cis/trans*. The ketones (**1a-l**) were reacted with hydroxylamine to form the oximes (**2a-l**), which were in turn reduced using $NaBH_4/MoO_3$ in methanol at 0 °C.[11] After the appropriate workup, the resulting amines (**3a-l**) were pure enough for ^{1}H-NMR characterization. Such reductive aminations result in mixtures of *cis* and *trans* isomers in a ratio varying from 60:40 to 95:5 (Table 4). The two isomers can be distinguished and quantified by ^{1}H-NMR analysis as described in the SI.

Table 4. Observed stereoselectivity for the reduction of the oximes using $NaBH_4$ and MoO_3.

Product	*cis* [%]	*trans* [%]
3a	62	38
3b	59	41
3d	88	12
3e	87	13
3f	88	12
3g	89	11
3h	95	5
3i	77	23
3j	90	10
3k	93	3
3l	85	15

In total, four different enzymes were screened: Wild type *Cv*-ATA,[20] the variant *Cv*-ATA W60C,[21] wild type ATA from *Vibrio fluvialis* (*Vf*-ATA) and ATA 113 (a commercial ATA from Codexis®).

Table 5. Bioamination of 4,4-disubstituted-cyclohexanones: conversions and *cis*/*trans* ratios.

Substrate	Solvent	*Cv*-ATA			*Cv*-ATA-W60			*Vf*-ATA		
		conv. [%]	*cis* [%]	*trans* [%]	*conv.* [%]	*cis* [%]	*trans* [%]	*conv.* [%]	*cis* [%]	*trans* [%]
1a	MTBE	50	98.6	1.4	15	98.7	1.3	3	98.6	1.4
	isooctane	60	98.3	1.7	-	-	-	56	96.8	3.2
1b	MTBE	55	95.1	4.9	79	99.6	0.4	3	98.8	1.2
	isooctane	83	97.6	2.6	-	-	-	80	95.7	4.3
1c	MTBE	45	68.5	31.5	10	0.5	99.5	8	20.7	79.3
	isooctane	70	21.0	79.0	-	-	-	85	37.0	63.0
1d	MTBE	55	24.3	75.7	22	4.0	96.0	10	18.0	82.0
	isooctane	28	12.1	87.9	-	-	-	30	7.1	92.9
1e	MTBE	50	1.7	98.3	21	0.9	99.1	13	7.7	92.3
	isooctane	-	-	-	-	-	-	40	12.0	88.0
1f	MTBE	60	1.5	98.5	26	0.6	99.4	14	1.8	98.2
	isooctane	-	-	-	-	-	-	47	0.3	99.7
1g	MTBE	46	0.4	99.6	15	0.2	99.8	9	< 0.1	> 99.9
	isooctane	-	-	-	-	-	-	50	0.2	99.8
1h	MTBE	58	< 0.1	> 99.9	33	0.6	99.4	3	0.4	99.6
	isooctane	-	-	-	-	-	-	50	0.2	99.8
1i	MTBE	41	77.7	22.3	19	77.5	22.5	10	77.1	22.9
	isooctane	-	-	-	-	-	-	47	77.5	22.5
1j	MTBE	15	0.8	99.2	10	13.8	86.2	3	4.8	95.2
	isooctane	-	-	-	-	-	-	30	1.7	98.3
1k	MTBE	n.c.	-	-	n.c.	-	-	3	0.9	99.1
	isooctane	n.c.	-	-	n.c.	-	-	18	0.6	99.4
1l	MTBE	35	7.1	92.9	22	0.6	99.4	14	0.5	99.5
	isooctane	15	2.7	97.3	-	-	-	29	0.2	99.8

Reaction conditions: 25 mM ketone; 50 mM (S)-PEA; 50 mM decane; 50 mM Brij® C10; 10 µL of enzyme preparation (0.95 U) in HEPES buffer (pH 8.2, 50 mM); isooctane or MTBE: final volume 1 mL. (n.c. = no conversion).

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The W60C variant of *Cv*-ATA shows an increased enantiospecificity for (*S*)-PEA compared to the wild type.

The ketone library (Figure 6) was explored using 25 mM of ketone, 50 mM of (*S*)-PEA, 50 mM of decane, 50 mM of Brij® C10, 1.0 % v/v HEPES buffer solution (50 mM, pH 8.2) containing 0.95 U of ATA. The final volume of the reaction solutions was adjusted to 1 mL using MTBE or isooctane as solvents. The mixtures were vigorously shaken at 37 °C and left to react for 48 hours (results in Table 5). Despite the preliminary solvent exploration had shown that isooctane was the most suitable solvent for these reactions, some of the synthesized ketones are poorly soluble in this solvent, and only MTBE was employed. The reported conversions were measured after 48 hours. At the end of the reaction, the solution was cooled to 4 °C to obtain phase separation. The organic supernatant essentially contains both the remaining substrates and the products, whereas salts, enzyme, cofactor and most of the surfactant are collected in the aqueous phase.

When ATA 113 was employed, no conversions were detected in any case. However, interesting results were obtained with the other ATAs. In general, the explored enzymes showed high *cis* selectivity towards 4-monosubstituted-cyclohexanones, whereas 4-substituted-4-hydroxyl-cyclohexanones are selectively transformed into *trans* amines. This apparent switch of selectivity is simply due to nomenclature: as a matter of fact, both the groups have the 4-alkyl chain positioned on the same side of the cyclohexene ring with respect to the amino moiety (Figure 7).

3a-c *cis* isomer

3d-l *trans* isomer

Figure 7. Comparison of the structure of the two groups of amines obtained after biotransformation.

The selectivity of the ATAs towards ketone **1i** is reversed, despite the similarity between phenyl and thienyl rings. In addition, the hydroxyl moiety seems not to affect the enzyme selectivity. In general, the biocatalytic approach leads to a good stereoselectivity. In addition, the ATA-catalyzed transaminations result in the production of the more unusual *trans* isomer as the major product, while the chemical approach using $NaBH_4$ and MoO_3 favors the *cis* isomer.

10.1002/adsc.201901434

Conclusion

A novel approach for the use of ATAs in the transformation of hydrophobic substrates was developed and employed, carrying out the reductive amination of a library of 12 cyclic ketones. This approach involves the use of surfactants for obtaining a macroscopically homogeneous water-in-oil emulsion, where the enzymatic preparation is finely dispersed in the organic phase. Such a system was characterized with dynamic light scattering analysis and different parameters were explored in order to optimize the process (*e.g.* temperature, solvents and type of surfactant). The highest conversions were obtained using isooctane or MTBE as solvent, Brij® C10 as surfactant at 37 °C and 1.0 % v/v of aqueous enzymatic preparation. Among the evaluated amino donors, only (*S*)-PEA was effective in this approach. Moreover, the solvent was proven to be an important parameter. MTBE dissolved the starting materials more efficiently than isooctane, even if the latter is better tolerated by the enzyme. In the end, this approach possesses many useful advantages. First, the use of organic solvents and surfactants permits to address the frequent solubility issues of hydrophobic substrates. At the same time, enzyme stability in the reaction mixture is remarkably high (stable for more than a week), the reaction workup is easy and practical since it mainly consists in cooling of the reaction mixture and followed by centrifugation to recover the final product. Lastly, this protocol can be employed with different substrates and ATAs.

Experimental Section

Reagents and instruments

All the necessary chemicals were purchased from Sigma-Aldrich. All solvents employed in reactions were of analytical grade and, where indicated, were dried on activated 4 Å molecular sieves prior to use. The solvents employed in HPLC analysis were of HPLC grade and the prepared eluents were filtered through a 0.45 µm membrane prior to use. Silica gel 60 F254 plates (Merck) were used for analytical TLC. Detection was achieved with UV light followed by ninhydrin, potassium permanganate, or cerium molybdate stains.

HPLC analyses were performed on a Jasco apparatus equipped with a diode array detector and a fluorimeter, fitted with a Kinetex 2.6 µm C18 100 Å column, length/internal diameter = 100/4.6 mm (Phenomenex). The excitation and emission wavelengths for fluorescence detection were 350 nm and 450 nm, respectively.

^{1}H and ^{13}C-NMR spectra were recorded on Bruker ARX 400 instrument operating at the ^{1}H resonance frequency of 400 MHz. Chemical shifts (d, ppm) are reported relative to tetramethylsilane (TMS) as the internal standard. All spectra were recorded in $CDCl_3$ or DMSO-d_6 at 305 K. Coupling constants (*J*) are reported in Hz. Mass spectra were acquired on BRUKER Esquire 3000 PLUS equipped with ESI Ion Trap LC/MS System.

GC-FID analyses were performed on a Hewlett Packard 5890 Series II gas chromatography apparatus equipped with a CP-ChiraSil-DEX CB 25 m x 0.32 mm x 0.25 mm or Chrompack CP-SIL 5CB column (25 m x 0.32 mm). The inlet and the detector were set to 250 °C. The following method was used: initial temperature 100 °C, hold for 0.5 min, 10 °C min^{-1} to 200 °C and hold 2-25 min.

UV spectra and absorbance measurements were performed on a Jasco V-560 UV/VIS spectrophotometer.

Dynamic light scattering (DLS) measurements were conducted on a Zetasizer Nano ZS instrument (Malvern, UK), at 37 °C, with an equilibration time of 120 s.

HPLC Conditions

The HPLC analyses of the cyclohexylamine derivatives were performed after derivatization with o-phthaldialdehyde (OPA) and *N*-protected-L-cysteine (*N*-acetyl-L-cysteine or *N*-*tert*-butyloxycarbonyl-L-cysteine, respectively NAC and TBC).[22] The OPA-NAC reagent was prepared by dissolving 8 mg of OPA and 10 mg of NAC in 1 mL of HPLC grade methanol, while OPA-TBC was prepared by dissolving 10 mg of OPA and 10 mg of TBC in 1 mL of HPLC grade methanol. Derivatizations were performed by adding 175 µL of borate buffer (0.4 M, pH 10.4) to 25 µL of samples and then adding 50 µL of OPA-NAC or OPA-TBC reagent. The samples were left to react for 1 minute in the dark before the injection. Due to the different nature of each amine, different HPLC conditions were employed; these are summarized in Table 5.

Table 5. HPLC running conditions for the analysis of the produced amines, after derivatization. A: acetate buffer 10 mM pH 5.2 (45%), MeOH (55%); B: MeOH (100%); C: acetate buffer pH 5.2 (95%), MeOH (5%)

	Derivatizing agent	Flow [mL min^{-1}]	A [%]	B [%]	C (%)	*cis* r.t. [min]	*trans* r.t. [min]
3a	OPA-NAC	0.85	87	13	-	22.9	23.9
3b	OPA-NAC	0.5	92.5	7.5	-	34.1	36.1
3c	OPA-TBC	0.85	87	13		26.7	24.2
3d	OPA-TBC	0.5	100	-	-	9.4	18.5
3e	OPA-TBC	0.5	100	-	-	13.1	28.3
3f	OPA-NAC	0.5	100	-	-	5.4	13.0
3g	OPA-NAC	0.5	100	-	-	9.2	27.2
3h	OPA-NAC	0.5	92.5	7.5	-	4.6	9.2
3i	OPA-NAC	0.5	92.5	-	7.5	7.5	16.1
3j	OPA-NAC	0.5	92.5	7.5	-	6.1	14.2
3k	OPA-NAC	0.85	70	30	-	13.9	42.3
3l	OPA-TBC	0.5	100	-	-	10.6	16.2

4-methyl-4-hydroxycyclohexanone (1d).
An ethereal solution (30 mL) of CH_3I (598 µL, 9.6 mmol) was added dropwise to a suspension of Mg turnings (233 mg, 9.6 mmol) in dry diethyl ether (2.5 mL) under vigorous stirring. The mixture was left to react at r.t. (a reflux condenser was added in order to control the reaction and prevent solvent evaporation) until complete dissolution of Mg; then, a solution of 1,4-cyclohexanedionone monoethylene ketal (1.5 g, 9.6 mmol) in dry diethyl ether (30 mL) was gradually added to the reaction mixture. The consumption of the ketone was followed by TLC. When the ketone was completely consumed, 25 mL of water were added to the reaction, the organic phase was separated and the aqueous phase was extracted with diethyl ether (2x30 mL). The organic phase was dried over anh. Na_2SO_4 and evaporated under reduced pressure obtaining 1.36 g of a pale yellow oil. 25 mL of aqueous 0.05 M HCl were added to the product and the resulting mixture was

stirred overnight at r.t. When the hydrolysis was complete the reaction medium was saturated with NH_4Cl and then extracted with AcOEt (4x25 mL). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The obtained pale yellow oil was purified by flash chromatography (AcOEt/Hexane/MeOH 2/8/0.1) obtaining 790 mg of a white solid corresponding to compound **1d** in 64% yield. ^{1}H NMR ($CDCl_3$): 1.33 (s, 3H), 1.61 (s, 1H), 1.75-1.87 (dt, 2H, *J*=5, 13.3), 1.89-1.98 (m, 2H), 2.15-2.24 (m, 2H), 2.70-2.74 (m, 2H).

According to the procedure described above, starting from 1,4-cyclohexanedionone monoethylene ketal and the opportune alkyl chloride or bromide, the following ketones were synthesized. Yields are calculated based on the starting ketale.

4-ethyl-4-hydroxycyclohexanone (1e).
White solid. 655 mg, yield 48 %.
1H-NMR ($CDCl_3$): 0.90-0.97 (t, 3H, *J*=7.5), 1.39 (s, 1H), 1.52-1.62 (q, 2H, *J*=7.5), 1.70-1.80 (dt, 2H, *J*=5.1, 8.4), 1.87-1.95 (m, 2H), 2.17-2.25 (m, 2H), 2.63-2.74 (m, 2H).

4-hydroxy-4-propylcyclohexanone (1f).
White solid. 252 mg, yield 17 %.
^{1}H-NMR ($CDCl_3$): 0.91-0.97 (t, 3H, *J*=7.2), 1.34-1.56 (m, 5H), 1.71-1.83 (m, 2H), 1.88-1.97 (m, 2H), 2.18-2.26 (m, 2H), 2.64-2.65 (m, 2H).

4-butyl-4-hydroxycyclohexanone (1g).
White solid. 392 mg, yield 24 %.
^{1}H-NMR ($CDCl_3$): 0.88-0.94 (t, 3H, *J*=7.4), 1.26-1.42 (m, 5H), 1.51-1.57 (m, 2H), 1.76-1.82 (dt, 2H, *J*=5.2,13.1), 1.88-1.97 (m, 2H), 2.17-2.24 (m, 2H), 2.64-2.73 (m, 2H). ^{13}C NMR ($CDCl_3$): 13.88, 23.10, 25.53, 36.76, 36.94, 42.22, 70.03, 212.40.

4-phenyl-4-hydroxycyclohexanone (1h).
White solid. 603 mg, yield 33%
^{1}H-NMR ($CDCl_3$): 1.722 (s, 1H), 2.17-2.26 (m, 2H), 2.29-2.52 (m, 4H), 2.87-3.00 (dt, 2H, *J*=5.2, 13.7), 7.29-7.34 (d, 1H, *J*=7.29), 7.38-7.43 (t, 2H, *J*=7.32), 7.53-7.56 (d, 2H, *J*=7.29)

4-hydroxy-4-(thiophen-2-yl)cyclohexanone (1i).
Yellowish solid. 999 mg, yield 53 %
^{1}H-NMR ($CDCl_3$): 2.18-2.35 (m, 4H), 2.70-2.85 (m, 4H), 6.89-6.93 (m, 1H), 6.95-6.98 (dd, 1H, *J*= 1.2, 3.6), 7.16-7.20 (dd, 1H, *J*= 1.2, 5.0). ^{13}C NMR ($CDCl_3$): 37.22, 39.38, 70.58, 122.54, 124.38, 126.88, 152.15, 211.10.

4-benzyl-4'-hydroxyl-cyclohexanone (1j).
White solid. 588 mg, yield 30 %
^{1}H-NMR ($CDCl_3$): 1.74-1.93 (m, 5H), 2.13-2.22 (m, 2H), 2.57-2.68 (m, 2H), 2.80 (s, 2H), 7.14-7.30 (m, 5H). ^{13}C NMR ($CDCl_3$): 36.79, 36.85, 48.63, 69.86, 126.90, 128.44, 130.39, 136.40, 211.78.

4-decyl-4'-hydroxyl-cyclohexanone (1k).
White solid. 977 mg. yield 40 %
^{1}H-NMR ($CDCl_3$): 0.84-0.88 (t, 3H, *J*=6.7), 1.14-1.42 (m, 17H), 1.49-1.56 (m, 2H), 1.71-1.82 (dt, 2H, *J*=5.2, 13.5), 1.88-1.97 (m, 2H), 2.17-2.26 (m, 2H), 2.62-2.75 (m, 2H). ^{13}C NMR ($CDCl_3$): 14.23, 22.58, 23.69, 29.23, 29.51, 30.08, 31.82, 36.84, 36.96, 42.60, 70.21, 212.01.

4-amino-1-methylcyclohexan-1-ol (3d).
A mixture of **1d** (70 mg, 0.55 mmol), hydroxylamine hydrochloride (57 mg, 0.82 mmol), sodium acetate (90 mg, 1.09 mmol), water (1 mL) and MeOH (4.5 mL) was stirred at r.t. for 1 h. Most of the solvent was evaporated under reduced pressure; the residue was resuspended in 5 mL water and extracted with AcOEt (4 x 10 mL). The combined organic fractions were dried over $NaSO_4$ and the solvent distilled off to give 79 mg of the corresponding oxime as a white solid, which was used in the next reactions without purification. To the obtained oxime, dissolved in MeOH (25 mL), MoO_3 was added and the resulting mixture was stirred at 0 °C. Then, $NaBH_4$ (209 mg, 5.52 mmol) was portion-wise added in 2 hours. The depletion of the oxime was followed by TLC. When the substrate was completely consumed, the solvent was evaporated under reduced pressure and the residue was suspended in ethyl acetate (10 mL); a solution of ammonium hydroxide (1 M, 5 mL) was added and the resulting mixture was filtered through a Celite pad. After phase separation, the aqueous phase was saturated with NaCl and extracted with AcOEt (4 x 10 mL). The combined organic fractions were dried over Na_2SO_4 and the solvent distilled off to obtain 50 mg of a yellowish oil (yield 71 %), which is a mixture of *cis/trans* isomer of the compound **3d**.
Yield 96%
^{1}H NMR (CDCl3): 1.13-1.20 (m, 3H), 1.30-1.79 (m, 9H), 2.50-2.59 (m, 1H, cis isomer), 2.70-2.80
(m, 1H, trans isomer). ESI-MS m/z 130 [M + H$^+$].

According to the procedure described above and starting from the corresponding ketones, the following amines were synthetized. Yields are calculated based on the starting ketone.

4-tert-butyl-cyclohexyl-1-amine (3a).
Yellowish oil. 57 mg, yield 81%
^{1}H-NMR ($CDCl_3$): 0.83-0.86 (s, 9H), 0.91-1.13 (m, 5H), 1.58.1.97 (m, 6H), 2.50-2.62 (m, 1H, *trans* isomer), 2.79-2.92 (m, 1H, *cis* isomer). ESI-MS m/z 156 [M + H$^+$].

4-phenyl-cyclohexyl-1-amine (3b).
Yellowish oil. 67 mg, yield 95%
^{1}H-NMR ($CDCl_3$):1.19-1.29 (m, 2H), 1.46-1.63 (m, 4H), 1.86-2.02 (m, 4H), 2.42-2.52 (m, 1H), 2.59-2.69 (m, 1H, *trans* isomer), 2.96-3.04 (m, 1H, *cis* isomer), 7.12-7.35 (m, 5H). ESI-MS m/z 176 [M + H$^+$].

4-amino-1-ethylcyclohexan-1-ol (3e).
Yellowish oil. 65 mg, yield 92%
^{1}H-NMR (CDCl3): 0.81-0.87 (t, 3H, J=7.5), 1.24-1.68 (m 14H), 2.51-2.60 (m, 1H, *cis* isomer), 2.76-2.84 (m, 1H, *trans* isomer). ESI-MS m/z 144 [M + H$^+$].

4-amino-1-propylcyclohexan-1-ol (3f).
Yellowish oil. 63 mg, yield 90%
^{1}H-NMR ($CDCl_3$): 0.80-0.91 (m, 3H), 1.15-1.80 (m, 16H), 2.53-2.63 (m, 1H, *cis* isomer), 2.75-2.85 (m, 1H, *trans* isomer). ESI-MS m/z 158 [M + H$^+$].

4-amino-1-butylcyclohexan-1-ol (3g).
Yellowish oil. 64 mg, yield 91%
^{1}H-NMR ($CDCl_3$): 0.82-0.94 (m, 3H), 1.12-1.86 (m, 18H), 2.52-2.64 (m, 1H, *cis* isomer), 2.85-2.97 (m, 1H, *trans* isomer). ESI-MS m/z 172 [M + H$^+$].

4-amino-1-phenylcyclohexan-1-ol (3h).
Yellowish oil. 67 mg, yield 95%
^{1}H-NMR (DMSO-d_6): 1.54-1.87 (m, 9H), 2.64-2.70 (m, 1H, *cis* isomer), 7.05-7.11 (t, 1H, *J*=7.3), 7.17-7.23 (t, 2H, *J*=7.3), 7.35-7.40 (d, 2H, *J*=8.1). ESI-MS m/z 192 [M + H$^+$].

4-amino-1-(thiophen-2-yl)cyclohexan-1-ol (3i).
Yellowish oil. 63 mg, yield 89%
^{1}H-NMR (DMSO-d_6): 1.51-1.70 (m, 4H), 1.71-1.82 (td, 2H, *J*=4.2, 13.4)1.88-1.95 (m, 2H), 2.59-2.69 (m, 1H, *cis* isomer), 2.74-2.82 (m, 1H, *trans* isomer), 2.61 (s, 2H), 2.58-2.65 (m, 1H, *trans* isomer), 6.80-6.84 (dd, 1H, *J*=3.6, 4.9), 6.84-6.87 (dd, 1H, *J*=1.2, 3.6), 7.09-7.11 (dd, 1H, *J*=1.2, 4.9). ESI-MS m/z 198 [M + H$^+$].

4-amino-1-benzylcyclohexan-1-ol (3j).
Yellowish oil. 66 mg, yield 96%

^{1}H-NMR (DMSO-d_6): 1.16-1.65 (m, 11H), 2.38-2.47 (m, 1H, *cis* isomer), 7.01-7.17 (m, 5H). ESI-MS m/z 206 [M + H^+].

4-amino-1-decylcyclohexan-1-ol (3k).
Yellowish oil. 57 mg, yield 65%
^{1}H-NMR ($CDCl_3$): 0.87-0.92 (t, 3H, *J*=6.7), 1.22-1.95 (m, 29H), 2.87-2.97 (m, 1H, *cis* isomer). ESI-MS m/z 256 [M + H^+].

4-amino-1-(((tetrahydrofuran-2-yl)oxy)methyl)cyclohexan-1-ol (31).
Yellowish oil. 43 mg, yield 61%
^{1}H-NMR ($CDCl_3$): 1.28-1.38 (td, 2H, *J*= 3.6, 13.0), 1.46-1.58 (qd, 2H, *J*= 3.3, 12.5, 23.7), 1.66-1.75 (m, 4H), 1.87-1.94 (bs, 3H), 1.95-2.01 (m, 2H), 2.58-2.68 (m, 1H), 3.19-3.27 (q, 2H, *J*=8.8), 3.75-3.91 (m, 4H), 4.09-4.14 (m, 1H). ESI-MS m/z 216 [M + H^+].

Enzyme preparation.
The genes encoding for the *Cv*-ATA-wt, *Cv*-ATA-W60C, *Cv*-ATA-F88A/A231F, *Cv*-ATA-W60C/F88A/A231F and *Vf*-ATA were inserted in a pET28a(+) vector including an *N*-terminal His_6-tag. The plasmids were transferred to the host BL21(DE3) *Escherichia coli* strain. Cells were grown at 37 °C in TB medium (Terrific Broth) supplemented with 50 mg/L kanamycin. Protein expression was induced at an OD_{600} ~ 0.7-0.9 by addition of IPTG (1 mM). After 24 h of incubation at 20 °C and 220 rpm, the cells were harvested by centrifugation (30 min, 8000 rpm, 4 °C) and resuspended in IMAC binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were disrupted by sonication and the cell debris was removed by centrifugation (30 min, 20,000 rpm, 4 °C) followed by filtration (0.45 μm). The purification process was performed on a Ni-NTA Sepharose column. For *Cv*-ATA-wt, *Cv*-ATA-F88A/A231F and *Vfl*-ATA the fraction containing ATA activity was dialyzed against HEPES buffer (50 mM, pH 8.2) on a PD10 column (GE Healthcare), according to the manufacturer's protocol. After addition of PLP (3 mM), the enzyme was incubated for 24 h at 4 °C. Thereafter, it was dialyzed against HEPES buffer (50 mM, pH 8.2) to remove the excess of PLP, after incubation at 37 °C for 1 h. For *Cv*-ATA-W60C/F88A/A231F and *Cv*-ATA-W60C the same procedure was applied using HEPES buffer (50 mM, pH 7.0). The protein concentration of the enzyme preparation was estimated by absorbance at 280 nm. The enzyme was stored at 4 °C and was incubated for 30 min at 37 °C prior to using.
Surfactant-protected lyophilized enzyme powders were prepared as follows: the surfactants, Brij® C10 (15 mg) and octyl-β-D-glucopyranoside (15 mg), were dissolved in 3 mL HEPES buffer (50 mM, pH 8.2) at 37 °C. Then, the enzyme was added (3 mL, 6 mg/mL). After mixing, the solution was rapidly frozen in liquid N_2 and lyophilized for the established time. For side-to-side comparison, half of the enzyme batch was lyophilized without the addition of surfactants.

Enzyme activity.
The enzymatic activity was assayed spectrophotometrically at 25 °C by monitoring the formation of acetophenone from PEA; 1 mL of reaction mixture containing 2 μg (or 150 μg) enzyme, 2.5 mM PEA (*S* or *R* depending on the enantiopreference of the enzyme), 2.5 mM pyruvate and HEPES buffer (50 mM, pH 8.2) was used. The reaction was monitored for 2 min following the absorbance increase at 245 nm (ε = 12 mM^{-1} cm^{-1}). One unit (1 U) was defined as the amount of enzyme converting 1 μmol of PEA in 1 min.

Bioaminations in neat solvents.
The solvents were dried over molecular sieves (4 Å) before use. 20 mg lyophilized *Cv*-ATA wild type, (*S*)-PEA (50 mM), compound **1a** (150 mM) and decane (50 mM) were dissolved in dry isooctane (1 mL). Conversions were evaluated by GC-FID following the concentration of acetophenone, while the *cis/trans* ratio of the formed amine was evaluated by HPLC. For the GC-FID, 20 μL samples were diluted in 0.5 mL of cyclohexane and then injected without further derivatization. HPLC analysis was performed at the end of each experiment; the reaction medium was evaporated under reduced pressure, then about 2.5 mg of the residue was dissolved in 1 mL HCl 0.05 M. The resulting solution was analyzed as previously described.

Bioaminations in disperse systems.

Firstly, the dry solvent (isooctane or MTBE) was heated to 37 °C. Secondly, the surfactant (50 mM) was added and dissolved. Then, ketone (25 mM), (*S*)-PEA (50 mM), decane (50 mM) and the enzymatic solution (1 % v/v) were sequentially added. 10 μL of the enzyme preparation contained 0.95 U of enzyme dissolved in HEPES buffer (50 mM, pH 8.2 or 7.0 depending on the type of enzyme). The enzyme solution was incubated at 37 °C for at least 30 minutes before use. The reaction mixture was vigorously shaken. Conversions were evaluated by GC-FID following the concentration of acetophenone while the *cis/trans* ratio of the formed amine was evaluated by HPLC. For the GC-FID analyses, 20 μL samples were diluted in 0.5 mL cyclohexane and then injected without any further derivatization. HPLC analyses were performed at the end of each experiment. Prior to the HPLC analysis, the reaction medium was evaporated under reduced pressure, then about 2.5 mg of the residue was dissolved in 1 mL HCl 0.05 M. The resulting solution was analysed as previously described.

When product isolation was needed, at the end of the reaction, the solution was brought to 4 °C, incubated for 30 min and centrifuged to facilitate the phase separation. The supernatant was evaporated and the residue was purified by column chromatography.

Acknowledgements

*The authors wish to deeply thank Prof. Francesca Baldelli-Bombelli (Politecnico di Milano) for DLS experiments and Flamma SpA (Chignolo d'Isola, ITALY) for the kind gift of substrate **11a**.*
This project was carried out in the frame of the COST Action CM1303 "SysBiocat" and dr. Fiorati could benefit from the funding for a "Short Term Scientific Mission" in Prof. Berglund's laboratories.

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10.1002/adsc.201901434

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