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Asymmetric Synthesis of 3-Substituted Cyclohexylamine Derivatives from Prochiral Diketones *via* Three Biocatalytic Steps

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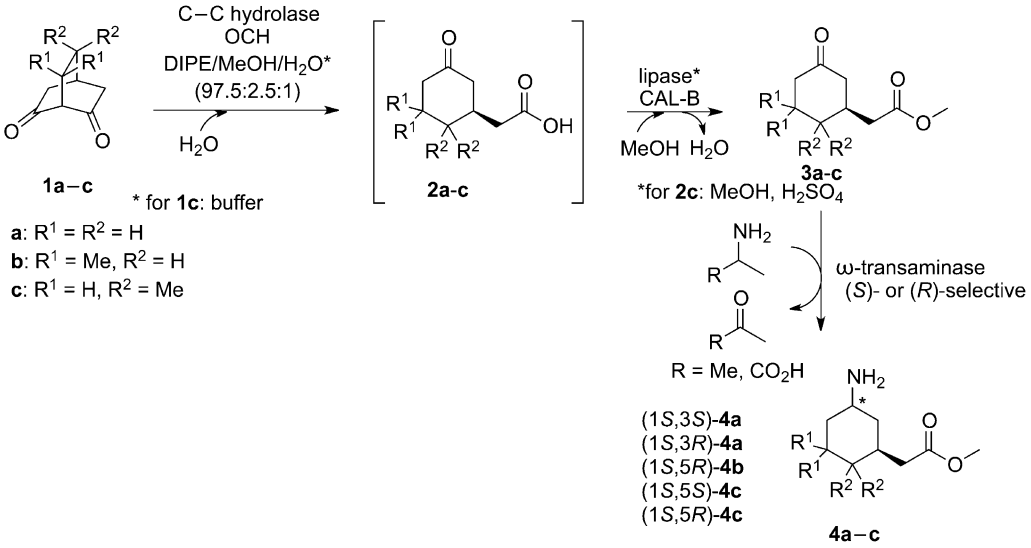
Abstract: Prochiral bicyclic diketones were transformed to a single diastereomer of 3-substituted cyclohexylamine derivatives *via* three consecutive biocatalytic steps. The two chiral centres were set up by a C–C hydrolase (6-oxocamphor hydrolase) in the first step and by an ω-transaminase in the last step. The esterification of the intermediate keto acid was catalysed by a lipase in the second step if possible. For two substrates the C–C hydrolytic step as well as the esterification could be run simultaneously in a one-pot cascade in an organic solvent. In one example, the reaction mixture of the first two steps could be directly subjected to bio-amination in an organic solvent without the need to change the reaction medium. Depending on the choice of the ω-transaminase employed and the substrate the *cis*- as well as the *trans*-diastereomers could be obtained in optically pure forms.

Keywords: asymmetric catalysis; biosynthesis; biotransformations; C–C hydrolase; ω-transaminases

Biocatalytic asymmetric transformations have become key steps for the preparation of optically pure compounds.[1] However, commonly only a single step in the synthetic route is performed by an enzyme. Exceptions are enzymatic cascade reactions,[2,3] where at least two enzymes work beside each other. This enables shorter reaction times, minimises work-up steps and reduces waste. Hence applying a sequence of stereoselective enzymatic reactions for the synthesis of compounds with two or more chiral centres[4] is an appealing option when starting from prochiral substrates. Recently, it was recognised that 2-(3-aminocyclohexyl)acetic acid derivatives (**4**), unnatural δ-amino acids bearing two chiral centres, serve as valuable building blocks in the synthesis of potential pharmaceuticals,[5] for example, as modulators for the multidrug-resistant protein MRP1.[6] However, catalytic and highly stereoselective methods for the preparation of 3-substituted cyclohexylamines in general are rare,[7,8] necessitating even resolution of enantiomers using chromatography.[9] Only in the cases of 3-aminocyclohexanol[10] or 1,3-cyclohexyldiamine[11] are more options available.

We envisaged that prochiral bicyclic diketones (**1a–c**) might serve as starting materials for the preparation of aminocyclohexylacetic acid derivatives **4** *via* a three-enzyme reaction sequence. In this sequence, the stereoselective hydrolysis of a C–C bond catalysed by a β-diketone hydrolase[12] would be the first step, followed by an esterification employing a lipase (Scheme 1). Finally the chiral amine moiety would be introduced by an ω-transaminase (ωTA).[13] Thus, the two chiral centres might be controlled on the one hand by the C–C hydrolase and on the other hand by the ωTA.

Since the C–C hydrolysis of substrate **1a** in buffer has been described previously,[14] **1a** was used as a model compound to test the reaction sequence. The enzyme employed for the first step was the 6-oxocamphor hydrolase (OCH) from *Rhodococcus* sp. NCIMB 9784, which catalyses the hydrolytic *retro*-Claisen condensation of non-enolisable, symmetrical β-diketones leading to optically pure keto acid **2a**.[14] Since the resulting keto acid **2a** was a non-substrate for amination by ωTAs, whereas the corresponding methyl ester **3a** was well accepted (*vide infra*), our emphasis was to combine the hydrolytic C–C cleavage with an esterification step in a simultaneous cascade. At a first glance the reaction conditions for these two steps look incompatible, since water is required for the hydrolysis, while water is detrimental for the esterification. Although water is a substrate for the hydrolytic step, it is also a product of the



Scheme 1. Three-enzyme sequence for the synthesis of 2-(3-aminocyclohexyl)acetic acid methyl ester derivatives (**4a–c**) bearing two chiral centres starting from prochiral diketones **1a–c**.

esterification step, thus formally, only catalytic amounts of water should be required due to its possible internal "reuse". Methanol was not a nucleophile accepted by the C–C hydrolase,[15] which would lead directly to the methyl ester. Therefore it was required to test the OCH in organic solvents in the presence of only a minimum amount of water: sufficient to promote enzymatic activity[16] and run the C–C hydrolysis but low enough to enable the methyl ester formation. OCH was recently shown to tolerate a broad variety of organic cosolvents up to 80% vv^{-1} maintaining in most solvents its excellent stereoselectivity.[15] Diisopropyl ether (DIPE) was the organic solvent best tolerated. In the presence of 80% vv^{-1} of DIPE the enzyme still retained 51% residual activity compared to aqueous buffer system. Methanol, on the other hand, inactivated the enzyme.

Although in the previous work DIPE was used up to 80% vv^{-1} as a cosolvent in the C–C hydrolysis, the conditions for the simultaneous one-pot reaction with the lipase-catalysed esterification needed to be tuned in order to minimise the competing ester hydrolysis catalysed by the lipase. Employing 2.5% vv^{-1} (1.4 M) of water in DIPE, OCH (lyophilised crude cell extract, 20 mg mL^{-1}) catalysed the hydrolysis of **1a** (50 mM) to **2a** with full conversion and perfect stereoselectivity (*ee* >99%) within three hours.

The esterification of (*S*)-**2a** (50 mM) was first studied separately, thus *Candida antarctica* lipase B was tested in DIPE:H_2O (97.5:2.5 vv^{-1}) with methanol. Commercial Novozyme 435 (10 mg mL^{-1}) enabled methyl ester formation leading to (*S*)-**3a** with 97% conversion in the presence of just 1% vv^{-1} of methanol within 3 h. Consequently, the C–C hydrolysis of **1a** was combined with the esterification in DIPE:H_2O:MeOH (97.5/2.5/1 $vv^{-1}v^{-1}$) leading to complete conversion within one hour (Figure 1). It is notable that the C–C cleaving step ran faster in the presence of the lipase-catalysed esterification, thus the intermediate keto acid (*S*)-**2a** was instantly esterified and was not detected by GC-MS analysis during the time course of the reaction. Furthermore, the enzymes could be recovered from the organic solvent and recycled without loss of activity or selectivity at least five times.

To test the amination step, enzymatically prepared (*S*)-**3a** was subjected to the ωTA catalysed amination using L- or D-alanine or 2-propylamine as an amine donor in aqueous buffer. In aqueous solution most ωTAs accept alanine as amine donor, and only few 2-propylamine. Employing alanine as amine donor, the formed coproduct pyruvate needs to removed for thermodynamic reasons either by transforming pyruvate to alanine employing an alanine dehydrogenase

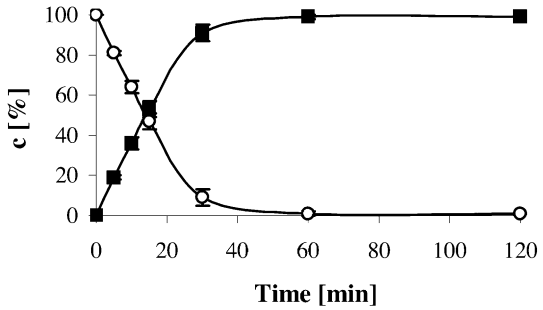


Figure 1. OCH (lyophilised crude extract, 20 mg mL^{-1}) and CAL-B (Novozyme 435, 20 mg mL^{-1}) catalysed conversion of diketone **1a** ○ (50 mM) to (*S*)-**3a** ■ in DIPE:H_2O:MeOH (97.5/2.5/1, $vv^{-1}v^{-1}$) at 30 °C, 120 rpm.

Table 1. Transaminase catalysed reductive amination of (*S*)-**3a**.[a]

Entry	ω-Transaminase	*c* [%]	*ee* [%]	*de* [%]	Product
1	*Vibrio fluvialis*	>99	>99	>99	(1*S*,3*S*)-**4a**
2	*Chromobacterium violaceum*	>99	>99	99	(1*S*,3*S*)-**4a**
3	*Arthrobacter citreus*	<1	n.a.	n.a.	n.a.
4	*Bacillus megaterium*	97	>99	>99	(1*S*,3*S*)-**4a**
5	*Alcaligenes denitrificans*	<1	n.a.	n.a.	n.a.
6	(*R*)-*Arthrobacter*	8	>99	97	(1*S*,3*R*)-**4a**
7	(*R*)-*Arthrobacter* variant[b]	96	>99	97	(1*S*,3*R*)-**4a**
8	*Aspergillus terreus*	<1	n.a.	n.a.	n.a.
9	*Hyphomonas neptunium*	<1	n.a.	n.a.	n.a.

[a] ω-Transaminase (lyophilised whole cells, 20 mg mL^{-1}) in phosphate buffer (100 mM, pH 7, 1 mM PLP) at 30 °C, 120 rpm, 24 h. L- or D-alanine (250 mM), LDH (90 U), GDH (30 U), NAD^+ (1 mM) and glucose (150 mM).
[b] 2-Propylamine (1 M) as amine donor in phosphate buffer (100 mM, pH 8) at 45 °C, 120 rpm, 24 h. n.a. not applicable.

(AlaDH) or by reduction to lactate with a lactate dehydrogenase (LDH).[13c] The AlaDH as well as the LDH requires NADH for reduction which is recycled, for example, with glucose and a glucose dehydrogenase (GDH). Various ωTAs were tested like the (*S*)-selective ω-transaminases from *Vibrio fluvialis* (His-VF),[17] *Chromobacterium violaceum* (CV),[18] *Arthrobacter citreus* (ArS),[19] *Bacillus megaterium* (BM)[20] and *Alcaligenes denitrificans* (AD)[21] and (*R*)-selective ω-transaminases from *Arthrobacter* sp. (wild-type ArR[22] and variant ArRmut11[23]), *Aspergillus terreus*[24] and *Hyphomonas neptunium*.[24] Best results were obtained with His-VF-ωTA leading to the optically pure diastereomer (1*S*,3*S*)-**4a** in >99% *ee* and >99% *de* (Table 1). For the diastereomer (1*S*,3*R*)-**4a** bearing an (*R*)-configured amine moiety, the (*R*)-selective enzyme variant from *Arthrobacter* sp. ArRmut11-ωTA turned out to be best suited. ArRmut11-ωTA was also tested for the amination of keto acid (*S*)-**2a**, however (*S*)-**2a** was a non-substrate.

Since the first two steps were performed in an organic solvent, it would be desirable to run also the subsequent third step in an organic environment, ideally in the same solvent as employed in the first two steps. The possibility to carry out the biocatalytic steps in solvents compatible with upstream and downstream chemistry reduces the extraction and solvent switching steps, which are often necessary for the conventional aqueous biotransformations. Thus keeping the same solvent can provide more efficient synthetic routes.[25] Asymmetric bio-amination in organic solvents employing crude cell extracts has been reported recently.[26]

The same methodology was applied here by subjecting the organic reaction mixture of the first two steps directly to the transamination step after removal of the hydrolases by filtration. Lyophilised crude extracts of His-VF-ωTA and ArRmut11-ωTA were pretreated in water saturated DIPE in order to equilibrate the amount of water for the catalyst before adding the substrate and the amine donor (2-propylamine, 3 equivalents). Employing ArRmut11-ωTA the reaction resulted in 91% conversion within 24 h leading to the (1*S*,3*R*)-**4a** diastereomer (*ee* >99%, *de* 98%).

The His-VF-ωTA-catalysed reaction proceeded more slowly, resulting in 71% conversions within 96 h giving optically pure (1*S*,3*S*)-**4a** (*ee* >99%, *de* >99%).

When synthesising racemic reference material **3a** *via* reduction of the imine formed between benzylamine and *rac*-**3a** using $NaBH(OAc)_3$, it was noticed that the *trans*-diastereomer (1*R**,3*R**) (96%) was formed preferentially, while only a minor amount (4%) of the *cis*-diastereomers (1*R**,3*S**)-**4a** was detected (Figure 2, trace 1).

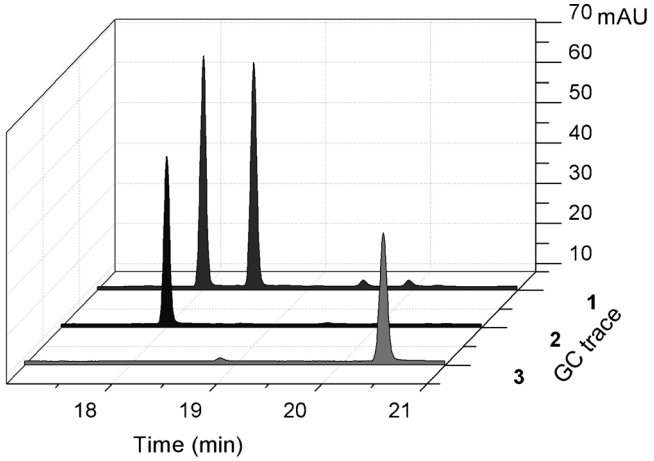


Figure 2. GC-trace 1: chemically prepared *all-rac*-**4a** (Chirasil-DEX CB column) after derivatisation (see the Supporting Information). Peaks from left to right: (1*S*,3*S*)-**4a**, (1*R*,3*R*)-**4a**, (1*R*,3*S*)-**4a** and (1*S*,3*R*)-**4a**. GC-trace 2: enzymatically prepared (1*S*,3*S*)-**4a** (His-VF-ωTA). GC-trace 3: enzymatically prepared (1*S*,3*R*)-**4a** (ArRmut11-ωTA).

Thus, the chemical reductive amination of keto ester **3a** preferably led to the *trans*-diastereomer, whereas the *cis*/*trans* selectivity can be controlled *via* the biocatalytic approach by the choice of the catalyst employed. When starting from (*S*)-**3a**, ArRmut11-ωTA gave access to the *cis*- while His-VF-ωTA produced the *trans*-stereoisomer.

To demonstrate the scope of the synthetic three-enzyme strategy for related compounds, substrates **1b** and **1c** were synthesised. Both substrates have never been tested before employing OCH. OCH hydrolysed both diketones to the corresponding δ-keto acids with perfect and very high stereoselectivity, respectively (*ee* >99% and *ee* = 99% for **2b** and **2c**, respectively) leading to the (*S*)-enantiomers, whereby **1c** was transformed faster than **1b**. In case of **1b**, >99% conversion was reached within four hours using 10 mM substrate concentration, whereas **1c** gave >99% conversion with the same biocatalyst loading and reaction time at 50 mM substrate concentration.

The simultaneous one-pot reaction system for C–C hydrolysis and esterification using OCH and CAL-B as catalysts led to >99% conversion of **1b** to **3b** (10 mM) within four hours. Interestingly, **2c** was not a substrate of CAL-B, so in the case of substrate **1c**, the C–C hydrolysis was first carried out in aqueous buffer, and the methyl ester was formed chemically.

Reductive amination of (*S*)-**3b** was tested with the transaminases identified as best suitable for (*S*)-**3a** (Table 1). In comparison to (*S*)-**3a** the keto ester (*S*)-**3b** was aminated slower, probably due to increased sterical hinderness. Nevertheless, optically pure (1*S*,5*R*)-**4b** was obtained in aqueous buffer (*ee* >99%, *de* >99%) (Table 2, entries 1 and 2). Interestingly, the (*S*)-selective ωTA His-Vf as well as the (*R*)-selective enzyme ArRmut11 yielded the same *trans*-product (1*S*,5*R*)-**4b**. When performing the reaction in organic solvent (DIPE), **3b** was not accepted as a substrate by these enzymes.

Keto ester (*S*)-**3c** was very well transformed by the ωTAs leading to >99% conversion and perfect stereoselectivity (*ee* >99%, *de* >99%) with both (*S*)- and (*R*)-selective enzymes in aqueous buffer leading to optically pure (1*S*,5*S*)-**4b** and (1*S*,5*R*)-**4c**, respectively (entries 5 and 6). ArRmut11-ωTA aminated (*S*)-**3c** also in organic solvent leading to 89% conversion within 24 h at 50 mM substrate concentration (entry 7).

All products **2**–**4** were also prepared on a preparative scale using 0.1–0.5 g of substrate. For instance, to get sufficient amounts of all above mentioned diastereomers of **4a–c**, the aminations of (*S*)-**3a–c** were successfully performed on 100-mg scales employing (*R*)- and (*S*)-selective ωTAs.

In conclusion, a biocatalytic synthetic route for the preparation of optically pure 3-substituted cyclohexylamine derivatives [methyl 2-(3-aminocyclohexyl)acetates] from prochiral bicyclic β-diketones was established employing three biocatalytic reaction steps. In the case of substrates **1a** and **1b**, the simultaneous cascade reaction catalysed by two hydrolases, namely a C–C hydrolase and a lipase, resulted in the corresponding methyl esters **3a** and **3b** in organic solvent (DIPE) containing 2.5% vv^{-1} water and 1% vv^{-1} methanol. The organic phase of the reaction with **1a** could be directly applied in the transaminase-catalysed reactions thus giving the diastereomers (1*S*,3*S*)-**4a** and (1*S*,3*R*)-**4a** by a one-pot approach. In the case of substrate **1c**, C–C-bond hydrolysis was catalysed in buffer, and the methyl ester of the crude keto acid was prepared chemically followed by transamination, which could successfully be performed in organic solvent as well as in buffer.

Table 2. Reductive amination of (*S*)-**3b** and (*S*)-**3c** by His-VF-ωTA and ArRmut11-ωTA.[a]

Entry	Substrate	ωTA	Solvent	*c* [%]	*ee* [%]	*de* [%]	Product
1	**3b**	ArRmut11	buffer[b]	69	>99	>99	(1*S*,5*R*)-**4b**[e]
2	**3b**	His-VF	buffer[c]	25	>99	>99	(1*S*,5*R*)-**4b**[e]
3	**3b**	ArRmut11	DIPE[d]	n.c.	n. a.	n. a.	–
4	**3b**	His-VF	DIPE[d]	n.c.	n. a.	n. a.	–
5	**3c**	ArRmut11	buffer[b]	>99	> 99	>99	(1*S*,5*R*)-**4c**
6	**3c**	His-VF	buffer[c]	>99	> 99	99	(1*S*,5*S*)-**4c**
7	**3c**	ArRmut11	DIPE[d]	89	> 99	>99	(1*S*,5*R*)-**4c**
8	**3c**	His-VF	DIPE[d]	n.c.	n. a.	n. a.	–

[a] Substrate concentration 50 mM, enzymes applied as lyophilised crude extracts (20 mg mL^{-1}).
[b] 2-Propylamine (1 M) as amine donor in phosphate buffer (100 mM, pH 8) at 45 °C, 120 rpm, 24 h.
[c] L-Alanine (250 mM) was used as amine donor in phosphate buffer (100 mM, pH 7, 1 mM PLP) at 30 °C, 120 rpm, 24 h. LDH (90 U), GDH (30 U), NAD^+ (1 mM) and glucose (150 mM).
[d] DIPE (H_2O sat.) was used as a solvent and 2-propylamine (150 mM) as amine donor. Reactions were shaken at 25 °C, 750 rpm, 24 h.
[e] Switch in Cahn–Ingold–Prelog priority. n.c. no conversion; n.a. not applicable.

Experimental Section

Expression and Preparation of the Enzymes

OCH was expressed in *E. coli* and the lyophilised crude extract was prepared as previously described.[15] The crude extract had a specific enzymatic activity of 9.0 $\mu mol_{\mathbf{1a}} min^{-1} mg_{crude\ enzyme}$ in buffer.[14] ω-TAs were expressed in *E. coli* BL21 (DE3) as previously described.[17b,22] Lyophilised cells containing overexpressed ωTAs were used as enzyme preparations in buffer. Cell-free extracts of the ω-TAs were prepared as described[26] for the experiments in organic solvent.

Simultaneous C−C Hydrolysis and Esterification

OCH (lyophilised cell free extract, 20 $mg\,mL^{-1}$), CAL-B (Novozyme 435, 20 $mg\,mL^{-1}$) and the diketone (**1a**, 50 mM; **1b**, 10 mM) were suspended in DIPE/H_2O/MeOH mixture (97.5:2.5:1, 1 mL) in an Eppendorf tube. The water content of the equilibrated reaction system was 5567 ppm. The samples were shaken in an orbital shaker at 120 rpm, 30 °C in that way that the tubes were in horizontal positions. Samples (100 μL) were diluted by EtOAc (300 μL) and the conversions were analysed by GC-MS and GC-FID (see the Supporting Information). In case the products **3a–c** were needed for the amination reactions in buffer, the reactions were stopped by filtering the hydrolases, and the solvents were removed under air flow to obtain a crude product. In case of the one-pot approach, hydrolases were removed by filtration, and the remaining solution was applied directly for the amination step in organic solvent.

Amination in DIPE with 2-Propylamine as an Amine Donor

This was carried out as previously described for MTBE as solvent.[26] ω-TAs (lyophilised crude extract, 20 $mg\,mL^{-1}$) were shaken in water-saturated DIPE (1 mL) in an Eppendorf tube for 15 min at 25 °C, 750 rpm in an Eppendorf shaker. Water (16 μL) was added, and the enzyme preparation was further shaken for 15 min. Substrate was added as a crude, purified material, or in a one-pot approach as a solution in DIPE/MeOH/H_2O (97.5:2.5:1), and 2-propylamine (150 mM) was added. The reactions were shaken at 25 °C, 750 rpm and samples (100 μL) were taken at intervals. The samples were diluted by EtOAc (300 μL) and the conversions were analysed by GC-FID as described in the Supporting Information.

Amination in Aqueous Buffer with 2-Propylamine as an Amine Donor

Lyophilised *E. coli* BL21 (DE3) cells containing the overexpressed (*R*)-selective variant from *Arthrobacter* sp. (ArRmut11) were rehydrated in phosphate buffer (100 mM, pH 8) containing 1 M isopropylamine. Since the harvested *E. coli* cells were resuspended in PLP (1 mM) containing phosphate buffer (100 mM, pH 7) before lyophilisation, no addition of PLP was necessary. After 15 min rehydration at 30 °C, 120 rpm, the substrate (50 mM) was added. Reaction mixtures were shaken at 500 rpm, 45 °C for 16 h in an Eppendorf shaker. The reactions were stopped by adding saturated $NaHCO_3$ solution (100 μL), and then were extracted by ethyl acetate (3 × 500 μL). After drying the combined organic layers over Na_2SO_4 the reactions were analysed by GC-FID (see the Supporting Information).

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