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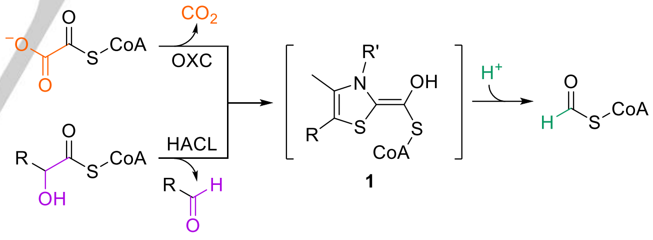
Oxalyl-CoA Decarboxylase Enables Nucleophilic One-Carbon Extension of Aldehydes to Chiral α-Hydroxy Acids

Simon Burgener,[a] Niña Socorro Cortina,[a] Tobias J. Erb*[a,b]

Abstract: *The synthesis of complex molecules from simple, renewable carbon units is the goal of a sustainable economy. Here we explored the biocatalytic potential of the thiamine diphosphate-dependent (ThDP) oxalyl-CoA decarboxylase (OXC)/2-hydroxyacyl-CoA lyase (HACL) superfamily that naturally catalyze the shortening of acyl-CoA thioester substrates through the release of the C_1-unit formyl-CoA. We show that the OXC/HACL superfamily contains promiscuous members that can be reversed to perform nucleophilic C_1-extensions of various aldehydes to yield the corresponding 2-hydroxyacyl-CoA thioesters. We improved the catalytic properties of Methylorubrum extorquens OXC by rational enzyme engineering and combined it with two newly described enzymes – a specific oxalyl-CoA synthetase and a 2-hydroxacyl-CoA thioesterase. This enzyme cascade enabled continuous conversion of oxalate and aromatic aldehydes into valuable (S)-α-hydroxy acids with enantiomeric excess up to 99%. Altogether our study showcases the potential to develop ThDP-catalyzed nucleophilic C_1-extensions as sustainable production platform for chiral building blocks.*

One of biotechnology's central goals is the synthesis of multicarbon compounds under mild and sustainable conditions from renewable resources. This requires biocatalysts that enable selective C-C bond formation ('carboligation') between two carbon units. Thiamine diphosphate (ThDP)-dependent enzymes display high catalytic and substrate promiscuity with respect to C-C bond breaking and forming reactions and they catalyze carboligation reactions at high rates and with excellent stereo- and enantioselectivity.[1] Several biocatalytic applications have been developed that rely on ThDP-dependent carboligases; notable examples are pyruvate decarboxylase (PDC),[2] benzoylformate decarboxylase (BFD),[3] benzaldehyde lyase [4] and transketolase (TK).[5] Their broad catalytic repertoire makes ThDP-dependent enzymes promising starting points for the development of biocatalysts for C-C bond forming reactions.

In the context of a methanol and/or formate-based economy, carboligations with one-carbon units are of particular interest.[6] The potential of ThDP-dependent enzymes for synthetic one-carbon fixation has been showcased by the computationally designed enzyme formolase, which condenses three formaldehyde molecules into dihydroxyacetone.[7] Here, we focused on the superfamily of ThDP-dependent oxalyl-CoA decarboxylase (OXC)/2-hydroxyacyl-CoA lyase (HACL). The OXC/HACL superfamily comprises of decarboxylating members (OXCs), as well as non-decarboxylating members (HACLs). Both catalyze the ThDP-dependent cleavage of formyl-CoA from their respective acyl-CoA thioester substrates (Scheme 1). OXCs catalyze the decarboxylation of the C_2-compound oxalyl-CoA to formyl-CoA,[8] whereas HACLs cleave a 2-hydroxyacyl-CoA into a fatty aldehyde that is shortened by a C_1-unit.[9] In OXC and HACL catalysis has been proposed to proceed through the same covalent intermediate **1** on the ThDP cofactor (Scheme 1).[9-10] After release of CO_2 or aldehyde, the remaining formyl-CoA moiety forms **1**. Analogous to other ThDP-dependent enzymes that form similar ThDP carbanion/enamine intermediates, we speculated that **1** can act as nucleophile in a carboligation reaction with an electrophilic carbon center, essentially reversing the native OXC/HACL reactions. This would enable nucleophilic C_1-extension reactions employing formyl-CoA or oxalyl-CoA as donor, which can in turn be produced from the cheap carbon sources formate[6b] and oxalate, respectively. Recently, Chou et al. demonstrated that HACL catalyzes the acyloin condensation of formyl-CoA with aldehyde acceptor substrates,[11] However, the study focused on HACL and formyl-CoA, thus the carboligation potential of OXC with oxalyl-CoA as donor remains unknown.



Scheme 1. OXC and HACL form the α-hydroxyl-CoA-ThDP carbanion/enamine intermediate (**1**) by decarboxylation of oxalyl-CoA and cleavage of a 2-hydroxyacyl-CoA, respectively. In the second half reaction **1** is protonated and released as formyl-CoA.

To further explore the carboligase potential within the OXC/HACL superfamily, we recombinantly produced human HACL ($HACL_{Hs}$), as well as OXC from *Methylorubrum extorquens* (OXC_{Me}), $HACL_{Hs}$ expressed very poorly at 25 °C. Lowering temperature to 15 °C and using the strain ArcticExpress, protein production was improved, but was still rather low (~2 mg protein per L culture), especially compared to OXC (~30 mg/L). The low expression and/or stability limit the use of human HACL for biocatalytic applications. To test their carboligation activity, the enzymes were incubated with formyl-CoA and various aldehyde acceptors. 2-Hydroxyacyl-CoA thioesters were analyzed by LC-MS and the products verified by MS/MS fragmentation (Figure S1). Product formation was detected in the presence of formaldehyde,

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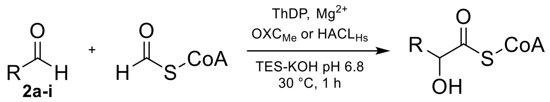
acetaldehyde, propionaldehyde, glycolaldehyde, succinic semialdehyde, benzaldehyde, and phenylacetaldehyde with both enzymes (Table 1). Glyceraldehyde, glyoxylate and acetone (not shown) were not accepted by OXC_{Me} and $HACL_{Hs}$. As expected from its physiological role as fatty acyl-CoA lyase, $HACL_{Hs}$ showed high activity with the aliphatic aldehydes acetaldehyde and propionaldehyde. On the other hand, OXC_{Me} showed very high activity with benzaldehyde, indicating a distinct catalytic spectrum between the two enzymes. In summary, $HACL_{Hs}$ and OXC_{Me} are able to perform nucleophilic C_1-extension reactions of different aldehydes with formyl-CoA as C_1-donor.

Next, we investigated whether both enzymes would also be able to perform decarboxylating carboligations with oxalyl-CoA as C_1-donor. We argued that the release of CO_2 should provide a strong thermodynamic driving force towards carboligation, analogous to the reactions reported for glyoxylate carboligase,[12] PDC,[13] BFD,[14] TK,[5a, 5b] and branched chain ketoacid decarboxylase.[15] $HACL_{Hs}$ possessed only very low oxalyl-CoA decarboxylation activity (k_{cat} < 1 min^{-1}, see Figure S2), in contrast to OXC_{Me} (k_{cat} = 98 ± 3 s^{-1}, see Table 2 & Figure S3), which confirms OXC_{Me}'s physiological function as oxalyl-CoA decarboxylase.

Table 1. Comparison of the aldehyde substrate scope of OXC_{Me} and $HACL_{Hs}$.[a]



Aldehyde	R	Product name	OXC_{Me}[c]	$HACL_{Hs}$[c]
2a	H	glycolyl-CoA	100	11
2b	Me	lactyl-CoA	74	100
2c	CH_2Me	2-hydroxybutyryl-CoA	5	100
2d	CH_2OH	glyceryl-CoA	1	100
2e	$CHOHCH_2OH$	erythronyl-CoA	n.d.[b]	n.d.[b]
2f	COOH	tartronyl-CoA	n.d.[b]	n.d.[b]
2g	$(CH_2)_2COOH$	2-hydroxyglutaryl-CoA	1	100
2h	Ph	mandelyl-CoA	100	3
2i	CH_2Ph	3-phenyllactyl-CoA	22	100

[a] The reaction contained **2a-2i** (10 mM), formyl-CoA (1 mM), OXC_{Me} or $HACL_{Hs}$ (5 µM). Products were analyzed by LC-MS after 1 h reaction time. [b] Product not detected. [c] Relative activity in %. Relative activity refers to the comparison of OXC_{Me} and $HACL_{Hs}$ for each aldehyde substrate.

Given the high activity towards benzaldehyde, we chose it as model substrate to study the decarboxylating carboligation of OXC_{Me} in more detail. When started with oxalyl-CoA and benzaldehyde, OXC_{Me} as expected produced mandelyl-CoA at a rate of 4 min^{-1} (Figure S4). However, mandelyl-CoA formation was preceded by formation of formyl-CoA, suggesting that OXC_{Me} first rapidly decarboxylated oxalyl-CoA into formyl-CoA, followed by slow carboligation of formyl-CoA with benzaldehyde (Figure S4).

Decarboxylation of oxalyl-CoA proceeds via formation of **1**, which can be either protonated and released as formyl-CoA or undergo a nucleophilic attack on benzaldehyde to form mandelyl-CoA. We speculated that mandelyl-CoA formation may be increased by suppressing the unwanted protonation reaction (and subsequent release of formyl-CoA) that competes with carboligation.

Structural, biochemical and theoretical studies on OXC from *Oxalobacter formigenes* (OXC_{Of}; 63% sequence identity to OXC_{Me}) demonstrated that protonation of **1** is mediated by a water molecule which is coordinated by several polar side chains, notably a tyrosine and a serine, which are conserved in OXC_{Me} (Tyr497 and Ser568).[10] In OXC_{Me} variants Y497A and S568A, formyl-CoA formation was decreased 20- to 50-fold, while the K_M of both variants was largely unaffected (Table 2 & Figure S3). When starting with oxalyl-CoA and benzaldehyde $OXC_{Me\text{-}Y497A}$ showed 5-fold increased mandelyl-CoA production rate (20 min^{-1}; Figure S4). The mutation Y497A thus increased the ratio of carboligation to decarboxylation by a factor of approximately 400 compared to the wild-type, suggesting that we successfully redirected activity of the enzyme towards carboligation by suppressing protonation of **1**.

Table 2: Steady-state kinetic parameters of oxalyl-CoA decarboxylation catalyzed by OXC_{Me}.[a]

Mutation	k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M (s^{-1} M^{-1})
wild-type	98 ± 3	105 ± 11	9.3×10^5
Y497A	1.32 ± 0.04	180 ± 17	7.3×10^3
S568A	5.53 ± 0.11	23 ± 2	1.1×10^5
Y497A S568A	0.32 ± 0.01	103 ± 15	3.1×10^3

[a] Michaelis-Menten graphs are shown in Figure S3.

Hydrolysis of mandelyl-CoA leads to mandelic acid, which serves as building block for various drugs as well as a resolving agent in chiral resolution processes.[16] We set out to identify a thioesterase capable of hydrolyzing the non-natural metabolite mandelyl-CoA. We tested PaaI, TesB and YciA from *E. coli*, which were shown to hydrolyze a broad range of acyl-CoA thioesters.[17] YciA showed relatively high mandelyl-CoA thioesterase activity (k_{obs} ≈ 1.5 s^{-1}), low activity with formyl-CoA (k_{obs} ≈ 0.06 s^{-1}) and no activity with oxalyl-CoA (Figure S5 & 1B). When used in combination with OXC_{Me}, the two enzymes formed an enzymatic cascade for the conversion of oxalyl-CoA and benzaldehyde into mandelic acid and free CoA (data not shown).

We noticed that subsequent regeneration of free CoA into oxalyl-CoA would allow us to close a catalytic cycle for the continuous production of mandelic acid (Figure 1A). To establish such a catalytic cycle, we obtained an oxalyl-CoA synthetase AMP-forming (OXS) homologue from *M. extorquens*,[18] and determined the enzyme's steady-state kinetic parameters (k_{cat} = 1.30 ± 0.02 s^{-1}; K_M(oxalate) = 9 ± 1 µM; Figure 1C). We then used OXS in combination with YciA and OXC_{Me} to continuously produce mandelic acid from oxalate and benzaldehyde. When we replaced OXC_{Me} by the Y497A variant, mandelic acid production rate increased 5-fold and the conversion increased 4-fold (Figure 1E). This was likely caused by decreased formation of the unwanted side product formyl-CoA (and its further hydrolysis by YciA). Chiral LC-MS revealed that (*S*)-mandelic acid was produced with enantiomeric excess of 97% (Figure 1D). Since YciA showed no stereospecificity in the hydrolysis of mandelyl-CoA (Figure 1B), the stereochemistry is exclusively determined by OXC_{Me}.

Next, we tested the substrate scope of the catalytic cycle by replacing benzaldehyde with substituted variants **2i**-**2k** (Figure 2A). Under limiting ATP concentrations (10 mM), the expected products **3a**-**3d** were formed at varying yields (57-93%) and *ee* (44-99%) (Figure 2B). Notably, also sterically demanding **2k** was converted to **3d** with high yield, albeit with moderate *ee*. The broad substrate scope of the OXS-OXC-YciA cascade was further confirmed by an extended screen, in which product formation was detected for ten other aromatic and three heteroaromatic aldehydes (**3e**-**3r**, Figure 2C). To test if the cascade can be scaled up, we performed the reaction on a semi-preparative scale (0.625 mmol **2h**). We added an ATP regeneration system comprising of creatine phosphate, creatine kinase and adenylate kinase. With catalytic amounts of ADP (0.5 mM) this five enzyme one-pot cascade produced **3a** with a yield of 53%. Taken together, these results indicate that the established enzyme cascade can be employed to produce various aromatic α-hydroxy acids with moderate to high (*S*)-selectivity.

We demonstrated that members of the OXC/HACL superfamily are able to catalyze C_1-carboligation reactions between **1** – formed either through decarboxylation of oxalyl-CoA or deprotonation of formyl-CoA – and several aldehydes to yield chiral 2-hydroxyacyl-CoA thioesters. These nucleophilic C_1-extension reactions expand the spectrum of ThDP-dependent enzymes as versatile biocatalysts for C-C bond forming reactions.[19]

What determines substrate specificity in the OXC/HACL superfamily? The observed variance in the aldehyde scope of OXC_{Me} and $HACL_{Hs}$ are likely caused by differences in the C-terminal domain, which forms a ‘lid’ on top of the active site.[10a] While the bottom part of the active site is virtually identical between OXC and HACL (with exception of Tyr133 and Glu134 in OXC_{Me} that are replaced by Phe and Gln in $HACL_{Hs}$), the C-terminal lid-domain shows a high variability between individual superfamily members. Further characterization of the OXC/HACL superfamily could reveal more carboligases with aldehyde preference for a desired application.

We engineered OXC_{Me} towards improved carboligation rate at the expense of formyl-CoA formation rate by replacing Tyr497 with Ala. Interestingly, the mutation Y497A did not affect the enantioselectivity for (*S*)-mandelyl-CoA. This is reminiscent of engineering efforts on PDC from *Zymomonas mobilis*, where Glu473 positions a water molecule that acts as proton donor for the ThDP carbanion/enamine intermediate. Mutating this amino acid to glutamine led to a 100-fold preference of carboligation over aldehyde release, under full retention of enantioselectivity.[13] Considering the high demand of mandelic acid and its derivatives in the (*R*) configuration,[16] it would be interesting to engineer OXC_{Me} towards inverted enantioselectivity. This has been achieved for other ThDP-dependent carboligases.[20]

The rational engineering of $OXC_{Me\text{-}Y497A}$ enabled a three enzyme cascade comprising of OXS, a newly identified oxalyl-CoA synthetase, and YciA, an efficient mandelyl-CoA thioesterase with only minor formyl- and oxalyl-CoA hydrolysis activities. The OXC_{Me}-mediated production of aromatic (*S*)-α-hydroxy acids in high *ee* from aldehydes and oxalate offers an alternative to hydrogen cyanide based syntheses catalyzed by nitrilases.[21] However, the requirements of CoA in catalytic amounts and an ATP regeneration system may limit the potential for a synthetic application on the larger scale. To this end, employing whole-cell catalysts may prove to be advantageous, providing not only the cascade enzymes but also ATP regeneration and a CoA pool without addition of purified enzymes and cofactors.[22]

Altogether, our study expands the spectrum of ThDP-dependent transformations by nucleophilic C_1-extensions, which gives access to α-hydroxy acids that are valuable chiral building blocks and showcases ways to establish *in vitro*- and *in vivo*-platforms for the continuous production of these compounds in the future.

Experimental Section

Experimental Details can be found in the Supporting Information.

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Keywords: oxalyl-CoA decarboxylase • thiamine diphosphate • C-C coupling • C1 building blocks • biocatalysis

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Figure 1. A) The OXS-OXC$_{Me}$-YciA cascade converts benzaldehyde and oxalate into mandelic acid and CO_2 under consumption of ATP. B) Thioesterase activity of YciA (2 µM) towards oxalyl-CoA, formyl-CoA and racemic mandelyl-CoA (0.5 mM each). The negative control without enzyme is shown in Figure S6. Error bars show standard deviation of two replicates. C) Michaelis-Menten graph of OXS with oxalate as substrate. D) Chromatograms of chiral HPLC analyzing the reaction products of the complete cascade with either wild-type OXC$_{Me}$ (wt) or Y497A. On top are commercial mandelic acid standards. E) Mandelic acid formation of the cascade over time. The reaction contained **2h** (25 mM), disodium oxalate (10 mM), ATP (10 mM), CoA (0.5 mM), OXS (5 µM), OXC (5 µM), YciA (2 µM). *ee* of (*S*)-mandelic acid is indicated for the last time point (22 h).

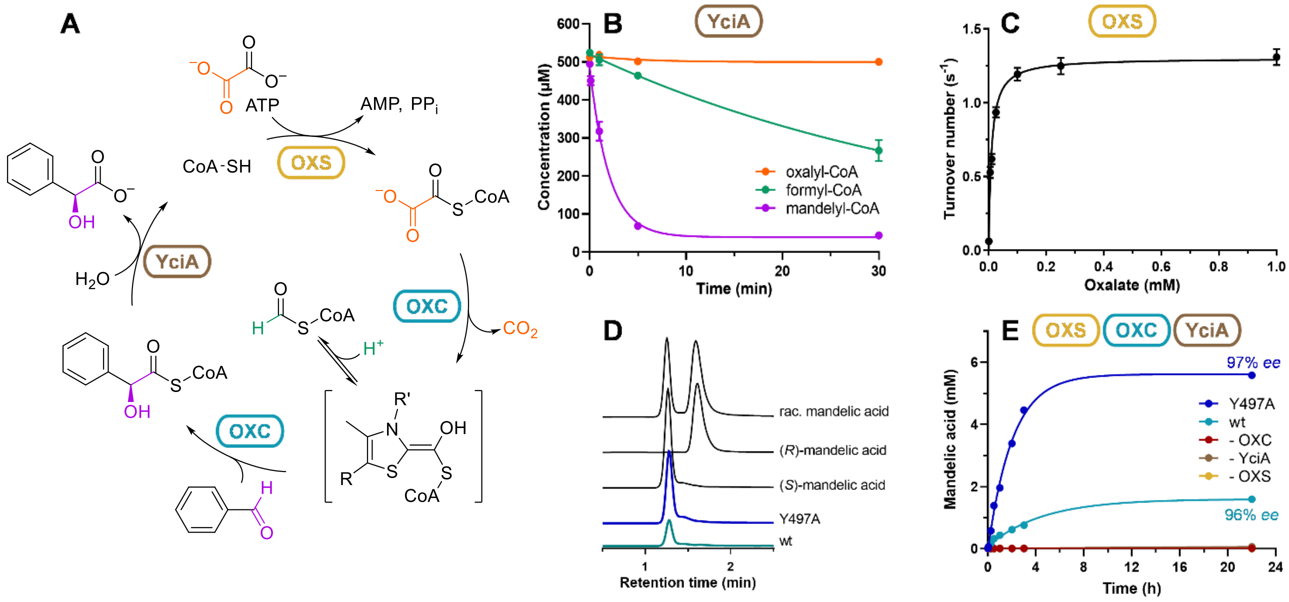

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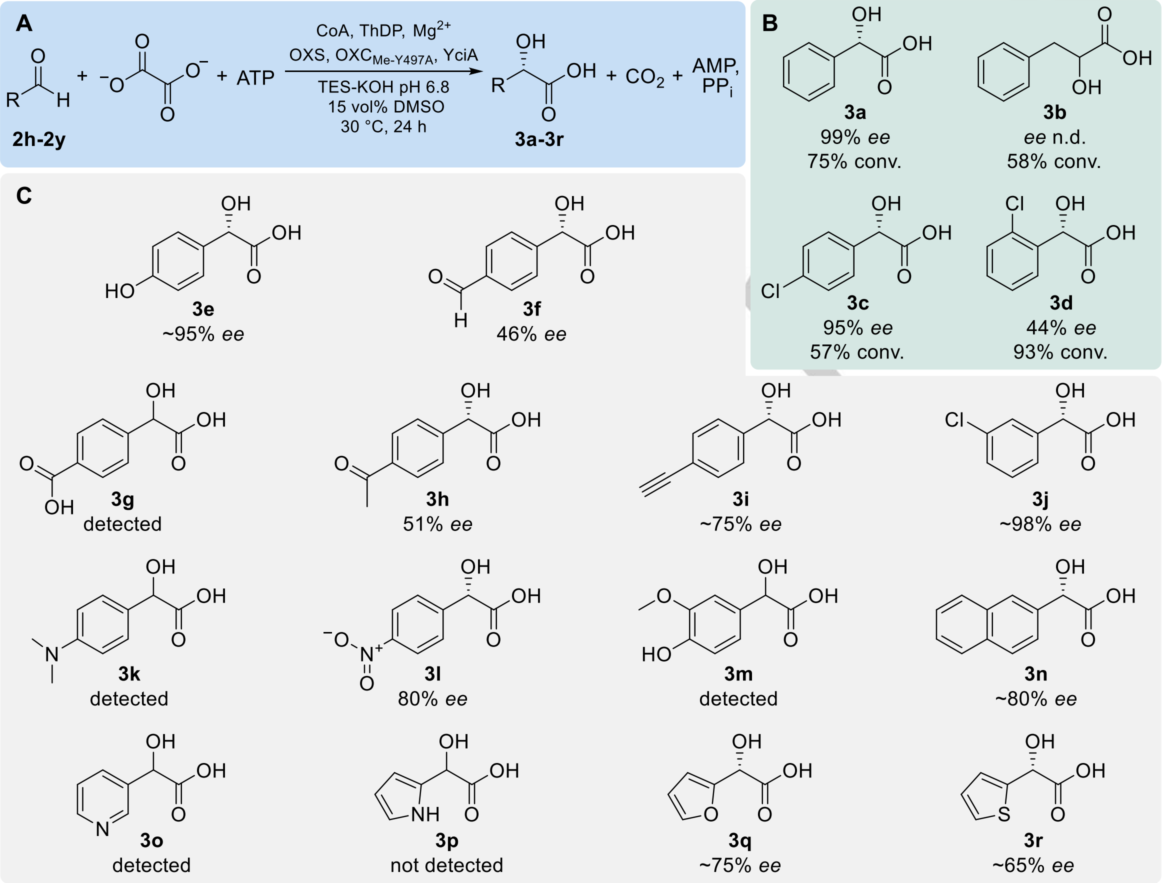
Figure 2. Scope of the OXS-OXC$_{Me\text{-}Y497A}$-YciA cascade for the synthesis of aromatic (*S*)-α-hydroxy acids. Chromatograms and time courses of the reactions are shown in Figure S7. A) The reactions contained **2h**-**2y** (25 mM), disodium oxalate (10 mM), ATP (10 mM), CoA (0.5 mM), OXS (5 µM), OXC$_{Me\text{-}Y497A}$ (5 µM), YciA (2 µM). Products were analyzed by chiral LC-MS after 24 h reaction time. *ee's* were estimated based on extracted ion counts. B) For **3a**-**3d** conversion and *ee* were quantified by comparison to a commercial standard. For **3b** separation of the enantiomers could not be achieved; n.d, not determined. C) **3e**-**3r** were analyzed qualitatively. Where chromatographic separation allowed, the *ee* was estimated, assuming identical ionization of the two enantiomers.



Graphical abstract