VIP Enzyme Catalysis

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Biocatalytic Access to α,α-Dialkyl-α-amino Acids by a Mechanism-Based Approach**

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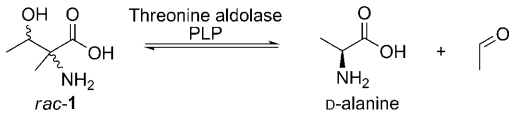
α,α-Dialkyl-α-amino acids[1] are important as building blocks of pharmaceuticals,[2a] as enzyme inhibitors,[2b] and as conformational modifiers of physiologically active peptides.[3] These compounds are not naturally occurring, and their synthesis is still a challenging task, comprising several steps and with difficult control of stereoselectivity.[4]

The application of an enzyme-catalyzed aldol reaction between an aldehyde and an amino acid provides a stereoselective pathway to β-hydroxy-α-amino acids. Aldolases applied for this purpose are tolerant towards the acceptor aldehyde, but they are quite stringent for the donor compound. Up to now only glycine has been accepted as a donor.[5] Acceptance of other amino acids would increase the possible product range to β-hydroxy-α,α-dialkyl-α-amino acids. Recently, α-methyl-3-nitro-β-phenylserine was obtained in low yield (12%) from the reaction of 3-nitrobenzaldehyde and D-alanine using a mutated alanine racemase (replacement of Tyr265 by Ala).[6] An α-methylserine hydroxymethyltransferase overexpressed in *E. coli* was applied to transform formaldehyde and D-alanine into α-methyl-L-serine in 64% yield and L-2-aminobutyric acid into α-ethyl-L-serine (55% yield).[7] No further examples were given.

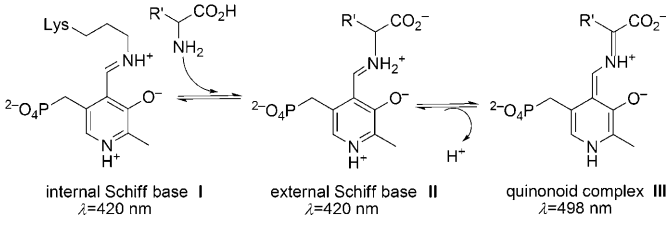
Recently a biocatalytic approach to enantiopure β-hydroxy-α-amino acids has been developed by application of threonine aldolases and investigated in detail in our group.[8] In a search for potential catalysts for the asymmetric synthesis of α,α-dialkyl-α-amino acids, we found two natural threonine aldolases that catalyze the cleavage of racemic α-methylthreonine (**1**) to produce acetaldehyde and D-alanine: an L-*allo*-threonine aldolase from *Aeromonas jandaei* (L-TA)[9] and a D-threonine aldolase from *Pseudomonas* sp. (D-TA; Scheme 1). The reactions proceeded with excellent



Scheme 1. Retro-aldol cleavage of α-methylthreonine **1** using L- and D-threonine aldolases.

enantioselectivity: only L-**1** was stereoselectively cleaved by L-TA and only the D isomers were accepted by D-TA (see the Supporting Information). Owing to the reversibility of aldol reactions, α-methylthreonine was produced in an aldol addition of acetaldehyde with an excess of alanine as donor catalyzed by either L-TA and D-TA.

To obtain more information on the donor-accepting properties of the two enzymes and to access to other β-hydroxy-α,α-dialkyl-α-amino acids, a mechanism-based approach was chosen. In threonine aldolases the cofactor pyridoxal phosphate (PLP) is bound to a lysine residue within the active site.[10] After entrance of the donor amino acid into the active site it is bound to PLP, and by proton abstraction a quinoid structure with an absorption maximum at 498 nm is formed (Scheme 2).[10] Besides glycine, only D-alanine and D-serine (but not the respective L enantiomers) form these complexes with the previously identified L-TA and D-TA and effect the corresponding spectral changes (Figure 1). D-cysteine shows only a weak band at 498 nm and an additional absorption maximum at 330 nm due to a thiozolidine formed from PLP and cysteine.[11]



Scheme 2. Formation of a quinoid complex between amino acid donors and the PLP cofactor in threonine aldolase.

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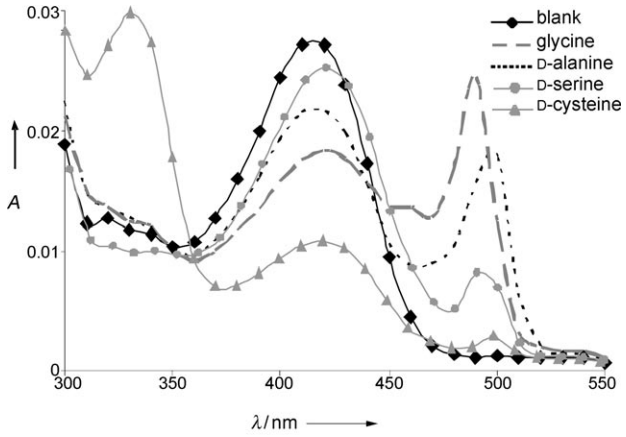
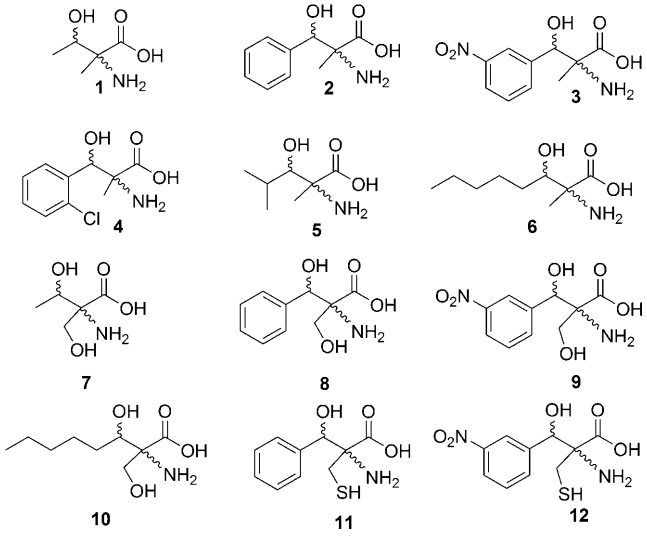


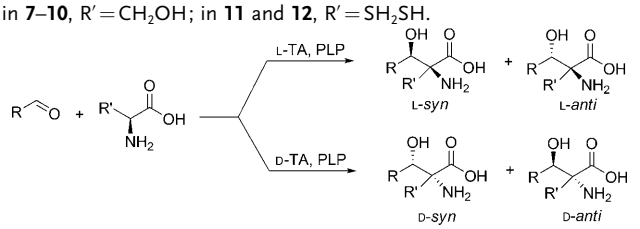
Figure 1. UV/Vis spectra of L-TA in the presence of glycine, D-alanine, D-serine, and D-cysteine in 50 mM phosphate buffer at pH 8.0.

Having identified potential donors, we synthesized β-hydroxy-α,α-dialkyl-α-amino acids starting from a range of aldehydes as acceptors and D-alanine, D-serine, or D-cysteine as donors (Scheme 3; Table 1). A wide range of L- and D-α-alkylserine derivatives were successfully produced with excellent enantiospecificity at the α-carbon atom (>99% *ee* at this center; Table 1). The acceptor specificity of L-TA is similar to that previously reported for the reactions when glycine was used as a donor.[8] The best substrates are aromatic aldehydes bearing electron-withdrawing groups which increase the electrophilic reactivity of the acceptor. For instance, 3-nitrobenzaldehyde was efficiently converted to L-**3** with 60% yield, whereas unsubstituted L-**2** was obtained with only 35% yield (Table 1, entries 2 and 3). A thermodynamic mixture of diastereomers ($de \leq 40\%$) was usually formed during the L-TA-catalyzed reactions. This can be explained by an unfavorable position of the equilibrium, which is reached very fast in this case.[12] On the other hand, the reactions with D-TA achieve the equilibrium state later



Scheme 3. β-Hydroxy-α,α-dialkyl-α-amino acids obtained in aldol reactions using L- and D-threonine aldolase.

Table 1: Stereoselective synthesis of β-hydroxy-α,α-dialkyl-α-amino acids using L- or D-threonine aldolase.[a] For R see Scheme 3; in **1**–**6**, R′ = CH_3; in **7**–**10**, R′ = CH_2OH; in **11** and **12**, R′ = SH_2SH.



	L-TA *Aeromonas jandaei*				D-TA *Pseudomonas* sp.		
Entry	Product	[%]	*de* [%][b]	Entry	Product	[%]	*de* [%][b]
1	L-**1**	20	46 (*anti*)	13	D-**1**	54	42 (*syn*)
2	L-**2**	35	6 (*anti*)	14	D-**2**	11	65 (*syn*)
3	L-**3**	60	7 (*anti*)	15	D-**3**	36	76 (*syn*)
4	L-**4**	24	35 (*syn*)	16	D-**4**	21	95 (*syn*)
5	L-**5**	6	26 (*anti*)	17	D-**5**	32	66 (*syn*)
6	L-**6**	58	8 (*anti*)	18	D-**6**	84	33 (*syn*)
7	L-**7**	6	65 (*anti*)	19	D-**7**	23	11 (*anti*)
8	L-**8**	10	40 (*anti*)	20	D-**8**	<1	n.d.
9	L-**9**	15	65 (*anti*)	21	D-**9**	<5	23 (*anti*)
10	L-**10**	30	45 (*anti*)	22	D-**10**	43	24 (*syn*)
11	L-**11**[c]	27	18 (*anti*)	23	D-**11**[c]	33	20 (*anti*)
12	L-**12**[c]	30	12 (*anti*)	24	D-**12**[c]	39	6 (*anti*)

[a] Reaction conditions: 1 mL reaction volume, 0.5 M D-alanine (products **1**–**6**), D-serine (products **7**–**12**), or D-cysteine (products **11**, **12**), 0.1 M corresponding aldehyde, PLP 50 μM, 30°C for L-TA, 10°C for D-TA, TA 4 U; conversions were determined after 24 h by HPLC and ^{1}H NMR analysis. [b] *ee* and *de* values were determined after precolumn derivatization by HPLC analysis on a reversed-phase column; in all reactions >99% *ee* was determined for reactions of both L-TA and D-TA (see the Supporting Information). [c] Determined by ^{1}H NMR analysis; n.d.: not determined.

and, as a result, products with a high diastereomeric ratio (up to 95% *de*) and moderate yields were obtained in the kinetically controlled mode. D-TA shows a broad flexibility for the acceptor carbonyl compound, among which aliphatic aldehydes were the best substrates. Optically pure long-chain D-**6**—a possible precursor for myriocin and sphingofungines E and F[13]—was obtained in 84% yield and 33% diastereomeric excess. Reactions with D-serine and D-cysteine usually gave lower yields than reactions of their α-methyl analogues (Table 1, entries 7–12 and 19–24).

Our results represent the first example of a biocatalytic asymmetric aldol synthesis of α-substituted serine derivatives using threonine aldolases. Moreover, our finding offers the possibility of accessing both enantiomers by choosing either L-TA or D-TA.

Reactions catalyzed by natural enzymes often suffer from low diastereoselectivities; this can be overcome by enzyme engineering, for example by rational protein design and directed evolution to enhance the utility of the biocatalyst. Another promising alternative would be the removal of the hydroxy group from the product β-hydroxy-α,α-dialkyl-α-amino acids by hydrogenation[19] such that optically pure L- or D-α,α-disubstituted α-amino acids would be obtained.

Finally, to find a possible explanation for the donor specificity in the tested aldolases, we constructed the homology model of L-TA from *A. jandaei* based on the known

crystal structure of L-TA from *Thermatoga maritima*[14] and a phenylserine aldolase from *Pseudomonas putida*.[15] Neither of these two enzymes can accept donors other than glycine. However, the comparison of their structures with the homology model of L-TA from *A. jandaei* does not show clear differences within the substrate-binding site that could explain the donor specificity in the investigated L-TA. It seems that donor specificity might be a consequence of more complex interactions.

The strict specificity of L-TA from *A. jandaei* towards the D isomer of a donor can be explained by modeling both enantiomers of alanine into the active site of the homology model (Figure 2). The amino acid forms a Schiff base with the PLP cofactor; in this structure the C_α–H bond must be perpendicular to the plane of the PLP ring in order to be cleaved.[16] In this case the α proton of D-alanine (which corresponds to the pro-2*S* proton in glycine) is abstracted most probably by a water molecule, which appears to be activated by the negatively charged PLP phosphate, His85, and His128 on the *re* face of the cofactor, opposite to the PLP-binding lysine Lys199. The substrate aldehyde enters the active site from the same side at which the C–C bond formation maintains the configuration of the α-carbon. His85 and possibly His128 are likely candidates for the further protonation of the hydroxy group at C_β of the thus-formed L product.[14] On the other hand, when L-alanine is bound to PLP, the deprotonation of C_α must occur from the *si* face of PLP. However, no residues were found in this area that could serve as the catalytic bases. Consequently, only the D isomer of a donor can be deprotonated and forms a quinoid complex with the cofactor. A more detailed discussion of the mechanism proposed is given in the Supporting Information.

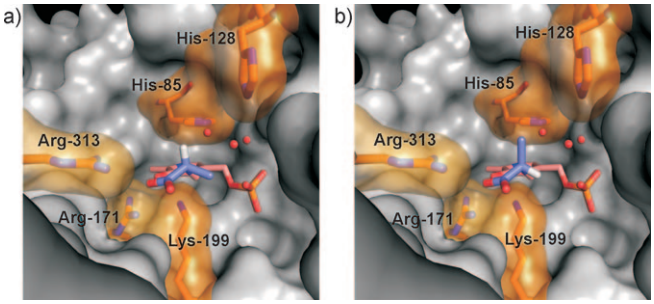


Figure 2. The active site of the L-TA *Aeromonas jandaei* model: a) complex with D-alanine (the α proton points towards the potential catalytic base); b) complex with L-alanine (the α proton points away from the potential catalytic base).

At present no crystal structure exists for D-threonine aldolase, but its similarity to bacterial alanine racemase (AR) was postulated based on the amino acid sequence.[17] Analogous to AR from *Bacillus stearothermophilus* (but in contrast to the situation in L-TA described above) the mechanism of catalysis of D-TA might involve two catalytic bases, which are located on opposite faces of PLP.[18] Thus, the α proton would be abstracted from D-alanine by a base located on one side of the PLP ring, whereas the substrate binding and the protonation of the β-hydroxy group would occur on the other side yielding a D-configured product. The second base must be located rather far from C_α to avoid the racemization of alanine.

In summary, we have found the first natural threonine aldolases that accept other donors than glycine, for example, alanine, serine and cysteine. The strict specificity for D-amino acids as donors exhibited by both L-TA and D-TA provided additional insight into mechanistic differences regarding the location of active site bases. Our findings increase the substrate range of aldolases and opens new routes for the unique and simple biocatalytic synthesis of highly valuable enantiopure L- or D-α-alkylserine derivatives. Moreover, the high enantioselectivity of these enzymes can be used for the kinetic resolution of chemically produced DL-*syn* or DL-*anti* mixtures of β-hydroxy-α-quaternary amino acids to obtain pure diastereomers. Genetic engineering of these threonine aldolases to enhance diastereoselectivities and optimization of reaction protocols will be the options to further improve the outcome of these biocatalytic transformations.[20]

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