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Threonine aldolases—an emerging tool for organic synthesis

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Abstract—In a systematic study, 21 ring-substituted benzaldehydes were reacted with glycine under catalysis with a L-threonine aldolase (LTA) from *Pseudomonas putida* and a D-threonine aldolase (DTA) from *Alcaligenes xylosoxidans* to form the corresponding β-hydroxy-α-amino acids **1**–**18**. DTA proved to be highly selective with ee's >99% (D) and de's up to 99% (*syn*). Two thiamphenicol precursors were synthesized utilizing DTA on a preparative scale. LTA-catalyzed reactions led to ee's >99% (L) but low to moderate de's (20–50%, *syn*).

1. Introduction

β-Hydroxy-α-amino acids and the corresponding amino alcohols are valuable precursors for active ingredients of pharmaceuticals and agrochemicals.[1–4] Furthermore, these compounds are ubiquitous in nature and important as both biological amino acids (threonine, serine, and 3-hydroxyproline) as well as key structural units in numerous bioactive natural products like antibiotics (chloramphenicol,[5,6] glycopeptides, e.g., vancomycin, ristocetin),[7,8] antifungal agents (sphingofungins),[9,10] immunosuppressants (mycestericins),[11] HIV-inhibitory and cytotoxic depsipeptides (papuamides,[12] callipeltin E[13]), anticancer agents (polyoxypeptin A),[14] neurotrophic agents (lactacystin),[14,15] and anti-inflammatory agents (cyclomarins).[16–18] In addition, they are useful chiral intermediates due to their ability to undergo a variety of transformations.

Since the end of the 1980s, tremendous efforts have been devoted to the synthesis of optically pure β-hydroxy-α-amino acids using chemical approaches such as Sharpless dihydroxylation,[19] Sharpless epoxidation,[15] Sharpless aminohydroxylation,[20] dynamic kinetic resolution via ruthenium/rhodium catalyzed dehydrogenation of *N*-substituted α-amino-β-keto esters,[21–27] aziridine ring-opening,[28] aza-Claisen rearrangements of allylic acetimidates,[29] addition of imides to aldehydes,[30] organocatalytic asymmetric aldol reactions of α-amino aldehydes,[31] catalytic glycine-derived silicon enolates,[32] chiral ammonium salts,[33,34] ammonium ylides,[35] radical bromination of protected amino acids forming *trans*-oxazolidinones as intermediates,[36] and numerous others.

Although each of these approaches represents an elegant methodology, there still remain some limitations such as insufficient stereoselectivity, expensive precursors or the need of stoichiometric amounts of chiral auxiliaries. In contrast, the direct enzymatic synthesis of β-hydroxy-α-amino acids from glycine and appropriate aldehydes is a onestep process under mild conditions with minimal protection needs for sensitive functionalities and hence may complement the established chemical methods.[37,38] This was first shown for whole cell fermentation back in 1975[39] and 1987.[40] Since then several threonine aldolases (TAs, EC 4.1.2.5)[41] and serine hydroxymethyl transferases (EC 2.1.2.1),[42–45] one L-phenylserine aldolase (EC 4.1.2.26),[46] and one D-3-hydroxyaspartate aldolase (EC 4.1.3.14)[47] have been discovered. Threonine aldolases use pyridoxal-5′-phosphate (PLP) as a co-factor[48,49] to promote the physiological degradation of threonine forming glycine and acetaldehyde. In the reverse reaction, PLP activates glycine in a non-physiological aldol reaction (Scheme 1). The low acidity of the C_α-protons prevents competing chemical background reactions. Genes encoding L-threonine aldolase (LTA) have been found in plants,[50] vertebrates,[51] several bacteria, yeasts, and fungi.[41] Two X-ray structures of LTAs are described in the literature.[52,53] In contrast, only a few D-threonine aldolase genes (DTA) have been reported so far.[41]

A decade ago, L- and D-threonine aldolases have been studied for the biocatalytic synthesis of β-hydroxy-α-amino

Keywords: Lyase; Threonine aldolase; Biocatalysis; Amino acid.
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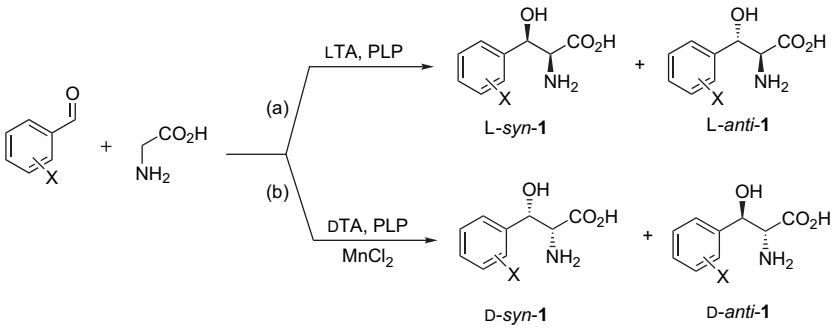


Scheme 1. Threonine aldolase catalyzed synthesis of (a) L-phenylserine L-**1** and (b) D-phenylserine D-**1**.

acids using various aldehydes and glycine. The reaction proceeded with complete stereospecificity at the α-carbon.[54–56] However, these investigations suffer from modest yields and low diastereoselectivities. These obstacles were overcome for β- and γ-benzyloxy aldehydes as was shown in the synthesis of monobactams[57] and mycestericin D and F.[58,59] Recently, L-phenylserine aldolase (from *P. putida*)[60] and D-3-hydroxyaspartate aldolase (from *Paracoccus denitrificans* IFO 13301)[61] were characterized with a similar substrate range compared to LTA and DTA, respectively, but very low diastereoselectivities for the produced β-hydroxy-α-amino acids. Additionally, the structure of L-phenylserine aldolase was obtained (pdb-entry: 1v72). Interestingly, the high structural similarity of PLP enzymes was shown by a novel engineered DTA, which was obtained by a single point mutation of alanine racemase (EC 5.1.1.1).[62]

Surprisingly, most enzymatic studies have focused on the kinetic resolution of chemically produced DL-*syn* mixtures (max. 50% yield)[63–65] and not on the more promising aldol reaction creating two stereogenic centers (max. 100% yield).[54–58,66] Herein, we report on an improved enzymatic procedure using D-threonine aldolases from *A. xylosoxidans*,[65] which leads to high enantio- and diastereoselectivity, whereas LTA from *P. putida*[67] gave only moderate selectivity. Both enzymes were also tested on benzaldehyde derivatives, which had been described as unfavored substrates.[56] The reactions were completed after 1–4 h with generally high analytical yields, although strongly depending on the reaction conditions.

2. Results and discussion

2.1. Enzyme overexpression and properties of LTA and DTA

Recombinant *Escherichia coli* L- and DTA expression strains based on the arabinose inducible pBAD plasmid system (Invitrogen) were obtained as published.[68] Threonine aldolase activity was measured by an NADH coupled assay with yeast alcohol dehydrogenase (Sigma). In this assay, L- or D-threonine is converted into glycine and acetaldehyde by the action of TA. Then, by the action of yeast alcohol dehydrogenase, the released acetaldehyde is reduced to ethanol with simultaneous oxidation of NADH to NAD^+ monitored by the decrease of absorbance at 340 nm. Recombinant overexpression of LTA yielded 16,000 units from 1 L cell culture. The crude enzyme extract prepared by cell disruption and centrifugation had a specific activity of 20 U/mg total protein (Biorad). Approximately 4800 U (D-threonine) of overexpressed DTA was obtained from 1 L cell culture and the specific activity was determined for the crude enzyme to be 8 U/mg.

2.2. Assignment of absolute configuration

The analytical yields and diastereoselectivities were monitored by HPLC and NMR. The assignment for *syn*- and *anti*-phenylserine derivatives **1**–**18** was based on the coupling constant between the α- and β-protons, which is slightly larger for the *syn*- than for the *anti*-isomer.[56,69] Further confirmation was obtained from the shift of the α-proton, which is lower for *syn* than for *anti*-products.[56] L- and D-threonine aldolases have been shown to be highly selective for the stereoconfiguration at the α-carbon.[56,61] This was demonstrated by HPLC using well-established derivatization methods for the analysis of amino acids on an achiral RP18 column (*o*-phthaldialdehyde OPA/2-mercaptoethanol achiral derivatization for *syn*/*anti* determination and OPA/*N*-acetyl-cysteine chiral derivatization for the separation of all four diastereoisomers).[70,71] Additionally, chiral HPLC analytics using Crownpack® CR(+) allowed the assignment for L- and D-isomers due to the fact that the D-amino acid always elutes first.

2.3. Temperature profile and timescale for the synthesis reaction

For the synthesis of D-phenylserine D-**1** employing DTA, the rate of the aldol reaction was studied from 5 to 40 °C (Table 1). As it was shown before, a high excess of glycine shifts the equilibrium toward the phenylserine side and thus 10 equiv of glycine was utilized (1 M glycine, 100 mM benzaldehyde) throughout the entire study.[56] Lowering the temperature to 5 °C resulted in an increase of diastereoselectivity (entry 1, up to 98%) as well as in a higher analytical yield

Table 1. DTA-catalyzed synthesis of D-*syn*-**1** at different temperatures

Entries	*T*/(°C)	Time/(h)	Analytical yield/(%)	de/(%)
1	5	4	79	98
2	20	2	72	92
3	30	1	43	91
4	40	1	45	73

Conditions: 1 mL buffer solution (pH 8) containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM), $MnCl_2$ (50 μM), and DTA (23 U); ee >99% (D) for all reactions; all results obtained by HPLC.

(79%) after 4 h. Rising the temperature to 20 and 30 °C resulted in a slightly lower de (entries 2 and 3) whereas at 40 °C, the selectivity is significantly decreased (entry 4). It is assumed that a lower temperature reduces the reaction rate and as a consequence, the formation of the kinetic product is favored. To the best of our knowledge, our results represent the first example of D-*syn*-**1** being produced in an enzyme-catalyzed procedure with high diastereoselectivity and analytical yield. No similar effects were detected for LTA at temperatures below 40 °C and after 2 h the maximum analytical yield of L-*syn*/*anti*-**1** was obtained (85% yield, de 20% *syn*).

2.4. pH dependency

LTA and DTA reversibly catalyze the formation and cleavage of **1**. The mechanism published by John indicates that the reaction is sensitive to pH as general base catalysis is involved.[48] Hence, the pH dependency of LTA and DTA was determined in order to obtain information of the possible range of process optimization (Fig. 1). LTA is stable between pH 5.5 and 8 but shows a clear decrease of activity at pH 5.0. On the contrary, DTA shows little activity at pH 6.0. However, by increasing the pH to 9.5 the analytical yield of D-**1** was doubled retaining high diastereoselectivity as compared to pH 8.0.

2.5. Co-solvents

The tolerance of LTA and DTA concerning co-solvents was tested on the synthesis of **1** (Table 2). Surprisingly, the solvents employed did not improve the performance of the reaction as it had been shown for other threonine aldolases.[56] Nevertheless, DMSO and DMF were both tolerated by LTA and DTA up to 20 and 30%, respectively (entries 1 and 2). LTA was less sensitive toward co-solvents compared to DTA as demonstrated by CH_3CN and EtOH, which were tolerated up to 10 and 20%, respectively (entries 3 and 4). TBME (up to 30% v/v) was shown to be the only water-immiscible co-solvent, which allowed running the LTA-catalyzed reaction (entry 5) without any loss of performance. All other co-solvents such as THF, Et_2O, toluene, and cyclohexane inactivated the enzymes irreversibly (entry 6).

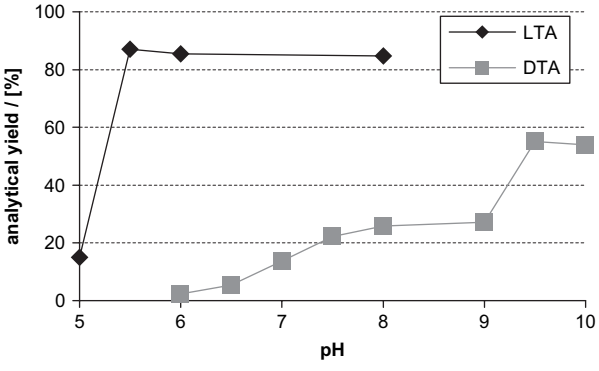


Figure 1. Synthesis of (a) L-*syn*/*anti*-**1** utilizing LTA and (b) D-*syn*/*anti*-**1** employing DTA in different buffers; Conditions: 1-mL solution containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM) at 25 °C; (a) LTA (77 U), ee >99% (L) and de 25% (*syn*) for all reactions; 2 h; (b) $MnCl_2$ (50 μM) and DTA (23 U); ee >99% (D) and de >90% (*syn*) for all reactions; 1 h; all results obtained by HPLC.

Table 2. Synthesis of **1**: amount (% v/v) of co-solvent used without any loss of activity and selectivity; LTA: analytical yield 40%, de 20% (*syn*); DTA: analytical yield 65%, de 85% (*syn*)

No.	Co-solvent	Co-solvent % (v/v) LTA	Co-solvent % (v/v) DTA[a]
1	DMSO	30	30
2	DMF	20	20
3	EtOH	20	<10
4	CH_3CN	10	<10
5	TBME	30	<10
6	THF, toluene, Et_2O, cyclohexane	<10	<10

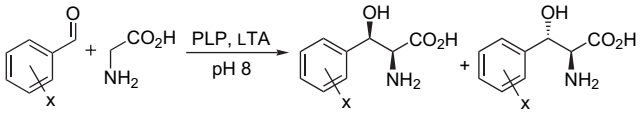
Conditions: 1 mL solution containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM), and LTA (77 U) or DTA (23 U) at 25 °C; ee >99% (D) for DTA and ee >99% (L) for LTA; LTA: 1 h; DTA: 2 h; analytical yield and de determined by HPLC.
[a] Additional: $MnCl_2$ (50 μM).

2.6. Synthesis of phenylserine derivatives 1–18

Threonine aldolases have been tested on a variety of aldehydes for the synthesis of β-hydroxy-α-amino acids in the past.[54–56] Nevertheless, a detailed study on benzaldehyde derivatives would help to understand the influence of aryl substituents for further modeling of the active sites of LTA and DTA. For the enzymes investigated no structural data exist to date whereas three structures of LTAs from other microorganisms are available. Additionally, some industrially relevant precursors for bioactive compounds, for example, thiamphenicol were synthesized and isolated.

2.6.1. L-Threonine aldolase. Following the preliminary studies, the substrate range for LTA investigated was extended to various benzaldehyde derivatives and the results were compared with benzaldehyde (Table 3, entry 1). Fluorobenzaldehydes showed good analytical yields and no significant change in selectivity (entries 2–4). 2-Chlorobenzaldehyde gave high analytical yield and increased de of 52% (entry 5). However, L-**6** and especially L-**7** were only obtained with significant lower analytical yields and de's (entries 6 and 7). All bromobenzaldehydes showed a similar decrease in analytical yield from *ortho* to *meta* and *para* compared to the chloroderivatives (entries 8–10 vs 5–7). L-**9** was obtained with similar de's compared to L-**5** (entry 5 vs 9). Apparently, the steric demand of 2-chloro and 3-bromobenzaldehyde improves the selectivity significantly. The low analytical yields of L-**7** and L-**10** may be explained by the low solubility of the corresponding aldehydes. L-**11**–**13** and the sulfonamide compound L-**15** showed high analytical yield with low selectivity whereas the methylsulfonylbenzaldehyde gave an increased selectivity of de 53% (entries 11–15). L-**11**–**15** are precursors for thiamphenicol and chloramphenicol isomers[7] and L-**16** is a precursor for the amino alcohol norfenefrin.[72] L-**16** was obtained with moderate analytical yield and selectivity (entry 16). Similarly, L-**17**—which can be converted to octopamin[73]—was synthesized although with low analytical yield and selectivity (entry 17). L-3,4-Dihydroxyphenylserine is an utmost important target. It is used as a drug against Parkinson's disease but can also be used for the synthesis of noradrenaline.[74,75] Since 4-hydroxybenzaldehyde was a poor substrate, it was not surprising that neither 3,4-dihydroxy- nor 3,5-dihydroxybenzaldehyde or 4-hydroxy-3-(hydroxymethyl)benzaldehyde was accepted by LTA (entries 18, 20, and 21). The

Table 3. LTA-catalyzed synthesis of L-phenylserine derivatives L-**1**–**18**



L-*syn* **1-18** L-*anti* **1-18**

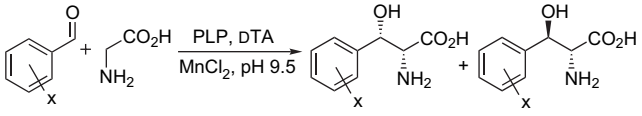
Entries	X	Analytical yield (%)[a]	de (%)[a]	Entries	X	Analytical yield (%)[a]	de (%)[a]
1	H L-**1**	85	20	12	3-NO_2 L-**12**	74	21
2	2-F L-**2**	68	35	13	4-NO_2 L-**13**	79	24
3	3-F L-**3**	64	27	14	4-$MeSO_2$ L-**14**	68	53
4	4-F L-**4**	51	29	15	2-NH_2SO_2 L-**15**	92[b]	24[b]
5	2-Cl L-**5**	90	52	16	3-OH L-**16**	56	51
6	3-Cl L-**6**	69	30	17	4-OH L-**17**	11[b]	36[b]
7	4-Cl L-**7**	57	17	18	3,4-OH	<1[b]	n.d.
8	2-Br L-**8**	79	34	19	3,4-O(Me) L-**18**	15	26
9	3-Br L-**9**	63	55	20	3-MeOH-4-OH	<1[b]	n.d.
10	4-Br L-**10**	47	14	21	3,5-OH	<1[b]	n.d.
11	2-NO_2 L-**11**	99	32				

Conditions: 1 mL solution containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM), and LTA (77 U) at 25 °C; 1–2 h; ee >99% (L) for all reactions.
[a] Determined by HPLC.
[b] Determined by ^{1}H NMR.

latter two substrates serve as starting material for the synthesis of levabuterol and terbutaline, respectively.[76,77] To eliminate the unfavorable effects of both hydroxy groups, piperonal was applied as protected 3,4-dihydroxybenzaldehyde and furnished the corresponding product L-**18** with low analytical yield and selectivity (entry 19). This strategy renders dihydroxybenzaldehyde compounds applicable to the TA technology.

2.6.2. D-Threonine aldolase. Consequently, the same benzaldehyde derivatives were tested also on D-threonine aldolase. D-**1** was obtained with good analytical yield and excellent de (Table 4, entry 1). All fluoro-substrates gave similar analytical yields but only D-**2** and D-**4** were obtained with comparable de's (entries 2–4). Similar effects were found for chloro- and bromobenzaldehydes (entries 5–10). The *meta* compounds D-**6**,**9** were gained with highest analytical yield, the *meta* D-**6**,**9** and *para* products D-**7**,**10** gave good de's. However, the analytical yields of 2- and 4-halogenated compounds D-**5**,**7**,**8**,**10** were modest and the C_β-selectivities for both *ortho*-substrates were lower compared to benzaldehyde. Thus, the active site of DTA seems to be more rigid compared to LTA, complicating the accommodation of bulky benzaldehydes, especially with *ortho*-substituents. This effect can also be seen for 2-nitrobenzaldehyde, which only gave D-**11** with low analytical yield and decreased selectivity (entry 11). On the contrary, the other nitro-compounds were obtained with good (D-**12**) and very high (D-**13**) de's (entries 12 and 13). Both the methylsulfonium and sulfonamide benzaldehydes were converted with similar analytical yields and very high *syn*/*anti* ratios (entries 14 and 15). 3-Hydroxybenzaldehyde was well accepted

Table 4. DTA-catalyzed synthesis of D-phenylserine derivatives D-**1**–**18**



D-*syn* **1-18** D-*anti* **1-18**

Entries	X	Analytical yield (%)[a]	de (%)[a]	Entries	X	Analytical yield (%)[a]	de (%)[a]
1	H D-**1**	79	98	12	3-NO_2 D-**12**	90	80
2	2-F D-**2**	68	95	13	4-NO_2 D-**13**	31	75
3	3-F D-**3**	54	81	14	4-$MeSO_2$ D-**14**	63	99
4	4-F D-**4**	42	91	15	2-NH_2SO_2 D-**15**	53[b]	>90[b]
5	2-Cl D-**5**	27	67	16	3-OH D-**16**	76	86
6	3-Cl D-**6**	60	85	17	4-OH D-**17**	15[b]	70[b]
7	4-Cl D-**7**	26	86	18	3,4-OH	<1[b]	n.d.
8	2-Br D-**8**	6	35	19	3,4-O(Me) D-**18**	16[b]	46[b]
9	3-Br D-**9**	43	71	20	3-MeOH-4-OH	<1[b]	n.d.
10	4-Br D-**10**	12	74	21	3,5-OH	<1[b]	n.d.
11	2-NO_2 D-**11**	18	65				

Conditions: 1 mL solution containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM), $MnCl_2$ (50 μM), and DTA (23 U) at 5 °C; 4 h; ee >99% (D) for all reactions.
[a] Determined by HPLC.
[b] Determined by ^{1}H NMR.

by DTA forming D-**16** whereas 4-hydroxybenzaldehyde furnished the corresponding product D-**17**, similar to the LTA-catalyzed reaction, with low analytical yield (entries 16 and 17). All dihydroxy compounds (entries 18, 20, and 21) remained unconverted. However, piperonal as protected analog of the unfavored 3,4-dihydroxybenzaldehyde gave D-**18** with low analytical yield and selectivity (entry 19).

2.7. Separation of derivatized *syn*/*anti*-1

Unfortunately, the low diastereoselectivity of LTA is a severe drawback for preparative application. Hence, new strategies involving selective coupled reactions and genetically improved enzymes are under investigation. For larger scale, recrystallization of the *syn*/*anti*-mixture has been applied and pure products were obtained.[56] Small amounts of *syn*/*anti*-**1** derivatives can be separated by a two-step derivatization involving an initial esterification[78] and subsequent BOC protection of the amino group.[79] The diprotected phenylserines were separated by flash chromatography yielding enriched *syn*- and *anti*-products.

2.8. Thiamphenicol

Several pharmaceuticals can be found in the literature, which contain the physiologically essential β-hydroxy-α-amino functionalities, for example, thiamphenicol (D-*syn*-2-dichloracetamido-1-(4-methylsulfonylphenyl)-propan-1,3-diol) and analogs (D-*syn*-2-dichloracetamido-1-(4-sulfamoylphenyl)-propan-1,3-diol). Hence, 4-formyl-benzenesulfonamide was synthesized as precursor using standard reactions.[80–82] Finally, the corresponding phenylserine derivatives D-*syn*-**14**,**15** were synthesized using DTA and isolated in good yields, absolute stereocontrol of the α-carbon (ee >99%) and excellent stereocontrol even for the β-carbon (Scheme 2). Further conversion to yield the desired thiamphenicol enantiomer can easily be achieved using standard transformations.[83] Additionally, the physiologically active enantiomer L-**14** was isolated in good yield (75%) but low de (20% *syn*).

2.9. Different donors

Aldolases are known to be flexible for the acceptor but rigid for the donor substrate. To clarify the situation for the enzymes in our hands we investigated whether LTA and DTA act on donor substrates such as D- and L-alanine, DL-leucine, glycine ethylester, glycine amide, and ethanolamine together with benzaldehyde or acetaldehyde. Deplorably, no conversion to the corresponding β-hydroxy-α-amino derivatives was detected.

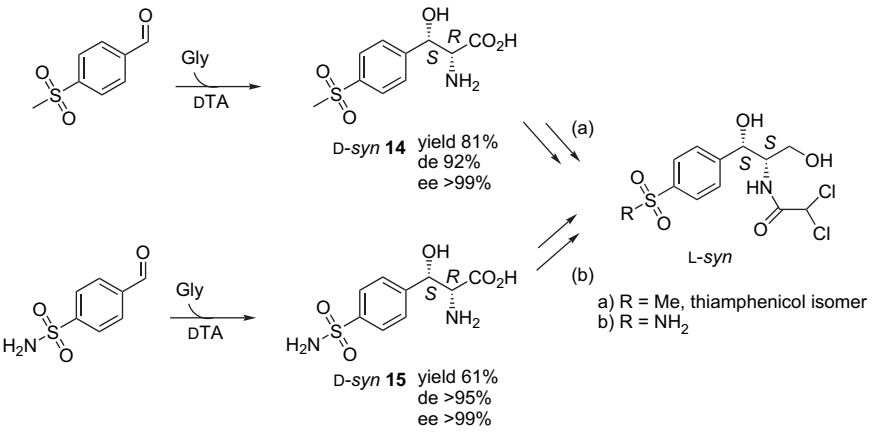
3. Conclusion

In summary, both enzymes—LTA from *P. putida* and DTA from *A. xylosoxidans*—were tested on a variety of benzaldehyde derivatives as acceptor substrates and the reaction conditions were optimized. DTA accepted all benzaldehydes excluding the dihydroxy substrates and furnished D-*syn* products with ee's >99% and de's up to 99% (*syn*). To outline the synthetic applicability of these reactions, a precursor of a thiamphenicol isomer and an analog were synthesized and isolated on a preparative scale with high yields. In case of LTA, the same benzaldehyde derivatives were well-accepted substrates and gave the corresponding amino acids with high analytical yields and ee's.

4. Experimental

4.1. General experimental procedures

All reagents and solvents were obtained from commercial sources and appropriately purified, if necessary. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 500 (^{1}H 499.82 MHz, ^{13}C 125.69 MHz) or on a Varian GEMINI 200 (^{1}H 199.98 MHz, ^{13}C 50.29 MHz) using the residual peaks of $CDCl_3$ (^{1}H: δ 7.26, ^{13}C: δ 77.0), D_2O (^{1}H: δ 4.79) or DMSO-d_6 (^{1}H: δ 2.50, ^{13}C: δ 40.2) as references. H_2O/D_2O-NMR samples were taken directly from the aqueous solution, diluted with D_2O (1:1) and recorded using H_2O presaturation.[84] Analytical HPLC was carried out with a Hewlett Packard Series 1100 HPLC using a G1315A diode array detector. If not otherwise noted, a Purospher® STAR RP18 (250 mm, 5 μm) column was used for analysis. Analytical yields and de's of β-hydroxy-α-amino acids were determined by HPLC after derivatization with *ortho*-phthaldialdehyde/2-mercaptoethanol (OPA/MCE, achiral derivatization, de determination) or OPA/*N*-acetyl cysteine



Scheme 2. Synthetic route to (a) a thiamphenicol isomer and (b) a thiamphenicol analog.

(OPA/NAC, chiral derivatization, ee determination).[70,71] DL-*anti*-**1**[85] and DL-*syn*-phenylserine derivatives[86] were synthesized as reference materials using published methods. All phenylserine derivatives are known compounds and thus, no characterization using high-resolution MS or microanalysis was undertaken. Mostly, diastereomeric mixtures were obtained.

4.2. Overexpression

Both LTA and DTA were expressed from pBAD constructs containing the genuine coding DNA sequences under the control of the *ara* promotor. *E. coli* Top10F' cells containing the pBAD-LTA*Pp*12565 (LTA) or the pBAD-DTA*Af* (DTA) plasmids were cultivated on 2xTY media supplemented with 100 mg/mL ampicilline. Overnight cultures (100 mL) in 300 mL flasks were inoculated with single colonies and grown at 37 °C. Main cultures (330 mL) in baffled 1000 mL flasks were inoculated with 3 mL of the preculture and grown at 37 °C for approximately 4 h to an OD_{600} of 1.5. Temperature was then lowered to 28 °C and induced with L-arabinose at a final concentration of 0.002%. Cultivation was continued overnight and cells harvested by centrifugation for 15 min at 5000×*g*. After resuspension of the pellets in 0.1 M sodium phosphate buffer the cells were disrupted by ultrasonic treatment. The crude lysate was cleared by centrifugation at 20,000×*g* for 1 h and the supernatant (cell-free extract) stored frozen at −20 °C until use.

4.3. Activity assay

The assay mixture contained D- or L-threonine (50 mM), HEPES–NaOH buffer (100 mM, pH 8), PLP (50 μM), $MnCl_2$ (only for DTA, 100 μM), NADH (200 μM), yeast alcohol dehydrogenase (30 U, Sigma), and 50 μL of diluted cell-free extract (1:100 for DTA, 1:1000 for LTA) in a final volume of 3 mL. The reactions were started by the addition of the diluted cell-free extract and recorded by the decrease of absorbance at 340 nm in a spectrophotometer. One unit of the enzyme is the amount of enzyme that catalyzes the formation of 1 μmol of acetaldehyde (1 μmol of NADH oxidized) per minute at room temperature under above-described conditions.

4.4. General procedure for the synthesis of L-phenylserine derivatives

4.4.1. L-Phenylserine L-1. To a solution of LTA (77 U) and PLP (13 ng, 50 nmol) in 1 mL buffer (KH_2PO_4, 50 mM, pH 8.0) benzaldehyde (10 mg, 0.1 mmol) and glycine (75 mg, 1.0 mmol) were added. The reaction mixture was stirred at rt for 1 h to give L-*syn*/*anti*-**1**; analytical yield 85%; de 20% (*syn*); ee *syn*>99% (L), *anti*>99% (L); HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=73:27, 0.8 mL/min, t_{syn}=16.3 min, t_{anti}=21.6 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, $t_{D\text{-}syn}$=11.5 min, $t_{L\text{-}syn}$=15.4 min, $t_{D\text{-}anti}$=18.1 min, $t_{L\text{-}anti}$=21.4 min; NMR data were consistent with those reported.[87]

4.4.2. L-2-Fluorophenylserine L-2. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=11.9 min, t_{anti}=13.9 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=8.6 min, $t_{L\text{-}syn}$=10.4 min, $t_{D\text{-}anti}$=12.2 min, $t_{L\text{-}anti}$=13.9 min; NMR data were consistent with those reported.[87]

4.4.3. L-3-Fluorophenylserine L-3. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=14.1 min, t_{anti}=17.5 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=11.1 min, $t_{L\text{-}syn}$=14.1 min, $t_{D\text{-}anti}$=15.3 min, $t_{L\text{-}anti}$=17.6 min; ^{1}H NMR (500 MHz, D_2O): δ 3.71 (d, 0.63H, *syn*, *J*=4.5 Hz), 3.88 (d, 0.37H, *anti*, *J*=4.0 Hz), 5.10 (d, 0.63H, *syn*, *J*=4.5 Hz), 5.16 (d, 0.37H, *anti*, *J*=4.0 Hz), 7.00 (m, 3H), 7.25 (m, 1H).

4.4.4. L-4-Fluorophenylserine L-4. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=13.8 min, t_{anti}=17.7 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=10.4 min, $t_{L\text{-}syn}$=13.4 min, $t_{D\text{-}anti}$=15.5 min, $t_{L\text{-}anti}$=17.9 min; NMR data were consistent with those reported.[87]

4.4.5. L-2-Chlorophenylserine L-5. HLPC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=15.4 min, t_{anti}=19.3 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=11.3 min, $t_{L\text{-}syn}$=13.3 min, $t_{D\text{-}anti}$=19.6 min, $t_{L\text{-}anti}$=23.0 min; ^{1}H NMR (500 MHz, D_2O): δ 3.91 (d, 1H, *J*=3.5 Hz), 5.31 (d, 0.24H, *anti*, *J*=3.5 Hz), 5.48 (d, 0.76H, *syn*, *J*=3.5 Hz), 7.30 (m, 4H).

4.4.6. L-3-Chlorophenylserine L-6. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=22.5 min, t_{anti}=27.8 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=20.4 min, $t_{L\text{-}syn}$=26.8 min, $t_{D\text{-}anti}$=27.4 min, $t_{L\text{-}anti}$=32.5 min; ^{1}H NMR (500 MHz, D_2O): δ 3.70 (d, 0.65H, *syn*, *J*=4.5 Hz), 3.87 (d, 0.35H, *anti*, *J*=4.0 Hz), 5.07 (d, 0.65H, *syn*, *J*=4.5 Hz), 5.13 (d, 0.35H, *anti*, *J*=4.0 Hz), 7.20 (m, 4H).

4.4.7. L-4-Chlorophenylserine L-7. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=69:31, 0.8 mL/min, t_{syn}=17.3 min, t_{anti}=21.4 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=22.9 min, $t_{L\text{-}syn}$=30.4 min, $t_{D\text{-}anti}$=32.5 min, $t_{L\text{-}anti}$=39.1 min; NMR data were consistent with those reported.[88]

4.4.8. L-2-Bromophenylserine L-8. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=16.9 min, t_{anti}=21.7 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=79:21, 0.8 mL/min, $t_{D\text{-}syn}$=12.6 min, $t_{L\text{-}syn}$=14.8 min, $t_{D\text{-}anti}$=23.3 min, $t_{L\text{-}anti}$=27.4 min; ^{1}H NMR (500 MHz, D_2O): δ 3.91 (d, 0.67H, *syn*, *J*=3.5 Hz), 3.93 (d, 0.33H, *anti*, *J*=3.0 Hz), 5.23 (d, 0.33H, *anti*, *J*=3.0 Hz), 5.44 (d, 0.67H, *syn*, *J*=3.5 Hz), 7.08 (t, 0.33H, *anti*, *J*=8.0 Hz), 7.11 (t, 0.67H, *syn*, *J*=8.0 Hz), 7.23 (t, 0.33H, *anti*, *J*=8.0 Hz), 7.29 (t, 0.67H, *syn*, *J*=8.0 Hz), 7.35 (t, 0.33H, *anti*, *J*=8.0 Hz), 7.44 (d, 1H, *J*=8.0 Hz), 7.48 (d, 0.67H, *syn*, *J*=8.0 Hz).

4.4.9. L-3-Bromophenylserine L-9. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=69:31, 0.8 mL/min, t_{syn}=18.0 min, t_{anti}=21.9 min; OPA/NAC: buffer pH 8

(50 mM KH_2PO_4)/CH_3CN=76:24, 0.8 mL/min, $t_{D\text{-}syn}$=12.2 min, $t_{D\text{-}anti}$=15.1 min, $t_{L\text{-}syn}$=15.5 min, $t_{L\text{-}anti}$=17.8 min; ^{1}H NMR (500 MHz, D_2O): δ 3.69 (d, 0.77H, *syn*, *J*=4.5 Hz), 3.87 (d, 0.23H, *anti*, *J*=4.0 Hz), 5.07 (d, 0.77H, *syn*, *J*=4.5 Hz), 5.12 (d, 0.23H, *anti*, *J*=4.0 Hz), 7.18 (m 2H), 7.40 (m, 2H).

4.4.10. L-4-Bromophenylserine L-10. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=69:31, 0.8 mL/min, t_{syn}=20.9 min, t_{anti}=25.6 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=76:24, 0.8 mL/min, $t_{D\text{-}syn}$=13.8 min, $t_{L\text{-}syn}$=18.0 min, $t_{D\text{-}anti}$=18.2 min, $t_{L\text{-}anti}$=21.4 min; NMR data were consistent with those reported.[89]

4.4.11. L-2-Nitrophenylserine L-11. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=12.9 min, t_{anti}=13.8 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, $t_{D\text{-}syn}$=13.5 min, $t_{L\text{-}syn}$=15.2 min, $t_{D\text{-}anti}$=18.8 min, $t_{L\text{-}anti}$=20.6 min; ^{1}H NMR (500 MHz, D_2O): δ 3.94 (d, 0.66H, *syn*, *J*=4.0 Hz), 3.98 (d, 0.34H, *anti*, *J*=3.0 Hz), 5.54 (d, 0.34H, *anti*, *J*=3.0 Hz), 5.71 (d, 0.66H; *syn*, *J*=4.0 Hz), 7.39 (m, 1H), 7.64 (m, 2H), 7.94 (m, 1H).

4.4.12. L-3-Nitrophenylserine L-12. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=15.5 min, t_{anti}=18.8 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, $t_{D\text{-}syn}$=19.8 min, $t_{L\text{-}syn}$=24.3 min, $t_{D\text{-}anti}$=26.1 min, $t_{L\text{-}anti}$=29.7 min; ^{1}H NMR (500 MHz, D_2O): δ 3.75 (d, 0.61H, *syn*, *J*=4.5 Hz), 3.91 (d, 0.39H, *anti*, *J*=4.0 Hz), 5.18 (d, 0.61H, *syn*, *J*=4.5 Hz), 5.25 (d, 0.39H, *anti*, *J*=40 Hz), 7.44 (t, 0.39H, *anti*, *J*=8.0 Hz), 7.47 (t, 0.61H, *syn*, *J*=8.0 Hz), 7.57 (d, 0.39H, *anti*, *J*=8.0 Hz), 7.65 (d, 0.61H, *syn*, *J*=8.0 Hz), 8.03 (m, 1H), 8.07 (s, 0.39H, *anti*), 8.14 (s, 0.61H, *syn*).

4.4.13. L-4-Nitrophenylserine L-13. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=16.2 min, t_{anti}=19.9 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, $t_{D\text{-}syn}$=21.3 min, $t_{L\text{-}syn}$=26.6 min, $t_{D\text{-}anti}$=28.1 min, $t_{L\text{-}anti}$=31.0 min; ^{1}H NMR (500 MHz, D_2O): δ 3.74 (d, 0.62H, *syn*, *J*=4.5 Hz), 3.92 (d, 0.38H, *anti*, *J*=4.0 Hz), 5.19 (d, 0.62H, *syn*, *J*=4.5 Hz), 5.24 (d, 0.38H, *anti*, *J*=4.0 Hz), 7.41 (d, 0.76H, *anti*, *J*=8.0 Hz), 7.48 (d, 1.24H, *syn*, *J*=8.0 Hz), 8.05 (d, 0.76H, *anti*, *J*=8.5 Hz), 8.08 (d, 1.24H, *syn*, *J*=8.0 Hz).[88,90,91]

4.4.14. L-3-(4-Methylsulfonylphenyl)serine L-14. HPLC: OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=71:29, 0.8 mL/min, t_{syn}=5.8 min, t_{anti}=6.7 min; OPA/NAC: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, $t_{D\text{-}syn}$=6.1 min, $t_{L\text{-}syn}$=6.8 min, $t_{D\text{-}anti}$=8.0 min, $t_{L\text{-}anti}$=8.1 min; ^{1}H NMR (200 MHz, D_2O): δ 3.19 (s, 3H), 3.86 (d, 0.76H, *syn*, *J*=4.4 Hz), 4.04 (d, 0.24H, *anti*, *J*=3.6 Hz), 5.31 (d, 0.76H, *syn*, *J*=4.4 Hz), 5.38 (d, 0.24H, *anti*, *J*=3.6 Hz), 7.61 (m, 2H), 7.91 (m, 2H).[83,91]

4.4.15. L-2-Amino-3-hydroxy-3-(4-sulfamoylphenyl)-propionic acid L-15. The analytical yield was calculated by measuring the ratio of the remaining glycine and the product, and the ratio of *syn*/*anti* was determined by integration of the corresponding C_α-proton of the diastereomers; ^{1}H NMR (500 MHz, D_2O): δ 3.73 (d, 0.62H, *syn*, *J*=4.0 Hz), 3.91 (d, 0.38H, *anti*, *J*=3.5 Hz), 5.16 (d, 0.62H, *syn*, *J*=4.0 Hz), 5.22 (d, 0.38H, *anti*, *J*=3.5 Hz), 7.40 (d, 0.76H, *anti*, *J*=8.5 Hz), 7.47 (d, 1.24H, *syn*, *J*=8.5 Hz), 7.72 (d, 0.76H, *anti*, *J*=8.5 Hz), 7.75 (d, 1.24H, *syn*, *J*=8.5 Hz).

4.4.16. L-3-Hydroxyphenylserine L-16. HPLC: LiChrospher® 100 RP-18 column (250 mm, 5 µm), OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=81:19, 0.8 mL/min, t_{syn}=8.1 min, t_{anti}=12.1 min; ^{1}H NMR (500 MHz, D_2O): δ 3.70 (d, 0.75H, *syn*, *J*=4.5 Hz), 3.86 (d, 0.25H, *anti*, *J*=4.0 Hz), 5.06 (d, 0.75H, *syn*, *J*=4.5 Hz), 5.09 (d, 0.25H, *anti*, *J*=4.0 Hz), 6.75 (m, 3H), 7.13 (m, 1H).

4.4.17. L-4-Hydroxyphenylserine L-17. (See Section 4.4.15) ^{1}H NMR (500 MHz, D_2O): δ 3.66 (d, 0.68H, *syn*, *J*=5.0 Hz), 3.84 (d, 0.32H, *anti*, *J*=3.5 Hz), 5.00 (d, 0.68H, *syn*, *J*=5.0 Hz), 5.07 (d, 0.32H, *anti*, *J*=3.5 Hz), 6.53 (d, 0.64H, *anti*, *J*=9.0 Hz), 6.72 (d, 1.36H, *syn*, *J*=8.0 Hz), 7.06 (d, 0.64H, *anti*, *J*=9.0 Hz), 7.12 (d, 1.36H, *syn*, *J*=8.0 Hz).[40,91]

4.4.18. L-3,4-Methylenedioxyphenylserine L-18. HPLC: LiChrospher® 100 RP-18 column (250 mm, 5 µm), OPA/MCE: buffer pH 8 (50 mM KH_2PO_4)/CH_3CN=76:24, 0.8 mL/min, t_{syn}=8.9 min, t_{anti}=11.8 min; ^{1}H NMR (500 MHz, D_2O): δ 3.65 (d, 0.63H, *syn*, *J*=4.5 Hz), 3.82 (d, 0.37H, *anti*, *J*=4.5 Hz), 5.05 (m, 1H), 5.79 (d, 1H, *J*=4.5 Hz), 6.70 (m, 3H).[91,92]

4.5. General procedure for the synthesis of D-phenylserine derivatives

4.5.1. D-Phenylserine D-1. To a solution of DTA (23 U), $MnCl_2$ (6 ng, 50 nmol), and PLP (13 ng, 50 nmol) in 1 mL buffer (glycine 100 mM/NaCl 50 mM, pH 9.5) benzaldehyde (10 mg, 0.1 mmol) and glycine (75 mg, 1.0 mmol) were added. The reaction mixture was stirred at 5 °C for 3 h to give D-*syn*-**1**; analytical yield 79%; de 98% (*syn*); ee *syn*>99% (D), *anti*>99% (D).

4.5.2. D-3-(4-Methylsulfonylphenyl)serine D-14. To a solution of glycine (2.25 g, 30 mmol), PLP (0.4 mg, 1.5 µmol), $MnCl_2$ (0.18 mg, 1.5 µmol), and DTA (690 U) in 30 mL buffer (glycine 100 mM/NaCl 50 mM, pH 9.5) 4-(methylsulfonyl)benzaldehyde (500 mg, 2.7 mmol) was added and stirred at rt. After 2 h, the opaque, slightly yellow solution was diluted with 300 mL MeOH and stored at 4 °C overnight. Precipitated glycine was filtered off (1.8 g, 80% of starting glycine) and the filtrate was evaporated. The crude product was purified on a silica column (CH_2Cl_2/MeOH/NH_3 (30% in H_2O) gradient 75:20:5–10:10:1) to yield 569 mg D-*syn*-**14**; yield 81%; de 87% (*syn*); ee *syn*>99% (D), *anti*>99% (D); for HPLC and ^{1}H NMR, see Section 4.4.14; ^{13}C NMR (125 MHz, D_2O): *syn*: δ 43.5, 60.7, 71.0, 127.5, 127.9, 138.8, 146.5, 171.8.

4.5.3. D-2-Amino-3-hydroxy-3-(4-sulfamoylphenyl)-propanoic acid D-15. To a solution of glycine (1.13 g, 15 mmol), PLP (0.2 mg, 0.75 µmol), $MnCl_2$ (0.09 mg, 0.75 µmol), and DTA (345 U) in 15 mL buffer (glycine

100 mM/NaCl 50 mM, pH 9.5) was added 4-formylbenzenesulfonamide (250 mg, 1.3 mmol) and stirred at rt. After 4 h, the opaque, slightly yellow solution was diluted with 150 mL MeOH and stored at 4 °C overnight. Precipitated glycine was filtered off (0.59 g, 52% of starting glycine) and the filtrate was evaporated. The crude product was purified on a silica column (CH_2Cl_2/MeOH/NH_3 (30% in H_2O) gradient 75:20:5–10:10:1) to yield 205 mg D-*syn*-**15**; yield 61%; de >95% (*syn*); for ^{1}H NMR, see Section 4.4.15; ^{13}C NMR (125 MHz, D_2O): *syn*: δ 60.9, 71.4, 126.5, 127.2, 141.1, 145.1, 172.6.

4.6. L-3-(4-Methylsulfonylphenyl)serine L-14

To a solution of glycine (2.25 g, 30 mmol), PLP (0.4 mg, 1.5 μmol), and LTA (2300 U) in 30 mL buffer (KH_2PO_4, 50 mM, pH 8.0) was added 4-(methylsulfonyl)benzaldehyde (500 mg, 2.7 mmol) under stirring at rt. The opaque, slightly yellow solution was diluted with 300 mL MeOH after 2 h and incubated at 4 °C overnight. Excess glycine precipitated and was filtered off (1.8 g, 80% of starting glycine) and the filtrate was evaporated. The crude product was purified on a silica column (CH_2Cl_2/MeOH/NH_3 (30% in H_2O) gradient 75:20:5–10:10:1) to yield 526 mg L-*syn*/*anti*-**14**; yield 75%; de 2(*syn*); ee *syn*>99% (L), *anti*>99% (L); for HPLC and ^{1}H NMR, see Section 4.4.14; ^{13}C NMR (125 MHz, D_2O): *syn*+*anti*: δ 43.4, 43.5, 59.6, 60.7, 70.7, 71.0, 127.5 (2), 127.6, 127.9, 138.8 (2), 144.7, 146.5, 170.8, 171.8.

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