Enzymatic Synthesis of β-Hydroxy-α-amino Acids Based on Recombinant D- and L-Threonine Aldolases

Teiji Kimura, Vassil P. Vassilev, Gwo-Jenn Shen, and Chi-Huey Wong*

Contribution from the Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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Abstract: To exploit the enzymatic method for the synthesis of β-hydroxy-α-amino acids, the genes coding for the *Escherichia coli* L-threonine aldolase (LTA; EC 2.1.2.1) and *Xanthomonus oryzae* D-threonine aldolase (DTA) were cloned and overexpressed in *E. coli* through primer-directed polymerase chain reactions. The purified recombinant enzymes were studied with respect to kinetics, specificity, stability, additive requirement, temperature profile, and pH dependency. DTA requires magnesium ion as a cofactor, while LTA needs no metal ions. These enzymes work well in the presence of DMSO with concentration up to 40%, and DMSO-induced rate acceleration of LTA-catalyzed reaction was observed. Both enzymes use pyridoxal phosphate coenzyme to activate glycine to react with a wide range of aldehydes. LTA gave *erythro*-β-hydroxy-α-L-amino acids with aliphatic aldehydes and the *threo* isomer with aromatic aldehydes as kinetically controlled products. On the other hand, DTA formed *threo*-β-hydroxy-α-D-amino acids as kinetically controlled products with aliphatic and aromatic aldehydes but the diastereoselectivity was lower than that of LTA. Under optimal conditions, several β-hydroxy-α-amino acid derivatives (3-hydroxyleucines, γ-benzyloxythreonines, γ-benzyloxymethylthreonines, and polyoxamic acids) have been stereoselectively synthesized on preparative scales using these enzymes. Also, the tandem use of DTA and phosphatases has made possible the synthesis and separation of D-*allo*-threonine phosphate and D-threonine.

Introduction

β-Hydroxy-α-amino acids constitute an important class of compounds as naturally occurring amino acids (threonine, serine, and 3-hydroxyproline) and as components of many complex natural products possessing a wide range of biological activities such as antibiotics and immunosuppressants (e.g., vancomycin, echinocardin D, cyclosporin, katanosin, polyoxin D, empedopeptin, and other peptide conjugates).[1] (2*S*,3*S*)-3-Hydroxyleucine, for example, is a key component of several natural peptide antibiotics, while the 2*S*,3*R* isomer is found to be a constituent of lysobactin,[1d] a macrocyclic peptide lactone antibiotic. Recently, a novel neurotrophic agent, (+)-lactacystin, was synthesized from (2*R*,3*S*)-3-hydroxyleucine, and a multistep procedure for the synthesis of all four stereoisomers has been developed.[1d] The D-isomers are also biologically significant, as they have been recently found in mature mammals.[2] In addition, these amino acids are useful building blocks in synthetic, combinatorial (e.g., for making libraries of β-lactam and vancomycin antibiotics analogues, Scheme 1), and medicinal chemistry.[3]

A number of methods have been reported for the chemical synthesis of β-hydroxy-α-amino acids; of particular interest are those based on chiral glycine enolate,[4a] glycine Shiff base,[4b] asymmetric dihydroxylation,[4c] and aminohydroxylation[4d] of olefins. On the other hand, enzymatic synthesis of these compounds from glycine and aldehydes may complement the existing chemical methods as the enzymatic synthesis is a one-step process with minimal protection for substrates under mild conditions. In fact, the syntheses of β-hydroxy-α-amino acids using serine hydroxymethyl transferases (SHMTs) or threonine aldolases have been reported,[1a,5d] but the SHMT from pig liver[1a] and D-threonine aldolase (DTA) from *Arthrobacter sp.*[5c] pro-

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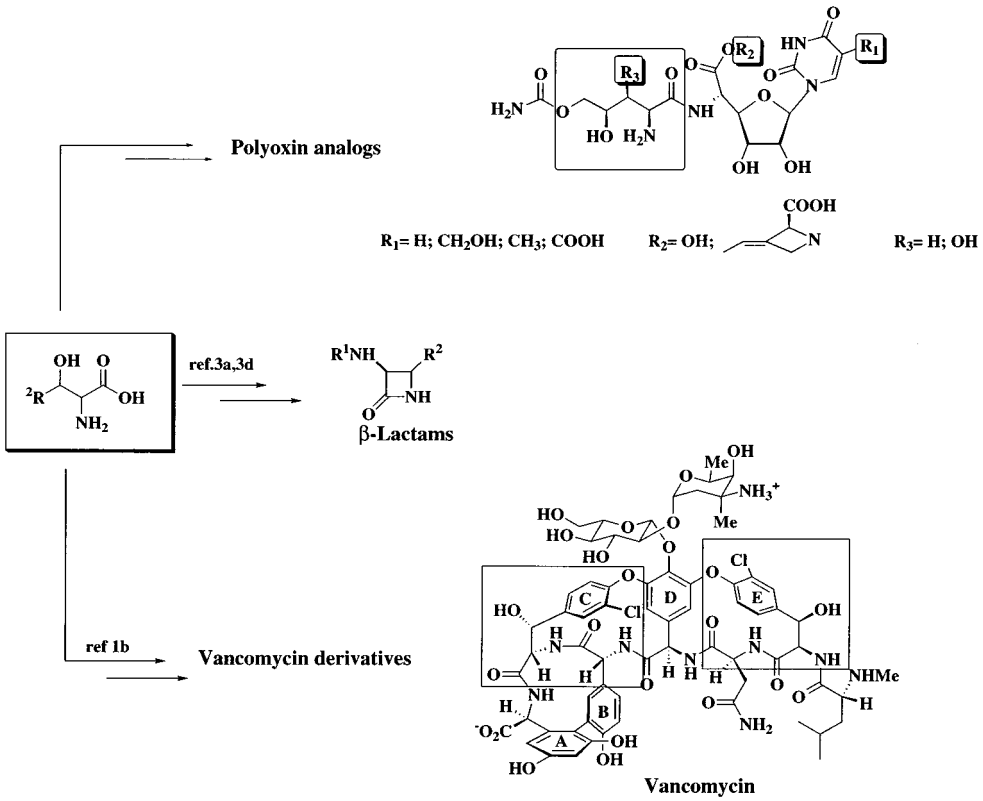
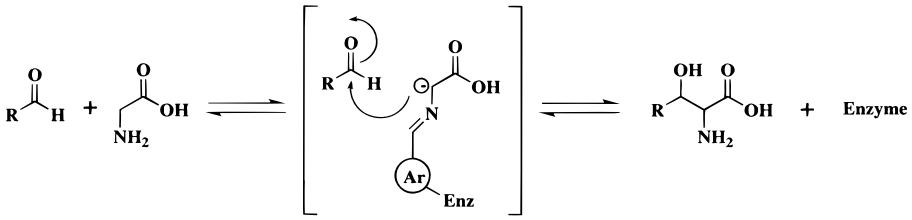
Scheme 1. β-Hydroxy-α-amino Acid as a Synthon



Scheme 2. Threonine Aldolase-Catalyzed Reaction



vided β-hydroxy-α-amino acids with virtually no stereoselectivity at the β-carbon. L-Threonine aldolase (LTA) isolated from *Candida humicola*[5a] and *Streptomyces amakusaensis*,[5b] however, stereoselectively gave β-hydroxy-α-amino acids with a limited number of substrates. To further exploit the synthetic utility of threonine aldolases, we have overproduced in *Escherichia coli* the recombinant D- and L-threonine aldolases from *Xanthomonus oryzae* and *E. coli*, respectively. Both enzymes use pyridoxal phosphate cofactor to activate glycine for the aldol addition reaction (Scheme 2).

Results and Discussion

Overexpression and Properties of L-Threonine Aldolase (LTA) and D-Threonine Aldolase (DTA). Since the genes coding for LTA from *E. coli*[6] and DTA from *X. oryzae*[7] have been cloned and sequenced, they were selected for overexpression of the corresponding enzymes.

On the basis of the retro-aldol cleavage reaction of threonine and *allo*-threonine, the specific activities and kinetic constants of these two enzymes were determined. Recombinant LTA was overproduced approximately 100 units (U, 1 unit = 1 μmol of L-*allo*-threonine cleaved per min) from 1 L of cell culture. The crude enzyme prepared after cell disruption and centrifugation has a specific activity of 0.7 U/mg. Further purification was carried out with affinity chromatography (using HisTrap) to obtain the enzyme with a specific activity of 3.5 U/mg and >95% purity as judged by SDS-polyacrylamide gel electrophoresis (Figure 1). The K_m value for L-*allo*-threonine is 0.62 mM, but that for L-threonine has not been determined, as the rate is too slow and the K_m value would be too high. The LTA has no activity for D-threonine and D-*allo*-threonine. On the basis of the results, perhaps LTA should be named L-*allo*-threonine aldolase. On the other hand, recombinant DTA was overexpressed at the level approximately 2800 U (based on D-threonine) from 1 L of cell culture. The crude and purified enzymes (>95% purity) prepared by the aforementioned method have specific activities of 88 and 153 U/mg, respectively. The K_m value for D-threonine was 0.94 mM and that for D-*allo*-threonine was 0.99 mM. L-Threonine and L-*allo*-threonine are not substrates for the DTA. The crude enzyme extraction was thus used directly for synthesis.

Enzyme Stability. During the initial experiments, the stability problem was encountered. In the absence of the reducing agent dithiothreitol (DTT), greater than 70% of LTA and DTA activities were lost after storing the cell free extracts overnight at 4 °C. This result is consistent with the report by

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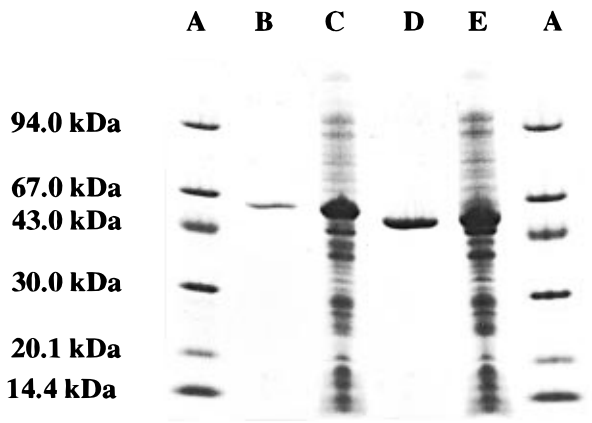

Figure 1. SDS gel electrophoresis: (A) molecular weight markers; (B) LTA after purification with His-Trap; (C) LTA, total protein from the recombinant cells; (D) DTA after purification with His-Trap; (E) DTA, total protein from the recombinant cells.

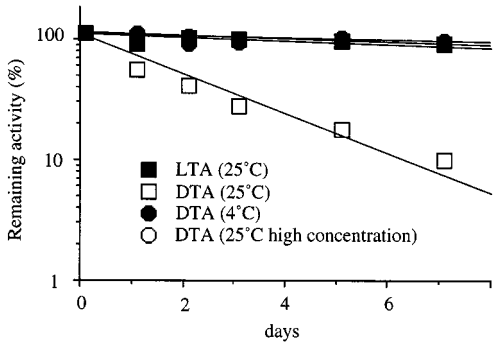

Figure 2. Stability of LTA and DTA.

Schirch et al.[8] that the LTA has one exposed sulfhydryl group. Both enzymes were, however, more stable at −78 °C for over 3 months in the presence of DTT. The enzyme stability was thus studied at room temperature for 1 week (Figure 2) in the presence of 10 mM DTT, and it was found that LTA (8 U/mL) retained more than 80% activity after 1 week, but DTA (5 U/mL) lost about one-half of its activity in approximately 1 day. However, when stored at 4 °C or at higher enzyme concentration (150 U/mL) at room temperature, DTA was stable and no significant loss of acvtivity was observed after 1 week.

Additive Requirement. It is well-known that a group of serine hydroxymethyl transferase and/or threonine aldolase requires pyridoxal-5-phosphate (PLP) as a cofactor.[9] In addition to the study of PLP dependency, we also investigated the requirement of metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Co^{2+}). DTA demands magnesium ion,[5c] while LTA needs no metal cofactor. Other divalent metal ions (e.g., Ni^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}) were not tested, as both enzymes contain a polyhistidine tag which would form a complex with the divalent cations.

To know which solvent can be used to help dissolve the substrates, several water-miscible organic solvents were used as cosolvent in the retro-aldol reactions. Surprisingly, DMSO (up to 30% concentration) accelerated the LTA-catalyzed reaction. In the case of DTA, although addition of DMSO does not drastically change the activity compared with LTA, the enzyme remained active in the presence of 40% DMSO (Figure 3). On the other hand, addition of 30% acetonitrile or DMF inactivated both enzymes.

The aldol reactions catalyzed by LTA and DTA were also studied in the absence and presence of additives (Table 1). Magnesium ions enhance the DTA-catalyzed aldol reaction.

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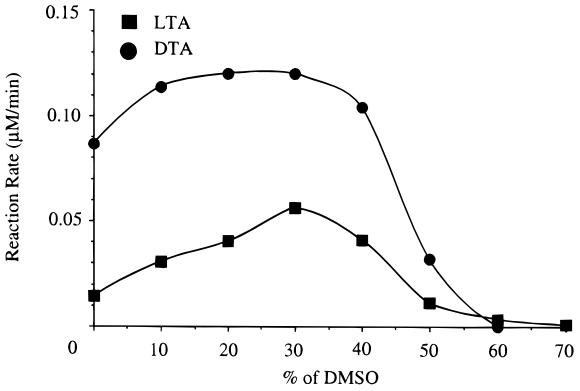
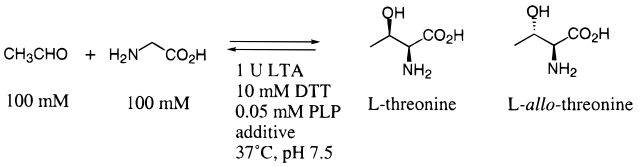
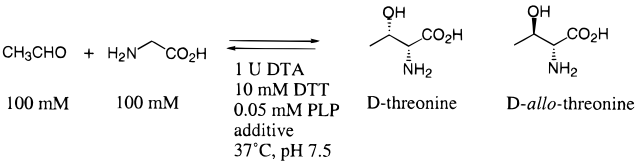


Figure 3. Effect of DMSO on LTA- and DTA-catalyzed retro-aldol reactions.

Table 1. Effect of Additives on LTA- or DTA-catalyzed Aldol Condesation of Acetaldehyde and Glycine



	3 h		24 h	
additives	yield (%)	*threo*/*erythro*	yield (%)	*threo*/*erythro*
none	18.0	<1/99	38.6	7/93
30% DMSO	34.5	<1/99	39.7	8/92



	3 h		24 h	
additives	yield (%)	*threo*/*erythro*	yield (%)	*threo*/*erythro*
none	10.2	63/37	2.3	61/39
10 mM $MgCl_2$	54.7	53/47	53.0	51/49
10 mM $MgCl_2$ + 30% DMSO	63.3	53/47	55.5	54/46

Although the addition of DMSO increased the rate for both LTA- and DTA-catalyzed aldol reaction, the product yields in the presence and absence of DMSO were basically the same when the reaction reached equilibrium. Regarding the stereoselectivity on β-carbon, LTA gave predominantly L-*allo*-threonine and DTA produced almost a 1:1 mixture of D-threonine and D-*allo*-threonine.

Temperature Profile. The rate of retro-aldol reaction was studied from room temperature to 70 °C. The reaction rate strongly depends on the reaction temperature. The LTA is not active at 25 °C, and the reaction rate at 50 °C is approximately 8 times faster than that at 37 °C. However, raising the temperature to 70 °C results in rapid enzyme inactivation. In the presence or absence of DMSO, the optimal reaction temperature for LTA is 50 °C and that for DTA is 37 °C. At 50 °C, DTA is rapidly inactivated. Both D-threonine and D-*allo*-threonine are equally good substrates for DTA.

The aldol reactions were further investigated at several temperatures. To obtain a kinetically controlled product, the reactions were terminated after 3 h in the presence of 1 U of enzyme, 100 mM of glycine, and 100 mM of aldehyde. In the case of LTA, increasing reaction temperature also increased the

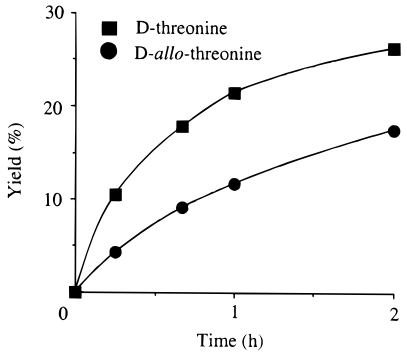


Figure 4. Time course of D-threonine and D-*allo*-threonine producted by DTA-catalyzed aldol reaction at 14 °C. The reaction mixture contains 1 U of DTA, 100 mM glycine, and 100 mM acetaldehyde.

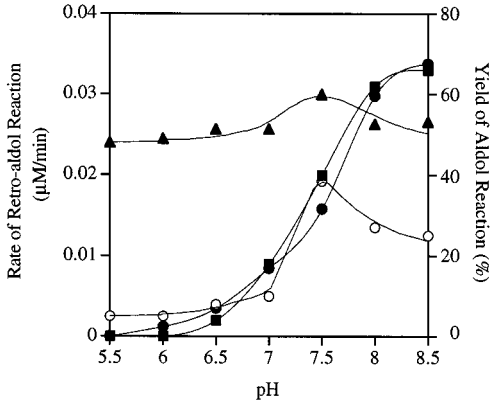


Figure 5. pH dependency of LTA- and DTA-catalyzed reactions. (■) LTA (retro-aldol): The reaction was carried out at 50 °C for 5 min in the presence of 30% DMSO. (●) DTA (retro-aldol): The reaction was carried out at 37 °C for 5 min in the presence of 10 mM $MgCl_2$. (▲) LTA (aldol): The reaction was carried out at 37 °C for 5 h in the presence of 30% DMSO. (○) DTA (aldol): The reaction was carried out at 37 °C for 5 h in the presence of 10 mM $MgCl_2$.

yield without affecting the diastereoselectivity. On the other hand, although raising the reaction temperature for DTA improved the yield, the diastereoselectivity decreased. This result indicates that DTA-catalyzed retroaldol reactions taken place within 3 h at 37 °C. In fact, when the reaction at 37 °C was terminated after 30 min, a higher diastereoselectivity was observed. To test if reducing the reaction temperature will minimize the formation of undesired isomer and give a better diastereoselectivity at β-carbon, enzymatic reactions at 14 °C were studied (Figure 4). Under the kinetically controlled approach at low temperature, the selectivity at β-carbon was improved and a 2:1 mixture of products in favor of the *threo* isomer was obtained.

pH Dependency. LTA and DTA reversibly catalyze aldol reactions. The proposed mechanism indicates that the reaction is sensitive to pH as a general base catalysis is involved.[9] To find an optimal pH for the synthesis direction, the pH dependencies of the synthetic and cleavage reactions were studied (Figure 5). In the cleavage reaction, both LTA and DTA exhibited the maximal activity over pH 8.0; however, both the LTA- and DTA-catalyzed aldol addition reactions gave the maximal yield at pH 7.5. All the synthetic reactions were thus carried out at pH 7.5.

Substrate Specificity. Both aldolases were found to accept a wide range of aldehydes as acceptors and only glycine as donor.[1,5] A systematic study of the substrate specificity for LTA- and DTA-catalyzed aldol reactions was carried out (Tables 2 and 3). The reaction yield and diastereoselectivity were monitored by NMR. In the addition reaction, a mixture containing glycine (0.1 M), an aldehyde (0.1 M), enzyme (1 U), DTT (10 mM), PLP (0.05 mM), and required additives was adjusted to pH 7.5 by 0.1 N NaOH. After incubation at 37 °C for 3 or 24 h, the yield and diastereoselectivity were calculated by integration of the corresponding protons for glycine and products (Table 4). The assignment for *threo*- and *erythro*-diastereomers was based on the coupling constant between the α- and β-protons which is usually slightly larger for the *threo*- than for the *erythro*-isomer.[1,10] In the case of LTA (Table 2), several alkyl aldehydes (entries 1−5) and an aroalkyl aldehyde (entry 7) gave *erythro*-β-hydroxy-L-amino acids. Extending the reaction time from 3 to 24 h increased the yield but reduced the diastereoselectivity, presumably due to the reverse reaction. Extending the alkyl chain or introducing a branch reduced the activity. The unsaturated alkyl aldehyde (entry 6) was not a substrate for the enzymes. Benzaldehyde was a weak substrate and was transformed to *threo*-phenylserine as a major isomer by LTA. Addition of DMSO to butyraldehyde or hydrocinnamaldehyde slightly increased the yield (entry 2A vs 2B and 7A vs 7B), but the effect was not as significant compared to acetaldehyde. The increased yield in the case of hydrocinnamaldehyde might be due to the acceleration effect and the increase of substrate solubility. Since isobutyraldehyde was a poor substrate, the amount of enzyme was increased (entry 5, condition c) to improve the yield, but the diastereoselectivity was dramatically decreased. Use of excess amount of glycine (condition D), however, increased the yield without loss of the diastereoselectivity. Aromatic aldehydes usually gave relatively high yields but low diastereoselectivity.

The DTA gave *threo*-β-hydroxy-D-amino acids as a major isomer in a kinetic mode of reaction regardless what aldehyde was used (entries 1−5, 7, and 8 in Table 3). However, the diastereoselectivities are lower than that of LTA reactions. Although extension of the reaction time to 24 h did not significantly increase the yield, the diastereoselectivities of some products obtained (entries 2, 4, and 7) were decreased, indicating that, like LTA, these DTA-catalyzed aldol reactions may have reached the equilibrium state after 24 h. Lengthening the alkyl chain reduces the reactivity, but aldehydes with less than six carbon length are still good substrates. Branching of the alkyl chain, however, did not affect the substrate reactivity for DTA, except that an unsaturated aldehyde was found not a substrate for DTA. Aromatic aldehydes are substrates (except *p*-hydroxybenzaldehyde), but their disastereoselectivities are relatively low. In general, DTA exhibited a wider substrate specificity than LTA.

We have also investigated the specificity of the aldolase reaction with chiral aldehydes. We selected the *R* and *S* isomers of isopropylidene glyceraldehyde (Tables 2 and 3, entries 16 and 17) for study as the products are stereoisomers of polyoxamic acid (Scheme 4), (+)-(2*S*,3*S*,4*S*)-2-amino-3,4,5-trihydroxypentanoic acid, which is a key constituent of polyoxins—a class of peptidyl nucleoside antibiotics.[1d] They have substantial activity against phytopathogenic fungi, and one representative, polyoxin J, is also effective against human pathogenic fungi *Candida albicans* by inhibiting chitin synthase,[1d] an essential enzyme for building fungal cell wall.

The results show that neither LTA nor DTA is stereospecific in reaction with the chiral aldehydes. Since both (*R*)- and (*S*)-glyceraldehydes are readily available,[11] all possible isomers of polyoxamic acid are obtained in good yields (not optimized), providing an opportunity for making new stereoanalogues of polyoxins.

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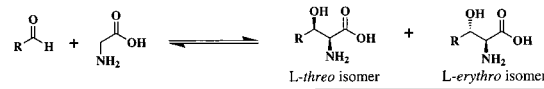
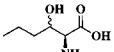
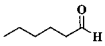
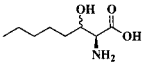
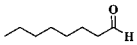
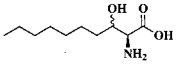
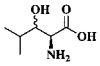
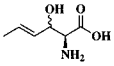
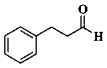
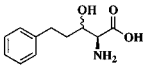
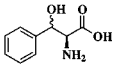
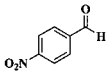
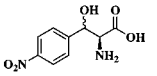
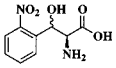
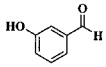
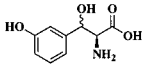
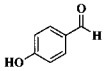
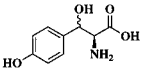
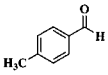
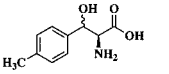
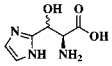
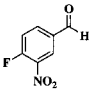
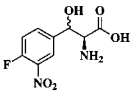
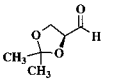
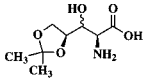
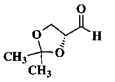
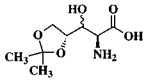
(11) (a) Hubschwerlen, C.; Specklin, J.-L.; Higelin, J. *Org. Synth.* **1995**, *72*, 1. (b) Schmid C. R.: Bryant, J. D. *Org. Synth.* **1995**, *72*, 6.

Table 2. Substrate Specificity of LTA-Catalyzed Aldol Reactions



Entry	Aldehyde	Product	Condition[a]	Reaction time (hr)	Yield[b] %	*Threo : Erythro*
1			A	3	18	<1 : 99
			A	24	39	7 : 93
			B	3	35	<1 : 99
			B	24	40	9 : 91
2			A	3	15	<1 : 99
			A	24	NT	NT
			B	3	18	3 : 97
			B	24	21	24 : 76
3			B	3	7	8 : 92
			B	24	16	37 : 63
4			B	3	0	-
			B	24	2[c]	28 : 72
5			B	3	0	-
			B	24	1	<1 : 99
			C	3	9	44 : 56
			C	24	15	46 : 53
			D	3	10	12 : 88
			D	24	NT	NT
6			B	3 or 24	0	
7			A	3	5[c]	19 : 81
			A	24	5[c]	17 : 83
			B	3	7[c]	23 : 77
			B	24	10[c]	28 : 72
8			B	3	3	71 : 29
			B	24	9	60 : 40
9			A	3 or 24	TR	-
			E	3	35	36 : 64
			E	24	53	47 : 53
10			A	3 or 24	TR	-
			E	3	46	32 : 68
			E	24	93	58 : 42
11			A	3 or 24	TR	-
			E	3	38	56 : 44
			E	24	43	73 : 27
12			A	3 or 24	0	-
			E	3	0	-
			E	24	0	-
13			A	3 or 24	TR	-
			E	3	17	40 : 60
			E	24	17	55 : 45
14			A	3 or 24	TR	-
			E	3	32	62 : 38
			E	24	40	66 : 34
15			A	3 or 24	TR	-
			E	3	19	25 : 75
			E	24	21	30 : 70
16			H	16	35	4 : 96
17			H	16	70	50 : 50

[a] Condition A: The reaction mixture containing 100 mM glycine, 100 mM aldehyde, 10 mM DTT, 0.05 mM PLP, and 1 U of enzyme in 1 mL of H_2O (pH 7.5) was incubated at 37 °C. Condition B: The same as A except that the reaction was carried out in 30% DMSO. Condition C: The same as B except that 6 U of LTA were used. Condition D: The reaction was carried out under the same condidtions as those of B except that 10 equiv of glycine was used. Condition E: The same as B except that 10 U of LTA and 5 equiv of glycine were applied. Condiditon H: Preparative scale synthesis (see the Expermental Section for details). NT = not tested. [b] The yield and *threo*/*erythro* ratio were determined by measuring the ^1H NMR and D_2O unless otherwise indicated. [c] Based on ^1H NMR measured in a 3:1 mixture of D_2O and CD_3OD.

Kinetically Controlled Synthesis of Chiral *β*-Hydroxy-α-amino Acids on a Preparative Scale. The aforementioned studies suggest that LTA gives *erythro*-*β*-hydroxy-α-L-amino acid and DTA gives *threo*-*β*-hydroxy-α-D-amino acids as kinetic products. Using an excess amount of glycine and shortening the reaction time to avoid the cleavage reaction of product was thus performed as a kinetically controlled reaction to improve the diastereoselectivity on a preparative scale (Table 5). First, isobutyraldehyde was selected as a substrate for the reaction, since the product 3-hydroxyleucine is an amino acid component of many antibiotics. One hundred units of LTA and 1 mmol of the aldehyde were used in this reaction because of the low affinity of isobutyraldehyde for LTA, while 10 U of DTA were used in the synthesis of the D-isomer. The reactions under the kinetically controlled condition provided (2*S*,3*S*)-3-hydroxyleucine with 92% de and 17% yield from LTA and (2*R*,3*S*)-3-hydroxyleucine with 86% de and 49% yield from DTA. Crystallization of the obtained products gave pure diastereoisomers. Thus the one-step enzymatic synthesis of chiral 3-hydroxyleucines proved to be effective on preparative scales compared with their chemical synthesis.[12]

Hydroxythreonine derivatives were chosen as the next targets, as the amino acids were used in the synthesis of sialyl Lewis x mimetics,[13] sphingosine,[14] monobactam antibiotics,[3a] and mycestericin D.[3b] Our previous report indicates that the L-threonine aldolase from *C. humicola* gave *β*-hydroxy-α-amino acids with good yield and high diastereoselectivity when an oxygen or nitrogen atom exists at the α-position of the substrate aldehyde.[5] Although the enzyme from *C. humicola* provided *γ*-benzyloxy-L-*allo*-threonine from α-benzyloxyacetaldehyde in good yield, it produced almost a 1:1 mixture of *γ*-benzyloxymethyl-L-threonine and *γ*-benzyloxymethyl-L-*allo*-threonine from *β*-(benzyloxy)propionaldehyde. The LTA from *E. coli* gave *γ*-benzyloxy-L-*allo*-threonine (88% de, 36% yield) and *γ*-benzyloxymethyl-L-*allo*-threonine (88% de, 10% yield) with higher diastereoselectivity. The DTA gave *γ*-benzyloxy-L-*allo*-threonine or *γ*-benzyloxymethyl-D-threonine (80% yield, 40% de; 45% yield, 29% de) in good yield but low diastereoselectivity under the condition. However, shortening the reaction time dramatically increased the diastereoselectivity. In addition, crystallization of the obtained products improved the diastereomeric excess to more than 80%.

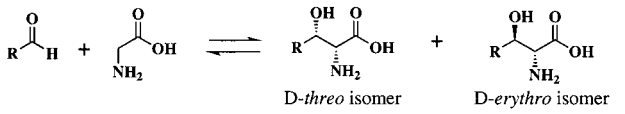
Synthesis and Separation of Thermodynamic Products. The kinetically controlled reaction using the LTA or DTA was used to obtain the products stereoselectively. However, in certain cases, since the recombinant LTA or DTA provided a

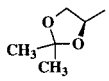
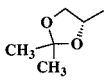
(12) (a) Sunazuka, T.; Nagamitsu, T.; Tanaka, H.; Omura, S. *Tetrahedron Lett.* **1993**, *34*, 4447. (b) Corey, E. J.; Lee, D.-H.; Choi, S. *Tetrahedron Lett.* **1992**, *33*, 6735. (c) Caldwell, C. G.; Bondy, S. S. *Synthesis* **1990**, 34. (d) Jung, M. E.; Jung, Y. H. *Tetrahedron Lett.* **1989**, *30*, 6637.

(13) Uchiyama, T.; Vassilev, V. P.; Kajimoto, T.; Wong, W.; Huang, H.; Lin, C.-C.; Wong, C.-H. *J. Am. Chem. Soc.* **1995**, *117*, 5395.

(14) Bertozzi, C. R.; Cook, D. G.; Kobertz, W. R.; Gonzalez-Scarano, F.; Bednarski, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 10639.

Table 3. Substrate Specificity of DTA-Catalyzed Aldol Reactions



Entry	Aldehyde (R)	Condition[a]	Reaction time (hr)	Yield[b] %	*Threo* : *Erythro*
1	CH_3	F	3	60	53 : 47
		F	24	50	53 : 47
2	*n*-C_3H_7	F	3	37	67 : 33
		F	24	36	55 : 45
3	*n*-C_5H_{11}	F	3	31	61 : 39
		F	24	23	64 : 36
4	*n*-C_7H_{15}	F	3	3[c]	69 : 31
		F	24	3[c]	50 : 50
5	$(CH_3)_2CH$	F	3	26	84 : 16
		F	24	25	85 : 15
6	CH3CH=CH	F	3	0	-
		F	24	0	-
7	$Ph(CH_2)_2$	F	3	16[c]	87 : 13
		F	24	16[c]	66 : 34
8	Ph	F	3	11	74 : 26
		F	24	10	73 : 27
9	*p*-NO_2-Ph	F	3 or 24	TR	-
		G	3	72	58 : 42
		G	24	88	55 : 45
10	*o*-NO_2-Ph	F	3 or 24	TR	-
		G	3	89	72 : 28
		G	24	71	64 : 36
11	*m*-OH-Ph	F	3 or 24	TR	-
		G	3	54	74 : 26
		G	24	55	73 : 27
12	*p*-OH-Ph	F	3 or 24	0	-
		G	3	0	-
		G	24	0	-
13	*p*-CH_3-Ph	F	3 or 24	TR	-
		G	3	17	47 : 53
		G	24	25	43 : 57
14	2-Im	F	3 or 24	TR	-
		G	3	34	55 : 45
		G	24	60	61 : 39
15	(*p*-F)(*m*-NO_2)-Ph	F	3 or 24	TR	-
		G	3	42	76 : 24
		G	24	49	65 : 35
16		H	16	73	70 : 30
17		H	16	84	88 : 12

[a] Condition F: The reaction mixture containing 100 mM glycine, 100 mM aldehyde, 10 mM DTT, 0.05 mM PLP, 10 mM $MgCl_2$, and 10 U of DTA in 1 mL of H_2O (ph 7.5) was incubated at 37 °C. Condition G: The same as F except the 5 equiv of glycine was applied and the reaction was carried out in 30% DMSO. Condition H: Preparative scale synthesis (see the Experimental Section for details).

threo and *erythro* mixture of products under the thermodynamic condition, separation of the products would be necessary, if a single isomer is desirable. Similar to the resolution of D- and L-phosphothreonine by acid phosphatase,[15] we have attempted the kinetic resolution of *erythro*- and *threo*-*β*-hydroxy-α-amino acid using phosphatase. As a result, D-*allo*-threonine was predominantly cleaved by acid phosphatase and D-threonine was cleaved by alkaline phosphatase. On the other hand, L-threonine was cleaved by acid and alkaline phosphatase faster than L-*allo*-threonine (Table 6).

On the basis of this finding, the synthesis of D-*allo*-threonine, which is the most expensive of the four isomers, was performed by the coupled enzymatic reaction of DTA and acid phosphatase (Scheme 3). Aldol condensation of acetaldehyde and glycine by DTA gave about a 1:1 mixture of D-*allo*-threonine and D-threonine (determined by NMR). Addition of EtOH to the reaction mixture removed about 95% of excess glycine. The mixture containing both isomers of D-threonine and D-*allo*-threonine and glycine was directly phosphorylated according to the method of Neuhaus and Korkes.[16] When the reaction mixture was passed through an ion exchange column, both isomers of *O*-phospho-D-threonine and *O*-phospho-D-*allo*-threonine were easily separated from glycine (70% yield for two steps). The resolution by acid phosphatase was carried out at pH 5.3 in citric buffer at 37 °C for 5 days. Purification of the reaction mixture on Dowex 50W-X8 (H^+) afforded diastereomerically pure D-*allo*-threonine in 45% yield. Compared to the other known methods[4c17] (e.g., via epoxidation/N_3 opening,[17a] dihydroxylation/cyclic sulfate/ azide opening,[4c] and D-hydantoinase hydrolysis[17b]) for the synthesis of D-*allo*-threonine, this method seems not to have a clear advantage. However, it provides two separable diastereoisomers in a straightforward manner and may be useful for the synthesis of isotopically labeled products, as the labeled substrates are readily available.

Conclusion

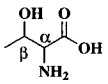
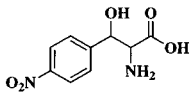
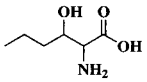
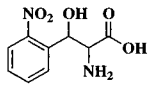
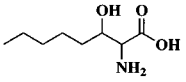
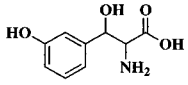
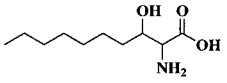
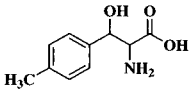
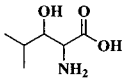
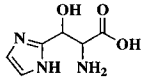
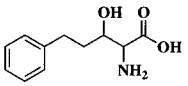
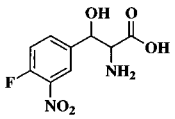
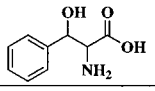
In summary, the LTA from *E. coli* and the DTA from *X. oryzae* were overexpressed in *E. coli* and their synthetic utilities were investigated. These enzymes accept a wide range of aldehydes as acceptors, providing a useful route to either D- or L-α-amino acids containing an epimeric mixture of the *β*-carbinol center as thermodynamic products. LTA gave mainly *erythro*-*β*-hydroxy-α-L-amino acids from aliphatic aldehydes and *threo*-*β*-hydroxy-α-L-amino acid from aromatic aldehydes, while DTA gave *threo*-*β*-hydroxy-α-D-amino acids as main kinetically controlled products. The stereoselective synthesis of some valuable *β*-hydroxy-α-amino acids with high yield and high diastereoselectivity has been achieved. In addition, a chemoenzymatic procedure with the tandem use of DTA and acid phosphatase has been developed for the synthesis of D-*allo*-threonine from the 1:1 mixture of diastereomeric products. It appears that this simple enzymatic approach to the synthesis of α-amino-*β*-hydroxy acids (albeit in certain cases the selectivity at the *β*-carbon is relatively low) may find use in the combinatorial (or rational) synthesis of various antibiotics analogues and enzyme inhibitors for new drug discovery.

Experimental Section

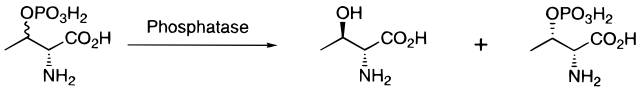
General. All chemicals were purchased from commercial sources as reagent grades. Histidine-tag protein affinity chromatography was performed on a HisTrap (Pharmacia biotech). Ion-exchange chroma-

(15) Scollar, M. P.; Sigal, G.; Klibanov, A. M. *Biotechnol. Bioeng.* **1985**, *27*, 247.
(16) Neuhaus, F. C.; Korkers, S. *Biochem. Prep.* **1958**, *6*, 75.
(17) (a) Pons, D.; Savignac, M.; Genet, J.-P. *Tetrahedron Lett.* **1990**, *31*, 5023. (b) Keil, O.; Schneider, M. P.; Rasor, J. P. *Tetrahedron: Asymmetry* **1995**, *6*, 1257. (c) Genet, J.-P.; Juge, S.; Mallart, S. *Tetrahedron Lett.* **1988**, *29*, 6765; Beaulieu, P. L. *Tetrahedron Lett.* **1991**, *32*, 1031; Morell, J. L.; Fleckenstein, P.; Gross, E. J. *J. Org. Chem.* **1977**, *42*, 355.

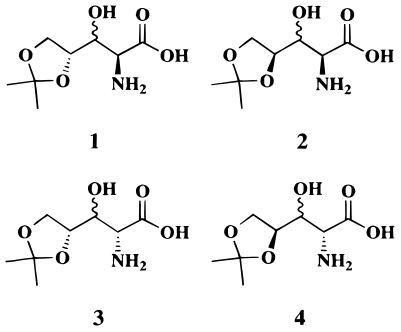
Table 4. Chemical Shifts and Coupling Constants of β-Hydroxy-α-amino Acids

	Threo		*Erythro*			*Threo*		*Erythro*	
	H_α (ppm)	$J_{\alpha\beta}$ (Hz)	H_α (ppm)	$J_{\alpha\beta}$ (Hz)		H_α (ppm)	$J_{\alpha\beta}$ (Hz)	H_α (ppm)	$J_{\alpha\beta}$ (Hz)
	3.54	4.9	3.79	3.9		3.91	4.4	4.09	3.8
	3.64	4.4	3.83	3.5		4.12	4.0	4.16	3.0
	3.55	4.8	3.74	4.0		3.86	4.0	4.01	3.97
	3.58	5.0	3.78	4.4		3.85	4.4	4.01	4.2
	3.80	3.5	3.92	3.1		4.06	5.4	4.15	3.0
	3.58	4.8	3.77	3.8		3.88	4.7	4.06	3.7
	3.91	4.5	4.07	4.1					

Scheme 3. Phosphatase-Catalyzed Synthesis of d-*allo*-Threonine



Scheme 4. Structure of 2-Amino-3-hydroxy-4,5-*O*-isopropylidenepentanioc Acids Synthesized on a Preparative Scale

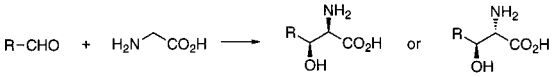


tography was conducted on Dowex-1, 50−100 mesh (Sigma), and Dowex 50W-X8, 20−50 mesh (Bio-Rad). UV−visible spectrum was recorded on a Beckman DU 650. SDS-PAGE was carried out on Pharmacia Phast System. ^{1}H NMR spectra were recorded at 250, 400, and 500 MHz on Bruker AMX-250, AMX-400, and AMX-500, respectively. Column chromatography was conducted on silica gel 60, 230−240 mesh (Mallinckrodt), and ODS Lichroprep RP-18, 40−63 μm (EM Science).

Plasmids for LTA and DTA. The *E. coli* K12 and *X. oryzae* IAM 1657 DNAs were isolated according to the method described by Maniatis et al.[18] PCR amplification was performed in a 100-μL reaction mixture containing 1 μL (1.5 mg) of DNA template, 200 nmol of the corresponding primers (5′-ATATTGGATCCTTAAAGCGTAAATGAAC and 5′-GCTTTGAATTCTTATGCGTAAACCGGGTAACG for

(18) Maniatis, T.; Fritsh, E. F.; Sanbrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor: Plainview, NY, 1989.

Table 5. Kinetically Controlled Preparative Enzymatic Synthesis of Chiral β-Hydroxy-α-amino Acids



R	Enzyme	Reaction Time[a]	Yield (%)	diastereoselectivity (% de)
$(CH_3)_2CH$	LTA	5 h	17	92
$BnOCH_2$	LTA	4h	36	88
$BnO(CH_2)_2$	LTA	3h	10	88
$(CH_3)_2CH$	DTA	3 h	49	86
$BnOCH_2$	DTA	4h	80	40
$BnO(CH_2)_2$	DTA	3h	45	29
		1h	37	58
		25min	35	64
$BnOCH_2$	LTA[b]	16h	78	84
$BnO(CH_2)_2$	LTA[b]	16h	53	6

[a] See the Experimental Section. [b] From *C. humicola*.

E. coli and 5′-ATATTGGATTCCCAGGAAGTCATACGC and 5′-GCTTTAGATCTTTAGCGCGAAAAGCCGCGCGC for *X. oryzae*), 200 μM of dNTPs, 5 μL of DMSO, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 U of *Thermus aquaticus* DNA polymerase (Stratagene Co.). The reaction mixture was overlaid with mineral oil and subjected to 36 cycles of amplifications. The cycle conditions were set as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min, and elongation at 72 °C for 1.5 min. The PCR products were purified by phenol extraction. The DNA inserts obtained from PCR amplification were purified on 0.8% agarose gel. These DNA bands corresponding to 1.2 kb were cut and purified with QIAEX gel extraction kit (Qiagen Co.)

Table 6. Hydrolysis of *O*-phosphothreonines by Phosphatase

	hydrolysis (%) after 24 h	
	acid phosphatase[a]	alkaline phosphatase[b]
O-phospho-L-threonine	19.0	6.2
O-phospho-L-*allo*-threonine	1.5	1.6
O-phospho-D-threonine	1.5	8.0
O-phospho-D-*allo*-threonine	14.3	0.3

[a] 0.1 U of acid phosphatase in 100 mM citrate buffer (pH 5.6) at 37 °C for 22 h. [b] 0.1 U of alkaline phosphatase in 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 100 mM glycine buffer (pH 10.4) at 37 °C for 22 h.

and eluted with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5). The LTA-DNA and DTA-DNA were twice digested with BamH I and EcoR I (for the *E. coli* gene), and with BamH I and Bgl II (for the *X. oryzae* gene) (Boehringer Mannheim Biochemical Co.) for 2 h at 37 °C, respectively. The digested DNAs were then recovered by phenol extraction and ethanol precipitation (70% of final ethanol concentration containing 10% of 3 N NaOAc, pH 5.2) and purified by agarose (0.8%) gel electrophoresis as described above. These DNAs were used as inserts. The vectors (pRSETA, Invitrogen Co.) were also digested and recovered with ethanol precipitation after phenol extraction. The restriction-enzyme digested vectors were further purified on agarose gel (0.8%) as described above. The insert was then ligated with the corresponding vector with T4 DNA ligase. The expression plasmids constructed in this way were then transformed into BL 21 strain (Stratagen Co.) for LTA and *E. coli* Sure strain (Stratagen Co.) for DTA and plated on LB agar plates containing 250 μg mL^{-1} ampiciline. A part of the grown colony was picked and dissolved with 50 μL of colony lysing solution (20 mM Tris-HCl, 1% Triton, 2 mM EDTA, pH 8.5). The solution was heated at 95 °C for 5 min. By using 1 μL of the resultant solution as the template, PCR amplification was carried out as described above. After analysis of 0.8% agarose gel to confirm the presence of the gene insert, the positive colony was selected. For LTA, the selected colony was directly used for protein expression. For DTA, the plasmids were isolated from the selected colony using the QIAprep-spin Plasmid Kit (Qiagen Inc.), transformed into *E. coli* AD494 (DE3; Stratagen Co.), and plated on LB agar plates containing 250 μg mL^{-1} ampiciline. One colony, which was reconfirmed to be positive by PCR and 0.8% agarose gel analysis, was randomly selected and used for protein expression.

Preparation of LTA and DTA. To express the desired proteins, the positive clone was grown in 100 mL of LB medium containing 250 μg mL^{-1} ampiciline at 37 °C with shaking (220 rpm). After the cell growth reached a point where the turbidity was about 0.5 as measured by the absorbance at 600 nm (OD_{600}), 20 mL of this culture was transferred to fresh LB medium (1 L) containing 250 μg mL^{-1} ampiciline and incubated with shaking until $OD_{600} = 0.4-0.5$, then 50 μL of 0.5 M IPTG was added to induce the expression of the target protein. The cultures were shaken for 6 h at room temperature for LTA and at 30 °C for DTA. The cells were harvested by centrifugation (10000*g*, 30 min, 4 °C). The cell pellets were suspended in Tris buffer (20 mM, pH 8.0) containing 10 mM dithiothreitol (DTT) and disrupted by a French pressure cell at 16 000 lb/in.^{2} The cell debris were removed by centrifugation at 23000*g* for 30 min, and the supernatant (cell-free extraction) was used for enzymatic synthesis without further purification. For kinetic studies, the enzymes were purified on prepacked histidine-tag affinity chromatograph (HisTrap) according to the manual. The active fractions, which were eluted with 300 mM imidazole, were pooled and dialyzed in 4 L of 20 mM Tris buffer (pH 8.0) containing 10 mM DTT and stored at −78 °C.

Enzyme Assay for Retro-Aldol Reaction and Kinetics. The LTA- and DTA-catalyzed retro-aldol reaction of threonine or *allo*-threonine to produce glycine and acetaldehyde was spectrophotometrically monitored by the increase of NADH absorption at 340 nm in a 1 cm light path cuvette using a coupled enzymatic reaction in which acetaldehyde was oxidized to acetic acid by NAD-aldehyde dehydrogenase (AD). To the reaction mixture containing 1 mM NAD, 1 U of AD, 100 mM KCl, 10 mM DTT, 0.05 mM PLP, and 100 mM Tris buffer (pH 8.5) were added the enzyme solution and additives. The reaction was initiated by the addition of substrate (threonine or *allo*-threonine). The initial velocity was estimated during the first 5 min. The background was calculated by measuring the OD_{340} of the reaction mixture before adding the substrate. One unit of the enzyme activity is defined as the amount of enzyme required to produce 1 μmol of NADH per minute. Unless otherwise indicated, the assay for LTA and DTA was performed in 30% DMSO at 50 °C and with 10 mM $MgCl_2$ at 37 °C.

For kinetic studies, initial velocities were measured at various concentrations of threonine or *allo*-threonine (0.125−3 mM). In the case of LTA, 0.06 U of LTA were added to the reaction mixture containing 1 mM NAD, 1 U of AD, 100 mM KCl, 10 mM DTT, and 0.05 mM PLP in 100 mM Tris buffer containing 30% DMSO (pH 8.5). After preincubation of the mixture at 50 °C for 5 min, the reaction was initiated by adding 1 μmol of substrate (L-threonine or L-*allo*-threonine) and monitored by measuring the OD_{340} at 50 °C for 5 min. In the case of DTA, 0.04 U of DTA was added to the reaction mixture containing 1 mM NAD, 1 U of AD, 100 mM KCl, 10 mM DTT, 0.05 mM PLP, and 10 mM $MgCl_2$ in 100 mM Tris buffer (pH 8.5). After preincubation of the mixture at 37 °C for 5 min, the reaction was initiated by adding 1 μmol of substrate (D-threonine or D-*allo*-threonine) and monitored by measuring the OD_{340} at 37 °C for 5 min. The accumulated data were fitted into Lineweaver−Burk plot to calculate the K_m values. Specific activities were calculated by dividing the enzyme activity by the protein concentration which was determined by the Protein-Assay kit (Pharmacia Biotec).

Enzyme Assay for the Aldol Reaction. The LTA- and DTA-catalyzed aldol condensation of glycine and the aldehyde acceptor to provide the product β-hydroxy-α-amino acid was monitored by NMR. A reaction mixture containing 1 U of enzyme, 100 mM glycine, 100 mM aldehyde, 0.05 mM PLP, 10 mM DTT, and optional additives in 1 mL of H_2O was adjusted to pH 7.5 with 0.1 N NaOH. After the reaction mixture was incubated at 37 °C for a period of time, the reaction mixture was directly applied to Dowex-1 (OH^- form; 3 mL) and washed with 50 mL of water. Elution with 20% acetic acid in H_2O gave the aldol product and unreacted glycine. The yield was calculated by measuring the ratio of the remaining glycine and the product, and the ratio of the *threo* to *erythro* product was determined by integration of the corresponding α-proton intensities of the product. Unless otherwise indicated, LTA- and DTA-catalyzed reactions were performed in 30% DMSO at 37 °C or with 10 mM $MgCl_2$ at 37 °C, respectively.

Enzyme Stability Study. LTA and DTA were incubated at room temperature or 4 °C in a 20 mM Tris buffer (pH 8.0) containing 10 mM DTT. At different period of time, 10-μL aliquots were removed and assayed for the remaining activity as described above.

pH Dependency. For the retro-aldol reactions, both enzymes were assayed in a 500 mM Tris-maleic acid buffer from pH 5.5 to pH 8.5 as described above. For LTA-catalyzed aldol reactions, the reaction mixture containing 1 U of LTA, 100 mM glycine, 100 mM acetaldehyde, 0.05 mM PLP, and 10 mM DTT in 50 mM phosphate buffer with 30% DMSO from pH 5.5 to pH 8.5 was incubated at 37 °C for 5 h. For DTA-catalyzed aldol reactions, the reaction mixture containing 1 U of DTA, 100 mM glycine, 100 mM acetaldehyde, 0.05 mM PLP, 10 mM DTT, and 10 mM $MgCl_2$ in 100 mM citric acid buffer from pH 5.5 to pH 6.5 and in 100 mM Tris buffer from pH 7.0 to pH 8.5 was incubated at 37 °C for 5 h.

Hydrolysis of *O*-Phosphothreonines. Four isomers of *O*-phosphothreonines (*O*-phospho-L-threonine, *O*-phospho-L-*allo*-threonine, *O*-phospho-D-threonine, and *O*-phospho-D-*allo*-threonine) were individually prepared according the method of Neuhaus et al.[16] The 1-mL solution containing 1 μmol of *O*-phosphothreonine and 0.1 U of wheat germ acid phosphatase in 100 mM citric acid (pH 5.6), or 1 μmol of *O*-phosphothreonine and 0.1 U of calf intestine alkaline phosphatase in 100 mM glycine, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$ (pH 10.4), was incubated at 37 °C. After 22 h, 50-μL aliquots were withdrawn from the reaction mixture and assayed for inorganic phosphate according to the method of Lanzetta et al.[19]

(19) Lanzetta, P. A.; Alvarez, L. J.; Reinach, P. S.; Candia, O. A. *Anal. Biochem.* **1979**, *100*, 95.

General Procedure for the Synthesis of *erythro*-*β*-Hydroxy-L-α-amino Acid by LTA. To a 50-mL solution of 1 M glycine, 10 mM DTT, and 0.05 mM PLP containing 30% DMSO (pH 7.5) was added 1 mmol of an aldehyde and 100 U of LTA. The reaction mixture was gently shaken at 37 °C for a period of time. After the mixture was boiled at 100 °C for 10 min to terminate the reaction, 200 mL of EtOH was added to the reaction mixture and the mixture was incubated at 4 °C for 10 h. The precipitate was collected and washed with 50 mL of EtOH to recover glycine (95−82%). The filtrate was concentrated in vacuo and applied to Dowex-1 (OH^- form, 50 mL). After being washed with 200 mL of H_2O, the product containing glycine was eluted with 20% acetic acid. The collected fractions were concentrated in vacuo, and the residue was purified on ODS (H_2O) to give the desired product.

(2*S*,3*R*)-3-Hydroxyleucine: 17% yield, 92% de before crystallization; ^{1}H NMR (D_2O, 500 MHz) δ 0.93 (3 H, d, J = 6.5 Hz, CH_3), 0.94 (3 H, d, J = 6.5 Hz, CH_3), 1.90 (1 H, dsep, J = 9.1 and 6.5 Hz, C*H*(CH_3)$_2$), 3.49 (1 H, dd, J = 9.1 and 3.1 Hz, β-H), 3.87 (1 H, d, J = 3.1 Hz, α-H).

(2*S*,3*R*)-2-Amino-4-(benzyloxy)-3-hydroxybutyric Acid (*γ*-Benzyloxy-L-*allo*-threonine): 36% yield, 88% de before crystallization; ^{1}H NMR (CD_3OD/D_2O = 65/35, 500 MHz) δ 3.70 (2 H, d, J = 4.0 Hz, C*H*$_2$OBn), 3.84 (1 H, d, J = 4.3 Hz, α-H), 4.26 (1 H, dd, J = 4.3 and 4.0 Hz, β-H), 4.55 (2 H, s, OC*H*$_2$Ph), 7.29−7.38 (5 H, m, Ph).

(2*S*,3*R*)-2-Amino-5-(benzyloxy)-3-hydroxypentanoic Acid (*γ*-Benzyloxymethyl-L-*allo*-threonine): 10% yield, 88% de before crystallization; ^{1}H NMR (D_2O, 400 MHz) δ 1.74−1.89 (2 H, m, *CH*$_2$CH$_2$-OBn), 3.68−3.73 (2 H, m, CH$_2$*CH*$_2$OBn), 3.81 (1 H, d, J = 3.7 Hz, α-H), 4.26 (1 H, dt, J = 9.5 and 3.7 Hz, β-H), 4.55 (1 H, d, J = 11.4 Hz, *CH*$_2$OBn), 4.58 (1 H, d, J = 11.4 Hz, *CH*$_2$OBn), 7.37−7.46 (5 H, m, Ph).

Crystallization of the above three products from EtOH−H_2O gave diastereomerically pure products (based on the NMR signal of α-H) in ∼62−65% yield.

General Procedure for the Synthesis of *threo*-*β*-Hydroxy-D-α-amino Acid by DTA. To a 50-mL solution of 1 M glycine, 10 mM DTT, 0.05 mM PLP, and 10 mM $MgCl_2$ in H_2O (pH 7.5) was added 1 mmol of an aldehyde and 10 U of DTA. The reaction mixture was gently shaken at 37 °C for a period of time. After the reaction mixture was boiled at 100 °C for 10 min to terminate the reaction, 200 mL of EtOH was added and the mixture left at 4 °C for 10 h. The precipitate was collected and washed with 50 mL of EtOH to recover glycine (95−89%). The filtrate was purified on ODS (H_2O) to give the desired product.

(2*R*,3*R*)-3-Hydroxyleucine: 49% yield, 86% de before crystallization; ^{1}H NMR (D_2O, 500 MHz) δ 0.89 (3 H, d, J = 6.8 Hz, CH_3), 0.94 (3 H, d, J = 6.8 Hz, CH_3), 1.69 (1 H, oct, J = 6.8 Hz, C*H*(CH_3)$_2$), 3.65 (1 H, dd, J = 6.8 and 3.5 Hz, β-H), 3.66 (1 H, d, J = 3.5 Hz, α-H).

(2*R*,3*R*)-2-Amino-4-(benzyloxy)-3-hydroxybutyric Acid (*γ*-Benzyloxy-D-threonine): 80% yield, 40.0% de before crystallization; ^{1}H NMR (D_2O, 500 MHz) δ 3.65−3.70 (2 H, m, *CH*$_2$OBn), 3.70 (1 H, d, J = 3.6 Hz, α-H), 4.26 (1 H, ddd, J = 8.9, 4.4, and 3.6 Hz, β-H), 4.65 (2 H, s, OC*H*$_2$Ph), 7.34−45 (5 H, m, Ph).

(2*R*,3*R*)-2-Amino-5-(benzyloxy)-3-hydroxypentanoic Acid (*δ*-Benzyloxymethyl-D-threonine): 35% yield, 64% de; ^{1}H NMR (D_2O, 400 MHz) δ 1.77−1.85 (1 H, m, *CH*$_2$CH$_2$OBn), 1.92−2.01 (1 H, m, *CH*$_2$CH$_2$OBn), 3.62 (1 H, d, J = 4.5 Hz, α-H), 3.68−3.73 (2 H, m, CH$_2$*CH*$_2$OBn), 4.23 (1 H, dt, J = 9.6 and 4.5 Hz, β-H), 4.57 (2 H, t, J = 11.6 Hz, *CH*$_2$OBn), 7.38−7.46 (5 H, m, Ph).

Crystallization of the above three products from EtOH−H_2O gave diastereomerically pure products (in ∼60−65% yield), based on the NMR signal of α-H.

D-*allo*-Threonine. To a 50 mM solution of 1 M glycine, 10 mM DTT, 0.05 mM PLP, and 10 mM $MgCl_2$ in H_2O (pH 7.5) were added 1 mmol of acetaldehyde and 10 U of DTA. The reaction mixture was gently shaken at 37 °C for 21 h. To the reaction mixture was added 200 mL of EtOH and the mixture left at 4 °C for 3 h. The precipitate was collected and washed with 50 mL of EtOH to recover glycine (87.6%). The filtrate was concentrated and applied to Dowex 1 (OH^- form; 50 mL). After washing with 200 mL of H_2O, the product containing glycine was eluted with 20% acetic acid. The fraction was collected and evaporated in vacuo. The residue was suspended with toluene and evaporated in vacuo. This procedure was repeated three times to remove H_2O. To the residue containing a 7:1:1 mixture of glycine, D-threonine, and D-*allo*-threonine (based on NMR analysis) (185 mg) was added chlorophosphoric acid,[18] and the mixture was heated at 60 °C for 2 h. To the solution was added 0.5 mL of H_2O and 1.5 mL of 1 N HCl, and the mixture was heated at 100 °C for 15 min. The mixture was then directly applied to Dowex 1 (OH-form; 50 mL) and washed with 1 L of H_2O. Glycine and unreacted threonine were eluted with 0.5 M formic acid, and the crude phosphorylated products were eluted with 0.7−1.0 M formic acid. The fractions were collected and evaporated in vacuo to give a 1:1 mixture of *O*-phospho-D-threonine and *O*-phospho-D-*allo*-threonine (74.9 mg).

***O*-Phospho-D-*allo*-threonine:** ^{1}H NMR (500 MHz, D_2O) δ 1.43 (3 H, d, J = 6.5 Hz, CH_3), 4.12 (1 H, brs, α-H), 4.81−4.88 (1 H, m, β-H).

***O*-Phospho-D-threonine:** ^{1}H NMR (500 MHz, D_2O) δ 1.35 (3 H, d, J = 6.5 Hz, CH_3), 4.28 (1 H, brs, α-H), 4.71−4.78 (1 H, m, β-H).

Wheat germ acid phosphatase (10 U) was added to a 10-mL solution of the crude phosphorylated product (45 mg, 0.226 mmol) in 100 mM citrate buffer (pH 5.6). The reaction mixture was shaken at 37 °C for 5 days and then applied to Dowex 50W-X8 (H^+ form; 50 mL). Unreacted *O*-phospho-D-threonine was recovered by washing with H_2O (11.6 mg, 43%), and D-*allo*-threonine was eluted with 3 N NH_4OH. The desired fraction was evaporated in vacuo and recrystallized with H_2O−MeOH to give pure D-*allo*-threonine (12.1 mg, 45%), which had an ^{1}H NMR spectrum identical to that of the authentic sample.

General Procedure for the Synthesis of 4,5-*O*-Isopropylidene-Protected Polyoxamic Acids. To a solution of the enzyme (approximately 500 U of LTA or 11 000 U of DTA)[20] in 50 mM potassium phosphate buffer (LTA) or 50 mM Tris (DTA), pH = 7.5 (25 mL), was added 3.8 g (51 mM) of glycine, 10 mg (40 μM) of PLP, 50 mg (0.3 mM) of DTT, and 500 mg of $MgCl_2 \cdot 6H_2O$ (for DTA).

To this solution (DTA) was added 1.14 g (8.76 mM) of (*R*)-isopropylideneglyceraldehyde.[11] After sonication for about 10 s, the mixture became homogeneous and was shaken at 37 °C for 16 h. After precipitation of excess glycine with ethanol (125 mL), filtration, and evaporation of the solvent, the total crude product was suspended in water and applied to a reverse phase RP_{18} column. Elution with water gave 1.5 g (84% yield) of the desired product **3** (Scheme 4) as a diastereomeric mixture (see Tables 2 and 3).

(2*S*,3*R*,4*S*)-2-Amino-3-hydroxy-4,5-*O*-isopropylidenepentanoic Acid. To prepare the diastereomeric product, compound **2** (0.249 g) (Scheme 4) was recrystallized from EtOH−H_2O (2:1 v/v) to give 0.12 g of the title compound in a diastereomerically pure form: mp 205−206 °C (dec) (EtOH−H_2O); $[\alpha]_D$ = + 18° (*c* 1.17 H_2O); ^{1}H NMR (D_2O, 400 MHz) δ 4.41 (1H, ddd, J = 7.8, 6.5, and 4.7 Hz, C4H), 4.18 (1H, dd, J = 9.0 and 6.5 Hz, C3H), 3.98 (1H, dd, J = 9.0 and 4.7 Hz, C4H*H*), 3.98 (1H, dd, J = 7.8 and 3.0 Hz, C4*H*H), 3.94 (1H, d, J = 3.0 Hz, C2H), 1.43 (3H, s, CH_3), 1.36 (3H, s, CH_3); ^{13}C NMR δ (D_2O, 400 MHz) 173.3, 112.7, 77.65, 73.4, 68.7, 59.1, 28.0, 26.3; FAB HRMS calcd for $C_8H_{15}NO_5$ 206.1028 (MH^+), found 206.1035.

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(20) The amount of the enzyme units is increased to obtain a reasonable yield because isopropylideneglyceraldehyde is unstable at room temperature ($t_{1/2}$ = 24 h). The product is, however, quite stable.