Biocatalysis

DOI: 10.1002/anie.201411484

Engineered L-Serine Hydroxymethyltransferase from *Streptococcus thermophilus* for the Synthesis of α,α-Dialkyl-α-Amino Acids**

*Karel Hernandez, Igor Zelen, Giovanna Petrillo, Isabel Usón, Claudia M. Wandtke, Jordi Bujons, Jesús Joglar, Teodor Parella, and Pere Clapés**

Abstract: *α,α-Disubstituted α-amino acids are central to biotechnological and biomedical chemical processes for their own sake and as substructures of biologically active molecules for diverse biomedical applications. Structurally, these compounds contain a quaternary stereocenter, which is particularly challenging for stereoselective synthesis. The pyridoxal-5′-phosphate (PLP)-dependent L-serine hydroxymethyltransferase from Streptococcus thermophilus ($SHMT_{Sth}$; EC 2.1.2.1) was engineered to achieve the stereoselective synthesis of a broad structural variety of α,α-dialkyl-α-amino acids. This was accomplished by the formation of quaternary stereocenters through aldol addition of the amino acids D-Ala and D-Ser to a wide acceptor scope catalyzed by the minimalist $SHMT_{Sth}$ Y55T variant overcoming the limitation of the native enzyme for Gly. The $SHMT_{Sth}$ Y55T variant tolerates aromatic and aliphatic aldehydes as well as hydroxy- and nitrogen-containing aldehydes as acceptors.*

The α,α-disubstituted α-amino acids are central to biotechnological and biomedical chemical processes, e.g., the synthesis of biologically active molecules with beneficial biomedical application in fields as diverse as immunology (e.g., myriocin, Figure 1), infectious diseases (e.g., the fungicides sphingofungines E and F, Figure 1), cardiovascular diseases (e.g., DOPA decarboxylase inhibitors), and neurology (e.g., lactacystin).[1]

The synthesis of chirally pure α,α-dialkylated β-hydroxy-α-amino acids remains a challenging task, because it requires the construction of stereogenic tetrasubstituted carbon centers with a strict control of stereoselectivity.[1f,h,2] Biocatalytic methods have become increasingly significant due to their potential to confer controlled stereoselectivity and efficiency under mild aqueous conditions.[3]

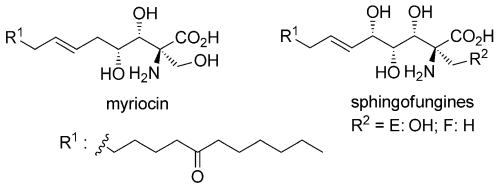


Figure 1. Structures of myriocin and sphingofungines E and F.

The most concise enzymatic route toward the preparation of optically pure α,α-dialkylated β-hydroxy-α-amino acids involves the stereoselective addition of an α-substituted α-amino acid to an aldehyde. Aldol additions of Gly to aldehydes have been previously achieved using Gly-dependent aldolases,[4] but biocatalytic aldol additions of other α-amino acids are rare.[3a,d–f] Furthermore, most of them are marred by low catalytic efficiency and poor diastereomeric control over the incoming β-hydroxy group.

We have cloned and characterized a novel pyridoxal-5′-phosphate (PLP)-dependent L-serine hydroxymethyltransferase from *Streptococcus thermophilus* ($SHMT_{Sth}$; EC 2.1.2.1).[5] $SHMT_{Sth}$ was found to catalyze aldol additions of Gly to aldehydes with good stereoselection under kinetic control (4 °C).[6]

[*] Prof. Dr. P. Clapés
Biotransformation and Bioactive Molecules Group
Instituto de Química Avanzada de Cataluña IQAC-CSIC
Jordi Girona 18–26, 08034 Barcelona (Spain)
E-mail: pere.clapes@iqac.csic.es
K. Hernandez, Dr. J. Bujons, Dr. J. Joglar, Prof. Dr. P. Clapés
Dept Química Biológica y Modelización Molecular
Instituto de Química Avanzada de Cataluña, IQAC-CSIC
Jordi Girona 18–26, 08034 Barcelona (Spain)
I. Zelen
Instituto de Biología Molecular de Barcelona, IBMB-CSIC
Baldiri Reixach 13–15, 08028 Barcelona (Spain)
Prof. Dr. I. Usón
Institució Catalana de Recerca i Estudis Avançats (ICREA)
Instituto de Biología Molecular de Barcelona, IBMB-CSIC
Baldiri Reixach 13–15, 08028 Barcelona (Spain)
G. Petrillo
Dept Química Biológica y Modelización Molecular
Instituto de Química Avanzada de Cataluña, IQAC-CSIC
Instituto de Biología Molecular de Barcelona, IBMB-CSIC (Spain)
C. M. Wandtke
Institut für Anorganische Chemie, Universität Göttingen
Tammannstr. 4, 37077 Göttingen (Germany)
Dr. T. Parella
Servei de Ressonància Magnètica Nuclear
Departament de Química
Universitat Autònoma de Barcelona, Bellaterra (Spain)

[**] This work was supported by the Spanish MINECO (CTQ2012-31605 and CTQ2012-32436, BFU2012-35367), Generalitat de Catalunya (2009 SGR 00281), ERA-IB MICINN, PIM2010EEI-00607 (EIB.10.012. MicroTechEnz-EIB., www.fkit.unizg.hr/miten), and COST Action: CM1303 Systems Biocatalysis. K.H. acknowledges the CSIC for a JAE Predoctoral contract program. G.P. acknowledges the Generalitat de Catalunya and Biochemize S.L. for a DI Predoctoral contract.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411484.

The α-methylserine hydroxymethyltransferase from *Paracoccus sp.* AJ110402 ($MSHMT_{Pc}$, EC 2.1.2.7) catalyzes the interconversion between α-methyl-L-serine and D-Ala in the presence of tetrahydrofolate.[3e] Interestingly, $SHMT_{Sth}$ wild type catalyzes the same reaction using L-Ser and Gly as substrates (Figure 2) sharing 50% sequence identity with $MSHMT_{Pc}$ (see Figure S1 in the Supporting Information, SI).

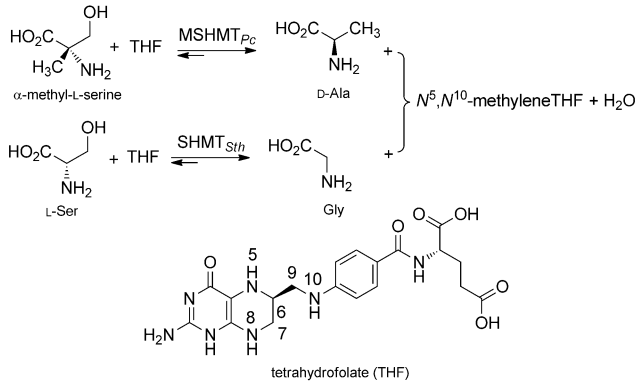
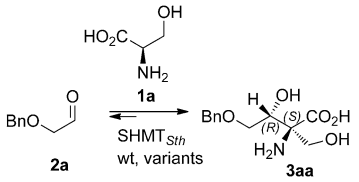


Figure 2. $MSHMT_{Pc}$ and $SHMT_{Sth}$ catalyze the interconversion of α-methyl-L-Ser to D-Ala and L-Ser to Gly, respectively, in the presence of tetrahydrofolate (THF).

The alignment of the $MSHMT_{Pc}$ and $SHMT_{Sth}$ protein sequences revealed three residues that are presumably involved in the donor selectivity: T60, H70, and, to a lesser extent, T236 of $MSHMT_{Pc}$, which correspond to Y55, Y65, and H229 in $SHMT_{Sth}$ (Figure S1).[3e] Hence, the $SHMT_{Sth}$ variants Y55T, Y65H, H229T, Y55T/Y65H, Y55T/H229T, Y65H/H229T, and Y55T/Y65H/H229T were constructed, overexpressed, purified to homogeneity, and tested as catalysts for the aldol addition of D-Ser (**1a**) to **2a** (Table 1); such activity is absent in the wild-type enzyme. Under identical reaction conditions, Y55T gave the best results (37%) with full stereoselectivity; that is, (2*S*,3*R*) configured diastereoisomer **3aa** (Figure 4, compound numeration: first letter referring to the substituent of R^1 of the amino acid donor and second one to the R^2 of the aldehyde acceptor).

Table 1: $SHMT_{Sth}$ wild type and variants as catalyst for the aldol addition of D-Ser (**1a**) to **2a**.[a]



$SHMT_{Sth}$ catalyst	Aldol adduct **3aa** [%][b]
wild type	–[c]
Y55T	37 (50[d])
Y65H	–[c]
H229T	–[c]
Y55T/Y65H	10
Y55T/H229T	8
Y65H/H229T	–[c]
Y55T/Y65H/H229T	21

[a] Reaction (1 mL), D-Ser (0.1 mmol), **2a** (0.1 mmol), PLP (0.3 μmol), pH 6.5 (the amino acid is the system buffer), DMF or DMSO (20% v/v), $SHMT_{Sth}$ catalyst (2 mg protein). [b] Aldol product formed after 24 h. [c] Not detected. [d] Reaction optimized (see Figures S2–S7): water/DMSO 1:1, [aldehyde]/[D-Ser] 1:4, pH 6.5 at 25 °C.

A focused library of mutants (94 clones) was generated by site-saturation mutagenesis at residue Y55 (i.e., NNK degeneracy, Figures S8 and S9). D-Ser and D-Thr were screened as putative donor substrates. We hypothesized that if D-Ser was tolerated, then D-Ala could also be a good donor. Using **2a** as acceptor, two new variants, Y55S and Y55C, consistently exhibited aldol activity on D-Ser (Figures S10 and S11, Table S2), whereas no active clones were found for D-Thr. The analogue of D-Ser, D-Cys, undergoes an irreversible side reaction with the PLP bound to $SHMT_{Sth}$ furnishing a thiazolidine and deactivating the enzyme (Figures S12A and S13).[7] $SHMT_{Sth}$ Y55T, Y55S, and Y55C did not show retroaldol activity toward L-Thr (Figure S14), which is consistent with the results reported for the SHMT from the *B. stearothermophilus* variant Y51F.[8]

The best donor for $SHMT_{Sth}$ variants and the wild type was D-Ala (Figure 3). $SHMT_{Sth}$ Y55T was the best catalyst for D-Ala and D-Ser donors, whereas the wild-type enzyme was the best for Gly but was inactive with D-Ser.

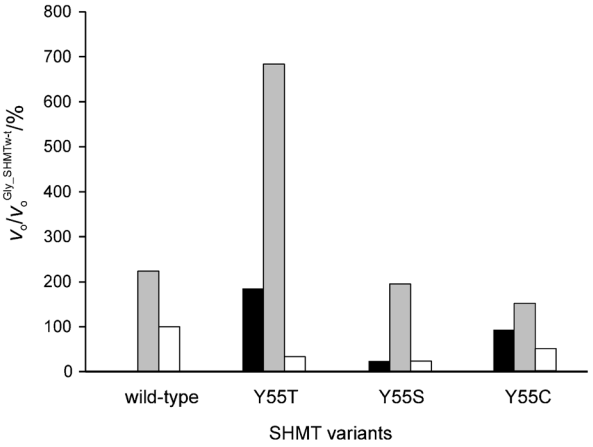


Figure 3. Relative initial reaction rates in % ($v_o/v_o^{Gly\_SHMT\,wt}$) of the addition of D-Ser (**1a**) (black), D-Ala (**1b**) (grey), and Gly (**1c**) (white) to **2a** catalyzed by $SHMT_{Sth}$ variants and the wild type; v_o = initial reaction rate; $v_o^{Gly\_SHMT\,wt}$ = initial reaction rate of the aldol addition of **1c** to **2a** for wild-type $SHMT_{Sth}$.

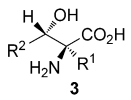
Surprisingly, the $SHMT_{Sth}$ wild type could also catalyze the aldol addition of D-Ala to **2a**. This could not be anticipated because when D-Ala is incubated with $SHMT_{Sth}$ wild-type a transamination took place yielding pyridoxamine phosphate, pyruvate, and apo-$SHMT_{Sth}$ (Figure S15).[9] The aldolase activity was regained when the acceptor aldehyde was present (Figures S16 and S17). The transamination reaction was reduced by 10-fold for $SHMT_{Sth}$ Y55T, Y55S, and Y55C variants suggesting the key role of Y55 in controlling the catalytic promiscuity of $SHMT_{Sth}$ (Figure S15).

The presence of a cosolvent is of paramount importance for obtaining good conversions, even with water-soluble aldehydes. Indeed, product formation at practical rates was only observed in water/DMSO 1:1 as demonstrated for the addition of D-Ala to **2m** (Figure S7(1)–S7(2)). Cosolvent molecules may cluster in the active site of enzymes affecting the enzyme–substrate interactions in different ways, for example, by altering the rate of conversion.[10]

Next, the four $SHMT_{Sth}$ biocatalysts were screened for the aldol additions of **1a**, **1b**, and **1c** to aldehydes **2a**–**p** (Figure 4). $SHMT_{Sth}$ Y55T and Y55C were the best catalysts for aldol additions of D-Ser, whereas the wild type was the best for Gly additions (Figure 4). For D-Ala additions, $SHMT_{Sth}$ wild type or Y55T were the best, with the former being remarkably active with aldehydes **2l**–**n**. Both Y55S and Y55C variants were generally less efficient than Y55T and the wild type, confirming the initial rational redesign of the biocatalyst. Aromatic aldehydes with electron-withdrawing groups, **2l** and **2n**, led to high-yielding reactions, whereas for those with electron-donating groups, **2o** and **2p**, no aldol product was detected. Benzaldehyde (**2j**) was a weak substrate, which is consistent with previously reported studies.[11]

Aldol additions of D-Ser (**1a**) to aldehydes catalyzed by $SHMT_{Sth}$ Y55T were highly (95:5) to fully diastereoselective, within the limits of NMR detection (Table 2). Mechanistically, the approach of any electrophile to the enzyme–donor complex invariably takes place at the *si*-face (i.e., always *S*-configured C2 stereocenter) with no exceptions known so

Table 2: Reaction conversion, yields of isolated product, and diastereomeric ratios for the aldol addition of **1a**–**c** to aldehydes **2** catalyzed by $SHMT_{Sth}$ Y55T and $SHMT_{Sth}$ wild type.



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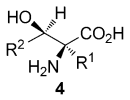

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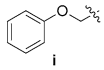
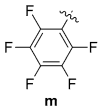
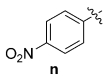
R^1	CH_2OH (**a**) $SHMT_{Sth}$ Y55T		CH_3 (**b**) $SHMT_{Sth}$ wild type		$SHMT_{Sth}$ Y55T		H (**c**) $SHMT_{Sth}$ wild type	
R^2	Conv.[a] (yield)[b] [%]	d.r.[c] **3/4**	Conv.[a] (yield)[b] [%]	d.r.[c] **3/4**	Conv.[a] (yield)[b] [%]	d.r.[c] **3/4**	Conv.[a] (yield)[b] [%]	d.r.[c] **3/4**
 **a**	47 (28)	>95:5	24 (21)	95:5	61 (36)	>95:5	–	–
 **b**	32 (20)	>95:5	27 (11)	72:28	45 (22)	95:5	15 (5)	67:33
 **c**	44 (21)	>95:5	44 (18)	93:7	39 (23)	>95:5	–	–
 **d**	31 (16)	>95:5	25 (9)	91:9	38 (6)	>95:5	–	–
 **g**	22 (14)	87:13	–	–	34 (22)	86:14	–	–
 **h**	31 (23)	95:5	44 (31)	65:35	52 (35)	85:15	18 (10)	49:51
 **i**	40 (22)	>95:5	40 (21)	86:14	71 (20)	95:5	33 (13)	50:50
 **j**	–	–	25 (16)	42:58	–	–	29 (10)	37:63
 **k**	12 (5)	95:5	35 (20)	40:60	24 (12)	71:29	29 (15)	50:50
 **l**	14 (9)	95:5	55 (29)	29:71	48 (26)	76:24	59 (48)	33:67
 **m**	–	–	94 (31)	8:92	43[d]	8:92	86 (65)	87:13
 **n**	21 (15)	95:5	83 (51)	44:56	61 (43)	92:8	60 (56)	50:50

[a] Conversion: percentage of aldol adduct (**3** + **4**) produced. [b] Yield of isolated product (**3** + **4**). [c] d.r.: diastereomeric ratio assessed by NMR spectroscopy (see SI). Products were enantiomerically pure (see text). [d] Reaction was performed at analytical scale.

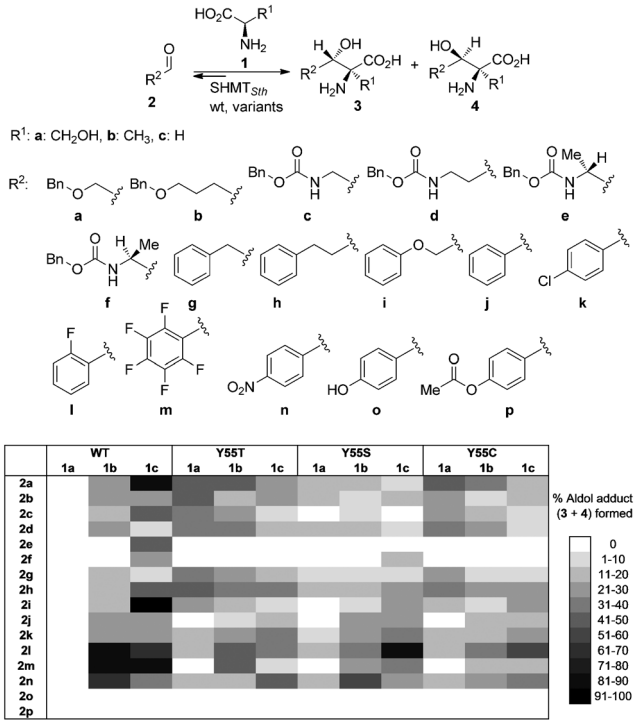


Figure 4. Screening of the SHMT$_{Sth}$ wild type and variants Y55T, Y55S, and Y55C as catalysts for the aldol additions of **1a–c** to aldehydes **2a–p**. For the numerical values, see Tables S4–S6.

far.[3d,6,12] This will ensure the full enantioselectivity of the reactions catalyzed by SHMT with any donor/acceptor combination. Moreover, the aldol adducts obtained were always *anti* (**3**) with respect to the α-amino and the β-hydroxy functions (i.e., always *si*→*re* attack) (Figure 5 and SI). Only

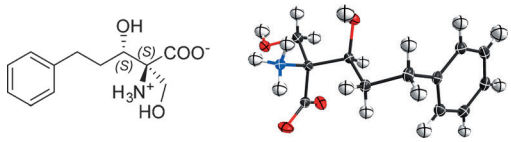


Figure 5. X-ray diffraction analysis for the determination of the absolute configuration of **3ah** as example. ORTEP-type plot displaying one molecule with 50% probability ellipsoids.[13]

aldehyde **2g** gave moderate diastereoselectivity (87:13 **3ag**/**4ag**). Additions of D-Ala (**1b**) to aldehydes proceeded for both the SHMT$_{Sth}$ wild type and SHMT$_{Sth}$ Y55T, thus allowing to compare the stereochemical outcome for identical substrates (Table 2). The SHMT$_{Sth}$ Y55T, unlike the wild type, catalyzed the additions with high stereoselectivity (i.e., *anti* (**3**), except for the acceptor **2m** for which the *syn* product (**4**) was the major product). SHMT$_{Sth}$ wild type provided *syn*/*anti* mixtures in most cases, the *anti* being the major adduct, whereas the *syn* was predominantly formed with aromatic aldehydes. The high selectivity of the SHMT$_{Sth}$ Y55T variant for D-Ala and D-Ser can be explained by mechanistic considerations which are consistent with the computational models described below and in Figure S96. On the other hand, additions of Gly catalyzed by wild-type SHMT$_{Sth}$ showed moderate to low diastereoselectivity and conversions (Table 2).

To characterize the structural frame of the catalytic system, we determined the X-ray structure of apo-SHMT$_{Sth}$ wild type (PDBID-4WXB), in complex with Gly-PLP (PDBID-4WXF), and with a mixture of L-Thr-PLP and Gly-PLP (PDBID-4WXG) (Figures S91–S94). Models of external aldimines (Figure S95) were generated for the SHMT$_{Sth}$ (Figures 6 and S96) based on the structure of the wild-type L-Thr-PLP complex (Figures 6A and S93).

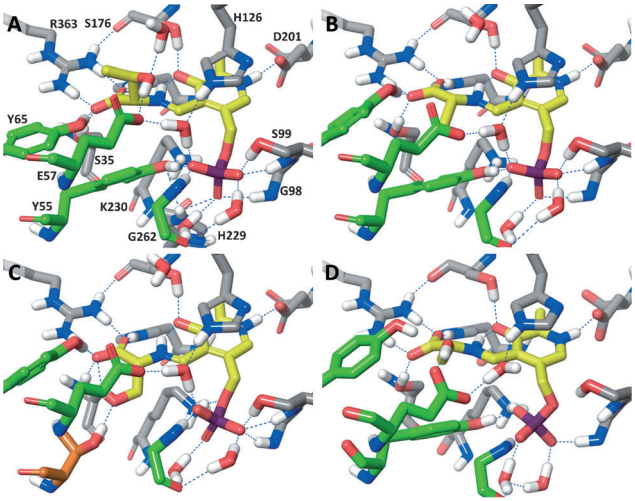


Figure 6. Modeled structures of the active site of SHMT$_{Sth}$ wild type with the bound external aldimines of L-Thr (**A**), D-Ala (**B**), and D-Ser (**D**), and of the SHMT Y55T variant with the bound external aldimine of D-Ser (**C**). Aldimines (yellow), protein residues (grey or green, meaning that they belong to different protein monomers), and the mutated T55 residue (**C**) (orange). Models were built from the corresponding X-ray structure (Figure S93).

These models suggest that D-Ala could form the aldimine intermediate (Figure S95 **I**) with wild-type SHMT$_{Sth}$ (Figure 6B) as that observed for the L-Thr-PLP complex. In this conformation, the hydrogen on the α-carbon (H–Cα), which is removed to form the quinonoid intermediate (Figure S95 **II**), is located at 4.4 Å of the E57 and at 3.9 Å of a water molecule, which appears to be activated by H bonds to the PLP-phosphate and E57 and H126. The D-Ser aldimine model for the Y55T variant (Figure 6C) shows that the space generated is partially occupied by the hydroxymethyl group of D-Ser, which establishes H bond interactions with the hydroxy group of T55 and the carboxylate group of D-Ser. In this conformation, the H–Cα would be even closer to residue E57 (3.0 Å) and to the water molecule (3.3 Å). Hence, one of these could operate as the catalytic base that triggers the quinonoid formation. Preliminary results with E57 and H126 variants (Table S14) showed that none of these two residues is essential for activity. Thus, the crystallographic water seems the most plausible candidate to mediate the H–Cα proton abstraction.[3d] The structure of wild-type SHMT$_{Sth}$ suggests that D-Ser could also form the aldimine I complex (Figure 6D). However, as compared with the Y55T variant, here

the hydroxy group of D-Ser would adopt an opposed orientation, stabilized by H bonds with residues E57 and Y65 and hindering the access to the H–Cα atom, which would be at > 5 Å from the E57 and water molecule. The lack of D-Ser reactivity with wild-type $SHMT_{Sth}$ could thus be due to the steric hamper caused by the hydroxy group of D-Ser, sitting between the proposed catalytic water and the H–Cα atom.

In summary, we have discovered the $SHMT_{Sth}$ Y55T variant for the efficient, highly enantio- and diastereoselective synthesis of α,α-disubstituted β-hydroxy-α-amino acids, which was so far unprecedented for glycine utilizing aldolases.

Received: November 27, 2014
Revised: December 18, 2014
Published online: January 21, 2015

Keywords: aldol reaction · amino acids · C–C bond formation · enzyme catalysis · quaternary stereocenters

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[13] CCDC 1032540 contains the supplementary crystallographic data for compound **3ah**. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. The Flack parameter is $x = -0.037(24)$, indicating the high enantiomeric discrimination power of the analysis for *S* configuration.

