

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

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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_{St}; 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_{St} Y55T variant overcoming the limitation of the native enzyme for Gly. The SHMT_{St} 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 cen-

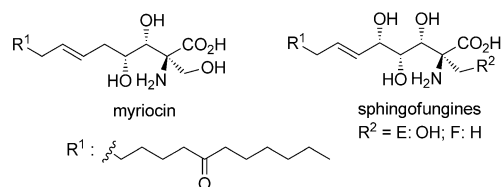


Figure 1. Structures of myriocin and sphingofungines E and F.

ters 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]

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_{St}; EC 2.1.2.1).^[5] SHMT_{St} was found to catalyze aldol additions of Gly to aldehydes with good stereoselection under kinetic control (4°C).^[6]

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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).

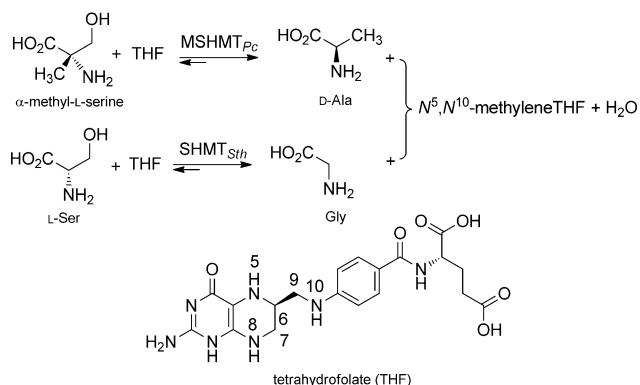


Figure 2. MSHMT_{Pc} and SHMT_{Sth} catalyze the interconversion of α -methyl-L-Ser to D-Ala and L-Ser to Gly, respectively, in the presence of tetrahydrofolate (THF).

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

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 μ mol), 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.

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 R¹ of the amino acid donor and second one to the R² of the aldehyde acceptor).

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. steartophilus* 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.

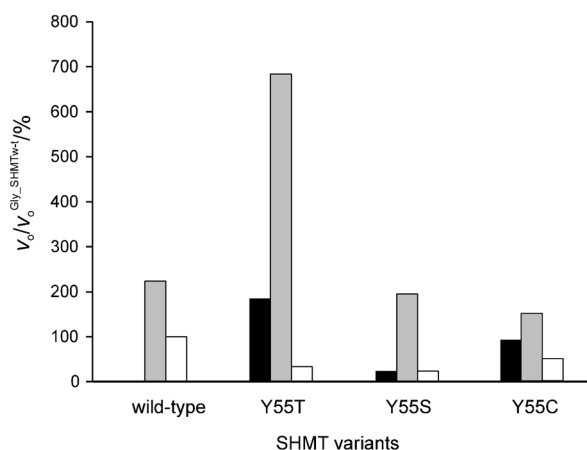


Figure 3. Relative initial reaction rates in % ($v_0/v_0^{\text{Gly-SHMT wt}}$) of the addition of D-Ser (**1a**) (black), D-Ala (**1b**) (grey), and Gly (**1c**) (white) to **2a** catalyzed by SHMT_{Sth} variants and the wild type; v_0 = initial reaction rate; $v_0^{\text{Gly-SHMT wt}}$ = initial reaction 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.

R ¹	CH ₂ OH (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
	47 (28)	> 95:5	24 (21)	95:5	61 (36)	> 95:5	–	–
	32 (20)	> 95:5	27 (11)	72:28	45 (22)	95:5	15 (5)	67:33
	44 (21)	> 95:5	44 (18)	93:7	39 (23)	> 95:5	–	–
	31 (16)	> 95:5	25 (9)	91:9	38 (6)	> 95:5	–	–
	22 (14)	87:13	–	–	34 (22)	86:14	–	–
	31 (23)	95:5	44 (31)	65:35	52 (35)	85:15	18 (10)	49:51
	40 (22)	> 95:5	40 (21)	86:14	71 (20)	95:5	33 (13)	50:50
	–	–	25 (16)	42:58	–	–	29 (10)	37:63
	12 (5)	95:5	35 (20)	40:60	24 (12)	71:29	29 (15)	50:50
	14 (9)	95:5	55 (29)	29:71	48 (26)	76:24	59 (48)	33:67
	–	–	94 (31)	8:92	43 ^[d]	8:92	86 (65)	87:13
	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.

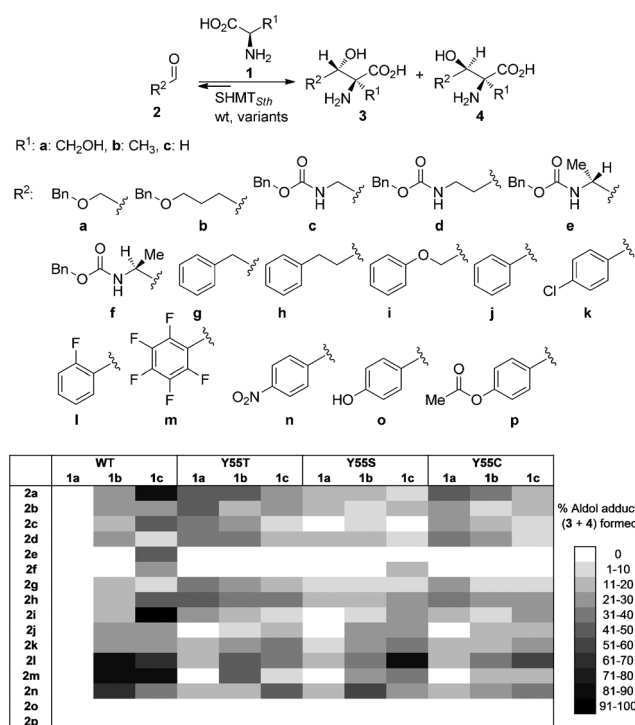


Figure 4. Screening of the SHMT_{Sth} wild type and variants Y55T, Y55S, and Y55C as catalysts for the aldol additions of **1a–c** to aldehydes **2a–p**. For the numerical values, see Tables S4–S6.

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

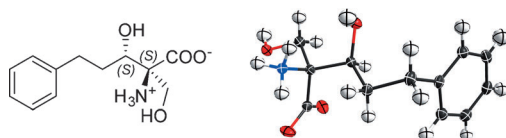


Figure 5. X-ray diffraction analysis for the determination of the absolute configuration of **3ah** as example. ORTEP-type plot displaying one molecule with 50% probability ellipsoids.^[13]

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).

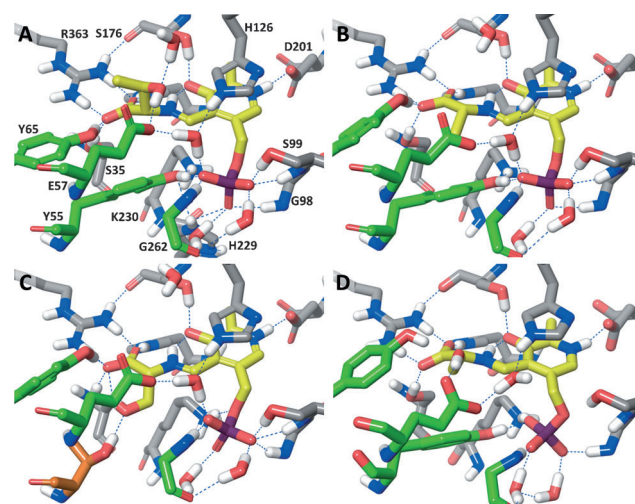


Figure 6. Modeled structures of the active site of SHMT_{Sth} wild type with the bound external aldimines of L-Thr (**A**), D-Ala (**B**), and D-Ser (**D**), and of the SHMT Y55T variant with the bound external aldimine of D-Ser (**C**). Aldimines (yellow), protein residues (grey or green, meaning that 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-C α), 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-C α 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-C α 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-C α atom, which would be at > 5 Å from the E57 and water molecule. The lack of D-Ser reactivity with wild-type SHMT_{Sh} 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-C α atom.

In summary, we have discovered the SHMT_{Sh} 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.

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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.