An Engineered Tryptophan Synthase Opens New Enzymatic Pathways to -Methyltryptophan and Derivatives.

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β-Methyl-α-amino acids are important building blocks in the synthesis of peptidomimetics and other pharmaceuticals (for examples see Scheme 1A & S1).[1] The introduction of a β-methyl substituent into synthetically modified peptides reduces the conformational freedom of the amino acid side chain, reducing the entropic penalty of binding to a biological receptor, resulting in higher affinity and improved biological activity.[2] In light of this, β-branched synthetic peptides have been used to develop improved opioid agonists, somatostatin receptor agonists, cholecystokinin B receptor agonists, glucagon receptor antagonists and AT4 receptor antagonists.[3,4] Nature has also adopted the strategy of introducing β-methyl-α-amino acids into peptides, alkaloids and other bioactive natural products (for examples see Scheme 1A & S2),[4] which presumably also serves to pre-organise the amino acid side chain for tighter binding to molecular targets in vivo.

The prominence of β-methyl-α-amino acids in synthetic peptides, natural products and other valuable compounds has resulted in the development of many methods for the synthesis of β-methyl amino acids including: β-lactone ring opening, alkylation of imines using secondary sulfonates,[3,3] sigmatropic rearrangements; the use of chiral auxiliaries and/or kinetic resolutions.[5] Many of these and other approaches require laborious multi-step synthetic procedures, use deleterious reagents and often fail to provide enantiomerically pure products. In contrast, there have been relatively few enzymatic approaches used in the preparation of β-methyl-α-amino acids, despite the inherent advantages of enzymes which include high stereoselectivity and cleaner, more benign aqueous reaction conditions. Previously, we elucidated the biosynthesis of β-methylglutamic acid, which involved the conditions. Previously, we elucidated the biosynthesis of β-methyl-α-amino acids, including: β-lactone ring opening, alkylation of imines using secondary sulfonates;[3,3] sigmatropic rearrangements; the use of chiral auxiliaries and/or kinetic resolutions.[5] Many of these and other approaches require laborious multi-step synthetic procedures, use deleterious reagents and often fail to provide enantiomerically pure products. In contrast, there have been relatively few enzymatic approaches used in the preparation of β-methyl-α-amino acids, despite the inherent advantages of enzymes which include high stereoselectivity and cleaner, more benign aqueous reaction conditions. Previously, we elucidated the biosynthesis of β-methyltryptophans (β-mTrp) are precursors in the biosynthesis of bioactive natural products and are used in the synthesis of peptidomimetic based therapeutics. Currently β-mTrp is produced by inefficient multi-step synthetic methods. Here we demonstrate how an engineered variant of tryptophan synthase from Salmonella (StTrpS) can catalyse the efficient condensation of L-threonine and various indoles to generate β-mTrp and derivatives in a single step. Although L-serine is the natural substrate for TrpS, targeted mutagenesis of the StTrpS active site provided a variant (βL166V) which can better accommodate L-Thr as a substrate. The condensation of L-Thr and indole proceeds with retention of configuration at both α- and β-positions leading to (2S,3S)-β-mTrp. The integration of StTrpS (βL166V) with L-amino acid oxidase, halogenase enzymes and palladium chemocatalysts provides access to further D-configured and regioselectively halogenated or arylated β-mTrp derivatives.
In this paper we demonstrate how an engineered variant of tryptophan synthase (TrpS) can efficiently produce a range of enantiomerically pure (2S, 3S)-L-β-methyltryptophan (β-mTrp) derivatives. Enzyme cascades utilizing the TrpS variant with L-amino acid oxidase (L-AAO) and halogenase enzymes provide access to more diverse L- and D-β-mTrp analogues. TrpS is a potential for pharmaceutical synthesis. Moreover, PLP-dependent amino acid oxidase (L-AAO) and halogenase enzymes provide access to more diverse L- and D-β-mTrp analogues. TrpS is a promising biocatalyst for the formation of enantiomerically pure β-mTrp derivatives.

We wished to explore the possibility of utilizing threonine as a substrate for TrpS instead of serine, to generate β-mTrp (Figures 1A & 3). Accordingly, StTrpS was overproduced in E. coli BL21(DE3) and the resulting cell-free extract was incubated with indole and a ten-fold excess of L-Thr. The progress of the reaction was followed by HPLC (Figure S3) revealing the formation of a new product peak, β-mTrp 1, which was observed to reach a maximum conversion of 60% (± 4%) after 5 hours (Figure S4). No production of β-mTrp 1 was evident in control assays when indole and L-Thr were incubated with standard BL21 lysate lacking StTrpS (Figure S3). Subsequent scale up, followed by solvent and solid-phase (C18) extractions led to the isolation of β-mTrp in a 54 % yield, demonstrating for the first time that the wild type StTrpS can be harnessed to generate a β-methyl amino acid.

While L-Thr is accepted as a substrate by StTrpS, the activity and isolated yields of L-β-mTrp are low compared to the wild type reaction with L-Ser, which gives over 90% of L-Trp under the same conditions. The published crystal structure(s) of StTrpS show that a leucine residue (βL166) is in close proximity to the β-position of the α-aminoacylate species. With L-Thr as a substrate, the introduction of a methyl group in the corresponding (E)-2-amino-2-butenolate intermediate could lead to a steric hindrance with the side chain of βL166 (Figure 1B). With this in mind, a StTrpS mutant was generated which replaced βL166 with the less bulky valine. Cell lysate experiments carried out with this new mutant (βL166V) showed improved activity compared to the wild-type with the conversion of indole to β-mTrp reaching 98% (±0.2%) within 3 hours (Figure S4). SDS-Page analysis suggests that both wild-type and βL166V lysates contain a similar amount of StTrpS protein (Figure S5). However, to facilitate direct comparison of the wild-type StTrpS and the βL166V mutant, the β subunits were expressed as hexahistidine fusion proteins and purified by metal affinity chromatography. The β-subunit was expressed individually to prevent the formation of heterologous mixtures of αβ2 and β2 complexes. Since the αβ2 is more active than the β2 such unquantified mixtures could bias comparative activity assays between the wild-type and the mutant. The subsequent kinetic analysis revealed that the activity of the βL166V mutant was ten-fold higher than the wild-type (Table 1). Presumably the increase in the size of the active site in the βL166V mutant allows it to better accommodate L-Thr as a substrate (Figure 1C). An additional mutant was generated to further expand the space within the active site, βL166A, but although this showed a five-fold improvement over the wild-type it was only half as active as βL166V. Replacing βL166 with alanine may provide too much space within the active site, possibly allowing either the indole or the (E)-2-amino-2-butenolate intermediate to adopt a suboptimal conformation within the active site, leading to less efficient conjugate addition. It is worth noting that although the measured kcat of βL166V with L-Trp is low, the enzyme functions in a slow but steady fashion, remaining stable and active within a crude cell lysate for several days, meaning good quantities of product can still be isolated. Also the rate of the αβ2 tetramer present in the cell lysate will be higher than that of the purified β2 complex observed here. Analysis of the kinetics with the natural substrate L-serine shows that activity towards serine is reduced in both of the two mutants. These results indicate that the βL166V mutant is a promising biocatalyst for the formation of enantiomerically pure β-mTrp derivatives.
Previous stereochemical studies with TrpS using labelled (2S,3R)- and (2S,3S)-[3-^3H]-serine show that the dehydration and addition of indole to form L-Trp proceed with retention of configuration at C3 as well as at C2 (Figure 1A). Based on this information, combined with analysis of X-ray crystal structures of STrpS\(^\text{[13]}\) (Figure 1B), we predicted that the condensation of L-Thr and indole catalysed by STrpS would proceed with indole addition to the Re face of the β-carbon in an (E)-2-amino-2-butenolate intermediate (iv, R = CH\(_2\)) resulting in (2S,3S)-β-mTrp 1. As anticipated the configuration of the β-mTrp generated by both the wild-type STrpS and the BL166V mutant was confirmed to be (2S,3S) by comparison of optical rotation and NMR data (Figure S6), including a \(^{1}J\(_{\text{H,H}}\) coupling constant of 7.4 Hz, with data from literature.\(^\text{[14]}\) The enantiomeric purity of the β-mTrp 1 was further assessed using L-amino acid oxidase (LAAO). Incubation of β-mTrp 1 with LAAO resulted in >98% conversion of 1 to the corresponding α-keto acid as determined by HPLC (Figure S7). Conversely when the β-mTrp 1 was incubated with D-amino acid oxidase (DAAO) under identical reaction conditions no α-keto acid was formed (Figure S7).

To further explore the scope of the BL166V mutant, biotransformations were carried out with a range of halogenated, methylated and methoxylated indoles (Scheme 2). This showed that the mutant could be used to generate β-mTrp derivatives (1-9) with substituents in either the 2-, 4-, 6- or 7-positions of the indole ring. The % conversions to various β-mTrp derivatives varied from 40-96% and may be further improved by recycling the unreacted indole for further biotransformation.\(^\text{[12a]}\) The preparation of enantiomerically pure L-β-mTrp derivatives in a single biotransformation offers significant advantages over the existing synthetic routes to these compounds. At least six different synthetic routes to β-mTrp derivatives have been reported (Figure S8), emphasising the importance of these compounds, and each synthesis requires between five and eight steps, including deleterious reagents, the use of expensive chiral auxiliaries, chiral catalysts or resolution steps with low overall yields.

While 2-, 4-, 6- or 7-substituted indoles were accepted by STrpS (BL166V), along with L-Thr, 5-substituted indoles proved to be very poor substrates for the enzyme. To address this we choose to explore the possibility of using the flavin-dependent tryptophan-5-halogenase PyrH to derivatise CS of the indole moiety of β-mTrp 1. In addition to regioselectively halogenating L-Trp,\(^\text{[16]}\) PyrH has been shown to be promiscuous and can halogenate a number of other aromatic compounds.\(^\text{[17]}\) The tryptophan halogenases are relatively unstable and inefficient biocatalysts. However, recent studies have shown that productivity of a tryptophan-7-halogenase ( RebH) can be considerably improved through the generation of cross-linked enzyme aggregates (CLEAs).\(^\text{[18]}\) Accordingly a CLEA was prepared containing PyrH, the flavin reductase Fru (for recycling the FADH\(_{2}\) cofactor), and alcohol dehydrogenase (for NADH regeneration). Using this CLEA, the β-mTrp 1 from STrpS could be converted to 5-chloro-β-mTrp 10 in 62% yield using only MgCl\(_2\), O\(_2\) (from air) and isopropanol as stoichiometric reagents (Scheme 3). This represents a 40% overall yield of the two enzymatic reactions from the indole starting material. Substitution of MgCl\(_2\) with NaBr, as inorganic halide donor, allowed the similar preparation of 5-bromo-β-mTrp 11 in 41% yield. Recently our laboratory and the Sewald group both showed how halogenase enzymes can be integrated with palladium-catalyzed cross-coupling chemistry, in one-pot reactions, to affect the regioselective arylation or alkenylation of C-H positions of aromatic scaffolds.\(^\text{[19]}\) Following this approach, we were able to affect the direct C5-arylation of β-mTrp 1 to give 5-phenyl β-mTrp 12, in a one-pot reaction with 63% yield, using the PyrH-CLEA to generate intermediate aryI bromide (11) and sSSHos and Na\(_2\)PdCl\(_2\) to catalyse cross-coupling with phenyl boronic acid. These results, coupled with our previous studies,\(^\text{[19e]}\) indicate that combination of STrpS (BL166V), with halogenases and transition metal catalysis can open the way to more highly modified β-mTrp derivatives that would be difficult to prepare directly from a functionalised indole precursor using TrpS.

While the exquisite stereoselectivity of TrpS is a major advantage, access to other diastereoisomers would be desirable. For example the D-configured epimer of 1, (2R,3S)-β-mTrp 13, is present in peptidomimetic drug candidates for diabetes, such as L-779,976 (Scheme 1A).\(^\text{[11c,d]}\) Given that we showed (2S,3S)-β-mTrp 1 is a substrate for LAAO we envisaged affecting stereoinversion at the α-position to give (2R,3S)-β-mTrp 12, via the non-selective reduction of the imine intermediate formed from the LAAO oxidation of 1. Such cyclic oxidation-reduction procedures have been used successfully in the deracemization or epimerisation of other α-amino acid substrates.\(^\text{[20]}\) Accordingly 1 was incubated with LAAO in the presence of an excess of ammonia-borane, using the established conditions,\(^\text{[20]}\) resulting in the formation of the (2R,3S)-epimer 13 in 93% yield. The H NMR of 13 clearly indicates the expected change in chemical
shifts and coupling constant between the α and β protons (Figure S6). The NMR and other analytical data are also in agreement with the literature.\[74\] Furthermore incubation of 13 with DAAO led to complete oxidation, whilst no reaction was observed with LAAO (Figure S7). The overall yield for the two step enzymatic preparation of (2R,3S)-β-mTrp 13 from indole is ca. 66%.

![Diagram of enzymatic preparation of (2R,3S)-β-mTrp](image)

**Scheme 3.** Diversification of β-mTrp 1 using: Halogenase (PyrH) catalysed C5-chlorination & bromination; C5-arylation using an integrated one-pot halogenase-Suzuki-Miyaura cross-coupling procedure\[17\] and biocatalytic stereoinversion (isolated yields).

In order to generate β-mTrp with opposite configuration at C3, we envisaged utilising L-allo-threonine (2S,3S-Thr) as a substrate for TrpS. Based on the structure and mechanism of StTrpS (Figure 1), L-allo-Thr would, if accepted, be predicted to generate the (Z)- rather than (E)-2-amino-2-butenoate intermediate (iv), leading to (2S,3R)-β-mTrp. However, we found that L-allo-Thr is not a substrate for StTrpS or the mutants we prepared. Given that the L-allo-threonine aldolase (LATA) can be used to produce L-allo-threonine from glycine and acetaldehyde,\[21\] engineering of TrpS to accept L-allo-threonine as an alternative amino acid substrate is an attractive future goal.

In summary we have demonstrated that tryptophan synthase (StTrpS) can utilise threonine, along with indole, in the preparation of enantiopure (2S,3S)-β-mTrp. Rational mutagenesis of StTrpS β-subunit changing Leu166 to Val, which is likely to better accommodate larger L-Thr substrate, provided significant advantages over the synthetic procedures, to a range of functionalised β-mTrp derivatives, which have proven useful building blocks for drug synthesis.\[14\] During revision of this manuscript, we became aware of a recent publication describing an evolved mutant of the tryptophan synthase β-subunit, from the thermophile *Pyrococcus furiosus* (PfTrpB), that can also be used to produce β-mTrp.\[20\] Kinetic parameters were not determined in this alternative study, which precludes a direct quantitative comparison between the PfTrpB and StTrpS variants. However, unlike the PfTrpB variant which requires high temperature (75 °C) for optimal catalytic activity, the StTrpS mutant described here efficiently catalyses condensation of indoles with L-Thr at ambient temperature which is preferred for biocatalytic processes.

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**Keywords:** Biocatalysis • Tryptophan synthase • Enzyme cascades • β-Methyl-α-amino acids • Peptidomimetics

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A mutant tryptophan synthase (StTrpS) catalyses the condensation of 2-, 4-, 6- & 7-substituted indoles with threonine to give (2S,3S)-β-methyltryptophans (β-mTrp), which are important building blocks in drug synthesis. Addition of L-amino acid oxidase (LAAO) gives (2R,3S)-β-mTrp. Halogenase (Hal) enzymes also provide halogenated β-mTrp derivatives that can be arylated in a one-pot chemobiotransformation.

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