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Asymmetric β-methylation of L- and D-α-amino acids by a self-contained enzyme cascade

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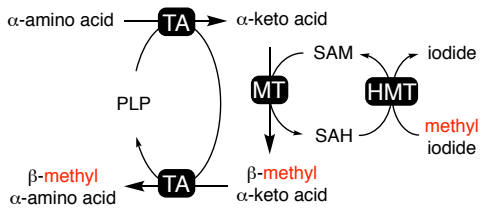
Abstract. This report describes a modular enzyme-catalyzed cascade reaction that transforms L- or D-α-amino acids to β-methyl-α-amino acids. In this process an α-amino acid transaminase, an α-keto acid methyltransferase and a halide methyltransferase cooperate in two orthogonal reaction cycles that mediate product formation and regeneration of the cofactor pyridoxal-5'-phosphate and the co-substrate S-adenosylmethionine. The only stoichiometric reagents consumed in this process are the unprotected L- or D-α-amino acid and methyl iodide.



Introduction. The development of new ways to make molecules is a central objective in chemical research. In the comparison of competing synthetic strategies, criteria such as substrate scope, reaction specificity, conversion efficiency and reagent economy are critical.[1] The number of preparative steps that require isolation of intermediates is a central parameter. Hence, integration of multiple elementary steps into cascade reactions has emerged as a promising approach to shorten synthetic routes.[2] Biocatalysis is particularly well suited for the design of cascading reactions.[3] Most enzymes have evolved to participate in multistep pathways and to process their substrates at low steady-state concentrations without accumulation of unstable intermediates.[4] However, other aspects that are also related to the cellular origin of enzymes make their application in chemical synthesis challenging. In particular, enzymes that catalyze group-transfer and redox reactions depend on co-substrates or cofactors such as nicotinamides (NAD(P)), nucleoside triphosphates (ATP, GTP), acyl-Coenzyme A, S-adenosylmethionine (SAM), pyridoxal 5-phosphate (PLP) or riboflavins (FMN, FAD). In a living cell regeneration of these reagents is coupled to central metabolism. In order to exploit these enzymes *in vitro* it is therefore necessary to engineer artificial minimal metabolisms that afford co-substrate regeneration instead.

Pioneering work on oxidoreductase biocatalysis has shown that cofactor regeneration can indeed be implemented to drive difficult transformations without the support of a living cell.[5] [3a] These systems generally depend on cyclic regeneration of NAD(P)H either by consuming sacrificial hydrogen donors or acceptors, or by shuttling hydrides from the substrate to the final product in a process known as hydrogen-borrowing.[5a, 5c, 6] Emerging technologies that enable regeneration of ATP, acyl-CoA or SAM indicate that this approach may be extended to ligations and group-transfer reactions.[7] Capitalizing on this idea we have developed an enzyme-based process that enables stereoselective production of L- or D-β-methyl-α-amino acids (*β*-Me-α-aas) from unprotected L- or D-α-amino acids and methyl iodide.

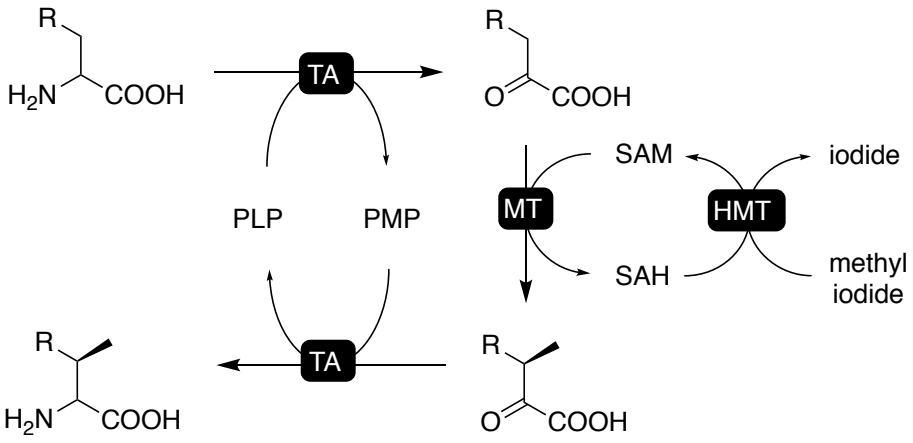


Figure 1. General scheme for stereoselective production of L- and D-β-methyl-α-amino acids by cascade biocatalysis: i) α-amino acids are oxidized to α-keto acids by PLP-dependent L- or D-α-amino acid transaminases (L-TA or D-TA); ii) α-keto acids are methylated by SAM-dependent methyltransferases (MT); iii) β-methyl-α-keto acids are reduced to the corresponding L- or D-β-methyl-α-amino acids by the PMP-containing TA. S-adenosylhomocysteine (SAH) is remethylated to SAM by a halide methyltransferase (HMT).

Results and discussion. The design of this system was motivated by the considerable potential of *β*-Me-α-aas as building blocks in bioactive compounds, and by the inherent difficulty associated with their chemical synthesis. L-*β*-Me-α-aas are common constituents of natural products, including the clinical antibiotic daptomycin (Supplementary Figure 1).[8] The value of L-*β*-Me-α-aas as building block in bioactive compounds is documented by the dramatic effect – the magic methyl effect – of strategically placed methyl groups on bioactivity and stability.[9] Despite this potential, application of *β*-Me-α-aas in research and development has been limited, not the least because their stereoselective synthesis remains challenging.[10] A more direct biocatalytic approach to produce *β*-methyl-tryptophan derivatives has been reported.[11] However, because this process depends on tryptophan synthases for stereoselective carbon-carbon bond formation, the substrate scope is limited to indole-containing L-α-amino acids.

The methodology described in the following is more general. In a way, this system is a bionic replica of the natural biosynthesis of L-*β*-Me Leu.[12] First, the substrate α-amino acid is oxidized to the corresponding *α*-keto acid by a PLP-dependent L-amino acid transaminase (L-TA, Figure 1). The *α*-keto acid is methylated by a SAM-dependent α-keto acid methyltransferase (MT) forming a new stereocenter with R-configuration. Finally, the methylated α-keto acid reclaims its amino group from the pyridoxamine (PMP)-containing L-TA to form a L-*β*-Me-α-aa. Because the reaction does not contain additional amine acceptors, the steady state concentration of the two α-keto acids is limited by the initial concentration of PLP. As a consequence, the accumulating product is the stable *β*-Me-α-aa instead of the *β*-methylated α-keto acid, which is prone to racemization.[7b] The third enzyme in the cascade is a halide methyltransferase. This enzyme regenerates SAM by stereoselective S-methylation of S-adenosylhomocysteine (SAH) using methyl iodide as methyl donor.[7b]

To realize this concept we produced the branched chain L-amino acids transaminase (IlvE) from *E.coli*, the α-keto acid methyltranferase (SgvM) from *Streptomyces griseoviridis,*[12] and the halide methyltransferase (HMT) from *Burkholderia xenovorans* in the SAH nucleosidase-deficient strain *E. coli* Δmtn (DE3). The purified enzymes (40 µM IlvE, 40 µM SgvM and 20 µM HMT) were combined with 2 mM of L-norvaline (**1**), 4 mM methyl iodide, 40 µM SAH, 40 µM PLP in a 100 mM phosphate buffer (pH 8.0) and incubated at 25 °C. This reaction converted 52 % after 24 hours and 75 % after 48 hours of L-norvaline to L-allo-isoleucine (**11**) as inferred by ^{1}H NMR spectroscopy (Table 1, entry 1). The diastereomer L-isoleucine (**11a**) was detectable as a minor product of 2 %.

IlvE and SgvM are both characterized by significant substrate promiscuity and may therefor support the synthesis of other L-*β*-Me-α-aas.[13] Indeed, the same enzyme composition converted of 89 % L-leucine (**2**) to (2S, 3R)-3-methyl-leucine (**12**) (entry 2) with a diastereomeric ratio of 98:2. α-amino acids with longer and branched alkyl chains (entries 3 and 6) or with alkenyl side chains (entries 4, 5 and 7) were

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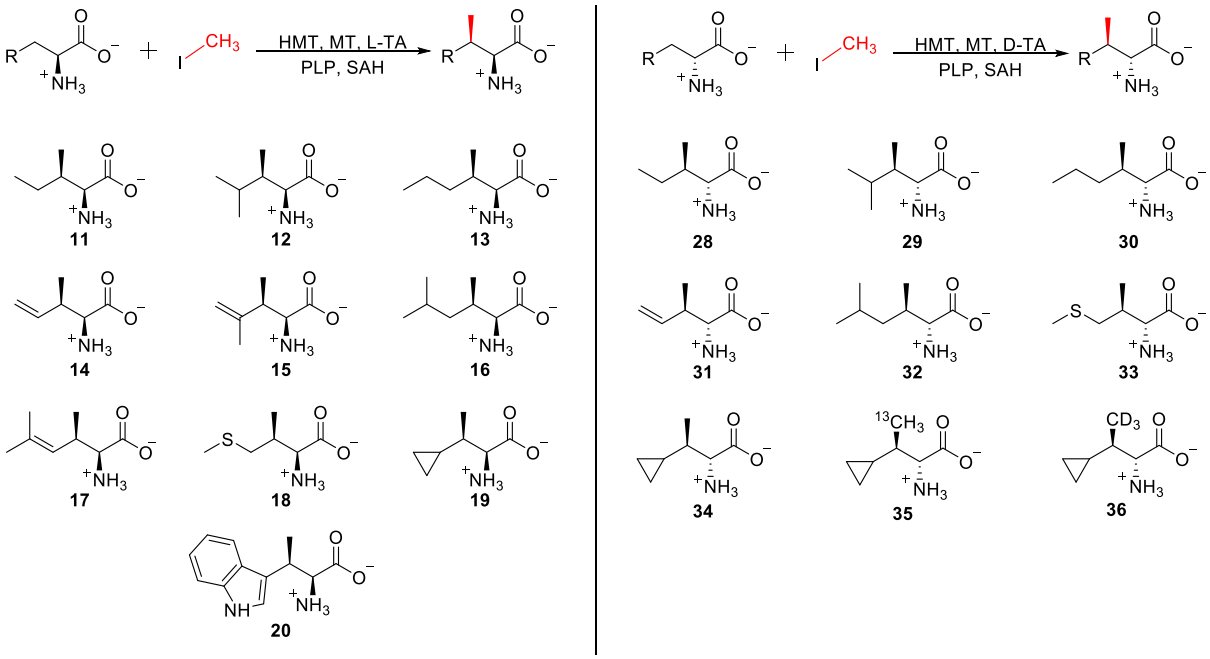

Scheme 1

Table 1. Substrates and products of the methylation cascade.[a]

Entry	Substrate	TA	MT	Product	Conversion	3R:3S
1	L-norvaline (**1**)	IlvE	SgvM	**11**	52 %	98:2
2	L-leucine (**2**)	IlvE	SgvM	**12**	89 %	98:2
3	L-norleucine (**3**)	IlvE	SgvM	**13**	80 %	95:5
4	L-allylglycine (**4**)	IlvE	SgvM	**14**	83 %	93:7
5	L-4,5 dehydroleucine (**5**)	IlvE	SgvM	**15**	87%	96:4
6	L-2-amino-5-methyl hexanoic acid (**6**)	IlvE	SgvM	**16**	82 %	98:2
7	L-2-amino-5-methyl Hex-4-enoic acid (**7**)	IlvE	SgvM	**17**	70 %	99:1
8	L-methionine (**8**)	IlvE	SgvM	**18**	47 %	99:1
9	L-cyclopropylalanine (**9**)	IlvE	SgvM	**19**	96 %	99:1
10	L-tryptophan (**10**)	MarG	MarI	**20**	>95 %	92:8
11	D-norvaline (**21**)	D-TA	SgvM	**28**	50 %	98:2
12	D-leucine (**22**)	D-TA	SgvM	**29**	39 %	99:1
13	D-norleucine (**23**)	D-TA	SgvM	**30**	90 %	95:5
14	D-allylglycine (**24**)	D-TA	SgvM	**31**	> 95 %	92:8
15	D-2-amino-5-methyl hexanoic acid (**25**)	D-TA	SgvM	**32**	89 %	91:9
16	D-methionine (**26**)	D-TA	SgvM	**33**	82 %	95:5
17	D-cyclopropylalanine (**27**)	D-TA	SgvM	**34**	> 95 %	98:2
18[b]	D-cyclopropylalanine (**27**)	D-TA	SgvM	**35**	> 95 %	98:2
19[b]	D-cyclopropylalanine (**27**)	D-TA	SgvM	**36**	> 95 %	98:2

[a] All entries correspond to 2 ml reactions containing 4 µmol of substrate in 100 mM phosphate buffer, at pH 8.0 and at 25 °C. All proteins were purified from E. coli Δmtn (DE3) over Ni(II)-NTA agarose. The identity of the product, and the conversion efficiency and stereoselectivity of the reaction were inferred by ^{1}H-NMR, ^{13}C-NMR, 2D NMR and high-resolution electrospray ionization mass spectrometry (HRESIMS, Supplementary figures 3 – 41).[b] Isotope labeled methyl iodide ($^{13}CH_3I$ or CD_3I) was used to produce compounds **35** and **36**.

also methylated with similar efficiency and stereoselectivity. Notably, compound **17** is a naturally occurring building block in cyclomarins, a class of anti-inflammatory cyclic peptides.[14] L-methionine (**8**) was also accepted as a substrate (entry 8) and methylated to produce **18**. The comparatively bulky L-cyclopropylalanine (**9**) was converted to product with outstanding efficiency (96 % conversion) and stereoselectivity (99:1, entry 9). To demonstrate that this cascade can be used for preparative synthesis, we scaled this reaction to 200 ml from which we isolated 0.33 mmol of **19** (47 mg, 82% yield). One-step purification by ion exchange chromatography was facilitated by the completeness of the reaction, the high stereoselectivity and the simplicity of the reaction mixture. By comparison, a synthetic scheme connecting **9** to **19** using traditional synthesis requires at least five steps, including protection, installation of a directing group, stereoselective methylation of the protected α-amino acid and deprotection.[10a]

Because of the modular architecture of this cascade reaction, its substrate scope could be expanded simply by using TAs and α-keto acid methyltranferases with different substrate specificities. These enzymes may be engineered variants of IlvE and SgvM,[15] or naturally evolved homologs. Enzymes known for their involvement in the biosynthesis of other L-*β*-Me-α-aas such as L-*β*-Me-Glu, L-*β*-Me-Trp, L-*β*-Me-Arg and L-*β*-Me-Phe provide an obvious starting point for diversification.[8b, 8c, 16] As an illustration, we tested the ability of the Trp-specific transaminase MarG and the indole-3-pyruvate-specific methyltransferase MarI to produce L-*β*-Me-Trp (**20**) in the context of our *in vitro* cascade.[8b] Indeed, a cascade reaction containing these two enzymes in combination with HMT, SAH, PLP and methyl iodide in phosphate buffer converted over 95 % of Trp to **20** (entry 10).

β-methylated D-α-amino acids (D-*β*-Me-α-aa) have not yet been observed as components of natural products but may be used to optimize and diversify existing therapeutics. Therefore we surmised that our technology could be used to produce some of these unexplored compounds. In the first step of the cascade reaction described above the L-α-amino acid is deaminated to an achiral α-keto acid. The stereoinformation, along with the amino group and the reduction equivalent is temporarily stored in the transaminase, while the methyltranferase acts on an achiral substrate. Hence, replacing the L-TA with a D-TA should allow the cascade to convert D-amino acids to D-*β*-Me-α-aas. To test this idea, we produced a D-TA with a substrate scope that nearly mirrors that of IlvE.[17] The cascade containing D-TA, SgvM, HMT, PLP, SAH and methyl iodide converted D-norvaline (**21**) and D-leucine (**22**) to compounds **28** and **29** with moderate efficiency (entries 11 and 12) but with excellent stereoselectivity. Residues with more bulky side chains were methylated with significantly higher efficiency (entry 13 - 17). Finally, as a demonstration that this methylation cascade could be used to produce isotope-labeled *β*-Me-α-aa from comparatively cheap reagents we also produced ^{13}C- and ^{2}H-isotopologs of **34** using $^{13}CH_3I$ or CD_3I as methylation agents (entries 18 and 19).

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Conclusions. We have developed an enzyme-catalyzed one-pot process for stereoselective production of L- or D-*β*-Me-α-aa. The high methyl-transfer potential of methyl iodide provides the free energy required to drive this transformation to near completion. The completeness of the reaction, the high stereoselectivity and the simplicity of the reaction mixture facilitates purification of the products. The modularity of this system, compounded with the diversity of known D-TAs and L-TAs and the growing abilities to modify the substrate specificity of enzymes by engineering bode well for the general applicability of this method for the synthesis of *β*-Me-α-aa. The use of methyl iodide as a reagent is an inherent weakness of this methodology. The toxicity and volatility of this reagent may complicate scale-up efforts. However, it is important to note that the limited choices of methylation agents is a general problem in organic chemistry. We are optimistic that future developments will eliminate this problem by introducing more benign reagents to methylation biocatalysis.

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Author contribution.

C.L. and F.P.S. contributed to this paper as follows: original conception and planning (F.P.S & C.L.); empirical work (C.L.); data analysis and interpretation (C.L. & F.P.S.); writing of the manuscript (F.P.S & C.L.).

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