Enzymatic Synthesis of L-Methionine Analogues and Application in a Methyltransferase Catalysed Alkylation Cascade

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Abstract: *Chemical modification of small molecules is a key step for the development of pharmaceuticals.* S*-adenosyl-*L*-methionine (SAM) analogues are used by methyltransferases (MTs) to transfer alkyl, allyl and benzyl moieties chemo-, stereo- and regioselectively onto substrates, enabling an enzymatic way for specific derivatisation of a wide range of molecules.* L*-Methionine analogues are required for the synthesis of SAM analogues. Most of these are not commercially available. In nature,* O*-acetyl-*L*-homoserine sulfhydrolases (OAHS) catalyse the synthesis of* L*-methionine from* O*-acetyl-*L*-homoserine or* L*-homocysteine, and methyl mercaptan. Here, we investigated the substrate scope of ScOAHS from* Saccharomyces cerevisiae *for the production of* L*-methionine analogues from* L*-homocysteine and organic thiols. The promiscuous enzyme was used to synthesise nine different* L*-methionine analogues with modifications on the thioether residue up to a conversion of 75%. ScOAHS was combined with an established MT dependent three-enzyme alkylation cascade, allowing transfer of in total seven moieties onto two MT substrates. Ethylation was nearly doubled with the new four-enzyme cascade, indicating a beneficial effect of the in situ production of* L*-methionine analogues with* Sc*OAHS.*

S-adenosyl-L-methionine (SAM) is an ubiquitous enzyme cofactor and serves as nature's methylation agent throughout the kingdoms of life.[1] Chemically, it consists of an adenosine and an L-methionine moiety with a positively charged sulfonium, activating the methyl group and other adjacent carbons.[2] Regio- and stereoselective transfer of the methyl group onto biomolecules and small molecule substrates is catalysed by methyltransferases (MTs).[3–5] Due to their versatile substrate range, MTs are desired tools for biotechnology.[3,6] SAM analogues with modifications at the alkyl substituent of the sulfonium further diversify possible products that can be synthesised using wildtype MTs.[3] Labelling of substrates with functional groups for click reactions and photocleavable groups are useful tools for in vivo tagging and subsequent downstream processes.[5,7] While there are many possible applications, the instability and the costs of the cofactor, as well as the availability of SAM analogues are still an obstacle. Enzymatic cascades for supply and regeneration of the cofactor are currently being investigated to improve the applicability.[8] In nature, as well as biomimetic alkylation cascades, SAM is synthesised from L-methionine and ATP by L-methionine adenosyltransferases (MATs).[4] SAM analogues (**1**) can be synthesised in the same way by the usage of L-methionine analogues.[7] Some MATs showed promiscuity towards variations of the alkyl chain, *e.g.* for L-ethionine or *S*-allyl-L-homocysteine.[9] Mutations in the active site led to MAT variants accepting larger residues, *e.g.* the MAT variant of *Methanocaldococcus jannaschii* (PC-*Mj*MAT).[7,10,11] Other methods use 5´-Cl-5´-deoxyadenosine (5´-CldA) and L-methionine analogues, or *S*-adenosyl-L-homocysteine (SAH, **3**) and haloalkanes to synthesise SAM analogues.[12–14] The SAM-derived, often inhibitory byproduct SAH can be irreversibly degraded by a methyl-thioadenosine/ SAH nucleosidase (MTAN) yielding *S*-ribosyl-L-homocysteine (**4**) and adenine (**5**).[4,15] Apart from L-ethionine, most L-methionine analogues are not commercially available and have to be synthesised chemically prior to the biocatalytic conversion from *e.g.* L-homocysteine (**6**) and haloalkanes.[7,9] An application containing an MT dependent alkylation cascade combined with enzymatic synthesis of L-methionine analogues was not established so far. In nature, L-methionine can be synthesised by direct sulfurylation of *O*-acetyl-L-homoserine (**7**) with methyl mercaptan.[16] This reaction is catalysed by pyridoxal phosphate (PLP) dependent *O*-acetyl-L-homoserine sulfhydrolases (OAHS, EC 2.5.1.49). The OAHS of *S. cerevisiae* (*Sc*OAHS) was also reported to exchange the γ-thiol of L-ethionine with methyl mercaptan, synthesising L-methionine (s. Figure S 1).[17] A few OAHS have been shown in different studies to produce L-methionine analogues from *O*-acetyl-L-homoserine or L-methionine.[17–20] Based on this information, we assumed that *Sc*OAHS is a good candidate for the production of L-ethionine and potentially other L-methionine analogues.

The gene of *Sc*OAHS (*scmet17*) from *S. cerevisiae BY4742* was cloned into an overexpression vector, the protein was heterologously produced in *E. coli* BL21Gold and purified *via* Ni^{2+} affinity chromatography (Figure S 2). Literature suggested a thiol transfer ability for *Sc*OAHS, allowing the elimination of the γ-terminal group of L-homocysteine or L-methionine and a subsequent attack of a thiol to form an L-methionine analogue (Figure S 1).[16] We first analysed the formation of L-ethionine from L-homocysteine or L-methionine with ethyl mercaptan (Figure S 3). Product formation was observed using both substrates. *Sc*OAHS was crystallised to gain insights into the mechanism of substrate binding, the structure was solved to a resolution of 2.3 Å, and confirmed the alpha fold prediction (Figure S 4, S 5 and Table S 3). Of the 444 *Sc*OAHS protein residues, 400 residues could be modeled but several loop regions were not resolved in

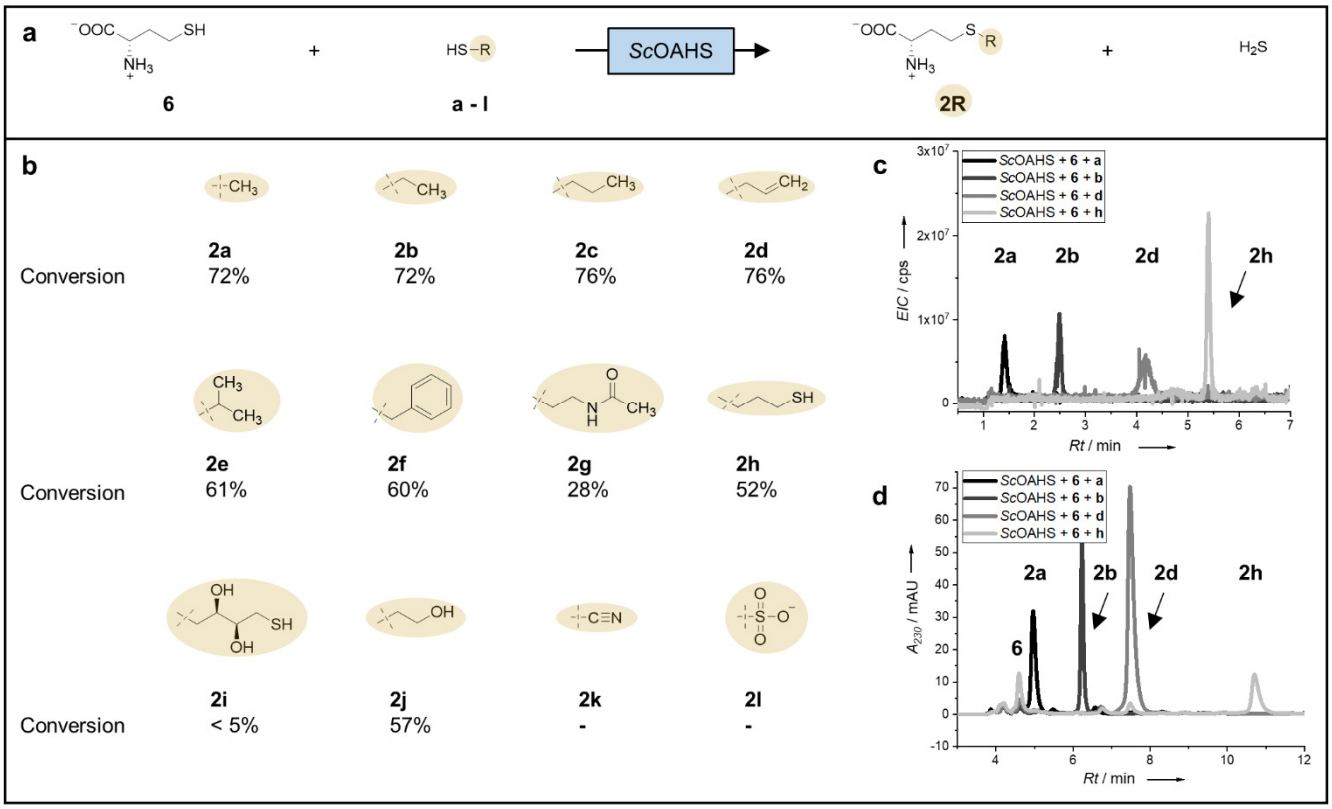

Figure 1. Substrate scope of *Sc*OAHS. **a** Enzymatic synthesis of L-methionine analogues from thiols and L-homocysteine. **b** Chemical structures (**R**) of the L-methionine analogues **2a** – **2l** synthesised, and conversions. **c** LC-MS analysis of the enzymatic production of selected examples: **2a** (m/z = 150.1), **2b** (m/z = 164.1), **2d** (m/z = 176.1), **2h** (m/z = 210.1). **d** HPLC-UV (λ = 230 nm) analysis of the conversion of L-homocysteine (**6**) to **2a**, **2b**, **2d** and **2h**.

the electron density map including loops likely involved in substrate binding (Figure S 4 and S 5). We therefore used the alpha fold structure, for docking experiments (Figure S 5).[21]Docking of the external aldimine state of L-homocysteine with CB-DOCK2[22] suggested a spacious active site, able to accommodate bulky substrates (Figure S 6). In the following experiments, different organic and inorganic thiols were used to analyse the production of the corresponding L-methionine analogues starting from L-homocysteine (Figure 1). The presence of the products was confirmed by LC-MS and the conversion was measured by the decrease of L-homocysteine *via* HPLC-UV (Figure S 7 and S 8). Methyl (**a**), ethyl (**b**), propyl (**c**), and *iso*-propyl (**e**) mercaptan were accepted with yields from 60% - 75%. Small hydrophilic thiols such as β-mercaptoethanol (**j**), 1,3-propyl dimercaptan (**h**) were converted with > 50% conversion, while the larger dithiothreitol (DTT, **i**) resulted in low conversion (4%). Unsaturated thiols such as allyl- (**d**) or benzyl (**f**) mercaptan were converted with yields of 76% and 60%, respectively. The inorganic compounds thiocyanate (**k**) and thiosulfate (**l**) were not converted. These results demonstrate the high promiscuity of *Sc*OAHS towards many organic thiols and the production of the corresponding L-methionine analogues.

These have to be converted to SAM analogues for utilisation in MT-dependent reactions. Production of the corresponding cofactors was tested with *Ec*MAT (for SAM formation) or PC-*Mj*MAT (for SAM analogue formation) in combination with *Sc*OAHS, L-homocysteine and the corresponding thiols. Analysis was performed with LC-MS and the masses of **1a** – **1d** and **1h** were found (Figure S 9). The high concentrations of the thiols and the additional catalytic step could influence cofactor synthesis. Therefore, production of SAM (**1a**) and *S*-adenosyl-L-ethionine (SAE, **1b**) from methyl mercaptan and ethyl mercaptan in combination with *Sc*OAHS was compared to the production from L-methionine and L-ethionine, as both are commercially available. Formation of SAM was similarly fast in both cases in the first hour (Figure S 10 and S 11). Afterwards, production of SAM proceeded faster starting with methyl mercaptan. SAE production with PC-*Mj*MAT led to 25% higher conversion after 24 h starting from ethyl mercaptan, compared to L-ethionine (Figure S 11).

Next, *Sc*OAHS was coupled with the previously described three-enzyme alkylation cascade (Figure 2). Having tested the broad substrate scope of *Sc*OAHS, analysis of the usage of the created L-methionine analogues for MT-dependent transfer reactions was performed. To analyse the applicability of the four-enzyme alkylation cascade, anthranilate *N*-MT from *Ruta graveolens* (*Rg*ANMT) and catechol *O*-MT from *Rattus norvegicus* (*Rn*COMT) were used. The enzymes were tested with anthranilate (AA, **8**) for *Rg*ANMT and 3,4-dihydroxy benzaldehyde (DHBAL, **9**) for *Rn*COMT. The identity of the products was confirmed by LC-MS and the conversion was analysed by UV-HPLC (Figure S 12 - S 17).

For methylation (**8a**, **9a**), conversion of > 98% was achieved for both MTs. Ethylation resulted in conversions of 38% and 41% for **8b** and **9b**, respectively. The masses of the propylated (**8c**, **9c**,

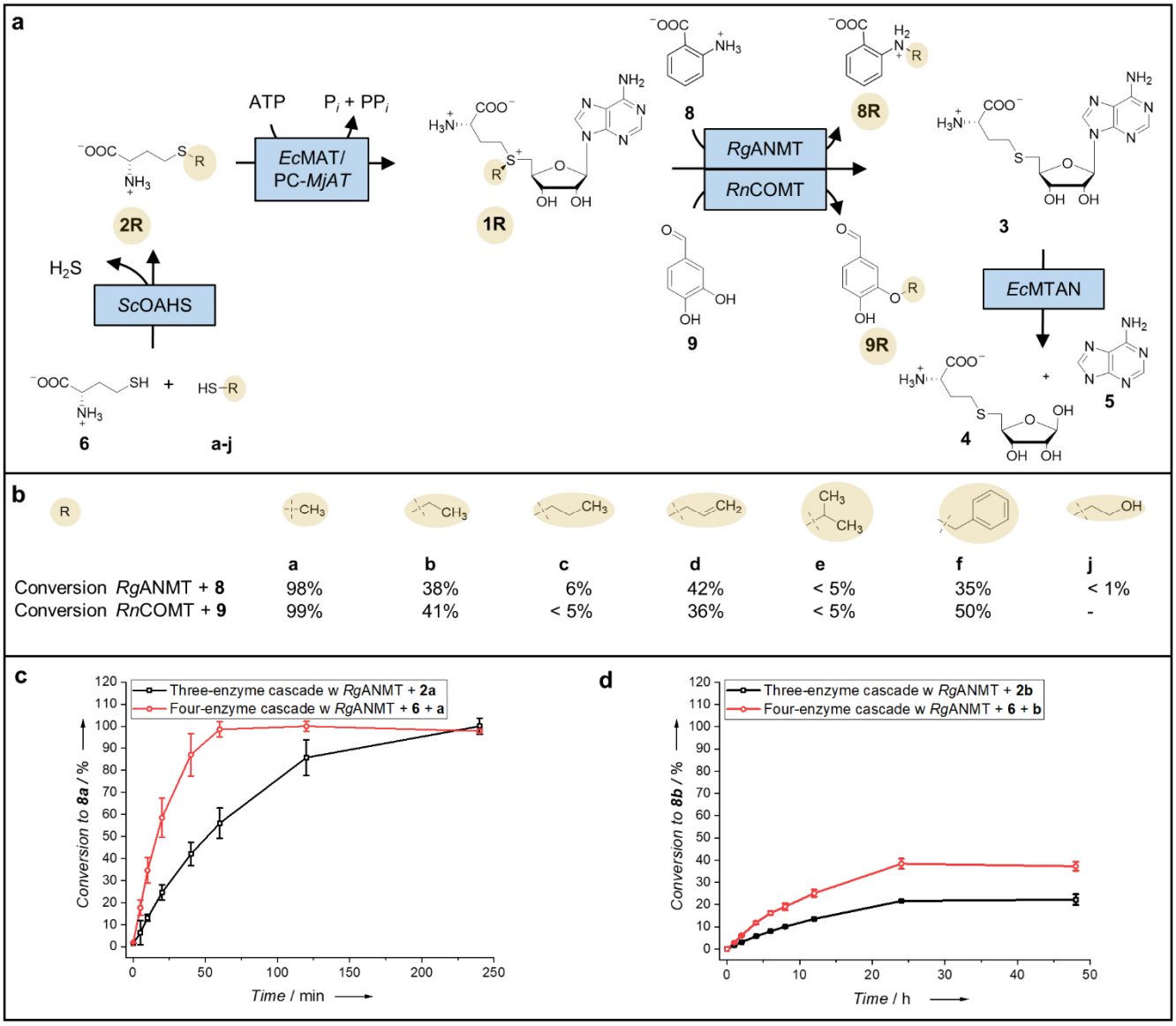

Figure 2. Alkylation with the four-enzyme cascade. **a** Scheme of the four-enzyme cascade with *Rg*ANMT or *Rn*COMT. For methylation *Ec*MAT was used, for all other alkylations PC-*Mj*MAT was used. **b** Transferable residues with the four-enzyme cascade are shown in combination with the conversions. Note: Vanillin (**9a**) and *iso*-vanillin (**9a´**) were quantified together and were present in a ratio of 5: 1. For all other moieties only one regioisomer was detected. **c** Comparison of the three-enzyme cascade and the four-enzyme cascade by monitoring the methylation of **8** to **8a,** and for **d** the ethylation of **8** to **8b** with *Rg*ANMT.

conversion 4% - 6%) and *iso*-propylated products (**8e**, **9e**, conversion 1.7 - 2.7%) were also found for both MTs. The elongated alkyl chains were transferred with lower conversions, compared to the ethyl group which could be reasoned by a steric hindrance.[23] Furthermore, a higher amount of the SAM analogue **1c** was formed than **1e**, also leading to lower alkylation with *iso*-propyl mercaptan(Figure S 9). In addition, introduction of functional groups through the MT reaction was investigated. While four L-methionine analogues with new functionalities could be synthesised enzymatically, only traces of hydroxy ethylated AA (**8j**) was detected *via* LC-MS. Hence, introduction of nucleophilic groups through MTs seems to be possible. However, the unstable **1j** was not found, presumably due to an internal cleavage of the cofactor. Future investigations will show if higher yields can be achieved through stabilisation of the SAM analogue and enzyme engineering. The unsaturated substrates allyl and benzyl mercaptan were transferred with both MTs with conversions of 35% - 50% (Figure 2). The mass for the SAM analogue **1f** was not found, possibly due to the low solubility of the SAM analogue. Allyl and benzyl SAM analogues offer a stronger nucleophilic activation of the attacked carbon compared to the saturated counterpart. The sp^2-hybridised ß-carbon of the unsaturated residues can further stabilise the sp^2 hybridised transition state during the S_N2 reaction as described by *Dalhoff et al.*.[23]

Finally, performance of the four-enzyme cascade was analysed in comparison to the three-enzyme cascade. Methylation and ethylation of the MT substrates from the corresponding thiols was compared to starting with L-methionine or L-ethionine. With the four-enzyme cascade full methylation was observed within 1 h for *Rg*ANMT, the three-enzyme cascade took 4 h (Figure 2). For *Rn*COMT, the four-enzyme cascade was comparable to the three-enzyme cascade (Figure S 16). Ethylation with the four-enzyme cascade showed a steeper slope than the three-enzyme cascade, leading to a 1.8 fold higher ethylation to **8b** and a 1.6

fold higher ethylation to **9b**. These results suggest that the in situ generation of L-ethionine has a beneficial effect on the performance of the alkylation cascade, potentially also for the transfer of other moieties.

With this work a functional and flexible one-pot alkylation cascade was demonstrated. *Sc*OAHS is a promiscuous enzyme that can be used for the straight-forward enzymatic synthesis of L-methionine analogues from L-homocysteine and organic thiols. The substrate range includes small to branched aliphatic residues, hydrophilic residues with functional groups and unsaturated residues. Most of the L-methionine analogues are to our knowledge not commercially available and can now be produced enzymatically from "off-the shelf" reagents. The in situ produced L-methionine analogues could be used to synthesise SAM and six derivatives. Seven products with transferred moieties from the SAM analogues were detected for the MTs tested. Nevertheless, utilisation of *Sc*OAHS requires the use of thiols and results in the release of H_2S, an environmentally critical gas. One possibility to reduce the environmental impact could be the deployment of *O*-acetyl-L-homoserine instead of L-homocysteine, which we will analyse in future work. The application of this four-enzyme cascade can help to easily screen substrate scopes for MATs and MTs without prior production and purification of the L-methionine analogues. Furthermore, only the L-amino acid analogue is produced, which eliminates interactions with the stereoisomers. This concept could also be applied in the future for the generation of SAM analogues with SalL. The four-enzyme cascade can further be evolved to become a tool for diversification of molecules at specific chemical positions, which can help at late stage optimisation of pharmaceutically active compounds. Regeneration of L-homocysteine from the MT byproduct SAH, can further reduce costs and improve the productivity.[8,24,25]

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