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Site-specific Bioalkylation of Rapamycin by the RapM 16-O-Methyltransferase

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The methylation of natural products by S-adenosyl methionine (AdoMet, also known as SAM)-dependent methyltransferase enzymes is a common tailoring step in many biosynthetic pathways. The introduction of methyl substituents can affect the biological and physicochemical properties of the secondary metabolites produced. Recently it has become apparent that some AdoMet-dependent methyltransferases exhibit promiscuity and will accept AdoMet analogues enabling the transfer of alternative alkyl groups. In this study we have characterised a methyltransferase, RapM, which is involved in the biosynthesis of the potent immunosuppressive agent rapamycin. We have shown that recombinant RapM regioselectively methylates the C16 hydroxyl group of desmethyl rapamycin precursors in vitro and is promiscuous in accepting alternative co-factors in addition to AdoMet. A coupled enzyme system was developed, including a mutant human enzyme methionine adenosyl transferase (MAT), along with RapM, which was used to prepare alkylated rapamycin derivatives (rapalogs) with alternative ethyl and allyl ether groups, derived from simple S-ethyl or S-allyl methionine analogues. There are two other methyltransferases RapI and RapQ which provide methyl substituents of rapamycin. Consequently, using the enzymatic approach described here, it should be possible to generate a diverse array of alkylated rapalogs, with altered properties, that would be difficult to obtain by traditional synthetic approaches.

Introduction

The natural product rapamycin 1, first isolated from Streptomyces hygroscopicus NRRL 5491 (reclassified as Streptomyces rapamycinicus sp. nov.), is a polyketide macrolide antibiotic and a potent immunosuppressant that is widely used in the clinic. Rapamycin’s mechanism of action derives from its binding to the FKBP12 immunophilin, with the resultant rapamycin-FKBP12 complex acting to inhibit downstream signalling pathways involving the protein kinase mTOR (mammalian target of rapamycin). Rapamycin analogues (rapalogs) have been approved for the treatment of a variety of diseases, demonstrating the versatility and wide-ranging biological activity of this natural product. For instance, the semi-synthetic derivative Everolimus possesses efficacy against a number of disorders such as renal angiomyolipoma, neuroglial and neuroendocrine tumours, and breast cancer. Other rapalogs possess a shorter half-life for reduced systemic immunosuppression, or have displayed neuroprotective abilities and show promising potential as treatment for ischemic stroke victims. In light of this, there has been considerable interest in the diversification of the rapamycin scaffold to create novel rapalogs with altered or improved physicochemical and pharmacological properties.

Rapamycin is a highly complex molecule and although total syntheses of rapamycin have been reported, these syntheses are unlikely to deliver the quantities of rapalogs that would be required for drug development. While semi-synthetic rapalogs have received FDA-approval, the scope and flexibility of this approach is limited with regards to amenable diversification of the rapamycin scaffold. The elucidation of the rapamycin biosynthetic gene functions has enabled methods including precursor-directed biosynthesis and mutasynthesis, to deliver rapalogs rapidly and in higher titres by fermentation using engineered strains. However, the modifications introduced by these approaches have so far been largely constrained to the cyclohexane starter unit or the pipecolic amino acid, with only a few examples of modifications made to the remainder of the rapamycin scaffold.

The polyketide synthase (PKS) enzymes, RapA, B and C along with the nonribosomal peptide synthetase pipecolate incorporating enzyme RapP are responsible for assembly of the macrocyclic intermediate prerapamycin. Post-PKS tailoring enzymes, including three O-methyltransferases RapM, I and Q, and two P450 monoxygenases RapJ and N, are required to
transform prerapamycin, the first PKS free intermediate, into the mature macrocycle 1 (Figure 1).

18 Gene knockout experiments suggest that RapI, M and Q are regiocomplementary AdoMet-dependent methyltransferases that methylate C39, C16 and C27 hydroxyl groups during the biosynthesis of rapamycin respectively. Given that some AdoMet-dependent methyltransferases have been shown to transfer alternative alkyl groups from AdoMet analogues, experiments suggest that RapI, M and Q are AdoMet-dependent methyltransferases have been shown to methylate C39, C16 and C27 hydroxyl groups during the biosynthesis of rapamycin.

The natural co-factor AdoMet, utilised by the majority of methyltransferases, is biosynthesised from ATP and l-methionine by the enzyme methionine adenosyltransferase in vivo. The wild-type rapM gene sequence was amplified from a pUC18-rapM construct (Biotica) and sub-cloned into the E. coli expression vector pET28(a) generating the expression construct pET28a-rapM (Figure S1A). The RapM protein was heterologously expressed in E. coli BL21 (DE3) cells using standard methods, and the soluble N-terminal His6-RapM fusion protein (Figure S1B) was subsequently purified using Ni-NTA affinity chromatography.

The in vitro activity of the RapM enzyme was evaluated using AdoMet (Sigma Aldrich UK) as a co-factor and prerapamycin (BC150) 2, which was previously isolated from an S. rapamycinicus deletion strain deficient in the genes encoding the post-PKS tailoring enzymes. In addition, 16-, 27-, 39-tri-O-desmethylrapamycin (BC231) 7 (Figure 2), derived from an engineered S. rapamycinicus strain lacking the methyltransferase-encoding genes rapI, M & Q, was also evaluated as a substrate for RapM. Analysis of the BC231 7 reaction by C3-reverse phase high performance liquid chromatography (RP-HPLC) (Figure 3A) revealed formation of a new product with a longer retention time, tR = 7.13 min, compared with the starting material tR of 5.99 min. The new product was isolated and high resolution mass spectrometry (HRMS) analysis revealed an observed m/z 908.5156 consistent with the [M+Na]+ ion for a mono-methylated derivative of BC231 (Table S1). Based on the proposed post-PKS tailoring steps (Figure 1), this new product was expected to be 16-O-methyl-BC231 7m. When prerapamycin (BC150) 2 was used as a substrate with RapM, C13-RP-HPLC analysis (Figure 3B) revealed two peaks for the substrate (tR = 6.77, tR = 7.67 min) reports have utilised synthetic AdoMet analogues as alkyl-donors. However, the chemoenzymatic route via MAT offers several advantages. Firstly, AdoMet analogues produced enzymatically using MAT are diastereomerically pure; conversely the synthesis of AdoMet analogues, from S-adenosyl-homocysteine and alkyl halides, generates a mixture of diastereomers at the sulphonium centre, of which only the (S)-configured diastereoisomer is accepted by methyltransferases. Synthetic AdoMet analogues also need careful purification to remove the unwanted (R)-configured diastereoisomer and residual S-adenosyl-homocysteine (SAH), both of which can inhibit methyltransferases. Additionally, some AdoMet analogues are unstable so an in situ enzymatic preparation, using MAT, can minimise the formation of degradation products. Finally, unlike AdoMet, methionine analogues are membrane permeable and can therefore be fed to cells possessing MAT to generate alternative AdoMet derivatives in vivo. Here we describe the first in vitro characterisation of a rapamycin O-methyltransferase, and its utilisation in coupled reactions with an improved variant of the human enzyme hMAT2A, creating alkylated derivatives of rapamycin at the C16-O-position.

Fig. 1. Proposed modification of prerapamycin 2 by tailoring enzymes. The first post-PKS intermediate 2 is assembled by three PKSs RapA, B and C and cyclised by the pipecolate-incorporating NRPS-like enzyme RapP. Subsequent modification of the macrocycle occurs by methylation at the 16, 27 and 39-O-positions by three O-methyltransferases RapM, RapQ and RapL. Two P450 monoxygenases RapJ and RapN serve to introduce a keto-group and hydroxyl-group at the C9 and C27 positions respectively. RapQ methylates only after hydroxylation at C27 by RapN.

Results and discussion

Characterisation of the RapM in vitro.

The in vitro activity of the RapM enzyme was evaluated using AdoMet (Sigma Aldrich UK) as a co-factor and prerapamycin (BC150) 2, which was previously isolated from an S. rapamycinicus deletion strain deficient in the genes encoding the post-PKS tailoring enzymes. In addition, 16-, 27-, 39-tri-O-desmethylrapamycin (BC231) 7 (Figure 2), derived from an engineered S. rapamycinicus strain lacking the methyltransferase-encoding genes rapI, M & Q, was also evaluated as a substrate for RapM. Analysis of the BC231 7 reaction by C3-reverse phase high performance liquid chromatography (RP-HPLC) (Figure 3A) revealed formation of a new product with a longer retention time, tR = 7.13 min, compared with the starting material tR of 5.99 min. The new product was isolated and high resolution mass spectrometry (HRMS) analysis revealed an observed m/z 908.5156 consistent with the [M+Na]+ ion for a mono-methylated derivative of BC231 (Table S1). Based on the proposed post-PKS tailoring steps (Figure 1), this new product was expected to be 16-O-methyl-BC231 7m. When prerapamycin (BC150) 2 was used as a substrate with RapM, C13-RP-HPLC analysis (Figure 3B) revealed two peaks for the substrate (tR = 6.77, tR = 7.67 min)
due to the presence of interconverting rotamers about the amide bond, along with new products with longer retention times which were predicted to be rotamers of 16-O-methyl-BC150 2m (t_R = 8.22, t_R = 9.36 min). The two product peaks for 2m were separated and HRMS revealed the same m/z for each, consistent with mono-methylated prerapamycin rotamers (Table S1). Additionally, the individual rotamers of both prerapamycin 2 and product 2m were separated by HPLC, left at 21 °C for 16 h, and then re-analysed again by HPLC, which revealed re-equilibration back to the original ratio of rotamers (Figure S2).

The hMAT2A mutant I322V utilises ATP and methionine as substrates prerapamycin (BC150) and BC231 7, generating methyl (e) ions, which result from the loss of CO_2, H_2O, and in the case of methylated products CH_3OH, can also be used to assign structures and identify the methylation site of RapM (Figures S3 & S4). The f_1 product ion containing only the C39-OH site was identical for 2 and 2m (m/z [M+Na]^+ 614.2), as well as both 7 and 7m (m/z [M+K]^+ 644.2), indicating that RapM has not methylated the C39-hydroxyl. The f_1 and f_2 ions were both observed with a +14 mass difference between 7 and 7m, which would be consistent with methylation at either the C16- or C27-hydroxyl groups of compound 7. However, the MS/MS analyses of 2 and 2m also revealed a +14 increase in the f_1 product ion. As prerapamycin 2 lacks the C27-hydroxyl, the presence of an additional methyl group in the f_1 fragment from 2m suggests that RapM is indeed the 16-O-methyltransferase for rapamycin (Figure S4). Furthermore, reactions of RapM with previously isolated compounds BC204 (16,27-bis-O-desmethyl-39-demethoxy-rapamycin) and BC207 9 (27-O-demethyl-39-demethoxy-rapamycin), showed conversion of 8 into 9, i.e. RapM catalyses methylation at the C16-hydroxyl (Figures 2 & S5A). The absence of activity of RapM with 9, a rapallog where the C16-hydroxyl is blocked by methylation but the C27-hydroxyl remains available, further confirms that RapM does not accept the C27-O-methylation site and is exclusive for the C16-position (Figure S5B).

Finally, the 16-O-methyl-BC231 compound 7m was analysed by proton-NMR (600 MHz), which revealed that 7m

**Confirmation of the regioselectivity of RapM.**

Based on gene deletion and complementation experiments with *S. rapamycinicus* the three methyltransferases RapM, Q and I, are predicted to methylate at the 16-, 27- and 39-O-positions respectively during rapamycin biosynthesis. However, as of yet there has been no in vitro characterisation of these methyltransferases. Therefore, to confirm the regioselectivity of RapM, the mono-methylated products 2m and 7m were subjected to tandem mass spectrometry revealing a fragmentation pattern consistent with rapalogs described previously (Figure 4). In addition to the key MS/MS product ions of 2, 2m, 7 and 7m (Table 1) a series of ions denoted as ‘e’ ions which resulting from the loss of CO_2, H_2O, and in the case of methylated products CH_3OH, can also be used to assign structures and identify the methylation site of RapM (Figures S3 & S4). The f_1 product ion containing only the C39-OH site was identical for 2 and 2m (m/z [M+Na]^+ 614.2), as well as both 7 and 7m (m/z [M+K]^+ 644.2), indicating that RapM has not methylated the C39-hydroxyl. The f_1 and f_2 ions were both observed with a +14 mass difference between 7 and 7m, which would be consistent with methylation at either the C16- or C27-hydroxyl groups of compound 7. However, the MS/MS analyses of 2 and 2m also revealed a +14 increase in the f_1 product ion. As prerapamycin 2 lacks the C27-hydroxyl, the presence of an additional methyl group in the f_1 fragment from 2m suggests that RapM is indeed the 16-O-methyltransferase for rapamycin (Figure S4). Furthermore, reactions of RapM with previously isolated compounds BC204 8 (16,27-bis-O-desmethyl-39-demethoxy-rapamycin) and BC207 9 (27-O-demethyl-39-demethoxy-rapamycin), showed conversion of 8 into 9, i.e. RapM catalyses methylation at the C16-hydroxyl (Figures 2 & S5A). The absence of activity of RapM with 9, a rapalog where the C16-hydroxyl is blocked by methylation but the C27-hydroxyl remains available, further confirms that RapM does not accept the C27-O-methylation site and is exclusive for the C16-position (Figure S5B).
exists as a mixture of amide rotamers, in line with previous NMR studies of rapamycin \textsuperscript{1}.\textsuperscript{47} From COSY and TOCSY experiments in DMSO-d\textsubscript{6}, most of the \textsuperscript{1}H signals for the major trans-isomer of \textit{7m} could be assigned (Table S2, Figures S6-S8) and are consistent with NMR data reported previously for rapamycin \textit{1} as described by Pagano.\textsuperscript{47} Notably a 3H singlet in the \textsuperscript{1}H NMR of \textit{2m} is evident at 3.06 ppm, which corresponds with the C16-\textsuperscript{O}-methyl signal assigned previously at 3.04 ppm, for rapamycin in DMSO-d\textsubscript{6}. This, along with the MS/MS experiments confirms the regioselectivity of the 16-\textsuperscript{O}-methyltransferase RapM.

![Fig. 4. MS\textsuperscript{3} fragmentation pattern for rapalogs, showing the defined product ions \textit{f}_1, \textit{f}_2, \textit{f}_3, and \textit{f}_4. The RapM site of methylation can be identified by comparing the \textit{f}_1 and \textit{f}_2 ions for rapalogs \textit{7}, \textit{7m} and \textit{2}, \textit{2m}.](image)

### Table 1. MS/MS of rapalogs BC150 (2) and BC231 (7)

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<th>Ion</th>
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<th>Observed m/z</th>
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Alkylation of rapalogs using MAT and RapM in tandem.

To effect regioselective alkylation of rapamycin, a one-pot coupled enzyme system with the human methionine adenosyl transferase hMAT2A and RapM was envisaged. The hMAT2A enzyme has been reported to be promiscuous,\textsuperscript{33,34} and further mutagenesis of active site residues around the binding pocket for the AdoMet methyl substituent has been shown to improve activity with methionine analogues possessing larger \textit{S}-alkyl substitutents.\textsuperscript{33} Based on this precedent a series of active site mutants of hMAT2A were created by site-directed mutagenesis of a hMAT2A expression vector pNIC28-Bsa4-hMAT2A that was kindly provided by Udo Oppermann (Oxford). Selected mutants were overproduced in \textit{E. coli} Rosetta\textsuperscript{TM} 2, purified by Ni-NTA affinity chromatography, and evaluated with \textit{L}-ethionine and \textit{S}-allyl-\textit{L}-homocysteine (SAhC) as substrates, revealing that the I322V mutation provides the highest efficiency with the methionine analogues (Figure S9).

The mutant hMAT2A (I322V) and RapM were then used in tandem to effect alkylation of prerapamycin \textit{2} and BC231 \textit{7} with ATP and \textit{L}-methionine, \textit{L}-ethionine or \textit{S}-allyl-\textit{L}-homocysteine, forming \textit{in situ} AdoMet, \textit{S}-adenosyl-\textit{L}-ethionine (SAE) or \textit{S}-adenosyl-\textit{L}-allyl-homocysteine (SAAH) respectively. The subsequent transfer of methyl, ethyl and allyl groups onto the rapalogs was analysed by RP-HPLC (Figure 5). The alkylation of BC231 \textit{7} in coupled assays with hMAT2A (I322V) and methionine analogues resulted in two new products ethyl-BC231 \textit{7e} (\textit{t}_R = 7.73 min) and allyl-BC231 \textit{7a} (\textit{t}_R = 9.18 min) with longer retention times than the methylated product \textit{7m}. HPLC analysis of the coupled reactions with prerapamycin \textit{2} revealed new products ethyl-BC150 \textit{2e} (\textit{t}_R = 9.18 min) and allyl-BC150 \textit{2a} (\textit{t}_R = 10.28 min). As with the corresponding methylated product \textit{2m}, the ethyl and allyl derivatives also result in dual peaks on HPLC analysis consistent with the presence of rotamers.

The minor amounts of methylated products observed in reactions utilising \textit{L}-ethionine or \textit{S}-allyl-\textit{L}-homocysteine are presumably due to the presence of residual active site-bound AdoMet from co-elution during hMAT2A (I322V) and RapM protein purification. HRMS of the new alkylated products \textit{2e}, \textit{2a}, \textit{7e} and \textit{7a} was consistent with mono-alkylated derivatives (Table S1) and UV analysis showed that all the new products possessed the distinctive rapamycin \textit{\lambda}_{\text{max}} at 267, 277 and 288 nm due to the triene chromophore. Finally partial assignment of the \textsuperscript{1}H and \textsuperscript{13}C NMR signals for the major rotamer of ethyl-BC231 \textit{7e} using COSY, TOCSY, NOESY and HSQC experiments was also consistent with the expected structure of \textit{7e} (Table S3, Table S4, Figures S10-S14). The efficiency of alkylation of the prerapamycin \textit{2} and BC231 \textit{7} in the coupled hMAT2A/RapM assays is clearly reduced with increasing size of the alkyl group on the sulphonium centre of the AdoMet analogue. Full conversion of \textit{7} and 75% conversion of \textit{2} to the methylated rapalogs was achieved for the assays with \textit{L}-methionine, whereas ethylation and alkylation reactions proceed to 19\% (\textit{2e}), 51\% (\textit{7e}), and 10\% (\textit{2a}), 33\% (\textit{7a}), under the same conditions (Figure S15).
produced at similar levels to that observed in the tandem assay (Figure S17). To explore this further, competitive assays with AdoMet and SAAH (Figure S16) were isolated and then separately enzymatically prepared AdoMet, independent of hMAT2A, minimising degradation and possible subsequent inhibition of RapM. These observations were used to improve the productivity of the tandem reaction with SAAH (Figure S19); by decreasing the concentration of hMAT2A (I322V), with RapM in 7.5-fold excess, yields of 7a can be increased to 72%. Presumably the excess RapM, relative to hMAT2A, prevents the accumulation of SAAH, minimising degradation and possible subsequent inhibition of RapM. Finally, the RapM methyltransferase was assayed against a range of concentrations of the best substrate BC231 7 (2.5-250 µM) with a set concentration of AdoMet (500 µM), to give the kinetic constants $K_m = 48.9 \pm 7.1 \mu M$ and $k_{cat} = 1.47 \pm 0.072$ min$^{-1}$. The relatively low catalytic rate of RapM is unsurprising given the nature of the methylation reaction; RapM is a tailoring enzyme for a complex polyketide natural product, and there is unlikely to be an evolutionary drive for a catalytically efficient methyltransferase, given that the intricate assembly process of the polyketide is more likely to be the limiting factor in overall rapamycin production. The lower relative activity of RapM with AdoMet and the natural precursor BC150 2 compared with BC231 7, an engineered compound possessing a C9 keto group that is not a natural intermediate (Figure S15), is also consistent with the proposed timing of tailoring steps in rapamycin biosynthesis. The proposed optimum pathway (Figure 1), 18 suggests RapM methylation occurs after oxidation at C9 by RapJ. Whilst a preferred route may have evolved to ensure each tailoring enzyme acts on its optimal substrate, the fact that BC150 2 is a substrate for RapM indicates that multiple minor parallel pathways are most likely operative, resulting in a diverse range of intermediary compounds all leading to the final most highly modified product rapamycin.

Conclusions

In summary, we have characterised the O-methyltransferase activity of RapM, confirming its specificity for methylation of the 16-OH position of rapamycin. The ability of RapM to transfer alkyl groups onto substrates including a biosynthetic precursor prerapamycin and an engineered rapalog BC231 has been shown, leading to alkyl-diversification of this clinically important polyketide. In addition, the catalytic constants for the enzyme when assayed with BC231 have been determined with AdoMet.

The enzymatic alkyl-diversification of rapamycin offers an attractive route to generating rapalogs with altered physicochemical and biological activity. Indeed, previous studies have noted that structural changes to rapamycin at the C16-position can be well tolerated, and in addition, that groups attached to the C16-hydroxyl group can be localised to a specific region of the rapalog bound FKBP-mTOR complex. Moreover, the regioselective attachment of orthogonal chemical handles to the rapamycin scaffold opens up the possibility of in vivo labelling of the rapamycin:FKBP complex, facilitating studies of the interactions of rapamycin with protein targets. Alternatively, the immobilisation of rapamycin offers a new route to coating clinical apparatus such as stents, or allows functional assays of rapamycin in complex with its protein binding partner(s). Given that methyltransferases are one of the most common classes of enzymes found in secondary metabolism, the methods described in this paper could be applied to a wide range of other complex bioactive natural product scaffolds.
Experimental

Construction of pET28a-rapM. The wild-type rapM gene sequence was PCR amplified using Phusion HF DNA polymerase (New England Biolabs) from a pUC183rapM construct (Biotica)\textsuperscript{16} with the primers His\textsubscript{6}rapM F and R (Table S5) to introduce Ndel and Xhol restriction sites at the 5’ and 3’ ends respectively. The gel-purified PCR product was doubly digested with Ndel and Xhol restriction enzymes (New England Biolabs) and ligated into a linearised pET28a(+) vector (Novagen) which had been similarly digested. The resultant construct pET28a-rapM was verified by nucleotide sequencing (GATC Biotech).

Site-directed mutagenesis of hMAT2A. The construct pNIC28-Bsa4-hMAT2A (provided by Udo Oppermann, Oxford) was used as a template for site-directed mutagenesis to generate hMAT2A mutants with expanded methionine-binding pockets as described by Wang and co-workers\textsuperscript{33}. Primers carrying the degenerate codon KYT (Table S5) were designed to generate mutants at the N117, V121 and I322 amino acid positions with either alanine, serine, valine or phenylalanine replacements. The mutants were verified by nucleotide sequencing (GATC Biotech).

Overexpression and purification of His-tagged RapM and hMAT2A. Transformant cells of E. coli BL21 (DE3) (pET28a-rapM) or Rosetta\textsuperscript{TM} 2 (pNIC28-Bsa4-hMAT2A) were cultivated (37 °C, 200 rpm agitation) in LB supplemented with kanamycin (pET28a-rapM, 50 µg/mL) or kanamycin and chloramphenicol (pNIC28-Bsa4-hMAT2A, 50 µg/mL and 25 µg/mL). The cells were allowed to reach a density of OD\textsubscript{600} 0.6-0.8 before protein expression was induced with the addition of isopropyl β-D-thiogalactopyranoside (IPTG, 0.5 mM). The cells were further cultivated for 4 h at 30 °C, 200 rpm before the cells were harvested by centrifugation. The pellet cells were resuspended in lysis buffer (5 mL per pellet from 800 mL culture; RapM lysis buffer: 50 mM Tris HCl pH 8.5, 500 mM NaCl, 5% glycerol (v/v); hMAT2A lysis buffer: 50 mM Tris HCl pH 8, 50 mM NaCl, 10% glycerol (v/v)). The cells were lysed by sonication and the lysates clarified by centrifugation. The soluble lysate was then applied over a Ni\textsubscript{3}NTA column (Biotica) and ligated into a linearised pET28a(+) vector (Novagen) which had been similarly digested. The resultant construct pET28a-rapM was verified by nucleotide sequencing (GATC Biotech).

Enzyme activity assays. For activity assays with the commercially purchased co-factor AdoMet (Sigma Aldrich), the RapM methyltransferase was assayed with the rapalogs BC231 7, BC150 2, BC204 8 or BC207 9 as follows: 1 mM DTT, 3 mM MgCl\textsubscript{2}, 1 mM AdoMet, 0.22 mM rapalog, 10 µM RapM, in 20 mM phosphate buffer pH 7.4. The reaction mixes were incubated at 30 °C with 800 rpm agitation, quenched at 30 and 60 min time points with an equal volume of methanol and centrifuged to remove precipitated proteins (13,000 x g, 4 °C, 10 min). The reactions were subsequently analysed by C\textsubscript{8} (rapalogs 7, 8 and 9) or C\textsubscript{18} (rapalog 2) RP-HPLC.

The hMAT2A assays were run as follows: 1 mM DTT, 3 mM MgCl\textsubscript{2}, 1.5 mM ATP, 1.5 mM 1-methionine (or analogue), 10 µM hMAT2A, in 20 mM phosphate buffer pH 7.4. The reactions were incubated at 30 °C, 800 rpm for 30 min, quenched with an equal volume of methanol and clarified by centrifugation (13,000 x g, 4 °C, 10 min). The assays were monitored by HILIC HPLC and the AdoMet analogues verified by ES\textsuperscript{+} LC-MS.

For coupled assays of RapM with hMAT2A, the assays were set up accordingly: 1 mM DTT, 3 mM MgCl\textsubscript{2}, 1 mM ATP, 1 mM 1-methionine (or analogue), 15 µM hMAT2A (I322V), 0.22 mM rapalog, 15 µM RapM in 20 mM phosphate buffer pH 7.4. The reaction mixes were incubated at 30 °C with 800 rpm shaking, quenched after 30 and 60 min time points with an equal volume of methanol and centrifuged to remove precipitated proteins (13,000 x g, 4 °C, 10 min). The reactions were subsequently analysed by C\textsubscript{8} (rapalogs 7, 8 and 9) or C\textsubscript{18} (rapalog 2) RP-HPLC.

NMR of 16-O-methyl BC231 and 16-O-ethyl BC231. Compounds 7m and 7e were prepared by purifying over C\textsubscript{18} RP-HPLC and collecting fractions containing the product peaks. The fractions were dried under a stream of N\textsubscript{2} and then in vacuo. The dried samples were reconstituted in DMSO-d\textsubscript{6} in a glove bag filled with nitrogen, and transferred to a DMSO-matched Shigemi tube (300 µL sample volume). The NMR spectra were recorded at a temperature of 298 K on a Bruker Avance AMX 600 MHz spectrometer equipped with a 5 mm inverse triple resonance cryoprobe. Spectra processing was performed using Topspin 3.1 software (Bruker).

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Notes and references

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Electronic Supplementary Information (ESI) available: For additional experimental information and supplementary figures see DOI: 10.1039/b000000x/