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Title: A Tandem Enzymatic sp2-C-Methylation Process: Coupling In situ S-Adenosyl-L-Methionine Formation with Methyl Transfer

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A Tandem Enzymatic sp^2-*C*-Methylation Process: Coupling *In Situ* S-Adenosyl-L-Methionine Formation with Methyl Transfer

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Abstract: A one-pot, two-step biocatalytic platform for the regiospecfic *C*-methylation and *C*-ethylation of aromatic substrates is described. The tandem process utilizes SalL (*Salinospora tropica*) for *in situ* synthesis of *S*-adenosyl-L-methionine, followed by alkylation of aromatic substrates using the *C*-methyltransferase NovO (*Streptomyces spheroides*). Application of this methodology is demonstrated by regiospecific methyl, ethyl and isotopically-labelled $^{13}CH_3$, $^{13}CD_3$ and CD_3 groups from their corresponding SAM analogues formed *in situ*.

Regiospecific, late-stage methylation is a powerful strategy for tuning the physical and biological properties of small molecules and biomacromolecules.[1] At present, synthetic methodologies that methylate C(sp^2)-H bonds are predominantly limited to transition metal-catalyzed strategies, which require elevated temperatures and the need for additives such as ligands and oxidants.[2–4] In addition, these methodologies are typically regioselective rather than regiospecific, which further restricts their scope.[5,6] In contrast, Nature routinely employs *S*-adenosyl-L-methionine (SAM) dependent methyltransferases (MTs) to methylate at *O*-, *N*-, *S*- and *C*- sites. Thus, MTs hold considerable potential for the development of a biocatalytic alkylation platform of small molecules.[7–9]

Of the various MTs available, *C*-MTs are particularly attractive as they enable C-C bond formation under mild conditions relative to traditional Friedel-Crafts reactions.[10,11] One *C*-MT exemplar is NovO, which methylates the C-8 position of **1a** in the biosynthesis of the antibiotic novobiocin to form **2a** (**Scheme 1a**).[12] We and others have shown that regiospecific methylation of the 8-position of **1b** is catalyzed by a novel His-Arg motif, which facilitates the deprotonation of the phenolic proton in the 7-position, followed by methylation at position 8 by SAM to form **2b**.[13,14] NovO is also effective in catalysing Friedel-Crafts alkylations using non-natural SAM analogues to alkylate **1b-d** regiospecifically.[8,11] Furthermore, NovO accepts non-natural substrates such as **3** to exclusively form **4** (**Scheme 1b**), which is currently only accessible by this process.[11]

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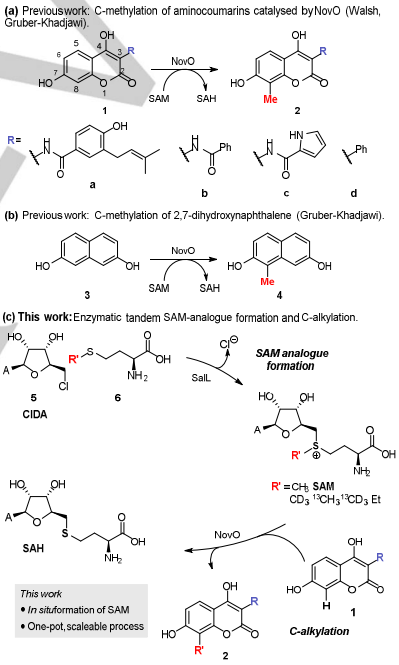
At present, the broader applicability of small-molecule MTs is hampered by the inherent instability of SAM (942 min at pH 8) as the corresponding methylating agent.[15,16] Additionally, the synthesis of SAM by chemical methods results in the formation of a diastereomeric mixture, which increases the cost of the process and has the potential for undesirable *C*-MT inhibition by the unwanted diastereomer.[17,18]



Scheme 1. Biocatalytic *C*-methylation of **(a)** coumarins (**1a-d**) and **(b)** 2,7-dihydroxynaphthalene (**3**) catalysed by NovO. Both studies require the formation of SAM prior to *C*-methylation. **(c)** Development of a one-pot, two-enzyme process involving *in situ* SAM formation (SalL) and C-methylation (NovO). SAM: *S*-adenosylmethionine. SAH: *S*-adenosylhomocysteine.

A powerful strategy that overcomes the need to prepare and isolate SAM and SAM analogues would produce the cofactor *in situ*, followed by alkyl transfer.[19–23] The enzyme SalL (*Salinospora tropica*) is known to form SAM from 5'-deoxy-5-chloroadenosine (CIDA, **5**) and methionine (Met, **6**).[24–28] As CIDA is readily prepared from adenosine in a one-pot process, the use of SalL offers an inexpensive and atom-efficient alternative to the use of methionine adenosyl transferases, which utilizes expensive ATP as the corresponding adenosyl donor and have been used previously for *in situ* SAM formation.[21,29–32]

At present, there have not been any reports on the utility of the SalL-catalysed SAM production in tandem with *C*-MTs in a one-pot procedure. Herein, we describe such a process for the methylation of small molecule aromatic scaffolds using SalL and the *C*-MT NovO (**Scheme 1c**). Additionally, we show that this

tandem strategy can be used to transfer ethyl groups and isotopically-labeled methyl groups.[33–35]

Initial investigations focused on determining the compatibility of SalL and NovO to operate in a one-pot process. The model substrate **1b** was used with an excess of ClDA (**5,** 2 eq.) and Met (**6,** 50 eq.) using an *E. coli* cell-free extract from the overexpression of NovO (**Figure 1a; Entry A**). Based on the previously reported kinetic parameters for SAM formation

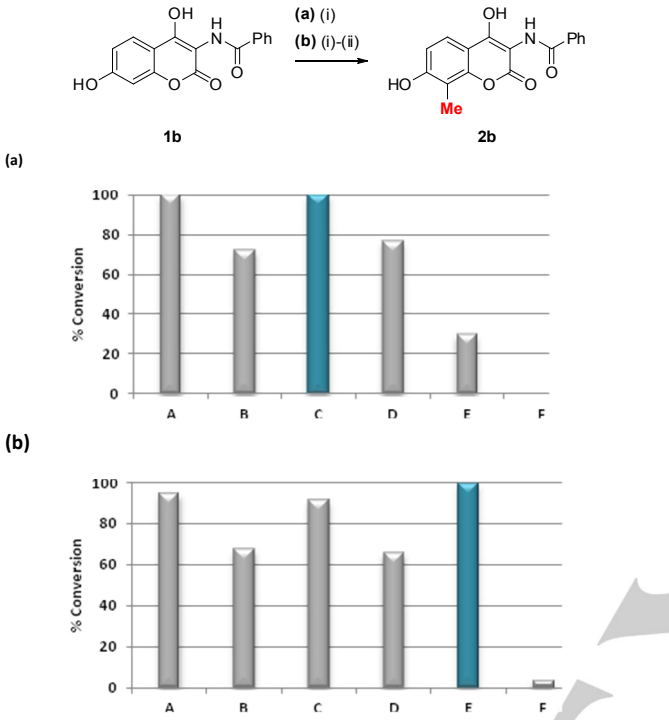


Figure 1. (a) Reaction optimization using substrate **1b**. *E. coli* cell lysates were used and % conversion determined by HPLC (area/area%) after 24 h. *Reagents and conditions*: (i) ClDA, Met, BSA, DTT, NovO cell lysate in 50 mM phosphate buffer, pH 6.5 resuspended at 10 mL/ g pellet, SalL cell free extract in 50 mM phosphate buffer, pH 6.8 resuspended at 5 mL/ g pellet. **A:** 2 eq. ClDA, 50 eq. Met; **B:** 1 eq. ClDA, 50 eq. Met; **C:** 2 eq. ClDA, 2 eq. Met; **D:** 2 eq. ClDA, 1 eq. Met; **E:** 2 eq. ClDA, no Met added; **F:** No ClDA or Met added. **(b)** Reaction optimization using purified enzymes. *Reagents and conditions*: (i) Met, SalL, BSA, 50 mM potassium phosphate buffer pH 6.5, ClDA, 37 °C, 8 hours. (ii) NovO, 37 °C, 16 hours. **A:** 2 eq. ClDA, 10 eq. Met, 0.5 μM MTAN added with NovO.; **B:** 2 eq. ClDA, 10 eq. Met; 2 uM SAHH added with NovO; **C:** 1.5 eq. ClDA, 1.5 eq. Met, 0.5 uM MTAN added with NovO; **D:** 1.1 eq. ClDA, 1.1 eq. Met, 0.5 uM MTAN added with NovO; **E:** 1.5 eq. ClDA, 2 eq. Met, 1 mM DTT and 0.5 uM MTAN added with NovO; **F:** 1.5 eq. ClDA, no Met added; 0.5 uM MTAN added with NovO. Optimised conditions highlighted in blue.

compared to ClDA formation, low levels of chloride present in the system were not anticipated to affect conversion levels.[36] No other sources of chloride were added to the system.

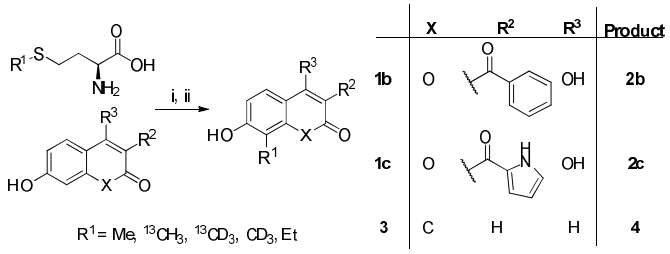
This resulted in quantitative conversion of **1b** into the methylated product **2b** in 24 hours. We then optimised the reaction conditions, firstly by minimising the number of equivalents of Met and ClDA relative to substrate **1b** (**Figure 1a** and **Table S1**)**.** When the number of equivalents of ClDA was reduced from 2 to 1.5 eq., a drop off in the formation of **2b** was observed (**Table S1**). Quantitative conversion was optimal with 2 equivalents of Met (**Figure 1a, Entry C**). Decreasing the amount of Met further to 1 eq. reduced the conversion of **1b** to **2b** to 77% (**Figure 1a**, **Entry D**). However, when the reaction was run without the addition of Met, 30% methylation of **1b** was observed (**Figure 1a**, **Entry E**). To determine whether this was due to residual SAM or Met present in the reaction mixture, the reaction was also run in the absence of ClDA (**Figure 1a**, **Entry F**). In this case, no conversion to **2b** was observed, which was indicative of background methylation being caused by residual Met present in the cell lysate and cell free extract (**Table S3**).

To address this issue, purified enzymes were used (**Figure 1b** and **Table S2**). Additionally, methylthioadenosine nucleosidase (MTAN) or SAH hydrolase (SAHH) was added to remove SAH from the reaction mixture, which is a known inhibitor of many SAM dependent MTs.[18,37,38] Since ClDA inhibits both MTAN[39] and SAHH[40], SAM was pre-formed *in situ* before the addition of NovO and MTAN or SAHH. Initially, the reaction was carried out using 2 eq. ClDA and 10 eq. Met with either MTAN or SAHH (**Figure 1b**, **Entries A** and **B**). Whilst only 68% conversion was achieved with SAHH, nearly quantitative methylation of **1b** was observed using MTAN as an additive. Further optimisation enabled the reduction of the number of equivalents of ClDA from 2.0 to 1.5 without loss of conversion to **2b** when 1 eq. DTT was added.[41] Carrying out the reaction in the absence of MTAN decreased the conversion by ~40%, confirming the role of MTAN decreasing by-product inhibition caused by SAH. Indeed, an IC_{50} value of 9.8 μM for ClDA has been reported for MTAN from *E. coli*, which was used in our study.[42] Finally, only 4% methylation was observed when the reaction was carried out in the absence of Met, which may be due to residual SAM bound in NovO (**Figure 1b** and **Table S2**).

With optimised conditions for a one-pot**,** tandem SAM formation *C*-methylation process in hand, we next explored the scalability of the methodology. The tandem process was carried out with Met, three isotopically labelled Met analogues and ethionine; using 20 mg of **1b**, **1c** or **3** in each case. To the best of our knowledge, this is the first time that a tandem process which involved the *in situ* formation of SAM has been used on preparative scale. For the transfer of an unlabelled methyl group crude *E. coli* cell lysate (NovO) and *E. coli* cell free extract (SalL) was used, whilst purified enzymes were employed for the transfer of isotopically labelled and ethyl groups. Moderate (65%) to excellent (88-100%) levels of conversion were obtained for transfer of CH_3, $^{13}CH_3$, CD_3 and $^{13}CD_3$, with **1c** showing quantitative conversion and isolated yields 76-91% in all cases (**Table 1**). High levels of conversion (88-97%) were also obtained for **1b**, although isolated yields were lower due to poor solubility of the corresponding products during work-up. Alkylation of **3** was also successful on this scale to form **4** in high conversions (69-87%) using purified SalL and NovO, whilst 65% of **4** was formed using the cell lysate.

In summary, we have demonstrated a scalable biocatalytic platform for a Friedel-Crafts alkylation using SalL for *in situ* cofactor SAM analogue synthesis from inexpensive starting materials. Furthermore, to the best of our knowledge this is the first example of using SalL for *in situ* cofactor production in tandem with a MT for the site specific *C*-methylation/alkylation of a small molecule. We envisage that our enzymatic platform could form the basis of a valuable biocatalytic tool for late-stage, regiospecific labelling of small molecules.[33,43–45]

Table 1. Preparative [(20 mg starting material [0.07 mmol **1b** and **1c**; 0.125 mmol **3**)] scale tandem enzymatic alkylation.



Substrate	CH_3	$^{13}CH_3$	CD_3	$^{13}CD_3$	Et
1b	97% (53%)[a]	95% (76%)	88% (52%)	90% (47%)	34% (18%)[b]
1c	100% (76%)[a]	100% (90%)	100% (80%)	100% (75%)	30% (27%)[b]
3	65% (46%)[a]	94% (83%)	91% (68%)	92% (85%)	7%[b]

[a] Reaction carried out using NovO cell lysate and SalL cell free extract and purified MTAN. [b] 32 hour pre-incubation with SalL. *Reagents and conditions*: (i) SalL (0.5 mol%), BSA (1 mg/mL), DTT (1 eq.), 50 mM potassium phosphate buffer pH 6.5, ClDA (**5**, 1.5 eq.), Met or Met analogue (2 eq.), 37 °C, 7 hours (CH_3, $^{13}CH_3$, CD_3 and $^{13}CD_3$) or 32 hours (Et). (ii) NovO (4 mol%), MTAN (0.05 mol%), 37 °C, 16 hours. Reactions carried out using 20 mg of substrate. Yields of isolated products in parentheses. Representative chromatograms for each substrate are shown in the Supporting Information.

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Keywords: methyltransferase • alkylation • biocatalytic • S-adenosylmethionine • multi-enzyme reaction

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Layout 1:

COMMUNICATION

Joanna. C. Sadler, Luke D. Humphreys, Radka Snajdrova, Glenn. A. Burley*

Tandem SAM: A one-pot, two-enzyme C-methylation process is described. Linking SAM production using SalL (*S. Tropica*) with the C-methyltransferase NovO (*S. Spheroides*) enables the synthesis a suite of methylated and ethylated cuomarin products.

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