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Communication

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Biosynthesis of branched alkoxy groups: iterative methyl group alkylation by a Cobalamin-Dependent Radical SAM Enzyme

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Supporting Information Placeholder

ABSTRACT: The biosynthesis of branched alkoxy groups like the unique *t*-butyl group found in a variety of natural products, is still poorly understood. Recently, cystobactamids were isolated and identified from *Cystobacter sp* as novel antibacterials. These metabolites contain an isopropyl group proposed to be formed using CysS, a cobalamin-dependent radical *S*-adenosylmethionine (SAM) methyltransferase. Here, we reconstitute the CysS-catalyzed reaction and demonstrate that it not only performs sequential methylations of a methyl group to form isopropyl groups but remarkably also *t*-butyl groups on *p*-aminobenzoate thioester substrates. To our knowledge, this is the first *in vitro* reconstitution of a cobalamin-dependent radical SAM enzyme catalyzing the conversion of a methyl group to a *t*-butyl group.

Natural products with branched alkoxy groups play an important role in the development of bioactive compounds. In particular, the *t*-butyl group has fascinated organic chemists for more than a century and has played a major role in mechanistic studies on organic substitution reactions and the design and characterization of theoretically interesting molecules such as the remarkable tetra *t*-butyl tetrahedrane.[1] While numerous branched alkoxy group substituted terpenes, polyketides and peptides have been identified, experimental studies on the biosynthesis of *t*-butyl groups are still at an early stage and many of the mechanistic proposals in the literature have not been adequately experimentally tested.[2]

For the ginkgolides and several other *t*-butyl substituted terpenes, the *t*-butyl group is formed by a double bond methylation using S-adenosylmethionine (Figure 1A).[3] Formation of the *t*-butyl group in the coumarin swietenone is proposed to involve carbocation insertion into a CH bond to give a cyclopropyl intermediate, which then undergoes acid mediated ring-opening (Figure 1B).[4] The biosynthesis of pivalic acid, a starter unit in the biosynthesis of *t*-butyl substituted polyketides, is mediated by a vitamin B_{12}-dependent enzyme (Figure 1C).[5-6] Very recently, the B_{12}/radical SAM mediated conversion of isopropyl glycine to *t*-butyl glycine in the polytheoamide propeptide was reported (Figure 1D).[7-8] The latter two enzymes are the only *t*-butyl biosynthesis enzymes that have been experimentally reconstituted.

Radical SAM enzymes use the 5′-deoxyadenosyl radical (5′-dA•), generated by reductive cleavage of SAM, to initiate a diverse set of radical reactions.[9] A subfamily of these enzymes combines adenosyl radical chemistry with methyl cobalamin chemistry enabling the methylation of non-nucleophilic centers in natural product biosynthesis.[10]

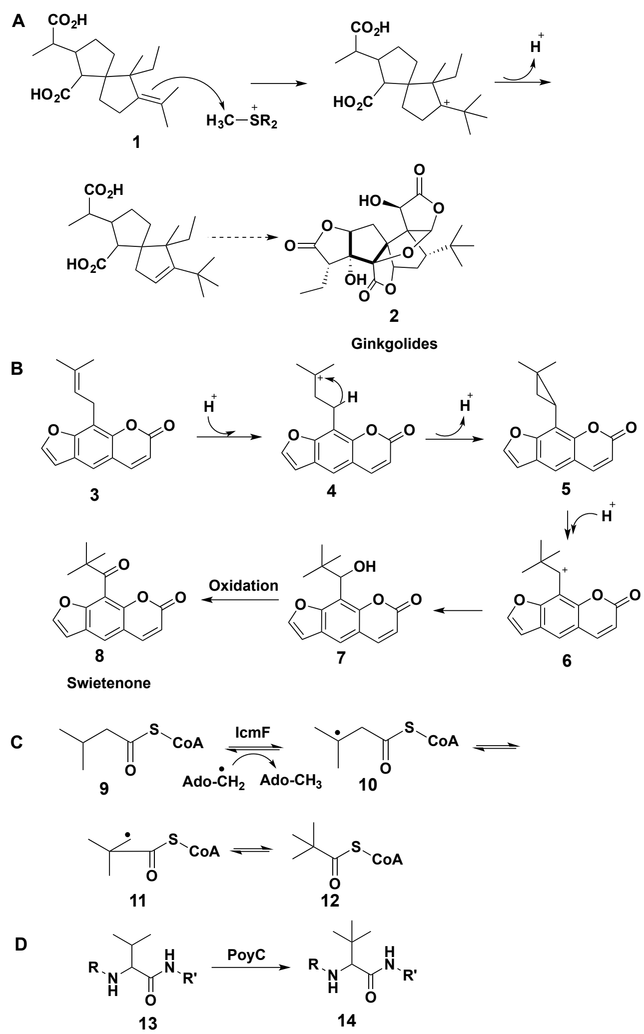

Figure 1. Mechanistic proposals for the formation of the *t*-butyl group in representative natural products.

Cobalamin-dependent radical SAM methyltransferases are experimentally challenging and are generally difficult to overproduce. Only a few systems have been reconstituted.[11] These include enzymes that catalyze phosphinic acid methylation (PhpK, L-phosphinothricin biosynthesis),[12] alcohol C-methylation (GenK, gentamicin biosynthesis[13] and Fom3, fosfomycin biosynthesis[14]), iterative C-methylation to form the ethyl group (ThnK, carbapenem biosynthesis)[15] and indole C-methylation (TsrM, thiostrepton biosynthesis).[16]

The cystobactamids **17** are a novel class of isopropyl substituted antibacterial compounds produced by myxobacteria.[17] The biosynthetic gene cluster has been identified and sequence analysis suggested that CysS is a cobalamin-dependent radical SAM methyltransferase, potentially involved in the iterative methylation of the 3-methoxy-4-aminobenzoic acid moieties of cystobactamid **15** (Figure 2). Some minor derivatives exhibit methyl, ethyl, isopropyl and *sec*-butyl groups (Stephan Hüttel and R.M., unpublished results) supporting the hypothesis of CysS being an enzyme iteratively adding methyl-groups to its substrate.

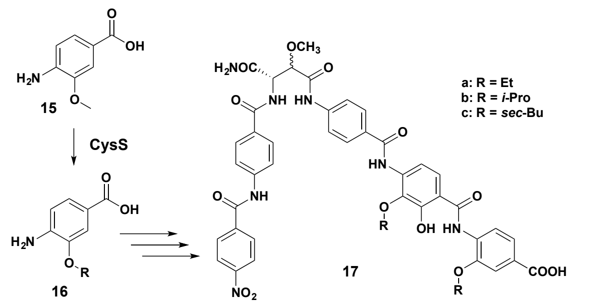

Figure 2. Proposed formation of the branched alkoxy groups of cystobactamids by CysS-catalyzed iterative methylations of a methyl ether.

To test this hypothesis, an *in vivo* labeling experiment using [^{13}C-Methyl]-*L*-methionine was performed to determine the origin of the isopropyl groups on cystobactamid 919-1 (**17b**). LC-MS analysis of the extracts showed a mass shift of +7 m/z indicating that the seven carbons from the methoxy and both isopropyl groups were from methionine and therefore most likely SAM derived (Figure 3).

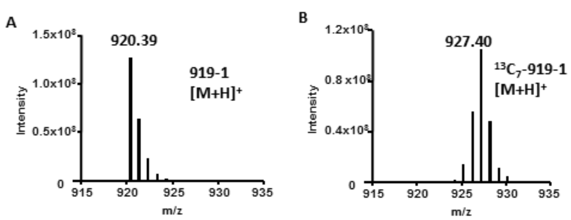

Figure 3. MS analysis of cell extracts containing (A) methionine and (B) [^{13}C-Methyl]-*L*-methionine showing the incorporation of up to seven ^{13}C in cystobactamid 919-1.

Here we describe the successful *in vitro* reconstitution of CysS and demonstrate that this enzyme can assemble isopropyl, *sec*-butyl and *t*-butyl groups by sequential methylations of a methyl group. To our knowledge, this is the first example of an isopropyl, *sec*-butyl and a *t*-butyl group biosynthesis from a methyl group using radical chemistry. Sequence analysis suggests that related chemistry is involved in the biosynthesis of other natural products such as SW-163G[18] and bottromycin.[19-20]

CysS was cloned into a pET28b vector and co-expressed with a plasmid encoding the *suf* operon ([4Fe-4S] biosynthesis)[21] in *E.coli* BL21 (λDE3). The protein was then purified, under anaerobic conditions, by Ni-NTA affinity chromatography. Cobalamin was not required in the growth medium for production of soluble protein. The UV-visible spectrum of purified CysS revealed a 420-nm shoulder, typical of a bound Fe/S cluster (Figure S1). Iron and sulfide analysis yielded 2.5 irons and 2.8 sulfides per monomer of CysS, demonstrating partial cluster formation in the overexpressed protein.

Several *p*-aminobenzoic acid (PABA) analogs were tested as substrates for CysS (Table S1). None gave the desired methylated product as indicated by LC-MS analysis. Further analysis of the cystobactamid biosynthesis cluster suggested the coenzyme A or the acyl carrier protein thioester of **15** (CysG)[17] as possible CysS substrates. To test this proposal, N-acetylcysteamine thioester **18** was synthesized and incubated with CysS, SAM, MeCbl, and flavodoxin/flavodoxin reductase/NADPH (Figure 4). LC-MS analysis of the resulting reaction mixture demonstrated the formation of the ethyl ether **19**. This was further confirmed by co-elution of the reaction product with a synthesized sample of **19** (Figure S2). When the ethyl ether **19** was incubated with CysS, the isopropyl ether **20** was detected by LC-MS analysis (Figure S2).

Pantetheinyl thioester **21** was a better substrate for CysS and iterative methylations to give the ethyl, isopropyl and the butyl ethers were detected by LC-MS analysis (Figure 5). Small amounts of the ethers **22a** and **22b** were detected in the absence of the reducing agent suggesting that some of the purified enzyme contained the reduced [4Fe-4S] cluster. To confirm the structures of **22a-c**, authentic samples of these compounds were synthesized. The enzymatic products matched the synthetic standards in terms of retention time, exact mass, and fragmentation pattern (Figure 5, Figure S3-5). In addition, CysS catalyzed the conversion of synthetic **22a** to **22b-d** and the conversion of synthetic **22b** to **22c, d** (Figure S6). The second component in the extracted ion chromatogram for the *t*-butyl ether **22c** (Figure 5C and D) was identified as the *sec*-butyl ether **22d** by co-migration with an authentic standard of **22d** (Figure S7).

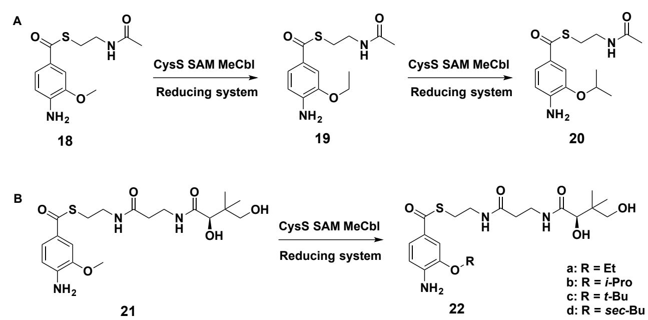

Figure 4. Identification of two substrates for CysS. A competition reaction with a 1:1 mixture demonstrated that **21** is 47 times more reactive than **18** (Supporting information).

Various [4Fe-4S] cluster reducing agents were tested in addition to the flavodoxin/flavodoxin reductase/NADPH. NADPH/methyl viologen, a commonly used electron source for cobalamin-dependent radical SAM enzymes, gave similar activity.[13, 15] However, dithionite or the combination of methyl viologen and dithionite gave a significantly lower activity.[22] Buffer thiols inactivate the substrate by trans thioesterification and need to be avoided.

Quantitative analysis of the enzymatic reaction mixture (CysS, methyl ether **21**, flavodoxin/flavodoxin reductase/NADPH, reaction run to completion) by LC-MS showed that 1 equivalent of enzyme undergoes >2 turnovers, generating around 2.0 equivalents of 5′-dA, 2.0 equivalents of SAH, 1.4 equivalents of ethyl ether **22a** and 0.3 equivalents of isopropyl ether **22b** (Figures 5 and 6, SI). The *t*-butyl ether **22c** was detected only when the concentration of the isopropyl ether **22b** was >23 μM. The ratio of 5′-dA to SAH was close to 1, suggesting that two molecules SAM were consumed for each methylation reaction and that the uncoupled production of 5′-dA is low. This is consistent with SAM functioning as the source of both the adenosyl radical and the methyl group and was further supported by LC-MS analysis of a reaction mixture containing CD_3-SAM which demonstrated CD_3 incorporation into the ethyl ether **22a** and the isopropyl ether **22b** (Figure 7).

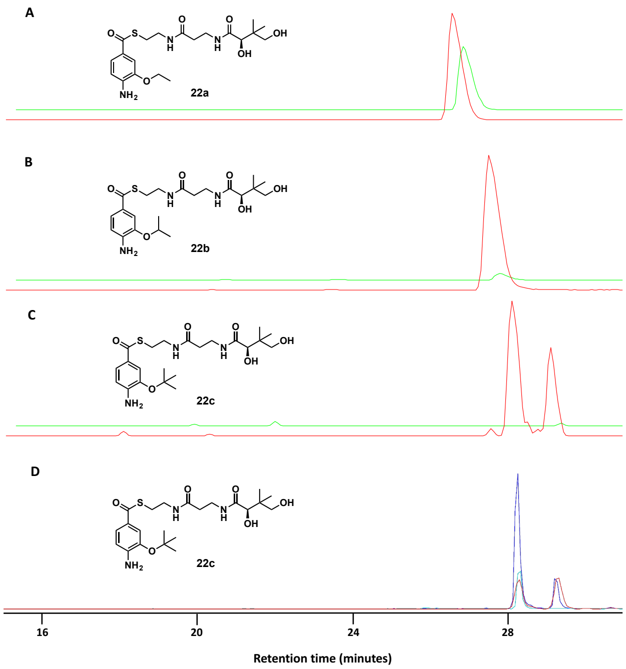

Figure 5. LC-MS analysis of the CysS-catalyzed iterative methylation of the methyl ether **21**. Red trace is for the complete reaction mixture. Green trace is for reaction mixtures where the reducing system (flavodoxin/flavodoxin reductase/NADPH) is absent. Ethyl ether **22a**, isopropyl ether **22b** were not formed in the control reactions lacking CysS, SAM or MeCbl. (A) Extracted Ion Chromatograms (EICs) of the ethyl ether **22a** $[M+H]^+$ (442.20±0.02). (B) EICs of the isopropyl ether **22b** $[M+H]^+$ (456.22±0.02). (C) EICs of the *t*-butyl ether **22c** $[M+H]^+$ (470.23±0.02). (D) EICs of $[M+H]^+$ (470.23±0.02) showing co-migration with a synthesized sample of **22c**. Cyan trace is the *t*-butyl ether standard. Blue trace is co-elution of the enzymatic product and synthetic standard. The second component in the extracted ion chromatogram for the *t*-butyl ether **22c** (panels C and D) was identified as the *sec*-butyl ether **22d** (Figure S7). The product ratio was determined by calibrating the signal intensity with known concentrations of standards (SI).

A mechanistic proposal for the CysS-catalyzed reaction, based on the proposed mechanisms for GenK[13] and ThnK[15], is shown in Figure 8. After initial formation of methylcobalamin by SAM mediated methylation, reductive cleavage of SAM by the $[4Fe-4S]^{+1}$ cluster generates the 5′-deoxyadenosyl radical. This abstracts a hydrogen atom from the methyl group of the substrate **21** to give radical **23**, which then undergoes a radical substitution with methyl cobalamin to give the ethyl ether **22a**. An analogous methyl transfer, by a radical substitution mechanism, has precedence in cobalamin model chemistry.[23] Regeneration of MeCbl from Cbl(II) can be achieved by reduction to Cbl(I) by the $[4Fe-4S]^{+1}$ cluster followed by SAM-mediated methylation. Repetition of this sequence results in the successive formation of the isopropyl, *t*-butyl and *sec*-butyl ethers of **21**. The *in vitro* ratio of branched alkoxy groups is likely to be different from the *in vivo* ratio because *in vivo* each methylation in the iterative sequence is in competition with the next step in the biosynthesis. This is not the case for the purified enzyme where no such competition exists thus allowing for the formation of higher levels of the *t*-butyl ether. Our studies on CysS suggest that *t*-butyl substituted cystobactamids, which have not yet been isolated, are likely to exist.

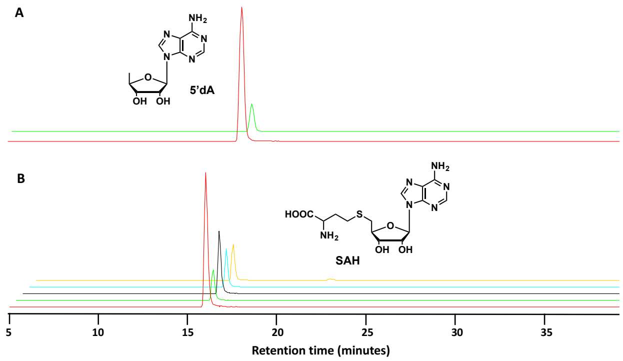

Figure 6. LC-MS detection of 5′-dA and SAH in the CysS-catalyzed iterative methylations of the methyl ether **21**. Red trace is for the complete reaction mixture. Green trace is for reaction mixtures where the reducing system (flavodoxin/flavodoxin reductase/NADPH) is absent. (A) EICs of 5′-dA $[M+H]^+$ (252.11±0.02). (B) EICs of SAH $[M+H]^+$ (385.13±0.02). Orange, green, cyan, and black traces are for reaction mixtures where either CysS, reducing system, MeCbl or substrate is absent. The product ratio was determined by calibrating the signal intensity with known concentrations of standards (SI).

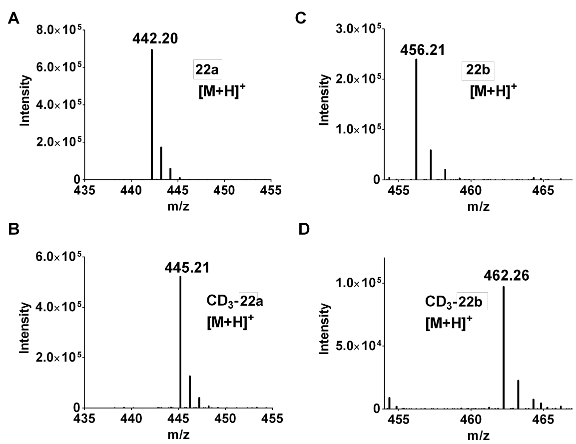

Figure 7. MS analysis of a reaction mixture in which CH_3-SAM is replaced with CD_3-SAM showing CD_3 incorporation into the ethyl and isopropyl ethers of **22a** and **22b** respectively. Panels A and C: Mass spectra of **22a** and **22b** formed from CH_3-SAM. Panels B and D: Mass spectra of **22a** and **22b** formed from CD_3-SAM.

In summary, we have elucidated the enzymology of a radical-mediated conversion of a methyl ether to a *t*-butyl ether. CysS is a cobalamin dependent radical SAM methyltransferase that catalyzes the iterative methylation of a substrate methyl ether to give ethyl, isopropyl, *t*-butyl and *sec*-butyl substituted products. Each methyl transfer is likely to proceed via hydrogen atom abstraction from the evolving carbon of the substrate followed by a radical substitution on methyl cobalamin. This biosynthetic strategy in principle enables the host myxobacterium to biosynthesize a combinatorial antibiotic library of 25 cystobactamid analogs. The analysis of the impact of these molecular decorations on bioactivity will be the task of future studies.

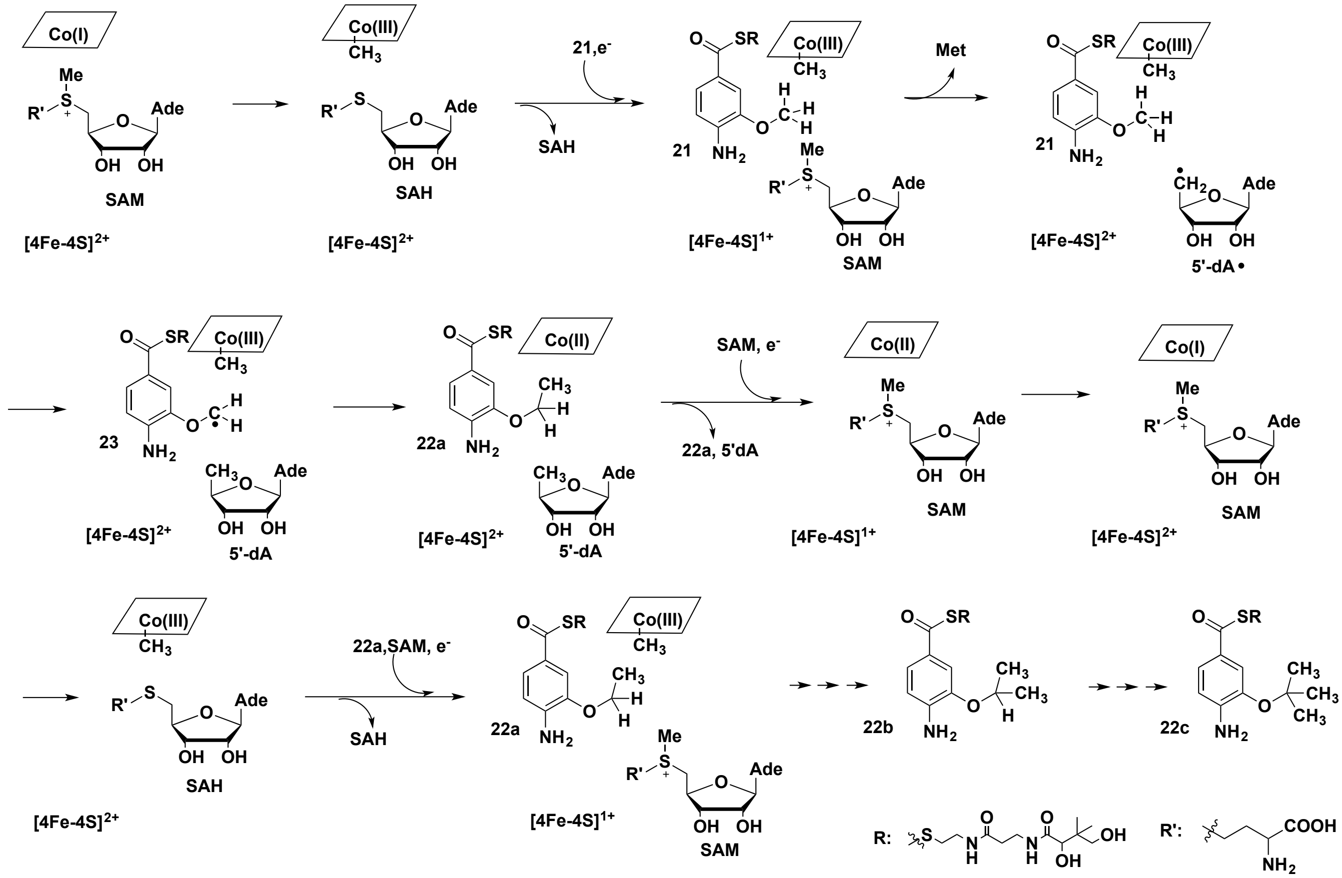

Figure 8. Mechanistic proposal for CysS-catalyzed iterative methylations to form branched alkoxy groups. The mechanism assumes two different SAM binding sites. It is also possible that SAM binds to a single site and that the position of the sulfonium moiety is altered by a protein conformational change.

ASSOCIATED CONTENT

Supporting Information

Experimental details regarding the labeling experiments, the expression and purification of CysS, the syntheses of substrates, NMR spectra and LC-MS analysis are described in the supporting information which is available, free of charge, via the Internet at http://pubs.acs.org.

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