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Biocatalytic Fluoroalkylation Using Fluorinated *S*-Adenosyl-L-methionine Cofactors

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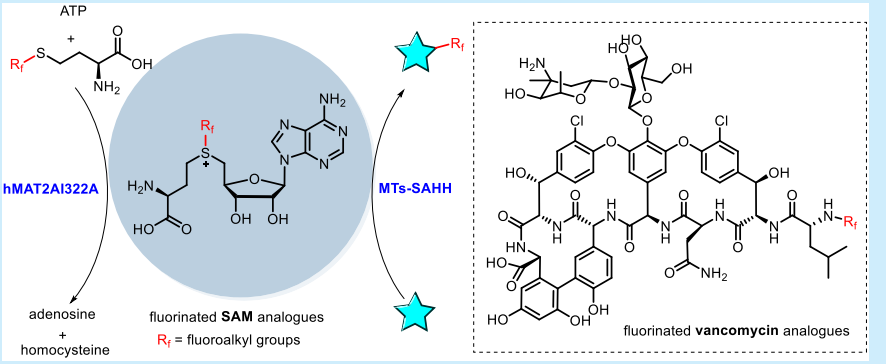

ABSTRACT: Modification of organic molecules with fluorine functionalities offers a critical approach to develop new pharmaceuticals. Here, we report a multienzyme strategy for biocatalytic fluoroalkylation using *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs) and fluorinated SAM cofactors prepared from ATP and fluorinated L-methionine analogues by an engineered human methionine adenosyltransferase hMAT2A^{I322A}. This work introduces the first example of biocatalytic 3,3-difluoroallylation. Importantly, this strategy can be applied to late-stage site-selective fluoroalkylation of complex molecule vancomycin with conversions up to 99%.

Fluoroalkyl groups have paramount applications in developing pharmaceuticals, agrochemicals, and advanced functional materials. In particular, the ability of fluorine functionalities to alter lipophilicity, metabolic stability, and pharmacokinetic profiles of drug candidates has significantly enriched the medicinal applications of organofluorine compounds.[1−5] Therefore, considerable efforts have been made to develop general and efficient methods to introduce fluoroalkyl groups into organic molecules.[6−9] Most of the developed methods rely on chemical catalysis using Lewis acids, organic molecules, and transition metals.[7,10−12] However, there is a noticeable lack of biocatalytic methodologies offering advantages in terms of mild reaction conditions, high efficiency, and selectivity. Limited examples including engineered aldolases-,[13−15] laccases-,[16] and cytochromes[17−19]-catalyzed fluoroalkylation reactions. One of the reasons is the lack of an efficient biocatalysis system and suitable fluoroalkylating reagents that can be recognized and utilized by multiple biocatalysts.

S-Adenosyl-L-methionine (SAM) and SAM-dependent methyltransferase (MT) systems are the most abundent and versatile catalysis systems in nature that selectively methylating small molecular metabolites, proteins, RNA, and DNA.[20−23] In addition to methylation utilizing SAM as a natural cosubstrate, some MTs have been shown to accept synthetic SAM derivatives bearing a broad range of fragments, such as alkyl, allyl, and propynyl groups, to facilitate non-native alkylation reactions.[24−34] Thus, replacing the SAM with its fluorinated SAM analogues would be a promising strategy for biocatalytic fluoroalkylation. However, there is only a proof-of-concept study, by Seebeck and co-workers, that reported coupling of MTs with halide methyltransferases (HMT) achieved fluoromethylation of selected small molecules, where HMT is responsible for the synthesis of fluorinated SAM from *S*-adenosyl-L-homocysteine (SAH) and fluoromethyl iodide.[35] The major obstacle is the accessibility of fluoroalkylating agents, i.e., *S*-fluoroalkylated SAMs, which are chemically complex, unstable, and lacking effective synthetic methods. Therefore, exploring alternative enzymatic approaches for the synthesis and utilization of fluorinated SAM cofactors that

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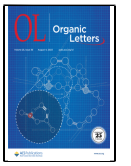

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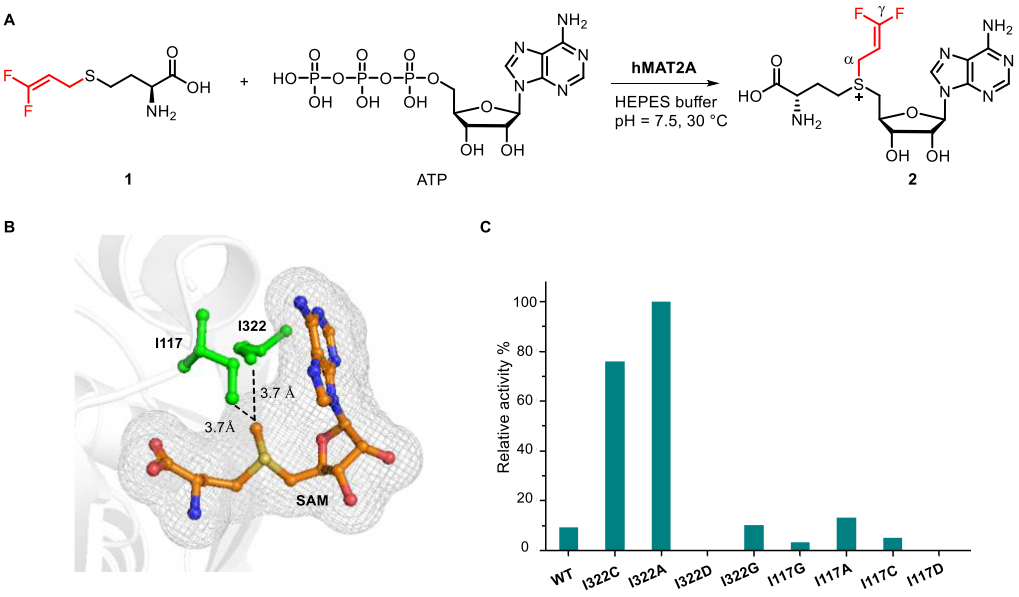

Figure 1. Directed engineering of hMAT2A for the production of **2**. (A) Reaction profile of the conversion of **1** and ATP to **2** by hMAT2A. (B) Crystal structure of hMAT2A (PDB: 2P02) with the ligand SAM. SAM and key residues interacting with the methyl of SAM are shown as sticks. (C) Determination of the activities of hMAT2A and its engineered variants. The relative activity of each enzyme was examined by quantitative analysis of the production of **2**.

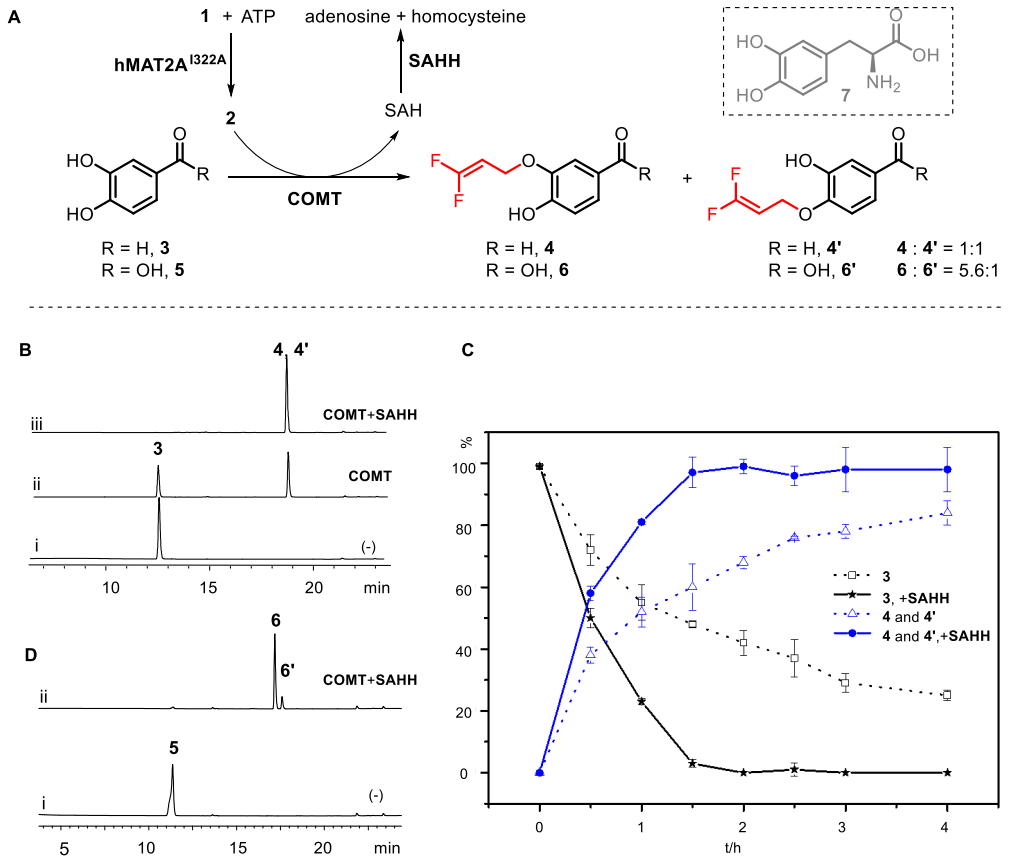

Figure 2. Biocatalytic *O*-fluoroalkylation of **3**, **5**, and **7**. (A) Reaction profiles. (B) Analysis of the production of **4** and **4′** by HPLC (λ = 280 nm). With the hMAT2A^{I322A}-free mixture as a control (i), reactions were performed by incubating **1**, ATP, **3**, hMAT2A^{I322A} and COMT in the absence (ii) or presence (iii) of SAHH for 2 h. (C) Time course of the **3** to **4** and **4′** transformation without (dotted lines) or with (solid lines) SAHH. (D) Analysis of the production of **6** and **6′** by HPLC (λ = 280 nm). With hMAT2A^{I322A}-free mixture as a control (i), reaction was performed by incubating **1**, ATP, and **5** with the hMAT2A^{I322A}-COMT-SAHH cascade for 5 h (ii).

allows the selective construction of novel organofluorides is highly desirable.

Methionine adenosyltransferase (MAT) is the primary catalyst for the biosynthesis of SAM from adenine triphosphate

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(ATP) and L-methionine (Met). Growing evidence shows that certain MATs or their engineered mutants can utilize Met analogues as substrates for the production of SAM analogues.[27,28,36,37] Herein, we introduce MAT as a new tool for the synthesis of fluorinated SAM cofactors from ATP and fluoroalkylated Met analogues, and coupling with different SAM-dependent MTs could effectively catalyze the fluoroalkylation of organic molecules and even complex clinical drugs.

Given that the sp^3-C−F bond directly attached to the sulfur atom of sulfonium salts is susceptible to hydrolysis,[35] we designed *S*-adenosyl-*S*-3,3-difluoroallyl-L-homocysteine (**2**), a SAM analogue containing inert sp^2-C−F bonds, as a new fluoroalkylating reagent for the initial test. First, we synthesized *S*-3,3-difluoroallyl-L-homocysteine (**1**) by S_N2' nucleophilic substitution using an L-homocysteine derivative and 3-bromo-3,3-diffuoropro-1-ene as substrates, and then used it as a fluoroalkyl donor for the screening of MATs. We examined several MATs toward the synthesis of a fluorinated SAM analogue from **1** and ATP. Specifically, we studied the human MAT I (hMAT1A) and MAT II (hMAT2A) catalytic alpha subunits,[38] and the archael thermophilic *Methanocaldococcus jannaschii* MAT (mMAT),[39] due to their reported promiscuous activities (Figure S1).[27] Out of three enzymes, only hMAT2A displayed slight activity toward the production of desired fluorinated SAM analogue **2** ($[C_{17}H_{23}F_2N_6O_5S]^+$ calcd: 461.1413, found: 461.1383) (Figure S2). We launched a rational engineering of hMAT2A in view of the possibility that protein engineering could enhance the enzyme activity. Considering the bulk difference between Met and its 3,3-difluoroallyl analogue **1**, we speculated that **2** formation may be enhanced by expanding the reaction pocket, especially the portion toward the methyl moiety of Met and/or SAM. Analyzing the cocrystal structure of SAM with hMAT2A (PDB: 2P02), we noticed that two key residues, i.e., I117 and I322, are located within 4 Å of the methyl moiety of SAM in the substrate-binding pocket, which may affect hMAT2A's activity toward **2** formation (Figure 1B). Thus, we systemically mutated these two residues into smaller or electron-rich amino acids (i.e., Gly, Ala, Cys, or Asp) individually, and screened the active variants by detecting **2**. Interestingly, previous studies have shown that the I117A mutation enables hMAT2A to recognize bulky alkynyl Met analogues;[26] however, this mutation has little effect on **1** recognition. In contrast, I322 residue mutations play a crucial role in generating fluorinated SAM analogue **2**, despite their ineffectiveness in alkynyl SAM analogues synthesis.[26] Among them, the I322A and I322C mutations dramatically enhanced the activity for fluorinated substrate **1**, which exhibited 9.0-fold and 7.0-fold increases in activity relative to wild-type hMAT2A, respectively (Figure 1C). The transformation from **1** to **2** was further improved by the optimization of reaction conditions, including buffer, pH, and temperature (Figures S3 and S4). However, the inherent instability of **2** prevented further isolation and related structural characterization (Figure S5). Therefore, we coupled the best active I322A variant with SAM-dependent MTs to examine the structure of **2** and its potential application in biocatalytic fluoroalkylation.

Catechol *O*-methyltransferases (COMTs) are among the most widely studied MTs, because of their biocatalytic potential for the *O*-methylation of catechol-containing structures.[40] We combined the $hMAT2A^{I322A}$ variant with an *O*-methyltransferase COMT from rat to catalyze the possible *O*-fluoroalkylation of catechol substructures (i.e., 3,4-dihydroxybenzaldehyde (**3**), 3,4-dihydroxybenzoic acid (**5**), and L-DOPA (**7**)). Incubation of **3** with the $hMAT2A^{I322A}$-COMT cascade resulted in the consummation of ∼70% **3** within 4 h, while simultaneously producing SAH and two other products (Figures 2 and S6), which were ultimately assigned as 3-*O*-3,3-difluoroallylated (**4**) and 4-*O*-3,3-difluoroallylated (**4′**) products by high-resolution electrospray mass spectrometry (HR-ESI-MS, $[C_{10}H_9F_2O_3]^+$ calcd: 215.0520, found: 215.0515) and ^1H, ^{13}C, and ^{19}F NMR spectroscopies (Figures S7−S10).

Interestingly, 3,3-difluoroallyl sulfonium salts have been studied as chemical *gem*-difluoroallylating reagents, with their γ-position showing stronger electrophilicity than their α-position, leading to the production of *gem*-difluoroallylated structures via formly S_N2' substitution at the more reactive γ-position (Figure S11).[41] However, in the $hMAT2A^{I322A}$-COMT system, no *gem*-difluoroallylated products but only the 3,3-difluoroallylated products **4** and **4′** were observed. Likely, methyltransferase COMT restricts the nucleophilic site to the less reactive α-position of **2**, exclusively producing 3,3-difluoroallylated products. The closer distance of the α-carbon atom than the γ-carbon atom to the reactive site of **3** in our docking results further supported this hypothesis (Figure S12). The resulted 3,3-difluoroallylated compounds contain a highly reactive carbon−carbon double bond due to the presence of strong electronnegative fluorine atoms, which could sever as a versatile synthetic handle for further postmodification.[42] Despite their application prospects, the synthesis of 3,3-difluoroallylated compounds remains a challenge.[43,44] Coupling of $hMAT2A^{I322A}$ with methyltransferases would be a promising strategy for the exclusive synthesis of 3,3-difluoroallylated compounds, where methyltransferase reshapes the intrinsic reactivity of 3,3-difluoroallyl sulfonium salts by directing the reaction site to the less reactive α-position. To enhance the desired transformation, SAH-hydrolase (SAHH) was employed, as coproduct SAH is an inhibitor of most if not all MTs.[45] As a result, substrate **3** was completely converted into 3- and 4-*O*-fluoroalkylated products within 90 min in >99% overall yield with a ratio of 1:1 (Figures 2C and S13−S14). **5** could likewise be fully converted into 3- and 4-*O*-fluoroalkylated products, but 3-*O*-fluoroalkylation is preferred, resulting in **6** and **6′** in a ratio of 5.6:1 (Figures 2D and S15−S17). In contrast, L-DOPA (**7**) failed to convert its 3,3-difluoroallylated products, probably due to a volumetric clash between the fluorinated SAM cofactor and L-DOPA.

In parallel, we also explored whether **2** could serve as a fluoroalkyl donor for *N*-3,3-difluoroalkylation. We chose the nicotinamide *N*-methyltransferase (NNMT) from *Macaca mulatta*, which catalyzes the 1-*N*-methylation of nicotinamide (**8**).[46] By incubation of NNMT and **8** with the $hMAT2A^{I322A}$-SAHH cascade, alkyl acceptor substrate **8** was exclusively converted into 1-*N*-3,3-difluoroallylated nicotinamide (**9**, $[C_9H_9F_2N_2O]^+$ calcd: 199.0677, found: 199.0674) as inferred by HPLC-ESI-MS (Figure S18), which was further validated by a synthetic standard, supporting the feasibility of biocatalytic *N*-3,3-fluoroalkylation.

Encouraged by the promising results of 3,3-difluoroalkylation on simple molecules, we further explored its application on complex substrates. Vancomycin is an important glycopeptide antibiotic which is considered as a last resort for the treatment of Gram-positive bacterial infections, especially those caused by resistant pathogens.[47] The introduction of fluoroalkyl groups into the vancomycin skeleton can provide new drug candidates with altered activity and/or target, as a

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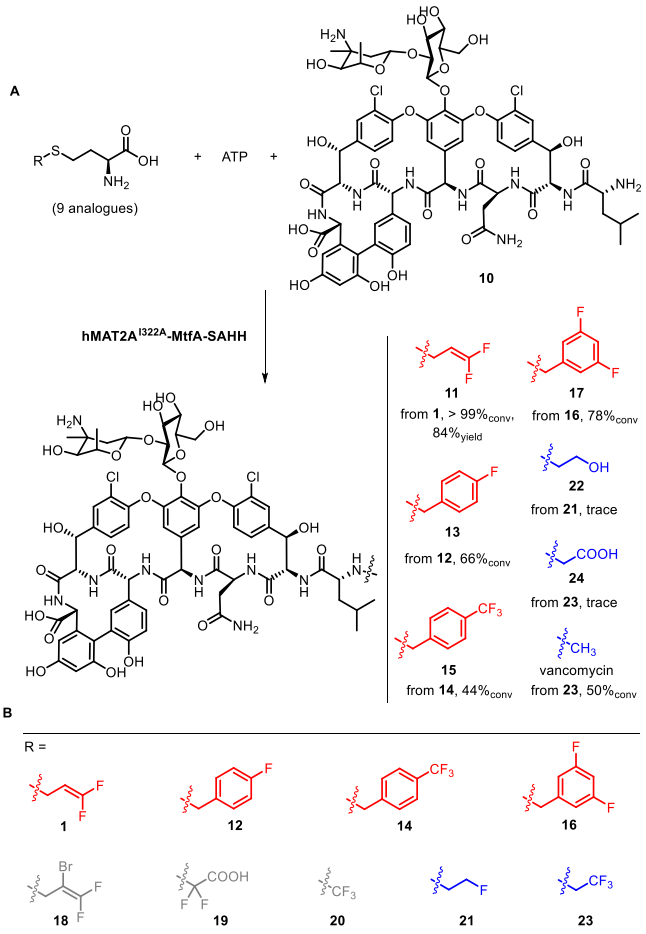
possible solution for emerging vancomycin-resistant strains. The *N*-methyltransferase MtfA from *Kibdelosporangium aridum* showed significant methylation activity against the N-terminus Leu of *N*-demethyl-vancomycin (**10**) to produce vancomycin.[48] The recombined enzyme MtfA and commercially available **10** were incubated with **1**, ATP, hMAT2A^{I322A}, and SAHH. Under the optimum conditions, **10** was completely transformed into a +76 Da (i.e., + 3,3-difluoroallyl) product **11** ($[C_{68}H_{77}Cl_2F_2N_9O_{24}]^{2+}$ calcd: 755.7213, found: 755.7206) (Scheme 1 and Figures S19 and S20). For structural

Scheme 1. Synthesis of Fluoroalkylated Vancomycin Analogues[a]



[a]Products (A) and fluoroalkyl donor substrates (B) of the hMAT2A^{I322A}-MtfA-SAHH cascade. The conversion was determined based on consumption rate of **10** by HPLC. In panel (B), the accepted substrates are colored (red for stable substrates and blue for unstable substrate in their Met- or SAM-level), while the unaccepted substrates are gray.

characterization, the hMAT2A^{I322A}-MtfA-SAHH cascade-catalyzed transformation was scaled up, leading to 12.6 mg of **11** from 16 mg of **10** with 99% conversion and 84% isolated yield. Purified **11** was further analyzed by 1D and 2D NMR spectroscopy. Overall, **11** and **10** are highly similar to each other in the ^{1}H and ^{13}C NMR spectra, where, however, a difference corresponding to the N-terminus Leu can be observed (Figure S21). Compared to the free amine of **10**, signal for free amine disappeared; meanwhile, signals for a 3,3-difluoroallyl were observed [i.e., δ_H 3.37(2H), 4.55−4.66(m, 1H), δ_C 62.9, 78.1−78.4(m), 155.3−157.9(m) and δ_F −89.3(m), −87.7(d, J = 45.1 Hz)], indicating the amine of **11** was substituted by the 3,3-difluoroallyl group. Together with HMBC, ^{1}H−^{1}H COSY correlations, and the HR-MS/MS spectrum (Figures S21 and S22), **11** was determined as *N*-3,3-difluoroallyl-vancomycin. Clearly, the hMAT2A^{I322A}-MtfA-SAHH cascade can site-selectively catalyze *N*-3,3-difluoroallylation of **10**, resulting in the fluorinated vancomycin analogue.

In addition to 3,3-difluoroallyl substrate **1**, we tested more fluorinated Met analogues with the hMAT2A^{I322A}-MtfA-SAHH cascade, aiming to create more fluorinated vancomycin analogues (Scheme 1). Several fluorinated *S*-benzyl-L-homocysteine substrates (i.e., **12**, **14**, and **16**) were converted into their fluorinated benzylvancomycins with 44−78% conversion, for which their structures were confirmed by HPLC-ESI-MS/MS and/or ^{19}F-NMR (Figures S23−S25). However, 2-bromo-3,3-difluoroallyl (**18**) and perfluoroalkyl Met analogues (i.e., **19**−**20**) remained unreactive due to their bulky substitution and/or strong electron-withdrawing properties hindering the synthesis of fluorinated SAM analogues by hMAT2A and its mutants. Other fluoroalkyl donor substrates, i.e., *S*-2-fluoroethyl-homocysteine (**21**) and *S*-2,2,2-trifluoroethyl-homocysteine (**23**), were consumed; however, they did not produce any detectable fluorinated vancomycin. Instead, a trace of *N*-2-hydroxyethyl-vancomycin (**22**, $[C_{67}H_{79}Cl_2N_9O_{25}]^{2+}$ calcd: 739.7282, found: 739.7300) was produced when using **21**, while *N*-carboxymethyl-vancomycin (**24**, $[C_{67}H_{77}Cl_2N_9O_{26}]^{2+}$ calcd: 746.7178, found: 746.7202) and vancomycin were produced when using **23** (Figures S26 and S28). We attributed the production of **22** to the instability of **21**, which was almost completely hydrolyzed in buffer to *S*-2-hydroxyethyl-L-homocysteine (**25**, $[C_6H_{14}NO_3S]^+$ calcd: 180.0694, found: 180.0684); a Met analogue could be recognized by hMAT2A (Figure S27). In contrast, **23** was stable in buffer, while its SAM product is destabilized by the sulfonium ion, resulting in fluorine hydrolysis to produce carboxymethyl SAM and further decarboxylated product SAM, leading to **24** and vancomycin production (Figure S28).

In summary, we have established a new biocatalytic approach for the fluoroalkylation of organic compounds using fluorinated SAM cofactors and SAM-dependent MTs. The key to success lies in accessing fluorinated SAM cofactors, which are prepared from ATP and fluorinated L-methionine analogues by the engineered human methionine adenosyltransferase hMAT2A^{I322A}. By coupling the hMAT2A variant with different SAM-dependent MTs and SAH hydrolase, some fluoroalkylated or alkylated organic compounds were synthesized with good efficiency and selectivity. Importantly, this strategy was applied to introduce a 3,3-difluoroallyl group and fluorine-containing benzyl groups into a drug molecule, vancomycin, with up to 99% conversion, showing the potential for the targeted modification of complex organic molecules. This approach enriches the toolbox in the synthesis of complex organofluorides, which will facilitate the development of fluorine-containing drugs.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.3c02028.

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Supporting Information and methods, results, Figures S1−S28, and Tables S1−S3. (PDF)

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Notes

The authors declare no competing financial interest.

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