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Non-enzymatic synthesis of *C*-methylated fluostatins: discovery and reaction mechanism†

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Two *C*-methylated fluostatins (FSTs) B3 (1) and B4 (2) were synthesized from flavin-mediated nonenzymatic epoxide ring-opening reactions of FST C. The structures of 1 and 2 were elucidated by HRESIMS, NMR, and ECD spectroscopic analyses. A subsequent ^{13}C labeling study demonstrated that the *C*-methyl groups of 1 and 2 were derived from DMSO and enabled the mechanistic proposal of a nonenzymatic *C*-methylation.

The methyl group is the simplest alkyl fragment appearing in small molecule drugs. The addition of methyl groups to pharmaceutical compounds often leads to notable enhancements in their lipophilicity, membrane solubility, bioavailability, and protection from enzymatic degradation *in vivo*.[1–3] In biological systems, methyl transfer is typically catalyzed by *S*-adenosylmethionine (SAM) dependent methyltransferases.[2,4] The molecular mechanism of SAM-dependent methylation generally entails a nucleophilic substitution taking place at the sulfonium methyl carbon of SAM, resulting in a variety of *C*-, *O*-, *N*- and *S*-methylated products,[4,5] or involves radical chemistry to yield C(sp^3)-methyl bonds (Fig. 1A).[6,7] In synthetic chemistry, considerable strategies have been developed to selectively and efficiently incorporate methyl groups into pharmaceutical scaffolds at both sp^2 and sp^3 carbon centres.[1] Traditionally, the C–H methylation of both sp^2 and sp^3 centres has primarily involved the deprotonation of acidic C–H bonds followed by alkylation using electrophilic methyl sources like methyl iodide (Fig. 1B).[8] Very recently, a bioinspired reaction *via* a hydrogen atom transfer (HAT)-SH^2 dual catalytic strategy has been developed for the direct C(sp^3)–H methylation of drug compounds.[9] Despite their sophisticated designs, these synthetic methods face challenges, including multi-step synthesis with low efficiency or the requirement of complex starting materials.[1]

Fluostatins (FSTs) are distinguished by their characteristic tetracyclic benzo[*a*]fluorene skeleton.[10,11,22] To date, approximately fifty analogues of FSTs have been identified, encompassing FST monomers,[11–15] the benzo[*cd*]indeno[2,1-*f*]indazole skeleton,[10] racemic aminobenzo[*b*]fluorenes,[13] a pentacyclic skeleton fused with both benzo[*b*]fluorine and a six-membered lactone ring,[16] and C–C or C–N coupled homo- or heterodimers.[15,17] Notably, all these FSTs feature a single *C*-methyl group at C-3. FSTs are frequently characterized by the presence of an epoxide group at C-2/C-3. Recently, the epoxide group in FST C (**9**) has been demonstrated to undergo flavin-mediated, nonenzymatic reductive and oxidative ring-opening reactions, leading to diverse products, including FSTs B1 (**3**),

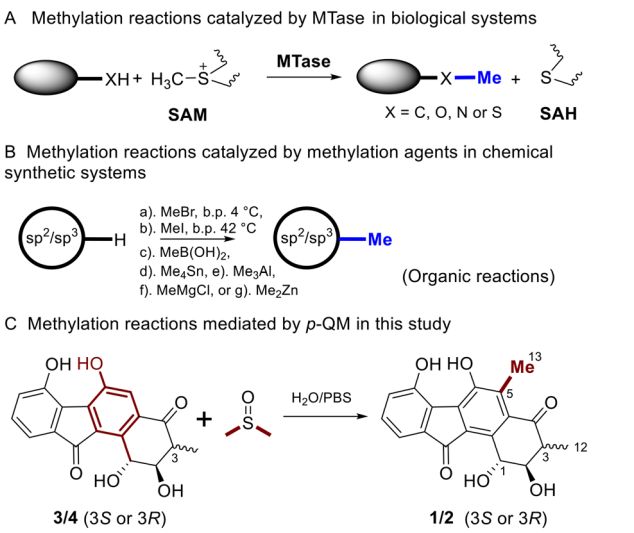

Fig. 1 Representative methylation reactions. (A) Enzymatic methylation reactions by methyltransferase (MTase). (B) Chemical methylation reactions involving various methylation agents in organic synthesis. (C) *C*-Methylation reaction of FSTs B1/B2 (**3**/**4**). The structures of FSTs B3/B4 (**1**/**2**) were determined in this study.

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B2 (**4**), C2 (**5**), C3 (**6**), C4 (**7**) and C5 (**8**) (Fig. 2A).[18] In this work, we identified two additional C_5-methylated FSTs, FSTs B3 and B4 (**1** and **2**), from the epoxide ring opening reactions. Subsequent experiments indicated that the C_5-methyl group was nonenzymatically derived from dimethyl sulfoxide (DMSO) (Fig. 1C). Herein, we report the isolation and structure elucidation of **1** and **2**, and propose a *C*-methylation mechanism.

In a previous study, we demonstrated that the α/β hydroxylase Alp1U can hydrolyze the epoxide of FST C (**9**), resulting in the chiral vicinal diol FST C1 and FST C2 (**5**).[19] More recently, we have described nonenzymatic epoxide ring opening reactions: the incubation of FST C (**9**) with flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH) resulted in the production of various redox products, including **3**–**8** (Fig. 2A and Fig. S1†), while the control assays of **9**, lacking either FAD or NADH, showed no conversions (Fig. 2A).[18] Furthermore, we provided evidence for the spontaneous tautomerization of **3** to **4** in buffers with neutral pH.[18] This phenomenon can be further amplified under alkaline pH conditions, yielding FST A.[18] Interestingly, in our further studies of the nonenzymatic epoxide ring opening reactions, we occasionally encountered two very minor products, FST B3 (**1**, yield 1%) and FST B4 (**2**, yield 1.2%; Fig. 2A).

To structurally characterize these minor products, **1** and **2** were isolated from a large-scale reaction of FST C (**9**), carried out in the presence of FAD and NADH, in a PBS buffer at pH 7.0. The molecular formula of **1** was established to be $C_{19}H_{16}O_6$ by HRESIMS (*m*/*z* 339.0874 $[M - H]^-$, calcd for 339.0881, Fig. S2†). The ^1H and ^{13}C NMR data of **1** were similar to those of FST B1 (**3**); the difference is that the H-5 in **3** was replaced by a methyl group. This assignment was supported by the HMBC correlations from H_3-13 to C-4a/C-5/C-6. Further detailed analysis of the 2D NMR data of **1** confirmed its planar structure (Fig. 2B). The relative configuration of **1** was assigned by NOESY correlations. The *trans*-configuration of H-1 and H-2 in **1** was deduced by the NOE correlations of

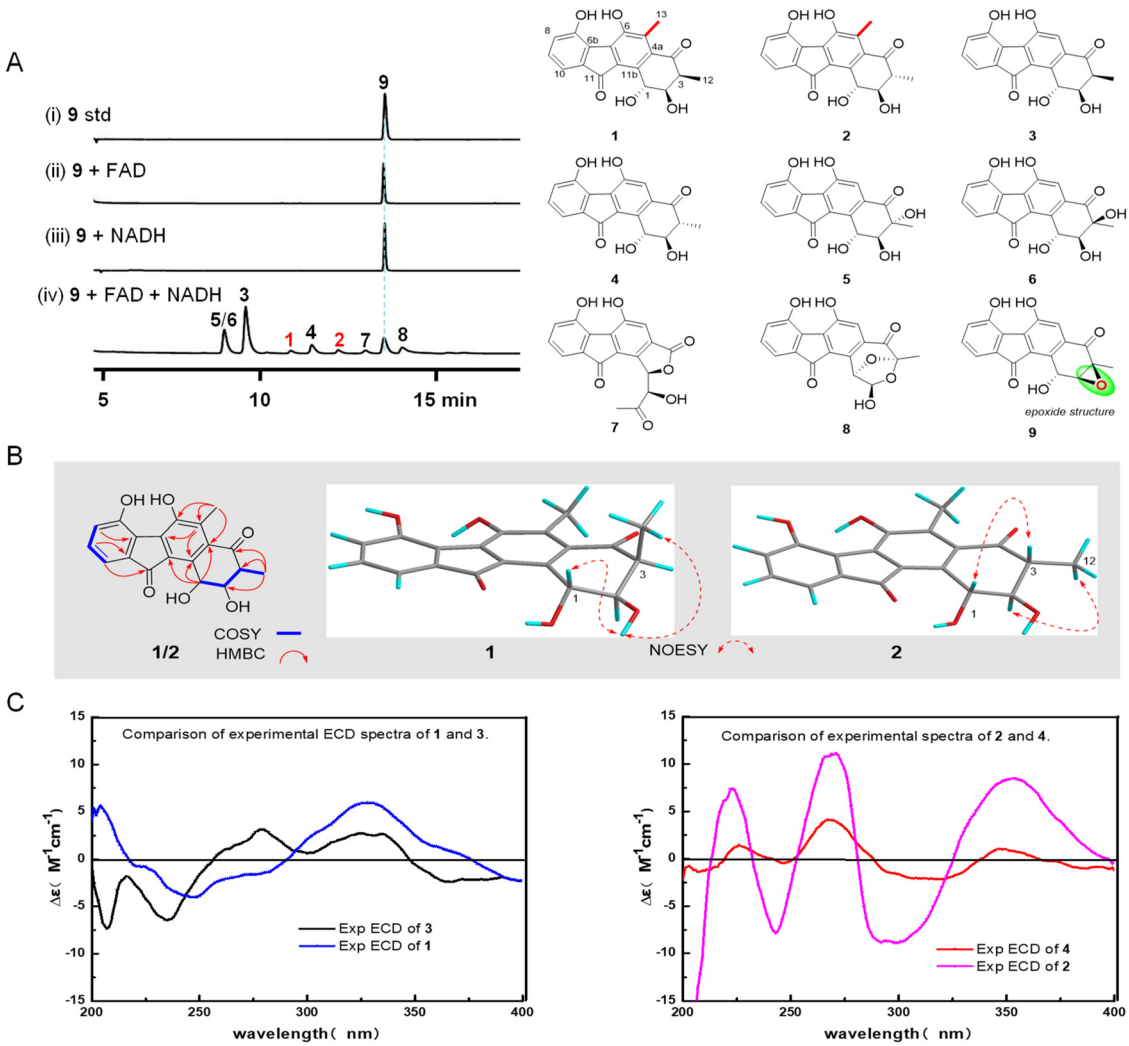

Fig. 2 (A) HPLC analysis of reactions involving FST C (**9**). (i) **9** std; (ii) **9** + FAD; (iii) **9** + NADH (pH 7.0); (iv) **9** + FAD + NADH in PBS buffer at 30 °C for 30 min. HPLC analysis was run with UV detection at 304 nm using a reversed phase C_{18} column. (B) The key HMBC, COSY, and NOESY correlations of **1**/**2**. (C) Comparison of experimental ECD spectra of **1**/**3** and **2**/**4**.

H-1/OH-2, and a *cis*-configuration of H-2/H-3 in **1** was suggested by the NOESY correlations of 2-OH/H3-12 (Fig. 2B). Based on comparison of the experimental ECD spectra of **1** and **3** (Fig. 2C), the absolute configuration of **1** was assigned as 1*R*,2*R*,3*S*. The molecular formula of **2** was established as $C_{19}H_{16}O_6$ by HRESIMS (*m*/*z* 339.0877 $[M - H]^-$, calcd for 339.0881, Fig. S3†), the same as that of **1**. The 1D and 2D NMR data of **2** (Table S1 and Fig. S3†) and **1** were highly similar, suggesting that **2** was a stereoisomer of **1**. The planar structure of **2** was confirmed to be identical to that of **1** by comparing their 2D NMR data. The observed NOESY correlations of H-1/H-3 and H-2/H_3-12 (Fig. 2B) indicated a *trans* configuration of H-1/H-2 and H-2/H-3 in **2**. Moreover, the absolute configuration of **2** was decided by comparison of the ECD spectra of **2** and **4** (Fig. 2C). Their almost identical cotton effects suggested that **2** should have the 1*R*,2*R*,3*R* configuration.

Since the compound **9** we used for the nonenzymatic epoxide ring opening reactions was dissolved in DMSO, we inferred that DMSO might play an essential role in the formation of the C_5-methylated products **1** and **2**. Additionally, the structural similarity between **1**/**2** and **3**/**4** suggested that ring-opening products **3**/**4** likely served as precursors for the formation of **1**/**2**. To verify this hypothesis, we conducted an experiment in which compound **3** was dissolved in DMSO and then incubated in PBS buffer (pH 7.0) for half an hour at 30 °C. As a result, traces of **1** were observed (Fig. 3A). A subsequent LC-MS analysis of the reaction products detected the presence of a molecular ion peak at *m*/*z* 339.7 ($[M - H]^-$) (Fig. 3B). Furthermore, **3** was dissolved in DMSO-d_6 and incubated in PBS buffer (pH 7.0). An LC-MS analysis then identified a +3 Da-shifted molecular ion peak at *m*/*z* 342.7 ($[M - H]^-$) for **1** (Fig. 3B), denoting the incorporation of three

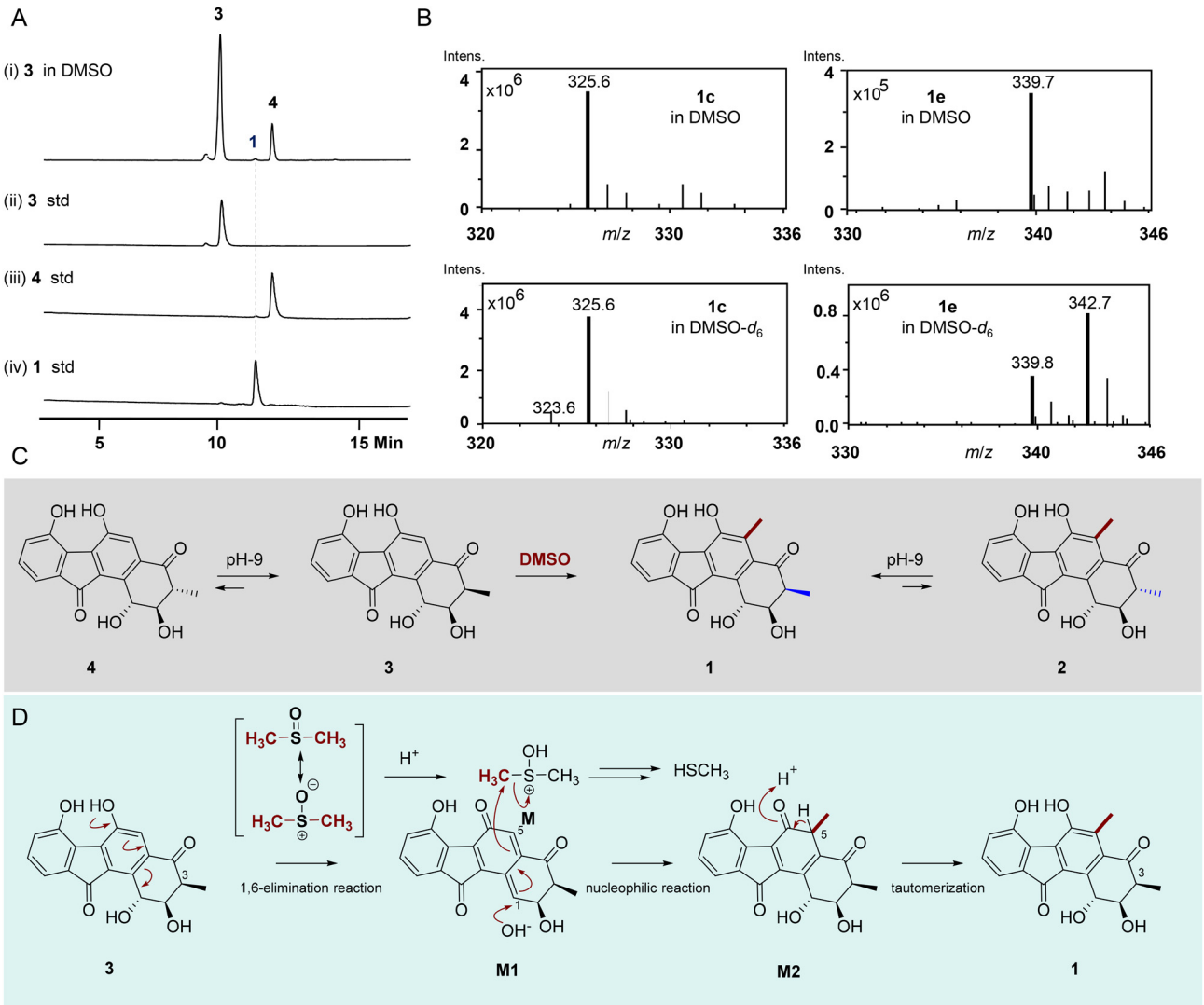

Fig. 3 Methylation reactions involving FST B1 (**3**) and the proposed mechanism. (A) HPLC analysis of reactions involving FST B1 (**3**). (i) **3** in DMSO; (ii) **3** std; (iii) **4** std; (iv) **1** std; in PBS buffer at 30 °C for 30 min. HPLC analysis was run with UV detection at 304 nm using a reversed phase C_{18} column. (B) LC-MS analysis of reactions in DMSO (control) or DMSO-d_6. (C) Potential inter-conversion of FST B1 (**3**) to FST B4 (**4**). (D) Proposed mechanisms for the methylation reaction of **3** in the presence of DMSO.

deuterons into the C_5-methylated **1**. This evidence strongly suggests that DMSO acts as a methyl donor in this methylation process, and **3** serves as a precursor. A similar reaction was observed between **4** and **2**. Given the previous research demonstrating the spontaneous conversion of **3** to **4**,[18] we also postulated that **1** might similarly convert to **2** under the same conditions. This proposal was subsequently validated by observing the facile transformation of **1** into **2** at pH 9 (Fig. 3C and Fig. S4†).

FSTs possess a unique tetracyclic benzo[*a*]fluorene skeleton.[11] The distinctive structural features of FSTs provide them with the ability to generate a reactive *para*-quinone methide (*p*-QM) intermediate through an autocatalytic process that involves the 1,6-elimination reaction in FSTs.[15] In a previous report, we described that a reactive *p*-QM-like intermediate undergoes coupling with a nucleophilic donor, leading to a diverse spectrum of C–C/C–N dimer FSTs.[15] Here, we propose that the formation of **1**/**2** follows a mechanism similar to the one previously described. Specifically, the reaction involves the following key steps (Fig. 3D): (i) DMSO undergoes protonation, forming an activated electrophilic species **M**;[20] (ii) **3** undergoes a 1,6-elimination reaction, yielding a transient *p*-QM intermediate **M1**;[15] (iii) OH^- participates in a nucleophilic reaction at C-1 of **M1**, facilitating electron transfer to C5 of **M1**; (iv) **M1** then undergoes a subsequent nucleophilic reaction with the methyl group of **M**, culminating in the formation of **M2**; and (v) **M2** undergoes tautomerization, leading to the formation of **1**.

Conclusions

In conclusion, C_5-methylated FSTs, **1** and **2**, were identified in the ring-opening reaction. Subsequent experimentation confirmed the chemical synthesis of **1** and **2** from precursors **3** and **4** in the presence of DMSO. The unique structural composition of *p*-QMs involves reactive carbonyl and olefinic moieties, facilitating resonance between neutral and zwitterionic structures.[21] This characteristic enables *p*-QMs to engage frequently in 1,6-conjugate addition reactions. Nonetheless, it is hypothesized that the acquisition of **1** and **2** is a result of the 1,4-addition of *p*-QM intermediates. This scenario presents a specific instance demonstrating the reaction pattern of *p*-QMs. Moreover, the study highlights the extension of methylation of C(sp^2)–H bonds under mild conditions, eliminating the need for metal and non-metal catalysts.

Author contributions

Bidhan Chandra De, C. Huang, and W. Zhang performed compound isolation and structure determination. Bidhan Chandra De and C. Yang carried out the reaction. Bidhan Chandra De, W. Zhang and C. Zhang wrote the manuscript. C. Zhang directed the research.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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