Flavin-enabled reductive and oxidative epoxide ring opening reactions

Bidhan Chandra De1,2,9, Wenjun Zhang1,2,3,4,9, Chunfang Yang1,2,3,4, Attila Mándi5, Chunshuai Huang1, Liping Zhang1,2,3, Wei Liu1, Mark W. Ruszczycky6, Yiguang Zhu1,2,3,4, Ming Ma7, Ghader Bashiri8, Tibor Kurtán5, Hung-wen Liu6 & Changsheng Zhang1,2,3,4

Epoxide ring opening reactions are common and important in both biological processes and synthetic applications and can be catalyzed in a non-redox manner by epoxide hydrolases or reductively by oxidoreductases. Here we report that flavostatins (FSTs), a family of atypical angucyclines with a benzofluorene core, can undergo nonenzyme-catalyzed epoxide ring opening reactions in the presence of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH). The 2,3-epoxide ring in FST C is shown to open reductively via a putative enol intermediate, or oxidatively via a peroxylated intermediate with molecular oxygen as the oxidant. These reactions lead to multiple products with different redox states that possess a single hydroxyl group at C-2, a 2,3-vicinal diol, a contracted five-membered A-ring, or an expanded seven-membered A-ring. Similar reactions also take place in both natural products and other organic compounds harboring an epoxide adjacent to a carbonyl group that is conjugated to an aromatic moiety. Our findings extend the repertoire of known flavin chemistry that may provide new and useful tools for organic synthesis.

Epoxides are important building blocks in organic synthesis and biosynthesis1. Due to the significant strain of the three-membered oxirane ring and polarized oxygen-carbon bonds, epoxides can readily undergo regioselective and stereoselective ring-opening under the control of an appropriate catalyst2. Epoxide ring opening generally proceeds via nucleophilic addition with inversion of stereochemistry; however, the regioselectivity can be sensitive to whether the reaction is conducted under acidic or alkaline conditions as shown in Fig. 1a. Accordingly, a number of nucleophiles have been developed to react with epoxides in order to yield 1,2-difunctionalized systems with trans stereochemistry4. Epoxide ring-opening can also be catalyzed by enzymes such as epoxide hydrolases (EHs) or oxidoreductases (ORs) (Fig. 1b)5. EHs catalyze the hydrolysis of epoxides to trans-vicinal diols6; ORs catalyze the stereoselective reduction of an epoxide to an alcohol (Fig. 1b)7.8.

Epoxides are also an important structural element found in a wide variety of natural products and reactive intermediates in biosynthetic pathways1. In particular, the flavostatins (FSTs, e.g. 1)9,10, kinamycins11,12, lomaviticins13,14, and nenestatins15,16 constitute a family of atypical angucyclines with a benzofluorene core (Supplementary Fig. 1). The...
The A-ring that can undergo further modifications such as acylation, epoxidation, glycosylation, and dimerization leading to significant structural diversity. The A-ring in atypical angucyclines are often characterized by a highly oxygenated membered A-ring (\( \alpha/\beta \)).

Naturally isolated FSTs (Supplementary Fig. 1), such as FST B12, pyr-oostatin C (1) to afford the two stereoisomeric FSTs C1 (2) and C2 (3) (Fig. 1c).

The \( \alpha/\beta \)-hydrolase FlsH from the FST pathway in the South China Sea-derived Micromonospora rosaria SCSIO N160 has been shown to catalyze deacylation of acyl FSTs; however, it is unable to catalyze hydrolysis of the epoxide in FSTs despite its homology to Alp1U. Naturally isolated FSTs (Supplementary Fig. 1), such as FST B12, pyrazolofluostatin A–C, and FST R have been proposed to derive from the corresponding epoxide precursors; however, given the activity of FlsH it is not entirely clear how the epoxide would be cleaved and what enzymes if any would be responsible.

In this work, the 2,3-epoxide ring in FST C (1) is shown to open nonenzymatically in the presence of flavin adenine dinucleotide (FAD, 4) and nicotinamide adenine dinucleotide (NADH) with reduced FADH2 (5) or C4a-hydroperoxylavin (FADOH, 6) serving as the reactive flavin species (Fig. 1c). The epoxide ring-opening reactions can thus proceed in a reductive manner to generate a putative enol intermediate, which is tautomered to products possessing a single hydroxyl group at C-2 (7 and 8), or is further modified by 6 to products with a vicinal diol at C-2 and C-3 (3 and 9).

The α/β-hydrolase Alp1U was previously shown to hydrolyze the epoxide of FST C (1) to afford FST C1 (2) and FST C2 (3) (Fig. 2a, traces i–iii). In contrast, the Alp1U homologue FlsH is unable to catalyze hydrolysis of the epoxide ring in 1 (Fig. 2a, trace iii). A possible explanation is that FlsH might require a protein partner or an exogenous cofactor to catalyze the epoxide hydrolysis. In order to test these hypotheses, several flavin reductases including Fre from E. coli were selected to examine their competence as putative protein partners for FlsH. It was found that incubation of FST C (1) with FlsH and Fre in the presence of NADH led to multiple products (Fig. 2a, trace vi). However, further experiments showed that formation of the same set of products was also observed in the absence of FlsH (Fig. 2a, trace vii), suggesting that Fre alone was able to mediate the same decomposition of FST C (1). Moreover, incubation of 1 with several other flavoenzymes including FisO2 (prejadomycin oxidase), TiaM (tiaconic halogenase), and XiaK (xiamycin N-hydroxylase) also led to the same result (Supplementary Fig. 2). Given that the activities of these flavoenzymes all rely on benzofluorene core of the kinamycins and lomaiviticins is further decorated with a diazo group endowing these compounds with potent antitumor activities that has attracted considerable attention. The typal angucyclines are often characterized by a highly oxygenated membered A-ring that can undergo further modifications such as acylation, epoxidation, glycosylation, and dimerization leading to significant structural diversity. The A-ring in atypical angucyclines are often characterized by a highly oxygenated membered A-ring (\( \alpha/\beta \)).

**Results**

**Nonenzymatic epoxide ring-opening reaction of FST C (1)**

The α/β-hydrolase Alp1U was previously shown to hydrolyze the epoxide of FST C (1) to afford FST C1 (2) and FST C2 (3) (Fig. 2a, traces i–iii). In contrast, the Alp1U homologue FlsH is unable to catalyze hydrolysis of the epoxide ring in 1 (Fig. 2a, trace iii). A possible explanation is that FlsH might require a protein partner or an exogenous cofactor to catalyze the epoxide hydrolysis. In order to test these hypotheses, several flavin reductases including Fre from E. coli were selected to examine their competence as putative protein partners for FlsH. It was found that incubation of FST C (1) with FlsH and Fre in the presence of NADH led to multiple products (Fig. 2a, trace vi). However, further experiments showed that formation of the same set of products was also observed in the absence of FlsH (Fig. 2a, trace vii), suggesting that Fre alone was able to mediate the same decomposition of FST C (1). Moreover, incubation of 1 with several other flavoenzymes including FisO2 (prejadomycin oxidase), TiaM (tiaconic halogenase), and XiaK (xiamycin N-hydroxylase) also led to the same result (Supplementary Fig. 2). Given that the activities of these flavoenzymes all rely on
Alp1U-catalyzed hydrolysis reaction. The second reaction product (Supplementary Fig. 3 and Table 1), which is also a product of the crystal X-ray analysis (Fig.2b, CCDC 2036399; Supplementary Table 3). The third product experimental and calculated ECD spectra carried out on the (1 absolute con

ECD methodology (Supplementary Fig. 5) and con

absolute con

mined to have the same planar structure as

Sp. TA-339112 and

planar structure as FST B, an FST naturally isolated from

Streptomyces

strain Acta 1383;13 however, the abso-

fication of the FST C decomposition products

facilitate the nonenzymatic decomposition of FST C (Fig.2a, traces viii & xii) and necessary (Fig.2a, traces ix xi) to serve as the actual catalyst of FST C (Fig.2a, traces vii). The product

was assigned by comparison of the

Comparison of experimental and calculated ECD spectra of 9

The X-ray crystal structures of 7 and 10. c The X-ray crystal spectrum of 9 (black line) compared with the B3LYP/TZVP PCM/McCN // ωB97X/TZVP PCM/McCN spectrum of (I,2R,3S)-9 (red line). The bars represent rotational strength values for the lowest energy solution conformers.

Identification of the FST C decomposition products

Reaction products (3 and 7–11) were isolated from a scaled up reaction of FST C (I) with FAD and NADH and structurally characterized as shown in Fig. 1c. The first product was identified to be FST C2 (3) (Supplementary Fig. 3 and Table 1), which is also a product of the Alp1U-catalyzed hydrolysis reaction. The second reaction product 7 (Supplementary Fig. 4 and Table 2) was established to have the same planar structure as FST B, an FST naturally isolated from Streptomyces sp. TA-339112 and Streptomyces strain Acta 1383; however, the absolute configuration of FST B was not determined. The (1R,2R,3S) absolute configuration of 7 was established by the solution TDDFT-ECD methodology (Supplementary Fig. 5) and confirmed by single crystal X-ray analysis (Fig. 2b, CCDC 2036399; Supplementary Table 3). The third product 8 (Supplementary Fig. 6 and Table 2) was determined to have the same planar structure as 7 and the (1R,2R,3R) absolute configuration of 8 was assigned by comparison of the experimental and calculated ECD spectra carried out on the (1R,2R,3R) stereoisomer (Supplementary Fig. 7). In order to distinguish the two stereoisomers of FST B, 7 and 8 were named FST B1 and FST B2 (Fig. 1), respectively. The product 9 (designated FST C, Fig. 1) was isolated with the same retention time as that of 3 during in the HPLC analysis with a reversed-phase C18 column. Analysis of the assay using a polar HPLC column allowed separation of these two species (Fig. 2a, trace xii). Finally, 9 was identified to be a stereoisomer of 3 by HRMS and NMR data (Supplementary Fig. 8 and Table 4), and the (1R,2S,3S) absolute configuration of 9 was established by TDDFT-ECD methodology (Fig. 2c, Supplementary Fig. 9).

Two additional products 10 and 11 were also identified and structurally characterized (Fig. 1c; Fig. 2a, traces vii–xii). The product 10, designated FST C4 (Fig. 1), was found to possess a contracted five-membered A-ring by NMR spectroscopic analysis (Supplementary Fig. 10 and Table 5) and was determined to have the (1R,2R) absolute configuration by single crystal X-ray analysis (Fig. 2b; Supplementary Table 6; CCDC 2129081). The product 11, designated FST C5 (Fig. 1), was structurally elucidated to have a bridged and expanded seven-membered A-ring (Supplementary Fig. 11 and Table 5). Assignment of the (1R,2S,3R) absolute configuration to 11 was based on its origin from 1 but otherwise remains tentative, because a definitive experimental characterization was not possible on account of its instability. Currently, there are no reports of 9–11 having been isolated from a natural source.
Labeling studies of the epoxide ring-opening reaction

To investigate the mechanism of the nonenzymatic epoxide ring opening reaction, FST C (1), FAD and NADH were co-incubated in PBS buffer (pH 7.0) prepared with deuterium oxide (D2O). Incorporation of a single deuterium into both 7 and 8 was indicated by the presence of a +1 Da-shifted molecular ion at m/z 326.7 ([M – H]–) for both reduced products (Supplementary Fig. 12). In contrast, no changes in the molecular weights of 3, 9, 10, or 11 (Supplementary Fig. 12) were observed. Subsequently, *H NMR and 13C NMR spectra were compared with those of 3, 9, 10, and 11 and the 3-Me doublet of 7 had collapsed to a singlet in 3, 9, 10, and 11 but not in 11. In support of this assignment, the COSY correlation between the H-2 and H-3 protons observed in 7 was absent in 11 (Supplementary Figs. 4, 13). Likewise, a singlet was also observed for the 3-Me along with the absence of any COSY correlation between H-2 and H-3 in 11 (Fig. 3a; Supplementary Figs. 13, 14). Moreover, the 13C NMR spectra of 3, 9, 10, and 11 showed broadening and reduced intensity of the signals from C-3, and δ-H/3C couplings at C-3 were also observed (Supplementary Figs. 13, 14). These observations demonstrate that a single solvent hydron is incorporated at C-3 during the reduction of 1 to both 7 and 8.

When FST C (1) was incubated with NADH and FAD under 18O2 (Fig. 3b, traces i–iii), LC-MS analysis of the reaction products revealed an increase of +2 Da in the molecular mass of the oxidized products (Supplementary Fig. 14). The δ-H/3C NMR spectra of 3, 9, 10, and 11 were isolated from a scaled up reaction (Fig. 3b, traces iii and iv). Further, the relative production of 3/9 and 10/11 versus 7/8 was O2 dependent, because incubation of FST C (1) with FAD and NADH after saturating the reaction buffer with O2 led to a significant increase in the levels of the former (3, 9–11) versus the latter (7 and 8); in contrast, saturating the reaction buffer with H2O led to 7 and 8 as the dominant products (Fig. 3b, traces iii and iv). To locate the exact position of 18O incorporation during the reaction (1 → 3/9), a scaled up reaction was performed by incubating 1, FAD, and NADH in 18O2-saturated PBS buffer (pH 7.0). Two products 3-18O ([M – H]– m/z 343.0718) and 9-18O ([M – H]– m/z 343.0722) (Fig. 3a; Supplementary Fig. 16) were isolated. NMR analysis showed that the 18O label was incorporated at C-3 of 9-18O (Supplementary Fig. 17). Specifically, comparison of the 13C NMR data for 9 and its 18O isotope 9-18O demonstrated a 3.8 Hz upfield shift of δ-C3 in the latter relative to that of 9 (Supplementary Fig. 18 and Table 4), supporting the 18O label at C-3 (i.e., 9-[18O]).
Probing parameters affecting the epoxide ring opening reactions

The pH dependence of the FAD/NADH-dependent decomposition of 1 was subsequently investigated by performing the reaction in buffers over the pH range 3–10. Following the incubation of 100 µM 1 with 100 µM FAD and 10 mM NADH for 2 h at 30 °C, 1 was more efficiently converted to the epoxide ring opened products with buffers in the pH range 5–7; however, the reaction was less efficient under more alkaline conditions at pHs 9–10 (Supplementary Fig. 19). FST B1 (7) was rather stable at pH 3–7; however, it readily converted to 8 at higher pH (Supplementary Fig. 20), where it also underwent oxidation to yield the previously reported natural product FST A (12) (Fig. 3b, trace v; Supplementary Fig. 20 and Table 1). FST B2 (8) was also stable at pH 3–6, and readily converted to 7 and 12 in more alkaline buffers (Supplementary Fig. 20). In contrast, FST C (1) was highly stable at pH 3–10, even in the presence of FAD or NADH alone (Supplementary Fig. 21) again demonstrating that decomposition of the epoxide in 1 requires the presence of both FAD and NADH.

A time course analysis of 100 µM 1 with 100 µM FAD and 10 mM NADH demonstrated the transient formation of an intermediary species 13 that vanished after longer incubation (Supplementary Fig. 22). A fixed initial concentration of 1 (100 µM) was also incubated with varying concentrations of FAD (0.1–1000 µM) and NADH (2.5 and 10 mM) for 30 min at 30 °C (Supplementary Fig. 22). The lifetime of the transient intermediate was significantly extended at decreased levels of FAD (Fig. 3b, trace vi). This allowed collection of the intermediate 13 followed by immediate injection on the HPLC demonstrating its conversion to 7 and 12 (Fig. 3b, trace vii). When the isolated intermediate 13 was immediately incubated with FAD under O2, the production of 7 and 12 was again observed (Fig. 3b, trace viii); however, if NADH was also included in the incubation, then 3 was instead observed as the major product with 12 as the minor product, and 7 was no longer observed (Fig. 3b, trace ix). The UV absorbance spectrum of the intermediate 13 was highly similar to those of 1, 3, and 7, and LC-MS analysis of the intermediate indicated an m/z 325.7 for the [M–H] ion (Supplementary Fig. 23). Based on these measurements, the intermediate is tentatively proposed to be the enol 13 (Fig. 3b), which is essentially a tautomer of 7 and 8 and thus has the same oxidation state as these two products.

While several attempts were made to isolate the intermediate in order to confirm its structural assignment as 13, only 7 was obtained as the major product along with another minor compound 14. This is consistent with the poor stability of the intermediate and its facile conversion to 7 (Fig. 3b, trace vii, Supplementary Fig. 24). Nevertheless, 14 could be characterized and was thus identified as the C-3 peroxide designated hydroperoxystafutin (Fig. 3a; Supplementary Fig. 25 and Table 5); however, poor recovery of 14 hampered collection of sufficient spectra for a complete characterization of its absolute configuration at C-3. Hydroperoxystafutin (14) was stable in water, even in the presence of FAD (Fig. 3b, traces x and xi). While the coinoculation of 14 with NADH alone led to multiple products 3, 9, 10 and 11, the inclusion of FAD with NADH did not change the product profile (Fig. 3b, traces xii and xiii; Supplementary Fig. 26). Given the observed conversion of 14 to both 3 and 9, compound 14 is likely a mixture of the (15S,23R) and (15S,23S) stereoisomers. The fact that isolation of 13 afforded a minor amount of 14 suggests that 14 is another intermediate that coelutes with 13 as opposed to being derived from 13. Furthermore, while 3/9 could be generated from both 13 and 14, 7/8 were only produced from 13, whereas 10/11 were exclusively derived from 14 (Fig. 3a).

When other flavin-related cofactors were considered, FMN and riboflavin in place of FAD were also found to efficiently mediate the decomposition of 1 in the presence of NADH (Fig. 3b, traces xiv-xvi). Isoulaqoxazine and NADH were also able to facilitate the formation of 3 from 1, albeit with a much lower efficiency (Fig. 3b, trace xvii). Only the products 3, 9–11 were observed using the cofactor FmNADH in the presence of the reducing system constructed from glucose 6-phosphate and FAD-dependent glucose 6-phosphate dehydrogenase (FGD)46–49, but notably no production of the reduced species 7 and 8 was observed (Fig. 3b, trace xviii). The observation of oxygen incorporation in the presence of F420 and NADH was unexpected, because reduced 8-deazaflavin has generally been reported to react very slowly with molecular oxygen60–62. Nevertheless, the results with flavin cofactor compatibility shown in Fig. 3b (traces xiv-xviii) were found to be reproducible (Supplementary Fig. 27). NADPH was also found to be an effective cofactor similar to NADH and thus support the epoxide ring opening reactions (Supplementary Fig. 19).

Generalization of the epoxide ring-opening reaction

To explore the generality of this reaction, an array of FST derivatives 15–21 (Fig. 4) were tested as possible oxirane reactants. Although FST F (15) is inert to Alp1U18,33, it could be efficiently converted in the presence of FAD and NADH to 1-O-methyl-FST C2 (22), 1-O-methyl-FST B1 (23), 1-O-methyl-FST C3 (24), and 1-O-methyl-FST B2 (25) (Fig. 4 and Supplementary Fig. 28), which were structurally characterized by NMR and ECD spectroscopic analysis (Supplementary Tables 7, 8 and Figs. 29–32), as well as single crystal X-ray diffraction analysis (e.g. 23, CCRF, CCDC 203641; Supplementary Fig. 29).

Similarly, 7-O-methyl-FST C (16), 6-O-methyl-FST C (17), FST D (18), FST S (19), and difluostatin H (DFST H, 20) could also undergo epoxide-ring opening reactions upon treatment with FAD/NADH to produce a set of multiple products related to 3, 7, 8, and 9 (Fig. 4, Supplementary Figs. 33–37). The major products from 16 and 17 were structurally characterized by 1D and 2D NMR as well as ECD spectroscopic analysis as FST M (26)53 and 6-O-methyl-FST B1 (27), respectively (Fig. 4, Supplementary Table 9 and Figs. 38, 39). The structures of the other products from reactions with 18–20 were deduced based on LC-MS analyses. Notably, FST Q (21), which differs from 16 in terms of a C-4 hydroxyl instead of a C-4 carbonyl, was unreactive with FAD/NADH (Supplementary Fig. 40) highlighting the essential role of the C-4 carbonyl in this chemistry.

The epoxides 28–37 (Fig. 4) were also tested as putative oxirane substrates. No activities were observed with 28–35 (Supplementary Figs. 41–46)54,55. In contrast, treatment of auxarthrol H (36)48 with FAD/ NADH led to multiple products (Supplementary Fig. 47), one of which was determined to be 38, which is a structural analog of auxarthrol F (Fig. 5, Supplementary Fig. 48 and Table 10)48, by comparison with the experimental and calculated ECD spectra of 36 and 38 (Supplementary Fig. 49). Reaction with menadione 2,3-epoxide (37) yielded three products (Supplementary Table 11 and Figs. 50–53) including 3-hydroxy-3-methyl-2,3-dihydronaphthalene-1,4-dione (39), 2-hydroxy-3-methyl-1,4-naphthoquinone (40), phthiocol, characterized by X-ray diffraction, CCDC 2036401; Supplementary Table 12, and 3-methyl-naphthalene-1,4-dione (41).

Two common structural features shared by all compounds that can react with FAD/NADH to undergo the epoxide ring opening reactions include (i) the presence of a carbonyl adjacent to the epoxide and (ii) the presence of an aromatic moiety adjacent to the carbonyl/epoxide pair (Fig. 4). The absence of a carbonyl group neighboring the epoxide ring in 21 and 28–31 may account for the observed lack of reactivity. While 36 and 37 underwent ring opening in the presence of FAD/NADH, no reaction was observed for 32–35 despite the presence of an epoxide/carbonyl pair in these compounds. This implies that conjugation of the carbonyl/epoxide pair to an aromatic system is also necessary for ring opening of the epoxide.

The commercial compound vitamin K1 2,3-epoxide (42) was also tested as a putative substrate, which features both an epoxide/carbonyl pair and an adjacent aromatic ring. However, incubation of 42 with FAD/NADH led to no changes in either its TLC retention profile or spectroscopic properties implying no reaction (Supplementary Fig. 48).
Fig. 4 | Expanded substrate and product profile for the FAD/NADH-mediated epoxide ring opening reactions and comparison with the flavoenzyme Rs05-catalyzed reactions. FST derivatives 15–20 could undergo epoxide-ring opening reactions upon treatment with FAD/NADH to produce multiple products related to 3, 7, 8, and 9. FST Q (21) was unreactive with FAD/NADH. The epoxides 28–35 displayed no activities with FAD/NADH. Treatment of auxarthrol H (36) or menadione 2,3-epoxide (37) with FAD/NADH yielded multiple products. The epoxides 42–46 were found to be unreactive with FAD/NADH. The oxidoreductase Rs05 catalyzed a reductive epoxide ring opening reaction on 43 to produce 44 during rishirilide biosynthesis and chalcone α,β-epoxide (46) was found to be a substrate of Rs05 to yield the single product 47.
Fig. 5. A recent study demonstrated that the flavoenzyme RsIO5 catalyzed a hydride-mediated reductive epoxide ring opening reaction to convert 43 to 44 during rishirilide biosynthesis (Fig. 4)\(^1\). The reaction proceeded in a manner that was highly similar to the conversion of 1 to 7. However, the production of 44 from 43 was confirmed to be strictly RsIO5-dependent, and 43 was unreactive in the presence of FMN and NADH\(^1\). Two additional commercial compounds trans-1,3-diphenyl-2,3-epoxypropan-1-one (45) and chalcone α,β-epoxide (46) were found to be unreactive with FAD/NADH; however, 46 but not 45 was accepted as a substrate by RsIO5 to yield the single product 47 (Supplementary Table 13 and Figs. 56, 57), which indicates that RsIO5 is specific for only one of the two enantiomers. Deuterium incorporation into 47 (Supplementary Fig. 58) was not observed from the RsIO5 reaction in buffered \(^2\)H\(_2\)O but was indeed observed when carrying out the RsIO5 reaction in a coupled assay either with the glucose dehydrogenase from Bacillus megaterium DSM 2894 (BmGDH) to provide (S)-[4-\(^2\)H]NADH\(^1\), or with the glucose dehydrogenase from Thermoplasma acidophilum ATCC 25905 (TaGDH) to provide (R)-[4-\(^2\)H]NADH\(^1\), respectively, employing \(D\)-[1-\(^2\)H]glucose as the deuterium donor (Supplementary Fig. 58)\(^2\). Previous studies demonstrated that the RsIO5-H172N mutant had a deficiency in FMN binding and was inactive with the native substrate 43\(^1\), suggesting the critical role of FMN in the RsIO5-catalyzed reaction. Taken together, a hydride from FMNH\(_2\) originating from NADH is proposed to be the effective nucleophile for RsIO5-catalyzed epoxide opening reactions.

In contrast, no deuterium incorporation into 7 was observed in reactions of 1 and FAD with either (S)-[4-\(^2\)H]NADH or (R)-[4-\(^2\)H]NADH generated as described above (Supplementary Figs. 58, 59). Therefore, nonenzymatic reduction of 1 to 7 in the presence of FAD/NADH differs from that of RsIO5 catalysis with respect to the ultimate origin of the C-3 hydrogen.

**Discussion**

Epoxide ring opening reactions are common and important in both biological processes and synthetic applications\(^3\). Nonenzymatic epoxide ring opening reactions are typically redox neutral proceeding through nucleophilic addition with the preferred regioselectivity and stereoselectivity contingent upon the nature of the epoxide and on the reaction conditions to generally yield products with a trans-vicinal diol\(^4\). Similar chemistry is encountered among the α/β-epoxide hydrolases that also generate trans-vicinal diol products\(^5\). For
example, the hydrolase AlpU catalyzes the epoxide hydrolysis of the
FST C (I) epoxide to afford 2 and 3, each of which exhibits a trans-
vinical diol (Fig. 1). In contrast, the flavoenzyme RsIOS was recently
reported to catalyze a reductive epoxide ring opening of rishirilide
thereby yielding a monohydroxylated product. The RsIOS chemistry
is thus distinctly different from that of the previously reported hydrolase reactions and instead represents hydrogenation of an
epoxide by reduced flavin, which is evident from the results using iso-
topically labeled substrates (Supplementary Fig. S8).

In this study, FAD/NADH is shown to induce the ring opening of
suitably activated epoxides leading to a variety of products with dif-
f erent redox states. Under anaerobic conditions epoxide ring opening
proceeds reductively in the presence of FAD/NADH via a mechanism
that may be unrelated to that of RsIOS catalysis. The resulting mono-
hydroxylated species, however, are highly susceptible to oxidation
by molecular oxygen resulting in quinones (e.g. 12) and vicinal alcohols in
reactions that can also be accelerated by the presence of FAD. Under
aerobic conditions, the epoxide ring opening appears to proceed
through the formation of an α-ketoperoxylated species which can be
reduced by NADH to again generate vicinal diols. Alternatively, the
peroxylated intermediate can undergo further rearrangement leading
to both ring contracted (e.g., the five-membered species 10) as well as
ring expanded (e.g., the seven-membered species 11) oxidized prod-
ucts.

On the basis of the accumulated experimental evidence, a puta-
tive mechanism is proposed for the FAD/NADH enabled nonenzymatic
epoxide-ring opening reactions (Fig. 5). Since both FAD and excess
NADH are required for the observed epoxide ring opening reactions,
NADH is proposed as the reductant with FAD serving as a redox cat-
ylist. When the reaction is conducted aerobically, O2 is proposed to act
as the oxidant. Given that NADH alone could not initiate the reaction,
the reduced flavin is proposed to be the species directly responsible
for the reduction of 1. It is possible that 1 is directly formed via a
hydride transfer from a reduced flavin species (e.g., N5-H of FADH2) to
C3 of 1 thereby opening the epoxide ring, which would resemble the
RsIOS catalyzed epoxide reduction reaction (Fig. 4). In this case, the
observation that the product 7 has its H-3 derived from solvent could
be explained by ready exchange of N5-H of the reduced flavin with
solvent. This would imply that 7 should be the immediate reduction
product prior to tautomerization to the observed intermediate 13.
However, a time course assay using 0.5 μM 1 with 10 μM FAD and 2 mM
NADH demonstrated that 13 accumulates first followed by conversion
1 to 7 and 8. In contrast, no significant accumulation of 13 was observed
in incubations of 7 under similar conditions (Supplementary Fig. 60).
These results imply that 13 is the initial reduction product rather than 7
or 8, and thus nonenzymatic reduction of 1 in the presence of FAD/
NADH does not proceed via direct hydride transfer to C-3 as is pro-
posed for RsIOS catalysis.

An alternative mechanism involves activation of the oxirane by
the adjacent carbonyl coupled to an aromatic ring and undergoes
reduction leading to an unstable intermediate. This intermediate is
unlikely to be 1,3,2-dimethyl-5-FST Q (48) (Fig. 4), which is the product of
a direct C-4 carbonyl reduction in 1, because the previously isolated
FST Q (21) is inert to the FAD/NADH reaction system (Supplemen-
tary Fig. 40). Instead, the intermediate during reduction of FST C (I)
is hypothesized to be an enol species such as 13, which is consistent
with the observation that 13 readily tautomerizes to yield 7 and 8.
This mechanism is also consistent with the incorporation of deuter-
um at H-3 of 7 and 8 when the reaction is carried out in 2H2O. Compound
13 is also expected to be susceptible to autoxidation to yield 3 and 9 in a manner that is sensitive to the presence of O2, FAD, and
NADH.

Formation of 13 may proceed via the addition of the nucleophilic
N-5 of the reduced flavin to 1. This model is supported by the fact that
no formation of 7 was observed using the reduced F420 cofactor, which
lacks the nucleophilic N-5 but is still able to serve as a hydride donor.
Nucleophilic addition of FADH2 to 1 may occur at either C-3 or C-4.
Given the presence of a sterically bulky CH3 group at C-3 of 1, C-4 is
proposed to be the likely site of nucleophilic attack, which is consistent
with the requirement for a carbonyl adjacent to the epoxide and thus
the different reactivity of 16 (C-4 carbonyl) versus 21 (C-4 hydroxy). Consid-
ering that N-5 in FADH2 possesses one proton and C-4 in 1 is a
trigonal carbonyl carbon, the redox reaction is proposed to happen via
an n→π* interaction. Specifically, the nucleophilic N-5 donates the lone-
pair (n) electron density into the trigonal carbene center (empty π*
orbital) along the Buri–Dunitz trajectory. Mixing of these orbitals is
thermodynamically favorable resulting in a covalent adduct 49
(Fig. 5). Subsequent N-1 deprotonation of the flavin moiety in 49 leads
to the breakage of the N-C4 linkage and release of the oxidized flavin
thereby completing the reduction of 1 to the enol intermediate 13 with
a ∆4 double bond (Fig. 5).

Intermediate 13 is highly unstable and undergoes facile tauto-
ermerization to incorporate a solvent hydron at the α-carbon (C-3) either
from the si face (the major route) or the re face (the minor route) of the
sp2 planar structure, to yield 7 or 8, respectively. This mechanism is
consistent with the site-specific incorporation of deuterium at C-3 in 7
and 8 from buffered 2H2O (Fig. 3a). Despite the 2,3-cis configuration of
7 compared to 8, the former appears to be the thermodynamically
favored tautomer with an equilibrium constant of approximately 7[7]/8 =
5.4 (Supplementary Fig. 61). Moreover, 7, 8 and the putative enol interme-
tiate 13 are also susceptible to autoxidation to yield 12 (Supplementary Fig. 62).

The putative enol intermediate 13 also appears to be sensitive to
molecular oxygen and can undergo autoxidation in the presence of
FAD and NADH. Reduced flavin is known to react with O2 to form the
covalent FADH2 adduct (FAD + FADOOH), and in some cases, H2O2
may be released from FADOOH to serve as the oxidant. However, nei-
ther 1 nor 13 can react with H2O2 (Supplementary Figs. 24, 63). Thus,
the intermediate 13 is proposed to react with the electrophilic C(4a)-
hydroperoxyl group of FADOOH to afford the C-3 monoxygenated
products (see Supplementary Fig. 64). Hydroxylation can occur either
from the si face (major route) or the re face (minor route) of the planar
double bond (∆4) to produce 3 or 9, respectively, with concomitant
release of the oxidized flavin (Fig. 5). The proposed mechanism is
supported by the observations that (i) the O incorporated into both 3
and 9 at C-3 originates in O2, and (ii) the conversion of 13 to 3 and 9
requires the presence of FAD, NADH and O2.

Isolation of intermediate 14 indicates that 3 and 9 can also be produced in an alternative way that does not involve 13. In the pre-
ence of molecular oxygen, the flavin (C4a)-peroxide (FADOH) may
serve as a nucleophile to regioselectively attack C-3 of 1 at either the si
face (major route) or the re face (minor route) to open the epoxide
ring, resulting in the putative adduct 50 (Fig. 3). The presence of the
C-4 carbonyl oxygen with electron-withdrawing effect may cause
changes of the electron density of C-3, to make C-3 a better site for
nucleophile substitutions than C-2 of the epoxide in 1. Deproton-
ation of the flavin N-5 in 50 breaks the C4=O–Covalt bond to release
14 and FAD (Fig. 5). The product 14 is a mixture of C-3 isomers and easily
reduced to 3 and 9 by NADH (Fig. 5, Supplementary Fig. 65). Coin-
cubation of 14 with NADH also leads to 10 and 11, which is proposed
to involve a Baeyer-Villiger reaction-like mechanism. The C-3 hydroper-
oxide in 14 may thus perform an intramolecular nucleophilic addition
at the C-4 carbonyl to form the putative intermediate 51, which can
carrier to generate 10 and 11. In the former case, intermediate 51
may undergo a Grieg rearrangement to yield a seven-membered lac-
tone ring, followed by an intramolecular backbiting addition of the
C-1 hydroxy at the C-4 carbonyl that leads to 10 after dehydration
(Fig. 5). Alternatively, the 1,2-dioxetane unit of 51 may undergo het-
erolytic cleavage, followed by Grob-like fragmentation to yield an
oxoheptanoate intermediate, which eventually generates the
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hemiacetel 11 after successive intramolecular cyclizations and dehydration.

In conclusion, the reductive epoxide ring opening is suggested to proceed mechanistically via a transient adduct (49) with an N-5-C-4' covalent bond that is formed between N-5 of the flavin cofactor and C-4' of the substrate FST C (Fig. 5). While tautomeration of the resulting product can yield 7 and 8, nucleophilic reaction with FADOOH can also afford the dial products 3 and 9. Oxidative epoxide ring opening of 1 produces the intermediate 14 via a putative C-4'-O-C-4' covalent adduct (50, Fig. 5). The canonical reactivity of flavin cofactors involves redox chemistry through N-5 hydride transfer or oxygen activation. Consequently, the nonenzymatic iodination of diverse aromatic compounds has been reported to catalyze the formation of covalent flavin-N-5-substrate adducts in a redox-neutral manner, with examples including the iminium-adducts in the catalytic cycle of UDP-galactopyranose mutase, flavin prenyltransferase, thymidylate synthase, and 2-haloacrylate hydratase.

The present study provides an example likely involving flavin-N-5 adduct formation during flavin-catalyzed nonenzymatic reactions (Fig. 5). Furthermore, in addition to serving as a nucleophile in mediating hydroxylation, epoxidation and Baeyer-Villiger oxidation in flavoenzyme catalysis, the flavin C(4a)-peroxide (FADOOH) is proposed to also facilitate the formation of peroxylated intermediates such as 14 (Fig. 5). Flavin and flavin derivatives are well known to participate as cofactors in a wide variety of reactions found in nature. Previous reports have shown that natural flavins can catalyze nonenzymatic oxidative decarboxylation of picolinic acid derivatives and the nonenzymatic ligation of diverse aromatic compounds. In this study, the chemistry of flavins is expanded to epoxide ring opening reactions in both a reductive and an oxidative manner to generate multiple ring-opened products. Consequently, the flavin chemistry identified in this study may be applicable to other epoxides and thus holds promise as a useful tool in organic synthesis.

Methods

Bacterial strains, compounds, and reagents

The strain Micromonospora rosaria SC50 was used for the isolation of FSTs. FST Q (21) was previously isolated from Streptomyces sp. PKU-MAO0045. Chemicals, enzymes, and other molecular biological reagents were purchased from standard commercial sources and used according to the recommendations of the manufacturer.

General analytical HPLC methods

HPLC analysis of reactions was generally carried out on an Agilent 1260 Infinity series instrument (Agilent Technologies Inc., USA) using a reversed phase C18 column (Kinetex® 5 μm C18 100 Å, LC Column 150 × 4.6 mm, Phenomenex, USA) or a polar column (Comixsep M, Phenomenex, USA). The chromatograms were obtained using a diode array detector at 254 nm. Chromatographic analysis was performed at a flow rate of 1 mL min⁻¹.

Expression, purification of recombinant enzymes, and general in vitro enzyme assays

The DNA fragment of RsOS (GenBank: KJ437438) was synthesized and cloned into pET28a to afford the plasmid pCSG8112 (Supplementary Table S14). When the cultures of E. coli BL21(DE3)/pCSG8112 in LB media containing kanamycin (50 μg mL⁻¹) were grown to an OD₆₀₀ of about 0.6 at 37 °C, the production of RsOS was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cultures were grown at 16 °C for an additional 12 h. The cells were then collected by centrifugation and were resuspended in the lysis buffer (20 mM Tris–Cl, 500 mM NaCl, and 5 mM imidazole, pH 8.0) for sonication. Purification of N-His-tagged RsOS was conducted using Ni-NTA affinity chromatography according to the manufacturer’s manual (Novagen, USA). After desalting with 10% glycerol, 1 mM EDTA, 50 mM NaCl, pH 8.0, at ~8 °C for further use. The expression and purification of FST, AlplU, FlsH, Xik, TiaM, and FlsOZ were performed similar as described for RsOS. The standard in vitro enzyme assays (AlplU, FlsH, Xik, TiaM, FlsOZ) contained 100 μM FST C (I) and 10 mM of purified recombinant enzyme in 50 mM PBS (10 mM NaH₂PO₄/Na₂HPO₄, pH 7.0) buffer (pH 7.0) in a total volume of 10 μL with incubation at 30 °C for 1 h. In general, the reactions were terminated by adding 10 μL of ice-cold MeOH and analyzed by HPLC using the general analytical HPLC method or subjected to LC-MS analysis.

Nonenzymatic reactions with flavins

A typical reaction mixture included 100 μM L, 100 μM FAD and 10 mM NADH in 50 mM PBS buffer (pH 7.0) in a total volume of 10 μL with incubation at 30 °C for 0.5–2 h. General analytical HPLC method or subjected to LC-MS analysis. For reactions under anaerobic conditions, the reaction buffers were flushed in N₂ for 5 min before adding L, cap-sealed using Parafilm and flushed with N₂ during incubation at 30 °C for 30 min. For reactions under aerobic conditions, the reaction buffers were flushed in O₂ for 5 min before adding L, cap-sealed using Parafilm and flushed with O₂ during incubation at 30 °C for 30 min. For isotope labeling reactions with ¹⁸O₂, each reaction was carried out in 50 mM PBS buffer (pH 7.0) prepared with deuterated water (D₂O). For isotope labeling reactions with ¹³C₂O₂, a reaction mixture containing 100 μM FAD and 10 mM NADH in 50 mM PBS buffer (pH 7.0) was degassed and flushed with ¹³CO₂ for 5 min; after the addition of 100 μM L, ¹³CO₂ gas was continuously bubbled into the reaction mixture for 2 h at room temperature with occasional shaking.

For determining the pH effects on the reactions, the assays were performed in a total volume of 100 μL containing 100 μM L, 100 mM FAD, and 10 mM NADH in 50 mM buffers with pH values ranging from 2 to 10 with incubation at 30 °C for 2 h. The buffers were prepared as follows: citric acid/Na₂HPO₄ buffer (pH 3–6); PBS buffer (pH 7); boric acid/borax buffer (pH 8–9); borax/NaOH buffer (pH 10). As controls, 100 μM L was incubated in different pH buffers (pH 3–10) in the presence of 100 μM FAD alone (10 mM NADH alone) at 30 °C for 2 h.

For time course assay, reactions were performed by incubating 100 μM L, 100 μM FAD, and 10 mM NADH in 50 mM PBS buffer (pH 7.0) at 30 °C with sampling at different time points of 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 25, and 30 min. For assaying the effects of different FAD/NADH concentrations, reactions were carried out with 100 μM L in 50 mM PBS buffer (pH 7.0) with varying concentrations of FAD and NADH. The first set of reactions were conducted with 100 μM L, 1 mM FAD, and 2 (or 5 or 10 mM) NADH. The second set of reactions were performed with 100 μM L, 10 mM FAD, and 2 (or 5 or 10) mM NADH. The third set of reactions were carried out with 100 μM L, 100 μM FAD, and 2 (or 5 or 10) mM NADH. The reaction mixtures were incubated in 50 mM PBS buffer (pH 7.0) at 30 °C for 30 min.

To test cofactor compatibility, assays containing 100 μM L, 100 μM FAD (or FMN/riboflavin/isoalloxazine) and 10 mM NADH in 50 mM PBS buffer (pH 7.0) in a total volume of 100 μL were incubated at 30 °C for 30 min. For testing F₄₂₀ as a cofactor, reactions containing 100 μM L, 200 μM F₄₂₀, 2.5 mM glucose-6-phosphate, 10 μM F₄₂₀-dependent glucose-6-phosphate dehydrogenase (FGD) in 50 mM Tris–HCl buffer (pH 7.5) in a total volume of 100 μL were incubated at 30 °C for 1 h.
The FAD/NADH-mediated epoxide ring opening reactions were tested with various substrates including both FST-related substrates 15–21 and FST-nonrelated substrates 28–37, 42, 45, and 46. A typical reaction mixture contains 100 μM substrate (15–21, 28–37, 42, 45, or 46), 100 μM FAD and 10 mM NADH in 50 mM PBS buffer (pH 7.0) in a total volume of 100 μL.

To test the reactivity of H2O2 with FST C (1), the assays were performed in a total volume of 100 μL containing 100 μM 1 and 30% H2O2 in 50 mM buffers at pH 3.0 (or pH 7.0 or pH 10.0) by incubation at 30 °C for 2 h.

Preparation and isolation of 3, 7–11, 22–27 and 38–41
A scaled up reaction of 1 in a total volume of 1 L was performed in 50 mM PBS buffer (pH 7.0) containing 100 μM 1, 10 mM NADH and 100 μM FAD at 30 °C for 2 h with occasional shaking. The reaction was quenched by the addition of an equal volume of ice-cold butanone and centrifuged at 2057 × g for 20 min at 4 °C. The reaction mixtures were then extracted with an equal volume of butanone three times and the solvents were removed under vacuum on an ice bath. The crude extracts were dissolved in 1.5 mL MeOH and subjected to preparative HPLC using an Agilent Eclipse XDB-C18 column (250 mm × 9.4 mm, 5 μm; Agilent technology Ltd., USA) with an isocratic elution gradient of 70% A (H2O with 0.8% formic acid) and 30% B (MeCN) at a flow rate of 2.5 mL min⁻¹.

In vitro RslO5 assays, isolation of 47 and deuterium incorporation studies
A standard RslO5 in vitro reaction mixture contained 100 μM trans-1,3-diphenyl-2,3-epoxypropan-1-one (45) or chalcone α,β-epoxide (46), 10 μM RslO5 and 5 mM NADH in 50 mM PBS buffer (pH 7.0) in a total volume of 100 μL and incubated at 30 °C for 2 h. Control reactions were run in parallel by omitting FAD or NADH in the assays. Co-incubation of 100 μM 14 in 30% H2O2 were also performed in a total volume of 100 μL. The reaction mixtures were incubated at 30 °C for 30 min. The reactions were quenched after adding 100 μL of ice-cold MeOH and were analyzed by HPLC using a polar column following the general analytical HPLC method.

Quantum chemistry calculations
Mixed torsional/low-mode conformational searches were carried out by means of the Macromodel 10.8.011 software using the MMFF with an implicit solvent model for CHCl3 applying a 21 kJ/mol energy threshold. Geometry re-optimizations of the resultant conformers were carried out at the ωB97X/TZVP level with the PCM solvent model for MeCN. TDDFT ECD calculations were performed with Gaussian 09 for ECD using various functionals (B3LYP, BH&HLYP, CAM-B3LYP, PBE0) and the TZVP basis set with the same solvent model as in the preceding DFT optimization step. ECD spectra were generated as the sum of Gaussians with 3000 and 3600 cm⁻¹ half-height widths, using...
dipole-velocity-computed rotational strength values\textsuperscript{79}. Boltzmann distributions were estimated from the oB97X energies. The MOLEKEL software package was used for visualization of the results\textsuperscript{80}.

**X-ray crystallographic analysis**
A single crystal of 7, 10, and 23 was obtained in a mixed solvent of MeOH and water, and single crystals of 40 were obtained in a mixed solvent of CH\textsubscript{3}CN and H\textsubscript{2}O. The suitable crystals were selected, and the crystal data were recorded on an XtaLAB AFC12 (RINC): Kappa single diffractometer, with Cu K\textalpha{} radiation (\(\lambda = 1.54184 \pm\)) Å). The crystals were kept at 99.9(7) K for 7, 99.8(9) K for 23, 100.00(10) K for 10 and 100.01(12) K for 40 for during data collection. Using Olex\textsuperscript{2}\textsuperscript{81}, the structure was solved with the ShelXT\textsuperscript{12} structure solution program using intrinsic phasing and refined with the ShelXL refinement package using least squares minimization.

**Data availability**
The data and code in this manuscript have been appropriately deposited into a public data repository or provided in the supplementary information for public availability. Crystallographic data can be obtained free of charge for 7 (CCDC 2036399), 10 (CCDC 2129081), 23 (CCDC 2036400), and 40 (CCDC 2036401) via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033. The GenBank accession number for DNA fragment of rs\textsubscript{605} is KJ437438.1 (https://www.ncbi.nlm.nih.gov/nuccore/KJ437438.1).

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Author contributions

B.C.D., W.Z., C.Y., H.C., L.P., and W.L. performed epoxide opening reactions, compound isolation, and structure determination. A.M. and T.K. conducted the ECD calculations. M.M. and G.B. contributed key compound and reagents. B.C.D., W.Z., M.W.R., Y.Z., T.K., H.w.L., and C.Z. analyzed the data and wrote the manuscript. C.Z. and H.w.L. directed the research. B.C.D. and W.Z. contributed equally to this work.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Hung-wen Liu or Changsheng Zhang.

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