Letter

Transformation of streptonigrin to streptonigrone: flavin reductase mediated flavin-catalyzed concomitant oxidative decarboxylation of picolinic acid derivatives

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Transformation of streptonigrin to streptonigrone: flavin reductase mediated flavin-catalyzed concomitant oxidative decarboxylation of picolinic acid derivatives

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ABSTRACT: In the flavin reductase-catalyzed reducing condition, a mild and efficient α-hydroxylation and decarboxylation procedure using natural flavins as a catalyst and atmospheric oxygen as an external oxidizing agent has been successfully developed and applied to the synthesis of streptonigrone from streptonigrin. This reaction was not only achieved with streptonigrin analogues, but also structurally diverse electron-rich picolinic acid derivatives. The hydroxylation and decarboxylation may take place in a concerted manner.

Streptonigrin (**1**), streptonigrone (**2**), and lavendamycin (**3**), a group of highly functionalized alkaloids, constitute a natural product family called "streptonigrinoids" with other related congeners (Figure 1).[1] Streptonigrinoids have drawn considerable scientific attention due to their diverse and remarkable bioactivities.[2] For instance, both **1** and **3** are known as antibacterial and antifungal agents,[3] while **2** inhibits the NO-dependent activation of human guanylyl cyclase despite the lack of antimicrobial activity.[4]

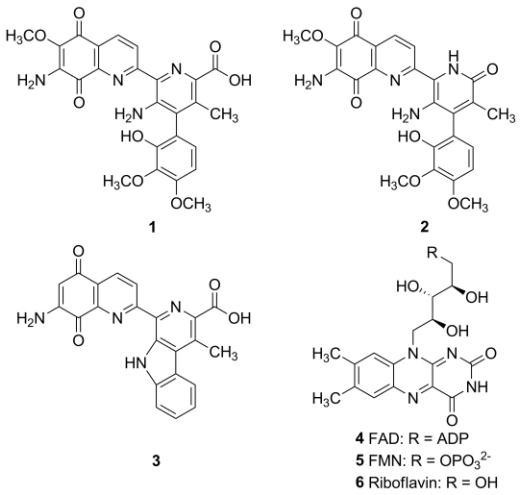
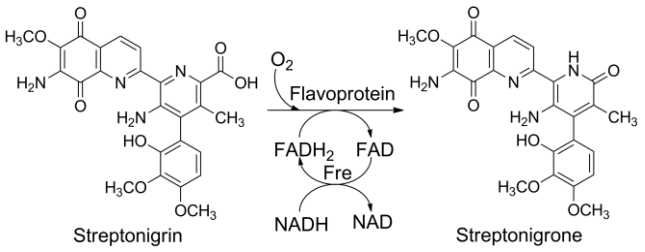


Figure 1. Chemical structures of streptonigrin (**1**), streptonigrone (**2**), lavendamycin (**3**), FAD (**4**), FMN (**5**), and riboflavin (**6**).

Structurally, both **1** and **3** contain a pyridine-2-carboxylic acid (picolinic acid) moiety while **2** comprises a 2-pyridone moiety instead. Genetic and biochemical studies have demonstrated that **3** is a biosynthetic intermediate of **1**.[5] However, the relationship between **1** and **2** remains obscure due to the lack of full understanding of their biosynthesis. **2** has been discovered as a minor component in streptonigrin-producing strains and prepared conventionally by chemical synthesis with a low yield.[1a,6] Based on a comparison of their structures, it has been proposed that **2** arises biosynthetically by decarboxylation and hydroxylation of **1**. This reaction is most likely catalyzed by a flavoenzyme similar to two-component flavin-dependent monooxygenases such as salicylate-1-monooxygenase or 6-hydroxynicotinate-3-monooxygenase (Scheme 1).[7]



Scheme 1. Proposed conversion of streptonigrin to streptonigrone catalyzed by a flavoprotein.

To find suitable conditions for the conversion of **1** to **2**, a range of flavoenzymes (including StnD, $StnH_2$ and $StnH_3$ Figure S1) from the biosynthetic gene cluster of **1** was evaluated. Based on the reactions catalyzed by two-component flavin-dependent monooxygenases, the test reaction mixtures included **1**, flavin adenine dinucleotide (**4**, FAD), nicotinamide adenine dinucleotide in reduced form (NADH), and the *Escherichia coli* flavin reductase Fre in phosphate buffer (Supporting Information). Reactions were initiated by addition of flavin reductase Fre which catalyzes the reduction of FAD to supply $FADH_2$ for the activation of molecular oxygen using NADH as a hydride donor.[8] Surprisingly, **1** could be converted to **2** without any other flavoprotein, but flavin reductase Fre, FAD, and NADH are required (Figure S2). The identity of **2** was confirmed by NMR analysis and high-resolution mass spectrometry (HR-MS, Figures S3 and S4). The molecular

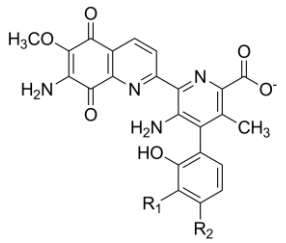
weight of **2** increased by two mass units when reactions were carried out in ^{18}O-labeled oxygen ($^{18}O_2$) instead of atmospheric oxygen ($^{16}O_2$), demonstrating that the keto group of **2** originates from molecular oxygen (Figure S4). In recent decades, catalysis of diverse reactions by flavins or flavin mimics had been reported, in particular the oxidation of amines and sulfur groups,[9] Baeyer-Villiger reactions,[10] the aromatization of dihydro-pyridines and benzothiazolines,[11] the hydrogenation of olefins[12] and the oxidation of aryl aldehydes.[13] To the best of our knowledge, no precedence of a concurrent hydroxylation/ decarboxylation catalyzed by a natural flavin utilizing atmospheric oxygen as an oxidant has been reported.[14]

To explore the potential of natural flavins to catalyze the combined hydroxylation/decarboxylation reaction, **1** was utilized as a standard substrate to optimize the reaction conditions. First of all, the effects of the catalyst concentration and reaction time on the reaction efficiency were tested. All reactions were carried out with 100 μM **1** and 5 mM NADH as the hydride donor for the reduction of FAD to generate $FADH_2$ at 25°C for 5 min. The reactions were monitored by HPLC. Moderate conversion of 30% was observed with 1 μM FAD and the conversion could be increased to 60% with 10 μM FAD, while higher FAD concentrations (100 μM and 1 mM) would make reactions reach a maximum conversion of about 75% (Figure S5). However, by allowing a prolonged reaction time of 10 min, **1** could be almost completely converted to **2** with 10 μM FAD as the catalyst (Figure 2). Subsequently, the effect of the pH of the reaction buffer was examined and the highest conversions were found in a pH range of 7.0-9.0. Lowering the pH significantly impaired the reaction system and hardly any conversion of **1** to **2** was detected at pH values below 6.0. On the other hand, decomposition of **1** was observed at pH values above 10.0 (Figure S6). Finally, the impact of the reaction temperature on the reaction was evaluated. When the flavin reductase Fre was used to generate $FADH_2$, almost complete conversion of **1** to **2** was observed at temperatures lower than 50°C, while no conversion occurred at temperatures higher than 60°C probably due to inactivation of the flavin reductase which terminated the supply of $FADH_2$ (Figure S7). Furthermore, decomposition of **2** started at temperatures higher than 40°C (Figure S7). Consequently, a standard reaction condition was defined for all follow-up reactions using 10 μM FAD as a catalyst in a phosphate buffer at pH 7.5 for 10 min at room temperature. Under this standard condition, the time-dependent reactions were performed. The increase of **2** nearly perfectly matched the decrease of **1**, and also matched the trend of the NADH consumption. However, about 80% NADH was converted to NAD within 2 min, while only about 50% **2** was formed at the same period of time (Figure S8). These observations suggested that the most of the generated $FADH_2$ from NADH catalyzed by Fre was not used to perform the oxidation of **1**.

The requirement for the flavin reductase Fre in this reaction seems to be a limitation for potential applications in organic synthesis. Therefore, alternative reducing agents were tested as potential substitutes of Fre in the reduction of FAD to its reduced form $FADH_2$. It has been reported previously that the flavin reductase can be replaced by dithiothreitol (DTT) for reactions catalyzed by two-component flavin-dependent monooxygenases.[15] Similar replacement of the flavin reductase with DTT for the coupled hydroxylation/ decarboxylation was possible, but the reaction efficiency was about two-fold lower than under the standard conditions (Figure S9). Furthermore, reaction attempts using sodium sulfide Na_2S (1 and 10 mM) and sodium dithionite $Na_2S_2O_4$ (1 and 10 mM) as reducing agents could only produce trace amounts of **2** (Figure S9).

Alternative oxidants like hydrogen peroxide or peroxy acids are known for some flavin-catalyzed oxidations such as the Baeyer-Villiger reaction and Dakin reaction.[16] Therefore, hydrogen peroxide, *m*-choroperoxybenzoic acid, and *tert*-butyl hydroperoxide were tested as potential oxidants. Strikingly, none of them could be utilized as an oxidant to convert **1** to **2** both with and without FAD (Figure S10). Furthermore, the natural flavins such as flavin mononucleotide (**5**, FMN) and riboflavin (**6**) were tested and found to be capable of catalyzing the conversion of **1** to **2** with similar efficiency as FAD (Figure S11). All of these natural flavins have a reduced form that is capable of activating atmospheric oxygen as the actual oxidizing agent. In summary, these results indicated that the flavin is essential for the catalytic hydroxylation/ decarboxylation of streptonigrin (**1**) and the actual oxidizing agent is $FADH_2$-activated molecular oxygen. This resembles other flavin-catalyzed oxidations.[14]

In the next step, the substrate scope of this natural flavin-catalyzed reaction was further explored. Similar to streptonigrin, all streptonigrin analogues could be converted to their corresponding streptonigrone analogues with similar conversion rates (Figure 2 and Figures S12-16). Interestingly, this reaction also worked for lavendamycin (**3**) where the carboxylic acid is attached to a tricyclic pyrido[3,4-*b*]indole moiety (Figures 1 and S16), albeit with lower efficiency (53% conversion). The reaction product of **1b** was characterized by ^1H NMR spectrum (Figure S17) and HR-MS, but other reaction products were confirmed by HR-MS due to the limited amounts available of the substrates.



Substrate	Conv.[a]
1a, $R_1 = R_2 = OCH_3$,	96%
1b, $R_1 = OCH_3$, $R_2 = H$,	96%
1c, $R_1 = OCH_3$, $R_2 = OH$,	91%
1d, $R_1 = OH$, $R_2 = H$,	80%

Figure 2. FAD-catalyzed hydroxylation/decarboxylation of streptonigrin analogues under standard reaction conditions. [a]Conversion rates were determined by HPLC based on the ratio of the peak area of the substrate after reaction to that of a control.

Overall, the observed substrate scope implicated that the natural flavin-catalyzed hydroxylation/decarboxylation may be applicable to a wider range of picolinic acid substrates. Therefore, several commercially available picolinic acids were tested as putative substrates (Figure 3). The conversion was monitored by HPLC via comparison with authentic standards (Figure S18-23). Firstly, amino-substituted picolinic acids were tested that bear structural similarity to the multi-substituted pyridine-2-carboxylic acid moiety of streptonigrin.

Complete conversion was found for 3-aminopicolinic acid (**7**) and 84% conversion for 5-aminopicolinic acid (**9**), but no conversion was detected for 4- and 6-aminosubstituted picolinic acids **8** and **10** (Figure 3). Subsequently, various hydroxy-substituted picolinic acids were tested in the same manner. The highest conversion rate (58%) was found for 3-hydroxypicolinic acid (**11**), while no reaction product could be detected for 4- and 6-hydroxypicolinic acids **12** and **13**. These findings are in line with the reactivity patterns of electrophilic aromatic substitutions. Electron donating groups activate the ortho- or para-positions and consequently, disfavor the reaction in the meta-positions. Inspired by these results, the corresponding benzoic acids bearing hydroxyl or amino groups at the ortho position (**14** and **15**) and both ortho- and para-positions (**16**) were tested. Surprisingly, none of the expected products could be detected even though electrophilic aromatic substitution at the benzene ring should be favored due to its higher electron density. In order to examine whether the electron donating groups at the picolinic acid are necessary at all, picolinic acid (**17**) and pyridine-2,3-dicarboxylic acid (**18**) with a weak electron withdrawing ortho-substituent were tested, but no conversion could be detected by HPLC analysis, consistent with electrophilic aromatic substitutions. Moreover, no conversion was found for pyridin-3-amine (**19**) suggesting that the reaction requires the decarboxylation of the ipso-position as its driving force.

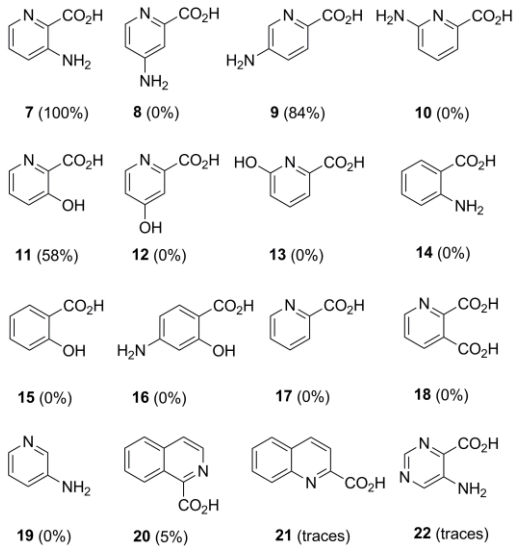
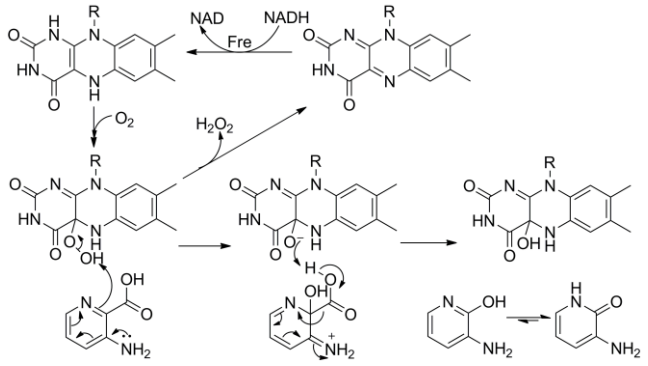


Figure 3. FAD-catalyzed hydroxylation/decarboxylation of other picolinic acid derivatives under standard reaction conditions. Conversion rates were determined by HPLC based on the ratio of the peak area of the substrate after reaction to that of a control.

Furthermore, other picolinic acid derivatives were tested: 5% conversion was observed for isoquinoline-1-carboxylic acid (**20**), and trace amounts of the hydroxylation/ decarboxylation products were detected when quinoline-2-carboxylic acid (**21**) and 5-aminopyrimidine-4-carboxylic acid (**22**) were used (Figure 3 and Figure S21-23). Although conversions are very low for **20** and **21**, it can be expected that this reaction might be successfully applied to structurally similar substrates that are activated by electron-donating groups.

Based on the formed products, reaction condition, and the tested substrates, we proposed that the reaction most likely follows the general addition-elimination mechanism of electrophilic aromatic substitutions (Scheme 2), because the involvement of radicals and superoxides can be ruled out by adding commonly used radical scavengers including hydroxylamine and 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) to the reaction mixture (Figure S24) and using hypoxanthine/xanthine oxidase and potassium superoxide to generate superoxides, instead of flavin, or adding superoxide scavenger SOD to the standard reaction mixture (Figure S25). The initial driving force of the oxidative decarboxylation process is anticipated to be the electron-donating properties of the $-NH_2$ group and the poor aromaticity of the pyridine moiety. In the first step, the attack at the flavin peroxide gives the resonance-stabilized arenium ion (σ complex), a dearomatized intermediate, as shown in Scheme 2. Subsequently, the resultant intermediate is deprotonated by flavin oxide and further undergoes a β-keto acid type decarboxylation under release of the 2-hydroxy-substituted pyridine which can tautomerize to the corresponding 2-pyridone. Re-introducing aromaticity may serve as an additional driving force for the decarboxylation. The arenium intermediate might be stabilized by interaction with the flavin C-4 keto group. This would give another possible explanation for the failure of the reaction with the benzoic acid derivatives where the stabilization of the σ complex might be inefficient due to the increased steric bulk of the additional hydrogen atom at the benzene ring. Indeed, the flavin peroxide is ready to decompose to release hydroperoxide and recycle flavin,[17] which is in line with our observation that only small portion of the consumed NADH was used to generate the product **2** (Figure S8).



Scheme 2. Possible mechanism of the electrophilic aromatic ipso-substitution with concomitant decarboxylation.

Among literature-known flavin-catalyzed reactions, our discovery of an electrophilic aromatic hydroxylation with concomitant *ipso*-decarboxylation of electron-rich picolinic acid derivatives catalyzed by natural flavins is the first example, compared to the two-step procedure for the transformation of picolinic acid into the corresponding 2-hydroxypyridines/2-pyridones via N-oxides catalyzed by lower aliphatic anhydrides and triethylamine.[18] The reaction developed here enables the scalable preparation of streptonigrone (**2**) from streptonigrin (**1**) that is available by fermentation, although it was carried out on a 30-mg scale (22 mg product purified yield, Supporting Information). Moreover, the substituted 2-pyridone moiety of the reaction products, has been used as a versatile core structure for drug design. For

instances, the 3-aminopyridin-2-one motif has the potential ability to form multiple hydrogen bonds or to act as a chelating ligand. Bioactive compounds including this important building block include a thrombin inhibitor,[19] an interleukin-2 inducible T-cell kinase inhibitor[20] and the human immunodeficiency virus type 1 reverse transcriptase inhibitor.[21] Furthermore, a series of substituted 3-hydroxy pridin-2(1H)-ones, 5-hydroxy-pyrimidin -4(3H)-ones and 3-hydroxyquinolin-2(1H)-ones are effective inhibitors of influenza endonuclease by acting as bimetal chelating ligands at the active site of the enzyme.[22] In addition, a number of functionalized 5-aminopyrimidin-4(3H)-ones are orally active inhibitors of human neutrophil elastase.[23]

In conclusion, an efficient and environmentally friendly electrophilic aromatic hydroxylation of picolinic acids with *ipso*-decarboxylation has been developed and applied to the conversion of streptonigrin to streptonigrone. The reaction utilizes atmospheric oxygen as the terminal oxidant which is activated by the reduced form of natural flavins in an aqueous solution. The reaction not only works for streptonigrin analogues, but can also be applied to other activated picolinic acid derivatives with electron donating groups to produce useful building blocks for bioactive compounds.

ASSOCIATED CONTENT

Supporting Information. Reaction procedures and analytical data are available free of charge via the Internet at http://pubs.acs.org..

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Notes

The authors declare no competing financial interest.

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