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Tandem Hydration of Diisonitriles Triggered by Isonitrile Hydratase in *Streptomyces thioluteus*

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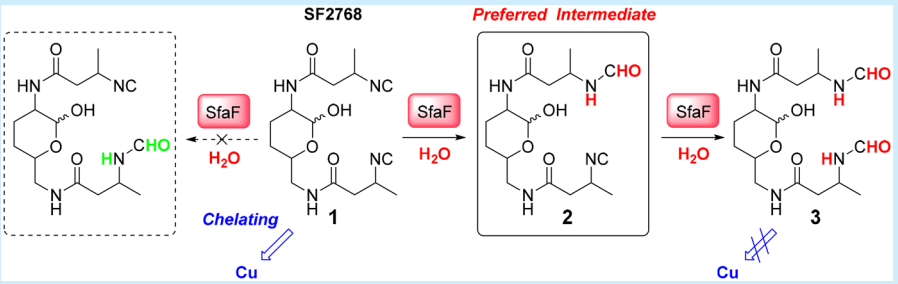
Supporting Information



ABSTRACT: The biosynthetic pathway of diisonitrile chalkophore SF2768 was identified in *Streptomyces thioluteus* through heterologous expression recently. Isolation and structure elucidation of the N-substituted formamides that coexisted with the diisonitriles implied that a hydration event was involved. *In vitro* enzymatic assays of an endogenous isonitrile hydratase suggested a rare sequential-hydration of the diisonitriles. Additionally, the results of Cu-CAS assays indicate that both partial and complete hydration led to the loss of the copper-chelating ability of SF2768.

Isonitrile natural products, a class of generally toxic and pungent substances, are usually discovered along with the corresponding N-substituted formamides.[1] Researchers once speculated that formamide was the biogenetic precursor of isonitrile when they initially observed the co-occurrence of an isonitrile−formamide pair in marine sponge *Halichondria*.[2] Afterward, the results of a series of preliminary assays implied that formamide, on the contrary, was biosynthesized from isonitrile.[3]

In 2001, Kobayashi and his colleagues discovered a novel isonitrile hydratase InhA (Q8G9F9.1) that hydrated isonitrile to the corresponding formamide from *Pseudomonas putida*,[4] offering the first enzymatic evidence for the hypothesis that formamide could be derived from isonitrile through biotransformation. Kobayashi also predicted that isonitrile hydratase could serve as a probe to clone the isonitrile biosynthetic gene since they are expected to be closely located in the genome in isonitrile-producing organisms.[4] Moreover, a highly conserved cysteine residue (Cys^{101}) in InhA family isonitrile hydratases was verified to play an essential role for catalysis.[5] In 2010, another isonitrile hydratase InhB (BAJ17399.1), which had no sequential similarity with InhA, was found to catalyze both isonitrile hydration and nitrile hydrolysis in *Arthrobacter pascens* by Kobayashi's group.[6] These investigations brought new insights into microbial isonitrile assimilation and detoxification.

Diisonitrile chalkophore SF2768 (compound **1**, Figure 1) was originally isolated from *Streptomyces* spp., and its structure was elucidated two decades ago.[7] Structurally, SF2768 possesses a pyran ring connected to two isonitrile moieties with amide bonds. One very intriguing and function-related, but to date rarely encountered structural feature of SF2768 is the diisonitrile functional group, which was observed only in a handful of bioactive natural products, including xanthocillin,[8] hazimycins,[9] and kalihinols.[10] In our previous study, the biosynthetic gene cluster (BGC) of SF2768 (*sfa*, genbank KY427327, Figure 2) was identified in *Streptomyces thioluteus* DSM40027, and the biosynthetic pathway was proposed based on metabolic profiles of the heterologous expression strains.[11] Herein, the formamides coproduced with diisonitriles were isolated and identified, leading to the discovery of a rare

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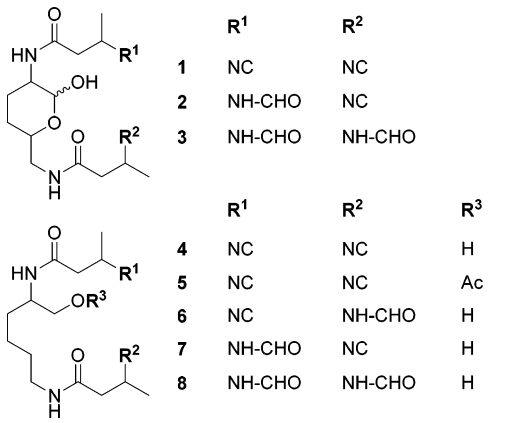

Figure 1. Structures of SF2768 (**1**) and its congeners.

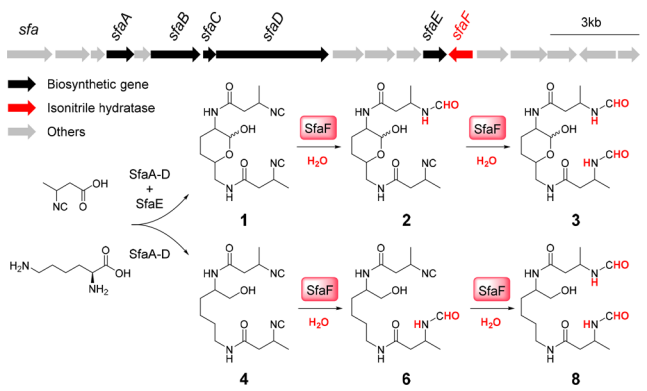

Figure 2. Isonitrile hydratase SfaF adjacent to the biosynthetic gene cluster *sfa* in *S. thioluteus* catalyzes the isonitrile groups of SF2768 (**1**) and its analog **4** to the corresponding N-substituted formamides.

isonitrile hydratase SfaF (previously Orf23, Genbank ATY72534) that catalyzed a sequential hydration.

Two heterologous expression strains, *Streptomyces lividans*::p13C and Δ*sfaE*, were constructed in the previous study, and their scaled-up fermentation was performed as described.[11] LC-MS metabolite analysis revealed that the peaks with $[M + H]^+$ at *m/z* 355 and *m/z* 341 were observed along with those of **1** (*m/z* 337) and **4** (*m/z* 323) in the fermentation broth of *S. lividans*::p13C and Δ*sfaE*, respectively, suggesting that a possible hydration event of **1** and **4** was concomitant with the diisonitriles biosynthesis (Figure S1). Thereafter, two hydrated analogs of **4**, compound **6** (obsd *m/z* 341.2190 $[M + H]^+$, calcd for $C_{16}H_{28}N_4O_4$) and **7** (obsd *m/z* 341.2188 $[M + H]^+$, calcd for $C_{16}H_{28}N_4O_4$), were isolated from Δ*sfaE* via a high-resolution mass spectrometry (HRMS) based fractionation, allowing the elucidation of their structures (Figure 1 and Figure S2). The putative hydrated **1** (obsd *m/z* 355.1972 $[M + H]^+$, calcd for $C_{16}H_{26}N_4O_5$, Figure S1) in *S. lividans*::p13C was not structurally characterized due to its low yield.

Notably, a putative isonitrile hydratase encoding gene *sfaF* was found to be located downstream of *sfaE*, an essential gene for the biosynthesis of SF2768, through a BLASTp search against the UniprotKB/Swiss-Prot database. SfaF showed a 32% identity to the previously verified isonitrile hydratase InhA, implying an enzymatic origin of those hydrated diisonitriles. To investigate whether the formamides were enzymatically generated by SfaF or converted spontaneously in an acidic environment,[5a,12] SF2768 (**1**), the final product of the biosynthetic cluster *sfa*, and its analog **4** were employed as the substrates *in vitro*. The enzymatic assays of SfaF were performed in a volume of 50 μL (50 mM Tris-HCl, 0.1 mM substrate, 35 μM SfaF, pH = 8.0) at 30 °C, and the boiled protein was utilized as the control. The reactions were terminated at different time points by adding 50 μL of cold acetonitrile and analyzed by LC-MS after filtration. The extracted ion chromatogram (EIC) traces of compounds in the time-course analyses illustrated that **1** and **4** were converted to the corresponding hydrated final products (Figure 3, blue

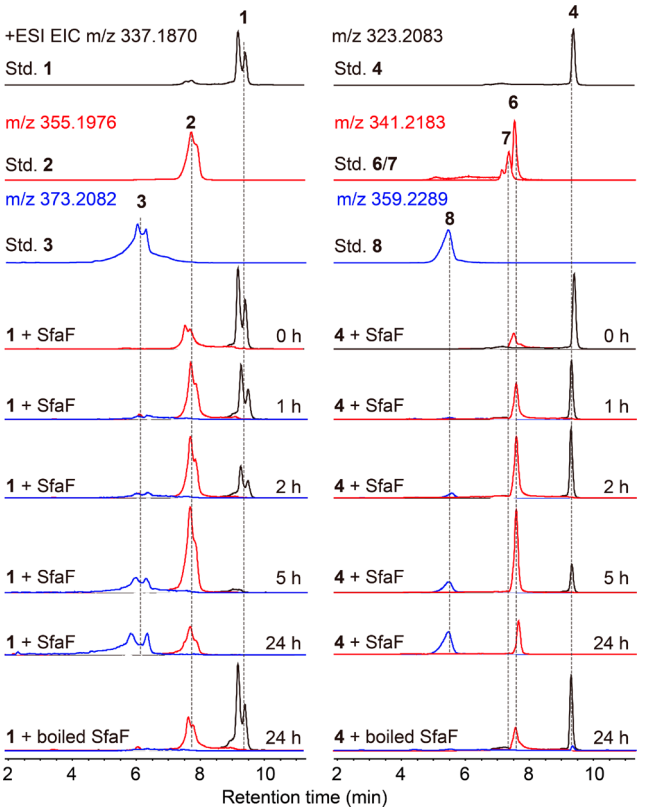

Figure 3. LC-MS analysis of the *in vitro* enzymatic assay of SfaF. Time-course analyses of the conversions of **1** into **3** (left) and **4** into **8** (right) by SfaF are illustrated by EIC overlays. The splitting peak patterns of **1**, **2**, and **3** likely reflect corresponding two anomers, respectively.

lines, *m/z* 373.2082 and 359.2289) through the partially hydrated intermediates (Figure 3, red lines, *m/z* 355.1976 and 341.2183). Subsequently, SfaF-catalyzed enzymatic reactions were carried out at large scale and the resultant products **2**, **3**, **6**, and **8** were used for structural elucidation after purification. The chemical structures and retention time of the metabolites unambiguously indicated that although SfaF eventually hydrated two isonitrile groups of the substrates (**1** or **4**) to N-substituted formamides, only one preferred intermediate (**2** or **6**) occurred during the corresponding catalytic process. Compound **7** cannot be yielded by SfaF, implying that a chemical transformation might be involved. To some extent, these findings coincide with Kobayashi's plausible hypothesis that isonitrile hydrating and synthesizing enzymes are expected to be coupled in isonitrile-producing organisms.[4]

Previous work exhibited the substrate promiscuity of InhA (*Pseudomonas putida*) and the dual function of InhB (*Arthrobacter pascens*).[4,6] To further characterize the substrate specificity of SfaF, two commercially available chemicals,

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cyclohexyl isocyanide (**9**) and cyclohexanecarbonitrile (**11**), were recruited as substrates *in vitro* in a volume of 500 μL consisting of 50 mM Tris-HCl (pH = 8.0), 1 mM substrate (dissolved in 20 μL acetonitrile and then added to the aqueous reaction), and 3 μM SfaF at 30 °C, respectively. The reactions were quenched by adding 500 μL of cold acetonitrile and analyzed by LC-MS after filtration as before. A time-dependent accumulation of the peak at *m/z* 128.1070 (the expected product hydrated **9**, namely *N*-cyclohexylformamide **10**) was observed (Figure 4A). Comparison based on retention time

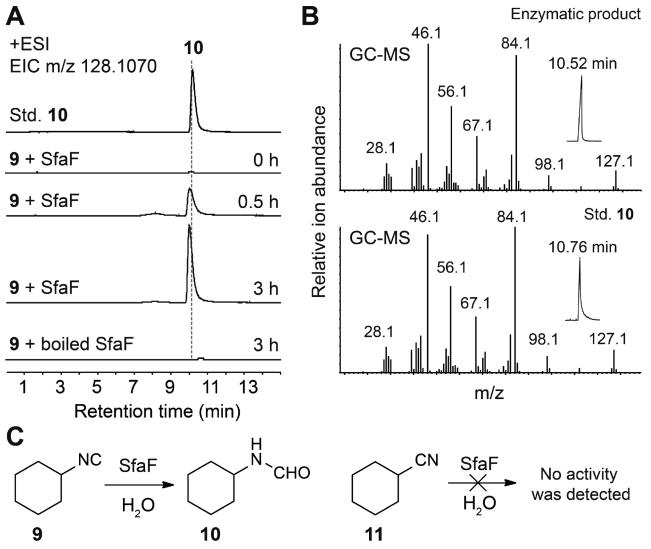

Figure 4. *In vitro* validation of hydration activity of SfaF against cyclohexyl isocyanide and cyclohexanecarbonitrile. (A) Time-course analysis of the conversion of cyclohexyl isocyanide **9** into *N*-cyclohexylformamide **10**. (B) GC-MS spectral comparison of the enzymatic product and *N*-cyclohexylformamide standard. (C) Substrate preference of SfaF for cyclohexyl isocyanide **10** over cyclohexanecarbonitrile **11**.

and the GC-MS spectrum of the enzymatically converted product and authentic **10** demonstrated that SfaF hydrated not only the native isonitriles **1** and **4** but also a chemically synthesized substrate (Figure 4B). In addition, no product was detected when **11** was utilized, signifying that SfaF did not perform a nitrilase activity such as InhB (Figure 4C).

To probe the catalytic efficiency of SfaF, the kinetic parameters were determined by a quantitative LC-MS-based assay at 30 °C (Figure 5A). SfaF showed a comparable K_M value toward **9** to InhA (K_M = 16.2 mM, k_{cat} = 16 s^{-1}, 20 °C),[4] while presenting a significantly lower k_{cat} value. This is possibly due to the differences in enzyme sequence (Figure S3A) or reaction condition such as temperature or buffer. The specificity constant (k_{cat}/K_M) for **1** was approximately 3-fold higher than that for the chemically synthesized **9**, indicating that the native substrate **1** was better consumed by SfaF. Previous site-directed mutagenesis experiments of two validated isonitrile hydratases from *Pseudomonas putida* and *Pseudomonas fluorescens* have established that the residues D17 and C101 were critical for the enzymatic catalysis.[4,5b] The conserved amino acid residues of SfaF were analyzed by aligning the target sequence with those of the verified isonitrile hydratases (Figure 5B). Although the amino sequence of SfaF is approximately one-tenth longer than the other two, the aspartic acid and cystine residues are conserved (Figure S3A). The mechanistic

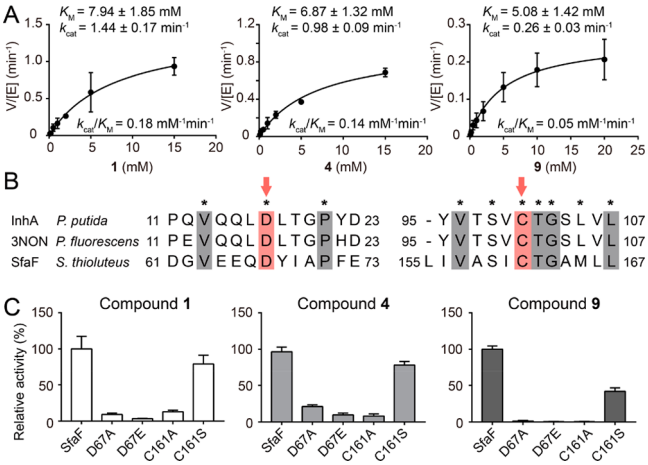

Figure 5. (A) Kinetic parameters of SfaF-catalyzed reactions. Error bars are exhibited as SEM. (B) Sequence alignment of three isonitrile hydratases from *P. putida* (InhA), *Pseudomonas fluorescens* (PDB ID: 3NON), and *S. thioluteus* (SfaF). (C) Catalytic activities comparison between SfaF and the mutants against three isonitrile substrates. Error bars represent SEM from six replicate trials.

roles of the conserved residues in SfaF were thus surveyed via site-directed mutagenesis and subsequent *in vitro* analysis. The hydration activities toward **1**, **4**, and **9** of four constructed mutants, D67A, D67E, C161A, C161S, were measured (Figure 5C). Consequently, compared to SfaF, the activities of D67A, D67E, and C161A were obviously reduced, which was in agreement with the previous work,[4,5b] whereas C161S remained active to a certain extent. Based on sequence alignment and the previous structural study of the homologous isonitrile hydratase,[5b] the plausible reason for the retained activity of C161S is that the hydroxyl group of serine is as excellent a nucleophile as cysteine thiolate that attacks the carbon of isonitrile. Future studies focused on the enzyme–substrate cocrystallization will shed more light on this hypothesis.

Compound **1** functions as a chalkophore that captures environmental copper for the producer due to its copper-chelating ability.[11] The results of copper chrome azurol S (Cu-CAS) assays revealed that copper chelating activities of **1** and **4** are approximately 30-fold higher than those of the corresponding partially hydrated diisonitriles (**2**, **6**, and **7**), while the difomamides (**3** and **8**) were completely inactive, indicating that the presence of two isonitrile groups is essential for diisonitriles to function as a chalkophore (Table 1). In addition

Table 1. Copper Chelating Activities (EC_{50}) of the SF2768 (1) and Its Derivatives

compound	isonitrile group	EC_{50} (μM)
1	2	82.2 ± 1.13[a]
2	1	2335.0 ± 66.3
3	0	–
4	2	62.5 ± 2.8[a]
5	2	84.6 ± 4.1[a]
6	1	2117.0 ± 27.7
7	1	2424.0 ± 389.1
8	0	–
EDTA	–	358.3 ± 25.9[a]

[a]These data are cited from the previous work.[11]

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to isonitrile assimilation/degradation, another possible physiological function of SfaF is to inactivate the overproduced or accumulated chalkophore **1** in cells by hydrating the functional group to prevent an endogenous disturbance of copper utilization, thus contributing to the copper homeostasis of *S. thioluteus*. Combined with the fact that SfaF, the "diisonitrile inactivator", is expected under different control from other biosynthetic enzymes in the pathway, this "self-resistance" hypothesis is also consistent with the high Michaelis–Menten constant and the modest catalytic efficiency of SfaF. Beyond that, SfaF is believed to play a role in chemical defense since isonitrile natural products secreted by microorganisms in the environment are generally antimicrobial due to their metal affinity that leads to enzyme inhibition.

In conclusion, a rare isonitrile hydratase that leads to a unique tandem hydration of the diisonitrile in *S. thioluteus* is reported. From this point of view, our studies complement the knowledge of isonitrile metabolism in microorganisms and enrich the toolbox for enzymatic reactions, thereby providing a mild method other than general acid catalysis for isonitrile hydration in aqueous media. Moreover, our case showed that isonitrile hydrating and biosynthetic enzymes can be closely located. Accordingly, more cryptic isonitrile-generating BGCs and underlying isonitrile natural products can be explored through targeted genome mining based on this strategy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b01341.

Experimental procedures and materials and spectral data of compounds (PDF)

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Notes

The authors declare no competing financial interest.

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