Organic & Biomolecular Chemistry

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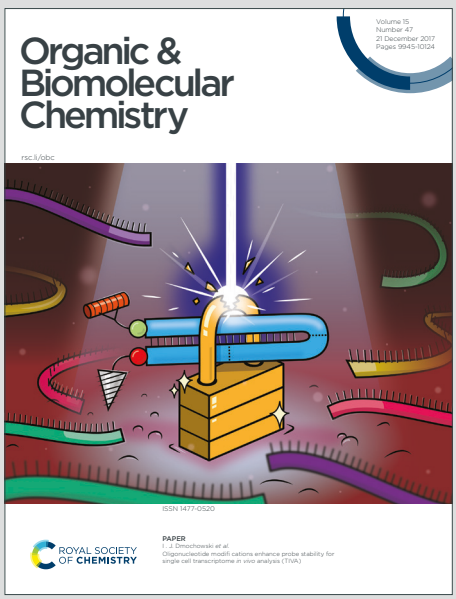

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DOI: 10.1039/D0OB00084A

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ARTICLE

Mutational Biosynthesis to Generate Novel Analogs of Nosiheptide Featuring Fluorinated Indolic Acid Moiety

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Nosiheptide (NOS) is a member of bicyclic thiopeptides possessing a biologically important indolic acid (IA) moiety appended onto the family-characteristic core system. The IA formation relies primarily on NosL, a radical S-adenosylmethionine (SAM) protein that catalyzes a complex rearrangement of the carbon side chain of L-tryptophan, leading to the generation of 3-methyl-2-indolic acid (MIA). Here, we establish an efficient mutational biosynthesis strategy for the structural expansion of the side-ring system of NOS. The *nosL*-deficient mutant *Streptomyces actuosus* SL4005 complemented by chemically feeding 6-fluoro-MIA is capable of accumulating two new products. The target product 6'-fluoro-NOS contains an additional fluorine atom at C6 of the IA moiety, in contrast with an unexpected product 6'-fluoro-NOSint that features an open side ring and a bis-dehydroalanine (Dha) tail. The newly obtained 6'-fluoro-NOS displayed equivalent or slightly reduced activities against the tested drug-resistant pathogens compared with NOS, while dramatically decreased water solubility compared with NOS. Our results indicate that the modification of IA moiety of NOS not only affect its biological activity but also affect its activity which will be key considerations for further modification.

Introduction

Natural products with diverse chemical structures and biological activities are an important source for drug discovery and development. However, most of the natural products are accompanied by biochemical drawbacks that need to be improved via directional structural modification. Benefitting from the maturing methodology in synthetic chemistry and the deepening understanding of biosynthetic paradigm, the combination of biological and chemical approaches has become an effective way to obtain natural product derivatives[1]. Nosiheptide (NOS) and thiostrepton (TSR) are two representative bicyclic thiopeptides, whose macrocyclic frameworks originate from the C-terminal core sequences of the ribosomally synthesized precursor peptides[2-4]. The core sequences then undergo precise and ordered post-translational modifications, including the incorporation of side-ring systems[5-8], and finally obtain the mature products. Specifically, NOS bears an indolic acid (IA) moiety within a 19-membered side-ring system, whereas in TSR, a quinaldic acid (QA) moiety is appended to the macrocyclic framework by forming a 27-membered side-ring system. Both the IA moiety of NOS and the QA moiety of TSR are derived from L-tryptophan. The IA formation relies primarily on NosL, a radical S-adenosylmethionine (SAM) protein that catalyzes a complex rearrangement of the carbon side chain of L-tryptophan and generates 3-methyl-2-indolic acid (MIA)[9, 10]. In contrast, the QA formation is initiated by TsrT, a radical SAM protein catalyzing the 2-methylation of L-tryptophan to produce 2-methyl-L-tryptophan[11], which will further go through the key expansion of an indole to a quinolone intermediate via the action of the pyridoxal-5'-phosphate-dependent protein TsrA and the flavoprotein TsrE[12, 13] (Fig. 1). Existence of the side ring can expand the chemical spaces available for biological activities, therefore, bicyclic thiopeptides seem to exhibit unique mode of action against pathogen infection[14]. It is reasonable to predict that changes in the IA- or QA-containing side-ring system may significantly affect the biological activities of the corresponding NOS or TSR[15, 16].

In our previous work, we have applied the strategy of precursor-directed biosynthesis to introduce structural diversity into the side-ring system of NOS. By feeding 5-fluoro-DL-tryptophan into the NOS-producing wild-type strain

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† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/D0OB00084A

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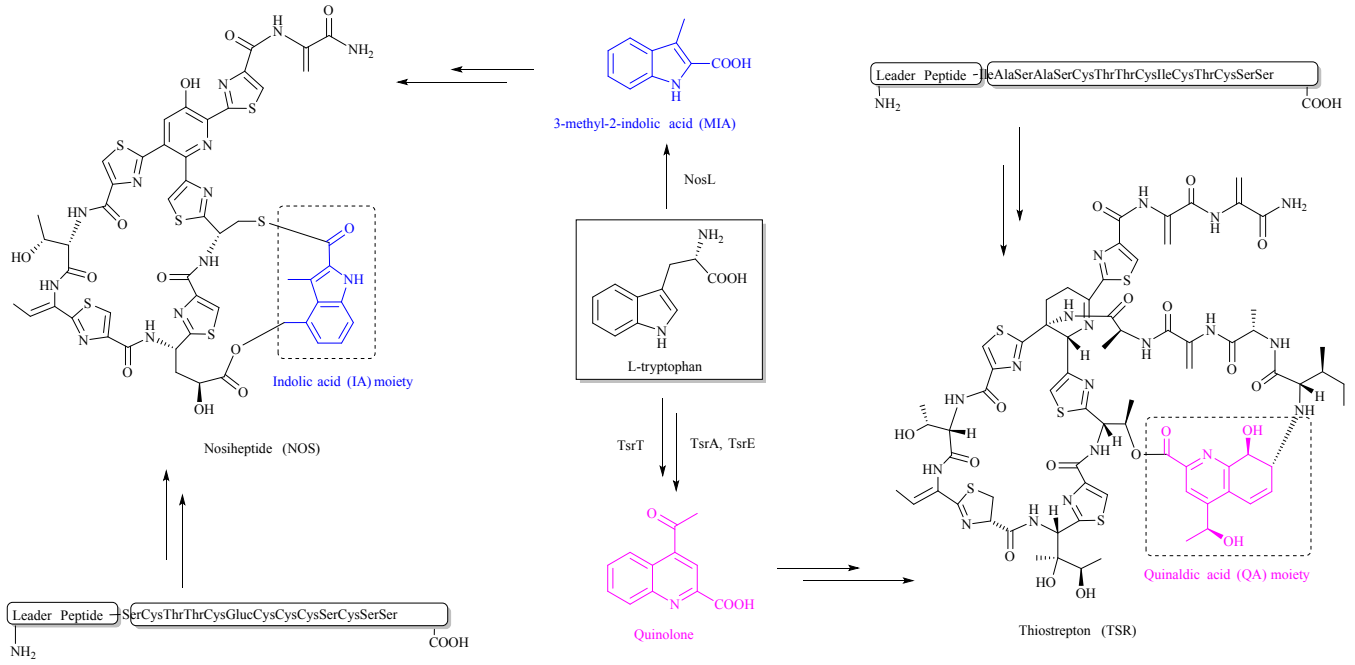

Figure 1. Biosynthetic paradigms of the bicyclic thiopeptides, NOS and TSR. The macrocyclic frameworks originate from the C-terminal core sequences of the precursor peptides[2-4], and the side-ring systems are constructed via the incorporation of the L-tryptophan-derived precursors[5-13].

Streptomyces actuosus, a new compound 5'-fluoro-NOS showing improved antibacterial activity was generated[10].

However, due to the competition of substrates in the native biosynthetic machinery, a mixture of NOS and 5'-fluoro-NOS were co-produced, leading to complex compound purification burden. Based on precursor-directed biosynthesis, mutational biosynthesis can utilize mutant strains deficient in a key aspect of the biosynthetic pathway and directionally produce natural product analogs via the supplementation of particular precursors[1]. By feeding the quinolone derivatives into a *tsrT*-deficient mutant strain, we have successfully obtained numbers of TSR analogs with significant activities against drug-resistant pathogens[17-19]. Since the competing native precursor was eliminated in this mutant strain, the resultant TSR analogs were prepared in high yields and efficiency. Considering the significant advantages of mutational biosynthesis, we herein use this strategy to expand the diversity of the NOS side ring via the establishment of an efficient operation procedure.

Results and discussion

MIA formation in the NOS biosynthetic pathway involves the NosL-catalyzed carbon side chain rearrangement of L-tryptophan, which is independent of the precursor peptide[9, 10]. Focusing on the biologically relevant but tunable MIA moiety, we have considered to introduce the pharmaceutically important fluorine onto the side ring of NOS by taking into account the electronic effect pertinent to drug design. Given the fact that 5-fluoro-L-tryptophan have been successfully introduced into the NOS biosynthetic pathway via the endogenous generation of 5-fluoro-MIA[10], it's reasonable to design the directional incorporation of exogenous 6-fluoro-MIA into an engineered NOS biosynthetic machinery.

For 6-fluorinated MIA preparation, an efficient two-step chemical synthesis route was developed. Briefly, 1-(2-bromo-4-fluorophenyl)ethan-1-one was reacted with ethyl isocyanoacetate in the presence of Cs_2CO_3 and CuI, resulting in 6-fluorinated indole-2-carboxylic acid ester in 90% yield. The 6-fluoro-MIA ester was further hydrolyzed by NaOH followed by acidification, which furnished 6-fluoro-MIA in 88% yield (Scheme 1). Our method can synthesize 6-fluoro-MIA in gram-scale, which is superior to the enzymatic conversion of fluorinated MIA by NosL[10].

We have previously constructed a *nosL*-deficient mutant strain, SL4005, in which the biosynthetic route to MIA was blocked[2]. NOS production was abolished in SL4005, but could

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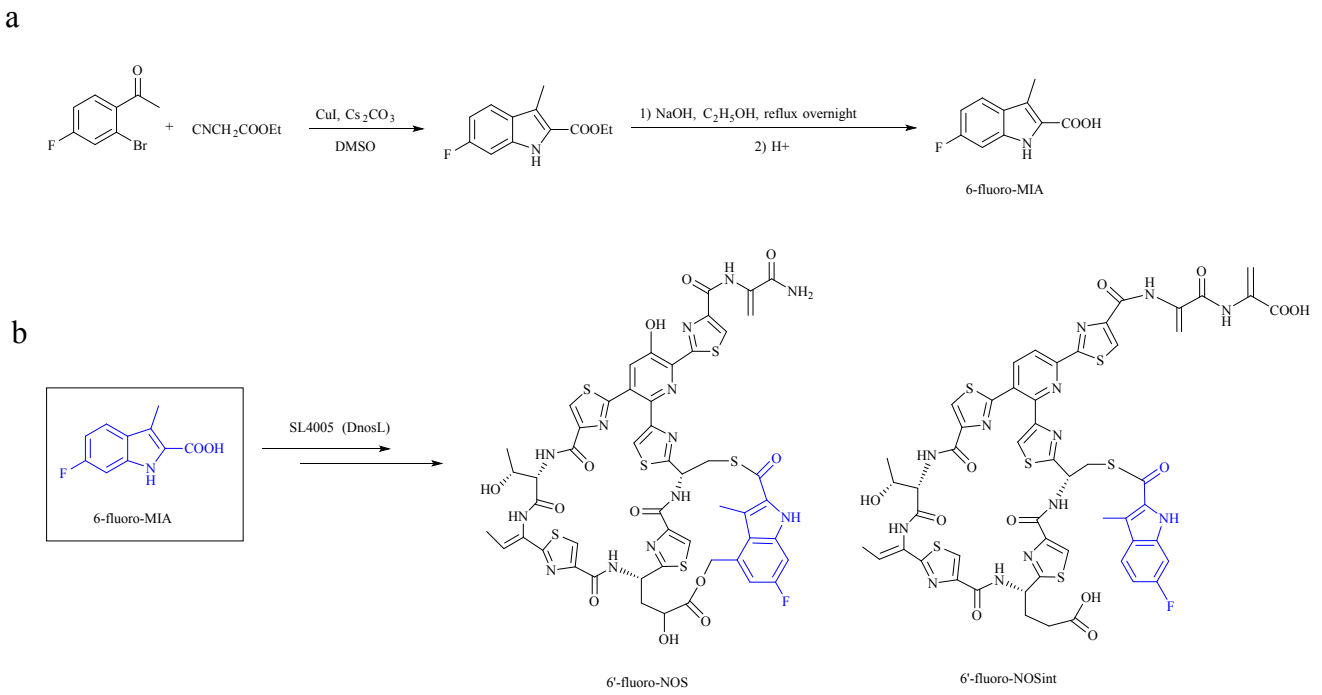

Scheme 1. Chemical synthesis route of the 6-fluorinated MIA (a) and mutational biosynthesis strategy for generating NOS analogs (b). The target product 6'-fluoro-NOS and the unexpected product 6'-fluoro-NOSint were co-produced in SL4005, a *nosL*-deficient mutant, complemented by chemically feeding 6-fluoro-MIA.

be restored by chemical feeding the synthetic MIA (Fig. 2).Therefore, SL4005 was served as an ideal host for the biosynthesis of NOS analogs by eliminating the interference of native precursor. Comparing to the methods described previously[2, 20], we further developed a procedure preferable in shortened fermentation cycle, improved conversion rate, and reduced separation difficulty. After sporulation, the *Streptomyces actuosus* spores were subjected to primary liquid fermentation, and 0.2 mM synthetic 6-fluoro-MIA was exogenously fed to the fermentation broth afterward. Notably, two new compounds, namely 6'-fluoro-NOS (~2 mg L^{-1}) and 6'-fluoro-NOSint (~4 mg L^{-1}) were efficiently produced by SL4005 complemented by chemically feeding 6-fluoro-MIA (Fig. 2).

The target NOS analog, 6'-fluoro-NOS, showed typical NOS-like UV absorption pattern (Fig. S1), suggesting the existence of the NOS bicyclic molecular skeleton. HR-ESI-MS datum established the molecular formula as $C_{51}H_{42}FN_{13}O_{12}S_6$ ($[M+H]^+$, calcd 1240.1457, obsd 1240.1459) (Fig. S2), which confirmed the introduction of a fluorine atom into NOS. Subsequent LC-MS/MS analysis further supported that the fluorine atom was incorporated into the IA moiety (Fig. S3). To fully elucidate the corresponding chemical structures, 6 mg 6'-fluoro-NOS was

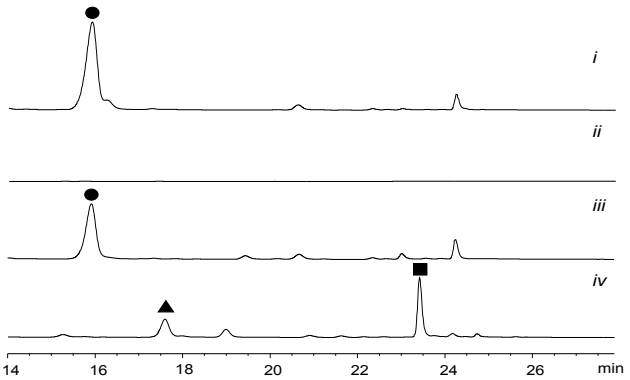

Figure 2. HPLC analysis of the fermentation products from the wild-type NOS-producing strain *Streptomyces actuosus* ATCC25421 (*i*), the *nosL*-deficient mutant strain SL4005 (*ii*), SL4005 complemented by chemically feeding MIA (*iii*) or 6-fluoro-MIA (*iv*). Solid dot (●) indicates NOS. Solid triangle (▲) indicates the NOS analog, 6'-fluoro-NOS. Solid square (■) indicates the NOS analog, 6'-fluoro-NOSint.

purified from 8 L fermentation broth and went through 1D and 2D NMR (^{1}H, ^{13}C and ^{19}F NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY) experiments (Fig. S6). The NMR data revealed signals typical for bicyclic thiopeptides and highly resembled to those of NOS, except for the appearance of an additional quaternary carbon (δ_C 160.5, Ind-C6) that suggested a difference in the IA moiety. In the ^{1}H NMR spectrum, two typical aromatic proton signals [δ_H 6.99 (H-5), 6.99 (H-7)] were observed. Besides, in the ^{19}F spectrum, chemical shift (δ_F) -114.9 was observed as well. Combining the above information with the HMBC correlations from H-5 to C-4, C-6, and C-7 in the IA moiety, the chemical structure of 6'-fluoro-NOS was determined, featuring an additional fluorine atom at C6 of the IA moiety (Scheme 1).

Besides 6'-fluoro-NOS, an unexpected compound, named as 6'-fluoro-NOSint, was co-produced. The UV absorption pattern of 6'-fluoro-NOSint was different from that of NOS (Fig. S1), indicating that changes might take place in the molecular skeleton. HR-ESI-MS datum established the molecular formula as $C_{53}H_{44}FN_{13}O_{12}S_6$ ([M+H]$^+$, calcd 1266.1613, obsd 1266.1604) (Fig. S4), implying the high correlation between 6'-fluoro-NOSint and 6'-fluoro-NOS. Given the LC-MS/MS datum that clearly provided a fragmental mass of the fluorinated MIA segment and a fragmental mass of a bis-dehydroalanine (Dha) segment (Fig. S5), we could basically verify that 6'-fluoro-NOSint was a 6-fluoro-MIA-S-conjugated, side-ring-opening NOS derivative with a bis-Dha tail (Scheme 1). The speculative chemical structure of 6'-fluoro-NOSint is highly similar to NOS analogue **4** which is isolated from a *nosN*-deficient mutant strain[5].

To fully elucidate the corresponding chemical structures, 8 mg 6'-fluoro-NOSint was purified from 4 L fermentation broth and went through 1D and 2D NMR (^{1}H, ^{13}C and ^{19}F NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY) experiments (Fig. S9). The NMR data highly resembled to NOS analogue **4** which is isolated from a nosN-deficient mutant strain, the appearance of an additional quaternary carbon (δC 160.5, 6-F-Ind-C6) that suggested a difference in the IA moiety. In the ^{1}H NMR spectrum, two typical aromatic coupling proton signals [δH 7.73 (H-4), 6.96 (H-5)] were observed. Similar to 6-F-Nos, in the ^{19}F spectrum, chemical shift (δF) -114.9 was also observed. Combining the above information with the HMBC correlations from H-5 to C-4, C-6, and C-7 in the IA moiety, besides with the ^{1}H-^{1}H COSY between H-5 and H-4, the chemical structure of 6'-fluoro-NOSint was determined, featuring an additional fluorine atom at C6 of the IA moiety (Fig. S11).

The biosynthesis of the side-ring structure requires the actions of NosI, -J, -K,-L, and –N[26]. NosN is a radical SAM protein that selectively build a C1 unit at the S-conjugated MIA moiety and form an ester linkage to establish the side ring system[5, 8]; besides, NosN was identified to close the side ring before NosO forms the pyridine ring[24]. Therefore, the introduction of a fluorine atom at C6 of MIA will not affect the enzymatic transformations of NosIJK; however, the introduction of a fluorine atom at C6 of MIA will interfere the methylation function of NosN, perhaps affect the electrical properties of MIA with electronegativity of F (4.0) higher than H (2.1) while atomic radius of F (0.71 Å) similar to H (0.79 Å), leading to the generation of a side-ring-opening intermediate. Our results show that the introduction of a fluorine atom at C6 of MIA deduce the electron cloud density of MIA and hence affect methylene radical electrophilic substitution of MIA, which is consistent with the proposed NosN reaction mechanism[24].

The side-ring-opening intermediate will go through sequential enzymatic transformations to forms the pyrinie ring by NosO; however, two oxidation events catalyzed by two cytochrome P450-like mono-oxygenases, NosB acts on Glu at its γ -position whereas NosC acts on the Pyr position, did not happen since these two steps occur at the tailoring stage, after the main scaffold is formed during NOS biosynthesis[25]. And the side-ring-opening intermediate also affect the enzymatic activity of NosA, a novel protein enabling the cleavage of the bis-Dha tail through the process of enamine dealkylation[21]. The side ring closure, catalyzed by NosN, will not only affect the tailoring stage enzymatic transformations but also affect the formation of mature NOS analog.

Table 1. Minimum inhibitory concentrations (MIC) values of 6'-fluoro-NOS, 6'-fluoro-NOSint and NOS against drug-resistant pathogens. Vancomycin was chosen as a control drug for the tests

Drug-resistant pathogen	MIC (μg mL^{-1})			
	6'-fluoro-NOS	6'-fluoro-NOSint	NOS	Vancomycin
MRSA ATCC43300	0.032	>0.128	0.032	1
VRE ATCC29212	0.032	>0.128	0.016	4
VRE ATCC51299	0.032	>0.128	0.016	64
VRE ATCC51559	0.016	>0.128	0.004	>128

Considering the importance of the IA moiety that can interact with A1067, a nucleobase of 23S rRNA that contributes to mutation-induced bacteria resistance[22, 23], 6'-fluoro-NOS, 6'-fluoro-NOSint and NOS were thus evaluated for their activities against a panel of Gram-positive drug-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA ATCC43300) and vancomycin- resistant *Enterococcus* (VRE ATCC29212, VRE ATCC51299, VRE ATCC51559) (Table 1). The newly obtained 6'-fluoro-NOS displayed equivalent or slightly reduced activities against the tested pathogens, which was in agreement with the dramatically decreased water solubility of 6'-fluoro-NOS (0.15 ± 0.02 μg mL^{-1}) compared with NOS (4.90 ± 0.11 μg mL^{-1}). The above results indicate that the modification of IA moiety of NOS not only affect the biological activity of NOS to drug-resistant pathogens but also affect the solubility of NOS, which may be an important bottleneck limiting the acquisition of bioactive new products. As our expected, the antibacterial activity of 6'-fluoro-NOSint became very poor, shed light on the closure of side ring on the biological activity of NOS. Side-ring-opening increases the degree of freedom of 6'-fluoro-NOSint, while affects the binding of 6'-fluoro-NOSint to the target at the same time.

Conclusions

We have successfully applied the strategy of mutational biosynthesis to generate the novel fluorinated analogs of NOS. Through the operation procedure we developed, the synthetic precursor analog, 6-fluoro-MIA, was efficiently fed into SL4005 that deficient in the endogenous MIA preparation, leading to the accumulation of the target product, 6'-fluoro-NOS. It is worth mentioning that 6'-fluoro-NOSint, an unexpected product featuring an open side ring and a bis-Dha tail, was produced simultaneously. Structure elucidation of 6'-fluoro-NOSint gives insight into the substrate selectivity of the enzymes responsible for MIA incorporation and NOS maturation. The bicyclic NOS analog 6'-fluoro-NOS displayed equivalent or slightly reduced activities against the tested drug-resistant pathogens, which might assign to the unforeseen reduction in water solubility. The closure of side ring not only affects physicochemical property of NOS but also interferes with substrate recognition and enzymatic transformation of the tailor enzymes. This work will deepen our understanding of natural products biosynthesis machinery and lay a foundation for the mutational biosynthesis of NOS analogs.

Experimental

Mutational biosynthesis of nosiheptide analogs: For sporulation, the *Streptomyces actuosus* strains were cultured on MS agar plates at 30°C for 3 days. The *S. actuosus* spores were inoculated into a 500-mL flask containing 100 mL of primary fermentation medium and incubated at 30℃ and 220 rpm for 30 hrs.

0.2 mL synthetic 6-fluoro-MIA (1mM dissolved in DMSO) was fed to 100 mL fermentation broth of *S. actuosus* SL4005, which would be incubated at 30℃ and 220 rpm for 48 hrs.
The fermentation broth was centrifuged, and the supernatant was discarded. The mycelia cake was soaked with acetone and sonicated for 30 min. The acetone sample was then centrifuged for HPLC analysis on an Agilent Zorbax column.

A total of 8 L fermentation broth was treated by the above method. The crude product was dissolved in tap water, and then extracted twice with an equal volume of n-butanol, and the organic phase was concentrated and purified by silica gel chromatography eluting with 100% CH_2Cl_2 followed by CH_2Cl_2-MeOH (100:1 to 100:10). The component 6'-fluoro-NOS was then collected and the organic solvent was evaporated to obtain a crude extract. The crude extract was further purified by semi-preparative HPLC performed on an Agilent 1100 with a Zorbax SB-C18 column (9.4 mm × 25 cm)

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported in part by grants from the National Natural Science Foundation of China (31972850, 21750004 and 21520102004), the Shandong Key Research Program (2019GSF108223), National Key Research and Development Program of China (grant No. 2018YFA0901903), Chinese Academy of Sciences (QYZDJ-SSW-SLH037 and XDB20020200), Science and Technology Commission of Shanghai Municipality (17JC1405100), Youth Innovation Promotion Association of the Chinese Academy of Sciences (2017303), and K. C. Wong Education Foundation.

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