Using Peptide Substrate Analogs to Characterize a Radical Intermediate in NosN Catalysis

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Contents

1. Introduction

2. Synthesis of NosN substrate surrogates and their deuterated analogs

2.1 Synthesis of 4,5,6,7-d_4 indolic acid **15**

2.2 Synthesis of building block I for SPPS

2.3 Solid phase peptide synthesis

2.4 HPLC purification

3. EPR sample preparation and data collection

4. EPR data simulation

5. Conclusions

Abstract

Nosiheptide is a ribosomally produced and post-translationally modified thiopeptide antibiotic that displays potent antibacterial activity *in vitro*, especially against Gram-positive pathogens. It comprises a core peptide macrocycle that contains multiple thiazole rings, dehydrated serine and threonine residues, a tri-substituted 3-hydroxypyridine ring and several other modifications. Among these additional modifications includes a 3,4-dimethyl-2-indolic acid (DMIA) moiety that bridges Glu6 and Cys8 of the core peptide to form a second smaller ring system. This side-ring system is formed by the action of NosN, a radical S-adenosylmethionine (SAM) enzyme that falls within the class C radical SAM methylase (RSMT) family. However, the true function of NosN is to transfer a methylene group from the methyl moiety of SAM to C4 of 3-methylindolic acid (MIA) attached in a thioester linkage to Cys8 of the core peptide to set up a highly electrophilic species. This species is then trapped by the side chain of Glu6, resulting in formation of a lactone and the side-ring system. The NosN reaction requires two simultaneously bound molecules of SAM. The first, SAM^{I}, is cleaved to generate a 5′-deoxyadenosyl 5′-radical, which abstracts a hydrogen atom from the methyl group of the second molecule of SAM, SAM^{II}. The resulting SAM^{II} radical is believed to add to C4 of MIA, affording a radical intermediate on the MIA substrate. Herein we describe synthetic approaches that allow detection of this radical by electron paramagnetic resonance (EPR) spectroscopy.

1. Introduction

Class C radical *S*-adenosylmethionine (SAM) methylases (RSMTs) operate on non-nucleophilic sp^2-hybridized carbons found most often during the biosynthesis of complex secondary metabolites. The chemical outcomes of the enzymes are one-carbon derived, and include methylation, lactonization, or cyclopropanation.(*1-3*) Recent mechanistic investigations on several class C RSMTs, including NosN,(*4, 5*) TbtI,(*6*) and C10P,(*7*) detail the requirement for two simultaneously bound SAM molecules during catalysis, which appears to be common to radical SAM (RS) enzymes designated as HemN-like, a group into which class C RSMTs fall.(*1, 8*) During catalysis, the first SAM molecule (SAM^I), which binds to the unique iron of the [Fe_4S_4] cluster, is reductively cleaved to methionine and a 5'-deoxyadenosyl 5'-radical (5'-dA•), which abstracts a hydrogen atom (H•) from the methyl group of the second SAM molecule (SAM^{II}). The resulting SAM^{II} methylene radical attacks the target sp^2-hybridized carbon of the substrate to yield a radical intermediate connected to SAM^{II} through its former methyl moiety. The formation of this radical intermediate is presumed to be common to all class C RSMTs, but it can be used to effect diverse outcomes, including methylation, lactonization or cyclopropanation, as dictated by the specific protein in which the adduct is formed.

NosN is an annotated class C RSMT that establishes the lactonic bond in the side-ring of nosiheptide (NOS, as shown in **Figure 1**), a thiopeptide natural product with potent *in vitro* activity against a variety of clinically relevant Gram-positive bacteria.(*9*) The biosynthetic gene cluster of NOS contains 16 genes, which include *nosA-nosP*. As shown in **Figure 1**, the biosynthesis of NOS starts from NosM, a ribosomal precursor peptide of 50 amino acids. Qiu et al. showed that NosM is first processed by NosF, G, and H to give intermediate **1**.(*10*) Next, NosK, an α/β-hydrolase,(*11*) recognizes this thiazole-bearing intermediate **1** and appends 3-methylindolic acid (MIA)—

generated from tryptophan via the action of NosL (*12*)—to the thiol group of Cys8 to give intermediate **2**.(*10*) Our previous studies suggest that NosN then recognizes **2** as its substrate and generates the side ring to give intermediate **3**.(*4, 5*) Although not experimentally verified, intermediate **3** is expected to be processed by NosD and NosE to generate the dehydrated serine and threonine residues, and then by NosO to form the major macrocycle through a [4+2] cycloaddition reaction.(*13*) Additional post-translational modifications give rise to the final nosiheptide structure.

As shown in **Figure 1**, the substrate for NosN is complex. Therefore, to study the NosN reaction mechanism, we designed a synthetic tripeptide **6** that integrates the minimal fragments necessary for forming the side ring, which includes the carboxylic side chain of Glu6 and MIA linked in a thioester bond to Cys8, as shown in **Figure 2A**.(*4*) Using this substrate surrogate, we established that the natural function of NosN is not to transfer a methyl group, but to transfer a methylene unit in such a way as to generate a highly electrophilic species that is trapped by the carboxylic group of Glu6. This step results in formation of the lactonic bond of nosiheptide concomitant with formation of the side ring. **Figure 2B** shows the current working hypothesis for catalysis by NosN. After addition of the SAM^{II} methylene radical to C4 of MIA, an unidentified base from NosN removes the C4 proton of the MIA radical intermediate, which promotes elimination of S-adenosylhomocysteine (SAH) as a co-product. The resulting radical intermediate then loses an electron, likely back to the iron-sulfur (Fe-S) cluster, affording an electrophilic methylene group that is attacked by the carboxylic group of Glu6 to close the ring.

To determine NosN's true substrate and when it acts during the biosynthesis of nosiheptide, we designed two substrate surrogates, compounds **8** and **10,** to be used as probes (**Figure 2C&D**).(*5*) Compound **8** is a surrogate for intermediate **2** in **Figure 1**, the species before

dehydration by NosD and -E (with truncation of the leader peptide), while compound **10** is the dehydrated version of **8**. These two compounds were used to assess whether NosN acts before or after dehydration by NosD and -E. Use of **8** in NosN reactions afforded the side-ring closed species as the sole product, while the use of **10** afforded two MIA-SAM adducts as major products. We posited that the more rigid structure of **10,** due to the dehydrated serines, impedes the base from removing the C4 proton of the radical intermediate, a step that allows the normal reaction to advance forward to product. After quenching the reaction, the radical species would either be oxidized or reduced by one electron, which would be followed by re-aromatization to give **11** (SAM adduct I) or protonation to give **12** (SAM adduct II) (**Figure 2D)**. The observation of the two SAM adducts suggested the possibility that the presumed radical intermediate would accumulate sufficiently to be detected by electron paramagnetic resonance (EPR) spectroscopy (**Figure 2B**). Although EPR spectroscopy has been a powerful tool to characterize radical intermediates in a variety of RS enzymes, no such characterization has been reported yet for class C RSMTs, mostly due to the complexity of their substrates. Herein, we provide synthetic strategies for generating substrate analogs, some with strategically placed isotopic labels, for studying the reaction mechanism of NosN.

2. Synthesis of NosN substrate surrogates and their deuterated analogs

Our EPR study of the NosN reaction requires compound **8**, compound **13** and its perdeuterated analogs **14** to assign the radical species observed. The chemical structures of compounds **13** and **14** are shown in **Figure 3**. Compound **13** is an analog of compound **8** but with no methyl group on C3 of MIA. Although not the natural substrate, compound **13** was designed because it is more convenient to incorporate deuterium atoms into indolic acid than MIA. As shown in **Figure 3A**,

the synthesis of compound **8**, **13**, and **14** was accomplished by solid phase peptide synthesis (SPPS), which requires two important building blocks, I and II. Herein, we detail our synthetic strategy in three sections: synthesis of deuterated indolic acid analogs **15**, synthesis of peptide building blocks, and solid phase peptide synthesis (SPPS).

2.1 Synthesis of 4,5,6,7-d_4 indolic acid **15**

Indolic acid and MIA, which are used to synthesize compound **8** and **13** respectively, are commercially available. By contrast, 4,5,6,7-d_4 indolic acid **15** must be accessed synthetically. The construction of the indole structures is achieved by an aerobic cross-dehydrogenative coupling between aniline and ethyl pyruvate, as reported by Xiao.(*14*) As shown in **Figure 4A**, we start from the corresponding perdeuterated aniline, which is commercially available.

1. **We found that scaling up the coupling reaction results a lower yield.** Thus, to obtain sufficient product, we usually prepare 10~20 parallel reactions at the scale described below (**Figure 4B**).

2. d_5-aniline (0.4 mmol, 1.0 equiv), $Pd(OAc)_2$ (9.0 mg, 0.04 mmol, 0.1 equiv), ethyl pyruvate (92.8 mg, 0.8 mmol, 2.0 equiv), acetic acid (96.0 mg, 1.6 mmol, 4.0 equiv), and 4A molecular sieve (80 mg) are added to DMSO (2.0 mL) in a 5 mL vial equipped with a stir bar. The reaction is bubbled with oxygen for 1 min then sealed. The reaction mixture is stirred at 70 ^{o}C for 12 h. After completion, the reaction mixtures are combined and diluted with ethyl acetate (250 mL) and washed thoroughly and sequentially with 0.1 M HCl (100 mL, twice), saturated aqueous sodium bicarbonate (100 mL, twice), water (100 mL), and brine (100 mL). The organic layer is dried over anhydrous sodium sulfate and concentrated *in vacuo*, and the resulting residue is purified by silica

gel flash chromatography (hexanes : ethylacetate = 20:1-5:1), giving compound **16** as a yellow solid.

3. Sodium hydroxide (200 mg, 5.0 mmol, 5 equiv) is added to the stirred solution of **16** (1.0 mmol, 1.0 equiv) in THF (7 mL)-MeOH (7 mL)-water (7 mL). The reaction mixture is stirred at room temperature for 1 h. After completion, the reaction mixture is diluted with ethyl acetate (70 mL) and washed thoroughly and sequentially with 0.1 M HCl (100 mL) and brine (100 mL). The organic layer is dried over anhydrous sodium sulfate and concentrated *in vacuo* to give compound **15** as a yellow solid.

2.2 Synthesis of building block I for SPPS

O- to N- acyl transfer during deprotection of Fmoc, which occurs in SPPS upon treatment with 20% piperidine in DMF, is common when there is an ester bond on the side chain of serine or threonine. To avoid this side reaction, it is necessary to minimize the number of deprotection steps during SPPS cycles. We therefore designed and used building blocks I and II for SPPS. Indolic acid and its deuterated analogs, as well as MIA, are introduced into building block I by solution phase synthesis prior to SPPS, as shown in **Figure 5**. Solution phase synthesis is also used to prepare building block II for SPPS, which is a tetra-peptide (**Figure 3)**. By using this strategy, we reduce the number of piperidine deprotections to one, which occurs between incorporation of building blocks I and II. We use Boc as the amino protecting group in building block II, because it can be removed concomitantly when cleaving the peptide from the resin. There are three unnatural amino acids used in solution phase synthesis and SPPS, including thiazolyl serine, thiazolyl glutamic acid, and thiazolyl threonine. The detailed preparation of these amino acids can

be found in a previous publication,(*15*) while the preparation of building blocks II and III can be found in another of our previous publications. (*5*)

2. N,N'-Dicyclohexylcarbodiimide (DCC, 124 mg, 0.6 mmol, 1.2 equiv) and 4-dimethylaminopyridine (DMAP, 6.1 mg, 0.05 mmol, 0.1 equiv) are added to the reaction mixture.
3. The resulting solution is stirred at room temperature for 1 h. The reaction will become cloudy because of the formation of dicyclohexylurea.
4. After completion, the reaction mixture is diluted with dichloromethane (50 mL) and washed thoroughly and sequentially with 0.1 M HCl (100 mL), saturated aqueous sodium bicarbonate (100 mL), and brine (100 mL). The organic layer is dried over anhydrous sodium sulfate and concentrated *in vacuo*, and the resulting residue is used for the next step without further purification.
5. The residue is dissolved in THF (20 mL), and tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 29 mg, 0.025 mmol, 0.05 equiv) and 1,3-dimethylbarbituric acid (78 mg, 0.5 mmol, 1.0 equiv) are added to the reaction. This reaction will remove the allyl ester to expose the carboxyl group for SPPS. The resulting reaction mixture is stirred at room temperature for 1 h. Next, the reaction mixture is diluted with ethyl acetate (100 mL) and washed thoroughly and sequentially with 0.1 M HCl (100 mL), saturated aqueous sodium bicarbonate (100 mL), water (50 mL), and brine (50 mL). The organic layer is dried over anhydrous sodium sulfate and concentrated *in vacuo* to give yellow foam which can be used for SPPS directly without further purification.

2.3 Solid phase peptide synthesis

We chose the Fmoc/^{t}Bu strategy to perform our SPPS. In detail, the amino group of each building block is protected by Fmoc, which is removed by treatment with 20% piperidine/DMF after each cycle of amide coupling. The side chains of the building blocks are protected by acid labile groups, such as tBu for hydroxyl and carboxyl groups (Ser, Thr, Asp, Glu), Pbf for guanidine groups (Arg), Boc for amino groups (Lys), and Trityl for thiol groups (Cys), and these are concomitantly removed when cleaving the peptide from the resin using a TFA cocktail. We chose 2-chlorotrityl chloride resin because we want our final product to be a free acid on the C-terminus. Rink Amide Resin can be selected if a C-terminal amide is preferred. Here we will detail how our SPPS was performed.

2.3.1 *Loading 2-chlorotrityl chloride resin*

Weigh 2-chlorotrityl chloride resin (0.75 meq/g, 2 g, 1.5 mmol) in a 50 mL solid phase peptide synthesis vessel and swell the resin in dichloromethane (20 mL) for 1 h. Drain the dichloromethane and introduce a solution of Fmoc-L-Ser(*t*Bu)-OH (2.9 g, 7.5 mmol, 5 equiv) and 2,4,6-collidine (2 mL) in dichloromethane (20 mL) into the vessel. Shake the vessel overnight, drain the reaction mixture and wash with dichloromethane three times.

2.3.2 *Capping 2-chlorotrityl chloride resin*

Introduce the capping solution (dichloromethane/methanol/*N,N*-diisopropylethylamine = 17 mL/2 mL/1 mL) into the vessel and shake for 1 h. Drain the capping solution and wash the resin with dichloromethane three times. Dry the resin under nitrogen flow. The obtained resin is 0.7 mmol/g.

2.3.3 *iterative/amide coupling/Fmoc removal*

We started at a 0.1 mmol scale (equals to 145 mg resin from previous step).

Removal of Fmoc is achieved by bubbling the resin in 20% piperidine in DMF (4 mL) for 10 min. Next, the resin is washed with DMF and dichloromethane. For amide coupling, a solution of the corresponding amino acid building block (0.4 mmol, 4.0 equiv) and HATU (152 mg, 0.4 mmol, 4.0 equiv) in DMF containing 20% N-methylmorpholine (5 mL) is added to the vessel and bubbled for 20 min. After completion, the reaction solution is removed and washed with DMF followed by dichloromethane. Repeat the deprotecting and amide coupling steps until all amino acids are incorporated. Generally, we do not recommend using a stir bar for the reaction because it may rupture the beads. Stirring can be achieved by bubbling, as shown in **Figure 6C**.

2.3.4 *Cleavage and deprotection*

After all amino acid building blocks are incorporated, cleavage cocktail (trifluoroacetic acid/H_2O/triisopropylsilane=9.5 mL/0.25 mL/0.25 mL) is added to the resin, and the resulting reaction mixture is shaken for 3 h. Next, the solution is collected in a 50 mL conical tube. The volume is reduced to ~4 mL under nitrogen flow and *t*-butyl methyl ether (40 mL, prechilled with ice) is added to precipitate the peptide. The white precipitate is pelleted by centrifugation and the residue is dried under a mild flow of nitrogen. The resulting residue is solubilized in water (10 mL), and the resulting solution is filtered before HPLC purification.

2.4 HPLC purification

The purification of crude peptides is performed on an Agilent 1260 Infinity II Preparative HPLC system using an Agilent 5 Prep-C18 (50 × 21.2 mm) column. The solvents for purification include 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), and detection is performed by UV monitoring at 254 nm. The detailed methods are listed below:

As shown in **Figure 7**, the peaks between the red lines are the desired compounds **8** or **13**. Iterative injections are performed to purify the entire crude peptide solution. Fractions are pooled and lyophilized to give a white powder as final products. The powder is re-dissolved in water to make a stock solution with a concentration of 5 mM. HPLC traces of compounds **14** are almost identical to that of compound **13** (data not shown).

3. EPR sample preparation and data collection

Our strategy to overproduce and purify NosN has been previously published.(*4*)

1. Bring stocks of NosN, SAM, substrate peptides, and sodium dithionite (DT) powder, together with a bottle of liquid nitrogen-cooled isopentane for sample freezing, into an anaerobic glovebox.

2. Made a fresh stock solution of DT (100 mM in 50 mM HEPES, pH 7.5) each time EPR samples are prepared.

3. Recipe for making EPR samples is shown in Table 3:

4. Mix all reagents shown in the table (excluding DT) in an Eppendorf tube. Initiate the reaction by addition of DT to the reaction mixture and quickly transfer the reaction into an EPR tube. Incubate the reaction in the EPR tube for given periods of time and freeze the sample in liquid nitrogen-cooled isopentane.

5. For compound **8**, an incubation time between 5-10 min gives the strongest EPR signals.

6. It is recommended to explore the time course of each sample to identify the best time to freeze the sample for the strongest signal. For compound **8**, we prepared samples at 15 s, 30 s, 2 min, 5

min, 10 min, and 60 min. As shown in **Figure 8**, samples prepared at 5 min and 10 min give the strongest signals. Thus, we chose 5 min as the standard reaction time for compound **8**.

7. The power dependence of the EPR signal was also explored, as shown in **Figure 9**. Attenuation of 40 dB (20 μW) gave the best signal, which represents the upper limit for suitable microwave power that allows an undistorted signal of this radical to be obtained at 60 K.

8. For compound **13** and its ^{2}H-labeled analog **14**, incubation times of 2 min are used. The comparison of EPR spectra of the labeled and unlabeled samples together with the simulated spectra are shown in **Figure 10**. Note that the modulation amplitude used to obtain these spectra is lowered to 0.2 mT to obtain the best spectroscopic resolution.

4. EPR data simulation

Complete loss of hyperfine structure in the EPR spectra of the intermediate upon ^{2}H-labeling of C4,C5,C6 and C7 agrees with the assignment of the observed signal to the aryl radical intermediate (see **Figure 2**). To simulate the EPR spectra obtained we adopted a simple model in which the ^{1}H/^{2}H nuclei on sp^{3}-hybridized carbons (e.g. C4) exhibit a predominantly isotropic hyperfine (HF) interaction (i.e. with principal components of the HF coupling $a_1 \approx a_2 \approx a_3$) and ^{1}H/^{2}H nuclei on sp^{2}-hybridized carbons (C5, C6 and C7) exhibit strongly rhombic hyperfine anisotropy ($a_1 > a_2 > a_3$). Taking this model as a starting point we performed a fit of the simulation to the natural-abundance EPR spectrum using the "*esfit*" routine of the EasySpin package in MATLAB. During fitting, we allowed to vary HF coupling constants, three g-values and the unitary linewidth. "*pepper*" from EasySpin with the first-order perturbation theory ("perturb1") option was used as the core simulation routine. This procedure afforded a satisfactory fit to the experimental data with a pseudo-Voigt unitary lineshape (0.8 mT and 0.15 mT Gaussian and Lorentzian line-width

parameters respectively) and an almost isotropic g-tensor (g_1=2.0029, g_2=2.0038, g_3=2.0041). Inclusion of three strong ^{1}H HF couplings was sufficient to interpret the spectra: A_1 = [84, 86, 93] MHz, A_2 = [20, 40, 47] MHz, A_3 = [13, 26, 38] MHz. Using these parameters, it was possible to interpret the ^{2}H spectra by scaling down the HF coupling constants by a factor of $g_n(H)/g_n(D)$=6.51 and slightly adjusting the linewidth parameters. It is worth noting, however, that a reasonable simulation of the natural-abundance EPR spectra can be also achieved with a set of almost completely isotropic HF couplings. While such an interpretation is not possible given that we have established the indole-based nature of the radical, it does highlight certain ambiguities associated with the interpretation of EPR spectra. Therefore, to fully characterize the electronic structure of the radical we need a comprehensive EPR analysis of various isotopologs obtained using synthetic procedures outlined above. This work is underway and will be published separately.

Conclusions

Class C RSMTs catalyze the formation of an adduct between the former methyl group of SAM and an sp^2-hybridized carbon of a substrate as a common intermediate. From this intermediate, reaction outcomes include methylation, lactonization, and cyclopropanation. Given the role that class C RSMTs play in the biosynthesis of bioactive natural products, determining the catalytic mechanism has the potential to shed light on how these molecules can be engineered to produce more effective therapeutics. The mechanistic study of NosN has been hindered by the complex nature of the substrate on which it acts. The methods described herein provide a facile synthetic

approach to generate NosN's substrate as a means to probe its reaction as well as others in the nosiheptide biosynthetic pathway.

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Figure and Table Legends

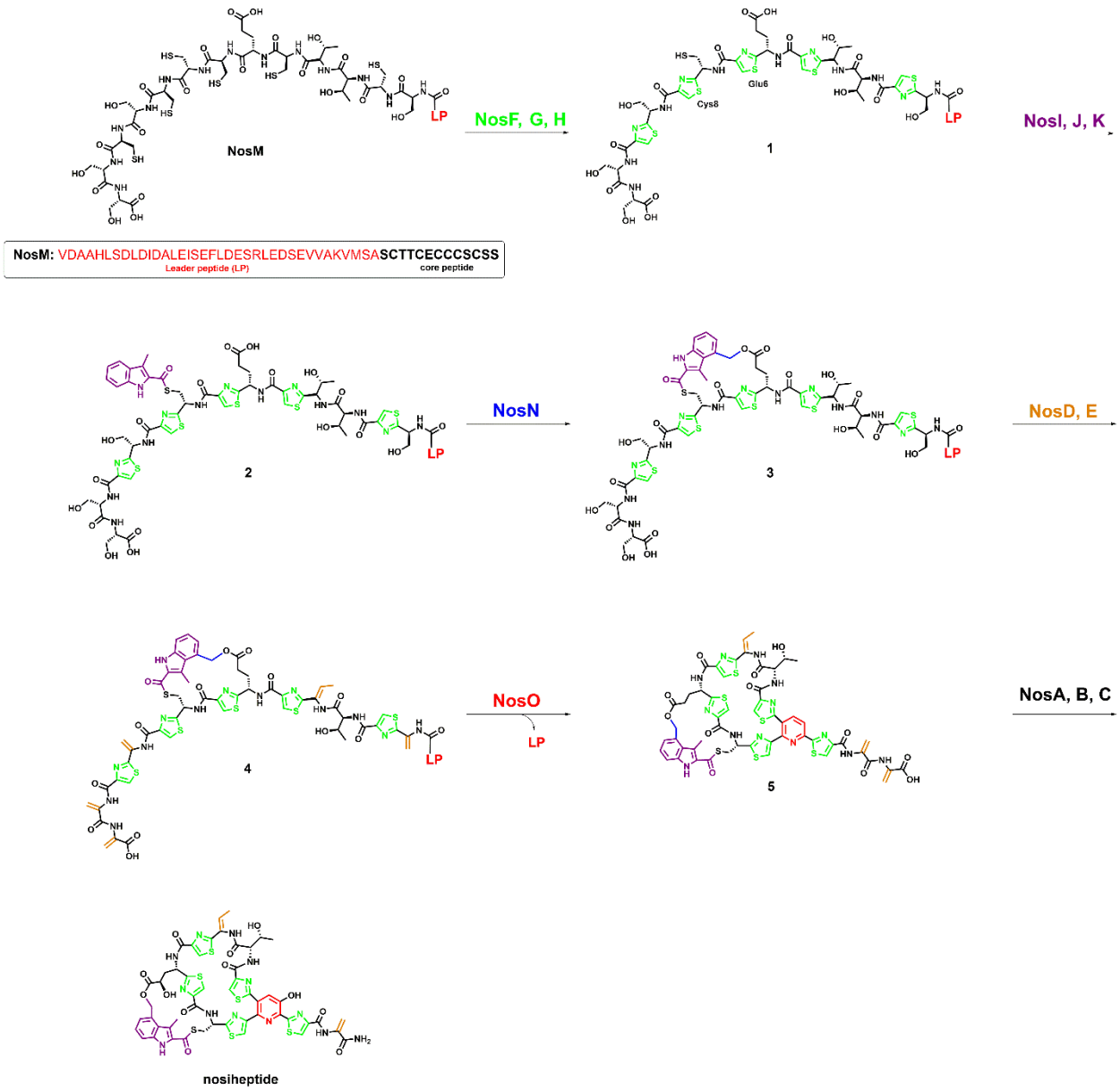


Figure 1. Biosynthetic pathway of nosiheptide

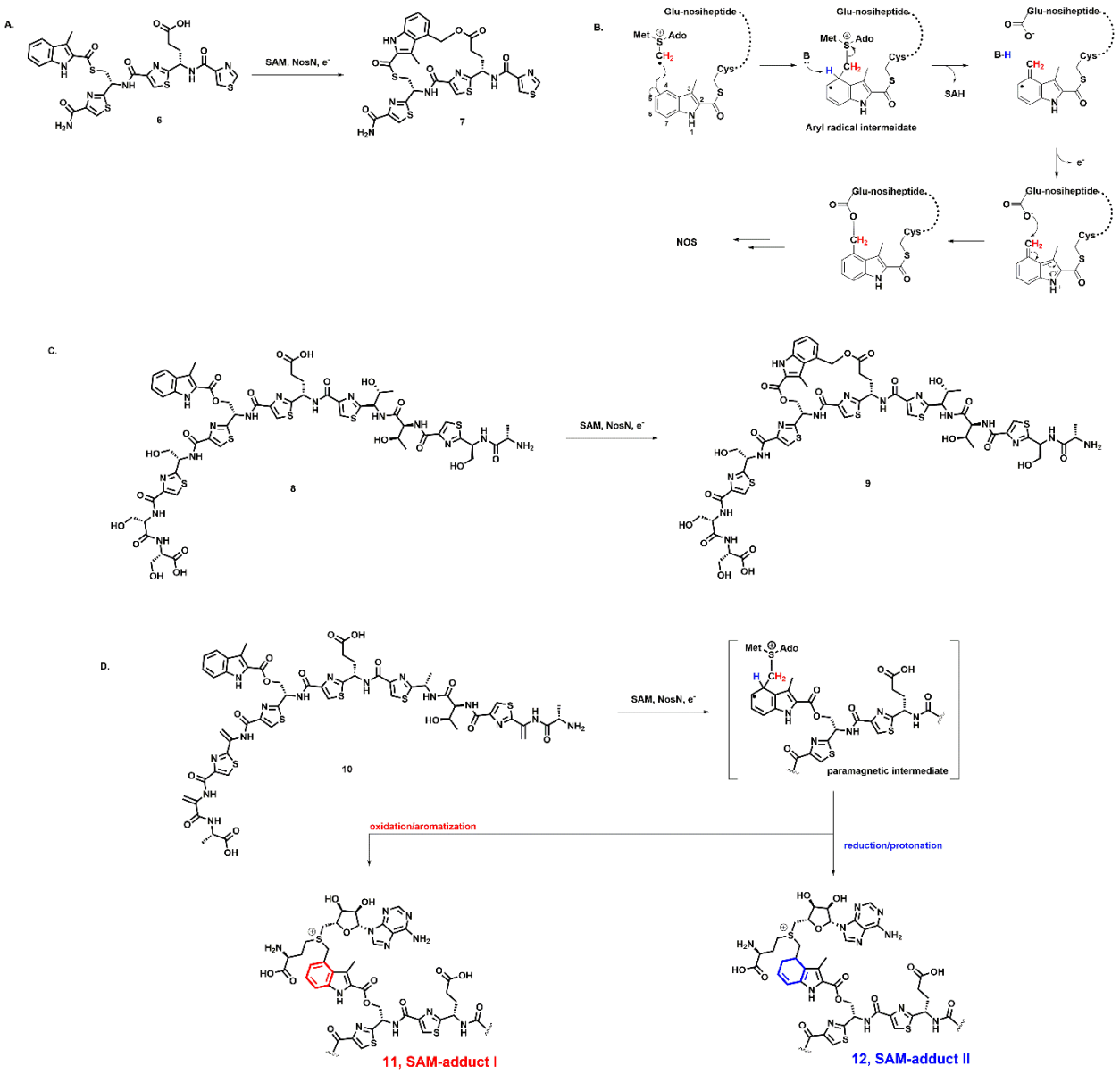


Figure 2. A) NosN catalyzes formation of the side-ring system on a tripeptide substrate surrogate; B) Proposed NosN catalytic mechanism; C) NosN catalyzed formation of the side-ring system on compound 8; D) compound 10 gives two SAM adducts from a presumed common radical intermediate.

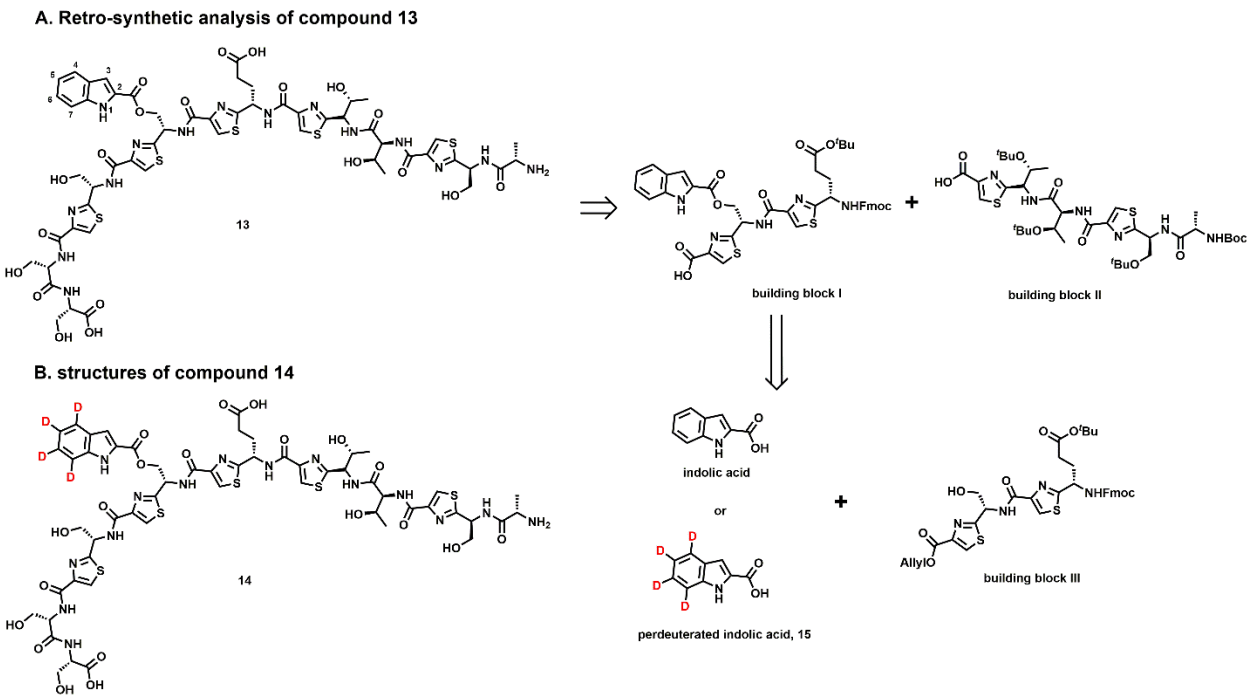
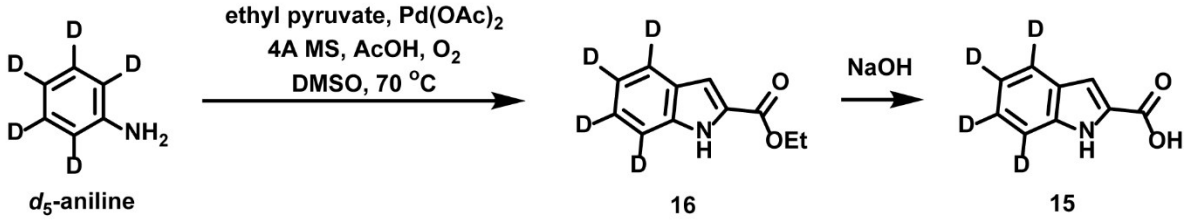


Figure 3. A) Retro-synthesis of peptide analogs; B) Chemical structures of compound **14** with deuteration on indolic acid to study the structure of the radical species.

A.



d_5-aniline 16 15

B.

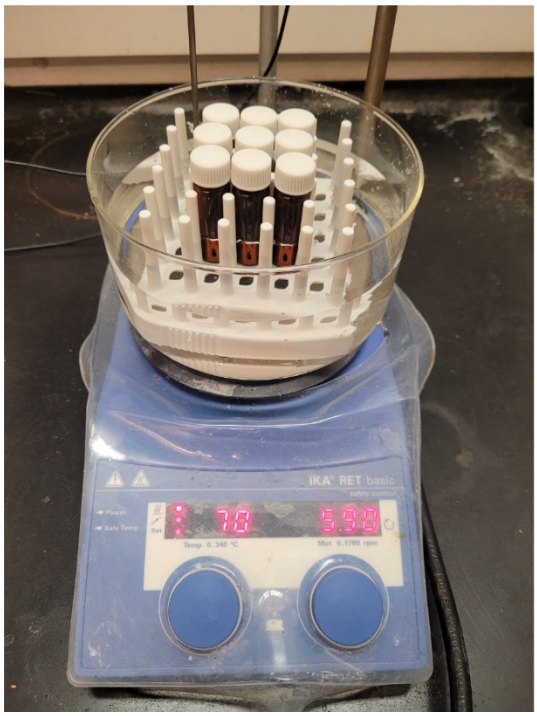


Figure 4. A) Synthesis of compound **15**; B) aerobic cross-dehydrogenative coupling set ups.

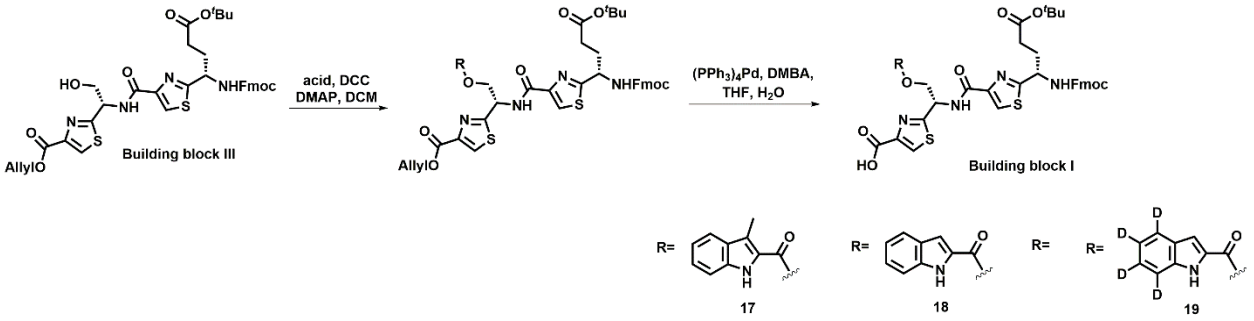


Figure 5. Solution phase synthesis of building block I for SPPS

1. Indolic acid (its deuterated analogs, or MIA (1 mmol, 2.0 equiv)) and building block III (359 mg, 0.5 mmol, 1.0 equiv) are dissolved in dichloromethane (10 mL) in a 50 mL flask with a magnetic stir bar.

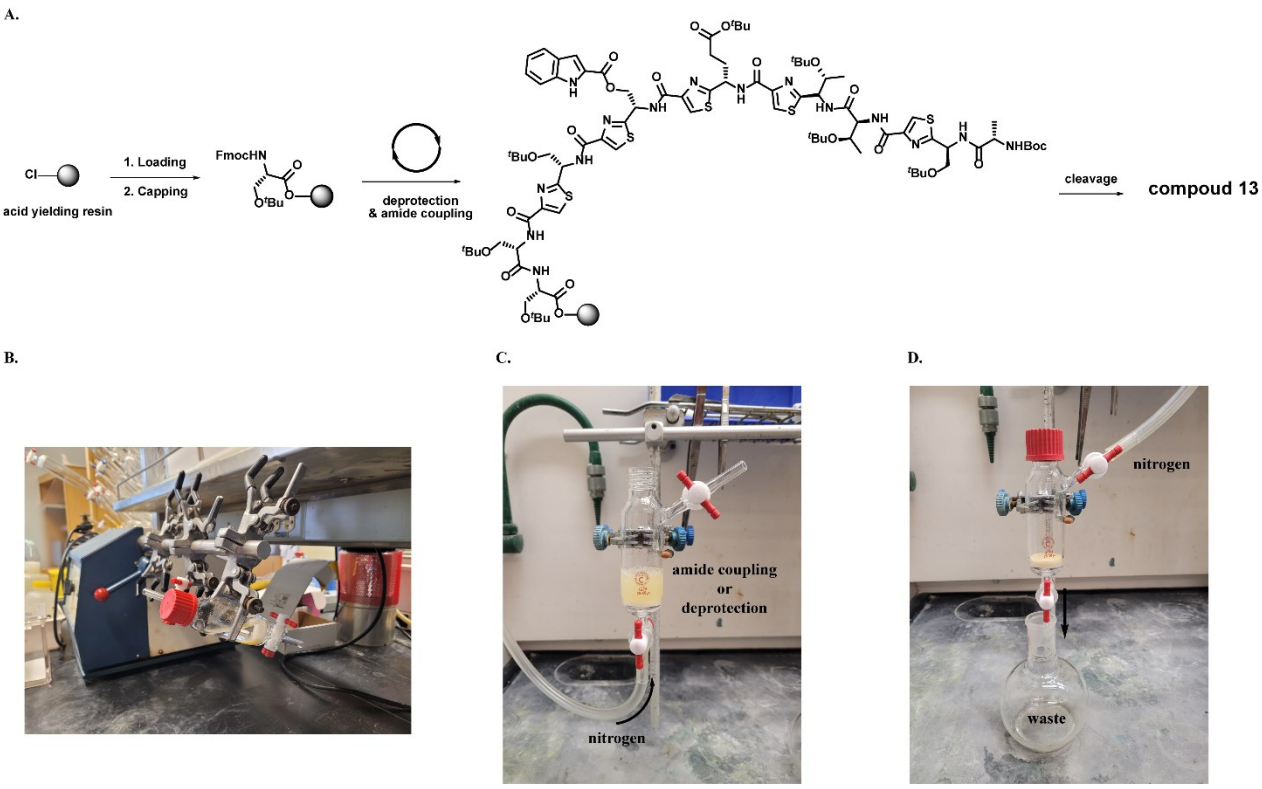


Figure 6. A) SPPS flow chart of compound 13; B) resin swelling, 1st amino acid loading, capping, and cleavage are performed by shaking; C) amide coupling and Fmoc removal reaction are stirred by bubbling; D) liquid phase is pushed out of the SPPS vessel with air or nitrogen to separate it from beads.

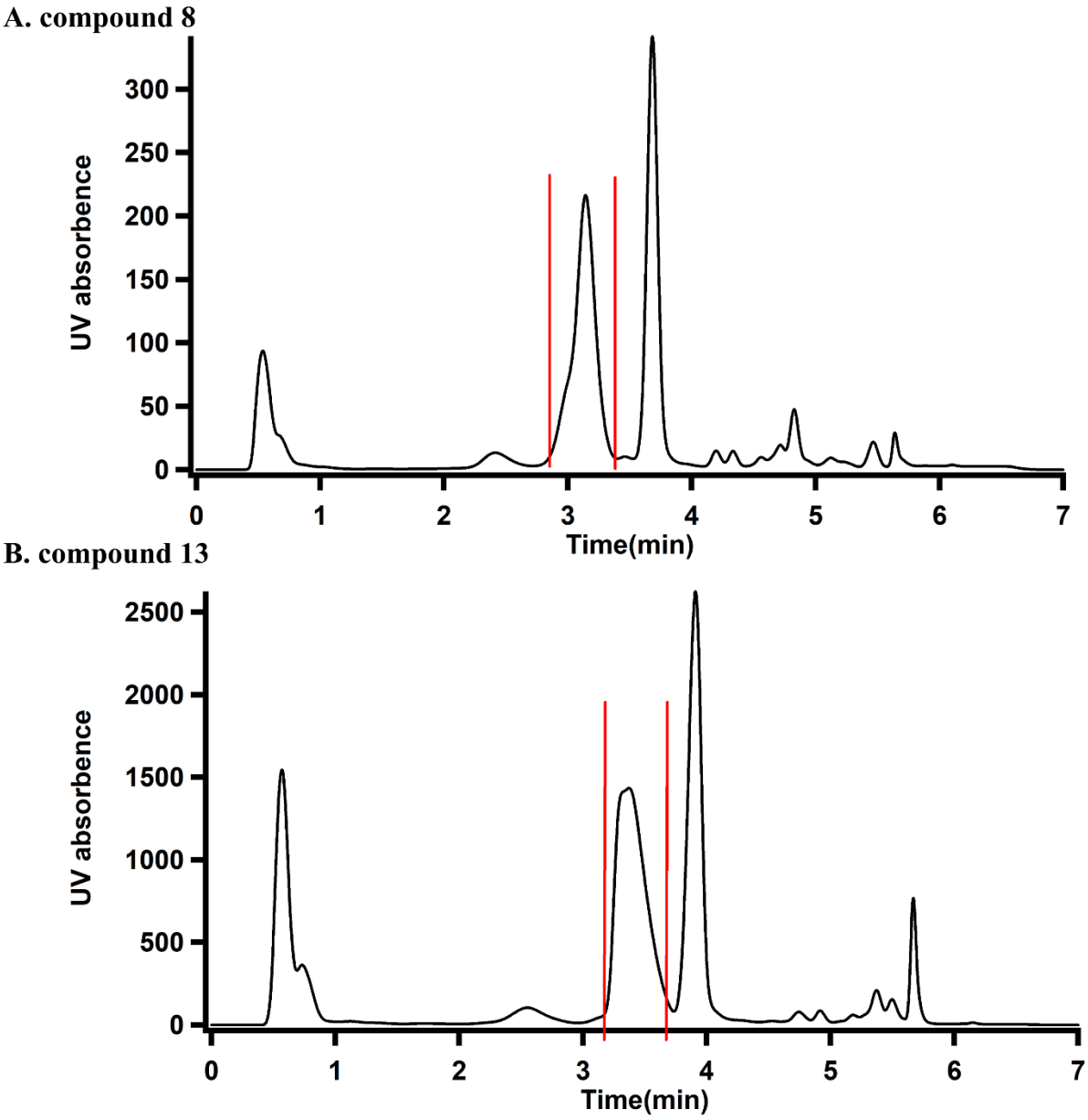


Figure 7. HPLC trace of compound **8** (A) and compound **13** (B). The red lines indicate the desired peak that were collected.

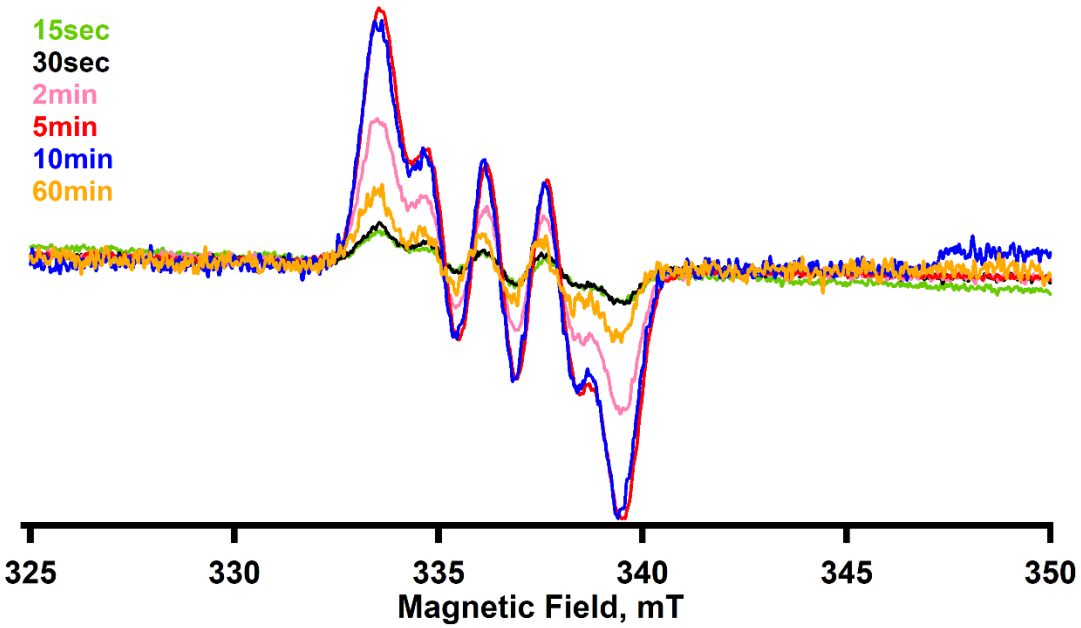


Figure 8. Time course EPR signals of compound **8**. Experimental conditions: Temperature, 80 K; microwave frequency, 9.436 GHz; microwave power, 1 mW (20dB attenuation); modulation amplitude, 1 mT; scan time of 60 s.

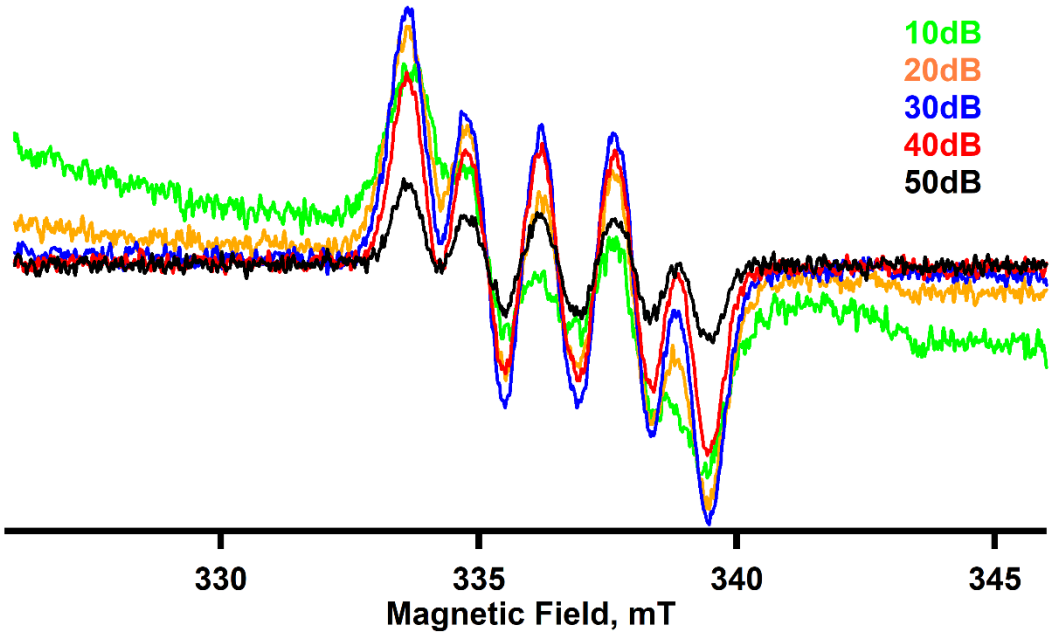


Figure 9. EPR signals of compound **8** at 5min using various attenuation numbers. Experimental conditions: Temperature, 60 K ; microwave frequency, 9.436 GHz; modulation amplitude, 1 mT; scan time of 60 s.

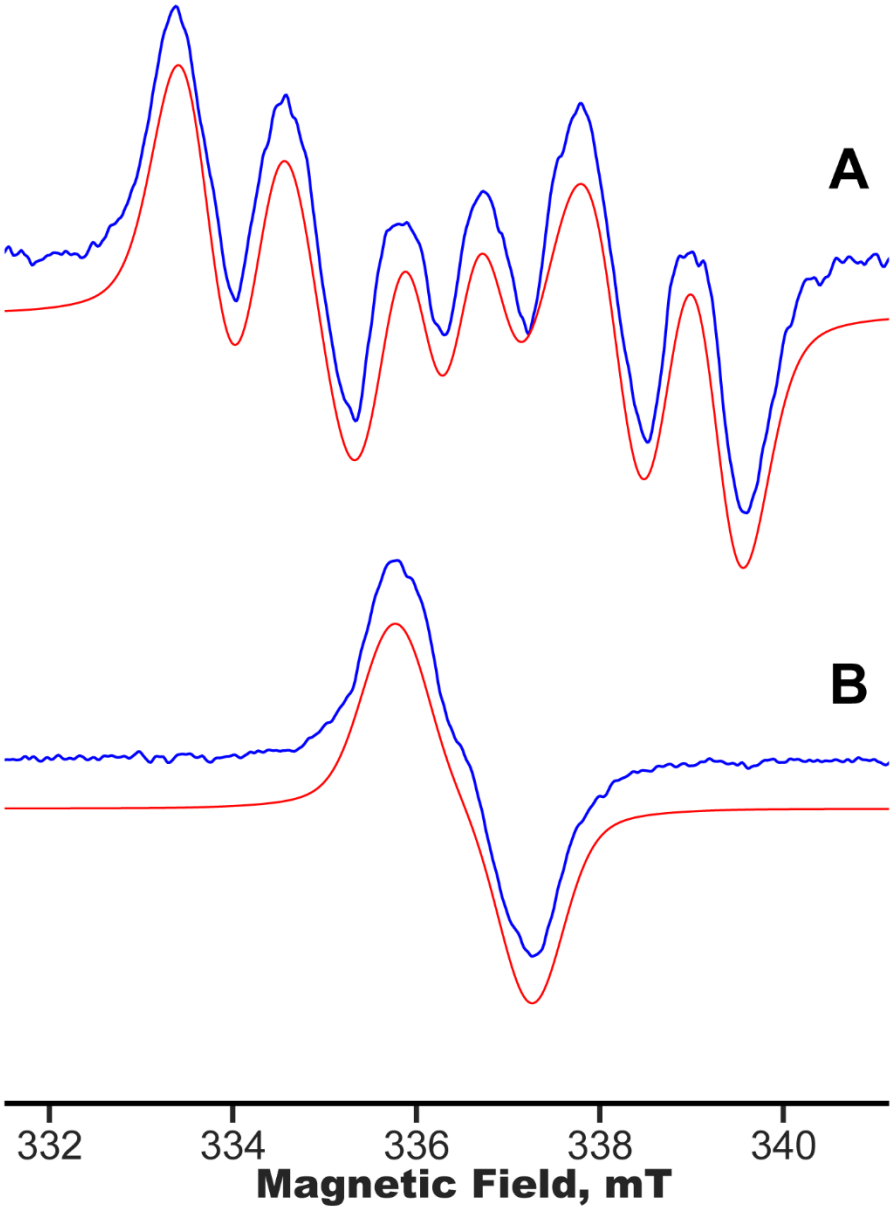


Figure 10. Simulation of the EPR spectra of radical intermediate obtained using natural-abundance substrate **13** (A) and ^{2}H-isotope labeled compound **14** (B). Experimental conditions: Microwave frequency, 9.436 GHz, Microwave power, 0.1 mW (30 dB attenuation), modulation amplitude, 0.2 mT and scan time, 60 s.