Tryptophan lyase (NosL): A cornucopia of 5′-deoxyadenosyl radical mediated transformations

Dhananjay M. Bhandari[†], Dmytro Fedoseyenko[†] and Tadhg P. Begley[*,†]

Supporting Information

Table of Contents:

Materials:
All chemicals were purchased from Sigma-Aldrich unless specified otherwise. LB medium (Lennox formulation) was from EMD Millipore. Kanamycin was bought from Teknova and IPTG was obtained from Lab scientific Inc. Chloramphenicol was from Fisher Scientific. HPLC and LC-MS solvents were purchased from EMD and were used without further purification. Bradford reagent was purchased from ThermoFisher Scientific. Histrap column was obtained from GE healthcare. Econo-Pack 10DG desalting columns were purchased from Bio-Rad. D_2O was purchased from Cambridge Isotope Laboratories. Large cultures were grown and overexpressed in 2.5L baffled ultra yield flasks from Thomson Instrument Company.

Overexpression and purification of NosL, mutants of NosL, FldA and FldR:
Gene of *nosl* from *Streptomyces actuosus* was used for all the studies. The overexpression and purification of all enzymes were performed as previously described. For NosL and its mutants, iron content was determined using the ferene assay while sulfide content was determined using the methylene blue assay (methods previously described)[1].

Enzymatic reaction conditions for NosL:
All enzymatic reactions of NosL and its mutants were carried out in an anaerobic chamber containing 95-97% nitrogen and 3-5% hydrogen. A typical enzymatic reaction was performed in 100 mM phosphate buffer, pH7.5 containing NosL (75-150 uM), dithionite (750 uM), L-tryptophan / substrate analog (400-500 uM) and SAM (600 uM). When assays were performed in the presence of the flavodoxin system, NosL was desalted using a Bio-Spin 6 column (Bio-Rad) into 100 mM phosphate buffer, pH 7.5. The components of the enzymatic reaction were NosL (75-100 uM), FldA (60 uM), FldR (40 uM), NADPH (750 uM), L-tryptophan / substrate analogue (500 uM) and SAM (600 uM). The enzymatic reactions were incubated at room temperature for 2-3 h. Protein was removed by ultrafiltration using 10 kda cut-off filters (VWR) and the reaction mixture was further analyzed by HPLC and LC-MS.

HPLC parameters:
An Agilent 1260 HPLC equipped with a quaternary pump was used. The system included a diode array UV-Vis detector and eluted compounds were detected by absorbance at 254, 260, 280, 288, 320, 340, and 475 nm. The parameters for the fluorescence detector were: excitation at 385 nm and emission at 484 nm. The HPLC analysis was performed on a ZORBAX Eclipse XDB-C18 column (15 cm x 4.6 mm, 5 µm particles, Agilent Technologies). Typical injection volumes were in the range of 10-50 µl. Data was processed using ChemStation ver. B.04.01 SP1 (Agilent technologies).

HPLC conditions:
A- Water
B- 100mM Potassium phosphate buffer, pH 6.6 OR 10 mM Ammonium acetate
C- Methanol

HPLC method:
0 min – 100% B, 5min – 100% B, 14 min – 7% A 70% B 23% C, 25 min – 25% A 0% B 75% C, 28 min - 25% A 0% B 75% C, 32 min - 100% B, 36 min - 100%B.

LC-MS parameters:
LC-ESI-TOF-MS was performed using an Agilent 1260 HPLC system equipped with a binary pump and a 1200 series diode array detector followed by a MicroToF-Q II mass spectrometer (Bruker Daltonics) using an ESI source either in negative mode or positive mode. The analysis was performed on an LC-18-T column (15 cm x 3 mm, 3 μm particles, Supelco). Typical injection volumes were in the range of 20-80 μl. The data was processed using DataAnalysis 4.0 SP1 (Bruker Daltonics).

LC conditions:
A- 5 mM ammonium acetate buffer, pH6.6
B- 75% Methanol and 25% Water.

LC method: (for negative mode on MS)
0min – 100% A, 7min – 100% A, 10min – 80% A 20%B, 27 min – 100%B, 29min – 100% B, 30min – 100% A, 40min – 100%A.

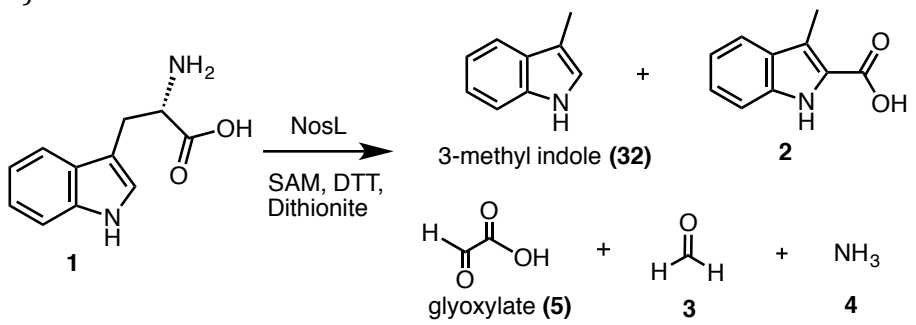
LC method: (for positive mode on MS)
0min – 100%A, 2min – 100%A, 4min – 80%A 20%B, 27min – 100%B, 29min – 100%B, 30min – 100%A, 40min – 100%A.

MS parameters: (for all LC-MS experiments)
Capillary, -4500 V; capillary offset, -500 V; nebulizer gas, 3.0 bar; dry gas, 10 L/min; dry gas temperature, 200 °C; funnel 1 RF, 250.0 Vpp; funnel 2 RF, 300.0 Vpp; ISCID, 0.0 eV; hexapole RF, 200 Vpp; quadrapole ion energy, 3.0 eV; collision cell, collision energy, 8.0 eV; collision RF, 150.0 Vpp, transfer time, 80.0 μs; prepulse storage, 5.0 μs.

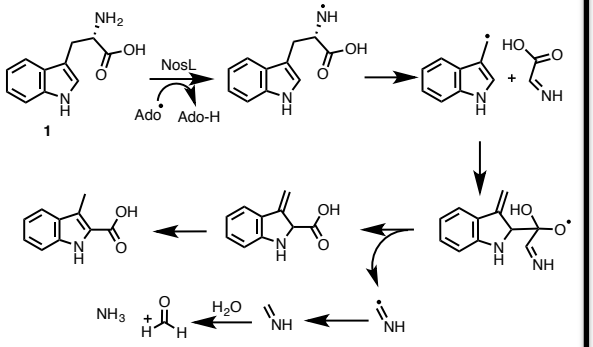
NMR analysis:
NMR spectra of all the synthetic samples were recorded on Bruker Avance III 400 MHz instrument. NMR characterization of samples collected by HPLC was performed on Bruker Avance III 500 MHz instrument with H-C-N cryoprobe in 3 mm Wilmad labglass (328-PP-7) high precision NMR tubes.

Summary of previous studies on the NosL-catalyzed reaction:

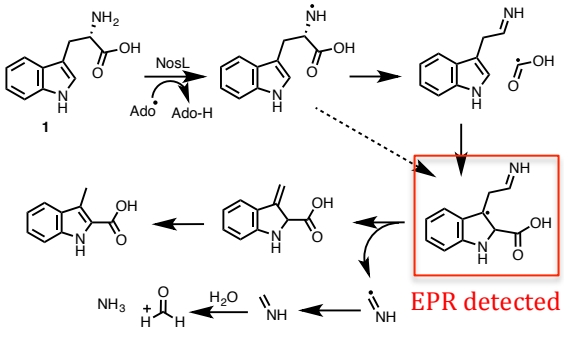
A)



B)



C)



D)

NosL variant	Product 1	Product 2
WT	3-methyl-2-indolic acid (**2**)	3-methylindole (**32**)
Y90F	3-methyl-2-indolic acid (**2**)	3-methylindole (**32**)
Y90A	3-methyl-2-indolic acid (**2**)	3-methylindole (**32**)
C200A	3-methyl-2-indolic acid (**2**)	3-methylindole (**32**)
S340A	3-methyl-2-indolic acid (**2**)	3-methylindole (**32**)
R323K	Indole-3-pyruvic acid (**11**)	adduct (**22**)

Figure S1: Summary of previous studies on NosL. A) Products derived from L-tryptophan (**1**) during the NosL-catalyzed reaction *in vitro*.[2] B) Mechanistic proposal involving C_α—C_β scission after generation of the nitrogen centered radical.[1] A product derived from late stage intermediate was reported with an analogue in which, the amino group of **1** is replaced by methyl group.[3] C) Mechanistic proposal involving C_α—$C_{carbonyl}$ scission was recently reported based on EPR characterization of the intermediate highlighted in red box.[4] D) Characterization of the active site mutants of NosL (including this study).[1] Several attempts to obtain steady-state parameters for NosL WT and its mutants were unsuccessful due to low turnover and poor reproducibility in the ratio of products generated.

Reaction of NosL Y90A with L-tryptophan (1):

We have previously demonstrated that the active site residue Y90 is not essential for the activity of NosL by testing NosL Y90F mutant. We further investigated the activity of NosL Y90A with L-tryptophan (**1**). As shown in Figure S2 only in the 'Full Rxn' sample do we see the formation of 3-methylindole (**32**) and 3-methyl-2-indolic acid (**2**). This suggests that the NosL Y90A mutant is also active and behaves similarly to the NosL WT enzyme.

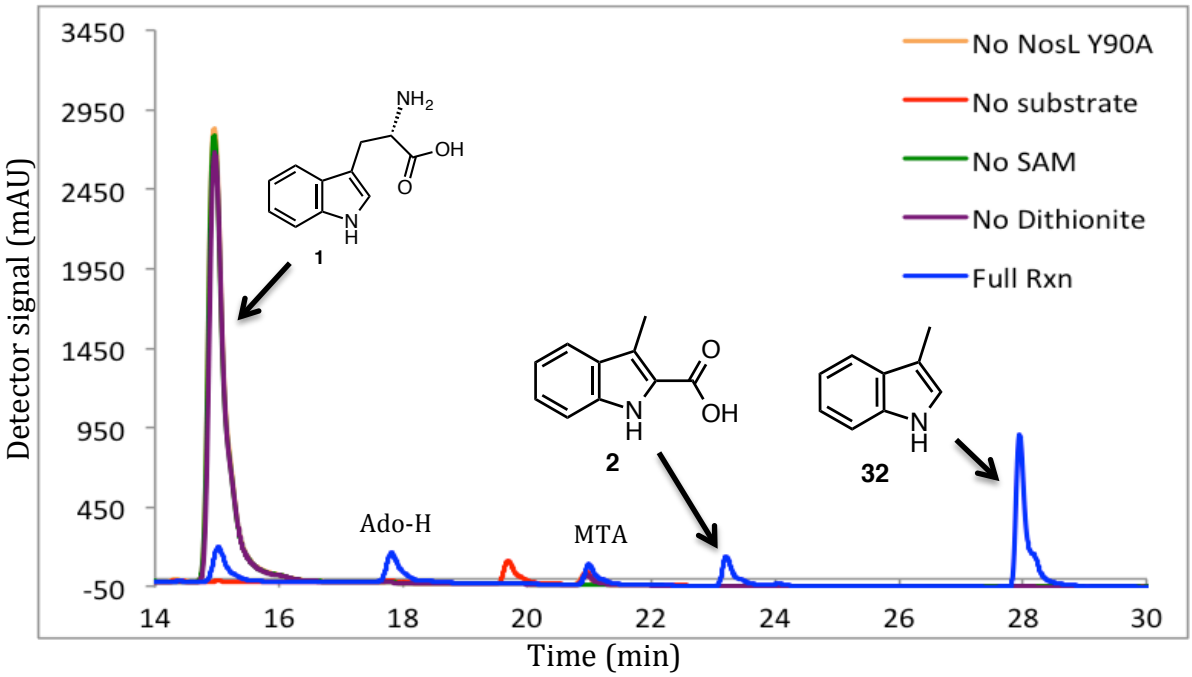


Figure S2: HPLC analysis of the NosL Y90A reaction with L-tryptophan (**1**) at 288 nm. Only in the 'Full Rxn' sample was consumption of the substrate and concomitant formation of 3-methylindole (**32**) and 3-methyl-2-indolic acid (**2**) observed.

Reaction of NosL Y90A with N_α-cyclopropyltryptophan (6):

We have previously reported the synthesis of N_α-cyclopropyltryptophan (**6**).[1] However, this analog is not a substrate for NosL WT. Since the cyclopropyl group is quite bulky, we made additional space in the active site by generating NosL Y90A mutant. As shown in Figure S3A, when the activity of NosL was investigated with **6**, we saw the formation of a new signal. This signal was identified as indole-3-pyruvic acid (**11**). The amino fragment was further identified as cyclopropylamine (**10**) by derivatization with NBD-F (**33**).

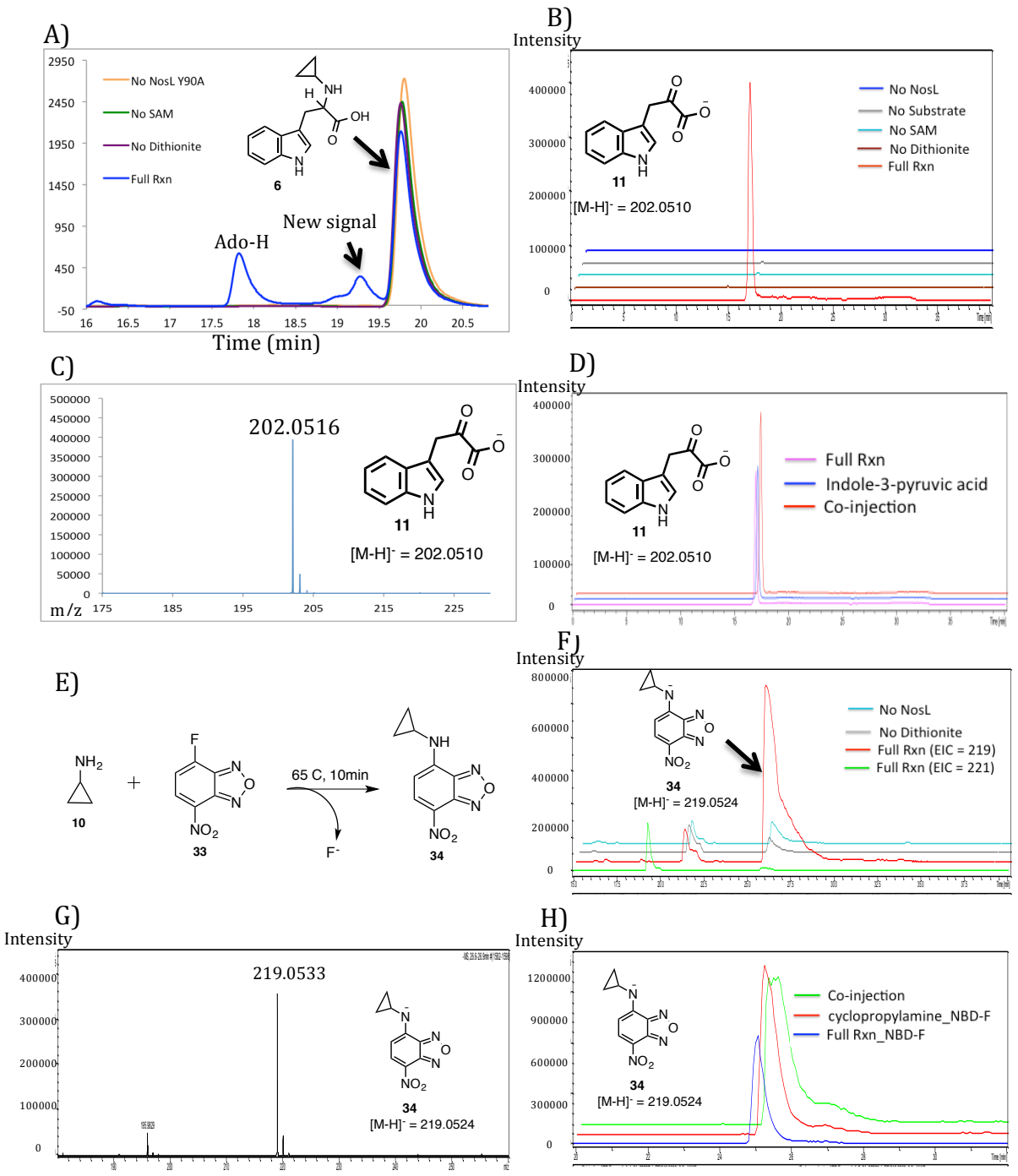


Figure S3: HPLC analysis of the NosL Y90A reaction with N_{α}-cyclopropyltryptophan. A) HPLC analysis at 280 nm indicates formation of a new signal in the 'Full Rxn' sample. B) LC-MS analysis showing EIC $[M-H]^-$ = 202.0 Da indicating the formation of indole-3-pyruvic acid (**11**) in the 'Full Rxn' sample. C) MS of indole-3-pyruvic acid (**11**) generated during the enzymatic reaction. D) LC-MS analysis showing EIC $[M-H]^-$ = 202.0 Da and co-injection with the authentic standard of **11** confirming its formation. E) Reaction scheme showing the trapping of cyclopropylamine (**10**) with NBD-F (**33**). F) LC-MS analysis after NBD-F treatment showing EIC $[M-H]^-$ = 219.0 Da and 221 Da suggesting the formation of the cyclopropylamine-NBD adduct (**34**) in the 'Full Rxn' sample. G) MS of **34** generated after NBD-F derivatization of the enzymatic reaction. H) LC-MS analysis after NBD-F treatment showing EIC $[M-H]^-$ = 219.0 Da and co-injection with NBD-F derivatized authentic standard of **10** confirming formation of **34.**

Analysis of 5'-deoxyadenosine (35) generated during the reaction of NosL Y90A with N_α-cyclopropyltryptophan (6).

When the reaction of NosL Y90A with **6** was carried out in the presence of 95% D_2O buffer, the 5'-deoxyadenosine (**35**) generated did not show incorporation of any deuterium. To identify the position of H-atom abstraction from the substrate, we synthesized **6** deuterated at the C_α position. C_α-deuterated **6** was synthesized with the previously described protocol with a slight modification.[1] $NaBD_3CN$ was used for reduction of the imine generated by the reaction of indole-3-pyruvic acid (**11**) and cyclopropylamine (**10**). When C_α-deuterated **6** isotopologue was used for the enzymatic reaction with NosL Y90A, it also generated indole-3-pyruvic acid (**11**) and cyclopropylamine (**10**) as products. However, analysis of the 5'-deoxyadenosine (**35**) generated during the enzymatic reaction showed incorporation of a single deuterium.

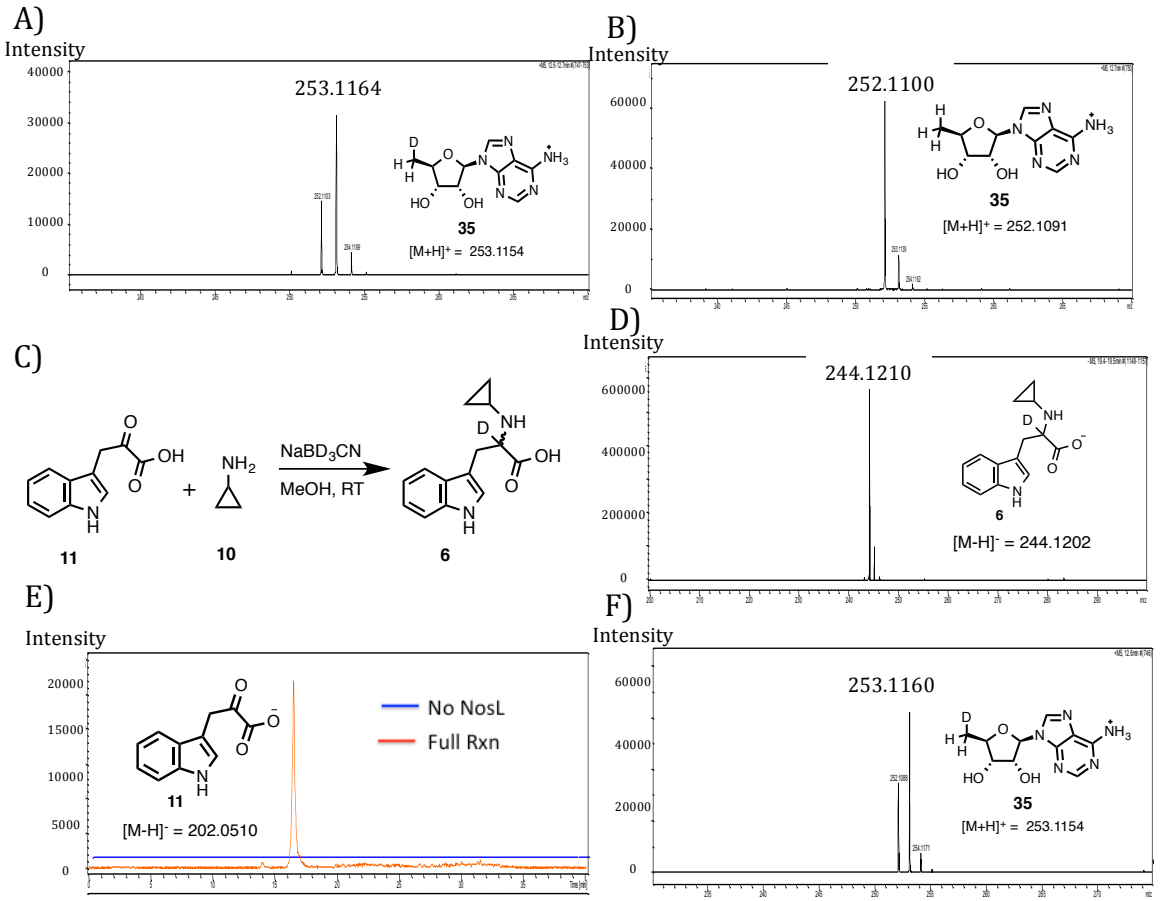


Figure S4: Analysis of the 5'deoxyadenosine generated during the NosL reaction with **6**. A) MS of 5'-deoxyadenosine (**35**) generated with NosL Y90A in the absence of any amino acid substrate (deuterated buffer). B) MS of 5'deoxyadenosine (**35**) generated during the reaction of NosL Y90A with **6** in 95% D_2O buffer. C) Scheme for the synthesis of C_α-deuterated-N_α-cyclopropyltryptophan. D) MS of the synthesized deuterated isotopologue. E) LC-MS analysis showing EIC $[M-H]^-$ = 202.0 Da confirming the formation of indole-3-pyruvic acid (**11**) in the 'Full Rxn' sample. F) MS of 5'-deoxyadenosine (**35**) generated during the reaction of NosL Y90A with C_α-deuterated-N_α-cyclopropyltryptophan.

The synthesis of the tryptophan methylene analogue (14):

Compound **14** was synthesized according to the previously reported procedure as shown in Figure S5A.[5] Briefly, in a dry round bottom flask, add 2 ml dry tetrahydrofuran (THF) and 130 mg (6 mmol) Mg turnings. Under an inert atmosphere, add 650 mg (6 mmol) of ethyl bromide (**36**) dissolved in 2 ml THF. Stir on an ice bath for ~30 min after all Mg turnings have dissolved. Add dropwise solution of 590 mg (5 mmol) indole (37) dissolved in 2 ml THF and stir for 30 min. Dissolve 1 g (5.5 mmol) of methylbromomethacrylate (**38**) and add it drop wise. Remove the ice bath and stir at room temp for 30 min. Quench the reaction with water. Extract the aqueous phase with ethyl acetate and dry over $MgSO_4$. The pure ester **39** was obtained after chromatography (silica, hexane:ethyl acetate = 90:10). To hydrolyze the methyl ester of **39**, dissolve the purified product in 5 ml of 80% ethanol. Add 300 mg KOH and stir for 5 h at room temp. Then acidify the solution to pH 1 and extract the compound using diethyl ether. Purify **14** using flash chromatography (dichloromethane:methanol = 95:5). NMR was recorded in MeOD.

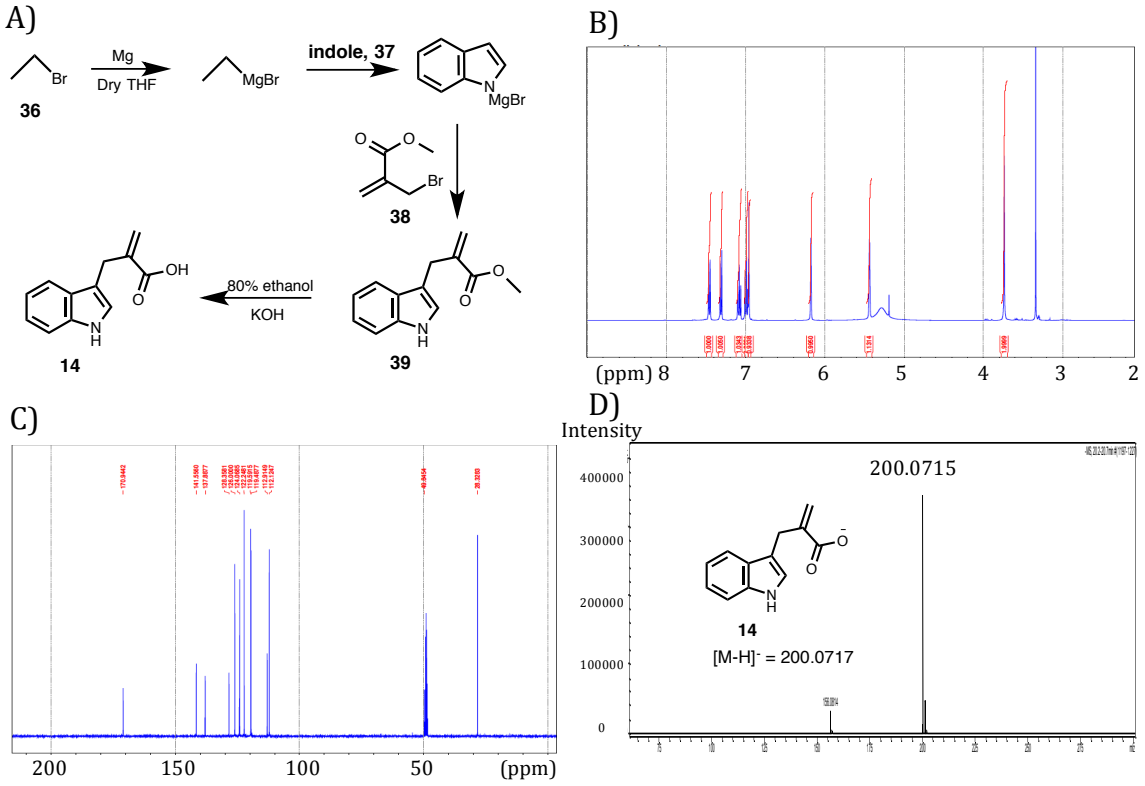


Figure S5: Synthesis and characterization of compound **14**. A) Synthetic scheme. B) ^{1}H-NMR. C) ^{13}C-NMR. D) MS.

Reaction of NosL with the tryptophan methylene analogue (14):

HPLC analysis of the reaction of **14** with NosL WT, revealed about 3% consumption of the substrate. LC-MS analysis demonstrated that 5'-deoxyadenosine (**35**) was not formed in the 'Full Rxn'. Three new signals were observed with a mass corresponding to adduct between the 5'-deoxyadenosyl radical and **14**. When this reaction was carried out in the presence of NosL R323K, the same three signals were observed at greatly enhanced levels (~10-fold).

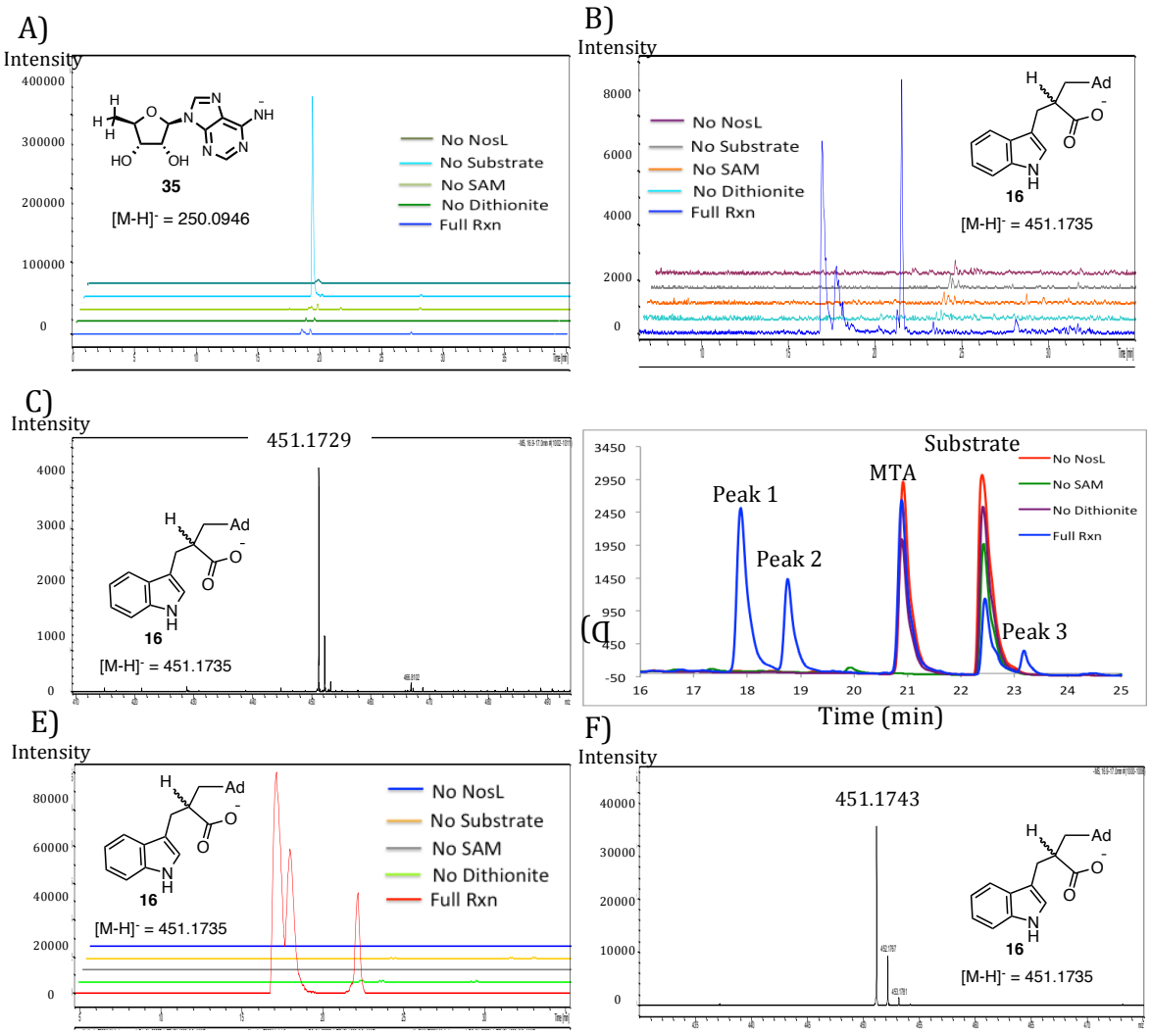
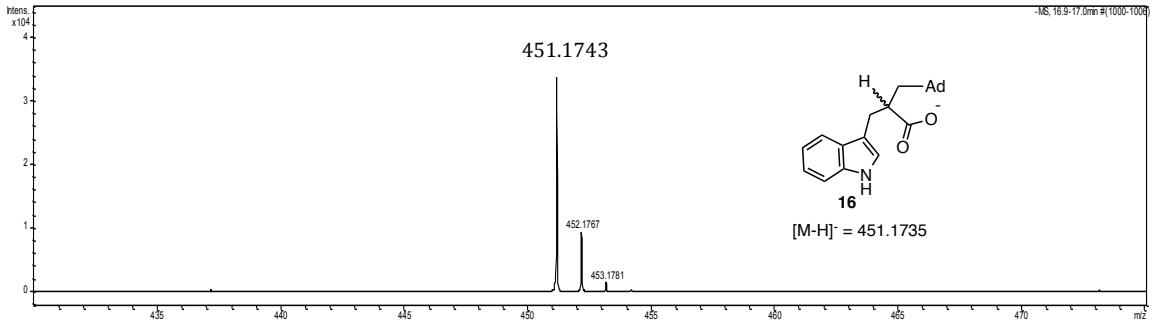


Figure S6: Analysis of the NosL reaction with **14**. A) LC-MS analysis showing EIC $[M-H]^-$ = 250.0 Da confirming the absence of **35** in the 'Full Rxn' sample with NosL WT. B) LC-MS analysis showing EIC $[M-H]^-$ = 451.1 Da suggesting the formation of three isomeric products corresponding to the mass of the adduct resulting from addition of the 5'-adenosyl radical to the **14** (one isomer shown). C) MS of the Peak 1 product generated in the 'Full Rxn' sample. D) HPLC analysis at 280 nm showing the enhanced formation of the three products when **14** is reacted with NosL R323K. E) LC-MS analysis showing EIC $[M-H]^-$ = 451.1 Da confirming that these three signals correspond to the mass of the adducts seen with NosL WT. F) MS of the Peak 1 product generated in 'Full Rxn' sample with NosL R323K.

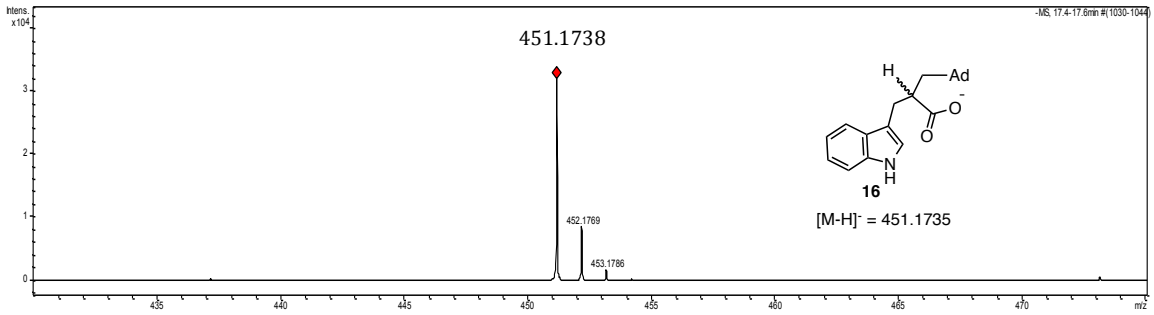
Reaction of NosL with 14 in 95% D_2O buffer:

When the reaction of **14** was carried out with NosL WT in 95% deuterated buffer, the Peak 1 and Peak 2 compounds (Figure S6D) did not show deuterium incorporation while the Peak 3 compound showed incorporation of a single deuterium.

A)



B)



C)

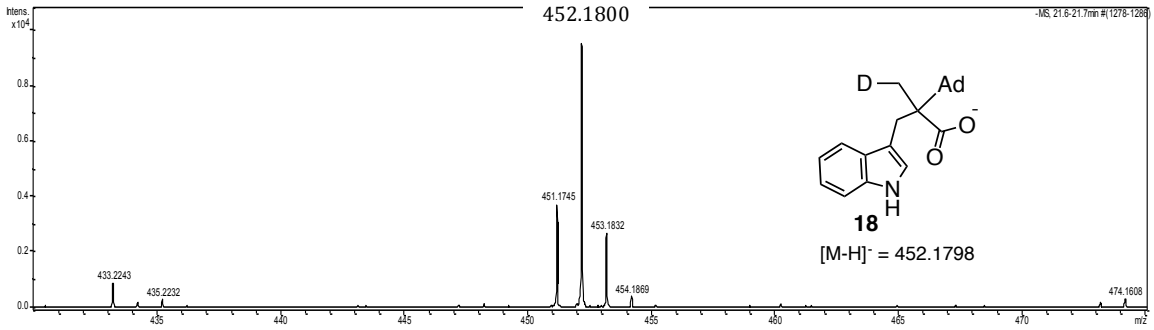


Figure S7: MS analysis of the product generated with the tryptophan methylene analogue (**14**) when the NosL catalyzed reaction is performed in 95% D_2O buffer. A) MS of the Peak 1 product does not show deuterium incorporation. B) MS of the Peak 2 product does not show deuterium incorporation. C) MS of the Peak 3 product shows incorporation of deuterium.

Reaction of NosL R323K with indole-3-pyruvic acid (11):

HPLC analysis of the reaction of indole-3-pyruvic acid (**11**) with NosL R323K revealed the formation of a new product. 5'-deoxyadenosine (**35**) was not observed in the 'Full Rxn' sample as previously observed with the tryptophan methylene analog (**14**) suggesting that the new product was generated by the addition of the 5'deoxyadenosyl radical to indole-3-pyruvic acid (**11**). In addition, LC-MS analysis demonstrated that this product had lost the carboxylate group.

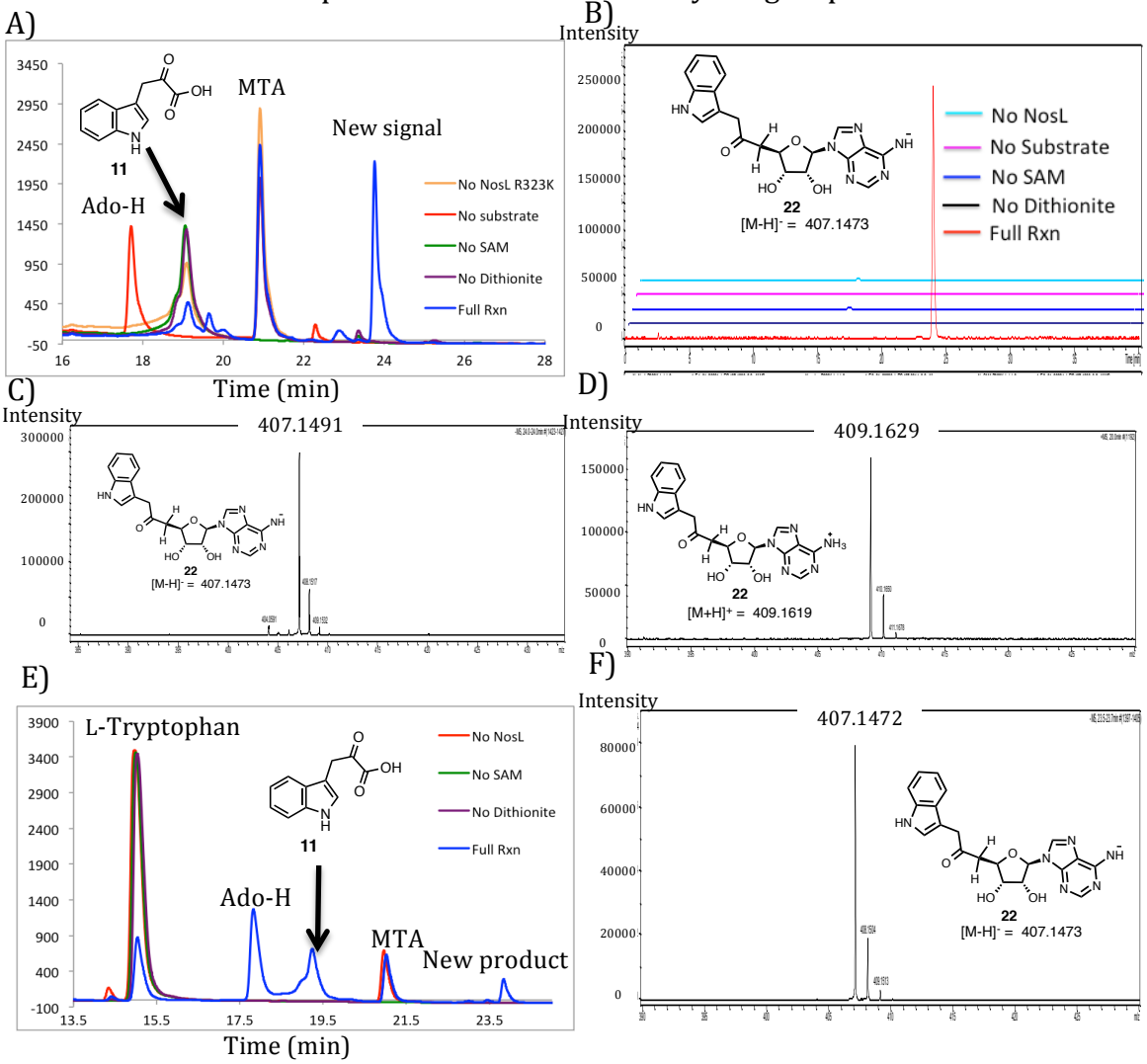
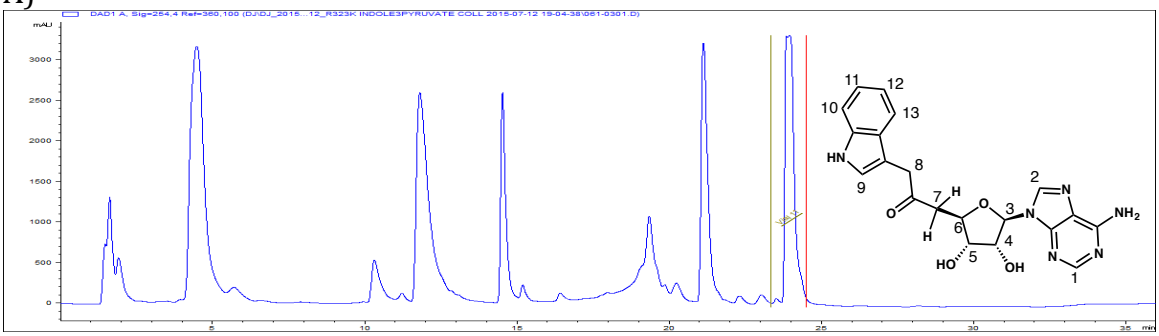


Figure S8: Analysis of the NosL R323K reaction with indole-3-pyruvic acid **(11).** A) HPLC analysis at 280 nm of the NosL R323K reaction with **11** showing consumption of substrate and formation of a new product only in the 'Full Rxn' sample. B) LC-MS analysis showing EIC $[M-H]^-$ = 407.1 Da suggesting the formation of an adduct between the 5'deoxyadenosyl radical and **11** with concomitant loss of CO_2. C) MS of the reaction product (new signal) in negative mode. D) MS of one of the reaction product (new signal) in positive mode. E) HPLC analysis of NosL R323K with L-tryptophan at 280 nm showing the formation of **11** and a new product, which we had previously reported as an unknown compound.[1] F) MS of this new product identical to the product formed when **11** was reacted with NosL R323K.

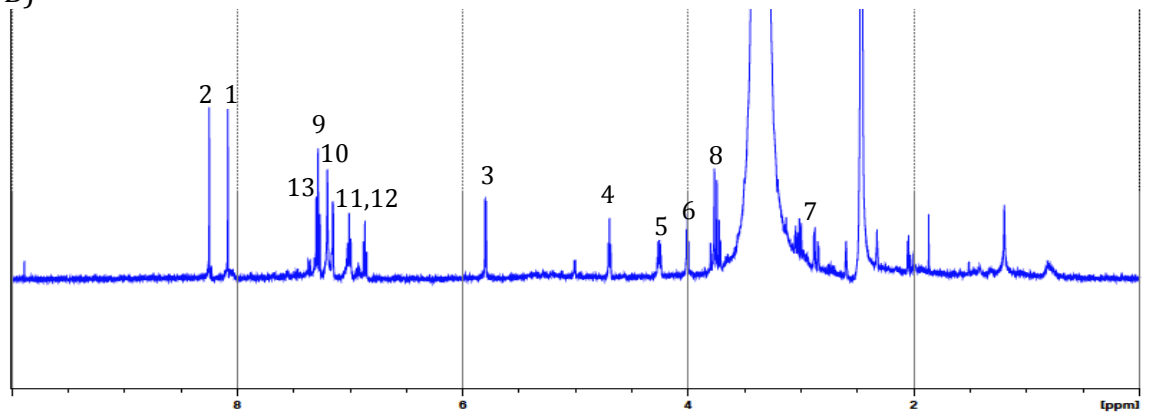
NMR characterization of the product generated when indole-3-pyruvic acid is used as substrate for NosL R323K:

To characterize the product, the reaction of indole-3-pyruvic acid with NosL R323K was scaled up and the product was purified by HPLC using 10 mM ammonium acetate as the elution buffer. The collected sample was lyophilized and dissolved in d_6-DMSO for NMR analysis.

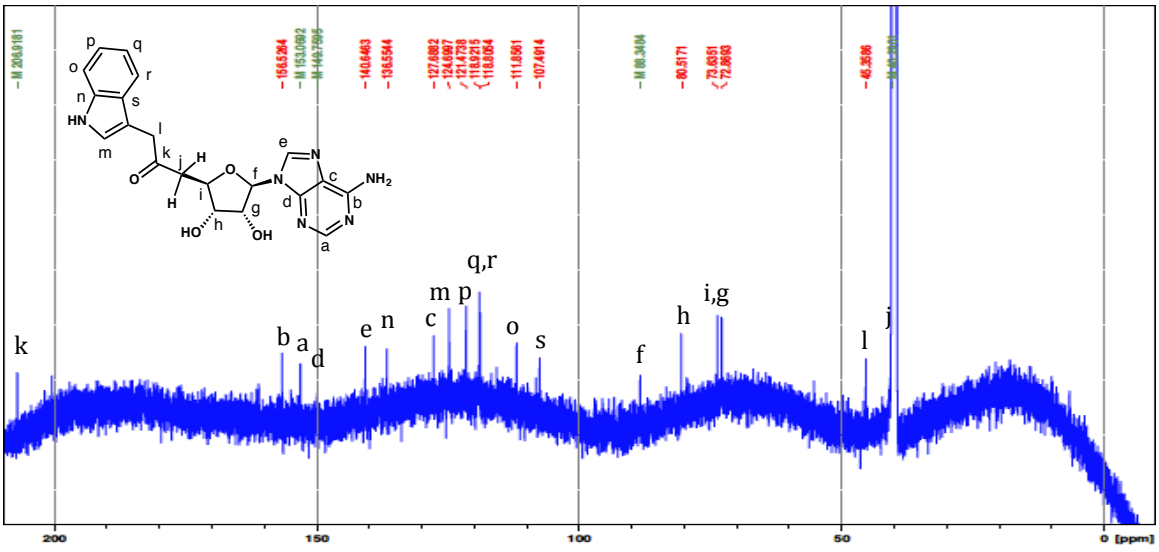
A)



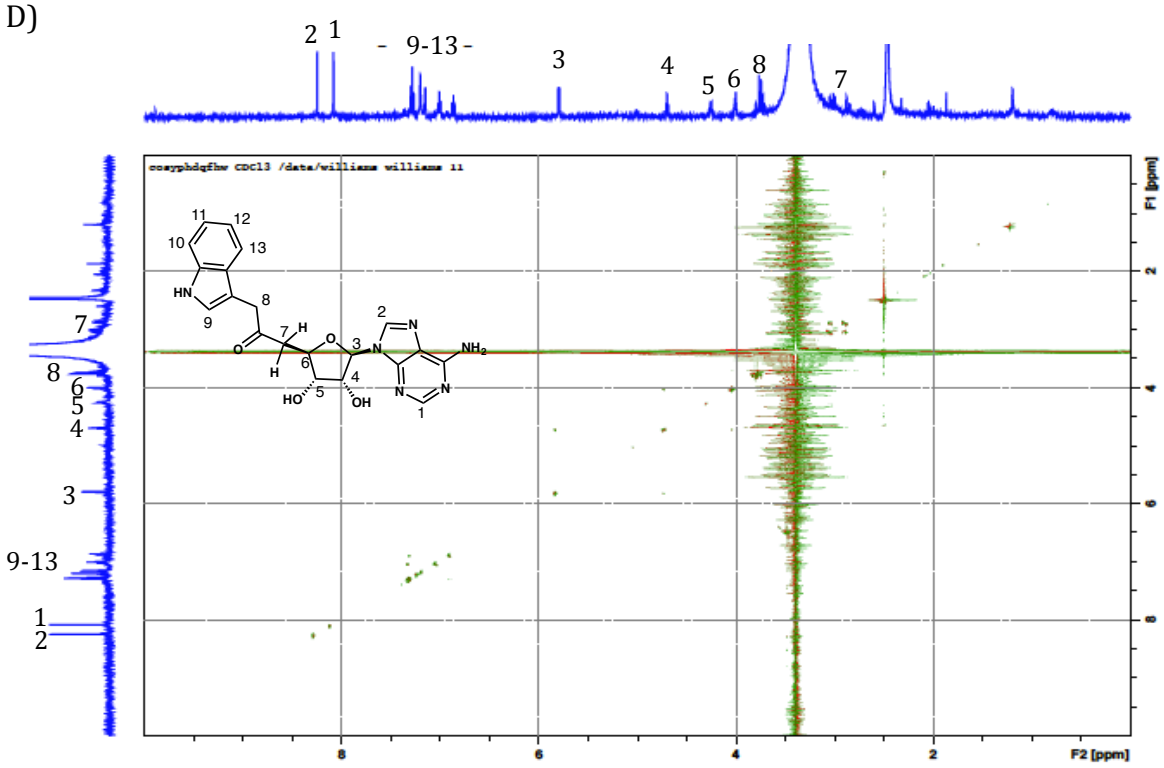
B)



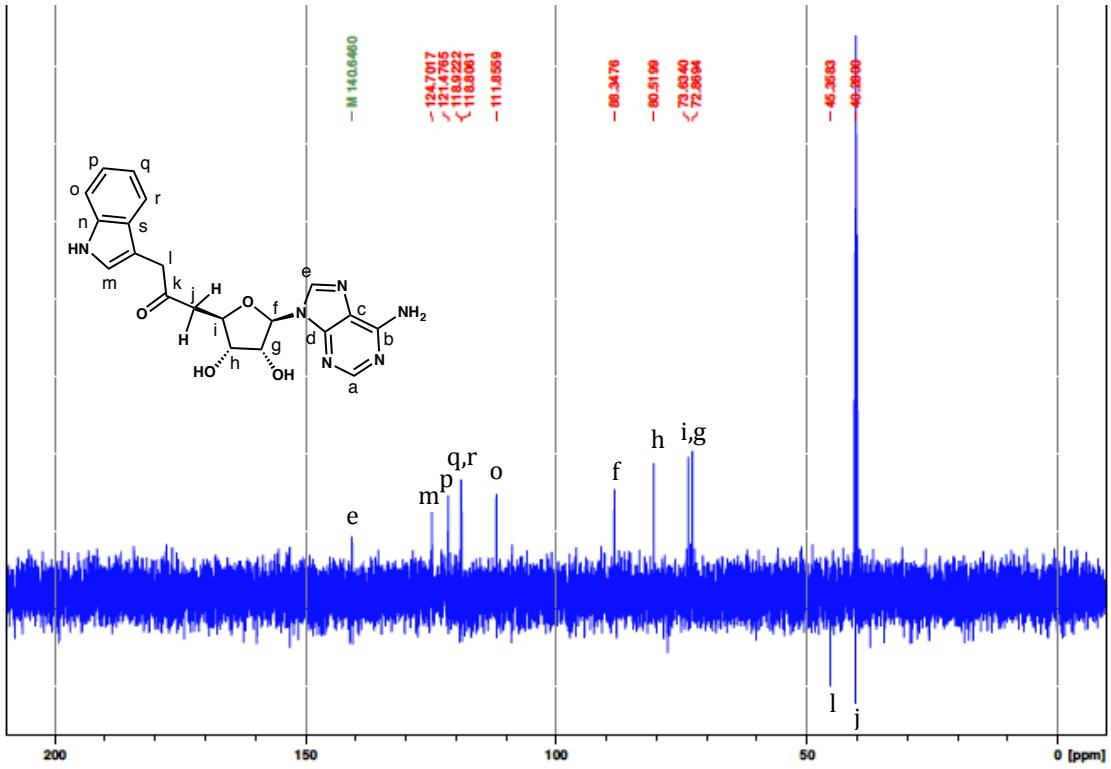
C)



D)



E)



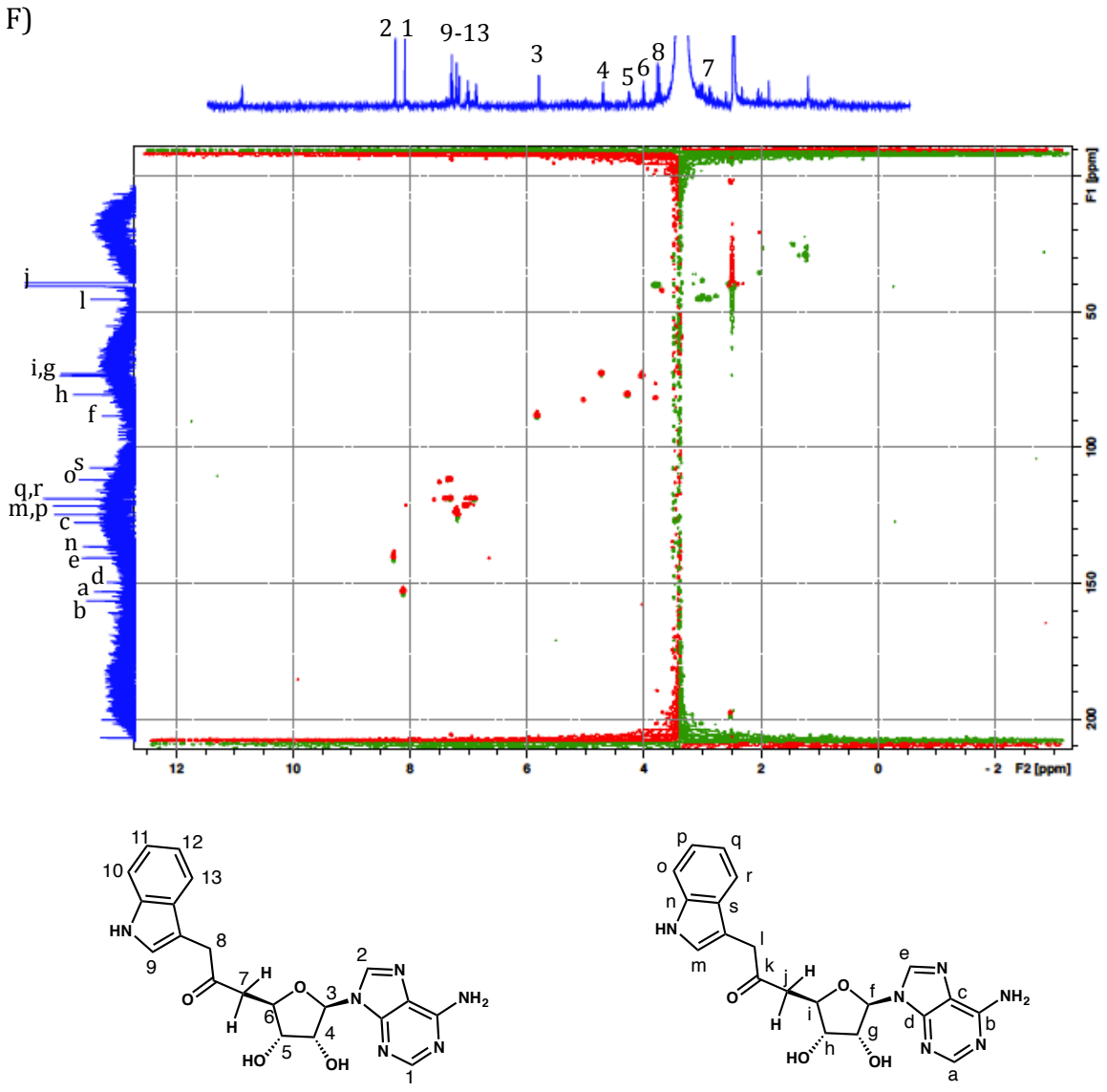


Figure S9: Characterization of the product generated by the reaction of NosL R323K with indole-3-pyruvic acid (**11**). A) HPLC analysis at 260 nm showing the collection of the signal corresponding to the product. B) ^{1}H-NMR of the collected product **22** in d_6-DMSO. C) ^{13}C-NMR of compound **22**. D) ^{1}H-^{1}H COSY-NMR of product **22.** E) DEPT-135 NMR spectrum of compound **22**. CH_2 are shown negative while CH and CH_3 are positive. F) ^{1}H-^{13}C HSQC-NMR of product **22**.

Characterization of 5'-deoxy-5'-thioadenosine (23):

The formation of a SAM-derived product, in the absence of the amino acid substrate, was observed with NosL Y90A. This product was formed in greater amounts than 5'-deoxyadenosine (**35**) with this variant. This new compound had an identical UV-Vis spectrum with SAM. LC-MS analysis gave a mass that was consistent with 5'-deoxy-5'-thioadenosine (**23**). This assignment was confirmed by treating the filtered enzymatic reaction mixture with iodoacetamide at pH 9 for ~1 hr at 30 °C.

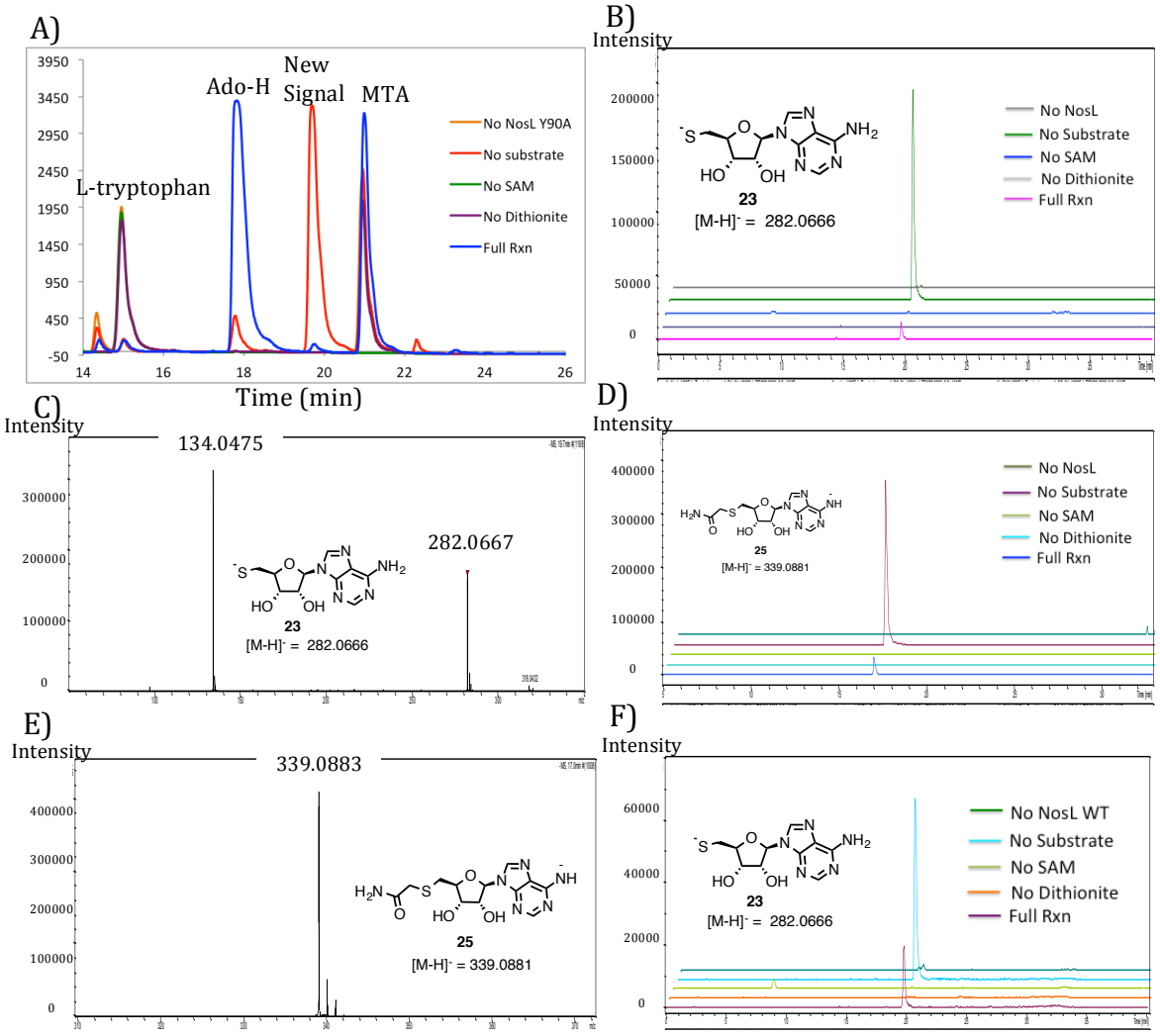
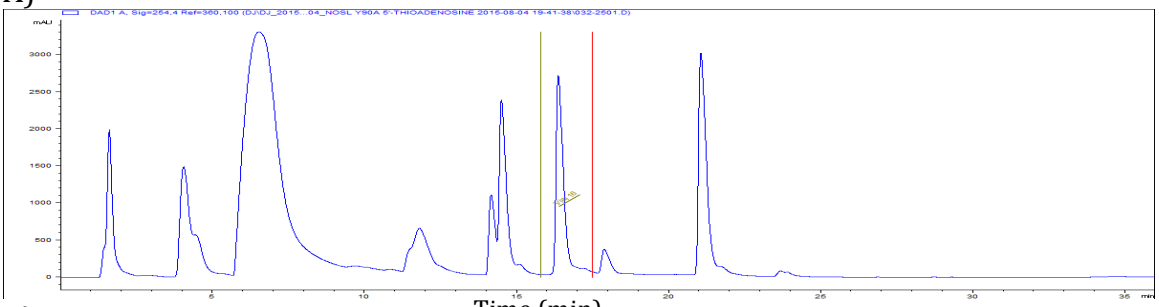


Figure S10: Analysis of the formation of 5'-deoxy-5'-thioadenosine (**23**). A) HPLC analysis at 260 nm of the NosL Y90A reaction with L-tryptophan (1). A new SAM-derived product is formed, in the absence of the amino acid substrate, that elutes at 20 min. B) LC-MS analysis showing EIC $[M-H]^-$ = 282.0 Da suggesting that this compound is **23**. C) MS of **23**. D) LC-MS analysis showing EIC $[M-H]^-$ = 339.0 Da confirming the derivatization of **23** with iodoacetamide. The signal of EIC $[M-H]^-$ = 282.0 Da disappeared after iodoacetamide treatment. E) MS of derivatized 5'-deoxy-5'-thioadenosine (**25**). F) LC-MS analysis showing EIC $[M-H]^-$ = 282.0 Da confirming formation of **23** in NosL WT. Similar results were obtained when FldA/R was used to reduce the [4Fe-4S] instead if dithionite.

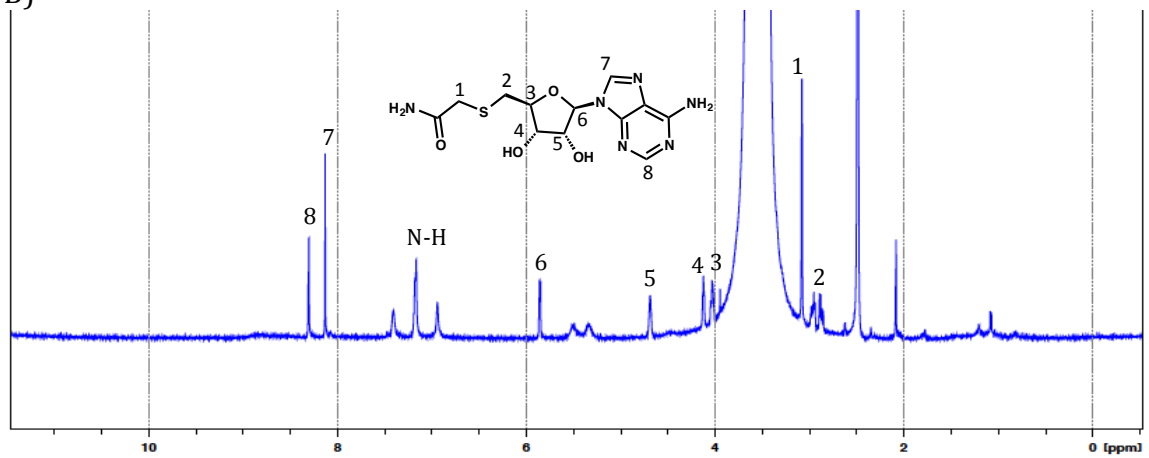
NMR characterization of the iodoacetamide derivatized 5'-deoxy-5'-thioadenosine (25):

The enzymatic reaction of NosL Y90A was scaled up and derivatized with iodoacetamide. The derivatized product was purified by HPLC, lyophilized and the NMR spectra were recorded in d_6-DMSO.

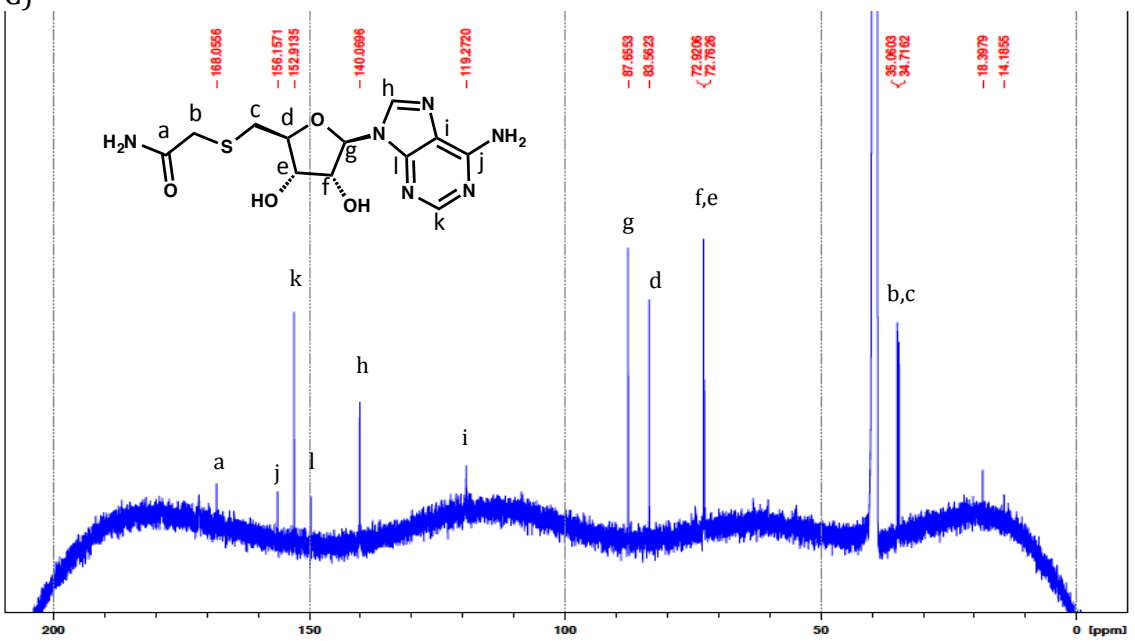
A)



B)



C)



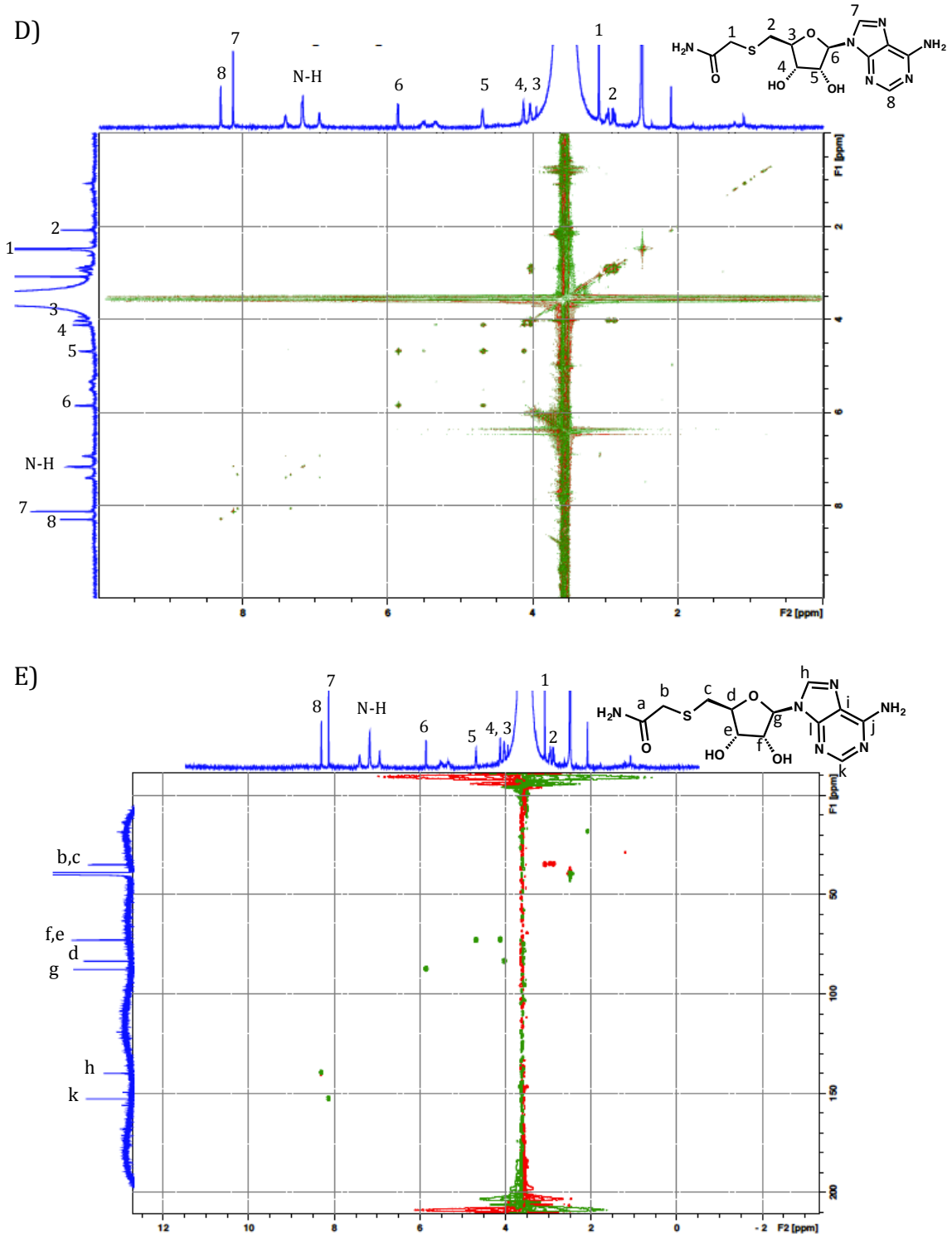
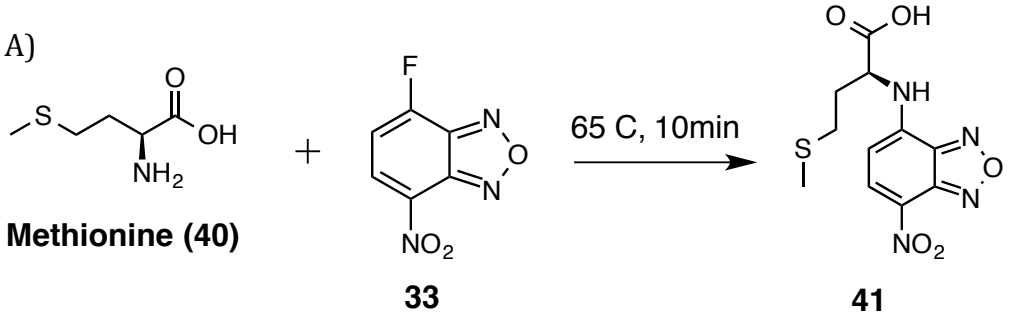


Figure S11: NMR characterization of the iodoacetamide derivative of 5'-deoxy-5'-thioadenosine (**25**). A) HPLC analysis at 260 nm of the iodoacetamide derivatized NosL Y90A derived product. The signal that was collected is highlighted between the red and the green vertical lines. B) ^{1}H-NMR of the collected product **25**. C) ^{13}C-NMR of **25**. D) ^{1}H-^{1}H COSY-NMR of compound **25**. E) ^{1}H-^{13}C HSQC-NMR of compound **25**.

The fate of the amino-acid fragment of SAM:

Methionine is the most likely byproduct of 5'-deoxy-5'-thioadenosine (**23**) formation from SAM. To look for this compound, the filtered enzymatic reaction mixture was treated with 2 mM NBD-F (**33**) at 65 °C for 10 min. before analysis by HPLC and LC-MS. Methionine (**40**) was the only derivatized amine detected.

A)



Methionine (40) **33** **41**

B)

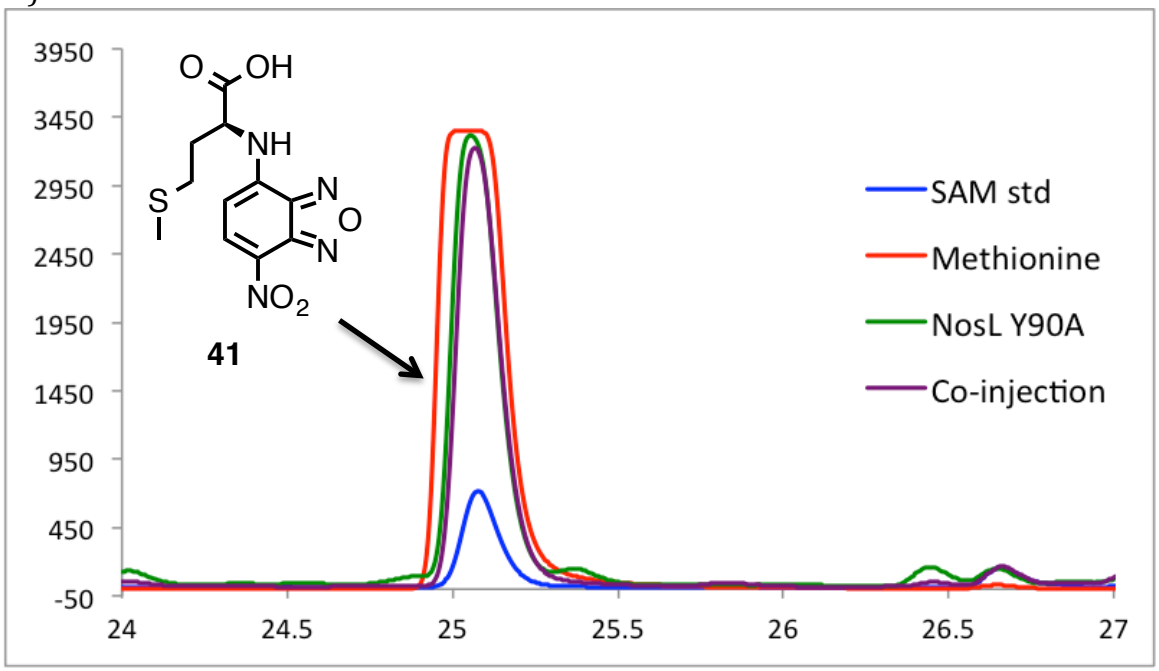


Figure S12: Analysis of the reaction mixture for SAM derived amine products. A) Scheme depicting the derivatization reaction between methionine and NBD-F. B) HPLC analysis at 475 nm of the enzymatic reaction after treatment with NBD-F. The observed reaction product comigrated with an authentic sample of **41.**

BioB catalyzed formation of biotin and 5'-deoxy-5'-thioadenosine (23)[6]

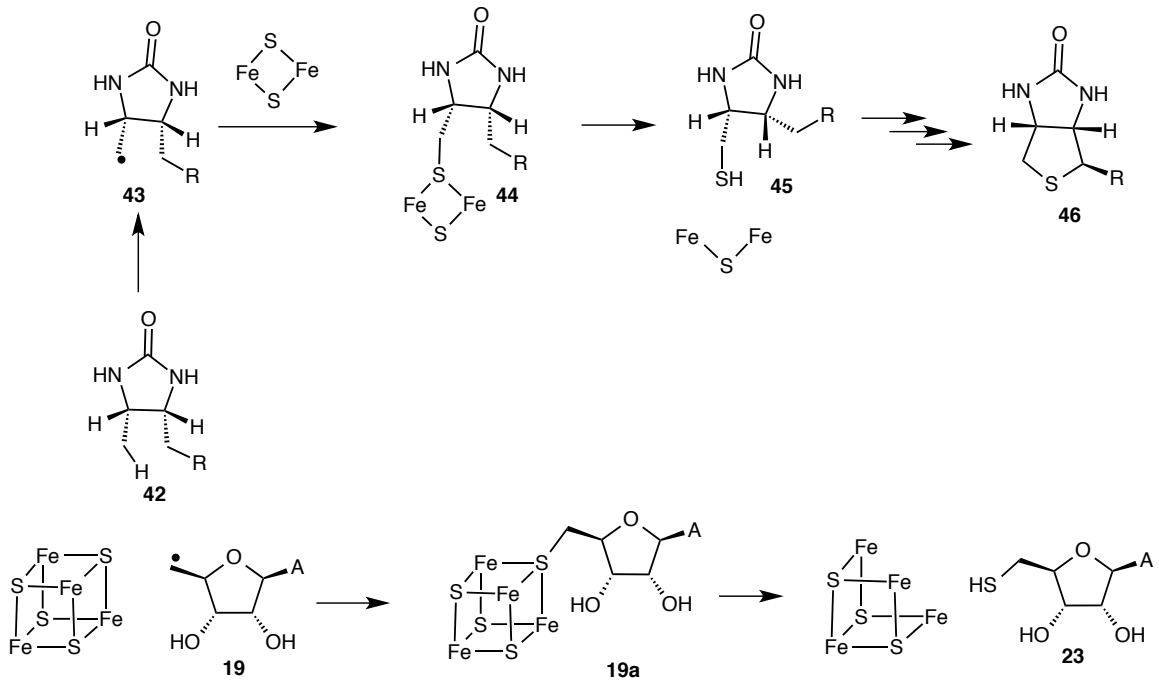


Figure S13: Mechanism for the formation of biotin and 5'deoxy-5'-thioadenosine by biotin synthase.

The synthesis of N-ethyl-L-tryptophan amide (49):

The amide was synthesized by a previously reported protocol with minor variation.[7] To a dry round bottom flask, add 10ml of 2M ethylamine (**48**) in methanol (20 mmol) and 1.3 g (5 mmol) of L-tryptophan methyl ester.HCl (**47**). The reaction mixture was stirred at room temp for ~3 days. After solvent removal, the product **49** was purified by flash chromatography (dichloromethane:methanol = 90:10, silica). Isolated yield ~50%. NMR was recorded in MeOD.

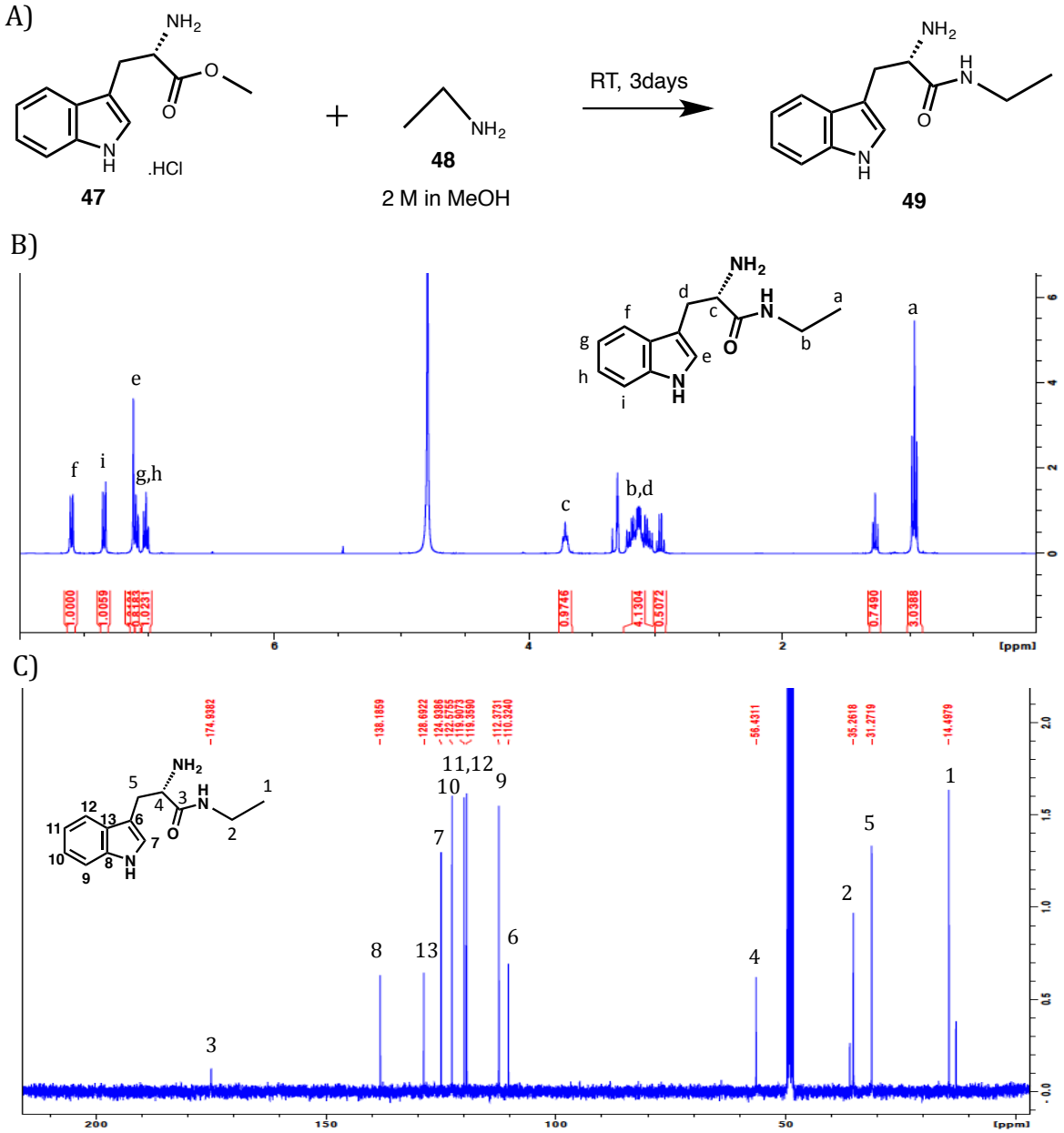


Figure S14: Synthesis of N-ethyl-L-tryptophan amide (**49**). A) Synthetic route. B) ^{1}H-NMR of the product showing a low level of ethylamine as an impurity. C) ^{13}C-NMR of the product.

Synthesis of the methyl and ethyl esters of D,L-indole-3-lactate:

The esters were synthesized by a previously reported protocol.[8] Polymer supported p-toluene sulfonic acid (**51**, 1.2 g, 1.5-2 mmol/g) was added to a solution of 0.5 g of D,L-indole-3-lactate (**50**) in 10ml of methanol, TLC indicated complete conversion after stirring at room temp for 3-5 days. Removal of the resin by filtration and the solvent by evaporation yielded pure product. The NMR was recorded in MeOD. The ethyl ester was prepared in an identical way using ethanol as the solvent.

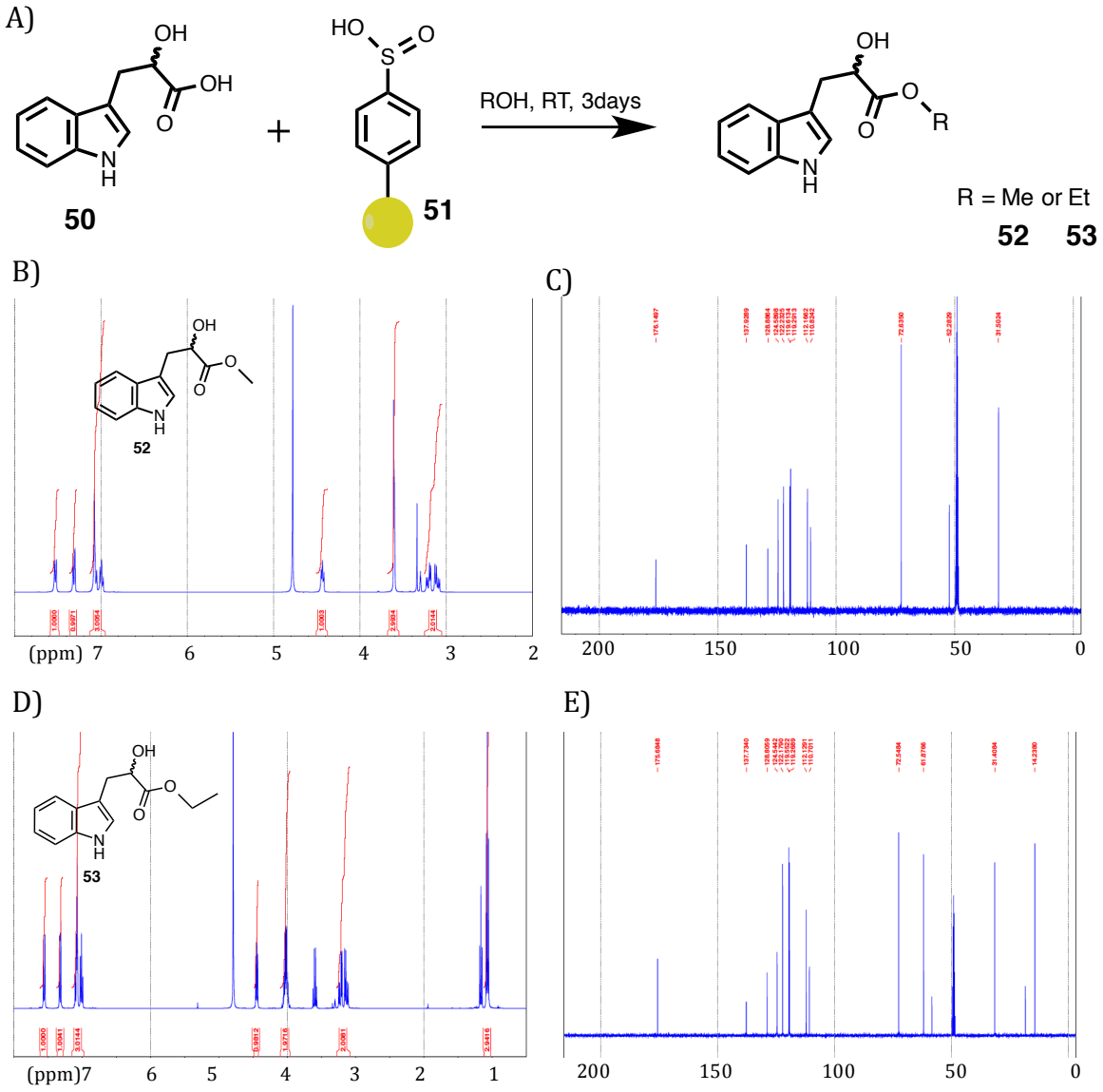
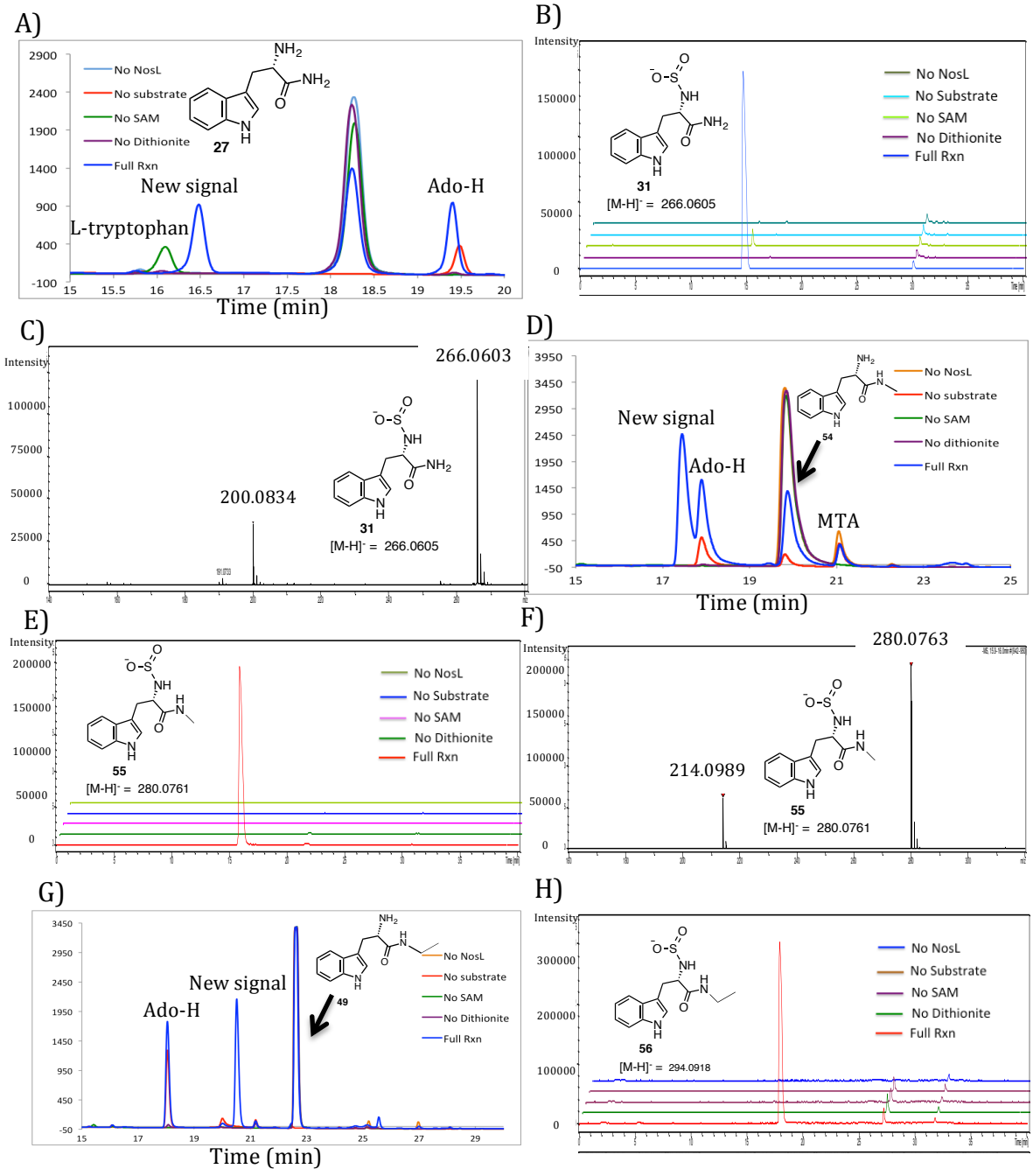


Figure S15: Synthesis of the methyl and ethyl esters of D,L-indole-3-lactate. A) Synthesis scheme. B) ^{1}H-NMR of D,L-indole-3-lactate methyl ester (**52**). C) ^{13}C-NMR of D,L-indole-3-lactate methyl ester (**52**). D) ^{1}H-NMR of D,L-indole-3-lactate ethyl ester (**53**). E) ^{13}C-NMR of D,L-indole-3-lactate ethyl ester (**53**).

Trapping of the amine radical with dithionite during the NosL reaction with L-tryptophanamide (27):

During the NosL-catalyzed reaction with L-tryptophanamide (**27**), hydrolysis to L-tryptophan is observed which is further converted to 3-methylindole and 3-methyl-2-indolic acid. HPLC analysis of this reaction revealed the formation of an additional product only in the 'Full Reaction' when dithionite was used as a reductant. LC-MS analysis suggested that this compound is the sulfuramidite **31**. Similar sulfuramidites were also observed when N-methyl and N-ethyl L-tryptophan amides were used as substrates. These sulfuramidites were not observed when FldA-FldR was used to reduce the [4Fe-4S].



I)

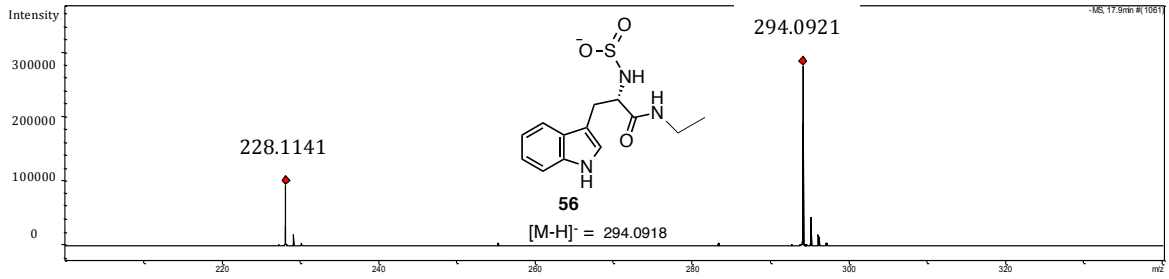


Figure S16: Analysis of the reaction of tryptophan amides with NosL. A) HPLC analysis at 280 nm of the NosL reaction with L-tryptophanamide (**27**). A new signal is seen only in 'Full Rxn' sample at 16.5 min. B) LC-MS analysis showing EIC $[M-H]^-$ = 266.0 Da suggesting that the new product is the sulfuramidite **31**. C) MS of the sulfuramidite **31**. D) HPLC analysis at 280 nm of the NosL reaction with N-methyl-L-tryptophanamide (**54**). A new signal is seen only in the 'Full Rxn' sample at 17.5min. E) LC-MS analysis showing EIC $[M-H]^-$ = 280.0 Da suggesting that the compound is sulfuramidite **55**. F) MS of the sulfuramidite **55**. G) HPLC analysis at 280 nm of the NosL reaction with N-ethyl-L-tryptophanamide (**49**). A new signal is seen only in the 'Full Rxn' sample at 20.5 min. H) LC-MS analysis showing EIC $[M-H]^-$ = 294.0 Da suggesting that the compound is the sulfuramidite **56**. I) MS of the sulfuramidite **56.**

Trapping of the amine radical with dithionite during the NosL reaction with esters of L-tryptophan:

When the NosL-catalyzed reaction was performed with the methyl and ethyl esters of L-tryptophan, we observed the corresponding sulfuramidite as previously observed with the amide analogues. Ester hydrolysis was also observed.

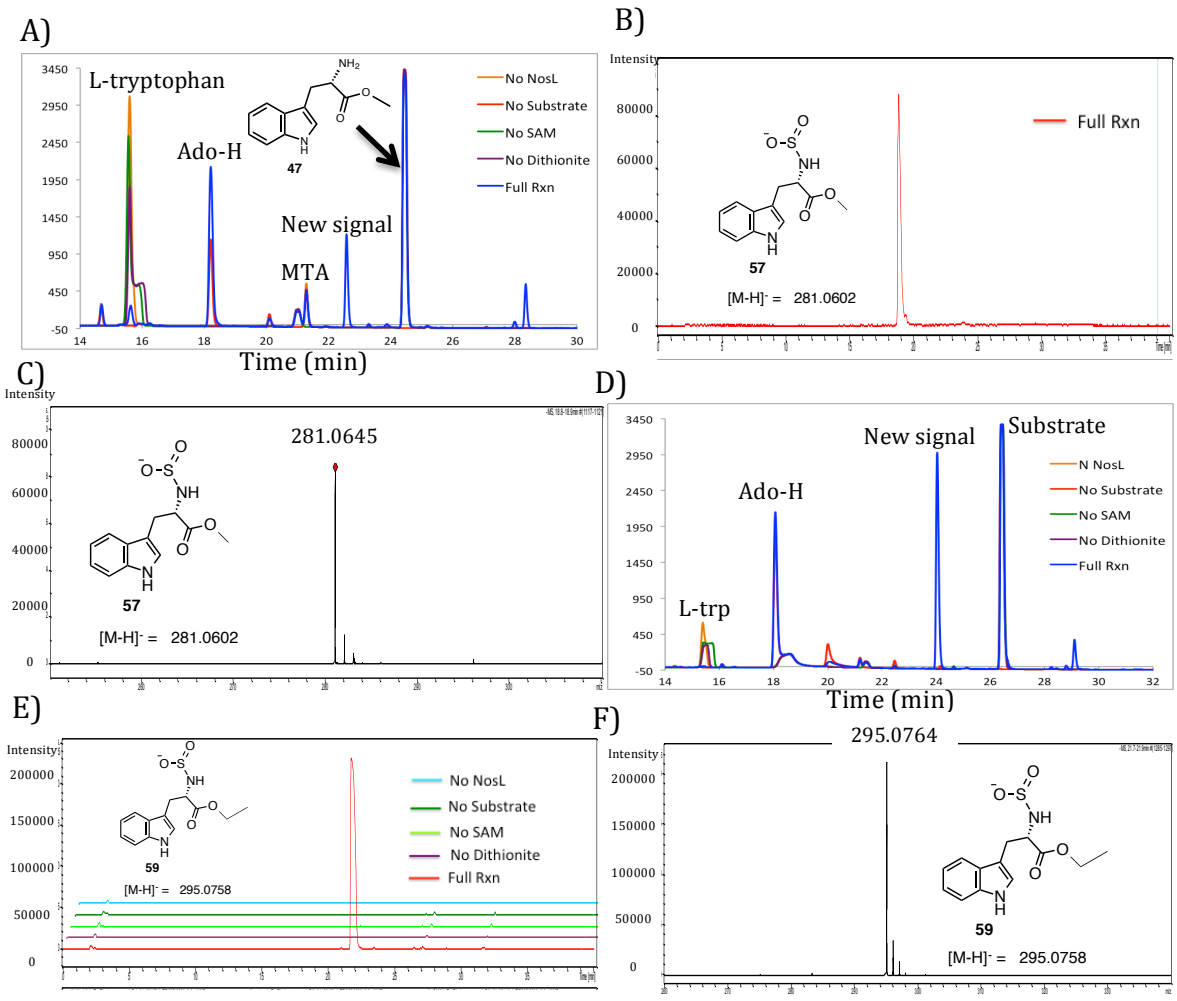


Figure S17: Analysis of NosL reaction with L-tryptophan esters. A) HPLC analysis at 280nm of the NosL reaction with L-tryptophan methyl ester (**47**). A new signal is seen only in the 'Full Rxn' sample at 22.5 min. B) LC-MS analysis showing EIC $[M-H]^-$ = 281.0 Da suggesting that the new product is the sulfuramidite **57**. C) MS of the sulfuramidite **57**. D) HPLC analysis at 280nm of the NosL reaction with L-tryptophan ethyl ester (**58**). A new signal is seen only in the 'Full Rxn' sample at 24.0min. E) LC-MS analysis showing EIC $[M-H]^-$ = 295.0 Da suggesting that the new product is sulfuramidite **59**. F) MS of the sulfuramidite **59**.

Radical trapping with dithionite during the NosL reaction with esters of D,L-indole-3-lactate:

We have previously demonstrated that NosL can abstract a hydrogen atom from the alcohol of D,L-indole-3-lactate (**50**).[1] When the NosL-catalyzed reaction was performed with the methyl and ethyl esters of D,L-indole-3-lactate, we observed the formation of the corresponding sulfites. In addition, the substrate esters underwent hydrolysis, generating indole-3-lactate. With the ester analogue, we also observed enhanced levels of 5'-deoxyadenosyl sulfinate (**62**).

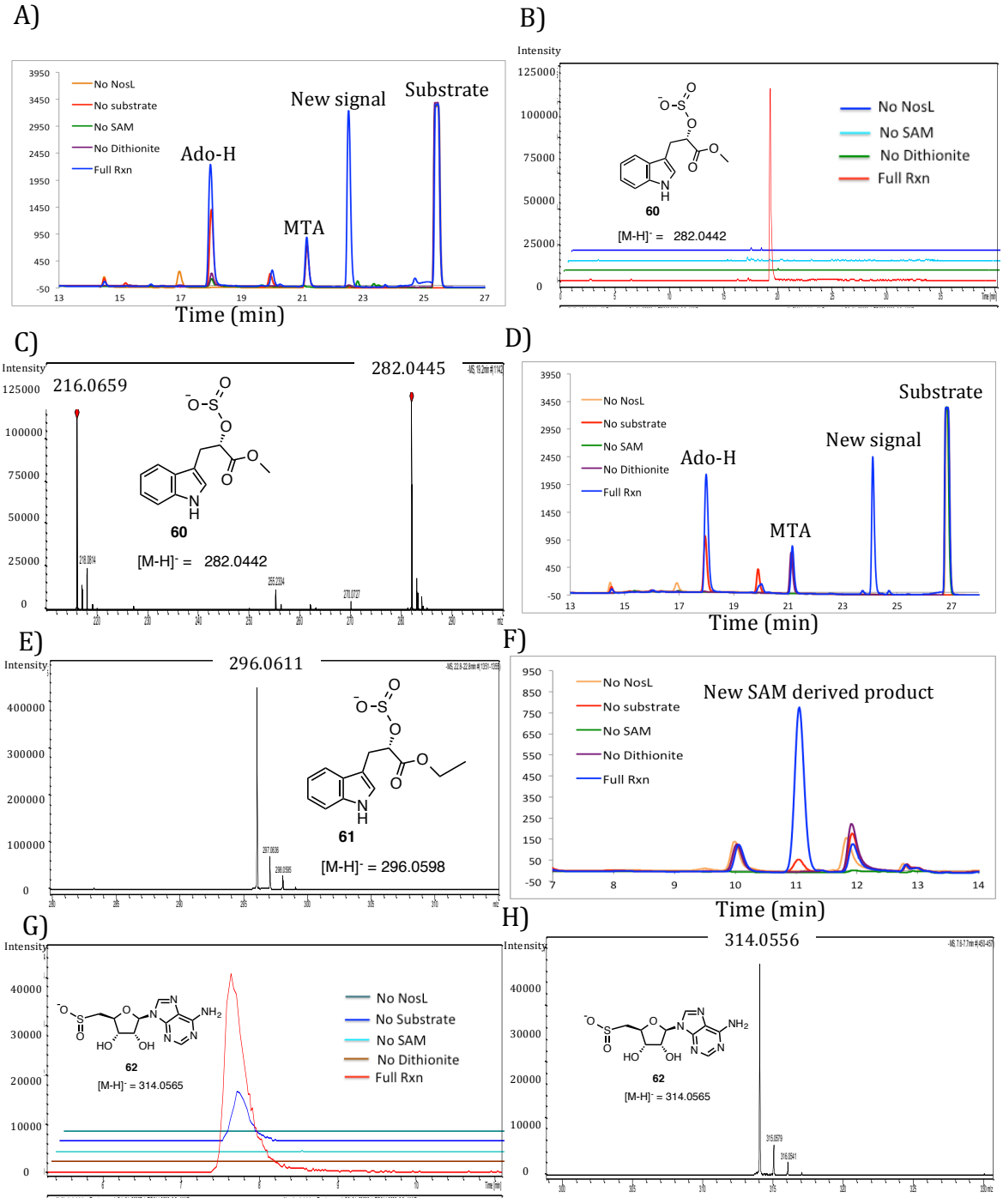


Figure S18: Analysis of the NosL reaction with esters of D,L-indole-3-lactate. A) HPLC analysis at 280 nm of the NosL reaction with D,L-indole-3-lactate methyl ester (**52**). A new signal is seen only in the 'Full Rxn' sample at 22.5 min. B) LC-MS analysis showing EIC $[M-H]^-$ = 282.0 Da suggesting that the new signal corresponds to the sulfite **60**. C) MS of the sulfite **60.** D) HPLC analysis at 280 nm of the NosL reaction with D,L-indole-3-lactate ethyl ester (**53**). A new signal is seen only in the 'Full Rxn' sample at 24.0 min. E) MS of the sulfite **61.** F) HPLC analysis at 260 nm of the NosL reaction with D,L-indole-3-lactate ethyl ester (**53**). A new SAM-derived signal is observed at 11.0min. G) LC-MS analysis showing EIC $[M-H]^-$ = 314.0 Da suggesting that the SAM derived product is 5'-deoxyadenosyl sulfinate (**62**). H) MS of 5'-deoxyadenosyl sulfinate (**62**).

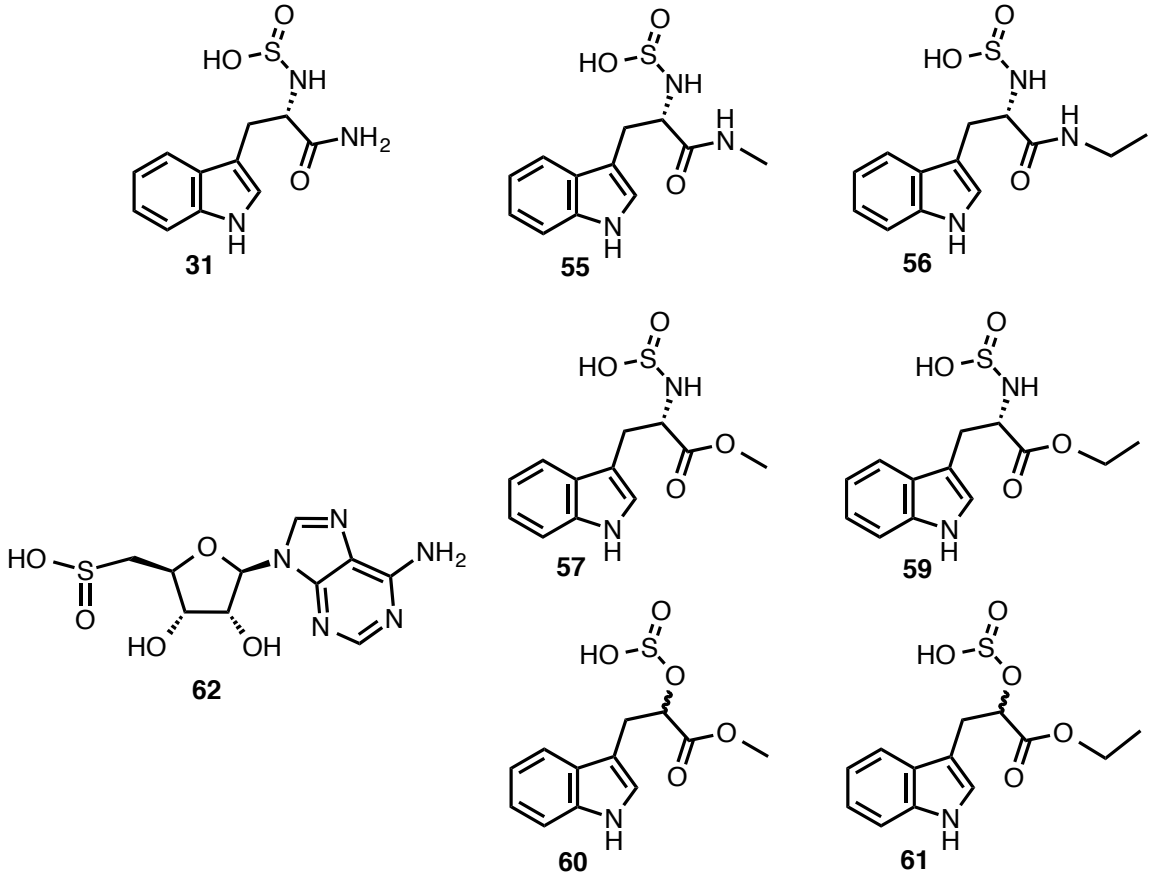
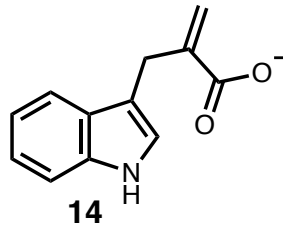


Figure S19: Structures of the dithionite adducts observed during the NosL-catalyzed reaction with the corresponding substrate analogues.

NMR chemical shifts of the compounds

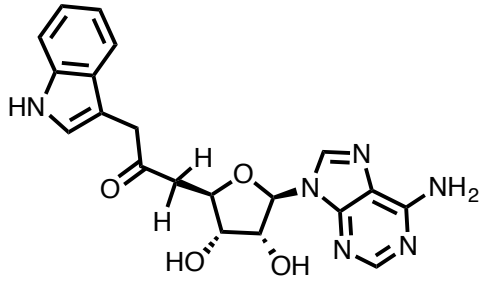
- Methylene analogue of tryptophan (**14**):



14

^{1}H NMR (400MHz, MeOD): 3.73 (s, 2H), 5.44 (m, 1H), 6.18 (m, 1H), 6.96 (s, 1H), 6.99 (td, 1H), 7.08 (td, 1H), 7.30 (dt, 1H), 7.45 (dt, 1H)
^{13}C NMR: 28.32, 112.12, 112.91, 119.48, 119.59, 122.24, 124.07, 126, 128.35, 137.88, 141.55, 170.94

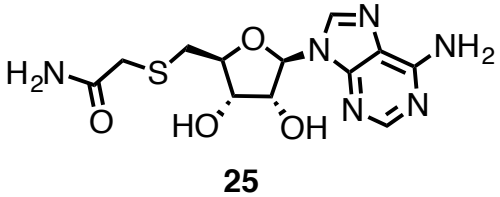
- Keto adduct (**22**):



22

^{1}H NMR (500MHz, d_6-DMSO): 2.9 (m, 2H), 3.65 (m, 2H), 4 (m, 1H), 4.12 (m, 1H), 4.65 (t, 1H), 5.8 (d, 1H), 6.76 (td, 1H), 6.99 (td, 1H), 7.1 (dt, 1H), 7.25 (s, 1H), 7.26 (dt, 1H), 8.06 (s, 1H), 8.11 (s, 1H)
^{13}C NMR: 40.28, 45.35, 72.86, 73.63, 80.51, 88.34, 107.49, 111.85, 118.80, 118.92, 121.47, 124.69, 127.68, 136.55, 140.64, 149.75, 153.07, 156.52, 206.91

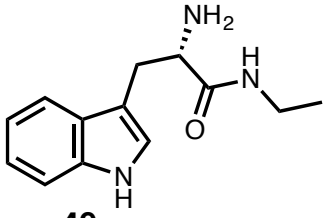
- Acetamide derivative of 5'-deoxy-5'-thioadenosine (**25**):



25

^{1}H NMR (500MHz, d_6-DMSO): 2.9 (m, 2H), 3.05 (s, 2H), 4.0 (m, 1H), 4.09 (t, 1H), 4.66 (t, 1H), 5.82 (d, 1H), 8.1 (s, 1H), 8.27 (s, 1H)
^{13}C NMR: 34.71, 35.06, 72.76, 72.92, 83.56, 87.65, 119.27, 140.07, 149.62, 152.91, 156.15, 168.05

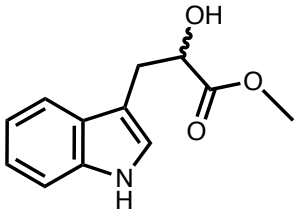
- N-ethyl-L-tryptophan amide (**49**)



49

1H NMR (400MHz, MeOD): 0.96 (t, 3H), 3.12 (m, 4H), 3.71(t, 1H), 7.01 (td, 1H), 7.09 (td, 1H), 7.11 (s, 1H), 7.33 (dt, 1H), 7.59 (dt, 1H)
^{13}C NMR: 14.49, 31.27, 35.26, 56.43, 110.32, 112.37, 119.35, 119.30, 122.57, 124.93, 128.69, 138.18, 174.93

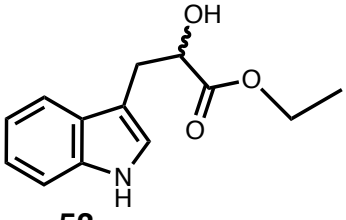
- Methyl-D,L-indole-3-lactate (**52**)



52

1H NMR (400MHz, MeOD): 3.16 (m, 2H), 3.6 (s, 3H), 4.43 (m, 1H), 6.99 (td, 1H), 7.06 (td, 1H), 7.07 (s, 1H), 7.31 (dt, 1H), 7.52 (dt, 1H)
^{13}C NMR: 31.50, 52.28, 72.63, 110.82, 112.16, 119.29, 119.61, 122.23, 124.58, 128.88, 137.92, 176.15

- Ethyl-D,L-indole-3-lactate (**53**)



53

1H NMR (400MHz, MeOD): 1.06 (t, 3H), 3.18 (m, 2H), 4.01 (m, 2H), 4.45 (dd, 1H), 7.01 (td, 1H), 7.07 (s, 1H), 7.08 (s, 1H), 7.32 (dt, 1H), 7.55 (dt, 1H)
^{13}C NMR: 14.23, 31.4, 61.87, 72.54, 110.70, 112.12, 119.27, 119.55, 122.18, 124.54, 128.80, 137.73, 175.68

References:

(1) Bhandari, D. M.; Xu, H.; Nicolet, Y.; Fontecilla-Camps, J. C.; Begley, T. P. *Biochemistry* **2015**, *54*, 4767.

(2) Zhang, Q.; Li, Y.; Chen, D.; Yu, Y.; Duan, L.; Shen, B.; Liu, W. *Nat Chem Biol* **2011**, *7*, 154.

(3) Ji, X.; Li, Y.; Jia, Y.; Ding, W.; Zhang, Q. *Angewandte Chemie International Edition* **2016**, *55*, 3334.

(4) Sicoli, G.; Mouesca, J.-M.; Zeppieri, L.; Amara, P.; Martin, L.; Barra, A. L.; Fontecilla-Camps, J. C.; Gambarelli, S.; Nicolet, Y. *Science* **2016**, *351*, 1320.

(5) Buddoo, S.; Rousseau, A. L.; Gordon, G. E. R.; Google Patents: 2009.

(6) Farrar, C. E.; Jarrett, J. T. *Biochemistry* **2009**, *48*, 2448.

(7) Teng, X.; Degterev, A.; Jagtap, P.; Xing, X.; Choi, S.; Denu, R.; Yuan, J.; Cuny, G. D. *Bioorganic & medicinal chemistry letters* **2005**, *15*, 5039.

(8) Nguyen, S. T.; Butler, M. M.; Varady, L.; Peet, N. P.; Bowlin, T. L. *Bioorganic & medicinal chemistry letters* **2010**, *20*, 5739.