Supplementary Materials

Nucleoside-linked Shunt Products in the Reaction Catalyzed by the Class C Radical S-Adenosylmethionine Methyltransferase NosN

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Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with a diode array detector. High resolution mass spectra (HRMS) were acquired using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc.). NMR spectra were recorded using Varian Inova 400 MHz or 500 MHz NMR spectrometers at the Nuclear Magnetic Resonance Facility at Fudan University. UV-vis spectroscopy analysis was performed on a 1900 double beam UV-vis spectrometer (Yoke Instrument Co. Ltd., Shanghai, China). PCR was performed on a Bio-Rad T100™ Thermal Cycler using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd, China).

Chemicals and Biochemicals

General chemical reagents were purchased from commercial sources and used without further purification unless otherwise specified. S-adenosyl-L-methionine (SAM) and sodium dithionite were purchased from Sangon Biotech Co. Ltd (Shanghai, China). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and Na_2S were from Adamas Reagent Co. Ltd (Shanghai, China). Homocysteine was from Tokyo Chemical Industry Co. Ltd (Shanghai). S-Adenosyl-L-[methyl-$^2H_3$]-methionine (d_3-SAM), S-guanosylmethionine (SGM), S-cytidinylmethionine (SCM), flavodoxin A (FldA) and flavodoxin reductase (Fpr) were all prepared previously in our lab.[1] Kanamycin and culture media were from Sinopharm Chemical Reagent Co. Ltd (China). Enzymes were from Takara Biotechnology (Dalian, China) or from Vazyme Biotech (Nanjing, China) unless otherwise specified.

Expression and purification of NosN

Chemically competent *E. coli* BL21 (DE3) cells were transformed with the NosN-expression plasmid (NosN-pET28a) constructed previously.[1] A single colony transformant was used to inoculate 4 mL culture of LB supplemented with 100 μg mL^{-1} kanamycin. The culture was grown at 37 °C for 16 h and was used to inoculate 2L of LB medium in a 5L flask containing 100 μg mL^{-1} kanamycin. Cells were grown at 37 °C and 220 rpm to an OD_{600}～0.6, and were then chilled on ice for 10 min. IPTG was then added to the culture to a final concentration of 200μM before additional 18-20 h of incubation at 18 °C. The cells were harvested by centrifugation at 4000 x g for 15 min at 4 °C.

Protein purification was performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 5 ppm of O_2. The pellet was resuspended in 30 mL of lysis buffer (40mM Tris, 200 mM NaCl, 10mM imidazole, 10% glycerol, pH 8.0) and was lysed by sonication on ice. Cell debris was removed via centrifugation at 21000 x g for 1 h at 4 °C. The supernatant was passed through a column containing 4 mL of high-affinity Ni-NTA resin (Qiagen Co. Ltd) pre-equilibrated with lysis buffer, and the column was then washed using 50 mL wash buffer (40 mM Tris, 200mM NaCl, 40 mM imidazole, 10% glycerol, pH 8.0). The protein fractions were collected using 10 mL of elution buffer (40 mM Tris, 200 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0). The desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit and analyzed by SDS-PAGE (12% Tris-glycine gel). Protein concentration was determined using a Bradford Assay Kit (Promega) using bovine serum albumin (BSA) as a standard.

Preparation of the reconstituted NosN

Reconstitution of the NosN [4Fe-4S] cluster was carried out in an anaerobic glove box by using standard procedures.[2] Dithiothreitol (DTT) was added to the purified protein solution to a final concentration of 5 mM. $Fe(NH4)_2(SO4)_2$ solution was then added slowly to a final concentration of 800 μM. After 15min incubation at the room temperature, Na_2S solution was added carefully to a final concentration of 800 μM. After further incubation on ice for 7-10 h, the resulting dark solution was subjected to desalt on a PD-10 (GE) column pre-equilibrated with the elution buffer

(40mM Tris, 25mM NaCl, 10 mM DTT and 10% (v/v) glycerol, pH 8.0). The eluted protein fraction was collected and concentrated, and was used directly for in vitro assay or stored at -80°C upon further use. Quantification of Fe and S atoms per molecule of protein was performed in duplicate according to the methods described previously.[3, 4]

Enzymatic assays of NosN

All NosN assays were performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 5 ppm of O_2. A typical assay was carried out by incubating 500 μM SNAC thioether substrate (**4** in the main text) with ～40 μM reconstituted NosN, 0.5M SAM, 1mM NADPH, 50 μM FldA, 20 μM Fpr in 40 mM Tris buffer (pH 8.0). Substrate **2** was initially dissolved in dimethyl sulfoxide (DMSO) and carefully added to the assay mixture. Reaction volumes were typically 200 μL and were maintained at room temperature (～25^{o}C) for 5 h prior to quenching, which was done by addition of formic acid to a final concentration of 5% (v/v). After removal of the protein precipitates by centrifugation, the supernatant was subjected to HPLC and/or LC-MS analysis. For single turnover reactions, the assay condition is the same except only 100 μM (instead of 1 mM) SAM was used.

Theoretical Calculations

Density functional theory (DFT) studies[5, 6] have been performed with the Gaussian 09 program[7] using the unrestricted B3LYP method[8, 9] and the 6-311+G(2d,p) basis set. The optimized structures were checked with harmonic vibration frequency calculations. The solvent effect was estimated with IEFPCM calculation[10, 11] with radii and non-electrostatic terms for SMD solvation model[12] in water (ε = 78.39) using the gas-phase optimized structures.

Fig. S1. SAM cleavage catalyzed by radical SAM enzymes. (A) Reductive cleavage of SAM can occur in two different ways. For most radical SAM enzymes, the SAM cleavage product is a dAdo radical and L-methionine (pathway i), whereas for Dph2 involved in diphthamide[13] biosynthesis, the SAM cleavage product is MTA and a 2-aminobutanoate radical (pathway ii). It should be noted that MTA is a product of SAM degradation[14] whereas dAdoH cannot be produced non-enzymatically from SAM. The apparent production of dAdoH suggested that the SAM cleavage in NosN catalysis occurs via pathway i. (B) Proposed mechanism for NosN catalysis. NosN produces a large amount of MTA in the reaction. However, the yield of homoserine lactone (HSL), the product of SAM degradation, is low in the reaction, and owing to its lability, whether it is a co-product or not remains to be validated. Production of homoserine can be observed by alkaline treatment of the reaction mixture, while accurate quantification of this product proved to be difficult because of the inefficient derivative methods. According to our DFT calculation, the pKa value of **3** is intrinsically low, therefore the conversion of **3** to **4** may possibly not require the assistance of a protein residue.

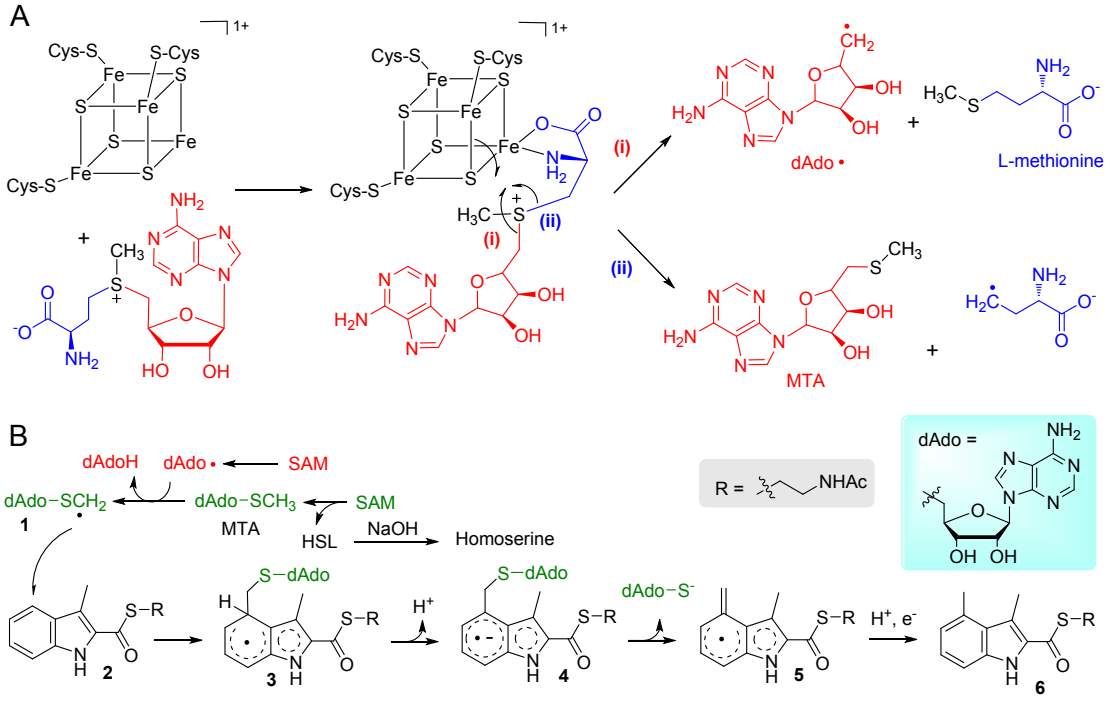


Fig. S2. HR-MS/MS analysis of **10d**, showing the collision induced dissociation (CID) fragments and the MS/MS spectrum.

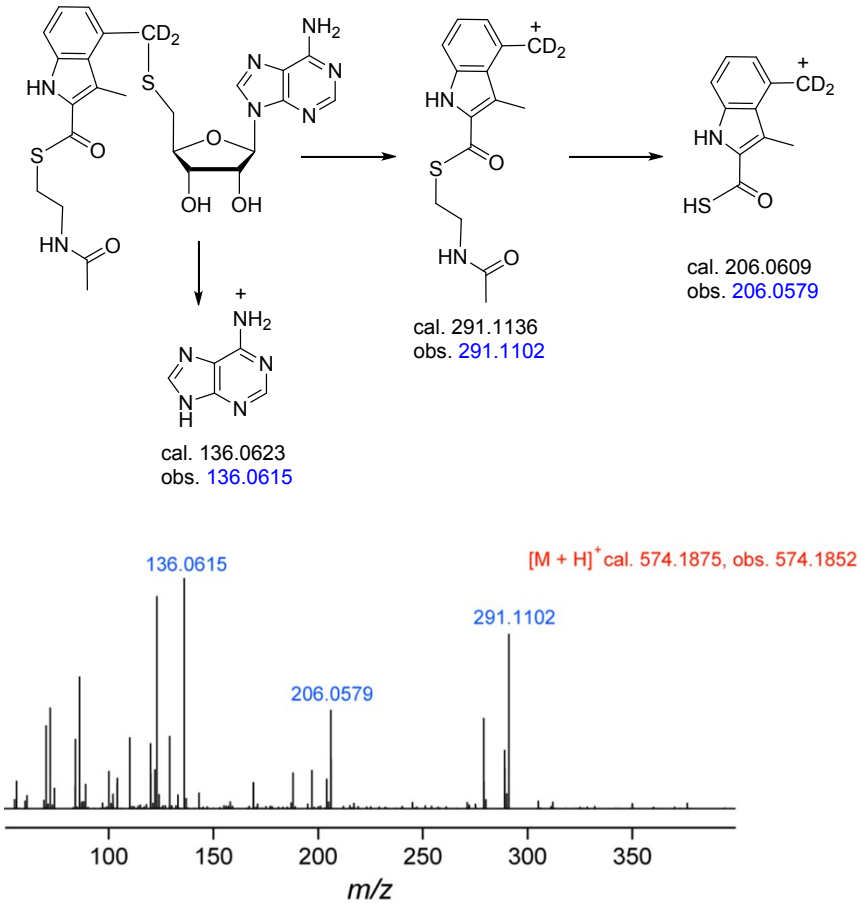


Fig. S3. Quantitative analysis of the methylated product **6** and the nucleoside-linked shunt products in NosN reaction with (A) SAM and (B) SGM. The reactions were carried out by incubating 40 μM reconstituted protein with 100 μM substrate (**2**), 500 μM SAM or SGM, 1 mM NADPH, 50 μM FldA, 20 μM Fpr in 40 mM Tris buffer (pH 8.0). Quantification of **10**, **11**, **12**, and **13** were done by comparing the MS/MS intensities of the fragment ions 136.1 and 152.1, respectively. The assays were performed in duplicate, and the standard deviations (S.D.) are shown by the error bars.

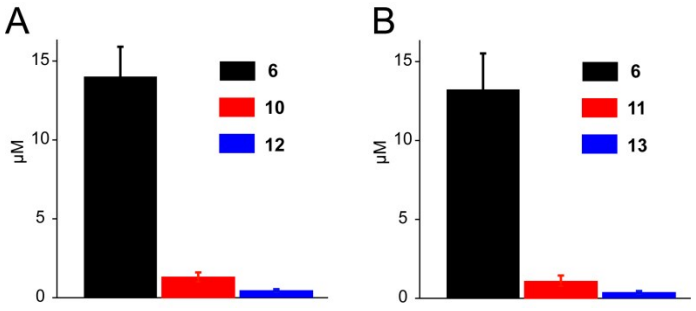


Fig. S4. The diverse fates of the radical intermediate **3** in NosN catalysis. The roughly estimated proportions of the methylated products (highlighted in yellow box) and nucleoside-linked shunt products (highlighted in green boxes) are shown in blue font.

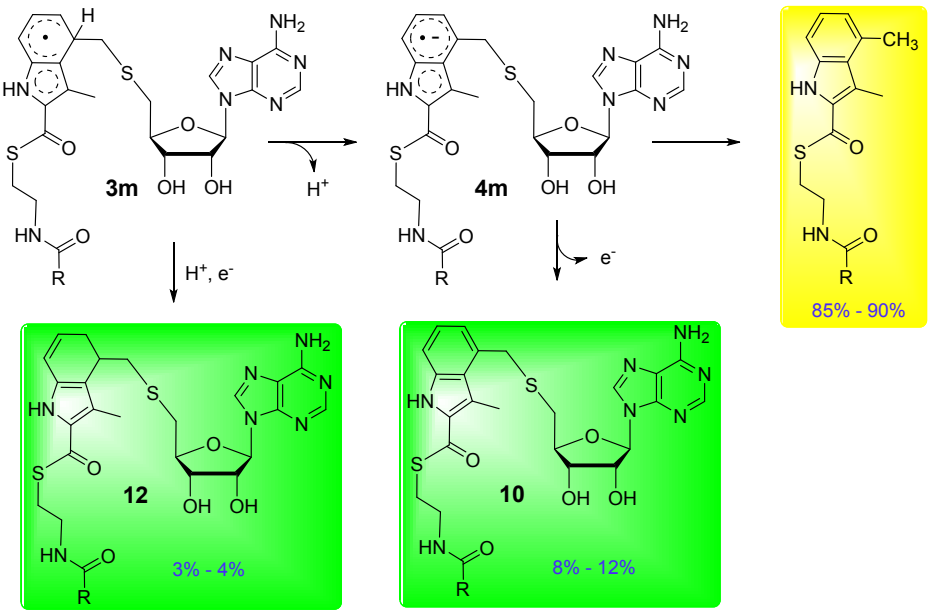
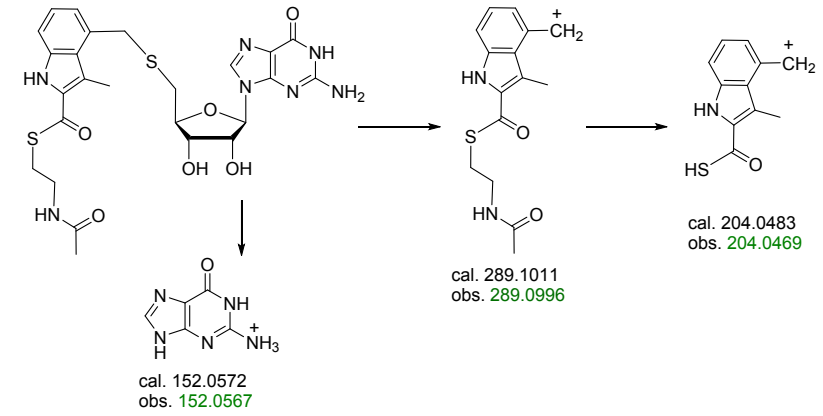


Fig. S5. HR-MS/MS analysis of **11**, showing the collision induced dissociation (CID) fragments and the MS/MS spectrum.



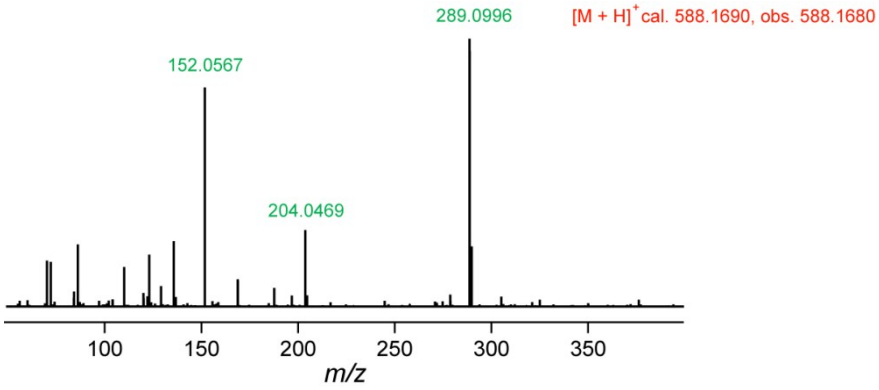


Fig. S6. Mulliken spin population of the products obtained by geometry optimization of **8m**. This result suggests that **8m** is intrinsically unstable and decomposes to a thiyl radical and a radical anion **9m**. DFT calculation was performed using an unrestricted B3lYP formalism and a 6-311+(2G, p) basis set using the SMD solvation model[12] in water.

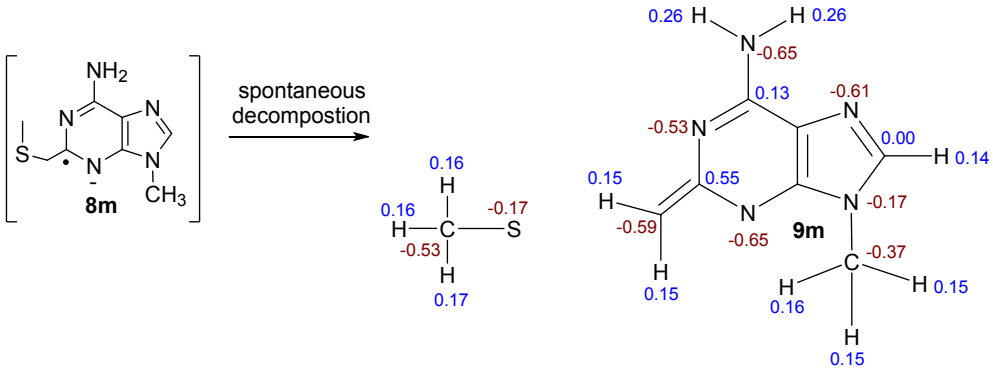


Fig. S7. Calculation of the pKa values of the DFT models **3m** and **7m**. The pKa calculation was performed using acetic acid as a reference according to the equation pKa = 4.75 + ΔG/2.303RT, where ΔG is the Gibbs free energy change (J/mol) of the reaction, 4.75 is the pKa value of acetic acid, R is the gas constant, and T is 298.15 K. We could not obtain the pKa value of **7m** because of the instability of **8m**, but it is apparently far larger than 6.4 because ΔG2 is far less than 0.

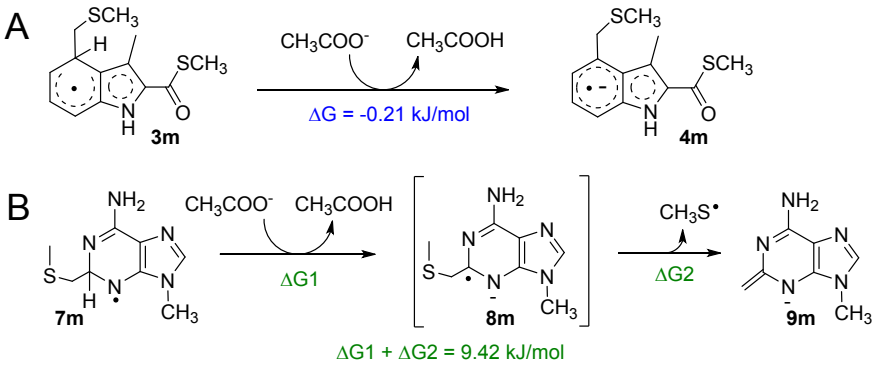
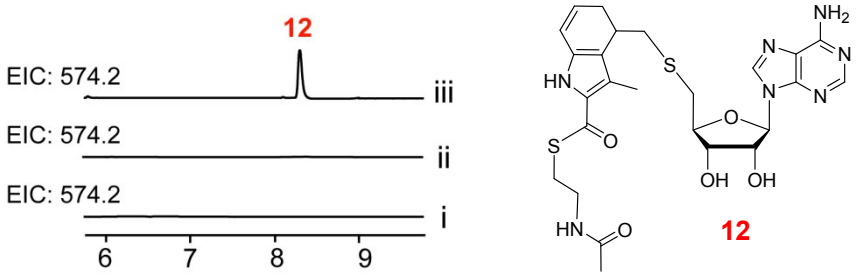


Fig. S8. Production of **12** in NosN reaction, showing the chemical structure of **12**, and the extracted ion chromatograms (EICs) of $[M + H]^+$ = 574.2 (corresponding to **12**) for (i) control reaction with the supernatant of boiled NosN, (ii) control reaction in which SAM was omitted, and (iii) NosN reaction with SAM.



Although only one peak is observed in the EIC in our analysis, **12** is likely a mixture consisting of different isomers, in which the reducing hydrogen residues on different positions of the indole ring. We proposed that the major component is **12** (shown above) because of two reasons: 1) **12** is obtained by reduction of the radical adduct **3** by a hydrogen equivalent, and DFT calculation of the chemical model shows that, in both HOMO and LUMO of **3m**, C5 and C7 have the highest spin density (see below), therefore the hydrogen equivalent should go to C5 or C7 upon **3** reduction; 2) the isomer with a C5 hydrogen is energetically favored over that with a C7 hydrogen, with a ΔG of -5400 J/mol for their interconversion (both geometry optimization and energies calculation were conducted at the B3LYP/6-311+G(2d,p)/SMD(water) level of theory).

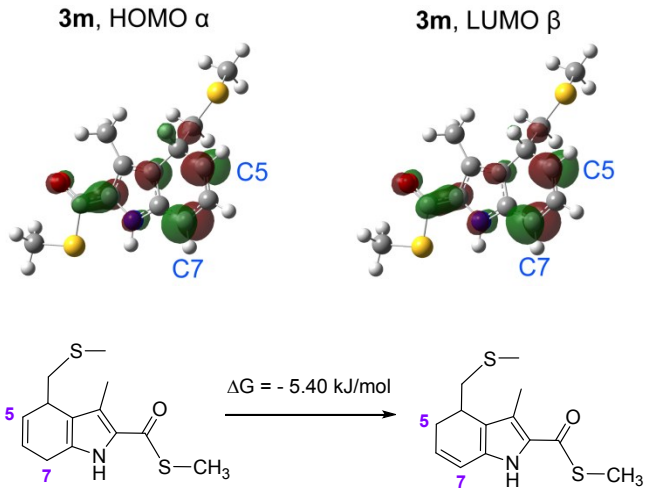
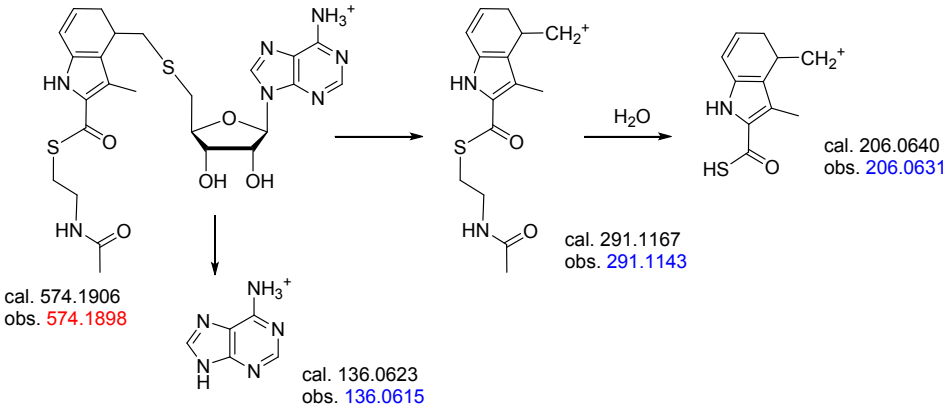


Fig. S9. HR-MS/MS analysis of **12**, showing the MS/MS spectrum and the collision induced dissociation (CID) fragments.



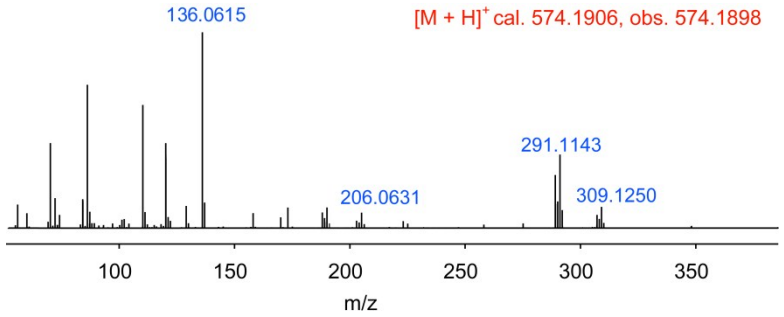
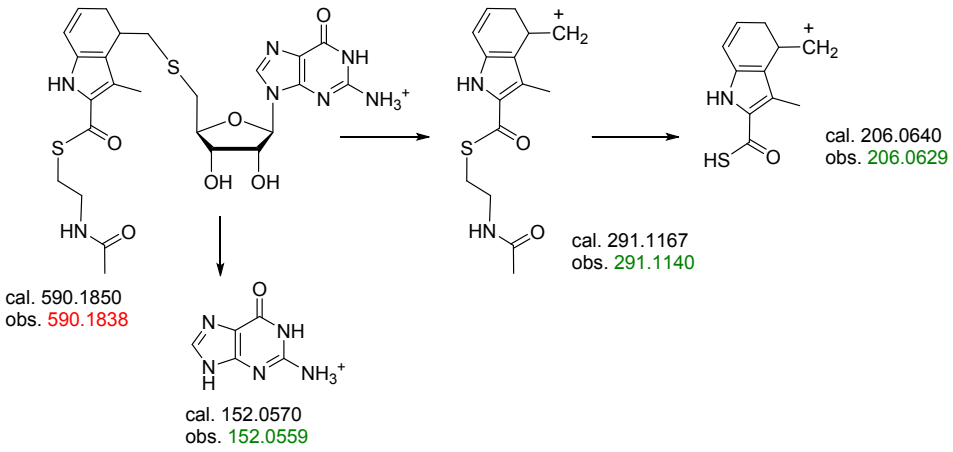
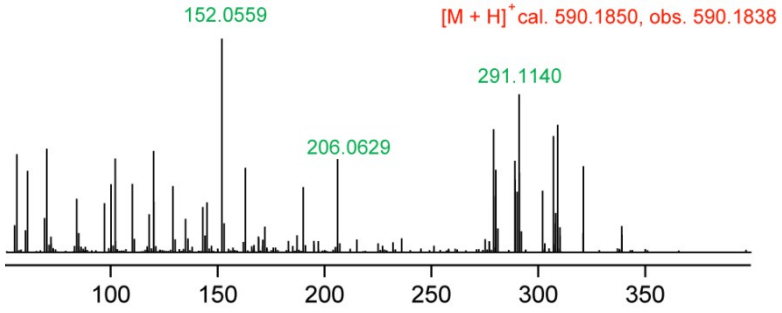


Fig. S10. HR-MS/MS analysis of **13**, showing the MS/MS spectrum and the collision induced dissociation (CID) fragments.





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