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Molybdopterin Biosynthesis: Trapping of Intermediates for the MoaA-Catalyzed Reaction Using 2′-DeoxyGTP and 2′-ChloroGTP as Substrate Analogues.

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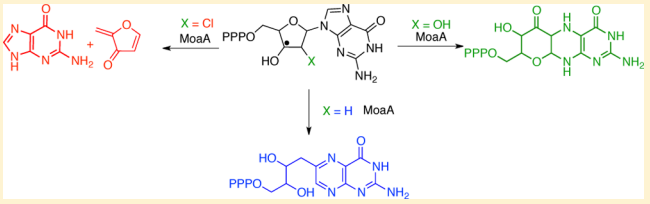
Supporting Information

ABSTRACT: MoaA is a radical S-adenosylmethionine (AdoMet) enzyme that catalyzes a complex rearrangement of guanosine-5'-triphosphate (GTP) in the first step of molybdopterin biosynthesis. In this paper, we provide additional characterization of the MoaA reaction product, describe the use of 2′-chloroGTP to trap the GTP C3′ radical, generated by hydrogen atom transfer to the 5′-deoxyadenosyl radical, and the use of 2′-deoxyGTP to block a late step in the reaction sequence. These probes, coupled with the previously reported trapping of an intermediate in which C3′ of the ribose is linked to C8 of the purine, allow us to propose a plausible mechanism for the MoaA-catalyzed reaction.



INTRODUCTION

Molybdopterin is a redox cofactor used by enzymes such as xanthine oxidase, sulfite oxidase, nitrate reductase, carbon monoxide dehydrogenase and formate dehydrogenase.[1] Previous studies have established that the C8 carbon of GTP **1** is inserted into the C2′-C3′ bond of the GTP ribose in a reaction catalyzed by MoaA–MoaC (Figure 1A).[2] MoaA is a radical SAM enzyme that utilizes two [4Fe-4S] clusters. EPR and structural studies show that these clusters interact with the purine of GTP and the amino acid of SAM.[3,4] A mechanistic proposal, based on the identification of the initially abstracted hydrogen atom and the trapping of intermediate **8** is outlined in Figure 1B.[5−7]

Previous studies suggested that **8** is the product of MoaA and that MoaC catalyzes its conversion to **15**.[7] Here we demonstrate that MoaA catalyzes the remarkable conversion of **1** to **15** and describe the successful use of 2′-chloroGTP **20** and 2′-deoxyGTP **30b** to further interrogate the mechanism of this complex reaction.

EXPERIMENTAL SECTION

Sources of Reagents and Supplies. AdoMet, GTP, 2′-deoxyGTP, *o*-(Pentafluorobenzyl) hydroxylamine (PFBHA), 3-mercaptobenzoic acid, sodium dithionite were obtained from Sigma-Aldrich. Alkaline phosphatase (10000 U/mL) was obtained from New England Biolobs.

High Performance Liquid Chromatography (HPLC) Conditions for the Isolation of the MoaA Reaction Product. Agilent 1260 series instrument; Supelcosil SPLC-18 column (25 cm x 44 mm, 5 μm)). The following gradient was used with (A) water, (B) 5 mM ammonium formate, and (C) methanol: 0 min, 100% B; 7 min, 10% A, 90% B; 12 min, 25% A/60% B/15% C; 17 min, 25% A/10% B/65% C; 19 min to 29 min, 100% B.

LC Conditions for MoaA Assays with 2′-ChloroGTP. Detection of Guanine. The following gradient was used with (A) 10 mM *N,N*-dimethylhexyamine, pH 6.4, (B) 75% methanol/25% water, Agilent Poroshell 120, EC-C18 column, 2.7 μm, 3 mm × 10 mm: 0 min, 100% A; 5 min,100% A; 15 min, 60% A/40% B; 27 min, 10% A/90% B; 36 min, 100% A.

LC Conditions for MoaA Assays with 2′-ChloroGTP. Detection of 3-Mercaptobenzoic Acid Derivatized Product. 1200 series Agilent (binary pump) LC conditions were as follows: (A) 5 mM ammonium acetate, pH 6.7, (B) 75% methanol, 25% water. Reverse phase column, Supelcosil LC18, 3 μm, 3 mm × 10 mm. The gradient was as follows: 0 min, 100% A; 7 min, 100% A; 15 min, 80% A/20% B; 20 min, 70% A/30% B; 26 min, 0% A/100% B; 28 min, 0% A/100% B; 29 min, 100% A.

LC Conditions for MoaA Assays with 2′-DeoxyGTP. Product Isolation and Coelution Experiment. HPLC (Supelcosil SPLC-18 column (25 cm × 10 mm, 5 μm)). Conditions were as follows with (A) water, (B) 5 mM ammonium formate, (C) methanol: 0 min, 100% B; 7 min, 10% A/90% B; 12 min, 25% A/60% B/15% C; 17 min, 25% A/10% A/65% B; 19 min to 29 min, 100% B.

Mass Spectrometry (MS) Parameters for All LC–MS Experiments. Capillary, −4500 V; capillary offset, −500 V; nebulizer gas, 3.0 bar; dry gas, 10.0 L/min; dry gas temperature, 200 °C; funnel 1 RF, 200.0 Vpp; funnel 2 RF, 200.0 Vpp; ISCID, 0.0 eV; hexapole RF, 200 Vpp; quadrapole, ion energy, 5.0 eV; low mass, 200 *m*/*z*; collision cell, collision energy, 10.0 eV; collision RF, 150.0 Vpp, transfer time, 100.0 μs; Prepulse storage, 5.0 μs. Data was processed with DataAnalysis ver. 4.0 SP4 (Bruker Daltonics, Billerica, MA).

Overexpression and Purification of MoaA. The overexpression and purification of MoaA has been described previously.[5]

Overexpression and Purification of MoaA from E. coli–MoaC Deletion Strain. The *E. coli* MoaC deletion strain was obtained from CGSC, Yale. This strain was rendered compatible for pET vectors using the λDE3 lysogenation kit. MoaA was overexpressed and purified as previously described.[5]

Received: March 15, 2014
Published: June 23, 2014

   dx.doi.org/10.1021/ja502663k | J. Am. Chem. Soc. 2014, 136, 10609−10614

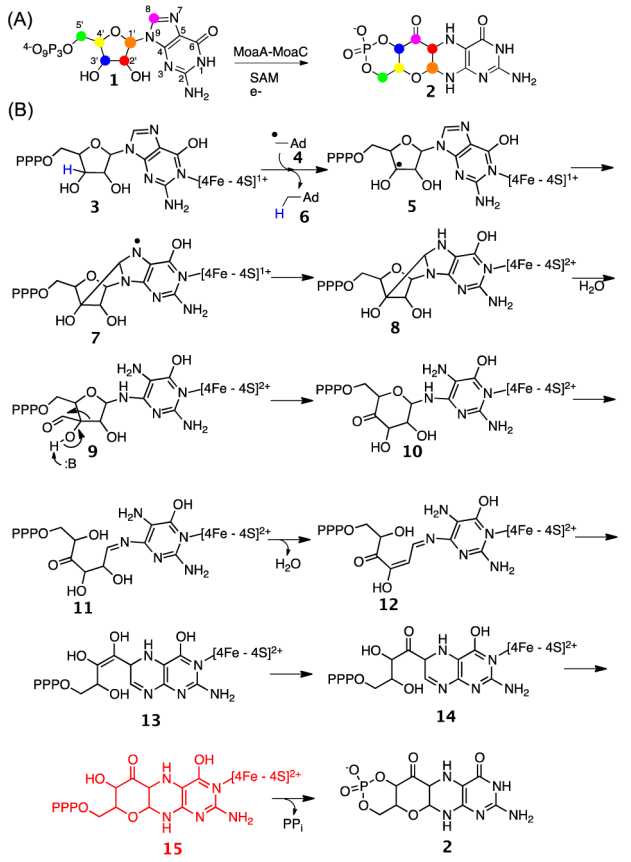


Figure 1. First steps in molybdopterin biosynthesis: (A) The carbon-labeling pattern for the conversion of GTP **1** to cyclic pyranopterin monophosphate **2**. (B) Initial mechanistic proposal for the MoaA/MoaC-catalyzed reaction.[5,6]

Isolation of the Product of the MoaA-Catalyzed Reaction. The reaction mixture consisted of 250 μM MoaA, 2 mM GTP, 3 mM AdoMet, and 10 mM dithionite and was incubated in an anaerobic chamber for 5 h at room temperature. The protein was then removed by ultrafiltration using a 10 kDa cutoff filter. The resulting small molecule pool was treated with 3 μL of alkaline phosphatases in the presence of 1 mM $MgCl_2$, incubated in the anaerobic chamber for an additional 3 h and quenched with 100 μL of oxygen-free KI/I_2 (5% I_2 (w/v) and 10% KI (w/v) in water).[5,8,9] The reaction mixture was then purified by HPLC. The fluorescent product eluting at 17 min was collected and dried using a vacuum centrifuge. Several such reaction mixtures were purified to yield sufficient product for NMR characterization. The dried samples were dissolved in 250 μL of a 90%:10% H_2O/D_2O mixture and analyzed by NMR (Bruker, 500 MHz).

MoaA reactions were also performed with MoaA overexpressed and purified from the *E. coli*−MoaC deletion strain. An identical fluorescent compound eluting at 17 min was observed (Supporting Information, Figure SI 36).

Hydroxylamine Derivatization of the Reaction Product. The reaction mixture consisted of 250 μM MoaA, 2 mM GTP, AdoMet, and 10 mM dithionite and was incubated in an anaerobic chamber for 5 h. The protein was then removed by ultrafiltration using a 10 kDa cutoff filter. The resulting small molecule pool was treated with 3 μL of alkaline phosphatase in the presence of 1 mM $MgCl_2$, incubated in the anaerobic chamber for an additional 3 h and quenched with 100 μL of oxygen free KI/I_2 (5% I_2 (w/v) and 10% KI (w/v) in water).[5,8,9] PFBHA (100 μL of 40 mM) was then added, and the mixture was heated at 65 °C for 1.5 h and analyzed by LC−MS. Control reactions, lacking MoaA, GTP, SAM, or dithionite were also run and similarly analyzed.

*MoaA-Catalyzed Reaction with 2′-ChloroGTP **20**.* 2′-ChloroGMP (3 mM) was synthesized and phosphorylated using NDP kinase (100 units), guanylate kinase (50 μM), and ATP (10 mM). Guanylate kinase was overexpressed and purified as described earlier.[5] This crude reaction mixture was used as a source of 2′-chloroGTP. For the MoaA-catalyzed reaction, 250 μM MoaA, 2 mM 2′-chloroGTP, 3 mM AdoMet, and 10 mM dithionite were mixed and incubated in the anaerobic chamber for 5 h. Controls were set up for this reaction in which either MoaA, AdoMet, dithionite, or NDP kinase/Guanylate kinase were absent. The small molecule pool was analyzed by LC−MS. For the trapping of the reactive 2′-Cl-ribose-derived product, all the above reactions were performed in dithiothreitol (DTT) free buffer. A 50 μL portion of the small molecule pool was treated with 3 μL of alkaline phosphatase followed by 3-mercaptobenzoic acid (50 μL of 100 mM solution) at 70 °C for 1 h.

*MoaA-Catalyzed Reaction with 2′-DeoxyGTP (**30b**).* MoaA (250 μM), 2 mM 2′-deoxyGTP, 3 mM AdoMet and 10 mM dithionite were mixed and incubated in the anaerobic chamber for 5h at room temperature. Controls were also set up in which MoaA, 2′-deoxyGTP, AdoMet or dithionite were absent. The protein was removed by ultrafiltration using a 10 kDa cutoff filter. The small molecule pool was treated with 3 μL of alkaline phosphatases in the presence of 1 mM $MgCl_2$ and incubated in the anaerobic chamber for 3 h. The reaction mixture was analyzed by LC−MS and the product was purified by reverse phase HPLC. The isolated product was concentrated using a vacuum centrifuge. For the coelution experiment 100 μM stock solutions of compounds **25** and **26** were made. Standards (50 μL) and 50 μL of the concentrated product were mixed and analyzed by HPLC.

*Synthesis of Compounds **25**, **26**, and 2′-ChloroGTP **20**.* The synthetic schemes and synthetic procedures for 2′-chloro GMP (the 2′*R* and the 2′*S* isomer), compound **25** and **26** are described in the Supporting Information.

RESULTS

Characterization of the MoaA Reaction Product. A time course for the product formation in the MoaA-catalyzed reaction is shown in Supporting Information, Figure SI38. Most of the product (approximately 70%) is formed in the first 90 min and there is no observed lag phase. The reaction was allowed to proceed for 5 h to maximize product formation. The protein was then removed by ultrafiltration, and the small molecule pool was treated with alkaline phosphatase to facilitate product purification by reverse phase HPLC. As the reaction product is highly oxygen sensitive, undergoing decomposition to a mixture of products, it was cleanly oxidized using KI/I_2 before exposure to air.[5,8,9] The resulting product was purified by HPLC as a fluorescent compound eluting at 17.4 min (Figure 2A). The UV−visible spectrum of the purified product shows the characteristic features of a pterin (Figure 2B).[2,10] Compound **25** was used as a standard to estimate the yield of product **15** formation at 30−34% (75−85 μM of product formed using 250 μM MoaA, assuming a single turnover enzyme). Multiple small-scale MoaA reactions were run to give sufficient product for characterization by LC−MS and NMR spectroscopy.

The LC−MS shows that the $[M + H]^+$ (280.1 Da) corresponds to the mass of compound **16** and **18** (Figure 2C). The ^1H NMR and the dqfCOSY and HSQC for the purified compound are shown in Figure 2D,E and Supporting Information, Figure S35. All are consistent with a mixture of compounds **16**, **17**, and **18**. The spectra are not consistent with an oxidation product derived from **8**.[7]

Hydroxylamine Derivatization of the MoaA Reaction Product. The oxidized reaction mixture prepared as described above was treated with PFBHA and analyzed for oxime

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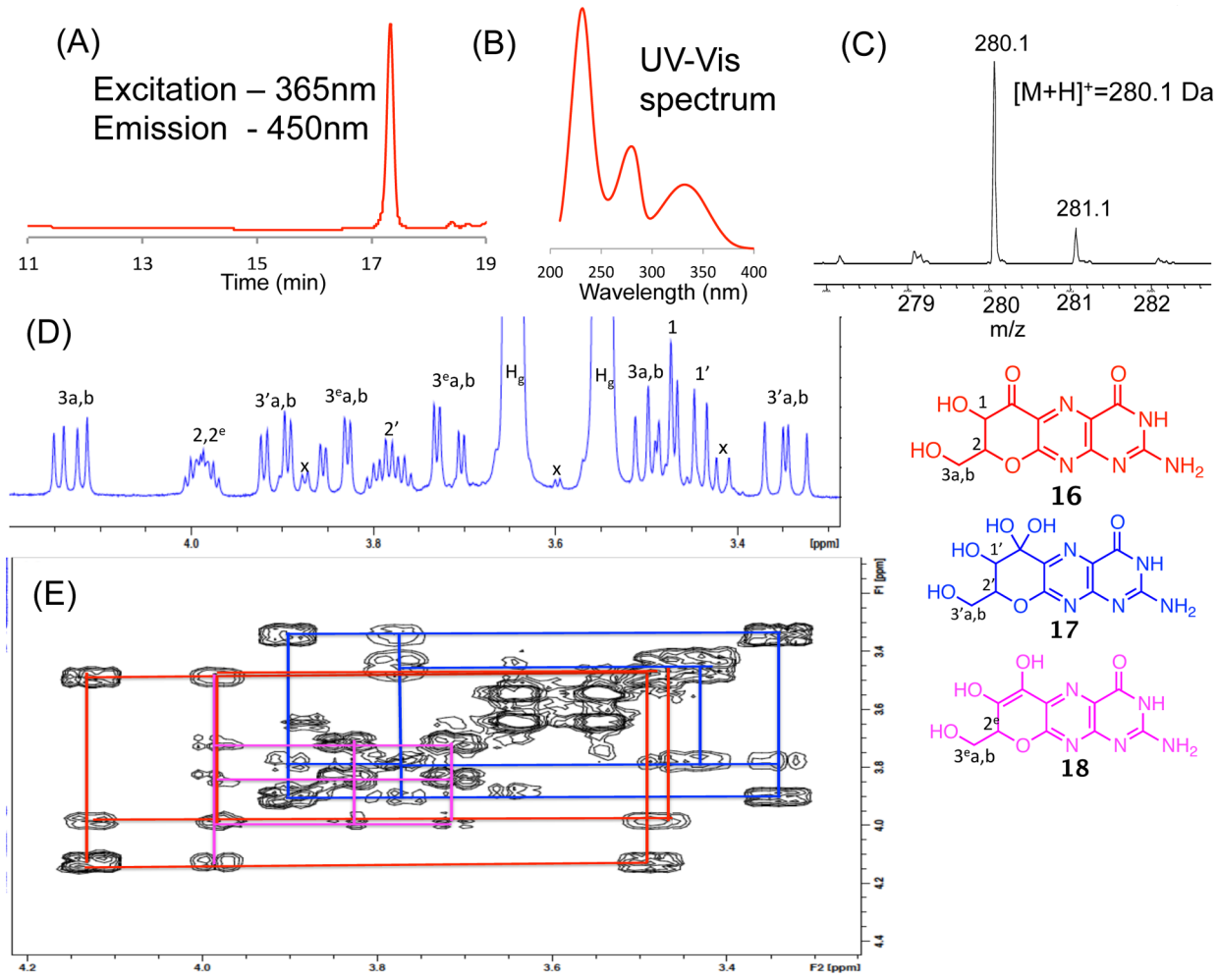

Figure 2. (A) MoaA assays with GTP are treated with phosphatase followed by oxidation with KI/I_2. The HPLC traces show the formation of the fluorescent product eluting at 17.4 min. (B) UV−vis spectrum of the product. The UV−vis spectrum of the compound was similar to that of a pterin.[2] (C) The mass spectrum $[M + H]^+$ of the product shown in the HPLC trace in Figure 2A. (D) ^{1}H NMR of the purified product. 1,2,3a,b are the signals for compound **16**, 2^e, 3^{e}a,b are the signals for compound **18**, and 1′,2′,3′ab are the signals for compound 17. X is an unknown impurity in the sample. (E) dqfCOSY of the purified product. The signals at 3.55 and 3.65 ppm are glycerol impurities.

formation by LC−MS. Figure 3 shows the extracted ion chromatograms for 475.1 Da which corresponds to the $[M + H]^+$ for the *E* and *Z* isomers of compound **19**. These were absent in controls where MoaA, GTP, AdoMet, or dithionite is absent.

MoaA Catalyzed Reaction with 2′-ChloroGTP 20. LC−MS and HPLC analysis of the MoaA/2′-chloroGTP reaction mixture demonstrated the formation of a new compound eluting at 3 min and identified as guanine **21** by comparison with an authentic standard (Figure 4A and B). Pterin was not detected in the reaction mixture.

The ribose-derived product **22** was trapped by treating the reaction mixture with 3-mercaptobenzoic acid. The extracted ion chromatogram for $[M-H]^-$ = 249.0 Da corresponded to the $[M-H]^-$ of compound **24**, the expected trapped product. An authentic standard of compound **24** was synthesized (Supporting Information, Figures SI19 − SI26) and found to be identical by LC−MS analysis to the trapped product (Figure 5).

MoaA-Catalyzed Reaction with 2′-DeoxyGTP (30b). The enzymatic reaction mixture was treated with alkaline phosphatase and analyzed by HPLC. To collect sufficient product, multiple reactions were run. A fluorescent product was observed at excitation wavelength of 365 nm and an emission wavelength of 450 nm. The UV−vis spectrum of the compound was similar to that of a pterin.[2,10] Oxidation with KI/I_2 was not necessary to stabilize this reaction product. LC−MS analysis revealed an $[M + H]^+$ = 268.1 Da. The UV−vis spectrum and LC−MS experiments suggested that structures **25** or **26** with undetermined stereochemistry at the purine-derived carbon. To resolve this, we synthesized both isomers (Figure S28−S32) and compared them with the enzymatic product by HPLC. This analysis demonstrated that the reaction product has the stereochemistry shown in **25** (Figure 6C). Compound **25** was used as a standard to estimate the yield of product **25** formation at 16−20% (40−50 μM of product formed using 250 μM MoaA, assuming a single turnover enzyme).

DISCUSSION

MoaA catalyzes a remarkable rearrangement reaction involving the insertion of the C8 carbon of GTP into the C2′-C3′ bond. MoaA is a radical SAM enzyme and uses the 5′-deoxyadenosyl radical to mediate this chemistry. Previous studies demonstrated that the 3′-hydrogen atom of GTP is abstracted by the 5′-deoxyadenosine radical and that intermediate **8** could be trapped as 2′,3′-dideoxy **8** using 2′,3′-dideoxyGTP as a substrate analogue. These studies were consistent with the

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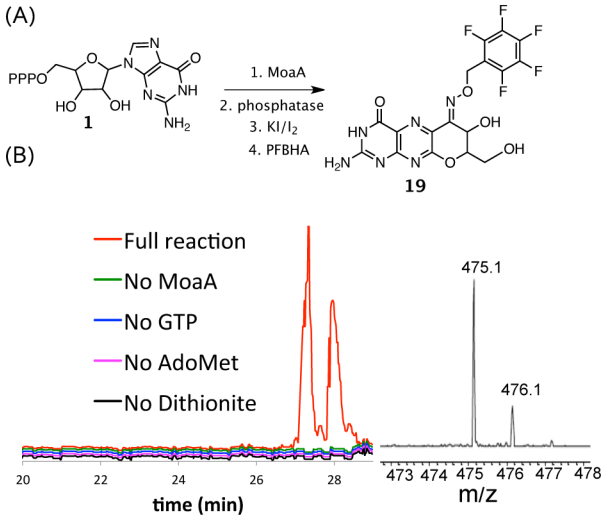

Figure 3. Trapping of the ketone-containing MoaA reaction product by oxime formation. (A) The MoaA reaction mixture was treated with phosphatase, oxidized with KI/I_2 and converted to the oxime with PFBHA. (B) Extracted ion chromatogram of the reaction mixture at 475.1 Da showing two peaks consistent with a mixture of the *E* and *Z* isomers of oxime **19** ($[M + H]^+$ of **19**(E+Z) = 475.1 Da).

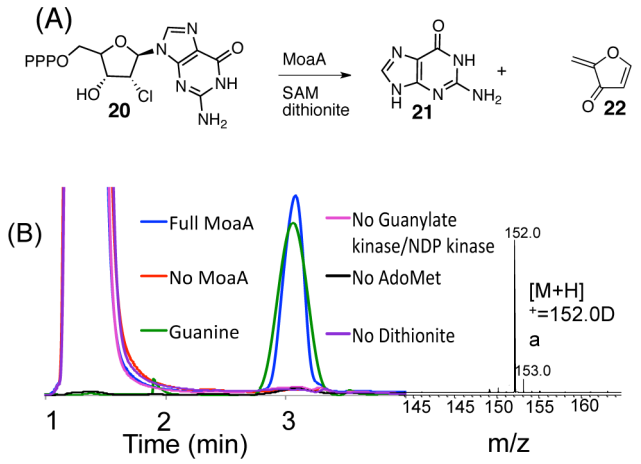

Figure 4. (A) The MoaA-catalyzed reaction of 2′-chloroGTP **20** results in the formation of guanine **21** and furanone **22**. (B) LC–MS traces to confirm the formation of guanine as one of the products.

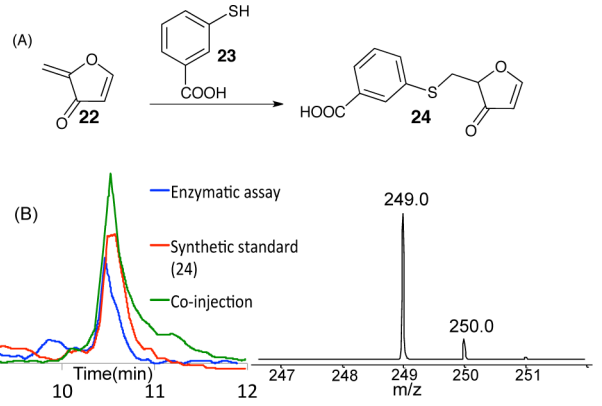

Figure 5. Analysis of the MoaA-catalyzed reaction of 2′-chloroGTP **20** for the ribose derived product: (A) The reaction mixture was treated with phosphatase followed by 3-mercaptobenzoic acid to yield **24**. (B) The chromatograms are extracted ion chromatogram 249.0 Da corresponding to the $[M-H]^-$ of **24**. Co-injection shows that that the 3-mercaptobenzoic acid derivative of the enzymatic product is identical to a synthesized sample of **24** by LC–MS analysis. Guanine was used as a standard to estimate the yield of product formation at 36–40% (90–100 μM of guanine formed using 250 μM MoaA, assuming a single turnover enzyme).

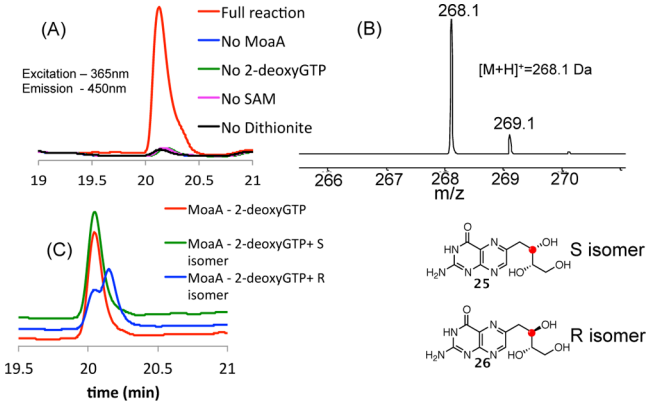

Figure 6. Analysis of the MoaA catalyzed reaction with 2′-deoxyGTP **30b**: (A) The HPLC chromatograms (fluorescence–excitation, at 365 nm; and emission, at 450 nm) show that a unique signal is seen only in the full reaction and not in the controls in which MoaA, GTP, AdoMet, or dithionite is absent. (B) $[M + H]^+$ of the unique signal in the full reaction. (C) HPLC co-injection experiment to determine the stereochemistry at the site of initial hydrogen atom abstraction.

mechanistic proposal shown in Figure 1. Independently, Kenichi Yokoyama's group found conditions under which MoaA releases **8**.[7] They directly characterized this intermediate and suggested it and not **15** was the product of MoaA. In our assays, we do not see a buildup of compound **8** (Supporting Information, Figure S33–S34).

Our first task therefore was to establish the identity of the MoaA reaction product (**8** or **15**). This was done by treating the reaction mixture with alkaline phosphatase followed by oxidation with KI/I_2 to cleanly convert the oxygen sensitive reaction product to a stable oxidized form. The resulting product was purified and identified by LC–MS and NMR analysis as a mixture of pterins **16**, **17**, and **18**. Product identity was further supported by the conversion of this mixture to the corresponding oxime **19**. Since it is very unlikely that KI/I_2 could catalyze the conversion of **8** to **15**, we conclude that **15** is the product of the MoaA reaction and that MoaC catalyzes the conversion of **15** to **2**. To exclude the possibility of MoaC contamination in our MoaA samples, we overexpressed MoaA in a MoaC deletion mutant of *E. coli*. The MoaA reaction product isolated from this strain had an identical retention time (Supporting Information, Figure SI36) to the product described in Figure 1A. This product structure was also confirmed by derivatization with hydroxylamine and LC–MS analysis (Figure SI37).

We next explored the use of 2′-chloroGTP **20** to trap radical **5**. This strategy was previously developed to study the radical intermediates formed by ribonucleotide reductase, DNA irradiation, and by radical-generating antibiotics.[11−13] In the event, treatment of 2′-chloroGTP **20** with MoaA generated guanine **21** and a ribose-derived product. This was identified as **22** by trapping with 3-mercaptobenzoic acid **23** followed by LC–MS analysis to demonstrate identity with a synthetic sample of **24**. A mechanistic proposal for this reaction is shown in Figure 7. We also tested the 2′-chloroGTP isomer where the

dx.doi.org/10.1021/ja502663k | J. Am. Chem. Soc. 2014, 136, 10609–10614

2′-chloro was in the *S* orientation as a substrate analogue. No reaction was observed.

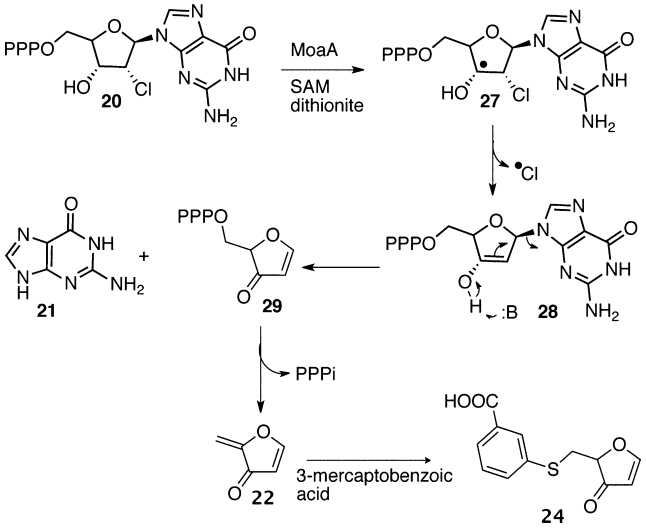

Figure 7. Mechanism of the MoaA catalyzed reaction of 2′-chloroGTP **20**. Loss of chloride from **27** followed by reduction is also possible.

Finally, we explored the use of 2′-deoxyGTP **30b** to probe the later steps of the reaction. The proposal in Figure 1 suggests that this compound might block the conversion of **8** to **15** and that any trapped species would elucidate the later steps in the MoaA-catalyzed reaction. In the event, treatment of **30b** with MoaA followed by phosphatase resulted in the formation of pterin **25**. KI/I_2 oxidation was not necessary to stabilize this compound. The stereochemistry at C3 of the 2,3,4-trihydroxybutyl substituent is assumed to be the same as that in the starting 2′-deoxy-GTP because this center does not participate in the reaction (Figure 1). The *S*-stereochemistry at C2 (the site of initial hydrogen atom abstraction) was determined by LC−MS comparison of the enzymatic product with synthetic standards. This suggests that the formation of **25** is primarily or exclusively occurring at the MoaA active site because nonenzymatic chemistry would result in the scrambling of stereochemistry at C3 of the pterin substituent. The formation of **25** is not consistent with the proposed conversion of **8** to **15** shown in Figure 1 and a revised mechanistic proposal for the MoaA-catalyzed reaction is outlined in Figure 8. When 2′-deoxyGTP is used as a substrate, we propose that **35b**, formed as shown in Figures 1 and 8, undergoes ring opening and two tautomerizations, to give **38b**. Conjugate addition followed by a double tautomerization would give **44**. Loss of water and a final tautomerization would give the observed enzymatic product **25**. For the native MoaA-catalyzed reaction, using GTP as the substrate (30a, X = OH), hydrogen atom abstraction from C3′ of GTP gives radical **31a** which then adds to C8 of the purine to give **32a**. Electron transfer from the purine liganded [4Fe-4S] cluster gives **33a**. Aminal hydrolysis to **34a** followed by a benzylic-like rearrangement gives **35a**. Ring opening followed by two tautomerizations gives **38a**, which is converted to **15** by a conjugate addition, water elimination, two tautomerizations, and a final ring closure. This mechanism is consistent with the previously reported regiochemistry of hydrogen atom abstraction by the 5′-deoxyadenosyl radical, with the trapping of **8** directly or using 2′,3′-dideoxyGTP and with the results described in this paper in which radical **31a** is trapped using 2′-Cl-GTP and 2′-deoxyGTP is converted to **25**.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information contains procedures for synthesis of reference compounds **24**, **25**, **26**, time course for MoaA reactions with GTP, and data for enzymatic assays with MoaA ($MoaC^-$). This material is available free of charge via the Internet at http://pubs.acs.org.

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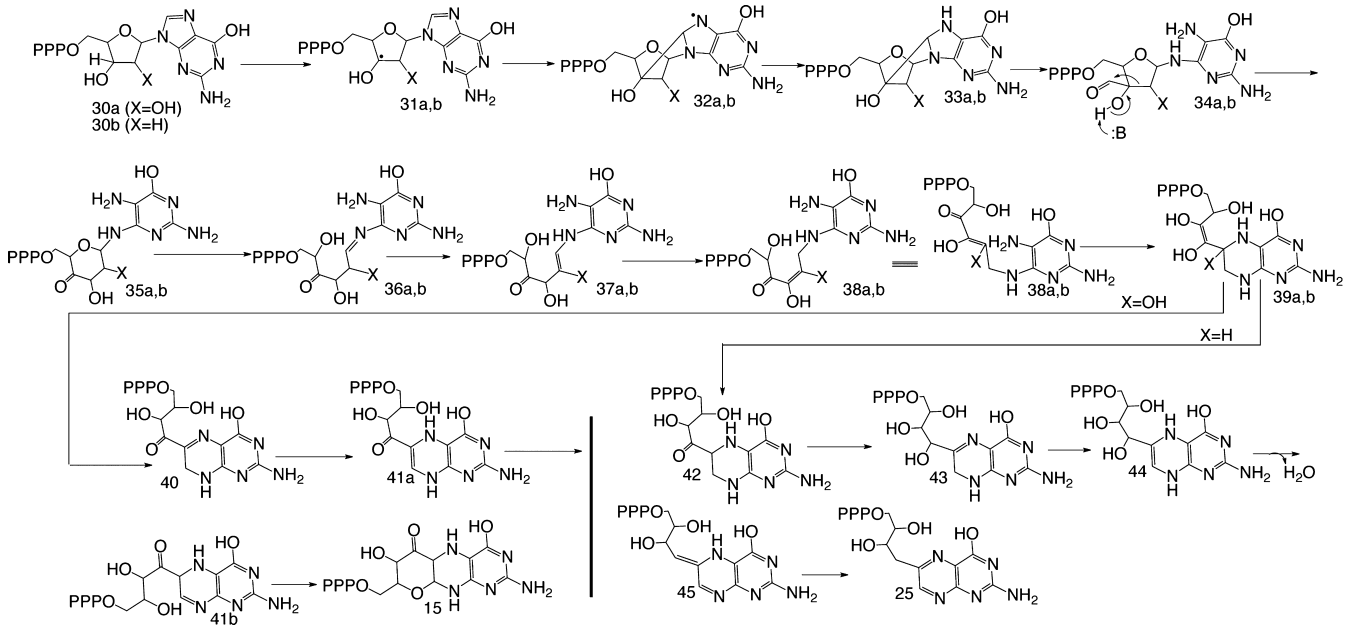

Figure 8. Mechanistic proposal for the MoaA-catalyzed conversions of GTP **30a** (also **1**) to **15** and 2′-deoxyGTP **30b** to **25**.

dx.doi.org/10.1021/ja502663k | *J. Am. Chem. Soc.* 2014, 136, 10609−10614

Author Contributions

[†]A.P.M. and S.H.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by a grant from the National Institutes of Health (DK44083) and by the Robert A. Welch Foundation (A-0034).

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