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Inhibition of the Fe₄S₄ Cluster-containing Protein IspH (LytB): EPR, Metallacycles and Mechanisms

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Abstract

We report the inhibition of the *Aquifex aeolicus* IspH enzyme (LytB, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate reductase, EC 1.17.1.2) by a series of diphosphates and bisphosphonates. The most active species was an alkynyl diphosphate having an IC₅₀ = 0.45 μM (*K*_i ~ 60 nM), which generated a very large change in the 9 GHz EPR spectrum of the reduced protein. Based on previous work on organometallic complexes, together with computational docking and quantum chemical calculations, we propose a model for alkyne inhibition involving π (or π/σ) “metallacycle” complex formation with the unique 4th Fe in the Fe₄S₄ cluster. Aromatic species had less activity and for these, we propose an inhibition model based on an electrostatic interaction with the active site E126. Overall, the results are of broad general interest since not only do they represent the first potent IspH inhibitors, they suggest a conceptually new approach to targeting other Fe₄S₄-cluster containing proteins that are of interest as drug and herbicide targets.

Introduction

The Rohmer, methyl-D-erythritol phosphate or non-mevalonate pathway is responsible for isoprenoid biosynthesis in most pathogenic bacteria, as well as in malaria parasites.^{1, 2} Since isoprenoids are essential for survival in these organisms and since the non-mevalonate pathway is absent in humans, enzymes that comprise the pathway are important anti-infective drug targets^{3, 4} and in previous work^{5, 6} it was shown that fosmidomycin (which inhibits the second enzyme in the pathway) gave very promising results in treating malaria. The structures and mechanisms of action of six of the eight enzymes present in the pathway have been known for some time, but deducing the structures (and mechanisms of action) of the last two enzymes has been more challenging. The penultimate enzyme is *E*-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) synthase (IspG, GcpE, EC1.17.7.1) which catalyzes the 2H⁺/2e⁻ reduction of 2-*C*-methylerythritol-2,4-*cyclo*-diphosphate (MEcPP, **1**) to HMBPP (**2**), while the terminal enzyme, HMBPP reductase (IspH, LytB, EC1.17.1.2) catalyzes the 2H⁺/2e⁻ reduction of HMBPP to isopentenyl diphosphate (IPP, **3**) and dimethylallyl diphosphate (DMAPP, **4**), in a ~5:1 ratio.

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The structure of IspG has not yet been reported, but there have been two structures published for IspH, one from *Aquifex aeolicus*,⁷ the other from *E. coli*.⁸ Both structures contain Fe₃S₄ clusters. However, based on the results of Mössbauer^{9,10} and EPR spectroscopy,¹¹ and from microchemical analyses,^{9, 11} we and others have concluded that Fe₄S₄ clusters are responsible for catalysis. The Fe₄S₄ cluster, while catalytically active, is thus relatively labile, as also found for example in aconitase and in pyruvate-formate lyase activating enzyme.¹² To date, there has been only one report of IspH inhibitors,¹³ with IC₅₀ values of ~1–2 mM having been obtained. Here, we report progress aimed at obtaining more potent inhibitors.

Based on the crystallographic, mutagenesis, catalytic activity and spectroscopic results described previously,^{7–11} we reasoned that there might be two distinct approaches to developing IspH inhibitors. In the first, it might be possible to develop “conventional” inhibitors which bind to amino-acids involved in catalysis. In previous work⁷ we proposed that a key residue involved in delivering H⁺ to HMBPP was the totally conserved E126, and the essential nature of this residue in catalysis has now been confirmed by site-directed mutagenesis.⁸ We reasoned that basic or cationic inhibitors might interact with this key residue, inhibiting the enzyme. The second hypothesis to test is that “unconventional” inhibitors, compounds that bind to the Fe/S cluster, might be developed. If the cluster is Fe₃S₄, this would be unprecedented and would thus, of course, not suggest what inhibitor structures to make. On the other hand, if the active species is Fe₄S₄ (as deduced from the IspH Mössbauer,^{9, 10} EPR¹¹ and catalytic activity^{9, 11} results), there is in fact precedent for the formation of organometallic species (i.e. containing Fe–C bonds) between Fe₄S₄ clusters and alkynes, which would be expected to act as IspH inhibitors. For example, in the early literature, McMillan *et al.*¹⁴ investigated the reduction of acetylene (C₂H₂) to ethylene (C₂H₄) by reduced Fe₄S₄ synthetic clusters, in particular [Fe₄S₄(SPh)₄]^{3–}, **5**. These workers proposed that an acetato complex reacted initially with C₂H₂ to form an organometallic species, **6**:

most likely containing a side-on (π/σ) acetylene unit, which was then *cis*-reduced to ethylene. Basically the same reduction, of diphenylacetylene (**7**) to *cis*-stilbene (**8**), was reported by Itoh.¹⁵

In these systems, the alkyne complex was not observed directly, but under controlled potential (electrochemical reduction) conditions, Tanaka *et al.*¹⁶ found evidence for a π complex of acetylene bound to [Fe₄S₄(SPh)₄]^{3–} and [Mo₂Fe₆S₈(SPh)₉]^{3–} clusters, as evidenced by quite large shifts in the C≡C vibrational Raman spectra. These workers also demonstrated that acetylene bound most strongly to reduced ([Fe₄S₄]⁺) clusters, and resulted in release of 1

SPh⁻. These results suggested to us that acetylenes might likewise bind to reduced IspH, forming organometallic (Fe-C) bonds. Interestingly, with low valent (Fe^I) complexes, alkynes have also been found to bind far more strongly than do alkenes,¹⁷ leading again to the possibility that alkynes could bind to Fe and be good IspH inhibitors, displacing the olefinic substrate, HMBPP.

This formation of a π (or π/σ) “metallacycle” complex would be very similar to that deduced for the binding of the HMBPP parent molecule, allyl alcohol (which lacks the Me and CH₂OPP substituents) to the FeMo cofactor in nitrogenase,¹⁸ illustrated schematically above (**9**) as the Fe₃MoS₃X cubane-like fragment. It also seemed possible that aromatic residues might interact with the Fe₄S₄ cluster, just as the cyclopentadienide ion does in model Fe₄S₄ clusters,¹⁹ such as **10** (as an η^6 as opposed to an η^5 species). Although the structures of complexes such as **6** have not been confirmed crystallographically, the structure proposed by McMillan et al.¹⁴ (**6**) is likely to involve the same type of bonding as found in many other organometallic complexes,²⁰ being described¹⁸ as a resonance hybrid of a pure π complex and a σ complex, the latter corresponding to a metallacycle.

Results and Discussion

In an effort to learn more about just what types of compound might bind to the Fe₄S₄ cluster in IspH, we first screened a series of small molecules, using EPR spectroscopy. The 9 GHz EPR spectrum of *A. aeolicus* IspH in the $g \sim 2$ region exhibits a broad spectrum (Figure 1a), very similar to that of the *E. coli* protein (Figure 1b) and is characteristic of an S=1/2 [Fe₄S₄]⁺ species.¹¹ The unliganded *A. aeolicus* IspH also has two small signals at $g \sim 5$ (Supporting Information Figure S1a), which likely indicate the presence of higher spin species. For purpose of comparison, the g -values for the systems studied here are presented in Supporting Information Table S1, together with g -values for related systems such as aconitase, and IspG (GcpE).

We first tested a series of small molecules and ions: CO, N₃⁻, MeCN and CN⁻, for cluster-binding in *A. aeolicus* IspH, but only CN⁻ was found to have any effect on the EPR spectrum, Figure 1c (and Supporting Information Figure S1b). The g -values observed were 2.08/2.05 and 1.94, Figure 1c, close to those observed previously with CN⁻ binding to the *Pyrococcus furiosus* 4Fe ferredoxin ($g_1 = 2.09$, $g_2 = 1.95$, and $g_3 = 1.92$), another 4Fe-4S cluster with 3 Cys ligands and a unique 4th Fe position,²¹ due we propose to end-on binding to the 4th Fe in IspH. There was also an increase in signal intensity in the $g \sim 2$ region (from ~20% to ~60% spin/protein), due to conversion of the higher spin state species to S=1/2.

We then tested a series of other small molecules that might be anticipated to bind in a sideways-on mode (as with proposed for acetylene in the model systems): propargyl alcohol, propargylamine and propiolic acid. All species resulted in large spectral changes, and by way of example the IspH + propiolic acid (HC≡CCO₂H) spectrum is shown in Figure 1d. These results indicated to us that, as with the Fe₄S₄ containing small molecules, acetylenes bind to IspH, forming π or π/σ complexes. With both CN⁻ as well as propiolic acid binding there also appear to be two bound ligand conformations or binding modes (e.g. hydrogen bonded or not), as evidenced by the splitting of the g_1 signals.

However, propiolic acid had poor activity in IspH inhibition, and we reasoned that acetylenic diphosphates (or the isoelectronic, cyano diphosphates) might interact more strongly with IspH, since there would be an increase in binding affinity due to their diphosphate moieties docking into the (inorganic) PPi site seen crystallographically.⁸ We thus synthesized four acetylenic diphosphates (**11–14**; Figure 2) and two cyanoalkyl diphosphates (**15, 16**) and tested them for their activity in IspH inhibition. IC₅₀ results are shown in Figure 2. The most active compound

was **12** with an $IC_{50} = 0.45 \mu\text{M}$ ($K_i \sim 60 \text{ nM}$; Figure 3a). As shown in Figure 3b, this compound can bind with its diphosphate group in the inorganic “P_i” site seen crystallographically,⁸ with the alkyne fragment interacting with the unique 4th Fe (identified from EPR, Mössbauer and catalytic activity results, and added computationally here as described previously⁷), with Fe-C bond lengths of $\sim 2.4 \text{ \AA}$, suggesting π -complex formation. There was a large change in the EPR spectrum of IspH in the presence of **12** (Figure 1e; Supporting Information Figure S1c) indicating a major change in the cluster’s electronic structure, consistent with these docking results. Moreover, there was evidence for only a single bound species. The shorter chain analog **11** was less active than was **12**, as was the longer chain analog **13**, and conversion of the bridging O to CH₂ (**17**) reduced the activity of **11** even more (Figure 2), due presumably to a disruption in H-bonding to the SXN motif. Likewise, the addition of a terminal CH₂OH (**14**) reduced activity, consistent with the known stronger binding of terminal versus substituted alkynes to low valent Fe complexes.¹⁷ The changes in g-values on addition of the acetylenes were generally similar, as expected, and as with CN[−] there was a $\sim 3\times$ increase in signal intensity in the g ~ 2 region (due to a high-to-low spin state conversion). We also tested the effects of the isoelectronic analogs (**15**, **16**) of the acetylenes ($-\text{C}\equiv\text{C}-\text{H} \rightarrow -\text{C}\equiv\text{N}$), cyanides, on IspH inhibition, but both compounds were far less active than their acetylenic counterparts.

These results clearly indicate that alkyne diphosphates can be good IspH inhibitors, with the most active species being $> 1,000\times$ more potent than previously reported IspH inhibitors.¹³ Since P_i itself and other diphosphates are only weak ($\sim 1 \text{ mM}$) IspH inhibitors,⁸ we conclude that ligand binding is driven by π (or π/σ) or η^2 -alkynyl complex formation with the Fe₄S₄ cluster, the same type of complex formation as suggested by Raman spectroscopy,¹⁶ for acetylene binding to an [Fe₄S₄(SPh)₄]^{3−} cluster. Moreover, the results of density functional theory calculations show that there is indeed Fe-C metal ligand bonding present in an [Fe₄S₄(SMe)₃(HC≡CCH₂OH)]^{2−} model system. We show in Figures 3c,d some typical MO results. The $\alpha\text{HOMO}-1$ shows the interaction of an Fe d orbital with the C≡C π orbital, in the plane of the Fe...C≡C fragment. The $\beta\text{HOMO}-1$ illustrates the interaction between an Fe d orbital with another type of C≡C π orbital, this time perpendicular to the plane of Fe...C≡C. If IspH, under these reducing conditions, contained an Fe₃S₄ cluster, there would be no obvious way in which acetylenes would bind tightly to the cluster (or the protein), while η^2 -alkynyl (i.e. π or π/σ) complex formation is predicted, based on previous work, and is supported by the DFT results.

We next investigated a series of cationic (or basic) diphosphates and (cationic) bisphosphonates, which are isosteres of the diphosphate group. In previous work²² we found that aromatic, cationic bisphosphonates (such as **18**) were potent ($K_i \sim 20 \text{ nM}$) inhibitors of the enzyme farnesyl diphosphate synthase since, as shown below:

their charge distribution mimics that expected for the allylic cation/diphosphate anion ion-pair.²³ In the case of IspH, similar allylic cations (shown above) have been proposed²⁴ to be involved in catalysis, so we tested both **18** and **19** for their activities in IspH inhibition. Neither had potent activity (**18**, $IC_{50} = 540 \mu M$; **19**, $IC_{50} > 1 mM$, Figure 2). We then tested six additional bisphosphonates (**20–25**, Figure 2) with 1-H, 1-Me or 1-OH backbone groups and pyridine, pyridinium or amino-pyridine side-chains. The most active compound ($IC_{50} = 67 \mu M$) was **21**, an amino-pyridine, expected to contain an amidinium-like (protonated) side-chain. Overall, however, the activity of these bisphosphonates was only modest, and based on Glide docking results using the “closed” IspH structure (PDB File #3F7T) it appeared that this might be due to the “branched” nature of the bisphosphonate backbone. We thus next synthesized a series of pyridinium diphosphates, **26–29**, which based on computational docking results using Glide²⁵ appeared to better fit the IspH active site. The most active compound in the series (Figures 2, 4a) was **26**, a *meta* pyridinium diphosphate having an $IC_{50} = 38 \mu M$, slightly more potent than the best bisphosphonate, **21**. When docked into the IspH active site, we find (Figures 4b, c) that when the diphosphate backbone binds to the “PPi” site seen crystallographically, the pyridinium H^N is only $\sim 1.9 \text{ \AA}$ from the E126 O (modeled as CO₂[−]), indicating the possibility of an H-bond or electrostatic interaction with this active site residue. The *para*-pyridinium analog (**27**) was less active ($IC_{50} = 149 \mu M$), and the *ortho*-pyridinium analog (**28**) was far less active ($IC_{50} = 1.2 mM$), correlating with the increased NH–O (CO₂[−]) distances of 3.47 Å and 4.28 Å, Figure 4c. Interestingly, all three aromatic rings also locate close to the 4th Fe, but the major decrease in activity seen as the ring nitrogens move away from the E126 carboxyl indicates the dominance of a Coulombic interaction, consistent with the lack of activity of the chloropyridine analog, **29**, which is expected to be far less basic than is **26**. These activity and docking results are consistent, then, with the pyridinium diphosphates binding to IspH with their PPi groups binding to the inorganic PPi site, while their aromatic rings interact with E126, as illustrated in Figures 4b, c. There was no major change in the EPR spectrum of **26** bound to the protein, Supporting Information Figure S1d, consistent with the lack of any π -complex interaction. We then sought to improve activity by varying the length of the CH₂ spacer in the side-chain: addition of 1 CH₂ group (**30**) resulted in an $IC_{50} = 9.1 \mu M$ ($K_i \sim 1.2 \mu M$), but addition of 2 CH₂ groups reduced activity (**31**, $IC_{50} = 463 \mu M$; Figure 2), with the pyridinium nitrogen in **30** again docking close to E126, while **31** did not. Taken together, these results argue against formation of a carbocation mechanism since for this, we would anticipate good activity for **28** (with maximum charge density separated by two carbons from the PPi backbone), while experimentally, maximum activity is found with **30** (with its NH⁺ located four carbons from the PPi backbone). That is, none of the cationic (**18**, **19**) or pyridine/pyridinium analogs of the putative carbocation transition states (or reactive intermediates) act as inhibitors, while the longer chain species that can bind to both the PPi site as well as E126, are the most potent inhibitors among the whole series of compounds (**18–31**) investigated. Notably, with both the alkyne as well as the cationic inhibitors, the side-chains are located (Figure 4d) close to the Fe₄S₄ cluster blocking, we propose, HMBPP access.

These results lead to the proposals for IspH inhibition shown in Figure 5. We propose that both alkyne as well as alkene diphosphates (i.e. HMBPP) bind to the Fe₄S₄ cluster at the unique 4th Fe, forming π (or π/σ) “metallacycles”, as shown in Figures 5a, b, the same type of binding reported previously for the HMBPP parent molecule (allyl alcohol) bound to the FeMo cofactor in nitrogenase.¹⁸ As shown in Figure 5b, the presence of such a metallacycle also immediately leads to a proposal for the IspH catalytic mechanism in which in a first H⁺/e[−] step, the HMBPP 4-OH is protonated and an η^1 -allyl intermediate and water form. This intermediate is then cleaved in a second H⁺/e[−] step, forming the DMAPP product, Figure 5b. IPP forms in the same way, after an η^1 -allyl (or η^3 -allyl) isomerization. This model is described in more detail elsewhere²⁶ and is supported by electron nuclear double resonance spectroscopy using [^u-¹³C] and [²H]-labeled HMBPP, which strongly suggest metal-ligand interaction. We thus propose that the alkyne as well as the cationic inhibitors block or displace HMBPP from binding

to the Fe_4S_4 cluster: the alkynes form π (or π/σ) “ η^2 -alkynyl” metallacycles, while the cationic inhibitors interact with the active site E126 carboxylate, again effectively blocking HMBPP binding.

Validation of the Mechanism of IspH Inhibition, and Catalysis

Finally, after review of this article, Grawert et al.²⁷ reported the results of new crystallographic structures of HMBPP, PPI, IPP and DMAPP bound to IspH²⁷ which help us validate the inhibition models proposed here. These workers now report that HMBPP binds as an alkoxide to the unique 4th iron (to the oxidized $[\text{Fe}_4\text{S}_4]^{2+}$ cluster), the same binding mechanism we proposed earlier.⁷ On irradiation in the x-ray beam, HMBPP is reduced, forming a deoxy species (bound to the Fe_4S_4 cluster), basically as we proposed based on EPR/ENDOR and docking results.²⁶ These x-ray results are of interest since they enable us to make a direct comparison between the x-ray and EPR/ENDOR/docking based methods,^{26, 27} helping us validate the use of inhibitor docking. We show in Figure 6a an alignment of the x-ray structure of HMBPP bound to IspH with that we proposed (Figure 1G in ref²⁶) for the initial HMBPP binding pose in which the O-4 can interact with the unique 4th Fe. There is a 0.32 Å rmsd between the heavy atoms (C, O, P) in the HMBPP ligand between the x-ray and our docking/EPR based structures, ~0.43 Å if we include the Fe, S atoms in the cluster, Figure 6a. On reduction, we proposed²⁶ that the 4-OH is removed from the ligand and the σ/π metallacycle is reduced, resulting in loss of water and formation of a η^1/η^3 -allyl complexes and thence, IPP and DMAPP.

The new crystallographic results are thus in good accord with those we proposed from our EPR, ENDOR and computational work, which gives confidence in the proposed ligand geometries we describe here for the bound inhibitors. As can be seen in Figure 6b, the alkyne group in propargyl diphosphate (**11**) bound to IspH²⁶ is located in essentially the same position as the deoxy product species seen crystallographically, where it can interact with the unique, 4th iron, forming a π or π/σ complex. The alkynes are much stronger inhibitors of IspH than are the alkenes (IPP, DMAPP),⁸ and indeed the ENDOR spectrum of **11** bound to IspH exhibits an ~6 MHz ^{13}C hyperfine interaction,²⁶ 2–3 times larger than the ^{13}C hyperfine interactions found for either allyl alcohol bound to nitrogenase,²⁸ or HMBPP bound to IspH.²⁶

Conclusions

Taken together, these results are of interest for several reasons. First, we find that cationic diphosphates are IspH inhibitors. However, rather than mimicking transition states/reactive intermediates, we propose that they interact with the active site E126, preventing HMBPP binding to the Fe_4S_4 cluster. Second, we find that alkyne diphosphates are quite potent (IC_{50} ~ 0.45 μM ; K_i ~ 60 nM) IspH inhibitors. This activity was predicted based on previous Raman and catalytic activity results with model systems, which have been interpreted as indicating π (or π/σ) “metallacycle” formation between Fe_4S_4 clusters and acetylene. That alkynes are, in fact, IspH inhibitors, also supports the idea that catalytically active IspH contains active-site Fe_4S_4 and not Fe_3S_4 clusters, since there is no obvious way in which an alkyne group would bind to the three exposed S^{2-} in such clusters. On the other hand, there is good literature precedent for alkynes binding to Fe_4S_4 clusters, supporting formation in IspH of π (or π/σ) complex formation. Third, the results of quantum chemical calculations indicate Fe-C metal ligand bonding with the acetylene group (in a $[\text{Fe}_4\text{S}_4(\text{SMe})_3(\text{HC}\equiv\text{CCH}_2\text{OH})]^{2-}$ model. Fourth, the formation of acetylene metallacycles is consistent with the observation that the HMBPP parent molecule, allyl alcohol, forms a metallacycle with the nitrogenase FeMo cofactor, which contains a related Fe/S (Mo, “X”) cluster and where the ligand forms Fe – C bonds with a single Fe. The observation that the IC_{50} for the most potent IspH alkyne inhibitor (IC_{50} = 0.45 μM ; K_i ~ 60 nM) is much smaller than the K_M for HMBPP binding (K_M = 5 μM) is also expected

based on organometallic precedent in which alkynes bind much more strongly (to low valent Fe complexes) than do alkenes.¹⁷ Fifth, we find that CN^- binds to IspH with the resulting spectrum quite strongly resembling that found with *P. furiosus* Fd, another protein with an unique 4th Fe. And finally, the recent revision of the earlier 3 Fe IspH structural/mechanistic models is in accord with our earlier mechanistic proposal of initial binding of the HMBPP 4-OH (as an alkoxide) to an $[\text{Fe}_4\text{S}_4]^{2+}$ cluster which, on reduction, forms an allyl complex which is then cleaved to form the IPP and DMAPP products.²⁶ This gives confidence in the models we propose here for alkyne inhibition. These results also open up the intriguing possibility that metallacycle complexes may act as reactive intermediates (or transition states) in both IspG as well as IspH catalysis, and that alkynes can be expected to be good inhibitors of other Fe_4S_4 cluster-containing enzymes which contains “unique” 4th Fe atoms.

Materials and Methods

A. *aeolicus* IspH protein purification

BL-21(DE3) cells expressing IspH from *A. aeolicus* were grown in LB media supplemented with 150 mg/mL ampicillin at 37 °C until the OD_{600} reached 0.6. Cells were then induced with 200 $\mu\text{g/L}$ anhydrotetracycline, then grown at 20 °C for 15 hours. Cells were harvested by centrifugation (9000 rpm, 8 min, 4 °C) and were kept at –80 °C until use. Cell pellets were resuspended and lysed in B-PER(Thermo Scientific, Rockford, IL) protein extraction reagent for one hour at 4°C, then centrifuged at 200,000 rpm at 4 °C for 15 min. The supernatant was applied to a Ni-NTA column equilibrated with 5 mM imidazole in pH 8.0 buffer containing 50 mM Tris-HCl and 150 mM NaCl. After washing with 20 mM imidazole, protein was eluted with 100 mM imidazole. Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5% glycerol, and 1 mM DTT, 4 times. The purified protein was flash-frozen in liquid nitrogen and stored at –80 °C until use.

E. coli IspH Protein Purification

BL21 DE3 (Invitrogen) cells harboring an *E. coli* IspH construct were grown in LB media at 37°C until the OD_{600} reached 0.6. Induction was performed with 200 ng/ml anhydrotetracycline at 20°C for 15 hours. Cells were harvested by centrifugation at 9000 rpm for 8 mins and stored at –80 °C. Cell pellets were resuspended and lysed in B-PER (Thermo Scientific, Rockford, IL) protein extraction reagent for about one hour at 4°C, then the lysate was centrifuged at 250,000 rpm for 30 mins. The supernatant was collected and loaded onto an IBA Strep-tag column equilibrated with buffer W (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). After washing with buffer W, protein was eluted using buffer E (buffer W containing 2.5 mM desthiobiotin). Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5% glycerol, and 1mM DTT, twice. The purified protein was flash-frozen in liquid nitrogen and stored at –80 °C until use.

Protein reconstitution

Both *A. aeolicus* and *E. coli* IspH proteins as isolated had a very small peak at 410 nm ($A_{280}/A_{410} < 0.02$), so were reconstituted for further studies. Before reconstitution, protein was transferred into a Coy Vinyl Anaerobic Chamber after being degassed on a Schlenk line. The following steps were performed inside the anaerobic chamber with an oxygen level < 2 ppm. In a typical reconstitution experiment, 10 mM DTT and ~ 0.5 mg of elemental sulfur were added to 3 mL 0.6 mM protein solution in a pH 8.0 buffer containing 50 mM Tris-HCl, 150 mM NaCl and 5% glycerol. After stirring for 1.5 hours, FeCl_3 was slowly added from a 30 mM stock solution to 6 equivalents. After 3 hours, an aliquot of the solution was centrifuged and a UV-VIS spectrum recorded. If the $A_{410\text{nm}}/A_{280\text{nm}}$ ratio was ≥ 0.38 , the protein was then desalted by passing through a PD10 column. If the ratio was < 0.38, more DTT, elemental sulfur and FeCl_3 were added and the sample incubated with stirring (for typically ~ 2 hours)

until the 410 nm/280 nm absorption ratio was ~ 0.38. The reconstituted protein was then concentrated by ultrafiltration, and the protein concentration determined by using a Bio-Rad (Hercules, CA) Protein Assay kit.

Enzyme inhibition assays

All assays were performed anaerobically at room temperature according to Altincicek et. al.²⁴ with minor modification. To a pH 8.0 buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, and 5% glycerol, sodium dithionite was added to 0.4 mM, methyl viologen was added to 2 mM, and IspH was added to 72 nM. For enzyme assays, various amounts HMBPP were added and the reactions were monitored at 732 nm. The initial velocities were fitted by using the Michaelis-Menten equation with OriginPro 8 (OriginLab Corporation, Northampton, MA) software. The activity of reconstituted *A. aeolicus* IspH tested under the conditions described above was 1.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ with a $K_M=7 \mu\text{M}$. For inhibition assays, various concentrations of inhibitor were added and incubated for 10 min, prior to addition of 34 μM HMBPP. Initial velocities at different inhibitor concentrations were then plotted as dose-response curves, and were fitted to the following equation, from which IC_{50} values were determined:

$$y = \frac{1}{1 + \left(\frac{x}{\text{IC}_{50}}\right)^{\text{slope}}}$$

where x is the inhibitor concentration and y is the fraction inhibition. K_i values were then deduced from the IC_{50} value by using the Cheng-Prusoff equation:²⁹

$$K_i = \frac{\text{IC}_{50}}{1 + \frac{[\text{S}]}{K_M}}$$

where $[\text{S}]$ is the HMBPP concentration, and K_M is the Michaelis constant.

EPR spectroscopy

Samples for EPR spectroscopy were typically 0.3 mM in IspH, and were reduced by adding 20 equivalents of sodium dithionite followed by incubating for 5 min. Glycerol was added to 42.5 % (v/v). EPR samples were frozen in liquid nitrogen after reduction. EPR spectra were collected at X-band using a Varian E-122 spectrometer together with an Air Products (Allentown, PA) helium cryostat. Data acquisition parameters were typically: field center = 3250 G; field sweep = 800 G; modulation = 100 kHz; modulation amplitude = 5 G; time constant = 32 ms; 60 sec per scan; 8 sec between each scan; and temperature = 15K. EPR spectral simulations were carried out by using the EasySpin program.³⁰

Docking calculations

For docking calculations, the IspH target protein (PDB: 3F7T) was prepared using the protein preparation wizard in Maestro 8.0.³¹ Water from the active site region was removed, as was the diphosphate ligand. The Fe_3S_4 cluster was reconstituted computationally to form the Fe_4S_4 species as described previously⁷ and hydrogen atoms were added to the protein. Hydrogen bonds were optimized to default values and an energy minimization in Macro-Model 9.5³² performed only on the protein hydrogens, using default parameters. A receptor grid large enough to encompass all crystallographically-observed binding sites was then generated from the prepared target protein. Geometry optimized ligands were docked using Glide²⁵ extra-

precision (XP) mode, and no other constraints were applied. In some instances, we also used the MMFF94 force-field³³ to effect further geometry optimization.

Density functional theory calculations

In order to gain a better understanding of the interaction between the propargyl diphosphate inhibitors and the Fe-S cluster, we used the published structure of the lowest energy form of allyl alcohol bound to the nitrogenase FeMo cofactor (structure 3 in ref.¹⁸), converting Mo → Fe, X → S and allyl → alkynyl as the initial structure. Geometry optimization was performed by using the pure density functional theory (DFT) method with a BPW91 functional,^{34, 35} a Wachter's basis (62111111/3311111/3111) for Fe,³⁶ 6-311G* for all the other heavy atoms, and 6-31G* for the hydrogens, using the Gaussian 09 program.³⁷ This method is similar to that used in the calculations of the ligand-bound nitrogenase structures¹⁸ and is the same as that we used previously to make accurate predictions of NMR hyperfine shifts, ESR hyperfine couplings, as well as Mössbauer quadrupole splittings and isomer shifts, in various iron-containing proteins and model systems.^{38–43}

Synthetic aspects

General methods—All reagents used were purchased from Aldrich (Milwaukee, WI). The purities of all compounds investigated were confirmed either via combustion analysis (for solid samples) or by using ¹H and ³¹P NMR spectroscopy at 400 or 500 MHz on Varian (Palo Alto, CA) Unity spectrometers. Cellulose TLC plates were visualized by using iodine or a sulfosalicylic acid-ferric chloride stain.⁴⁴ Propargyl methanesulfonate and but-3-ynyl methanesulfonate were synthesized according to a literature method.^{45, 46} The syntheses and characterization of compounds **18–25** have been described previously.^{22, 47–51} and the samples used here were from the same batches as described.

General procedure for preparation of diphosphates—Diphosphates were prepared using modified literature procedures.⁵² Typically, 0.5–1 mmol of halide or mesylate in a minimum amount of CH₃CN (0.4–0.6 mL) was added dropwise to a stirred solution of 2–3 equiv of tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3–6 mL) at 0°C, then the reaction mixture was allowed to stir for 2–24 h at room temperature and solvent removed under reduced pressure. The residue was dissolved in cation-exchange buffer (49:1 (v/v) 25mM NH₄HCO₃/2-propanol) and slowly passed over 60–100 mequiv Dowex AG50W-X8 (100–200 mesh, ammonium form) cation-exchange resin, pre-equilibrated with two column volumes of the same buffer. The product was eluted with two column volumes of the same buffer, flash frozen, then lyophilized. The resulting powder was dissolved in 50 mM NH₄HCO₃. 2-Propanol/CH₃CN (1:1 (v/v)) was added, and the mixture vortexed, then centrifuged for 5 min at 2000 rpm. The supernatant was decanted. This procedure was repeated three times, and the supernatants were combined. After removal of the solvent, lyophilization, then flash chromatography on a cellulose column, a white solid was obtained.

Synthesis of individual compounds

Prop-2-ynyl diphosphate (11)—Propargyl methanesulfonate (134 mg, 1 mmol) in CH₃CN (0.5 mL) was added dropwise to a stirred solution of 2.70 g (3.0 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (4 mL) at –20°C. The reaction mixture was then slowly warmed to room temperature over 2 h and solvent removed under reduced pressure. Flash chromatography on a cellulose column (4:1:2.4 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 47 mg (18 %) of a white solid. ¹H NMR (400 MHz, D₂O) δ 2.68 (s, 1H), 4.38 (d, J_{H,P} = 9.2 Hz, 2H); ³¹P NMR (162 MHz, D₂O) δ –10.10 (d, J = 20.7 Hz), –7.67 (d, J = 20.7 Hz).

But-3-ynyl diphosphate (12)—But-3-ynyl methanesulfonate (148 mg, 1 mmol) in CH₃CN (0.5 mL) was added dropwise to a stirred solution of 2.70 g (3.0 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (4 mL) at 0°C. The reaction mixture was allowed to warm to room temperature over 24 h and solvent removed under reduced pressure. Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 28 mg (10%) of a white solid. ¹H NMR (400 MHz, D₂O): δ 2.16–2.17 (m, 1H), 2.35–2.40 (m, 2H), 3.81–3.84 (m, 2H); ³¹P NMR (162 MHz, D₂O): δ –9.83 (d, J = 17.0 Hz), –7.82 (d, J = 15.9 Hz).

Pent-4-ynyl diphosphate (13)—Pent-4-ynyl methanesulfonate (162 mg, 1 mmol) was treated with 2.70 g (3 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (4 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 103 mg (35%) of a white solid. ¹H NMR (400 MHz, D₂O): δ 1.65–1.70 (m, 2H), 2.13–2.17 (m, 3H), 3.83 (q, J = 6.8 Hz, 2H). ³¹P NMR (162 MHz, D₂O): δ –9.64 (d, J = 20.9 Hz), –7.76 (d, J = 20.7 Hz).

4-Hydroxybut-2-ynyl diphosphate (14)—4-Chlorobut-2-yn-1-ol (104 mg, 1 mmol) was treated with 2.70 g (3 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (4 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded a white solid. ¹H NMR (400 MHz, D₂O): δ 4.07 (s, 2H), 4.31 (d, J_{H,P} = 6.8 Hz, 2H); ³¹P NMR (162 MHz, D₂O): δ –10.11 (d, J = 20.8 Hz), –8.85 (d, J = 20.7 Hz).

Cyanomethyl diphosphate (15)—2-Chloroacetonitrile (76 mg, 1 mmol) was treated with 2.70 g (3 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (4 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 45 mg (17%) of a white solid. ¹H NMR (400 MHz, D₂O): δ 4.56 (d, J = 10.4 Hz, 2H); ³¹P NMR (162 MHz, D₂O): δ –10.25 (d, J = 22.0 Hz), –7.37 (d, J = 20.7 Hz).

2-Cyanoethyl diphosphate (16)—3-Bromopropanenitrile (134 mg, 1 mmol) was treated with 1.80 g (2 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (4 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 31 mg (11 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 2.68 (t, J = 6.4 Hz, 2H), 3.96 (q, J = 6.4 Hz, 2H). ³¹P NMR (162 MHz, D₂O): δ –10.24 (d, J = 20.7 Hz), –6.21 (d, J = 20.7 Hz).

[[[Prop-2-ynyl] phosphinyl] methyl] phosphonic acid (17)—Propargyl methanesulfonate (134 mg, 1 mmol) was treated with 2.70 g (3 mmol) tris(tetra-n-butylammonium) hydrogen methanediphosphonate in CH₃CN (4 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 93 mg (35 %) of a white solid. ¹H NMR (500 MHz, D₂O): δ 2.00 (t, J_{H,P} = 12.0 Hz, 2H), 2.70 (t, J = 2.0 Hz, 1H), 4.37 (dd, J_{H,P} = 9.2 Hz, J = 2.0 Hz, 2H); ³¹P NMR (202 MHz, D₂O): δ 15.59 (d, J = 9.3 Hz), 20.06 (d, J = 10.7 Hz).

(Pyridin-3-yl)-methyl-diphosphate (26)—3-(Bromomethyl)pyridine (86 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (3:2 (v/v) 2-propanol/50 mM NH₄HCO₃) yielded 73 mg (45 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 4.80 (d, J_{H,P} = 7.2 Hz, 2H), 7.31 (dd, J = 7.6 Hz, J = 4.8 Hz, 1H), 7.81 (d, J = 7.6 Hz, 1H), 8.30 (d, J = 4.8 Hz, 1H), 8.42 (s, 1H); ³¹P NMR (162 MHz, D₂O): δ –9.74 (d, J = 22.0 Hz), –5.95 (d, J = 22.0 Hz).

(Pyridin-4-yl)-methyl-diphosphate (27)—4-(Bromomethyl)pyridine (86 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (3:2 (v/v) 2-propanol/50 mM NH₄HCO₃) yielded 65 mg (40 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 4.92 (d, J_{H,P} = 8.0 Hz, 2H), 7.41 (d, J = 6.0 Hz, 2H), 8.34 (d, J = 5.6 Hz, 2H); ³¹P NMR (162 MHz, D₂O): δ -9.66 (d, J = 20.7 Hz), -6.49 (d, J = 20.7 Hz).

(Pyridin-2-yl)-methyl-diphosphate (28)—2-(Bromomethyl)pyridine (86 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (3:2 (v/v) 2-propanol/50 mM NH₄HCO₃) yielded 56 mg (35%) of a white solid. ¹H NMR (400 MHz, D₂O): δ 4.91 (d, J_{H,P} = 7.2 Hz, 2H), 7.28 (t, J = 4.8 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.80 (t, J = 8.0 Hz, 1H), 8.28 (d, J = 4 Hz, 1H); ³¹P NMR (162 MHz, D₂O): δ -9.55 (d, J = 22.0 Hz), -6.02 (d, J = 19.4 Hz).

(6-Chloropyridin-3-yl)-methyl-diphosphate (29)—5-(Bromomethyl)-2-chloropyridine (103 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (3:2 (v/v) 2-propanol/50 mM NH₄HCO₃) yielded 70 mg (40 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 4.82 (d, J_{H,P} = 7.6 Hz, 2H), 7.29 (d, J = 8.4 Hz, 1H), 7.74 (dd, J = 8.4 Hz, J = 2.4 Hz, 1H), 8.20 (d, J = 2.4 Hz, 1H). ³¹P NMR (162 MHz, D₂O): δ -9.73 (d, J = 22.0 Hz), -5.81 (d, J = 22.0 Hz).

Pyridin-3-yl-ethyl-diphosphate (30)—3-(2-Bromoethyl) pyridine (93 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 42 mg (25 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 2.87 (t, J = 6.4 Hz, 2H), 4.00 (q, J = 6.4 Hz, 2H), 7.33 (t, J = 6.4 Hz, 1H), 7.79 (d, J = 8.0 Hz, 1H), 8.24 (s, 1H), 8.36 (s, 1H); ³¹P NMR (162 MHz, D₂O): δ -9.78 (1 d, J = 20.6 Hz), -7.07 (d, J = 20.6 Hz).

Pyridin-3-yl-propyl-diphosphate (31)—3-(3-Bromopropyl) pyridine (100 mg, 0.5 mmol) was treated with 1.35 g (1.5 mmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in CH₃CN (3 mL). Flash chromatography on a cellulose column (4.5:2.5:3.0 (v/v/v) 2-propanol/CH₃CN/50 mM NH₄HCO₃) yielded 57 mg (33 %) of a white solid. ¹H NMR (400 MHz, D₂O): δ 1.75–1.89 (m, 2H), 2.65–2.73 (m, 2H), 3.84 (t, J = 6.0 Hz, 2H), 7.34–7.36 (m, 1H), 7.74 (d, J = 8.0 Hz, 1H), 8.26 (s, 1H), 8.35 (s, 1H). ³¹P NMR (162 MHz, D₂O): δ -9.49 (d, J = 20.7 Hz), -6.87 (d, J = 21.9 Hz).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. 9 GHz EPR spectra of IspH at 15K reduced with $\text{Na}_2\text{S}_2\text{O}_4$. a) *A. aeolicus* IspH, no added ligands, microwave power = 1 mW. b) *E. coli* IspH, no added ligands, microwave power = 1 mW; c) *A. aeolicus* IspH + KCN, 10 equivalents, microwave power = 1 mW; d) *A. aeolicus* IspH + propionic acid, 10 equivalents, microwave power = 0.2 mW; e) *A. aeolicus* IspH + but-3-ynyl diphosphate (**12**), 20 equivalents, microwave power = 0.05 mW. Spectral simulations are shown by the dashed lines (g-values are compiled in Supporting Table S1, together with g-values for Fe_3S_4 proteins).

Figure 2.

Structures of the 21 compounds investigated as *A. aeolicus* IspH inhibitors together with (below the structures) their IC_{50} values.

Figure 3.

IspH inhibition by alkyne diphosphates. a) Dose-response curves for IspH inhibition by **12** ($IC_{50} = 450$ nM) and **13** ($IC_{50} = 6.5$ μM). b) Proposed docking model for **12** bound to the IspH active site: the diphosphate binds to the PPi site while the alkyne group forms a π (or π/σ) “metallacycle” complex with the unique 4th Fe in the Fe₄S₄ cluster, similar to that proposed for acetylene binding to model Fe₄S₄ clusters and allyl alcohol binding to a nitrogenase FeMo cofactor. c) α HOMO-1 for propargyl alcohol bound to a model Fe₄S₄ cluster, illustrating metal ligand interaction. d) as c) but β HOMO-1. The contour values are ± 0.02 au.

Figure 4.

IspH inhibition by pyridinium diphosphates. a) Dose-response curves for IspH inhibition by **26**, **27**, **28** and **30**. The IC₅₀ for the most potent inhibitor (**30**) is IC₅₀ = 9.1 μM; K_i ~ 1.2 μM. b) Glide docking pose for **26** bound to the IspH active site: the diphosphate binds to the PPI site while the pyridinium sidechain interacts with E126. c) View of the *ortho*, *meta* and *para*-pyridinium diphosphates (**26–28**) docked to IspH: the most potent inhibitor has the shortest H^N...E126 CO₂⁻ distance; the worst inhibitor has the longest distance. d) Superimposed docking poses for **12** and **26** showing that they both bind in the same region (preventing HMBPP binding).

Figure 5.

Mechanistic proposals for IspH catalysis and inhibition. a) π (or π/σ) metallacycle complex formation by alkyne inhibitors. b) IspH catalysis is which an η^2 -alkenyl metallacycle is reduced and protonated then dehydrates to form an allyl complex, which is then cleaved to form the DMAPP and IPP products. c) IspH inhibition by pyridinium diphosphates which block HMBPP binding to the 4th Fe.

Figure 6. Comparisons between x-ray structures and docking/EPR/ENDOR deduced structures for **a)**, HMBPP initial docking (PDB code 3KE8 and Ref. ²⁵, Fig 1G). **b)** propargyl diphosphate (docking) superimposed on deoxy complex (PDB ID code 3KE9 and Ref. ²⁵, Fig. 5).