APPENDIX B

MODELING THE [FEFE]-HYDROGENASE H-CLUSTER: COMBINING A PEPTIDE-SUPPORTED [FE_4S_4] CLUSTER AND A SYNTHETIC DIIRON SUBSITE MIMIC

Abstract

The synthesis of biomimetic model complexes of the full H-cluster of [FeFe]-hydrogenase was targeted. Intended to be a more synthetically modular approach than that reported by the Pickett group, the [Fe_4S_4] cluster would be supported by short peptides of variable amino acid composition containing a conserved CX_2CX_2C motif. The incorporation of the diiron subsite would then be accomplished via thioester exchange with a specialized model complex shown to work in the literature. The synthesis of a variety of peptides was accomplished though the solubility of the sequences proved problematic for characterization. [Fe_4S_4] incorporation into a peptide containing the sequence NH_2-GCIACGACGW-$(CO)NH_2$ could be confirmed spectroscopically. However, subsequent addition of the diiron subsite mimic yielded unclear results. Furthermore, the correct binding of the diiron unit to the cluster could not be confirmed. Instead, evidence for peptide acetylation resulting from thioester exchange with polar residues side chains was observed. This unanticipated complication in subsite incorporation resulted in the discontinuation of the project.

INTRODUCTION

As a fuel, molecular hydrogen is both carbon-neutral and renewable, if produced by solar-driven electrolysis.[1] Currently, steam reforming of hydrocarbons, primarily methane, is the dominant industrial method for hydrogen production.[2] This process is both energy intensive and ultimately dependent on fossil fuels, making it an ineffectual method of producing hydrogen for use as an alternative fuel. Current electrolysis cells typically require precious metals, typically platinum,[2] to function without high energetic penalties in the form of catalytic overpotentials. While inorganic materials such as molybdenum sulfide and phosphide have emerged recently as promising low overpotential electrocatalysts for hydrogen evolution, further research is required to continue to improve future devices.[3]

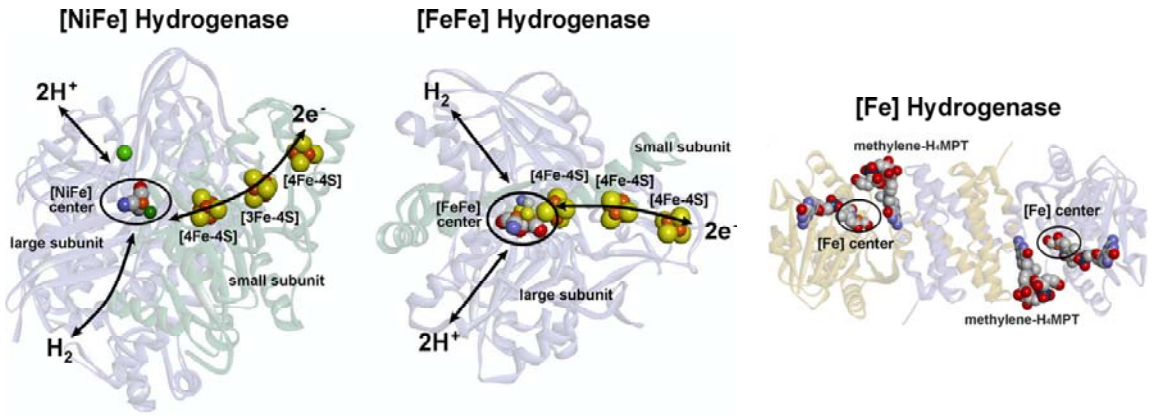


Figure 1. Protein crystal structures of the three classes of hydrogenases. Figure adapted from Lubitz,W. *Chem. Rev.* **2014**, *114*, 4081-4148.

Inspiration for suitable replacement proton reduction electrocatalysts can be found in nature. The hydrogenases are a family of enzymes capable of catalyzing the reversible interconversion of protons and electrons with molecular hydrogen.[4] These enzymes operate at negligible overpotentials and undergo thousands of turnovers per second while using only the earth-abundant metals iron and nickel.[4a] Protein X-ray crystallography studies have structurally characterized the active sites of three distinct classes of hydrogenase enzymes:

[FeFe]-, [NiFe]-, and [Fe]-only hydrogenase (Figure 1), which each show diverse metallic content and active site structure.[5] Biomimetic model complexes of each class of hydrogenase have sought to mimic the chemical properties and reactivity of their native enzymatic counterparts to better understand the biological systems and aid in the rational design of future electrocatalysts.[4] The enzymatic active site inorganic cofactor of [FeFe]-hydrogenase, dubbed the H-cluster, consists of [Fe_4S_4] cluster linked to an unusual diiron subsite by a bridging cysteine ligand (Figure 2, Left). The diiron subsite itself possess multiple, biologically unusual carbon monoxide and cyanide ligands and an azadithiolate ligand which bridges the two iron centers in a butterfly conformation with both sulfur donors. While two model complexes have been reported that incorporate both a diiron subsite model and a tethered redox cofactor (Figure 2, Right), H-cluster mimics remain uncommon in the literature.[6] Herein we discuss attempts to synthesize a six iron model complex of the H-cluster that utilizes and peptide to support a [Fe_4S_4] moiety that will be tethered to a diiron subsite mimic previously reported by Pickett and coworkers.[6b]

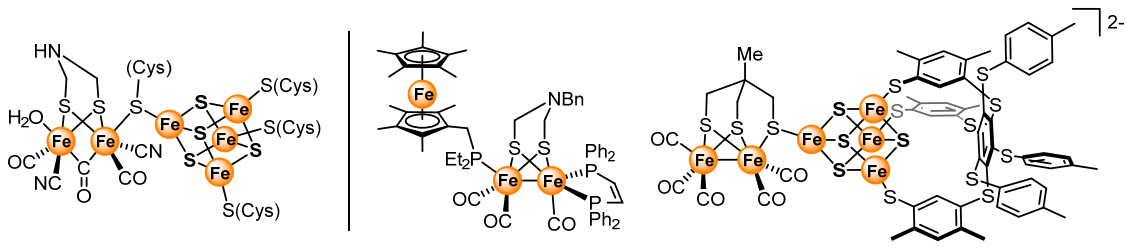
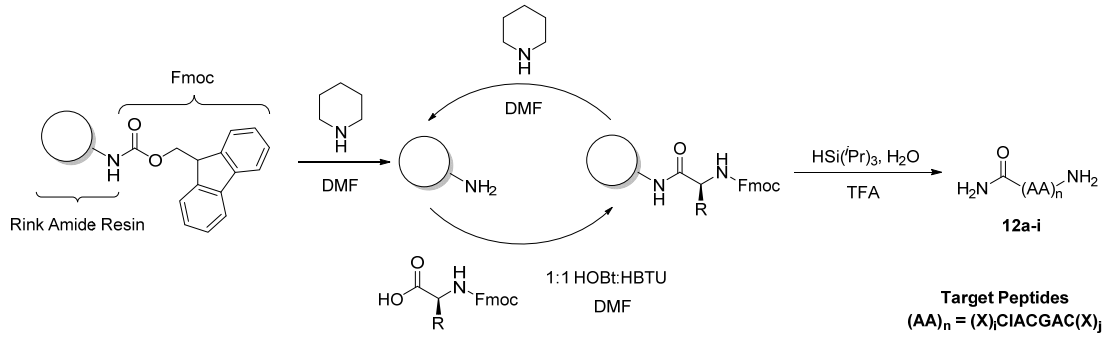


Figure 2. Schematic representation of the [FeFe]-hydrogenase active site (Left), and synthetic model complexes mimicking both the diiron subsite and [Fe_4S_4] cluster (Right).

RESULTS AND DISCUSSION

Section B.1 Peptide Synthesis

Scheme 1. General scheme for Fmoc-based solid phase peptide synthesis



9-fluorenylmethyloxycarbonyl-based (Fmoc) protection schemes were employed to produce tricysteine peptides on a rink amide resin using solid-phase peptide synthesis protocols (Figure 4).[7] Subsequent cleavage from solid support using trifluoroacetic acid yielded C-terminally amidated, unpurified peptides on a multiple 100 mg scale. Sequences of the general form NH_2-$(X)_i$CIACGAC$(X)_j$-$CONH_2$ based on literature peptides were designed to include an internal CX_2CX_2C motif previously shown to bind $[Fe_4S_4]$-clusters (Table 1). $(X)_j$ sequences generally consisted of one to two glycine residues followed by a tryptophan to serve as a convenient UV/Vis (ε_{280nm} = 5600 $M^{-1}cm^{-1}$) and ^{1}H-NMR handle, while $(X)_i$ sequences were typically a single glycine.

Unfortunately, the majority of peptides (**1a**-**1i**) synthesized possessed limited solubility making purification by reverse-phase high performance liquid chromatography (RP-HPLC) difficult (Table 1). N-terminally acetylated peptides were virtually insoluble in all solvents except DMF and DMSO. Ultimately, the literature peptide, **1h**, was selected for preparatory-scale RP-HPLC as it presented the highest solubility in aqueous-acetonitrile solvent systems and was known to efficiently bind $[Fe_4S_4]$-clusters. The limited solubility of most of these peptides is likely a reflection of the cluster-binding environment in ferredoxin proteins, which are often buried in hydrophobic portions of the protein away from bulk solvent. The identity

and purity of **1h** was confirmed by electrospray ionization mass spectrometry (ESI-MS) and ^{1}H-NMR spectroscopy.

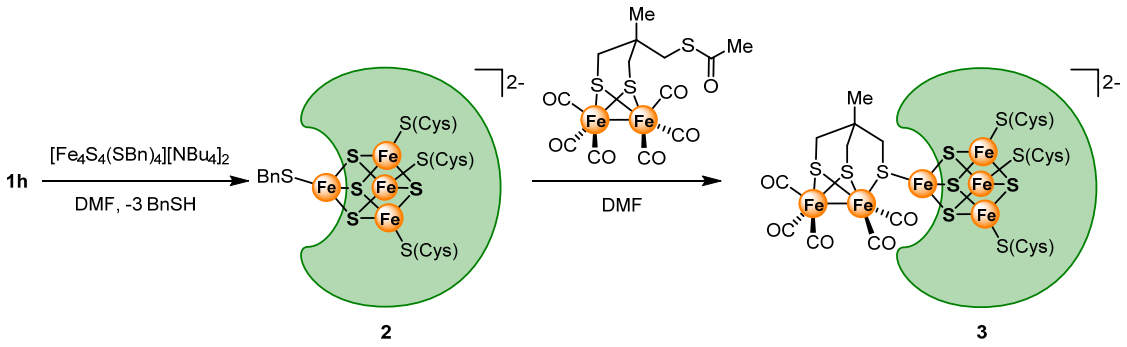
Table 1. Compiled list of synthesized peptides. ‡ Synthesized of glycine preloaded Wang resin.

Peptide #	N-term.	$(AA)_n$	C-Term.	Solubility
1a‡	*p*-F-aryl-C(O)-	GCIACGWCGG	-OH	<10mg/mL in DMF or DMSO
1b‡	Acetyl-	GCIACGWCGG	-OH	<10mg/mL in DMF or DMSO
1c‡	H-	GCIACGACGG	-OH	<10mg/mL in DMF or DMSO
1d	H-	GCIACGWCGG	$-NH_2$	<10mg/mL in DMF or DMSO
1e	H-	CGGCGGC	$-NH_2$	~20mg/mL MeCN:H_2O
1f	H-	CGGCGGCW	$-NH_2$	<10mg/mL in DMF or DMSO
1g	Acetyl-	CGGCGGCW	$-NH_2$	<10mg/mL in DMF or DMSO
1h	**H-**	**GCIACGACGW**	**$-NH_2$**	**~20mg/mL MeCN:H_2O**
1i	H-	RGCIACGACGW	$-NH_2$	~20mg/mL MeCN:H_2O

Section B.2 Attempted Assembly of Peptide-supported H-cluster Model Complexes

To assemble the peptide-supported H-cluster, a synthetic strategy analogous to that reported for the synthesis of **6** was adopted (Scheme 2).[6b] Previous studies of peptide-supported $[Fe_4S_4]$-clusters employed reconstitution protocols that took advantage of the CX_2CX_2C motif to self-assemble clusters from dissolved iron and sulfide salts under anaerobic conditions.[8] Tetracysteine peptides demonstrated near complete reconstitution yields, while omission of a single cysteine typically showed diminished efficiencies of ~50%.[9] To circumvent the reduced yields, use of a preformed cluster allowed for facile cluster binding via thiolate exchange. Similar strategies were previously shown by Holm to achieve near quantitative cluster incorporation in peptides.[10]

Scheme 2. Proposed synthetic scheme for the synthesis of a peptide supported H-cluster model complex

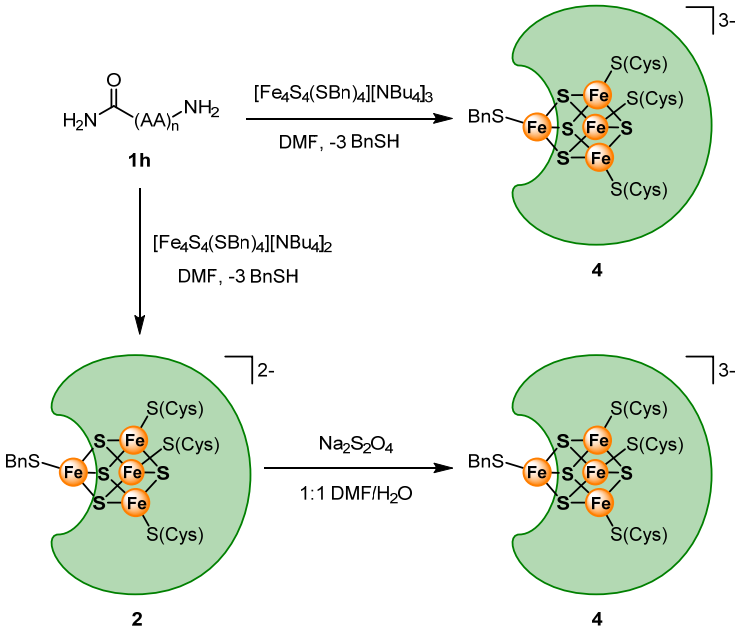


Preformed $[Fe_4S_4]$-clusters have been extensively studied,[11] and the benzyl thiolate and tetrabutylammonium variant, $[Fe_4S_4(SBn)_4][NBu_4]_2$, was readily synthesized. Reaction of **1h** with the preformed cluster produced a dark brown solid for which broadened ^{1}H-NMR resonances could be observed and assigned to benzyl thiolate and peptide protons (Scheme 3). ESI-MS confirmed the formation of **2** which showed signals corresponding to $[\mathbf{2}]^{2-}$ and $[\mathbf{2}\text{-}(SBn)]^{2-}$ ions. Electron paramagnetic resonance (EPR) spectroscopy of samples of **2** showed no signal confirming negligible degradation of the $[Fe_4S_4]^{2+}$ cluster to an EPR active $[Fe_3S_4]^{+}$ species. Furthermore, reduction with disodium dithionite produced **4** for which a rhombic, $S = {}^1/_2$, EPR signal with g-values of 1.88, 1.93, and 2.06 was observed (Figure 3), in close agreement with literature values for ferredoxin proteins.[12] An independently synthesized reduced $[Fe_4S_4]$-cluster precursor, $[Fe_4S_4(SBn)_4[NBu_4]_3$, produced an axial EPR signature with g-values of 1.93 and 2.04, supporting intact cluster binding to **4**.[12] Reactions of the reduced cluster precursor with **1h** directly yielded **4** without the use of chemical reductants (Figure 4).

With the $[Fe_4S_4]$-cluster bound to **1h**, incorporation of the diiron subsite was attempted. In literature reports a diiron subsite containing thiolate masked by an acetyl group was employed.[6b] Thioester exchange and subsequent loss of a carbon monoxide ligand formed the

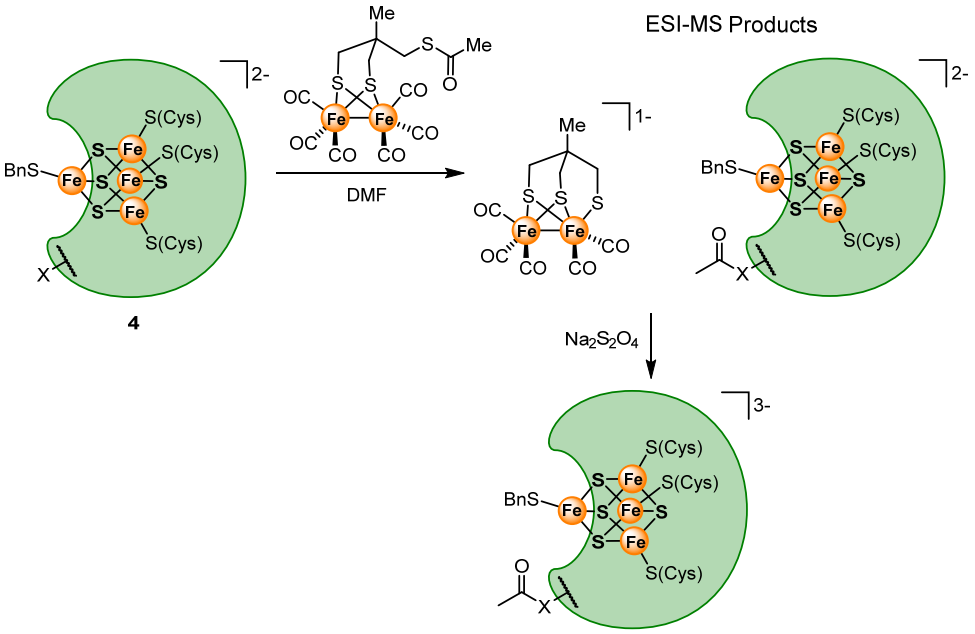
μ_2-thiolate to generate six Fe cluster and release the acetylated thiolate. A similar approach was attempted for the peptide-bound compound **2**. Despite repeated attempts, reactions of the diiron subsite with **2** never showed evidence of formation of the target complex, **4**. ESI-MS confirmed consumption of the diiron subsite complex, however only acetylated **2** and diiron decomposition products were detected (Scheme 4). These data suggest quantitative acetylation of peptide side chains or N-terminus of **2** without displacement of the benzyl thiolate.

Scheme 3. Synthesis of peptide-supported $[Fe_4S_4]$ clusters



EPR spectroscopy of reaction mixtures revealed an unusual feature centered around 3320 G, which could not be attributed to known $[Fe_4S_4]$-cluster degradation products (Figure 5). Upon reduction with disodium dithionite, the signal typical of a $[Fe_4S_4]^+$ cluster appeared (Figure 6), confirming the presence of an intact, peptide-bound cluster.

Scheme 4. Attempted incorporation of the diiron subsite to **4**



In theory, prior acetylation of the N-terminus of **1h** should be sufficient to minimize side reactions with the diiron subsite complex. However, the limited solubility of such a peptide would make RP-HPLC purification on reasonable scales challenging. Future peptides, will include additional polar amino acids to improve solubility while avoiding of sites capable of competing with the thioester exchange required for diiron linkage to the $[Fe_4S_4]$-cluster.

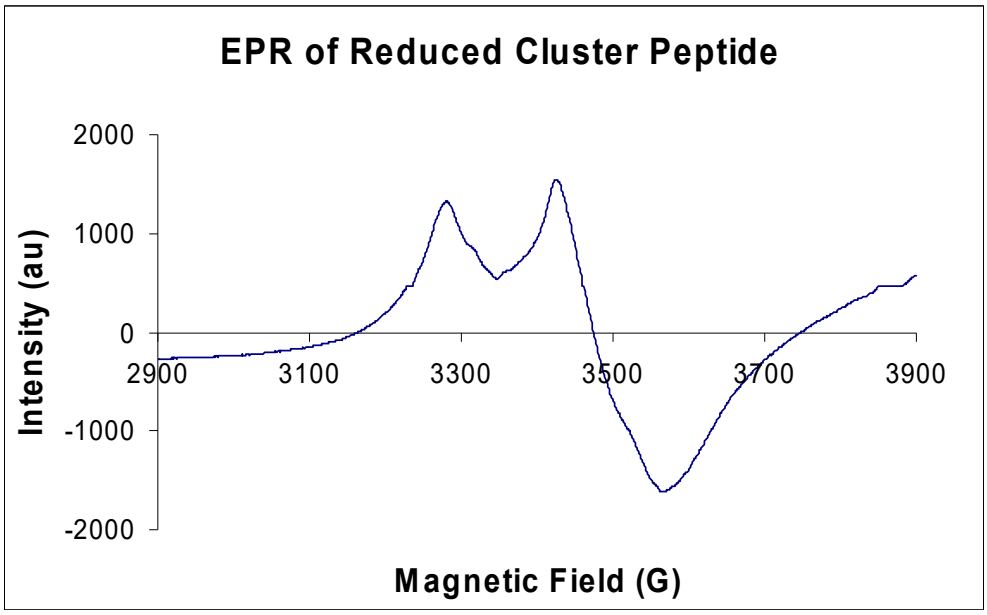


Figure 3. EPR spectrum of **4** produced by the metalation of **1h** with the reduced cluster precursor.

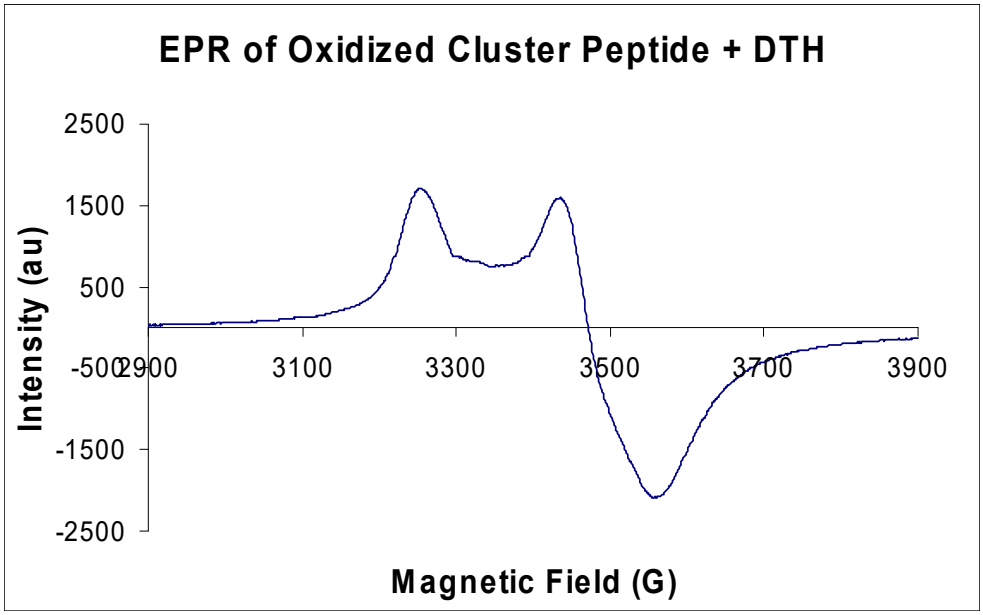


Figure 4. EPR spectrum of **4** produced by the reduction of **2** with dithionite.

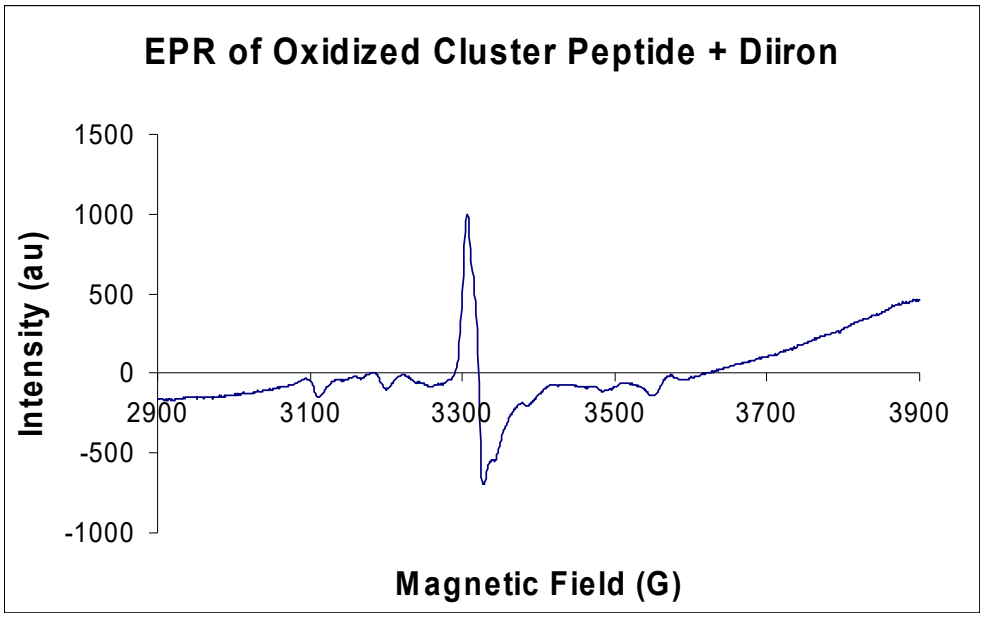


Figure 5. EPR spectrum of the reaction of **2** with the diiron subsite model complex.

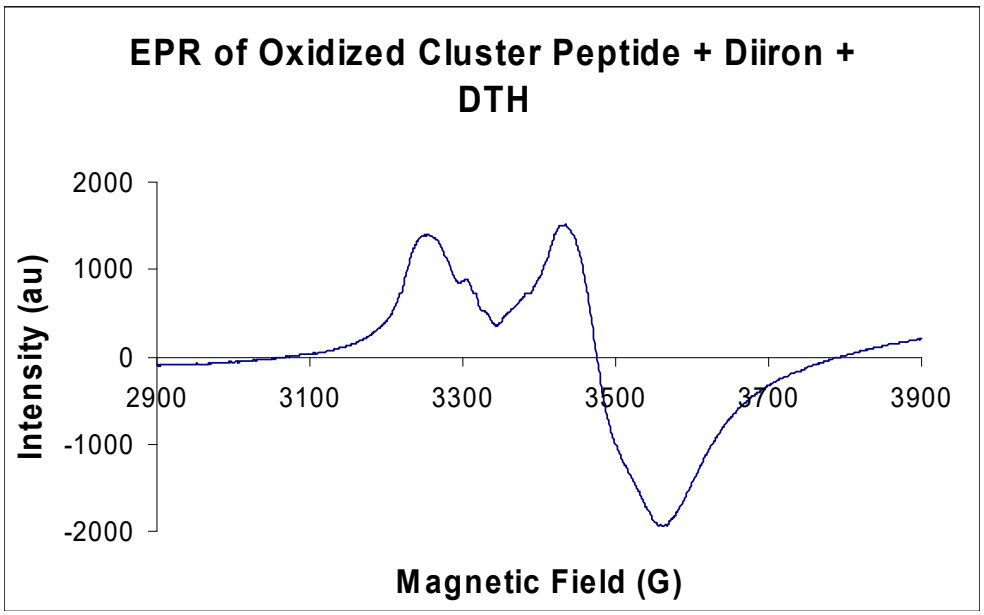


Figure 6. EPR spectrum of the reaction of **2** with the diiron subsite model complex following reduction with dithionite.

CONCLUSIONS

In conclusion, attempts to synthesize peptide-supported H-cluster model complexes was attempted. Peptide syntheses were successfully accomplished utilizing Fmoc-based approaches, however sequence optimization was required to access peptides of sufficient solubility to properly characterize and utilize in reactions. Incorporation of preformed [Fe_4S_4] clusters into the peptides proved successful with degradation not observed by EPR or ESI-MS. Unfortunately, attempts construct the full six Fe cluster proved unsuccessful as acetylation of the peptide backbone was interfering with thioester exchange with the last thiolate ligand coordinated to the [Fe_4S_4] cluster.

EXPERIMENTAL SECTION

General considerations.

Unless otherwise specified, all compounds were manipulated in a glovebox under a nitrogen atmosphere. Solvents for all reactions were dried by the Grubb's method. All purchased chemicals were used as ordered without further purification. Fmoc-protected amino acids, solid-phase resins and coupling reagents were purchased from Advanced Chemtech. Trifluoroacetic acid (TFA), triisopropyl silane (TIS), diisopropylethylamine (DIEA), and piperidine were purchased from Sigma Aldrich. HPLC-grade acetonitrile was purchased from VWR International. All deuterated NMR solvents were purchased from Cambridge Isotopes. ^{1}H-NMR and ^{31}P-NMR were recorded on a Varian 300 MHz with chemical shifts reported with respect to internal solvent at ambient temperatures unless otherwise noted. Mass spectrometry data was obtained using the Caltech Institute of Technology Mass Spectrometry Facility ESI-MS instrument. RP-HPLC was run on Agilent instruments utilizing a preparatory-scale C18 column. EPR experiments were run on an X-band Bruker instrument at 1 mmolar concentrations and 10 K unless otherwise noted. Other EPR instrument settings include microwave frequency 9.378 GHz, microwave power 5.115 mW, receiver gain 1.00e+003, modulation frequency 100 kHz, modulation amplitude 5.00 G, time constant 40.060 ms. $Fe_2(CO)_6CH_3C(CH_2S)_2CH_2SCOCH_3$,[6b] $[Fe_4S_4(SBn)_4][NBu_4]_2$,[11a] and $[Fe_4S_4(SBn)_4][NBu_4]_3$[12] were synthesized according to literature procedures.

Peptide synthesis and purification of Compound 1h

Peptide synthesis of **1a**-**1i** were done using identical methods. All peptide synthesis was done open to air using undried, reagent-grade solvents. Peptide synthesis conditions were

adapted from literature protocols.[18] Rink amide resin (850 mg, 0.5 mmol, 1 equiv) was added to a 50 mL peptide synthesis vessel (Chemglass, CG-1860-03) and swelled in *ca.* 40 mL of dichloromethane for 3 hours after which the solvent was removed by vacuum filtration. The resin was then washed 3 times with *ca.* 30 mL of DMF. Alternating Fmoc deprotection and amide coupling reactions were used to synthesize **1h** by the stepwise addition of amino acids. Fmoc deprotection was deemed complete by Kaiser test with *ca.* 20 mL of 20% (v/v) piperidine in DMF agitated on a shaker for 25-30 minutes, and the resin was washed 3 times with *ca.* 30 mL DMF, then 3 times with *ca.* 30 mL of dichloromethane, then 3 times again with DMF. For amide couplings, the Fmoc-protected amino acid (2 mmol, 4 equiv), 10 mL of freshly made 0.2 M solution of 1:1 HBTU:HOBt in DMF (2 mmol, 4 equiv), and 15 mL of a 0.2 M DIEA solution in DMF (3 mmol, 6 equiv) were mixed and allowed to stand at room temperature for 2 minutes. The activated amino acid was then transferred to the deprotected resin agitated on a shaker for 3 hours or until deemed complete by Kaiser test. Excess unreacted coupling reagents and amino acids were removed by washing 3 times with *ca.* 30 mL DMF, then 3 times with *ca.* 30 mL of dichloromethane, then 3 times again with DMF. Following the final Fmoc deprotection, peptides were cleaved from the resin support with 20 mL of a 95:2.5:2.5 TFA:TIS:H_2O stirred for 3 hours. A orange-yellow solution was collected by filtration and combined with TFA washes of the resin (*ca.* 10 mL) and the solvent removed *in vacuo*. The yellowish residue was triturated with 30 mL ether, then 3 times with 30 mL of hexanes, and then 3 more times with ether. The resulting off-white powder was carried on to RP-HPLC purification. Saturated solutions of crude **1h** (*ca.* 15-20 mg/mL) in 1:1 MeCN:H_2O acidified with 0.1% TFA were passed through 0.1 micron syringe filters (Pall Corporation). 2 mL injections of **1h** solution were separated on a preparatory-scale C18 column using a gradient from 0% solvent B-30% solvent B over 45 minutes at a flow rate of 20 mL/min,

where solvent A is 5:95 MeCN:H_2O acidified with 0.1% TFA and solvent B is 95:5 MeCN:H_2O acidified with 0.1% TFA. Fractions containing **1h** were combined and lyophilized to yield a *ca.* 40 mg fluffy white powder in 8.5% overall yield. ^{1}H-NMR (300 MHz, d_6-DMSO) δ 10.77 (s, 1H, Trp(N-H)), 8.57 (d, 1H), 8.23 (t, 1H), 7.55 (d, 1H), 7.40 (s, 1H), 7.29 (d, 1H), 6.53 (s, 1H), and multiple poorly resolved chemical shifts in both aliphatic and aromatic regions. MS (m/z): calcd. 939.12 $[M+H]^+$; found 939.2 $[M+H]^+$, 961.5 $[M+Na]^+$ (ESI-MS).

Synthesis of Complex 2

12h as the trifluoroacetate salt (10.0 mg, 9.5 μmol, 1 equiv) was stirred in 4 mL DMF for 30 minutes then was transferred to solution of $[Fe_4S_4(SBn)_4][NBu_4]_2$ (12.7 mg, 9.5 μmol, 1 equiv) in 1 mL of DMF. The resulting mixture was allowed to stir overnight at room temperature. Aliquots of the reaction mixture were used for characterization. ^{1}H-NMR (300 MHz, d_6-DMSO) δ 10.76 (broad, Trp(N-H)) and multiple, poorly resolved, broadened signals in both aliphatic and aromatic regions. MS (m/z): calcd. 705.0 $[M-2(NBu_4)]^{2-}$; found 705.2 $[M-2(NBu_4)]^{2-}$, 643.0 $[M-SBn-2(NBu_4)]^{2-}$ (ESI-MS).

Reduction of Complex 2 with $Na_2S_2O_4$.

500 μL of a freshly prepared 10 mmolar disodium dithionite solution in H_2O (5 μmol, 5 equiv) was added to a 500 μL aliquot of a 2 mmolar solution of **2** in DMF (1 μmol, 1 equiv). The mixture was stirred for 30 minutes before EPR aliquots were frozen. EPR g-values (2.06, 1.93, 1.88).

Synthesis of Complex 4

Analogous protocol used for the synthesis of **2** with the addition of the reduced cluster precursor $[Fe_4S_4(SBn)_4][NBu_4]_3$ (14.9 mg, 9.5 μmol, 1 equiv). EPR g-values (2.04, 1.92, 1.88). MS (m/z): calcd. 705.0 $[M\text{-}2(NBu_4)]^{2-}$; found 705.2 $[M\text{-}2(NBu_4)]^{2-}$, 643.0 $[M\text{-}SBn\text{-}2(NBu_4)]^{2-}$, 1652.2 $[M\text{-}(NBu_4)]^{-}$ (ESI-MS).

Reaction of Complex 2 with $Fe_2(CO)_6CH_3C(CH_2S)_2CH_2SCOCH_3$.

250 μL of a freshly prepared 2 mmolar solution of $Fe_2(CO)_6CH_3C(CH_2S)_2CH_2SCOCH_3$ in DMF was added to 250 μL of a 2 mmolar solution of **2** in DMF. The mixture was stirred for 30 minutes and aliquot was frozen for EPR. 5 equiv $Na_2S_2O_4$ was added. After stirring for an additional 30 minutes an aliquot was frozen for EPR.

Reaction of Complex 4 with $Fe_2(CO)_6CH_3C(CH_2S)_2CH_2SCOCH_3$.

250 μL of a freshly prepared 2 mmolar solution of $Fe_2(CO)_6CH_3C(CH_2S)_2CH_2SCOCH_3$ in DMF was added to 250 μL of a 2 mmolar solution of **2** in DMF. The mixture was stirred for 30 minutes and aliquot was frozen for EPR. 5 equiv $Na_2S_2O_4$ was added. After stirring for an additional 30 minutes an aliquot was frozen for EPR.

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