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**Enzyme Probes**

# Probing Inducible Nitric Oxide Synthase with a Pterin–Ruthenium(II) Sensitizer Wire**

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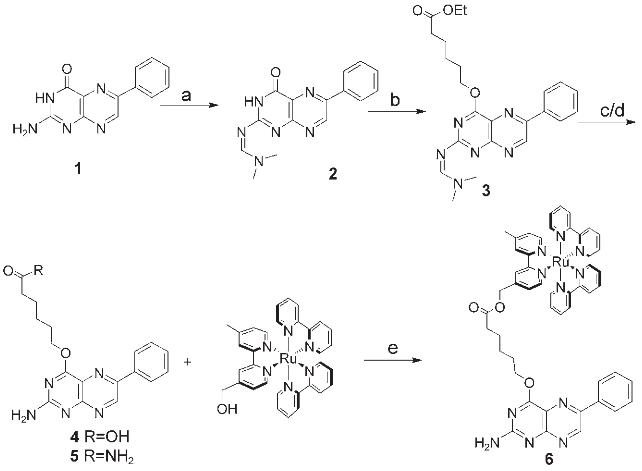
Nitric oxide synthase (NOS) is the primary biological source of the ubiquitous signaling molecule nitric oxide (NO). The enzyme utilizes tetrahydrobiopterin ($H_4B$) as an essential cofactor, which plays a key role in the catalytic conversion of L-arginine to citrulline and NO.[1] Pterin has been shown to serve both structural and catalytic roles in the enzyme by affecting the monomer–dimer transition, promoting protein stability, and forming a radical cation during catalytic turnover.[2]

We have developed redox-active sensitizer wires to probe the active sites of heme enzymes.[3] Herein, we describe a new class of redox-active wires that target the pterin binding site of NOS. The pterin group lies adjacent to the heme, and directly interacts with the catalytic center through hydrogen bonds to the heme propionate group. It is close in space, but physically distinct from the face of the heme containing the arginine binding site, allowing both cofactor and substrate to bind simultanously.[4,5] Thus, introducing a molecular wire at the pterin site may allow photochemical triggering of enzyme turnover, thereby offering an opportunity to study catalytic intermediates and to shed new light on the cofactor's role in catalysis.[6] Herein we report the design and synthesis of a ruthenium(II)–pterin wire, and investigate its interaction with the heme domain of murine inducible nitric oxide synthase ($iNOS_{heme}$).

The wire combines two essential moieties: an analogue of the cofactor to direct binding, and a redox-active sensitizer that can be used for light-induced charge injection to generate specific oxidation states of the heme (the pterin is believed to provide an electron to the heme during catalysis).[7] The 6-phenylpterin analogue was synthesized in the catalytically inactive, fully oxidized form (**1**, Scheme 1). This moiety has been shown to bind in a competitive manner with the fully reduced cofactor.[8] The tether was attached to the pterin ring at the 4-O position, which allows it to extend through the iNOS active-site channel that is normally occupied by solvent or other small molecules;[5] the linker length was chosen based on modeling studies. In addition, we incorporated a ruthenium(II)–diimine sensitizer that acts as a luminescent binding probe, and as an excited-state oxidizing or reducing agent.[9]



*Scheme 1.* Synthesis of pterin probes. Reagents and conditions: a) DMF dimethyl acetal (10 equiv), DMF, 50 °C, 2 h, 92 %; b) ethyl 6-bromohexanoate (5 equiv), $K_2CO_3$ (2 equiv), DMF, 4 h, 68 %; c) 1 M NaOH/DMF, 1 h, 82 %; d) $NH_3$/MeOH, 18 h, 85 %; e) (with **4**), 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (EDCI; 1.5 equiv), 4-dimethylaminopyridine (DMAP; 1.5 equiv), pyridine, 12 h, 68 %.

The pterin-linked wire **6** was synthesized from the oxidized 6-phenyl pterin (**1**; see Scheme 1), which was prepared according to the procedure of Storm et al. by an Isay condensation reaction.[10] The 2-amino functionality was protected as the (dimethylamino)methylene group to improve solubility and facilitate further synthetic modification.[11] Alkylation of the 4-hydroxy group was performed under standard Williamson ether synthesis conditions. This chemistry led to a combination of species alkylated at 4-O and 3-N which could be resolved using silica gel flash chromatography.[12] Cleavage of both the ester and (dimethylamino)-methylene protecting groups was accomplished in a single step with NaOH in DMF (1 M). The addition of the long-chain alkane improved solubility, allowing the final conjugation to the ruthenium(II) complex by esterification under standard conditions; alternatively, it was converted to the amide **5** for use as a model compound.[13]

Several spectroscopic methods were utilized to quantify wire binding to $iNOS_{heme}$. Upon binding $H_4B$, the iNOS heme

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undergoes a conversion from low spin to high spin, with a complete shift obtained after incubation with both pterin and L-arginine. Addition of wire **6** to iNOS$_{heme}$ affects a change in the UV/Vis spectrum that is consistent with partial conversion from the DTT-ligated bis(thiolate) complex into a high-spin state (Figure 1). This change is more subtle than observed with the natural cofactor, but has the same features in the difference spectrum, with a maximum at 400 nm and a minimum at 459 nm (see Supporting Information, Figure S1). These results are consistent with other synthetic pterin analogues that have been found to produce partial low-spin to high-spin shifts in optical spectra.[14]

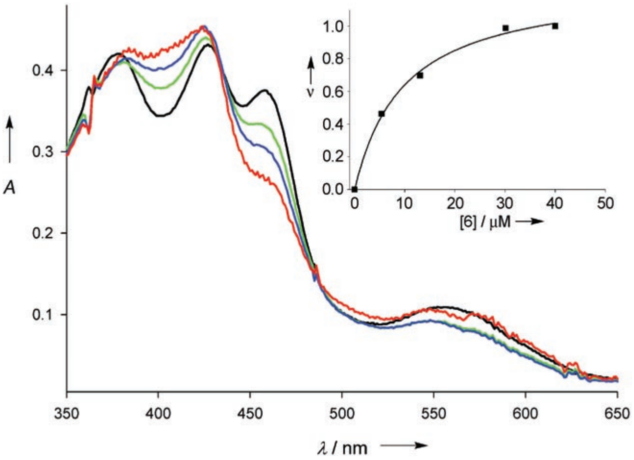

***Figure 1.*** Reconstitution of substrate- and $H_4B$-free iNOS$_{heme}$ with wire **6.** iNOS$_{heme}$ (ca. 10 μM) was incubated at 25 °C with the pterin analogue in the presence of 1 mM DTT, and the absorbance spectrum was taken after 2 h (10 μM, green, 30 μM, blue, 50 μM, red). The spectrum of the DTT-equilibrated sample (black, without wire) shows the formation of a bis(thiolate) heme. The inset shows the binding curve obtained from titration data, plotting the fractional difference in absorption $\nu$ ($\Delta\Delta A/\Delta\Delta A_{total}$, $\Delta\Delta A = \Delta A_{400} - \Delta A_{459}$) as a function of concentration.

The affinity ($K_d \approx 8$ μM) of **6** for the protein was obtained from a plot of $\nu = \Delta\Delta A/\Delta\Delta A_{total}$ versus concentration (see Figure 1, inset). Binding of the natural cofactor causes several structural and electronic modifications in the enzyme, including adjustments in the dimerization interface as well as the substrate and pterin binding pockets, and the binding is known to be quite slow.[15] The time dependence of the absorption changes in the difference spectra for wire **6** was analyzed, and the spectroscopic changes were complete within about 60 min, in good agreement with the rate of binding of the natural cofactor (see Supporting Information, Figure S2). Thus, the structural and electronic modifications within the enzyme upon binding wire **6** are fully consistent with those observed upon binding $H_4B$.

The binding of wire **6** to iNOS$_{heme}$ was confirmed by analysis of changes in ruthenium(II) emission. As shown in Figure 2, the emission centered at 610 nm is dramatically quenched upon addition of iNOS$_{heme}$, owing to Förster energy transfer. Furthermore, the ruthenium(II) emission is restored upon addition of the natural cofactor $H_4B$, or the higher-affinity pterin analogue 4-amino-$H_4B$ (4A$H_4$B), indicating that the interaction of **6** with the enzyme competes with pterin cofactors.

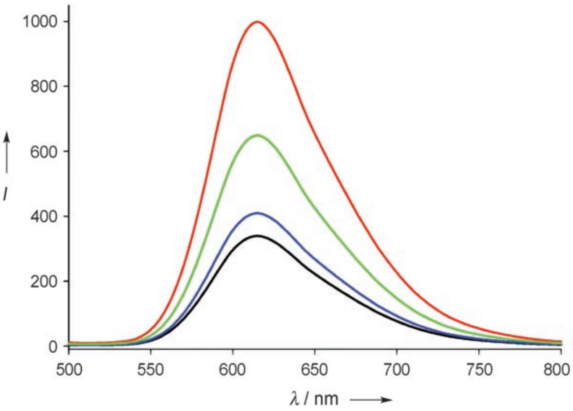

***Figure 2.*** The steady-state ruthenium(II) luminescence spectrum of **6** (10 μM, red) is quenched in the presence of equimolar iNOS$_{heme}$ (black); it recovers upon addition of 10 μM (blue) and 100 μM (green) 4A$H_4$B.

Time-resolved emission experiments also confirmed ruthenium(II)–pterin binding to the iNOS heme (see Supporting Information, Figure S4).[3] The emission decay of the wire is monoexponential in the absence of protein ($\tau = (368 \pm 2)$ ns), but becomes biexponential ($\tau_1 = 370$ ns, $\tau_2 = (40 \pm 4)$ ns) in the presence of iNOS$_{heme}$. Fits of the decays provided estimates of the ratio of enzyme-bound/free ruthenium(II), giving a dissociation constant of approximately 4 μM (see Figure 3). As in the steady-state experiment, addition of the cofactor analogue 4A$H_4$B triggers dissociation of the wire from the protein, and recovery of the longer-lived, unquenched species (see Supporting Information, Figure S7).

The affinity of **6** for iNOS$_{heme}$ appears to be determined by the pterin and its linker rather than the ruthenium(II) sensitizer. Both the pterin core **1** and intermediate wire **5**, lacking the ruthenium(II) complex, are inherently fluores-

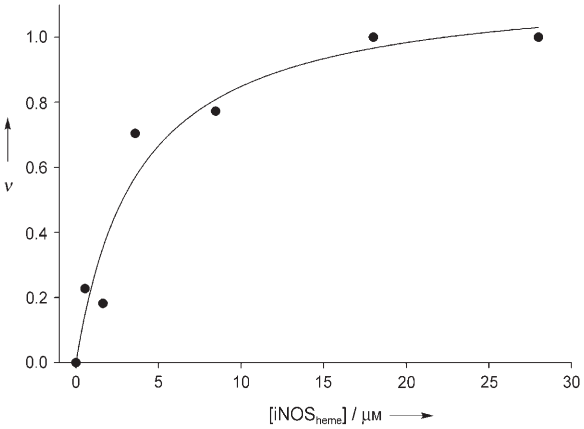

***Figure 3.*** The relative fraction of the fast phase of luminescence decay for compound **6** (taken as fraction bound) as a function of [iNOS$_{heme}$]$_{free}$. An estimate for the dissociation constant of **6** to iNOS$_{heme}$ was obtained by fitting the curve to a single-site binding model ($K_d = (3.8 \pm 1.4)$ μM), assuming that the saturation value of the fractional fast phase represents a 1:1 complex.

cent, and display marked quenching in the presence of protein (see Supporting Information, Figure S3). The efficient quenching of pterin fluorescence by the heme was used to characterize wire binding to the protein. The 4 μm dissociation constant for **6** is in agreement with that for compound **5** ((5.1 ± 1) μm), which was obtained by steady-state fluorescence quenching (see Supporting Information, Figure S8); it is significantly lower than the value (26 μm) reported for pterin **1**.[8] Thus, the addition of the tether rather than the ruthenium(II) complex is likely to be responsible for the increased binding affinity. Indeed, other pterin analogues have been reported that show enhanced binding upon alkylation at the 4-position,[8a,16] and wires designed for cytochrome P450 from *P. putida* ($P450_{cam}$) show higher affinities than substrate alone.[3b] It is likely that the additional hydrophobic contacts provided by the linker in the access channel are responsible for each of these enhanced affinities.

A model for the interaction of wire **6** with $iNOS_{heme}$ is shown in Figure 4. The pterin portion of the wire was overlaid with the $H_4B$ bound at the dimer interface, and the linker and ruthenium(II) conformations were adjusted to minimize steric conflict. The distance between the heme edge and the nearest diimine ring of the ruthenium(II) complex is 17 Å in this model, which agrees well with the value of (15.7 ± 1.4) Å obtained by assuming that the quenched (40 ns) component of the emission decay is attributable solely to Förster energy transfer to the heme in the wire–enzyme conjugate.[17]

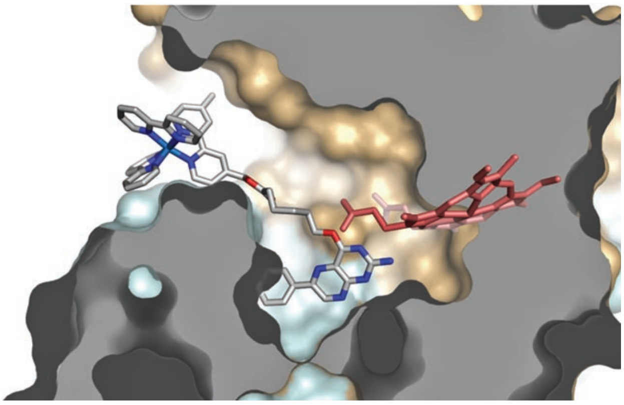

*Figure 4.* Structural model of wire **6** docked into $iNOS_{heme}$. Heme is shown in red; for **6**: gray C, red O, blue N, Ru.

Upon steady-state illumination at 450 nm, we observe reduction of the wire-bound enzyme in the presence of CO and the reductive quencher TMPD (*N,N,N′,N′*-tetramethylphenylenediamine). Changes in the UV/Vis spectra are dependent both on the concentration of the wire and the irradiation time. Photoreduction generates a species with an absorption maximum at 420 nm, rather than one with a peak at 450 nm associated with the cysteine-ligated $Fe^{II}(CO)$ form of the enzyme (see Figure 5). In a control experiment, reduction of the wire-bound enzyme with sodium dithionite produces a combination of P450 and P420 species, with the P420 species predominating.[18] In addition, experiments performed on the enzyme in the presence of 50 μm $H_4B$ demonstrate the instability of the P450 form, and show conversion into the P420 form with time or exposure to 450 nm light (see Supporting Information, Figure S9). It thus appears that photolysis of the CO-bound heme in the presence of the natural cofactor results in conversion into the five-coordinate form; it follows that any P450 species formed upon irradiation would be converted to the P420 form.

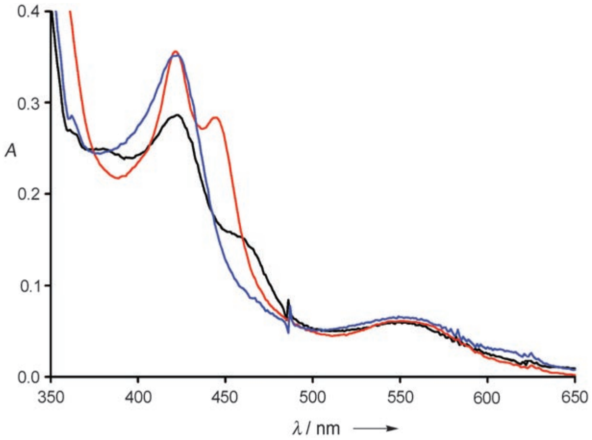

*Figure 5.* Photoreduction of $iNOS_{heme}$ using wire **6**. Illumination of the $iNOS_{heme}$–wire complex under an atmosphere of CO causes an increase in absorption at 420 nm, consistent with the formation of the reduced CO-bound P420 species (black line, 0 min, blue line, 30 min). 1 mm TMPD was used for the experiment; no spectral change was observed without TMPD, or without light. Chemical reduction with $Na_2S_2O_4$ in the dark results in production of a combination of P450 and P420 species (red line).

In summary, our pterin-based photoactive wire binds in competition with native pterins to the heme domain of murine iNOS. Charge injection from the metal complex should facilitate mechanistic investigations of the enzyme, and may help determine how the pterin acts as an essential redox-active cofactor at specific points of the catalytic cycle. Importantly, the wire also can be used as a fluorescent probe for rapid screening of small molecule inhibitors that target the pterin binding site.

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[12] A more direct approach to the 4-O species was attempted using Mitsunobu chemistry (see ref. [11]); however, it was found that this route also led to a combination of O- and N-alkylated species, but with an overall lower yield.

[13] The amide was used instead of the carboxylic acid to prevent any undesired reactions with the protein.

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[17] Förster energy transfer calculations were performed as described in ref. [3d]. See Supporting Information for further details.

[18] The instability of the P450 form of NOS is well documented; it is clear that the pterin plays a central role in determining the degree of conversion to the P420 form. Pterin-free NOS is converted over time completely to the P420 form, and variable fractions of the two forms are observed with subsaturating levels of $H_4B$ or synthetic pterin analogues. See refs [7b, 15], and J. Wang, D. J. Stuehr, D. L. Rousseau, *Biochemistry* **1995**, *34*, 7080 – 7087.