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Probing Heme Coordination States of Inducible Nitric Oxide Synthase with a Re(I)(imidazole-alkyl-nitroarginine) Sensitizer-Wire

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Abstract

Mammalian inducible nitric oxide synthase (iNOS) catalyzes the production of L-citrulline and nitric oxide (NO) from L-arginine and O₂. The Soret peak in the spectrum of the iNOS heme domain (iNOS_{oxy}) shifts from 423 to 390 nm upon addition of a sensitizer-wire, [Re^I-imidazole-(CH₂)₈-nitroarginine]⁺, or [ReC₈argNO₂]⁺, owing to partial displacement of the water ligand in the active site. From analysis of competitive binding experiments with imidazole, the dissociation constant (*K_d*) for [ReC₈argNO₂]⁺:iNOS_{oxy} was determined to be 3.0 ± 0.1 μM, confirming that the sensitizer-wire binds with higher affinity than both L-arginine (*K_d* = 22 ± 5 μM) and imidazole (*K_d* = 14 ± 3 μM). Laser excitation (355 nm) of [ReC₈argNO₂]⁺:iNOS_{oxy} triggers electron transfer to the active site of the enzyme, producing a ferroheme in less than ~1 μs.

INTRODUCTION

Nitric oxide (NO) is an important secondary signaling molecule that has diverse biological functions, including neurotransmission, blood pressure regulation, and immune response.^{1–3} All three isoforms of homodimeric nitric oxide synthase (NOS) (inducible, endothelial, and neuronal) catalyze the reaction between L-arginine and dioxygen to produce L-citrulline and NO. The N-terminal oxygenase domain contains binding sites for an iron protoporphyrin IX (heme), tetrahydrobiopterin (BH₄), and L-arginine.^{4–6} The C-terminal reductase domain, with binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and nicotinamide adenine dinucleotide phosphate (NADPH), is the source of electrons during the catalytic cycle.⁷ At the interface of the two domains is a flexible peptide linker containing the binding site for calmodulin (CAM).⁸

We have shown that several sensitizer-linked substrates bind with high affinity to the active-site channel of the oxygenase domain of inducible NOS (iNOS_{oxy}, with the reductase domain cleaved at the CAM linker site).^{9,10} Here we report that a Re(I) sensitizer coupled to nitro-L-arginine by an imidazole-alkyl molecular wire can be employed to trigger rapid photoreduction of the ferriheme in the active site of the enzyme. The Re-wire described here has the distinction that it bears a functional group structurally similar to the native enzyme substrate and supports rapid electron transfer into the heme active site.

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EXPERIMENTAL SECTION

General

Chemicals were obtained from Aldrich unless stated otherwise. Absorption spectra were recorded on an Agilent 8453 spectrometer. Steady-state luminescence was recorded using a Fluorolog Model FL3-11 fluorometer equipped with a Hamamatsu R928 PMT. Luminescence was measured following excitation at 355 nm; the PMT was equipped with a 405 nm long pass filter to remove scattered excitation light during data collection.

Bromooctylphthalamide (BrC₈phth)

Phthalamide (1.0 g, 6.8 mmol) was added to a flask containing 1.0 g (7.5 mmol) K₂CO₃ and 10 mL (0.5 M) DMF. Dibromohexane (Br₂C₈, 1.4 mL, 7.5 mmol) was added and stirred overnight (12 h) at room temperature. The reaction mixture was concentrated by rotary evaporation and purified by flash column chromatography (6:1 Hex:EtAc eluent). ¹H NMR (300 MHz, CDCl₃): δ (ppm) 1.2–1.4 (m, 8H), 1.6 (m, 2H), 1.9 (m, 2H), 3.4 (t, 2H), 3.7 (t, 2H), 7.7 (dd, 2H), 7.9 (dd, 2H).

Imidazolyl-octyl-phthalamide

Imidazole (200 mg, 3.2 mmol) was stirred in 5.0 mL (0.60 M) anhydrous DMF. Solid NaH (78 mg, 3.5 mmol) was added to the reaction and stirred for 2 h. BrC₈phth (1.0 g, 3.2 mmol) was added and stirred overnight at 40 °C. The reaction mixture was concentrated by rotary evaporation to remove DMF. Water (50 mL) and toluene (20 mL) were added to the oil in a separation funnel. The toluene layer was collected and product was further extracted from the water layer with another 20 mL toluene. This procedure was repeated 3 times. The organic layer was dried over MgSO₄; the MgSO₄ was removed by gravity filtration and the organic layer was concentrated by rotary evaporation, yielding product, a pale yellow solid (PhthC₈imid) (490 mg, 51% yield). ¹H NMR (300 MHz, CDCl₃): δ (ppm) 1.2–1.4 (m, 8H), 1.6 (m, 2H), 1.8 (m, 2H), 3.6 (t, 2H), 3.9 (t, 2H), 6.9 (s, 1H), 7.0 (s, 1H), 7.1 (s, 1H), 7.7 (dd, 2H), 7.9 (dd, 2H).

Imidazolyl-octyl-amine (1)

PhthC₈imid (270 mg, 0.83 mmol) and N₂H₂ (281 μL, 5.8 mmol) were stirred in 1.0 mL (0.8 M) EtOH for 2 h. A white precipitate formed and was removed by vacuum filtration through a filter frit. The solid was washed with more EtOH. The filtrate was collected and concentrated by rotary evaporation. The product (NH₂C₈imid) was carried onto the next step without further purification (300 mg, 100% yield). ¹H NMR (300 MHz, CDCl₃): δ (ppm) 1.2–1.4 (m, 8H), 1.6 (m, 2H), 1.8 (m, 2H), 2.6 (t, 2H), 3.9 (t, 2H), 6.9 (s, 1H), 7.0 (s, 1H), 7.1 (s, 1H).

Imidazolyl-octyl-BOC-nitroarginine

BOC-nitroarginine (1.0 g, 3.1 mmol) and **1** (1.2 g, 6.2 mmol) were stirred with DCC (1.3 g, 6.2 mmol) in anhydrous THF (45 mL) at 50 °C. White solid DHU precipitated and was removed by vacuum filtration through a frit containing celite. The filtrate was concentrated by rotary evaporation and purified by flash column chromatography (50:3 CH₂Cl₂:MeOH). The product was collected as a clear oil (800 mg, 50% yield). ¹H NMR (300 MHz, CD₃OD): δ (ppm) 1.2–1.8 (m, 18H); 1.5 (s, 9H); 3.2 (t, 2H); 4.0 (t, 2H); 6.9 (s, 1H); 7.1 (s, 1H); 7.6 (s, 1H). ¹³C NMR (300 MHz, CD₃OD): δ (ppm) 20–30, 39.9, 40.1, 53, 55, 80, 120, 129, 139, 159, 160, 172.

Imidazole-octyl-nitroarginine (2)

Imidazole-octyl-BOC-nitroarginine (700 mg, 1.4 mmol) was chilled to 0 °C in 15 mL CH₂Cl₂. TFA (2.1 mL) was added drop wise. Once the reaction was completed, an aqueous sodium bicarbonate work up was conducted. The organic layer was collected and concentrated.

The product was purified by flash column chromatography (90:1 CH₂Cl₂:MeOH) and collected as a clear oil (200 mg, 48% yield). ¹H NMR (300 MHz, CD₃CN): δ (ppm) 1.2–1.8 (m, 18H); 3.1 (t, 2H); 3.9 (t, 2H); 6.9 (s, 1H); 7.1 (s, 1H); 7.5 (s, 1H). ¹³C NMR (300 MHz, CD₃OD): δ (ppm) 24.5, 26.9, 27.1, 29.0, 29.2, 29.9, 30.5, 31.9, 32.5, 33.5, 39.9, 51.5, 120, 126.5, 128, 131, 134.

[Re(CO)₃(4,7-dimethylphenanthroline)(imid-C₈-NH-nitroarginine)]BF₄, [ReC₈argNO₂]BF₄

Re(CO)₃(dmp)(THF) (145 mg, 0.22 mmol) was stirred with 100 mg (0.25 mmol) of **2** in 10 mL 1:1 CH₂Cl₂:THF at 50 °C for 2 days. The yellow precipitate was removed by vacuum filtration through a filter frit and the filtrate was collected and concentrated by rotary evaporation. The product was purified by flash column chromatography (90:1 CH₂Cl₂:MeOH) and collected as a yellow solid (100 mg, 45% yield). ¹H NMR (300 MHz, CD₂Cl₂): δ (ppm) 1.0 (broad s, 1H); 1.2–1.8 (m, 18H); 3.0 (s, 6H); 3.2 (t, 2H); 3.4 (br s, 1H); 3.7 (t, 2H); 6.1 (br s, 1H); 6.4 (s, 1H); 6.7 (s, 1H); 7.3 (s, 1H); 7.9 (d, 2H); 8.3 (d, 2H); 9.4 (d, 2H). ¹⁹F NMR (300 MHz, CD₂Cl₂): δ (ppm) -151.1 (4F). ESI/MS (*m/z*)⁺: 880.3 [M]⁺ (calc 879.3).

Expression and Purification of iNOS_{oxy}

Escherichia coli JM109 competent cells and pCWori plasmid (ampicillin resistance, tac-tac promoter) were provided by M. Marletta (University of California, Berkeley). iNOS_{oxy} was overexpressed in *E. coli* following literature procedures^{11,12} with the following alterations: fresh cell pellets from 6 L of culture were resuspended in two rounds of 40 mL B-Per lysis buffer (Pierce) containing 10 µg/mL benzamidine, 5 µg/mL pefabloc, 1 µg/mL each of pepstatin, antipain, chymostatin, and chytrolysin, 100 µg/mL DNase, 100 µg/mL RNase, 500 µg/mL lysozyme, and 20 mM imidazole per liter of cells. Cells in lysis buffer solution were shaken for 1 h at 4 °C. The supernatant was collected after centrifugation and loaded directly onto a 5 mL His-Trap nickel column (Amersham). The column was washed with 20 column volumes of 20 mM imidazole in 50 mM sodium phosphate and 300 mM sodium chloride buffer at pH 8. The protein was then eluted with a linear gradient of imidazole (20 – 150 mM). The protein-containing fractions were concentrated to 5 mL over an Amicon Ultra filtration device (10,000 MWCO, Millipore) and loaded onto a Superdex 200 gel filtration column (HiLoad 26/60, Amersham).

Preparation of iNOS_{oxy} Samples

Before every experiment, a 100 µL aliquot of 100 µM iNOS_{oxy} was thawed on ice and filtered through a PD10 desalting column (BioRad). The PD10 column removed any excess imidazole bound to the protein during purification. The storing buffer was exchanged for a 50/50 mM KPi/KCl buffer at pH 7.4 by the same method. The protein concentration in the stock solution was 20 µM (in 1 mL buffer after the PD10 column). [ReC₈argNO₂]BF₄ was added from a 5 mM stock ethanol solution to protein solution, keeping the ethanol concentration <2% for every sample (final volume changes were <2% of the initial volume).

Dissociation Constants

Wire affinities were determined by saturation-binding experiments analyzed by three different methods: double reciprocal plots from steady-state absorption data, Scatchard analysis of steady-state fluorescence experiments,^{13,14} and extrapolated free-to-bound substrate ratios from transient luminescence decay traces. Equilibria from competitive-binding experiments involving imidazole (*K_d* = 14 ± 3 µM) and [ReC₈argNO₂]BF₄ were analyzed using a literature spectral perturbation method.^{15,16}

Transient Luminescence and Absorption Spectroscopy

All laser experiments were carried out in atmosphere-controlled, 1-cm pathlength cuvettes equipped with Kontes valves for pump and purge cycles. Transient luminescence and absorbance decay traces were obtained using a frequency-tripled Nd:YAG laser ($\lambda_{\text{ex}} = 355$ nm).^{17,18} The instrument has a response limit of approximately 10 ns. Transient luminescence decay were fit using a nonlinear least-squares algorithm according to the function

$$I(t) = c_0 + \sum_n c_n e^{-k_n t}$$

where $n = 1 - 2$ for mono- and bi-exponential decays, respectively; k_n is the luminescence decay rate constant for the bound- and free-wire at time t ; and c_n is the amplitude of bound- and free-wire. The amplitudes of the decay traces were used to determine the fractions of bound and free wire ($c_1/(c_1 + c_2) = [\text{Re}]_b/[\text{Re}]_o$; $c_2/(c_1 + c_2) = [\text{Re}]_f/[\text{Re}]_o$). The binding constant for the $\text{iNOS}_{\text{oxy}}:\text{[ReC8argNO}_2\text{]}^+$ complex was calculated from the following expression:

$$K_d = \frac{[\text{Re}]_f}{[\text{Re}]_b} \frac{[\text{P}]}{[\text{P}]_o - \left([\text{Re}]_o \times \left(\frac{[\text{Re}]_b}{[\text{Re}]_o}\right)\right)}$$

where $[\text{P}]_o$ and $[\text{S}]_o$ are the initial concentrations of protein and $[\text{ReC8argNO}_2]^+$, and $[\text{S}]_i$, $[\text{S}]_f$, $[\text{S}]_b$, are the initial, free, and bound concentrations of $[\text{ReC8argNO}_2]^+$.

RESULTS AND DISCUSSION

Synthesis of $[\text{ReC8argNO}_2]\text{BF}_4$

The imidazole-wire synthesis required six steps (Scheme 1). Deprotonation of the phthalamide ligand in the presence of dibromooctane produced bromo-octyl-N-phthalamide. Utilizing a NaH deprotonation reaction of the imidazole secondary amine, the bromo-octyl-N-phthalamide was coupled with imidazole to produce phthalyl-octyl-imidazole. The phthalamide protecting group was removed with hydrazine monohydrate, resulting in the amino-octyl-imidazole product. The alkylimidazole was then coupled with N^{ω} -BOC- N^{ω} -nitroarginine by reaction with DCC, followed by deprotection of the BOC group with TFA, affording the imidC8argNO_2 ligand. The final step of the synthesis was metalation of the ligand with $[\text{Re}(\text{CO})_3(4,7\text{-Me}_2\text{phen})(\text{THF})]\text{BF}_4$, yielding $[\text{ReC8argNO}_2]\text{BF}_4$.

Binding $[\text{ReC8argNO}_2]^+$ to iNOS_{oxy}

Addition of L-arginine to iNOS_{oxy} causes a blue shift in the heme Soret peak from 423 (six-coordinate, resting-state Fe(III)) to 390 nm (high-spin, five-coordinate Fe(III)).¹⁹ The heme Soret peak of the protein shifts from 423 to 390 nm, owing to partial displacement of the water ligand from the active site of iNOS_{oxy} , as occurs when arginine binds (Figure 1). Samples of iNOS_{oxy} with different concentrations of imidazole (5, 10, 30, and 50 μM) were titrated with $[\text{ReC8argNO}_2]\text{BF}_4$ (up to 100 μM). Absorption peak shifts (427 to 390 nm) confirmed that $[\text{ReC8argNO}_2]^+$ displaces imidazole from the active site. Double reciprocal plots are consistent with competition between $[\text{ReC8argNO}_2]^+$ and imidazole for the iNOS_{oxy} binding site (Figure 2). The K_d of $3.0 \pm 0.1 \mu\text{M}$ obtained for $\text{iNOS}_{\text{oxy}}:\text{[ReC8argNO}_2\text{]}^+$ is much smaller than those for arginine ($22 \pm 5 \mu\text{M}$) and imidazole ($14 \pm 3 \mu\text{M}$) conjugates with the enzymes.

$[\text{ReC8argNO}_2]^+:\text{iNOS}_{\text{oxy}}$ Structural Model

The location of $[\text{ReC8argNO}_2]^+$ in the iNOS_{oxy} active site was estimated by computer modeling (DS Viewer Pro software package; PDB code 1NOD)⁵ (Figure 3). The arginine end of the wire is parallel to the heme in an orientation similar to that in the arginine conjugate. Hydrogen bonding interactions between the guanidinium group of the wire and Gly³⁶⁵, Trp³⁶⁶, and Glu³⁷¹ residues are retained in the model. The Re(I) complex was placed at the opening of the

active-site channel, closely interacting with two tryptophan residues (Trp⁴⁹⁰ and Trp⁸⁴ estimated to be 2.98 and 4.96 Å from the phenanthroline ligand). The opening of the active-site channel is about 20 Å from the center of the heme.

[ReC₈argNO₂]⁺ Luminescence

The luminescence intensity of electronically excited [ReC₈argNO₂]BF₄ decreases upon addition of the wire in buffer to an iNOS_{oxy} solution (Figure 4). The luminescence maximum for ReC₈argNO₂ is at higher energy when the chromophore is bound in a protein environment (550 nm) than when it is in buffer solution (570 nm). Scatchard analysis for wire:protein ratios less than 4:1 yields a K_d of $2.0 \pm 0.1 \mu\text{M}$,²⁰ a value in line with that extracted from competitive binding experiments with imidazole ($K_d = 3 \pm 0.1 \mu\text{M}$). At wire:protein ratios greater than 4:1, a small amount of curvature is observed in the Scatchard plot, possibly indicating a second [ReC₈argNO₂]⁺ binding on iNOS_{oxy} (apparent K_d of $12 \pm 1 \mu\text{M}$).²⁰

Luminescence decay traces ($\lambda_{\text{ex}} = 355 \text{ nm}$, $\lambda_{\text{obs}} = 600 \text{ nm}$) were obtained for samples containing [ReC₈argNO₂]BF₄ in the absence of protein; Re(I)* decay was monoexponential with a lifetime of 500 ns.²⁰ Biexponential decay traces were obtained for samples with [ReC₈argNO₂]BF₄ in the presence of protein (Figure 5). The faster decay is attributable to * [ReC₈argNO₂]⁺ bound to protein ($\tau = 92 \text{ ns}$), and the slower component corresponds to uncomplexed * [ReC₈argNO₂]⁺ in buffer ($\tau = 500 \text{ ns}$). Luminescence decay traces were obtained for various ratios of [ReC₈argNO₂]BF₄-to-protein.²⁰ All transient luminescence traces of [ReC₈argNO₂]BF₄ in the presence of protein exhibited biexponential decays with fast ($\tau \sim 90 \text{ ns}$) and slow ($\tau \sim 500 \text{ ns}$) components.

[ReC₈argNO₂]⁺ Transient Absorbance

Single-wavelength transient absorption traces of solutions with varying [ReC₈argNO₂]BF₄:iNOS_{oxy} ratios are shown in Figure 6. As the wire concentration increases, the *Re(I) absorbance signal increases (~500 ns decay). A species with a much longer lifetime also is generated; a difference spectrum of this transient features a bleach in the 390 – 435 nm region, an isosbestic point at 438 nm, and an absorption increase maximizing near 445 nm (inset, Figure 6). The bleach at ~425 nm is attributable to reduction of the Fe(III); the increased absorption at 445 nm is consistent with the spectrum of substrate-bound Fe(II)-iNOS_{oxy}.²¹ Reduced, substrate-bound iNOS is reported to have a Soret maximum near 413 nm that exhibits a noticeable shoulder near 450 nm^{21–25} attributable to a high-spin ferrous heme. Indeed, the difference spectrum shown in the inset to Figure 6 resembles that resulting from dithionite titration of arginine-bound, BH₄-depleted Fe(III)-eNOS_{oxy}.²¹ Owing to overlapping signals from the excited-state decay of free [ReC₈argNO₂]⁺ ($\tau = 500 \text{ ns}$), we were not able to measure the kinetics of Fe(II) formation. We can nevertheless conclude that Fe(III) reduction is complete in less than ~1 μs , orders of magnitude faster than reduction by the reductase domain ($k_{\text{ET}} = 0.9 - 1.5 \text{ s}^{-1}$).^{26,27} It is important to note that the rate of the latter process may be limited by large-scale protein conformational changes.

Steady-state absorption spectra of iNOS_{oxy} (solid line, $\lambda_{\text{max}} = 423 \text{ nm}$) as well as [ReC₈argNO₂]⁺-bound iNOS_{oxy} recorded before (dotted line, $\lambda_{\text{max}} = 390 \text{ nm}$) and after (open circles, $\lambda_{\text{max}} = 445 \text{ nm}$) excitation are shown in Figure 7 (inset is the difference spectrum). After laser excitation, there is an intensity decrease in the 390 – 420 nm region and a slight increase near 445 nm, attributable to the disappearance of five-coordinate Fe(III) and appearance of Fe(II)-iNOS_{oxy}. The Fe(II) species persists for hours before reoxidation occurs, with return to the Fe(III) resting state.

We can only speculate on the mechanism of Fe(II) formation in [ReC₈argNO₂]⁺:iNOS_{oxy}. A first step could be reductive quenching of * [ReC₈argNO₂]⁺ by a nearby tryptophan residue

(Trp⁴⁹⁰ or Trp⁸⁴), generating Re(0) and a Trp radical cation. Direct, coupling-limited electron tunneling from Re(0) to Fe(III) (~20 Å) could proceed on the microsecond timescale.^{28,29} More complex redox chemistry, possibly involving Trp⁺ oxidation of one of the three tyrosines (Tyr⁴⁸³, Tyr⁴⁸⁴, Tyr⁴⁸⁵) near the heme (Figure 3), also may occur.⁹ Experiments with selected mutants are planned to test these mechanistic proposals.

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Figure 1. Absorption spectra of iNOS_{oxy} in buffer (7.5 μM, solid line) and with 1:1 [ReC₈argNO₂]⁺:iNOS_{oxy} (dotted line). The inset is the difference spectrum of the two absorption traces, showing a type I perturbation.

Figure 2.

Double reciprocal plots from competitive binding studies between imidazole and $[\text{ReC}_8\text{argNO}_2]^+$: $1 / (\text{Abs}_{427} - \text{Abs}_{390})$ versus $1 / [\text{ReC}_8\text{argNO}_2]^+$. iNOS_{oxy} ($2\mu\text{M}$) was titrated with $[\text{ReC}_8\text{argNO}_2]^+$ at a fixed concentration of imidazole for each sample: squares, 5 μM imidazole; diamonds, 10 μM imidazole; triangles, 30 μM imidazole; circles, 50 μM imidazole.

Figure 3.

Structural model of $[\text{ReC}_8\text{argNO}_2]^+:\text{iNOS}_{\text{oxy}}$: the arginine substrate is shown in green, the heme in olive, and the $[\text{ReC}_8\text{argNO}_2]^+$ wire in orange (based on the iNOS crystal structure, PDB Code 1NOD, ref. ⁵). The model was designed to maximize the overlap between arginine and the terminus of $[\text{ReC}_8\text{argNO}_2]^+$.

Figure 4. Steady-state luminescence traces for [ReC₈argNO₂]BF₄ in buffer (solid line) and 1:1 [ReC₈argNO₂]⁺:iNOS_{oxy} (dotted line).

Figure 5. Luminescence decay traces for 4.8 μM $[\text{ReC8argNO}_2]^+$ (dashed line), 1:1 $[\text{ReC8argNO}_2]^+:\text{iNOS}_{\text{oxy}}$ (solid line). Biexponential fit (dots) and residuals (dotted line above) for $\tau_1 = 92$ ns and $\tau_2 = 500$. The slow decay is free wire in solution and the fast decay is wire-bound to protein ($\lambda_{\text{ex}} = 355$, $\lambda_{\text{obs}} = 600$ nm).

Figure 6.

Transient absorption kinetics of varying concentrations of $[\text{ReC8argNO}_2]^+$ in $9.6 \mu\text{M}$ iNOS_{oxy} ($\lambda_{\text{ex}} = 355$, $\lambda_{\text{obs}} = 443$ nm, $[\text{ReC8argNO}_2]^+:\text{iNOS}_{\text{oxy}}$: magenta, 0:1; blue, 1:1; green, 2:1; cyan, 3:1; red, 4:1). The negative spike is due to scattered laser light. Inset: Transient difference spectra recorded at 1.5 (circles), 2.5 (triangles), and 3.5 μs (diamonds) after excitation ($[\text{ReC8argNO}_2]^+:\text{iNOS}_{\text{oxy}} = 4:1$).

Figure 7. Absorption spectra of $i\text{NOS}_{\text{ox}}$ (solid line), $[\text{ReC}_8\text{argNO}_2]^+$ bound $i\text{NOS}_{\text{ox}}$ before (dashed line) and after (open circles) excitation. The difference spectrum is shown in the inset ($\lambda_{\text{min}} = 423 \text{ nm}$; $\lambda_{\text{max}} = 445 \text{ nm}$).

Scheme 1.
Synthesis of $[\text{ReC}_8\text{argNO}_2]\text{BF}_4$.