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Ligand-Directed Approach to Activity-Based Sensing: Developing Palladacycle Fluorescent Probes That Enable Endogenous Carbon Monoxide Detection

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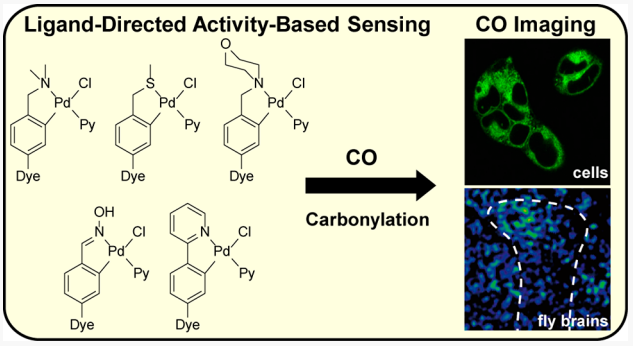
ABSTRACT: Carbon monoxide (CO) is an emerging gasotransmitter and reactive carbon species with broad anti-inflammatory, cytoprotective, and neurotransmitter functions along with therapeutic potential for the treatment of cardiovascular diseases. The study of CO chemistry in biology and medicine relative to other prominent gasotransmitters such as NO and H_2S remains challenging, in large part due to limitations in available tools for the direct visualization of this transient and freely diffusing small molecule in complex living systems. Here we report a ligand-directed activity-based sensing (ABS) approach to CO detection through palladium-mediated carbonylation chemistry. Specifically, the design and synthesis of a series of ABS probes with systematic alterations in the palladium-ligand environment (e.g., sp^3-*S*, sp^3-*N*, sp^2-*N*) establish structure−activity relationships for palladacycles to confer selective reactivity with CO under physiological conditions. These fundamental studies led to the development of an optimized probe, termed Carbon Monoxide Probe-3 Ester Pyridine (COP-3E-Py), which enables imaging of CO release in live cell and brain settings, including monitoring of endogenous CO production that triggers presynaptic dopamine release in fly brains. This work provides a unique tool for studying CO in living systems and establishes the utility of a synthetic methods approach to activity-based sensing using principles of organometallic chemistry.



INTRODUCTION

Carbon monoxide (CO) is an essential gasotransmitter that exhibits a diverse array of cytoprotective, anti-inflammatory, and signaling effects in living systems.[1−3] In addition to these roles in basic biology, CO is being increasingly recognized for its contributions to medicine as molecules with the capacity to release CO *in vivo*, including Carbon Monoxide Releasing Molecules (CORMs), are being evaluated as potential therapeutics for a broad range of cardiovascular diseases.[4−13] The biological and biomedical importance of CO motivates the need to develop new methods to monitor dynamic changes in CO fluxes with high selectivity and sensitivity in living systems on a molecular level. In this context, fluorescent probes offer a powerful set of chemical reagents for studying other transient biologically active small molecules, including the gasotransmitters NO[14−16] and H_2S[17−27] as well as H_2O_2,[28−43] presaging the use of this approach to decipher the chemistry of CO in biological settings.

As part of a larger program in our laboratory on activity-based sensing (ABS),[44−48] we and others have become interested in developing small-molecule probes for one-carbon species,[49−51] and we reported the development of Carbon Monoxide Probe-1 (COP-1),[52] which established the use of palladium-mediated carbonylation chemistry for CO detection with high selectivity over competing reactive oxygen, nitrogen, carbon, and sulfur species. In parallel with our initial report, the He and Chen laboratories described COSer,[53] a first-generation fluorescent protein sensor for CO. COP-1 has been independently applied to monitor and study CO released by CORMs in biological samples,[54−57] and its organometallic trigger has been adapted by others to develop various CO-responsive probes,[58−61] including fluorescent indicators for two-photon imaging,[62] Raman spectroscopy nanosensors,[63] and CO-responsive nanoparticles.[64] In addition, allyl ether-based fluorescent probes have emerged as an alternative strategy for CO-detection,[65−67] but the requirement for exogenous addition of free palladium salts to biological samples makes this three-component system more challenging to implement. These efforts have spawned

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broader strategies for exploiting the use of organometallic reactivity in cells.[68−77]

Despite previous successes with COP-1, this first-generation ABS probe exhibits shortcomings that limit its broader use in biological settings, including a hydrophobic nature that promotes punctate cellular staining, thereby effectively precluding visualization of substantial portions of the cell, as well as relatively poor cellular retention, making washing steps (to increase signal-to-noise responses) in imaging experiments more challenging. We sought to address these issues by turning our attention to studying the underlying fundamental organometallic chemistry of CO detection by systematically modulating the metal−ligand environment of the reactive palladacycle trigger. Specifically, using COP-1 as a starting point, tuning of the covalently attached amine ligand with sp^3-*S*, sp^3-*N*, sp^2-*N* substitutions and the chloro-dimer-bridge were evaluated to explore their influence on the reactivity of the CO probe. We adapted the most promising reaction trigger identified in each round of synthetic screening and explored further functionalization of the BODIPY fluorophore to increase aqueous solubility, cellular distribution, and retention properties. These foundational structure−activity studies resulted in the development of an optimized CO-probe, termed Carbon Monoxide Probe-3 Ester Pyridine (COP-3E-Py), which exhibits markedly increased solubility in aqueous media and improved cellular retention. We demonstrate the utility of COP-3E-Py for the detection of CO release from CORMs in live cells. Included is the successful visualization of endogenous release of CO during neural stimulation in fly brain preparations using electrical and chemical input, highlighting the potential of fundamental organometallic chemistry for chemical biology applications.

RESULTS AND DISCUSSION

Imaging Studies Reveal Differences in Cellular Localization for COP-1 and its Carbonylation COP-1′ Product. We initiated structure−activity studies with extensive characterization of COP-1 and the carboxylic acid product formed from reaction of COP-1 and CO, termed COP-1′. This postcarbonylation product is presumably formed following migratory insertion of COP-1 with CO to provide a Pd-acyl intermediate and subsequent reductive hydrolysis. Confocal microscopy images of HEK293T cells loaded with COP-1 showed punctate staining. This phenomenon was revealed to arise from lysosomal localization through a costaining experiment with lysosome marker LysoTracker Red DND-99. Interestingly, this experiment showed strong colocalization for COP-1 with LysoTracker Red DND-99 (PC: 0.792, M1:0.584) but not for the more polar carboxylic acid product COP-1′ (PC: 0.600, M1:0.158) (Figure 1). We initially hypothesized that COP-1 accumulates in acidic stores due to protonation of the basic directing group.[78] However, while this alkylamine functionality is shared by COP-1, COP-1′, and LysoTracker Red DND-99 (Figure 1h), the presence of an additional polar carboxyl group on COP-1′ mitigates lysosomal localization. We further investigated the reaction of COP-1 with H_2S by subjecting COP-1 to excess Na_2S in a suspension of CH_2Cl_2 and water. Subsequent LCMS analysis revealed that the product of this reaction is indeed the protodemetalation product (Figure S1).

Synthesis, Structure, and CO Reactivity of a Monomeric Analogue of COP-1. With data on COP-1′ showing that a more hydrophilic COP analogue could promote more even and diffuse cellular staining, we targeted the synthesis of a monomeric COP dye in hopes that the decreased molecular

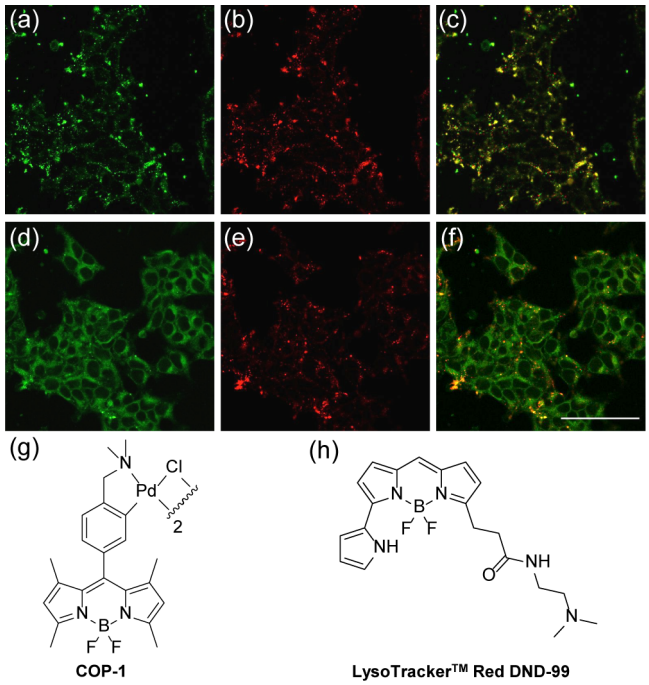

Figure 1. Colocalization of COP-1 and COP-1′ with LysoTrackerRed DND-99. (a−c) HEK293T cells were incubated with COP-1 (1 μM) and LysoTracker (50 nM) for 30 min, resulting in the observed cellular staining of (a) COP-1, (b) LysoTracker, and (c) overlay. (d−f) HEK293T cells were incubated with COP-1′ (1 μM) and LysoTracker (50 nM) for 30 min, resulting in the observed cellular staining of (d) COP-1′, (e) LysoTracker, and (f) overlay. Scale bar represents 80 μm. (g,h) Chemical structures of COP-1 and LysoTracker Red DND-99 showing shared alkylamine motifs.

weight would result in increased hydrophilicity and CO reactivity. To this end, we conducted a bridge-splitting reaction with pyridine as the σ-donating ligand to synthesize the monomeric probe COP-1-Py.[79] This reaction was conducted with a suspension of COP-1 in CH_2Cl_2, and upon addition of an equimolar amount of pyridine (1:1 with Pd) at room temperature, we observed an immediate increase in solubility in CH_2Cl_2 through the formation of a homogeneous solution (Figure 2a). The resulting COP-1-Py complex was isolated and behaved similarly *in vitro* to samples prepared from the μ-chloro dimer COP-1, reacting with CO to give the same COP-1′ product. Single crystals of COP-1, COP-1-Py, and the carbonylation product COP-1′ were obtained from slow evaporation of concentrated dichloromethane solutions and characterized by single crystal X-ray crystallography to provide molecular connectivity for all three compounds (Figure 2b). As anticipated, the palladacycles of COP-1 and COP-1-Py are perpendicular to the BODIPY plane and the palladium centers adopt a square planar geometry. The crystal structure suggests that COP-1 exists as a dimer with two μ-chloro bridges linking the palladium(II) centers, whereas COP-1-Py exists as a monomer with a single square-planar palladium center. A hydrogen-bonded zwitterion is observed for the COP-1′ carbonylation product after CO-induced palladium release. The acidic hydrogen of the ammonium group, located in the electron density map, is 1.636 Å from the nearest oxygen of the carboxylate moiety.

Modulation of the Directing Group Ligands on the COP Platform to Tune CO-Dependent Palladacycle Carbonylation Reactivity. Since dimeric COP-1 and

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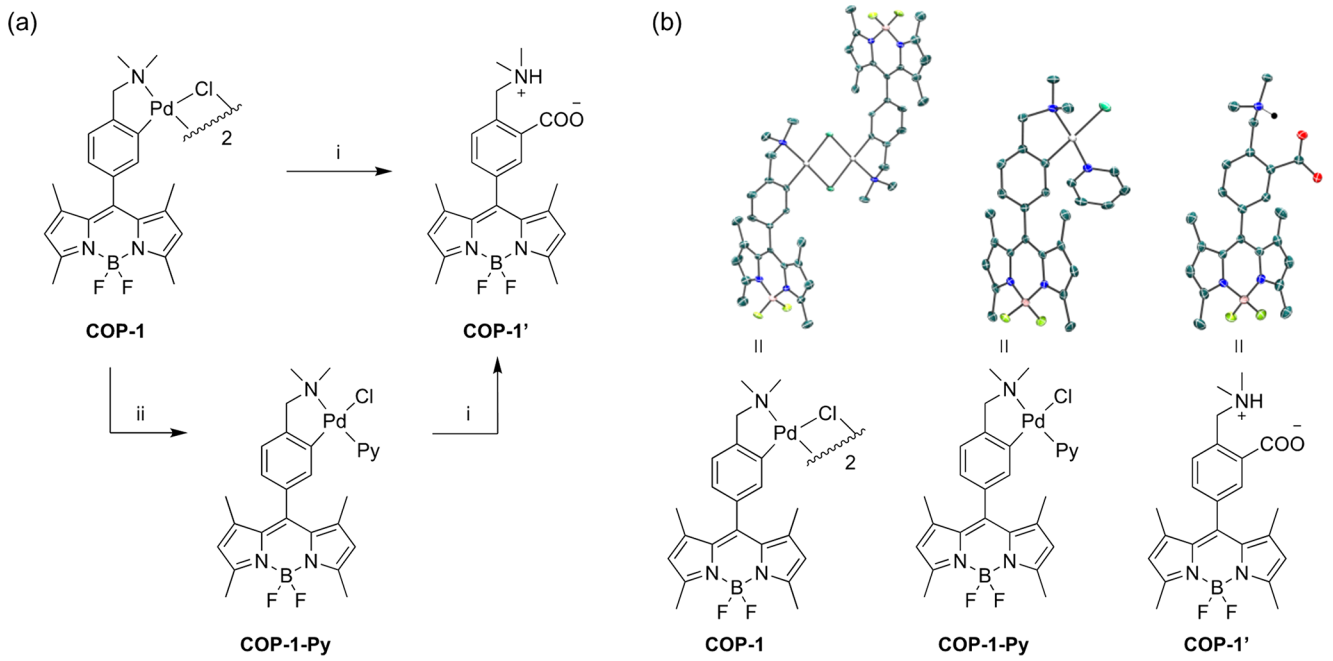

Figure 2. Synthesis and reactivity of the of the monomeric CO indicator COP-1-Py and crystal structures of COP-1, COP-1-Py, and COP-1′. (a) Reagents and conditions: (i) CO, wet CH_2Cl_2, 31 °C, 14 h; (ii) pyridine, CH_2Cl_2, rt, 5 h, 79%. (b) X-ray crystal structures: hydrogen atoms have been omitted for clarity, except for the H-bonding hydrogen in COP-1′. Thermal ellipsoids are shown at the 70% probability level.

monomeric COP-1-Py showed the same CO-dependent carbonylation reactivity, we decided to move forward and design monomeric palladacycles to tune CO reactivity by systematic modulation of the directing group ligand on palladium. In particular, we reasoned that the chelating heteroatom ligand, which is the directing group for the cyclopalladation reaction, does not exhibit fluxional behavior and is therefore expected to potentially have the most profound effect on CO reactivity. We therefore focused our efforts on varying the chelating heteroatom ligand and explored a set of sp^3-*S*, sp^3-*N*, and sp^2-*N* donors. Indeed, these types of directing ligands are known to facilitate the formation of palladacycles and to undergo CO insertion in an abiological experimental setting. We sought to explore varied σ-donating directing group ligands to evaluate its influence on both reactivity and subcellular localization. Finally, in addition to *N*-basic directing groups, we sought to characterize the potential of a sp^3-*S*-derived palladacycle for CO-sensing applications. Previous mechanistic studies in abiotic reaction environments suggest that these palladacycles undergo more facile CO-insertion compared to sp^3-*N*-derived palladacycles.[79,80] As such, we envisioned that this promotion of CO-insertion could potentially allow for faster reaction kinetics and improved selectivity of the probe scaffold over H_2S, a competing gasotransmitter and a substrate for an off-pathway protodemetalation.

An sp^3-*S*-derived CO-probe, termed COP-2-Py, and a comparably less-basic sp^3-*N*-derived morpholino congener to COP-1, termed COP-3-Py, were obtained via similar synthetic procedures as COP-1-Py (Scheme 1). In addition, an oxime-palladacycle probe, COP-4-Py, and a pyridine-based dye, COP-5-Py, were synthesized via newly developed synthetic routes (Scheme 1). Specifically, compounds **2** and **3** were prepared in a similar fashion to the COP-1 precursor, i.e. via a nucleophilic substitution of the benzylic chloride **1**. The oxime-containing synthon **7** was prepared from 1,4-phthalaldehyde by monoacetal protection (to differentiate the two aldehydes), BODIPY formation, and acetal hydrolysis to provide aldehyde **6**. Condensation with hydroxyl amine then afforded aldoxime **7**. 2-Pyridinyl **9** was prepared from the bromo BODIPY **8** via a Stille cross-coupling. All probes were prepared from their precursors via directed palladation, appropriate anion metathesis, and bridge-splitting with pyridine.

With this family of CO probes in hand, we evaluated their comparative reactivity with CO and H_2S *in vitro* as well as their subcellular localization and staining pattern in live HEK293T cells (Figure 3). Specifically, we evaluated the CO responses of these reagents in buffered aqueous solution using [$Ru(CO)_3Cl$(glycinate)] (CORM-3) as a CO source. COP-2-Py did not exhibit a significant fluorescent turn-on response with either CO or H_2S in phosphate buffered saline, suggesting that sp^3-*S*-palladacycles do not undergo facile CO-insertion under physiological conditions relative to sp^3-*N*-palladacycle congeners. Moreover, COP-4-Py and COP-5-Py were found to be less chemoselective for CO over H_2S than COP-1. Notably, both probes exhibit a stronger relative and absolute fluorescence response with H_2S compared to CO. Whereas COP-4-Py shows a continual rise in fluorescence turn-on response with H_2S (over the time course of the experiments), COP-5-Py exhibits a rapidly declining fluorescence turn-off response toward H_2S after 15 min. Some possible speculations for this observation are aggregation and/or precipitation of the expected nonpolar product from protodemetalation. In contrast to CO, which gives a carbonylated product upon activity-based sensing, H_2S is presumably undergoing protodemetalation due to its acidic nature and potential ability to form a metal-sulfide precipitate.[17] Of this series, COP-3-Py exhibits the strongest fluorescence response toward CO with minimal reactivity with H_2S, offering superior specificity over the first-generation COP-1 reagent. Taken together, these structure−activity relationships for a family of monomeric COP dyes suggest that sp^3-*N*-palladacycles appear to be best-suited for the development of palladacycle

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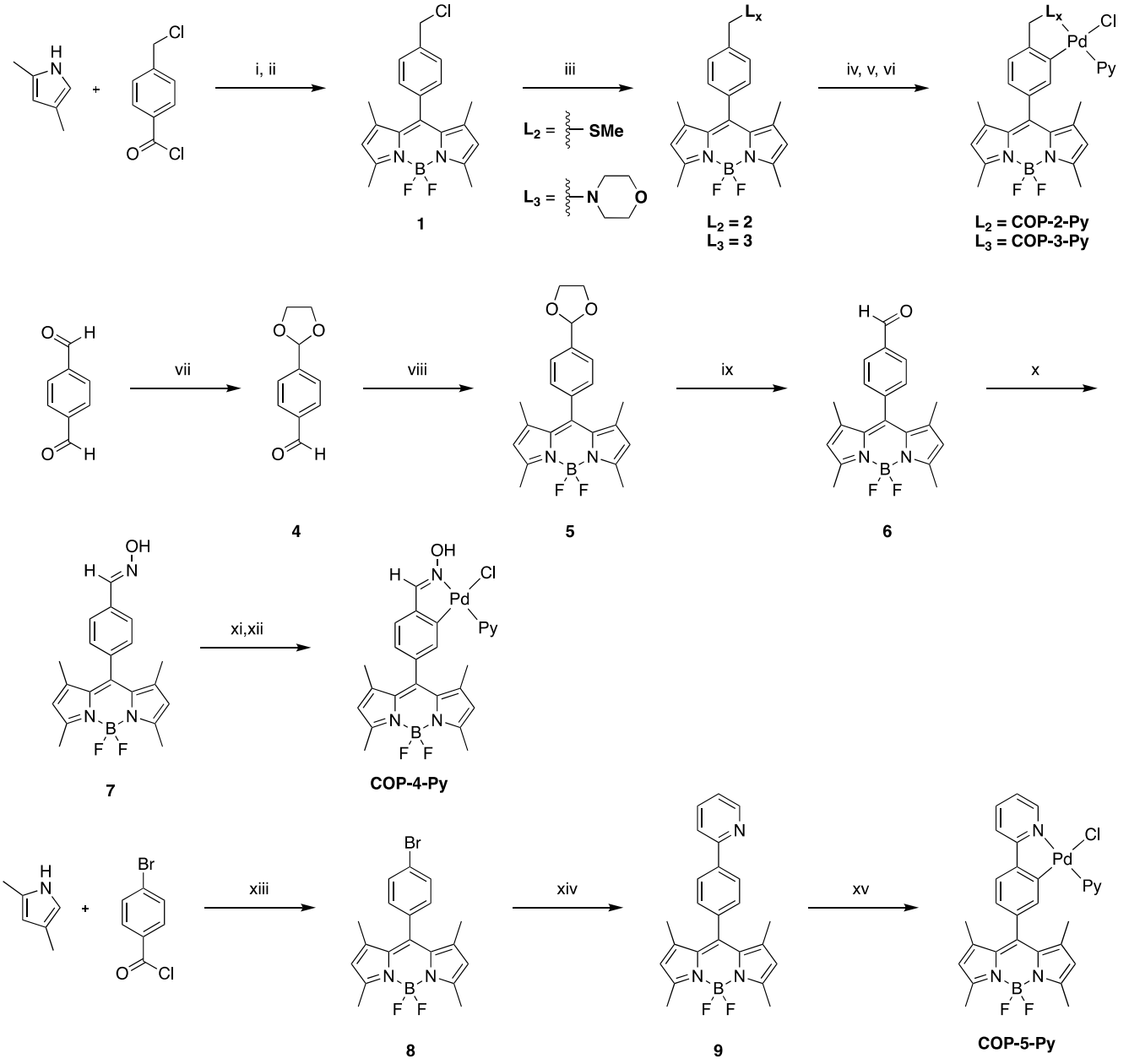
Scheme 1. Synthesis of COP-2-Py, COP-3-Py, COP-4-Py, and COP-5-Py[a]



[a]Reagents and conditions: (i) CH_2Cl_2, rt, 14 h; (ii) DIPEA, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 3 h, 28% (2 steps); (iii) L_2: NaSMe, EtOH, rt, 25 h, 45%; L_3: KI, K_2CO_3, CH_3CN, morpholine, 80−100 °C, 60 min, 92%; (iv) L_2: $Pd(OAc)_2$, AcOH, 120 °C, 55 min; L_3: $Pd(OAc)_2$, C_6H_6, 50 °C, 14 h; (v) LiCl, acetone, rt, 4 h; (vi) pyridine, CH_2Cl_2, rt, 5 h, 22% for L_2 (3 steps); 13% for L_3 (3 steps); (vii) ethylene glycol, *p*-toluenesulfonic acid, 130 °C, 4 h, 24%; (viii) 2,4-dimethylpyrrole, TFA, CH_2Cl_2,16 h, rt, then DIPEA, $BF_3{\cdot}OEt_2$, MePh, 45 °C, 50 min, 30%; (ix) acetone, 10% HCl, reflux, 2 h, 78%; (x) hydroxylamine hydrochloride, KOAc, EtOH, H_2O, 70 °C, 3 h, 91%; (xi) di-μ-chlorobis[2-[(dimethylamino)methyl]phenyl-C,N]-dipalladium(II), $CHCl_3$/AcOH, 50 °C, 16 h; (xii) pyridine, CH_2Cl_2, rt, 16 h, 69% (2 steps); (xiii) CH_2Cl_2, rt, 14 h, then Et_3N, $BF_3{\cdot}OEt_2$, rt, 20 h, 50%; (xiv) 2-(tributylstannyl)pyridine, toluene, $Pd(Ph_3P)_2Cl_2$, 110 °C, 48 h, 65%; (xv) $Pd(OAc)_2$, PhH, 50 °C, 14 h then LiCl, acetone, rt, 4 h then pyridine, CH_2Cl_2, rt, 74%.

activity-based sensing probes for CO that achieve improved selectivity over H_2S.

Evaluation of the staining patterns and localizations of all monomeric COP dyes showed observably less punctate staining (Figure 3e−h) relative to COP-1 (Figure 1). In addition to making probes less hydrophobic upon reduction in size from dimer to monomer, these results give some indication that less basic directing group ligands can decrease probe localization in acidic stores, presumably by lowering protonation under physiological conditions. However, the sp^3-*N* morpholino analogue COP-3-Py still exhibits minor punctate staining in HEK293T cells owing to its basic nitrogen donor. As such, we subjected COP-3-Py to a second round of structure optimization focusing on the fluorophore backbone.

Synthesis and Characterization of COP-3E-Py Bearing a More Hydrophilic BODIPY Core. The foregoing structure−activity studies led to the identification of a sp^3-*N* morpholino directing ligand on COP-3-Py as an improved activity-based sensing trigger for CO detection. To further optimize this scaffold, we next decided to functionalize the BODIPY core with

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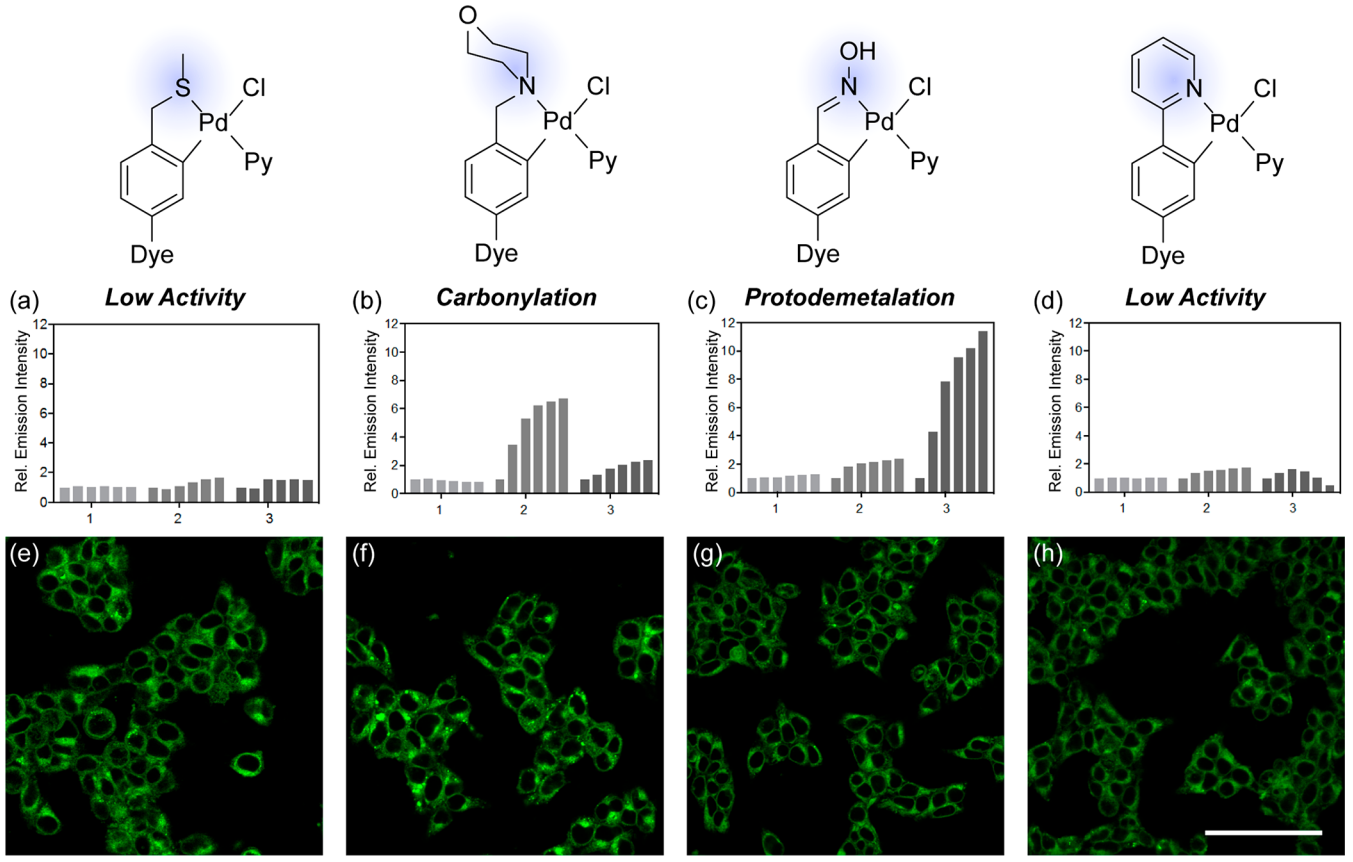
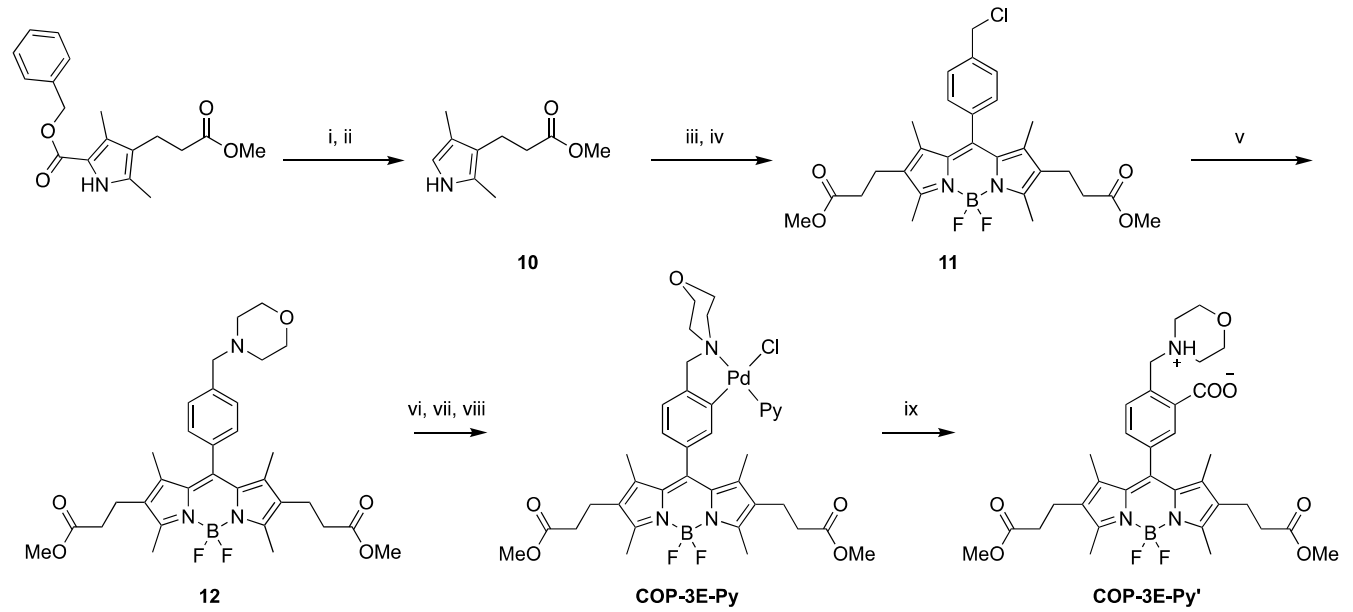


Figure 3. Evaluation of COP-2-Py, COP-3-Py, COP-4-Py, and COP-5-Py reactivity *in vitro* and *in cellulo*. (a–d) Fluorescent responses of the respective COP reagent (1 μM) to CO and H_2S. Bars represent integrated fluorescence intensities between 520 and 620 nm normalized to the respective probe in PBS at 0 min. Analytes were incubated at 50 μM concentration in pH 7.4 PBS at 37 °C and the bars correspond to the time points 0, 5, 15, 30, 45, and 60 min after addition of the respective analyte. Legend: (1) control; (2) CO; (3) H_2S. CORM-3 was used as a CO source. (e–h) Cellular staining of the respective probes (1 μM) on a HEK293T cell monolayer after 60 min of incubation show basal localization of the dyes. Images were acquired with individualized microscope settings and normalized for staining comparison. Scale bar represents 80 μm.

Scheme 2. Synthesis of COP-3E-Py and COP-3E-Py'[a]



[a]Reagents and conditions: (i) H_2, Pd/C, acetone, rt, 12 h; (ii) TFA, 40 °C, 10 min, 48% (2 steps); (iii) *p*-(chloromethyl)benzoyl chloride, CH_2Cl_2, rt, 14 h; (iv) DIPEA, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 3 h, 22% (2 steps); (v) KI, K_2CO_3, morpholine, CH_3CN, 60 °C, 3 h, 94%; (vi) $Pd(OAc)_2$, C_6H_6, 50 °C, 14 h; (vii) LiCl, acetone, rt, 4 h; (viii) Pyridine, CH_2Cl_2, rt, 5 h, 70% (3 steps); (ix) CO, wet CH_2Cl_2, 31 °C, 14 h, 92%.

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ester chains to increase aqueous solubility and hydrophilicity and improve cellular retention by exploiting native intracellular esterase activity.[81] The probe Carbon Monoxide Probe-3 Ester Pyridine (COP-3E-Py) was designed and synthesized according to Scheme 2. Initial deprotection of a commercially available pyrrole gave the more electron-rich pyrrole **10**. This intermediate was unstable due to rapid oxidation and was therefore used directly to form BODIPY **11** via condensation with 4-chlorobenzoyl chloride and complexation with BF_3. Nucleophilic substitution of chlorine with morpholine, directed palladium metalation, ligand exchange, and palladacycle-bridge-splitting gave access to the final COP-3E-Py probe. The carbonylated product COP-3E-Py′ was synthesized by reacting COP-3E-Py under a CO atmosphere in a solvent mixture of CH_2Cl_2 and water. In contrast to protodemetalation, the direct observation of carbonylated products formed in the presence of CO *in vitro* illustrate the unique nature of our ABS strategy.

In aqueous PBS solution, we observed an 11-fold fluorescence turn-on for COP-3E-Py (1 μM) upon addition of CORM-3 (50 μM) as a CO source within 60 min. Both the COP-3E-Py indicator (λ_{max} = 521 nm, λ_{em} = 535 nm, Φ_{fl} = 0.12, ε_{521} = 31 000 M^{-1} cm^{-1}) and its carbonylated product COP-3E-Py′ (λ_{max} = 521 nm, λ_{em} = 537 nm, Φ_{fl} = 0.51, ε_{521} = 43 000 M^{-1} cm^{-1}) exhibit a red shift compared to the parent COP-1 (λ_{max} = 499 nm, λ_{em} = 503 nm). Other potentially competing analytes, including reactive nitrogen, oxygen, and sulfur species, were tested at the same concentration (50 μM) for reactivity with COP-3E-Py (Figure 4). Only minimal responses were observed

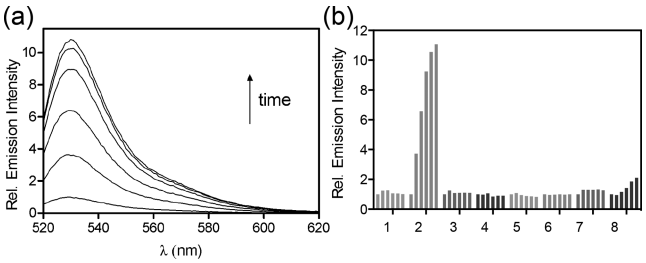

Figure 4. Turn-on fluorescence response to CO and analyte selectivity of COP-3E-Py in buffered aqueous solution. (a) Turn-on fluorescence response of COP-3E-Py (1 μM) to CORM-3 (50 μM) in pH 7.4 PBS at 37 °C (λ_{ex} = 515 nm, emission collected from 520−630 nm). Time points taken at 0, 5, 15, 30, 45, and 60 min after addition of CORM-3. (b) Fluorescence responses of COP-3E-Py (1 μM) to CO and other biologically relevant reactive small molecules. Bars represent integrated fluorescence intensity between 520 and 620 nm normalized to COP-3E-Py in PBS at 0 min. Analytes were incubated at 50 μM concentration in pH 7.4 PBS at 37 °C and the bars correspond to the time points 0 (or 1 for (8)), 5, 15, 30, 45, and 60 min after addition of the respective analyte. Legend: (1) control; (2) CORM-3; (3) H_2O_2; (4) NaOCl; (5) TBHP; (6) KO_2; (7) $ONOO^-$; (8) H_2S.

with other biologically relevant small molecules in this assay, showing the high specificity for activity-based sensing of CO by the COP-3E-Py palladacycle.

We next evaluated the ability of COP-3E-Py to image CO in biological samples and compared its performance to COP-1. Administration of CORM-3 to live HEK293T cells in the presence of COP-1 or COP-3E-Py yielded a robust fluorescent turn-on response in both cases (Figure 5). However, in contrast to COP-1, which accumulated in lysosomes, COP-3E-Py gave a more diffuse, homogeneous staining pattern in live cells. Moreover, dual-dye imaging experiments with COP-3E-Py

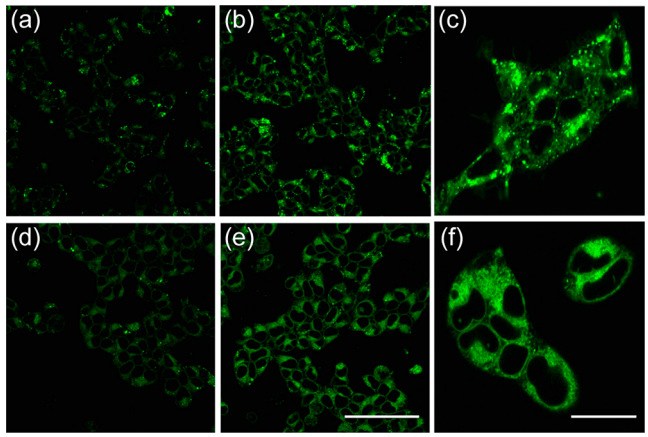

Figure 5. Live-cell imaging comparison between COP-1 and COP-3E-Py establishing improved diffuse staining of the more hydrophilic COP-3E-Py congener compared to lysosomal localization of COP-1. (a,b,d,e) CO-detection in live HEK293T cells using COP-3E-Py and COP-1. HEK293T cells were incubated with 1 μM COP 1 (a,b) or COP-3E-Py (d,e) for 120 min total, with negative control vehicle (a,d) or CORM-3 (50 μM) added for the positive control for the final 60 min (b,e). (c,f) Representative images taken at 63× for staining pattern comparison. Scale bar represents 80 μm (a,b,d,e) or 20 μm (c,f).

and ER-Tracker Red E34250 in the presence of CO indicate some colocalization with the ER (PC: 0.913, M1: 0.682, Figure 6).[9] Notably, the observation that this CO detection reagent can

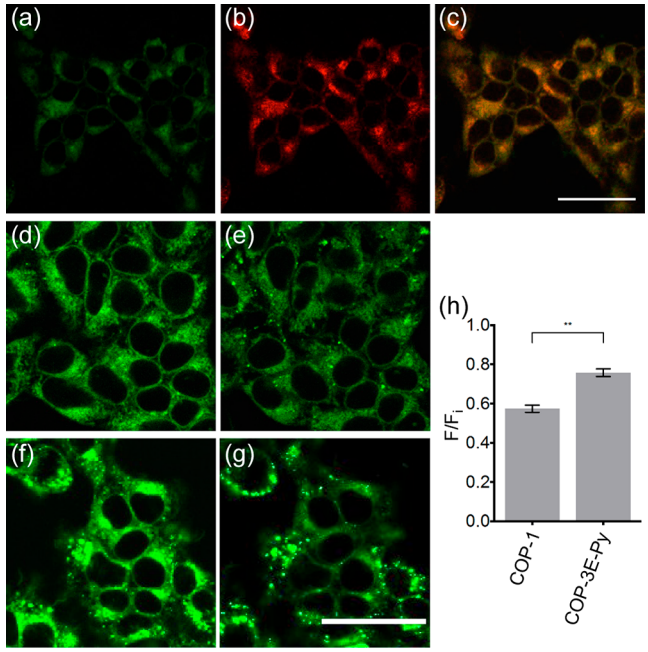

Figure 6. Live-cell imaging comparison between COP-3E-Py and COP-1 reagents showing superior retention of the COP-3E-Py dye after sample washing. Panels (a-c) show subcellular localization of COP-3E-Py. HEK293T cells were stained with COP-3E-Py (a, 1 μM) and ER-Tracker Red E34250 (b, 1 μM). (d−g) Confocal microscopy images of HEK293T cells with COP-3E-Py (d,e) or COP-1 (f,g) were taken before and after a buffer exchange was conducted. The images after buffer-exchange were recorded after an equilibration period of 10 min. (h) Mean fluorescence intensities of images acquired 10 min after buffer exchange as fractions of the initial fluorescence before buffer exchange. Error bars denote SEM (n = 3). $^{**}P < 0.002$. Scale bars represent 40 μm (for a−g).

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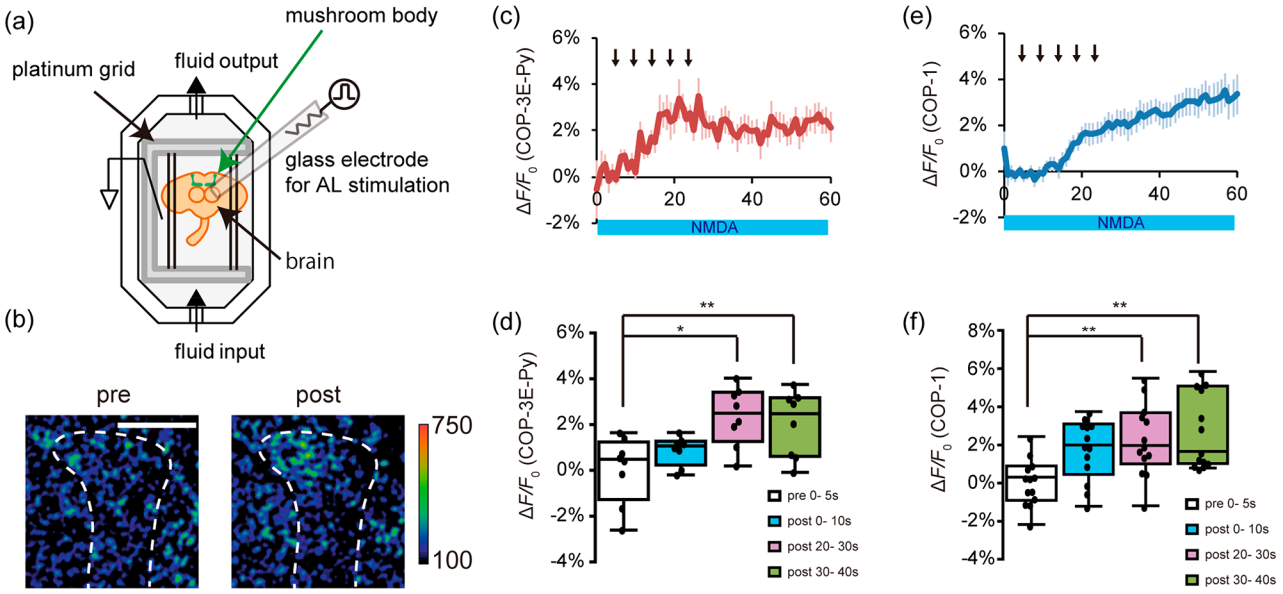

Figure 7. Endogenous CO release monitored with COP-3E-Py. (a) Schematic of the fly brain experiment. (b) Representative images of COP-3E-Py fluorescence observed at the $\alpha 3/\alpha' 3$ compartments of the mushroom body vertical lobes of the fly brain preparation. Scale bar represents 25 μm. (c–f) Trace and quantification of turn-on response after stimulation of the antennal lobe and NMDA stimulation using COP-3E-Py or COP-1 respectively. $^*p < 0.05$; $^{**}p < 0.01$; Bonferroni *post hoc* test.

monitor changes in CO fluxes associated with the ER is desirable owing to the ER-membrane localization of heme oxygenase-1 (HO-1) which a major endogenous source for production of CO.[82] As such, the increased hydrophilicity of COP-3E-Py relative to COP-1 affords improved detection of CO through broader distribution of the probe throughout the cell.

Next, we conducted comparative cellular retention assays with COP-1 and COP-3E-Py (Figure 6). Probes were loaded into cells and imaged, and then washed by a buffer exchange and imaged again. Under these conditions, the fraction of final fluorescence intensity relative to initial fluorescence intensity of COP-1 was 57%, with COP-3E-Py offering an improvement to 76% retention of fluorescence signal after washing (Figure 6). The observed increase in cellular retention for COP-3E-Py expands opportunities for using this reagent for CO detection in biological settings that benefit from protocols with washing steps. In order to assess the most useful time window for COP-3E-Py probe applications in biological samples, we conducted a cell viability experiment (Figure S3). After 3 h, a decrease in viability was observed, suggesting that this probe is most useful to monitor CO release on shorter time scales. These data establish that systematic modification of the activity-based sensing palladacycle trigger for selective tuning of CO reactivity as well as increasing hydrophilicity of the BODIPY core for improved cellular localization and retention leads to COP-3E-Py as an advanced reagent for biological CO detection with enhanced properties.

COP-3E-Py Enables Detection of Endogenous CO Release in Fly Brain Models. To showcase the utility of palladacycle COP-3E-Py for biological CO imaging applications, we sought to apply this reagent to observe endogenous CO signaling events. To this end, we utilized COP-3E-Py to monitor the postsynaptic release of CO in a fly brain model, as a novel role for CO as a retrograde messenger in noncanonical dopamine release was recently established.[83] Thus, dopamine release from dopaminergic neuron terminals on the mushroom body is induced by CO generated in the mushroom body itself, in which simultaneous activation of acetylcholine and glutamate receptors has taken place. Fly brains were isolated and the mushroom body vertical lobes were imaged with COP-3E-Py, with a washing step after probe incubation (Figure 7). Two different types of stimulation were employed to evoke dopamine release and neurotransmission in this biological model: (1) electrical stimulation of the antennal lobe (acetylcholine input) and (2) bath application with NMDA (*N*-methyl-D-aspartate; glutamate input). A clear increase in COP-3E-Py fluorescence was observed, confirming that this probe could monitor endogenous CO release mediated in this fly brain region.

CONCLUDING REMARKS

In summary, we have presented a ligand-directed approach to activity-based sensing of CO using organometallic reactivity. In-depth evaluation of the first-generation palladacycle probe COP-1 and systematic modulation of the ligand environment on palladacycle reactivity under physiological conditions laid a foundation for the development of next-generation molecular imaging probes for biological CO. The data revealed that sp^3-*N*-palladacycles are superior to other common classes of palladacycles investigated. Remarkably, sp^3-*N*-palladacycles undergo facile CO insertion and are also sufficiently stable toward protodemetalation under physiological conditions as shown by comparative reactivity studies with the competing gasotransmitter H_2S. Further functionalization of the core BODIPY fluorophore with hydrophilic ester substituents gave rise to the development of COP-3E-Py, an optimized scaffold with improved properties for CO detection, including more diffuse cellular staining and enhanced cellular retention. This advanced CO probe enabled detection of biological CO in both cell and fly brain models, as shown by monitoring CORM-3-mediated CO release in live HEK293T cells and visualization of endogenous CO release in fly brain preparations upon electrical and NMDA stimulation. In addition to providing a unique chemical tool for deciphering biological and biomedical contributions of CO, this study provides a starting point for

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the broader use of organometallic principles in the design of activity-based sensing probes for chemical biology applications.

EXPERIMENTAL SECTION

General Methods. Reactions utilizing air- or moisture-sensitive reagents were performed in oven- or flame-dried glassware under an atmosphere of dry N_2. Reagents from commercial sources were used as received without further purification. Reagents were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of reagent grade. COP-1,[52] COP-1′,[52] and CORM-3[84] were synthesized according to literature procedures. Silica gel P60 (SiliCycle) was used for all column chromatography purifications and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for thin layer chromatography. ^{1}H NMR and ^{13}C NMR spectra were acquired at 25 °C with Bruker AVB-400, AVQ-400, or AV-300 at the College of Chemistry NMR facility at UC Berkeley (UCB). Signals were internally calibrated to solvent peaks. High resolution mass spectrometry (ESI-MS) data were provided by the mass spectrometry department of UCB.

Synthesis of COP-1-Py. A round-bottom flask was charged with COP-1 (78.3 mg, 0.15 mmol, 1 equiv). CH_2Cl_2 (15 mL) and pyridine (14.5 μL, 0.18 mmol, 1.2 equiv) were added and the solution was stirred for 4 h. Hexanes were added, and the precipitate was collected with a Büchner funnel to yield COP-1-Py (80.9 mg, 0.15 mmol, 98%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (d, J = 5.4 Hz, 2H), 7.78 (t, J = 7.9 Hz, 1H), 7.36−7.29 (m, 2H), 7.09 (d, J = 7.5 Hz, 1H), 6.88 (d, J = 7.3 Hz, 1H), 5.93 (s, 2H), 5.92 (s, 1H), 4.06 (s, 2H), 2.94 (s, 6H), 2.50 (s, 6H), 1.42 (s, 6H).

Synthesis of 1. 4-(Chloromethyl)benzoyl chloride (5.00 g, 26.5 mmol, 1.0 equiv) was dissolved in dry CH_2Cl_2 (350 mL) and the solution was sparged with N_2 for 40 min. 2,4-Dimethylpyrrole (6.80 mL, 66.1 mmol, 2.5 equiv) was added, and the mixture was stirred for 16 h at rt. Dry diisopropylethylamine (23.0 mL, 132 mmol, 5.0 equiv) was added within 1 min, and stirring was continued at room temperature for 15 min. $BF_3{\cdot}OEt_2$ was added dropwise to the flask, and the mixture was allowed to stir at room temperature for 15 min. The flask was then equipped with a water-cooled condenser and heated to 50 °C for 50 min. The mixture was then allowed to cool to room temperature and concentrated *in vacuo*. The resulting residue was dissolved in CH_2Cl_2, washed with water, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification with flash chromatography on silica gel eluting with CH_2Cl_2:hexanes (50:50 → 60:40 → 75:25) to provide **1** (2.74 g, 7.35 mmol, 28%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum showed accordance with the literature.[52] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (d, J = 8.1 Hz, 2H), 7.26 (d, J = 8.1 Hz, 1H), 5.99 (s, 2H), 4.66 (s, 2H), 2.56 (s, 6H), 1.38 (s, 6H).

Synthesis of 2. **1** (558 mg, 1.50 mmol, 1.0 equiv) and NaSMe (210 mg, 3.00 mmol, 2.0 equiv) were dissolved in EtOH under an N_2 atmosphere. The mixture was stirred for 25 h at rt. It was diluted with CH_2Cl_2, washed with H_2O, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification with flash column chromatography with CH_2Cl_2:hexanes (60:40 → 100:0) yielded **2** (259 mg, 0.68 mmol, 45%) as a red solid. NMR: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.42 (d, J = 7.5 Hz, 2H), 7.22 (d, J = 7.6 Hz, 2H), 5.97 (s, 2H), 3.74 (s, 2H), 2.54 (s, 6H), 1.96 (s, 3H), 1.38 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 155.54, 143.10, 141.59, 139.30, 133.73, 131.54, 129.81, 128.18, 121.32, 38.01, 14.47. HRMS(ESI) calcd for $C_{21}H_{23}BF_2N_2S$ [M]: 384.1638; found: 384.1633.

Synthesis of COP-2-Py. A round-bottom flask equipped with a water condenser was charged with **2** (50.0 mg, 0.130 mmol, 1.05 equiv) and $Pd(OAc)_2$ (27.8 mg, 0.124 mmol, 1 equiv) under a nitrogen atmosphere. AcOH (2 mL) was added. The mixture was stirred in an oil bath at 120 °C for 55 min and then allowed to cool to room temperature. CH_2Cl_2 (10 mL) was added, and the solution was washed with H_2O (15 mL × 2). The organic layer was collected and concentrated *in vacuo*. The resulting crude solid was dissolved in a saturated solution of LiCl in acetone (3 mL) and was allowed to stir for 30 min at room temperature. Acetone was removed *in vacuo*, and the residue was dissolved in CH_2Cl_2. The mixture was passed through a plug of Celite and concentrated, yielding an orange solid. The solid was redissolved in minimal CH_2Cl_2, and hexanes were added. The resulting precipitate was collected by vacuum filtration to provide COP-2 (39 mg, 0.037 mmol). This material was suspended in minimal CH_2Cl_2. Pyridine (2.1 equiv relative to Pd) was added and the reaction mixture was allowed to stir for 5 min. CH_2Cl_2 was removed *in vacuo*, and the excess pyridine was removed by azeotroping with CH_2Cl_2 (×5). The resulting orange solid was collected in minimal CH_2Cl_2 and hexanes was added. The resulting precipitate was collected by vacuum filtration, to yield COP-2-Py (15 mg, 22%) as an orange solid. ^{1}H NMR (400 MHz, chloroform-*d*) 8.77 (d, J = 5.8 Hz, 2H), 7.81 (m, 1H), 7.37 (m, 2H), 7.18 (d, J = 7.8 Hz, 1H), 6.87 (d, J = 7.7 Hz, 1H), 6.14 (s, 1H), 5.93 (s, 2H), 4.38 (d, J = 14.2 Hz, 1H), 4.12 (d, J = 14.1 Hz, 1H), 2.74 (s, 3H), 2.49 (s, 6H), 1.41 (s, 3H), 1.38 (s, 3H). δ 155.3, 155.0, 153.4, 153.0, 152.4, 148.7, 143.2, 142.5, 142.2, 138.9, 133.5, 132.7, 131.4, 131.3, 125.6, 125.0, 124.3, 123.8, 121.1, 120.9, 48.6, 34.7, 25.3, 23.0, 20.7, 14.5. HRMS(ESI) calcd for $C_{25}H_{28}BF_2N_4PdS$ [M − Cl − Py + 2MeCN]$^+$: 571.1131; found: 571.1124.

Synthesis of 3. **1** (0.222 g, 0.596 mmol, 1.0 equiv), KI (0.198 g, 1.19 mmol, 2.0 equiv), and K_2CO_3 (0.165 mg, 1.19 mmol, 2.0 equiv) were suspended in CH_3CN (1.8 mL), and morpholine (260 μL, 2.98 mmol, 5.0 equiv) was added. The reaction mixture was heated via microwave irradiation to 80 °C for 5 min, then to 100 °C for 60 min and cooled to room temperature. The mixture was diluted with CH_2Cl_2 (60 mL), washed with water and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification with flash chromatography on silica gel eluting with a 4:1 mixture of hexanes/CH_2Cl_2 and running a shallow gradient from 0 to 5% EtOAc in this mixture yielded **3** (0.325 g, 0.768 mmol, 92%) as an orange solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.45 (d, J = 7.7 Hz, 2H), 7.22 (d, J = 7.8 Hz, 2H), 5.96 (s, 2H), 3.73 (t, J = 4.6 Hz, 4H), 3.59 (s, 2H), 2.54 (s, 6H), 2.46 (t, J = 4.5 Hz, 4H), 1.36 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.3, 142.9, 141.6, 138.6, 133.8, 131.4, 129.8, 127.9, 121.1, 121.1, 66.9, 62.9, 53.5, 14.5, 14.3. HRMS(ESI) calcd for $C_{24}H_{29}BF_2N_3O^+$ [M + H]$^+$: 424.2366; found: 424.2365.

Synthesis of COP-3-Py. Amine **3** (0.212 g, 0.501 mmol, 1.05 equiv), $Pd(OAc)_2$ (0.107 g, 0.477 mmol, 1.00 equiv), and benzene (5 mL) were added to a 20 mL vial under an atmosphere of N_2. The mixture was heated to 50 °C in an oil bath under stirring for 16 h. The solution was cooled to room temperature, concentrated *in vacuo*. The mixture was then dissolved in CH_2Cl_2 and filtered over a fritted funnel and concentrated *in vacuo*. A volume of 5 mL of a saturated LiCl solution in acetone was added, and the reaction mixture was stirred for 16 h. The reaction mixture was filtered over a fritted funnel with CH_2Cl_2 and methanol, concentrated *in vacuo*, and dissolved in CH_2Cl_2 (5 mL). Pyridine (81.0 μL, 1.00 mmol, 2.00 equiv) was added, and the reaction mixture was stirred 16 h at room temperature. The reaction mixture was concentrated *in vacuo*, suspended in CH_2Cl_2, and filtered over a Büchner funnel. Addition of hexanes led to the precipitation of a solid which was collected. This solid was again precipitated with CH_2Cl_2 and hexanes. The generated solids were again collected. The solids were dissolved in CH_2Cl_2 and filtered over a Büchner funnel. The mother liquor was concentrated *in vacuo* to provide COP3-Py as an orange solid (0.043 g, 0.063 mmol, 13%).[52] ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.90−8.79 (m, 2H), 8.78−8.70 (m, 2H), 7.77 (dddt, J = 7.5, 5.7, 3.7, 1.8 Hz, 2H), 7.33 (tdd, J = 10.5, 5.7, 1.4 Hz, 4H), 7.13 (d, J = 7.6 Hz, 1H), 6.89 (dd, J = 7.5, 1.6 Hz, 1H), 5.92 (s, 2H), 4.43 (s, 2H), 4.10 (ddt, J = 9.8, 7.3, 3.2 Hz, 4H), 3.99−3.85 (m, 2H), 2.84 (dt, J = 13.6, 4.3 Hz, 2H), 2.49 (s, 6H), 1.39 (s, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 155.1, 153.3, 153.1, 150.1, 146.5, 142.7, 142.4, 138.6, 138.5, 132.1, 131.3, 130.7, 125.4, 124.9, 124.2, 122.5, 120.9, 68.1, 62.4, 58.8, 14.5, 14.4, 14.3. HRMS(ESI) calcd for $C_{34}H_{38}BF_2N_5OPd^+$ [M + H]$^+$: 687.2167; found: 687.2167.

Synthesis of 4. A round-bottom flask was charged with terephthalaldehyde (25.5 g, 190 mmol, 1.00 equiv), ethylene glycol (11.2 mL, 200 mmol, 1.05 equiv), toluene (300 mL), and *p*-toluenesulfonic acid (1.81 g, 9.5 mmol, 0.05 equiv). A Dean−Stark trap was attached, and the reaction mixture was refluxed at 130 °C on an oil bath for 4 h. The oil bath was removed and the reaction mixture was concentrated under reduced pressure. The crude product was purified via flash chromatography on silica gel eluting with 20% CH_2Cl_2, 80%

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hexanes → 20% CH_2Cl_2, 12% ethyl acetate, 68% hexanes → 20% CH_2Cl_2, 16% ethyl acetate, 64% hexanes. The product mixture was refined with a second round of flash chromatography on silica gel eluting with 100% CH_2Cl_2 providing the desired product (8.20 g, 46.0 mmol, 24%) as a colorless liquid. Not all product containing fractions were further purified from the unprotected and diprotected mixture. An acquired ^{1}H NMR (600 MHz, $CDCl_3$) spectrum showed accordance to the literature.[85] ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.04 (s, 1H), 7.91 (d, J = 8.2 Hz, 2H), 7.66 (d, J = 8.1 Hz, 2H), 5.88 (s, 1H), 4.24−3.95 (m, 4H).

Synthesis of 5. A two-necked round-bottom flask was charged with dry CH_2Cl_2 (500 mL) which was sparged with N_2 for 15 min. Aldehyde **4** (2.07 g, 11.6 mmol, 1.0 equiv), 2,4-dimethylpyrrole (2.51 mL, 24.4 mmol, 2.1 equiv), and 1 drop of trifluoroacetic acid were added. The reaction mixture was stirred 16 h at room temperature. A slurry of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (2.63 g, 11.6 mmol, 1.0 equiv) in dry CH_2Cl_2 (150 mL) was added to the reaction mixture. Stirring was then continued for 60 min. The reaction mixture was concentrated *in vacuo* until approximately 100 mL of solvent was left. Dry toluene (300 mL) and dry diisopropylethylamine (10.1 mL, 58.0 mmol, 5.0 equiv) were added. $BF_3{\cdot}OEt_2$ (7.16 mL, 58.0 mmol, 5.0 equiv) was added, and the mixture was heated up to 50 °C in an oil bath for 45 min. The oil bath was removed, and the reaction mixture was concentrated *in vacuo*. The crude solid was dissolved in CH_2Cl_2, washed three times with H_2O, dried over Na_2SO_4, filtered over Celite, and concentrated *in vacuo*. Purification via flash chromatography on silica gel eluting with 60% CH_2Cl_2, 40% hexanes → 100% CH_2Cl_2 provided BODIPY **5** (1.39 g, 3.51 mmol, 30%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (d, J = 7.9 Hz, 2H), 7.31 (d, J = 7.8 Hz, 2H), 5.97 (s, 2H), 5.85 (s, 1H), 4.24−4.13 (m, 2H), 4.13−4.01 (m, 2H), 2.55 (s, 6H), 1.36 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 155.5, 143.1, 141.1, 138.8, 135.9, 131.3, 128.0, 127.4, 121.2,103.3, 65.4, 14.5, 14.5. HRMS(ESI) calcd for $C_{22}H_{23}BF_2N_2NaO^+$ $[M + Na]^+$ 419.1713; found: 419.1715.

Synthesis of 6. A 100 mL round-bottom flask equipped with a water-cooled condenser was charged with BODIPY **5** (0.793 g, 2.00 mmol, 1.0 equiv), acetone (20 mL), and a 10% HCl (aq) solution (1.0 mL). The reaction mixture was put under N_2 and refluxed on an oil bath for 2 h. The oil bath was removed, and the reaction mixture was concentrated *in vacuo*. The resulting residue was dissolved in CH_2Cl_2, and the organic phase was washed three times with H_2O, dried over Na_2SO_4, filtered over Celite, and concentrated *in vacuo*. Purification via flash chromatography on silica gel eluting with 60% CH_2Cl_2, 40% hexanes → 100% CH_2Cl_2 provided aldehyde **6** (0.554 g, 1.57 mmol, 78%) as an orange solid.[85] ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.10 (s, 1H), 8.02 (d, J = 7.8 Hz, 2H), 7.49 (d, J = 7.8 Hz, 2H), 5.99 (s, 2H), 2.55 (s, 6H), 1.34 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 191.5, 156.1, 142.7, 141.3, 139.6, 136.6, 130.7, 130.3, 129.0, 121.6, 14.6, 14.5. HRMS(ESI) calcd for $C_{20}H_{20}BF_2N_2O^+$ $[M + H^+]$ 353.1631; found: 353.1629.

Synthesis of 7. A round-bottom flask equipped with a water cooled condenser was charged with hydroxylamine hydrochloride (0.160 g, 2.30 mmol, 3.0 equiv), potassium acetate (0.340 g, 3.45 mmol, 4.5 equiv), ethanol (10 mL), H_2O (5 mL), and aldehyde **6** (0.280 g, 0.795 mmol, 1.0 equiv). The reaction mixture was put under N_2 and heated up to 70 °C in an oil bath for 3 h. The oil bath was removed and the reaction mixture was concentrated *in vacuo*. The resulting residue was dissolved in CH_2Cl_2 and concentrated *in vacuo*. CH_2Cl_2 was added and the organic phase was washed one time with H_2O, dried over Na_2SO_4, filtered over Celite, and concentrated *in vacuo*. Purification via flash chromatography on silica gel eluting with 100% CH_2Cl_2 → 5% ethyl acetate, 95% CH_2Cl_2 provided aldoxime **7** (0.266 g, 0.724 mmol, 91%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (s, 1H), 7.72 (d, J = 8.2 Hz, 2H), 7.32 (d, J = 8.1 Hz, 2H), 5.99 (s, 2H), 2.56 (s, 6H), 1.40 (s, 6H). ^{13}C NMR (101 MHz, acetone-d_6): δ 156.2, 148.8, 143.8, 142.4, 136.5, 135.0, 131.9, 129.3, 128.2, 122.1, 122.1, 14.7, 14.6, 14.6, 14.6. HRMS(ESI) calcd for $C_{20}H_{21}BF_2N_3O^+$ $[M + H]^+$ 368.1740; found: 368.1739.

Synthesis of COP-4-Py. A two-necked round-bottom flask equipped with a water-cooled condenser was charged with aldoxime **7** (0.119 g, 0.325 mmol, 1.0 equiv) and di-μ-chlorobis[2-[(dimethylamino)methyl]phenyl-C,N]dipalladium(II) (0.090 g, 0.163 mmol, 1.0 equiv). The mixture was dissolved in a 1:1 solution of $CHCl_3$/acetic acid (20 mL). The reaction mixture was heated to 50 °C in an oil bath for 16 h under stirring. The oil bath was removed, and the precipitates were collected on a Büchner funnel. The solids were transferred to a 20 mL vial and dissolved in 10 mL of CH_2Cl_2. Pyridine (52 μL, 0.65 mmol, 2.0 equiv) was added, the reaction mixture was stirred for 16 h at room temperature, filtered over Celite, and concentrated to 5 mL, and hexanes (5 mL) were added, which led to immediate precipitation. The precipitate was collected with a Büchner funnel to yield COP-4-Py (0.131 g, 0.223 mmol, 69%) as an orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.20 (s, 1H), 8.78−8.70 (m, 2H), 7.91 (s, 1H), 7.87 (tt, J = 7.7, 1.7, 1H), 7.49−7.41 (m, 2H), 7.32 (d, J = 7.6, 1H), 6.98 (dd, J = 7.6, 1.6, 1H), 6.15 (d, J = 1.5, 1H), 5.94 (s, 2H), 2.49 (s, 6H), 1.43 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 156.3, 155.4, 153.6, 152.5, 142.8, 142.4, 141.2, 139.0, 135.1, 130.9, 130.1, 126.3, 126.2, 124.7, 121.1, 14.5, 14.5. HRMS(ESI) calcd for $C_{25}H_{25}BClF_2N_4OPd^+$ $[M + H]^+$ 587.0807; found: 587.0807.

Synthesis of 8. 4-Bromobenzoyl chloride (6.14 g, 26.3 mmol, 1 equiv) was dissolved in dry CH_2Cl_2 (65 mL) in an N_2 atmosphere. 2,4-Dimethylpyrrole (5.04 g, 53.0 mmol, 2 equiv) was added, and the reaction mixture was stirred for 14 h at room temperature. Triethylamine (11.0 mL, 78.3 mmol, 3 equiv) was added dropwise and stirred for 15 min. $BF_3{\cdot}OEt_2$ (17.0 mL, 137 mmol, 5.3 equiv) was added dropwise, and the reaction mixture was stirred for 20 h. CH_2Cl_2 was added, and the organic layer was washed with water, dried over Na_2SO_4, and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel, eluting with 50% CH_2Cl_2 in hexanes. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (d, J = 7.9 Hz, 2H), 7.14 (d, J = 8.0 Hz, 2H), 5.99 (s, 2H), 2.54 (s, 6H), 1.40 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.91, 142.95, 140.07, 133.94, 132.49, 131.20, 129.86, 123.31, 121.53, 14.69. HRMS(ESI) calcd for $C_{19}H_{18}BBrF_2N_2$ [M]: 402.0709; found: 402.0707.

Synthesis of 9. Compound **8** (770 mg, 1.91 mmol, 1 equiv) was dissolved in toluene (25 mL), and 2-(tributylstannyl)pyridine (878 mg, 2.39 mmol, 1.25 equiv) was added under an N_2 atmosphere. $Pd(Ph_3P)_2Cl_2$ (67.0 mg, 0.10 mmol, 0.05 equiv) was added. The reaction mixture was heated to 110 °C for 48 h and cooled to rt, CH_2Cl_2 was added, and the mixture was filtered through Celite. It was concentrated and purified with flash column chromatography in CH_2Cl_2 → CH_2Cl_2 + 4% EtOAc to yield **9** as an orange solid (496 mg, 1.23 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (d, J = 4.6 Hz, 1H), 8.18 (d, J = 8.1 Hz, 2H), 7.83 (t, J = 9.1 Hz, 2H), 7.41 (d, J = 8.1 Hz, 2H), 7.30 (t, J = 5.3 Hz, 1H), 6.01 (s, 2H), 2.59 (s, 6H), 1.46 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.28, 155.59, 149.86, 143.20, 141.35, 139.98, 137.05, 135.67, 131.36, 128.55, 127.57, 122.72, 121.33, 120.65, 14.68. HRMS(ESI) calcd for $C_{24}H_{23}BF_2N_3^+$ $[M + H]^+$: 402.1948; found: 402.1941.

Synthesis of COP-5-Py. A round-bottom flask was charged with **9** (124 mg, 0.31 mmol, 1 equiv) and $Pd(OAc)_2$ (69.9 mg, 0.31 mmol, 1 equiv). The flask was flushed with nitrogen to create an N_2 atmosphere and protected from light. Benzene (20 mL) was added, and the reaction mixture was stirred for 14 h at 50 °C. CH_2Cl_2 was added, and the mixture was filtered over Celite and concentrated *in vacuo*. A volume of 20 mL of a saturated solution of LiCl in acetone was added and stirred at room temperature for 4 h. It was concentrated *in vacuo*, dissolved in CH_2Cl_2, and filtered over Celite to remove excess LiCl. It was dissolved in CH_2Cl_2(10 mL) and pyridine was added (25 uL, 1.2 equiv). COP-5-Py (142 mg, 0.23 mmol, 74%) was obtained by precipitation in hexanes as an orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.52 (d, J = 5.2 Hz, 1H), 8.85 (d, J = 5.0 Hz, 2H), 7.91−7.84 (m, 2H), 7.74 (d, J = 8.0 Hz, 1H), 7.60 (d, J = 7.8 Hz, 1H), 7.47−7.42 (m, 2H), 7.23 (t, J = 6.6 Hz, 1H), 7.02 (d, J = 8.9 Hz, 1H), 6.14 (s, 1H), 5.95 (s, 2H), 2.51 (s, 7H), 1.48 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.01, 155.48, 155.12, 153.48, 152.82, 152.55, 146.55, 143.08, 141.92, 139.20, 138.88, 138.75, 136.06, 131.45, 131.19, 126.18, 125.13, 124.56, 123.75, 122.78, 121.25, 118.88, 14.76. HRMS(ESI) calcd for $C_{31}H_{28}BF_2N_5Pd$ [M + MeCH/−Cl]: 625.1441; found: 625.1435.

Synthesis of 10. A round-bottom flask was charged with methyl 5-(benzyloxycarbonyl)-2,4-dimethylpyrrole-propionate (10.0 g, 31.8

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mmol, 1.0 equiv), palladium on carbon (0.20 g), and acetone (320 mL). A hydrogen atmosphere was established by flushing the flask with hydrogen gas and subsequent attachment of a hydrogen gas containing balloon. Stirring was continued for 12 h at room temperature. Palladium on carbon was filtered off over Celite. The reaction mixture was concentrated *in vacuo*, trifluoroacetic acid (32 mL) was added, and the reaction mixture was heated to 40 °C for 15 min. The oil bath was removed and $CHCl_3$ was added. The diluted reaction mixture was washed with H_2O and a saturated K_2CO_3 solution in H_2O. The aqueous phases were extracted with $CHCl_3$, and the pooled organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. Purification with flash chromatography on silica gel with CH_2Cl_2 gave **10** (2.8 g, 15.4 mmol, 48%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (s, 1H), 6.49 (s, 1H), 3.81 (s, 3H), 2.90 (t, 2H), 2.63 (t, 2H), 2.31 (s, 3H), 2.21 (s, 3H).

Synthesis of 11. A round-bottom flask was charged with 4-(chloromethyl)benzoyl chloride (1.46 g, 7.7 mmol, 1 equiv). **10** (2.8 g, 15.4 mmol, 2 equiv) was dissolved in dry CH_2Cl_2 (30 mL) and added under an N_2 atmosphere. The reaction mixture was stirred for 14 h at room temperature. DIPEA (6.7 mL, 38.5 mmol, 5 equiv) was added dropwise and stirred for 20 min. $BF_3{\cdot}OEt_2$ (4.8 mL, 38.5 mmol, 5 equiv) was added dropwise, and the reaction mixture was stirred for 3 h. CH_2Cl_2 was added, and the organic layer was washed with water, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by flash chromatography on silica gel, eluting with 20% EtOAc, 80% hexanes → 30% EtOAc, 70% hexanes. **11** (950 mg, 1.7 mmol, 22%) was obtained as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (d, 2H), 7.26 (d, 2H), 4.66 (s, 2H), 3.64 (s, 6H), 2.62 (t, 4H), 2.53 (s, 6H), 2.34 (t, 4H), 1.29 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.1, 154.4, 140.2, 139.4, 138.7, 135.6, 130.9, 129.4, 128.6, 51.8, 45.7, 34.3, 19.4, 12.7, 12.1. HRMS(ESI) calculated for $[C_{28}H_{32}BClF_2N_2NaO_4]^+$: 567.2004, found: 567.2005.

Synthesis of 12. A round-bottom flask was charged with **11** (950 mg, 1.7 mmol, 1 equiv), K_2CO_3 (470 mg, 3.4 mmol, 2 equiv) and KI (564 mg, 3.4 mmol, 2 equiv). CH_3CN (20 mL) and morpholine (741 μL, 8.5 mmol, 5 equiv) were added to the flask and the reaction mixture was stirred at 60 °C for 3 h. CH_2Cl_2 was added and the organic layer was extracted with water, dried over Na_2SO_4, and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel, eluting with a 100% hexanes →100% EtOAc gradient. **12** (968 mg, 1.6 mmol, 94%) was obtained as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45 (d, 2H), 7.19 (d, 2H), 3.72 (t, 4H), 3.61 (s, 6H), 3.60 (s, 2H), 2.60 (t, 4H), 2.51 (s, 6H), 2.46 (s, 4H), 2.32 (t, 4H), 1.27 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.07, 154.05, 140.80, 139.36, 134.45, 130.93, 130.04, 129.18, 128.40, 128.13, 66.86, 63.01, 53.49, 51.70, 34.22, 29.72, 19.34, 12.62, 11.90. HRMS(ESI) calculated for $[C_{32}H_{41}BF_2N_3O_5]^+$: 596.3102, found: 596.3104.

Synthesis of COP-3E-Py. A round-bottom flask was charged with **12** (968 mg, 1.6 mmol, 1 equiv) and $Pd(OAc)_2$ (347 mg, 1.5 mmol, 0.95 equiv). The flask was flushed with nitrogen to create an N_2 atmosphere and protected from light. Benzene (40 mL) was added, and the reaction mixture was stirred for 14 h at 50 °C. CH_2Cl_2 was added, and it was filtered over Celite and concentrated in vacuo. A volume of 60 mL of a saturated solution of LiCl in acetone was added and stirred at room temperature for 4 h. It was concentrated *in vacuo*, dissolved in CH_2Cl_2, and filtered over Celite to remove excess LiCl. Hexanes were added, and a precipitate was collected with a Büchner funnel. The precipitate was dissolved in CH_2Cl_2. Pyridine (13 μL, 0.15 mmol, 2.2 equiv) was added, and the solution was stirred for 4 h. Hexanes were added, and the precipitate was collected with a Büchner funnel to yield COP-3E-Py (90 mg, 0.11 mmol, 70%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.81 (d, 1H), 8.72 (d, 1H), 7.75 (m, 1H), 7.31 (m, 2H), 7.11 (d, 2H), 6.86 (d, 2H), 5.84 (s, 1H), 4.43 (s, 2H) 4.08 (t, 4H), 3.91 (m, 2H) 3.63 (s, 6H), 2.84 (m, 2H), 2.59 (t, 4H), 2.46 (s, 6H), 2.31 (t, 4H), 1.30 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.0, 153.7, 153.4, 153.3, 150.4, 146.6, 141.7, 139.2, 138.7, 138.6, 132.6, 131.0, 130.9, 129.0, 125.5, 125.1, 124.4, 122.7, 68.3, 62.6, 59.0, 51.8, 34.3, 19.4, 12.6, 12.1, 11.9. HRMS(ESI) calculated for $[C_{36}H_{45}BF_2N_5O_5Pd]$: 782.2517, found: 782.2513.

Synthesis of COP-3E-Py′. A round-bottom flask was charged with COP-3E-Py (60 mg, 0.073 mmol, 1 equiv). CH_2Cl_2 (20 mL) and water (1 mL) were added and the flask was flushed with CO. A CO-filled balloon was attached and the reaction mixture was stirred for 14 h at 31 °C while maintaining an atmosphere of CO. The reaction mixture was cooled to room temperature, transferred to a separatory funnel, and diluted with DCM (50 mL). The organic layer was washed with water, dried over sodium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography by elution with a EtOAc → 40% MeOH, 60% EtOAc gradient. COP-3E-Py′ (43 mg, 6.7 mmol, 92%) was isolated as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, 2H), 7.37 (d, 2H), 3.97 (s, 2H), 3.84 (s, 4H), 3.64 (s, 6H), 2.79 (s, 4H), 2.60 (t, 4H), 2.53 (s, 6H), 2.34 (t, 4H), 1.26 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 176.3, 173.2, 154.9, 138.9, 138.6, 137.1, 133.7, 132.4, 130.7, 129.7, 65.5, 61.9, 51.9, 51.6, 34.3, 29.8, 20.9, 19.4, 12.8, 12.4. HRMS(ESI) calculated for $[C_{33}H_{41}BF_2N_3O_7]^+$: 640.3000, found: 640.2995.

X-ray Crystallography. Single crystals were coated with Paratone-N hydrocarbon oil and mounted on Kaptan loops. Temperature was maintained at 100 K with an Oxford Cryostream 700 instrument during data collection at the University of California, Berkeley, College of Chemistry, X-ray Crystallography Facility. Samples were irradiated with Mo Kα radiation with λ = 0.71073 Å using a Bruker APEX II QUAZAR diffractometer equipped with a Microfocus Sealed Source (Incoatec IμS) and APEX-II detector. The Bruker APEX2 ver. 2009.1 software package was used to integrate raw data which were corrected for Lorentz and polarization effects.[86,87] A semiempirical absorption correction (SADABS) was applied.[44] Space groups were identified based on systematic absences, E-statistics, and successive refinement of the structures. The structures were solved using direct methods or the Patterson method and refined by least-squares refinement on F^2 and standard difference Fourier techniques using SHELXL.[88−90] Thermal parameters for all non-hydrogen atoms were refined anisotropically, and hydrogen atoms were included at ideal positions and refined isotropically. The ammonium hydrogen in structure COP-1′ was located in the electron density map.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS 220B 75 W xenon lamp and power supply, A 1010B lamp housing with an integrated igniter, switchable 814 photon counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for emission measurements were contained in a quartz cuvette with a path length of 1 cm and 1.5 mL cell volume (Starna, Atascadero, CA). CO-probes were dissolved in DMSO (2 mM) and diluted to a final volume of 1 μM in PBS or HEPES (Ca, Mg) buffer. The cuvette was placed in a water bath at 37 °C, and after temperature equilibration a t = 0 a spectrum was acquired. Subsequently 5 μL of a 10 mM solution of CORM 3 (final concentration: 50 μM) in DMSO was added and the cuvette was again placed in a water bath. Emission spectra were recorded by quickly removing the cuvette from the water bath, recording the spectrum and returning the cuvette to the bath. Spectra were taken at t = 0, 5, 15, 30, 45, and 60 min. COP-3E-Py was excited at 515 nm and emission spectra were recorded from 520 to 620 nm in 1 nm intervals with an integration of 0.1. For selectivity assay the CO-probes were treated with different compounds analogously to the treatment with CORM-3: At 37 °C the tested species were added to a final concentration of 50 μM to a 1 μM aqueous COP solution (PBS (Ca, Mg) buffer). Emission spectra were recorded by quickly removing the cuvette from the water bath, recording the spectrum, and returning the cuvette to the bath. Spectra were taken at t = 0, 5, 15, 30, 45, and 60 min. For the selectivity assay, 10 mM solutions of H_2O_2, TBHP, NaOCl, KO_2, $ONOO^-$, and H_2S were prepared and 5 μL was added to 1 mL of a 1 μM solution of COP-3E-Py in PBS.

Cell Culture and Confocal Microscopy in Live Cells. Cells were grown in the UC Berkeley Tissue Culturing Facility. HEK293T cells were cultured in Dubelcco's Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and incubated at 37 °C in 5% CO_2. One day before imaging, cells were passaged and plated in phenol red-free medium on 8 well chamber

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slides (Corning, Corning, NY) and grown to 70−80% (HEK293T) confluency. 100 μL of 3 μM respective CO-Probe in DPBS was prepared from a 0.4 mM COP in DMSO stock solution and added to chambers filled with 200 μL of medium. For CO-response studies in HEK293T cells, 100 μL medium was removed after 60 min of COP incubation and 1.5 μL of 10 mM CORM-3 in DMSO was added (1.5 μL of DMSO for the controls), mixed and readded to the respective chamber. Imaging was performed approximately 120 min after COP addition. Confocal fluorescence microscopy was performed with a Zeiss laser scanning microscope 710 equipped with a 40× water-immersion and 63× oil-immersion objective lens, with Zen 2009 software (Carl Zeiss). COP-1, and COP-1-Py to COP-5-Py were excited at 488 nm, and COP-3E-Py was excited at 488 or 514 nm. The emission was collected with a META detector. Hoechst 33342 and ER-Tracker Red E34250 were used at 1 μM concentration and excited at 405 nm. Image analysis was performed using FIJI (National Institute of Health). Colocalization coefficients where calculated with the plugin JACoP.

Detection of CO in Fly Brain Models. Brains were prepared for imaging as previously described.[91] Briefly, brains were dissected in ice cold 0 mM Ca^{2+} HL3 medium (in mM as follows: 70 NaCl, 115 sucrose, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 5 HEPES, pH 7.3, 359 mOsm) and placed in a recording chamber filled with normal, room temperature HL3 medium (the same recipe as above, containing 1.8 mM $CaCl_2$). COP-3E-Py was added to the buffer from a DMSO stock solution and used at 3 μM final concentration. The antennal lobe (AL) was stimulated (30 pulses, 100 Hz, 1.0 ms pulse duration) using glass microelectrodes. For NMDA stimulation, 200 μM NMDA, diluted in HL3 containing 4 mM Mg^{2+}, was bath-applied to the recording chamber. Fluorescent imaging was done with a confocal microscope (A1R, Nikon) with a 20×x water-immersion lense (numerical aperture 0.5; Nikon). F_0 was obtained by averaging the five sequential frames before stimulus onset.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c06405.

Experimental details, including characterization of compounds (PDF)

Crystallographic data for COP-1 (CIF)

Crystallographic data for COP-1-PY (CIF)

Crystallographic data for COP-1′ (CIF)

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Notes

The authors declare no competing financial interest.

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