FLUORESCENCE AS A TOOL FOR METHOD DEVELOPMENT, DETECTION OF TRACE ANALYTES, AND IMAGING

by

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Since the first report in 1845, fluorescence has evolved into a ubiquitous and useful phenomenon. Fluorescence has found utility in the areas of analytical, organic, biological, and physical chemistry, with uses ranging from detection of trace analytes, use in imaging biological processes and conformations, as well as use as a model substrate to study reactivity and various transformations. Herein, the uses of fluorescence will be discussed with respect to these examples. Development of a new paradigm for catalytic assays will be presented using fluorogenic and colorimetric substrates. This approach was employed in the detection of trace palladium in active pharmaceutical ingredients as well as in polymers prepared by palladium catalysis and ores collected from a mining site. Additionally, the kinetics of an aqueous Tsuji-Trost reaction were studied using a fluorogenic substrate, enabling collection of a high density of information in a high-throughput manner. A change in the turnover-limiting step was uncovered, dependent on substrate concentration. The development of the first-in-kind blinking fluorophore for attachment to bioactive molecules by click chemistry as well as studies of Pittsburgh Green as a blinking fluorophore for super-resolution imaging are presented herein. These three projects demonstrate the utility of fluorescence as a tool for varied studies across multiple fields.

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PREFACE

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Abbreviations:

 $\dot{A} = angstrom(s)$ Ac = acetyl aq = aqueous Ar = aryl br = broad (spectral) t-Bu = tert-butyl

B3LYP = 3-parameter hybrid Becke exchange/Lee-Yang-Parr correlation functional

°C = degrees Celsius

- calcd = calculated
- cat = catalytic
- cm = centimeter(s)
- $cm^{-1} = wavenumber(s)$
- δ = chemical shift in parts per million downfield from tetramethylsilane
- d = days; doublet (spectral)
- DCC = N, N'-dicyclohexylcarbodiimide
- DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone
- DFT = density functional theory
- DIBALH = diisobutylaluminum hydride
- DMF = dimethylformamide
- DMSO = dimethylsulfoxide
- eq = equation
- equiv = equivalent
- ESI = electrospray ionization
- Et = ethyl
- g = gram
- h = hours
- HRMS = high-resolution mass spectrometry
- HRP = horseradish peroxidase
- HOSu = *N*-hydroxysuccinimide

Hz = hertz

IgG = immunoglobulin G

IR = infrared

J = coupling constant (in NMR spectroscopy)

K = kelvin(s)

L = liter(s)

 $\mu = micro$

m = multiplet

- M = molar (mols per liter)
- M+ = parent molecular ion

Me = methyl

MHz = megahertz

min = minutes

- mm = millimeter(s)
- mM = millimolar (millimols per liter)

mol = mol(s)

mp = melting point

- MS = mass spectrometry
- MW = molecular weight
- m/z = mass to charge ratio
- N = normal (equivalents per liter)

nm = nanometer(s)

NMR = nuclear magnetic resonance

Nu = nucleophile

PBS = phosphate buffered saline

Ph = phenyl

- PMB = p-methoxybenzyl
- ppb = parts per billion
- ppm = parts per million
- Pr = propyl

*i*Pr = isopropyl

q = quartet

 R_f = retention factor (in chromatography)

rt = room temperature

s = singlet (spectral); second(s)

t = triplet (spectral)

t = time

T = temperature

TBTA = tris(benzyltriazolylmethyl)amine

TFE = 1, 1, 1-trifluroethanol

- THF = tetrahydrofuran
- TFP = tri(2-furyl)phosphine
- TLC = thin layer chromatography
- t_R = retention time (chromatography)

UV = ultraviolet

vis = visible

v/v = volume per unit volume (volume-to-volume ratio)

wt = weight

w/w = weight per unit weight (weight-to-weight ratio)

1.0 INTRODUCTION

Fluorescence is a phenomenon where a photon of light is absorbed by a light sensitive molecule, known as a fluorophore, which enters excited state followed by return to the ground state by emission of a lower energy photon. It was first reported in 1845 by Sir John Frederick William Herchel, upon observing excitation of a quinine solution by sunlight.¹ In the subsequent nearly 170 years that followed, fluorescence has become widely studied as a tool in chemistry, finding utility in detection of trace analytes through chemosensor and chemodosimeter development,²⁻⁶¹ kinetic studies of various reactions and processes,⁶²⁻⁷¹ as well as imaging, even going beyond the traditional limit of microscopes.⁷²⁻⁹⁶ As fluorescence is central to my graduate work and the projects presented herein, I will first discuss the basics of fluorescence and related instrumentation. The subsequent chapters will discuss the development of fluorogenic chemosensor systems, study of kinetics by a fluorogenic reaction, and manipulating the intrinsic properties of fluorophores discussed in this chapter.

1.1 INTRODUCTION TO FLUORESCENCE

Fluorescent molecules are aromatic molecules containing multiple conjugated π bonds and are able to convert the absorbed wavelength of light and release it in the form of light. This phenomenon is visualized through a Jabłoński diagram, first reported in 1933 (Figure 1-1).⁹⁷ The

blue arrow represents absorption of a given wavelength, leading to promotion to the singlet S_1 state. Spectroscopically, the observed emission of a molecule is the mirror image of the S_0 to S_1 absorption.⁹⁸ A non-symmetrical absorption-emission spectrum is indicative of excitation to higher vibrational energy levels of S_1 prior to relaxation and emission. This is explained by Kasha's rule, which states that fluorescence emission comes from the lowest energy S_1 state.^{99,100} Thus, in the event of excitation to higher vibrational frequencies, the molecule quickly relaxes to the lowest S_1 energy level (orange arrow). Relaxation to the triplet state (red arrow), through either intersystem crossing or reduction can lead to either non-emissive relaxation (black arrow) or phosphorescent emission (purple arrow), with the latter being quenched rapidly in solution.⁹⁸ Relaxation to the non-emissive triplet state will be discussed further in Chapter 4.



Figure 1-1. Representative Jabłoński diagram.

Common examples of fluorophores and their spectroscopic properties are shown in Figure 1-2, including quinine from which Herschel first reported the phenomenon of fluorescence¹ and the commonly used xanthenones fluorescein and rhodamine. As shown, the emission and absorption spectra of the fluorophores differ from as much as 110 nm for POPOP

and as little as 18 nm for fluorescein. This change in absorption-emission wavelengths is referred to as a Stokes shift.⁹⁸ An ideal fluorophore would have a large Stokes shift, to prevent overlap between the absorption and emission spectra, minimizing undesired crosstalk. For biological uses, a fluorophore should have a red-shifted spectrum; the red-absorbing fluorophores will emit closer to the near-IR region of the light spectrum, which has the maximum depth penetration of tissue.¹⁰¹ Additionally, the red-shifted absorption/emission avoids the use of potentially damaging UV excitation.



Figure 1-2. Common fluorophores and their spectral properties in indicated solvents.⁹⁸

Fluorescence is quantified through the measurement of quantum yield (Q.Y; Φ). A number close to unity corresponds to a near ideal emitting fluorophore, with emitted photons approaching photons absorbed (Equation 1-1). Emission wavelength and quantum yield are independent of absorption wavelength.^{100,102}

$$\boldsymbol{\phi} = rac{photons\ emitted}{photons\ absorbed}$$

Equation 1-1. Formula to calculate quantum yield.

Quantum yield can be measured indirectly using a reference standard under identical conditions, including excitation, fluorometer slit length, photomultiplier voltage, etc. to prevent addition of extra variables. These conditions will be further elaborated in Section 1.2. Equation 1-2 demonstrates how to calculate quantum yield using a known reference fluorophore, with *Int* referring to the area under the emission peak, A is the optical density at the excitation wavelength, n is the refractive index, with the subscript R denoting reference values¹⁰³

$$\phi_{sample} = \phi_R * \frac{Int}{Int_R} * \frac{1-10^{-A_R}}{1-10^{-A}} * \frac{n^2}{n_R^2}$$

Equation 1-2. Calculating quantum yield of a fluorophore.

Structurally, fluorescent molecules must be able to absorb appropriate wavelengths of light in an appropriately sized conjugated system. Aromatic hydrocarbons contain conjugated π systems and Φ increases with increasing conjugation. For example, observed quantum yields of benzene, upon irradiation with UV light, is low ($\Phi = 5.3 \times 10^{-3}$) bordering on non-fluorescent, whereas anthracene and pyrene display $\Phi = 0.25$ and 0.69 respectively under similar irradiation conditions.¹⁰⁴ Additionally, restricted conjugated systems tend to be more fluorescent, as absorbed energy cannot be lost through bond rotation, as demonstrated in Chapter 2.

Solvent also plays a key part in fluorescence measurements. Solvent effects are able to further shift emission to lower energy regions, owing to stabilization of the S_1 state by polar solvent molecules. Less polar fluorophores are notably less susceptible to this phenomenon.⁹⁸ Absorption is not affected by solvent polarity, as absorption/excitation occurs on the order of 10^{-15} s, faster than fluorophore or solvent motion.⁹⁸ Following Kasha's rule, where emission occurs

from the lowest energy state, indicating that solvent effects must be considered, including formation of internal charge transfer states and solvent relaxation for emission (Figure 1-3).



Figure 1-3. Jabłoński diagram representing emission shifts to longer wavelengths due to solvent effects

The effect of solvent on fluorophores is illustrated by 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). In aqueous solution, TNS is relative non-fluorescent whereas in non-polar solvents, it displays notable fluorescence, shifting to longer emission wavelengths.¹⁰⁵ The shift in emission wavelength in this example is nearly 125 nm, indicating that solvent has a large effect on emission from a fluorophore. Additionally, changing pH can have an effect on Φ and emission, as will be explained later.

1.2 INSTRUMENTATION UTILIZED IN FLUORESCENCE MEASUREMENTS

Fluorometers are instruments used for measurement of absorption and fluorescence of a sample. As stray light can interfere with measurements, fluorometers are designed to minimize incident light, storing samples in a dark cell. Excitation of the sample by a light source, such as an arc lamp, is controlled through appropriate filters and monochromators, to ensure desired excitation by a specific wavelength. Beer's law, indicating dependence on both length of the cell and sample concentration, governs the absorbance of a sample as shown in Equation 1-3, where A is the absorbance, ε is the extinction coefficient of the molecule, l is the length of the cell, and c is concentration of the analyte.

$A = \varepsilon lc$

Equation 1-3. Beer's Law

Monochromators are useful to filter out stray and incident light beyond what is desired. Light enters into the monochromator through an entrance slit, aimed at a collimator to minimize scattering. The refracted light is passed through a diffraction grating to disperse the various wavelengths followed by further reflection toward an exit slit through which only the desired wavelength is allowed to interact with the sample. A Czerny-Turner monochromator is shown in Figure 1-4 with various parts labeled.⁹⁸



Figure 1-4. Schematic of a Czerny-Turner monochromator

Following excitation, the resultant emission signal is measured through the use of a photomultiplier tube (PMT). The PMT consists of a thin metal film, the photocathode, and a series of dynodes to amplify the resultant signal. The PMT, held at a high negative potential, up to -2000 mV, absorbs the incident emission photons, expelling 5-20 electrons. The electrons in turn are pulled toward the next dynode, where a subsequent collision takes place, ejecting more electrons in sequence. As each dynode is held at gradually decreasing potentials, electrons are pulled from dynode to dynode until finally reaching the anode and reporting the signal (Figure 1-5). A single photoelectron can be multiplied by a factor of 10⁶. The resulting current at the anode is decoded into a readout by a computer in the form of arbitrary fluorescence units. In the event of a concentrated sample, the absorbance will increase (following Beer's law) leading to an overabundance of resulting electrons moving toward the anode and resulting in a non-linear

response. The ideal fluorometer should always have a constant excitation from the light source, and both emit and detect photons without discrimination by wavelength equally.⁹⁸



Figure 1-5. Schematic representation of a photomultiplier tube

Though fluorometers boast high sensitivity, allowing for the tuning of excitation and emission wavelengths with remarkable accuracy, they suffer from analysis time and repeated calibration. At the expense of sensitivity, smaller plate reader fluorometers have been developed to expedite analysis. Utilizing wider emission measurements ranging entire color regions instead of a single wavelength, samples can be excited by a desired wavelength and the emission measured faster than with a fluorometer. These smaller, more portable-fluorometers utilize black plates instead of cuvettes for sample analysis, and can accommodate over 100 samples/plate, allowing for rapid analysis of samples in parallel. For example, 96 samples can be measured in approximately 3-5 minutes and with some plate readers, even faster.

1.3 FLUORESCENCE QUENCHING

Restriction of absorption and emission of fluorophores enables control over their optical properties. Through quenching of the excited state through depletion, non-emissive relaxation, or stabilization of the ground state, fluorophores can be turned "off", that is, they exist in a dark state. There are various mechanisms of quenching, including promiscuous quenching by collision and specific fluorophore-quencher pairs that will be discussed.

1.3.1 Collisional quenching

Collisional quenching involves collision with a fluorophore in the exited state and a quencher, enabling non-radiative relaxation from the triplet state (Figure 1-6). The most promiscuous quencher is oxygen.¹⁰⁶ Able to travel up to 44 Å in the typical fluorescence lifetime of 4 ns in aqueous solution, dissolved molecular oxygen can collide with excited state molecules, quenching fluorescence.⁹⁸ To combat off-target incidental quenching, solvents can be degassed to removed dissolved oxygen. Similar to oxygen, collision with a heteroatom may induce intersystem crossing (ISC) and subsequent relaxation from the singlet state to the triplet state, leading to a dark state, as illustrated in Figure 1-6. The red arrow represents collision with a quenching species, such as oxygen, a quencher, or a heteroatom. The black arrows represent non-emissive relaxation or further ISC to return to the ground state without emission.



Figure 1-6. Representative Jabłoński diagram for quenching by collisional quenching.

Beyond collision, heteroatoms may also quench by transferring an electron to an excited state fluorophore, leading to the formation of a charge-transfer complexes known as an "exciplex", This complex does not have the characteristic emission of the fluorophore and usually has a new absorbance band corresponding to the complex formation. For example, as reported by the Knibbe group, exposure of anthracene to diethylaniline in non-polar solvent leads to the disappearance of the fluorescence band at 397 nm and gives rise to a long wavelength fluorescence band (Figure 1-7).¹⁰⁷



Figure 1-7. Exciplex formation with anthracene and diethylaniline.

Increasing solvent polarity leads to a disappearance of the exciplex band with retained fluorescence quenching, indicating that other mechanisms of quenching are likely operative, including photoinduced electron transfer (PET), where an electron is directly transferred from

the excited state.¹⁰⁷ Though quenching is often in short space, these exciplex formations require remarkably short-range interactions (<2 Å), indicating molecular contact is a crucial requirement for this type of quenching. Whereas exciplex formation with heteroatoms is proposed to occur through transfer of an electron from the amine to the fluorophore, electron scavengers such as Cu^{2+} , Pb^{2+} , and Mn^{2+} may also strip an electron from the fluorophore, inhibiting emission.¹⁰⁸ This type of quenching can be controlled by the addition of additives, such as glutathione or dithiothreitol, enabling control over quenching.⁷⁸

1.3.2 Static Quenching

Fluorophores may be quenched by stabilizing the ground state, a phenomenon also known as static quenching. Common mechanisms of static quenching are π stacking with fluorophores,¹⁰⁹⁻¹¹² where the π bonds of aromatic rings form noncovalent, attractive interactions, and restriction of absorption. Static quenching is characterized by a shift in the absorbance spectrum, as the molecule is no longer able to enter the excited S₁ state. Fluorescein and 6-hydroxy-9-phenyl-fluoron (HPF) will serve as a model system to illustrate this phenomenon. Fluorescein, at lower pH displays significantly lower quantum yield ($\Phi = 0.25-0.35$) than at higher pH when the dianion is formed ($\Phi = 0.9-1.0$; Figure 1-8a). HPF displayed a similar trend ($\Phi = 0.9-1$ to 0.20-0.25; Figure 1-8b). In acidic environments, the phenolic group is protonated, restricting resonance through the xanthenone ring, (Figure 1-8). Furthermore, the absorption spectrum of fluorescein is varied in lower pH environments,¹¹³ indicating a static quenching through stabilization of the ground state.⁹⁸ The carboxylic acid moiety of fluorescein is able to shift into a lactone conformation, which is non-fluorescent. The lactone spirocycle of fluorescein prevents

absorption in the visible region; however other mechanisms must be present to explain the loss of fluorescence in HPF in similar pH environments.



Figure 1-8. Structural conformations of fluorescein and 6-hydroxy-9-phenyl-fluoron.

Previous studies to determine the nature of this quenching concluded that the quenching and loss of quantum yield is due internal conversion (S_1 to S_0) state instead of ISC (S_1 to T_1);^{114,115} however, this does not account for the shifted absorbance spectrum observed in fluorescein. In both cases, extremely low pH environments lead to the formation of the trityl cation, restoring fluorescence to the system, supporting the claim that conjugation and resonance (as seen in the dianion of fluorescein) are required for appropriate absorption and resulting fluorescence. Similar methods for stabilization of the ground state will be demonstrated in Chapter 2, pertaining to chemodosimeter development and restriction through alkylation instead of protonation.

1.3.3 Intermolecular quenching

Intermolecular quenching is the effect of structural motifs to quench fluorescence. For example, in 2004, the Koide group demonstrated the effects of conformational changes on fluorescence utilizing a fluorescein derivative. Amine groups were installed and were able to quench fluorescence by PET: quenching was dependent on conformation of the amines (Figure 1-9).¹¹⁶



Figure 1-9. Conformation based intramolecular quenching of a fluorescein derivative.

Equatorial orientation of the piperazine placed the lone pair electrons farther from the π system of the ring, rendering them unable to interact; a ring flip to the axial conformation positioned the electrons closer to the π system, enabling PET to quench fluorescence (Figure 1-9). Treatment of the system with β -cyclodextrin, which binds to the anisole groups, restored fluorescence up to 4.6-fold confirming the lone pair interaction as the mechanism for quenching.

Intramolecular attack is also a useful approach to quench fluorescence. Nucleophilic attack to form a spirocycle can separate the aromatic rings of a xanthenone system, effectively quenching fluorescence. Re-opening of the spirocycle restores fluorescence (Figure 1-10). Similar to the fluorescenin lactone, the interruption of the conjugation mediates the absorbance, not only preventing fluorescence, but shifting the absorbance spectra of the molecule.^{91,117} The Koide group reported a reversible spirocyclization of a Pittsburgh Green derivative with a hydroxymethyl group as the intermolecular nucleophile as observed by broadening of NMR signals.¹¹⁸ Further study with green fluorescent fluorophore Pittsburgh Green revealed a pH dependence similar to fluorescein. At higher pH, a non-fluorescent spirocycle is formed in equilibrium with the fluorescent open form; at lower pH, the protonation of the phenol prevents fluorescence (Figure 1-10a).^{36,117} Notably, the fluorescence is pH-independent at pH 5-10, indicating the utility of Pittsburgh Green for biological imaging.¹¹⁷

In 2014, the Urano group studied the intermolecular attack to reversibly form spirocycles in various derivatives to understand what contributes to opening and closing stochastically (Figure 1-10b). The electrophilicity of the spirocycle carbon was directly tied to the LUMO of the molecule. A more stabilized LUMO combined with an appropriate nucleophile will lead to more favorable spirocycle formation and by extension, more effective quenching. Various factors contributed to the spirocycle opening and closing, including bridging atoms of the xanthenone and the nucleophile.^{91,119,120} The further utility of structural motifs for fluorescence applications will be demonstrated in Chapter 4.



Figure 1-10. Quenching fluorescence through spirocycle formation. (a) Quenching of Pittsburgh Green based on pH. (b) Screening a library of fluorophores to study intramolecular attack.

1.3.4 Förster Resonance Energy Transfer

Förster (sometimes referred to as fluorescence) resonance energy transfer (FRET) is a unique mechanism of quenching for a single fluorophore, in which the energy of the excited state fluorophore (donor) is transferred to another moiety (acceptor) without the emission of a photon (Figure 1-11; the red arrow refers to either emission of the acceptor or non-radiative relaxation); transmission occurs through dipole-dipole interactions instead.^{121,122}


Figure 1-11. Representative diagram of FRET.

From the first report of FRET by Förster in 1946,¹²² it has demonstrated utility as a "molecular ruler", due to remarkable distance sensitivity. Efficiency between a donor-acceptor pair, *E*, is measured relative to Förster distance R_0 , the distance at which energy transfer is 50% efficient, and the distance between the pair, *r* (Equation 1-4).

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

Equation 1-4. Efficiency of FRET.

A common distance between donor-acceptor pairs is 20 - 60 Å.⁹⁸ With this distance, FRET can measure distances between fluorophore-quencher pairs as well as fluorophore pairs, in which the observed emission is that of the acceptor. Furthermore, spectral overlap of the emission from the donor to the acceptor is required to ensure appropriate energy transfer. With ideal efficiency, the emission of the donor will be effectively ablated, appearing quenched.⁹⁸

Due to the inherent quenching of the donor fluorophore, FRET has found a wide variety of uses, including DNA hybridization studies¹²¹ and protein conformational studies.¹²³ In both

cases, each strand is tagged with either a donor or acceptor. As the species are separated or brought together, the efficiency of FRET changes with the distance between the pair, allowing facile determination of separation through observed fluorescence.

1.3.5 Photobleaching

Photobleaching is a quenching process in which a fluorescent molecule is unable to emit, due to photochemical degradation from either the singlet or triplet states. Once destroyed, the fluorophore is unable to absorb the required light for emission, putting it in a permanent dark state (Figure 1-12).¹²⁴ Photobleaching can occur from either the ground or excited state. Because of this, every excitation event possesses the possibility for photobleaching; on a sufficient time scale, all the fluorophores in a sample will be bleached. This prevents constant irradiation for data collection. One pathway is oxygen forming an adduct with the fluorophore, leading to photobleaching. Much work has been dedicated to the kinetics of photobleaching in confocal microscopy,¹²⁴⁻¹²⁸ highlighting the difficult to predict nature of the phenomenon. Mechanisms for photobleaching in biological samples are less well understood.¹²⁷



Figure 1-12. Jabłoński diagram of photobleaching

1.3.6 Summary

These representative examples demonstrate not only quenching of fluorescence as a function of naturally occurring interferences, such as dissolved molecular oxygen or spirocycle formation, but also through controlled additions of additives. The utility of strategic fluorescence quenching has use in a variety of areas, including chemosensor development and super-resolution imaging. In this document, the potential of a fluorescent readout will be demonstrated with respect to detection of trace analytes (Chapter 2), analysis of reaction kinetics (Chapter 3), and development of dyes for super-resolution imaging (Chapter 4).

2.0 AUTONOMOUS STALLING IN CATALYTIC FLUORESCENCE METHODS FOR DETECTION OF TRACE ANALYTES

A significant part of this chapter was published as: Koide, K.; <u>Tracey, M.P.</u>; Bu, X.; Jo, J.; Williams, M.J.; Welch, C.J. *Nat. Commun.* **2016**, *7*, 10691. In this work, I was responsible for synthesis, characterization, and optimization of the method, as well as validation with polymer and ore samples. Experiments with inductively coupled plasma mass spectrometry as well as further validation of the method including active pharmaceutical ingredients were carried out by X. Bu, J. Jo, M.J. Williams, and C. Welch of Merck & Co.

2.1 INTRODUCTION

Development of optical assays for facile quantification of trace analytes is an ever-expanding field. Target analytes range from trace metals¹²⁹ and biological signaling agents^{130,131} to chemical weapons.¹³² Whereas many analytical methods require intensive instrumentation, fluorescence-based chemosensors and chemodosimeters manifest a signal as a simple fluorescent readout. These readouts can be a shift in absorbance or emission wavelength or to the naked eye, a shift in color. In some cases, only a simple penlight is used to determine the strength of the signal.

Non-catalytic quantitative assays rely on a single turnover from the analyte for a chemical conversion or a reversible binding and have the benefit of time-independence: the signal does not change over time once the reaction or binding event is complete. A major drawback of non-catalytic systems is higher limits of quantification, as the signal is directly tied to analyte concentration. Catalytic assays forego time-independence in favor of continuous signal

generation. The resulting signal is tied to both analyte concentration and time elapsed. This leads to lower detection limits, as even a trace amount of analyte can produce strong signals given enough time.

The continuous nature of catalysis-based signal amplification presents some practical challenges to assay development. In metal catalysis-based assays, once the metal has entered into the catalytic cycle, the reaction will continue indefinitely until the substrate is consumed. In enzyme and enzyme-linked immunosorbent assays,¹³³ the reaction continues until the substrate is consumed or until a terminating reagent is added.¹³⁴ In either case, if an analyte is present in sufficient concentrations or is allowed to react unsupervised, the assay substrate will be consumed, preventing accurate quantitation. Additionally, when a catalysis-based assay is externally stopped, it cannot be restarted¹³⁴ and the assay must be repeated to obtain quantitative data. This limits the dynamic range of catalysis-based assays, usually within one to two orders of magnitude. Labor- and instrument-intensive methods, such as inductively coupled plasma mass spectrometry (ICP-MS) have detection ranges up to five orders of magnitude. An approach to catalysis-based assays could expand this dynamic range and allow for time independence would be beneficial to the development of future assays.

2.2 OPTICAL CHEMOSENSORS FOR THE DETECTION OF PALLADIUM

2.2.1 Coordination-based chemosensors for palladium

Chemosensors are molecules that reversibly respond to an analyte, usually by complexation or binding. Commonly, a fluorophore will be tagged with a binding site for an analyte that shifts or quenches fluorescence. Upon binding, the receptor can no longer interact with the parent fluorophore and the optical signal is generated. Thus, a common motif in chemosensors is a binding-induced conformation change, unmasking fluorescence.^{3,10,11,13,14,16,18,21,23,25} The softness of Pd²⁺ lends to the formation of strong interactions with soft heteroatoms, notably sulfur and nitrogen, allowing for cyclic binding motifs.⁵ Additionally, the π -philicity of Pd²⁺ allows for detection based on interactions with alkene and alkyne bonds.¹³⁵ Binding between the chemosensor and the analyte can have a varied stoichiometry, ranging from 2:1, 1:1, and 1:2 as indicated by a Job plot. This prevents lower detection limits, as the generated signal will not be strong enough to be detected. As the output signal from a chemosensor is based on interactions between the palladium and the receptor, removal can reverse the effect. Exposure of the system to a competing or chelating species such as EDTA or cyanide sequesters the palladium species and interrupts the complex formation.^{3,6,12,13,16,21-23,27} Selected examples of chemosensors for palladium will be discussed herein.

In 2010, Peng disclosed a rhodamine-based chemosensor manipulating the π -affinity of palladium. The closed spirocycle of **1.1** quenches the rhodamine fluorescence, affording a non-fluorescent colorless solution. Pd²⁺ binds to the allyl groups and thus opens the spirocycle, as visualized by an increase in fluorescence at 580 nm (Figure 2-1). Spirocycle opening also manifests in the formation of a bright purple color in solution. A Job plot of the system confirmed 1:1 stoichiometry proposed by **1.1+Pd²⁺**. When tested in the presence of other metals, mercury displayed quenching, likely due to interaction of the mercury with the alkenes over palladium. The reversibility of this chemosensor was demonstrated upon exposure to sulfide, upon which the fluorescence was quenched in a concentration-dependent manner, indicating the removal of the Pd²⁺ from the complex.¹²



Figure 2-1. Fluorogenic and colorimetric detection of Pd²⁺ by chemosensor **1.1**.

In 2014, Wang and Qin reported a similar rhodamine-based chemosensor exploiting the binding of palladium to an amide nitrogen and a thioether. 1.2, similar to 1.1, is colorless and non-fluorescent in solution due to the presence of the quenching spirocycle. Upon exposure to Pd^{2+} , the spirocycle is opened and red fluorescence is manifested with concomitant appearance of a purple color in solution. (Figure 2-2). Complex $1.2-Pd^{2+}$ was studied by mass spectrometry, confirming the 1:1 stoichiometry.²³ Furthermore, replacement of the sulfur atom with oxygen led to a decrease in responsiveness to Pd^{2+} , indicating the requirement for the soft sulfur atom to complex effectively to Pd^{2+} . Other chemosensors have taken advantage of this interaction, utilizing large crown moieties.^{8,9,27} 1.2 also demonstrated utility as a fluorimetric and colorimetric palladium chemosensor when dried onto strips of paper, successfully detecting Pd²⁺ concentrations as low as 2.5 µM in pure H₂O. Fluorescence measurements in 80:20 MeCN/H₂O found the detection limit for 1.2 to be 2.4 nM. Addition of 10 eq. CN⁻ lowered the fluorescence by 95.5%, indicating effective removal of Pd^{2+} from **1.2-Pd^{2+}**, restoring the spirocycle and quenching the fluorescence. In 2013, Zeng also reported a similar chemosensor utilizing a triphenylphosphine mojety to target palladium.³



Figure 2-2. Colorimetric and fluorimetric detection of Pd^{2+} by **1.2**.

The previous examples have both highlighted the binding to both electron-rich π -bonds (1.1) and to soft heteroatoms (1.2) to open a fluorescence-quenching spirocycle. Fluorescence "turn on" can also be achieved through binding to heteroatoms of a fluorophore without conformational change. Utilizing this, Zhou disclosed an acridine derivative in 2015 for Pd²⁺ detection. The precursor acridine derivative displayed only slight fluorescence at 434 nm. Upon addition of Pd²⁺, the fluorescence increased approximately 2.5-fold. The linear range was calculated to be from 0-1 μ M and the method successfully detected Pd²⁺ in the presence of other ions. The fluorescence enhancement was proposed to stem from binding between the hydroxyl groups and nitrogen of 1.3. This is supported by the observation that at high pH (>10), fluorescence decreased, likely due to deprotonation of the hydroxyl groups. A Job plot revealed a 2:1 stoichiometry of Pd²⁺ to 1.3, as shown in Figure 2-3. Exposure to EDTA led to sequestration of Pd²⁺ indicated by a decline in fluorescence.²⁷



Figure 2-3. Fluorogenic detection of Pd²⁺ by chemosensor **1.3**

Taken together, these examples demonstrate the use of reversible binding to detect trace palladium. As the signal is tied directly to concentration, detection limits tend to be higher, relying on higher concentrations of chemosensor to observe a stronger signal. As the signal generation is tied to complexation, the resultant output does not increase over time once the binding event is complete indicating time-insensitivity relative to catalysis-based methods, in which signal generation increases over time.

2.2.2 Catalysis-based chemodosimeters for palladium

Whereas coordination-based chemosensors rely on stoichiometric amounts of analytes and reversible binding to afford a quantifiable signal, chemodosimeters utilize an irreversible transformation for signal transduction. Czarnik reported the first instance of chemodosimetry in 1992, detecting Hg²⁺ and Ag⁺ through thioamide degradation.¹³⁶ As chemodosimeters are reaction based, signal generation is tied to the reaction kinetics: a faster reaction will produce an endpoint signal faster. Of note, catalytic systems will continue to produce a signal following multiple turnovers, indicating that the signal is tied to both analyte concentration and reaction

time. For palladium, catalysis-based assays typically rely on coupling, specifically a Sonogashira or Suzuki-Miyaura reaction, or cleavage of heteroatom bonds to unmask new interactions.^{29-35,38,39,41-43,45-61} In these cases, the palladium is the catalyst, leading to formation of new bonds, either through cyclization or cleavage. Select examples of palladium chemodosimeters will be discussed in this section.

A remarkable example of a reaction-based palladium chemodosimeter comes from the Pang group in 2012. Reporting the first near infrared (NIR) emissive palladium probe, they employed Pd^{2+} to mediate the cyclization of **1.4** to afford 2-(2'-hydroxyphenyl)-benzoxazole (HBO) derivative **1.5** (Figure 2-4). Upon exposure of **1.4** to Pd^{2+} , fluorescence emission at both 522 and 780 nm upon excitation at 360 nm was observed. The signal was both time and concentration dependent, reaching a maximum at 40 minutes with 1 equivalent of $PdCl_2$. Superstoichiometric amounts of $PdCl_2$ led to signal saturation.³⁰

Pang (2012)



Figure 2-4. Detection of Pd²⁺ by chemodosimeter **1.4** through benzoxazole formation

This example is particularly noteworthy, because whereas other chemodosimeters involve latent fluorescence of the starting material, this involves the direct synthesis of a fluorophore *in situ*, meaning any observed signal as indicative of reaction turnover. Additionally, without the addition of Pd²⁺, the 5-*endo*-trig cyclization is disfavored by Baldwin's rules,¹³⁷ further increasing the likelihood of any observed signal being indicative of the desired palladium-mediated cyclization instead of spontaneous cyclization. In the presence of Pd²⁺, aromaticity is the driving force of the reaction.

A very common reaction manipulated in palladium chemodosimetry is the palladiummediated cleavage of an allyl or propargyl ether/carbonate/carbamate from a fluorophore. Kinetic insight and the mechanism for the cleavage of an allyl ether will be discussed later in Chapter 3. The mechanism for depropargylation is proposed to proceed through two pathways, depending on the oxidation state of the palladium. Pd⁰ is proposed to proceed through a allenylpalladium complex followed by nucleophilic attack by water to liberate the desired heteroatom. Cleavage by Pd^{2+/4+} is proposed to undergo association to the alkyne followed by nucleophilic attack by water and subsequent elimination.^{45,49} In either cleavage, these examples have demonstrated significant sensitivity and responsiveness to various oxidation states of palladium.

Han and Wei recently disclosed the first chemodosimeter utilizing spontaneous cyclization following depropargylation, trapping the incipient enol from the propargyl ether cleavage. The non-fluorescent starting material **1.6** is converted to fluorescent coumarin derivative **1.7**. The fluorescence potential of the starting material is mitigated through internal rotation of the benzothiazole leading to non-fluorescent emission upon excitation (Figure 2-5).

Once the heterocyclic ring is formed, the rotation is restricted and energy release occurs through fluorescent emission.³² Han also disclosed a similar chemodosimeter, again utilizing a coumarin derivative in a separate publication.³¹



Figure 2-5. Palladium detection by chemodosimeter 1.6 through cyclization.

Another common type of palladium chemosensor involves the cleavage of an allyl bond through a Tsuji-Trost-type reaction. The removal of an allyl ether, carbamate, or carbonate reinstalls the electron density from the heteroatom, allowing for absorbance and subsequent fluorescence. A chemodosimeter based on a 2',7'-dichlorofluorescein derivative Pittsburgh Green was reported by Koide in 2007 and subsequently improved through later publications.^{19,20,36,37} Alkylation of 2',7'-dichlorofluorescein followed by reduction and re-oxidation afforded chemodosimeter allyl Pittsburgh Green ether (APE). Whereas the parent

fluorescein is green fluorescent, both the bis-allyl-2',7'-dichlorofluorescein and APE are not due to alkylation of the phenol, analogous to the pH dependence of fluorescein, as discussed previously. Initial investigation (Figure 2-6 "2007") tested the cleavage of the allylic ether upon exposure to palladium with Ph₃P as a ligand and reducing agent.²⁰ Subsequent improvements (Figure 2-6 "2010") to the method involved utilizing tri(2-furyl)phosphine (TFP) as a ligand and the addition of NaBH₄ as a reducing agent, lowering the detection limit into the sub-nM range and increasing metal specificity of the method.¹⁹



Figure 2-6. Fluorogenic detection of palladium with chemodosimeter APE

As a palladium detection method, APE has also employed for detection of residual palladium in flasks used for palladium-catalysis^{40,44}, reactivity of palladium nanoparticles, as well as kinetic studies of an aqueous Tsuji-Trost reaction as further described in this document.

2.2.3 Intracellular imaging of palladium in live cells

For more background on this area, please see the following review I co-authored: <u>M.P. Tracey</u>, D. Pham, K. Koide*, *Chem. Soc. Rev.* **2015**, *44*, 4769-4791 and references therein.¹³⁸

2.3 DISCONTINUOUS SIGNAL DEVELOPMENT IN A PALLADIUM-CATALYZED FLUOROGENIC AND COLORIMETRIC REACTION

2.3.1 Development and characterization of resorufin allyl ether

The Koide group previously reported a fluorogenic method for quantifying palladium in pharmaceuticals based on the palladium-catalyzed fluorogenic conversion of allyl Pittsburgh Green ether (APE) to Pittsburgh Green (Figure 2-6).^{19,20,37,139} Although this method showed excellent sensitivity and an ability to accurately quantify low-level palladium in real-world samples, we realized that a colorimetric version of the assay could allow even simpler, instrument-free access to low-level palladium measurements, a goal previously attempted by several other researchers with limited success.^{25,46}

Investigation of a number of candidate chromogenic substrates led to the preparation of yellow-colored resorufin allyl ether (RAE) in one step in 85% yield from commercially available purple-colored resorufin (Figure 2-7).



Figure 2-7. Synthesis of colorimetric chemodosimeter resorufin allyl ether (RAE).

Resorufin in 800 mM NH₄OAc in EtOH displayed a strong $\lambda_{abs} = 562$ nm; absorption at 572 nm by RAE was negligible. RAE displayed a $\lambda_{abs} = 460$ nm, whereas resorufin did not, indicating not only the change in sensitivity to excitation by green light (490-570 nm), but also a colorimetric shift from purple to yellow (Figure 2-8).



Figure 2-8. Absorbance profile of resorufin allyl ether and resorufin in 800 mM NH₄OAc. Both curves are normalized to 20 μ M of each compound.

2.3.2 Determination of reaction conditions for conversion of resorufin allyl ether to

resorufin

Attempts at palladium-catalyzed deallylation of RAE using the optimized conditions for APE TFP, NaBH4, DMSO/1.23 M phosphate pH 7 buffer (1:9)) were unsuccessful. However,

screening a variety of commercially available phosphines and additives (Figure 2-9) led to the identification of suitable conditions for carrying out the transformation (Figure 2-9, Figure 2-10, and Table 2-1). Initial optimization utilized benzylamine to raise pH, however, ammonium acetate was employed to create a slightly buffered system. Benzyalmine as an additive has recently shown utility in palladium-containing ore analysis in the Koide group with continuous signal generation (unpublished results).



Figure 2-9. Phosphines screened for conversion of RAE to resorufin.

Table 2-1. Raw data of fluorescence intensities. Darker green indicates stronger fluorescence signals relative to 400 μ M P1. Deallylation conditions were 200 μ M RAE, 40 ppb Pd²⁺, 300 μ M PhCH₂NH₂, 0 – 400 μ M phosphine, 10 mM NaBH₄, EtOH, 25 °C, 60 min, *n* = 1.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
25	1.1	0.4	0.8	1.6	1.2	0.9	2.5	0.9	2.3	3.6	4.5	1.0	0.7	0.9	0.6
50	1.3	0.4	1.8	1.2	1.3	1.0	3.1	0.7	2.3	3.4	4.4	1.3	0.7	1.1	0.8
100	1.3	0.5	0.5	1.4	1.3	1.1	3.4	0.9	2.5	3.4	4.4	1.0	0.8	1.0	0.7
200	1.1	0.2	.6	1.1	1.5	1.2	3.6	1.2	2.5	2.8	4.8	0.9	0.9	1.0	0.7
400	1.0	0.2	0.6	1.2	1.5	1.2	3.4	0.7	2.2	1.7	3.8	0.9	0.7	0.9	0.5
phos	phosphine														

in µM

Based on the data in Table 2-1, we chose to re-screen phosphines **P1** (triphenylphosphine), **P9** (tri(2-furyl)phosphine), and **P11** (tris(4-methoxyphenyl)phosphine) (Figure 2-9). Finding similar reactivity across all three, we selected TFP as the ligand for future development, as we have previously shown that we can store solutions in DMSO with 250 ppm butylated hydroxytoluene (BHT) for over 6 months without degradation.



Figure 2-10. Rescreening **P1**, **P9**, and **P11** from Table 2-1. Conditions for deallylation were 200 μ M RAE, 40 ppb Pd²⁺, 300 μ M PhCH₂NH₂, 0 – 400 μ M phosphine, 10 mM NaBH₄, EtOH, 25 °C, 60 min, *n* = 3. (a) **P1**, **P9**, and **P11** screened alongside one another; (b) Optimizing **P9** concentration dependence.

We found that RAE was selectively responsive to palladium over other metals tested (Ag, Au, Cd, Co, Cr, Fe, Hg, Mn, Ni. Pt, Rh, Ru, Zn, Sr, Ir, Cu; Figure 2-11a,b) and could detect palladium without interference from these metals, with the exception of Hg, where a small level of interference was observed (Figure 2-11c). When selectivity was measured by absorbance, higher values were observed in the presence of Au, Ag, and Hg owing to turbidity of the solution, although fluorescence measurement revealed that these were merely false positives (that is, these metals did not convert RAE to resorufin; Figure 2-11b). When exposed to palladium, the fluorescence signal increased linearly with respect to palladium concentration (Figure 2-12c), indicating a first-order relationship suitable for convenient quantification.



Figure 2-11. Metal selectivity for conversion of RAE to resorufin. (a) Metal selectivity compared to other metals. Conditions for deallylation: 29 μ M RAE, 100 μ M metal except Pd²⁺ at 10 μ M, 3 mM NaBH₄, 200 μ M TFP, 800 mM NH₄OAc, EtOH, 24 °C, 24 °C, 60 min, *n* = 3. Higher absorbances of Ag, Hg, and Au samples were due to opacity. The photograph was taken under ambient light (below). (b) Fluorescence intensities vs. metal of the reaction solutions from (a). (c) Metal selectivity with a mixture of Pd and other metals in a 1:10 ratio. Conditions: 29 μ M RAE, 100 μ M metal, 10 μ M Pd²⁺, 3 mM NaBH₄, 200 μ M TFP, 800 mM NH₄OAc, EtOH, 24 °C, 60 min, *n* = 3.

2.3.3 Competitive and reversible deactivation in a palladium-catalyzed reaction

The Pd-catalysed deallylation of APE in phosphate buffer was more effective in the presence of NaBH₄, which reduces higher oxidation states of Pd to catalytically active Pd^0 , but did not require this reducing agent as a critical component.¹⁹ In contrast, Pd^{2+} species (Pd(NO₃)₂) did not

catalyze the deallylation of RAE in NH₄OAc-containing EtOH without the reducing agent, with the amount of NaBH₄ dictating the duration of reaction (Figure 2-12a).



Figure 2-12. Dependence on reducing agent for deallylation of RAE to resorufin. (a) Reaction lifetime dictated by NaBH₄ concentration. Conditions: 29 μ M RAE, 10 ppb Pd²⁺, 200 μ M TFP, 800 mM NH₄OAc, 0 – 125 NaBH₄, EtOH, 25 °C. (b) Stalled deallylation reactions can be restarted by NaBH₄ addition. Conditions: 29 μ M RAE, 0.3 ppm Pd²⁺, 200 μ M TFP, 800 mM NH₄OAc, 0, 0.6, 1.2, 1.8, 2.4 mM NaBH₄, added as 2.5 M aliquots at indicated time points.

This novel NaBH₄-dependence boded well with our aim at competitively and reversibly deactivating catalysis-based assays, as detailed below. Lower concentrations of NaBH₄, ranging from 5–25 mM, led to stalling of the color-forming reaction within 30 s, presumably because of rapid consumption of the reductant, NaBH₄, combined with ongoing air-oxidation of catalytically active Pd⁰ to higher valent, inactive palladium species. In contrast, NaBH₄ concentrations in excess of 50 mM allowed the reaction to continue for several minutes. Importantly, the addition of more NaBH₄ could restart a stalled deallylation reaction (Figure 2-12b), affording a convenient way to trigger signal generation on demand.

Subsequently, we sought to gain insights into the reaction stalling to rationally expand this developing methodology. When tested, the palladium-catalyzed conversion of APE to Pittsburgh Green stalled in the presence of NH_4OAc but continued in a phosphate buffer (Figure 2-13), indicating that this stalling is not substrate specific and can be utilized in other palladium-catalyzed reactions.



Figure 2-13. Stalling APE deallylation. Conditions: 20 μ M APE, 10 ppb Pd²⁺, 80 μ M TFP, 600 mM HPO₄²⁼ or NH₄OAc, 25 °C, 20% v/v EtOH/H₂O

We next chose to test concentration dependence of NH₄OAc with respect to stalling. With 200, 400, 600 and 800 mM NH₄OAc followed by pH adjustment, the reactions stalled nearly at the same time, indicating a relative concentration independence for stalling (Figure 2-14a). The palladium-catalyzed deallylation reaction of RAE under a nitrogen atmosphere were found to stall more slowly than those carried out in open air (Figure 2-14b), suggesting that aerobic oxidation of Pd⁰ to higher order palladium species may account for the observed reaction stalling.



Figure 2-14. Effects of stalling with both concentration of NH₄OAc and O₂. (a) Various NH₄OAc concentrations do not have an effect on reaction lifetime. Conditions: 29 μ M RAE, 0.3 ppm Pd²⁺, 200 μ M TFP, 0 – 800 mM NH₄OAc, 0.6 mM NaBH₄, EtOH, 25 °C, n = 3. Samples were basified with 1 N NaOH prior to fluorescence reading to normalize. (b) O₂ effectively inhibits deallylation. Conditions: 29 μ M RAE, 200 μ M TFP, 1 mM NaBH₄, 100 ppb Pd²⁺, 800 mM NH₄OAc, EtOH, 25 °C, 15 min, n = 1.

In an effort to develop a simple, user-friendly colorimetric palladium quantification assay, we prepared a reagent cocktail combining all reaction components except NaBH₄ in a single solution (See Appendix A for details). This cocktail, which is stable for over 2 weeks when stored at 5 °C, can be dispensed as needed, simplifying application of the colorimetric method. The addition of either 20 μ l of a solution or 2–5 mg of a solid sample containing trace palladium to 1 ml of the reaction cocktail, followed by the addition of a NaBH₄ solution, generated color and fluorescence within 1 min. The color intensity was linearly correlated with palladium concentration, and the dynamic range and reaction time of the assay were tailored by adjusting the NaBH₄ concentration.

The power of this method is shown in Figure 2-15. Known concentrations of palladium afford widely different colors with a single concentration of NaBH₄, with the color persisting for 24 h (Figure 2-15a). Additionally, the NaBH₄ concentration can be tailored for palladium

concentrations. If a sample contains 1 ppb palladium, then 100 mM NaBH₄ is required to observe a color change (Figure 2-15b). If a sample contains 10 ppm palladium, then no NaBH₄ is added to observe a color change. Thus, palladium concentrations ranging from 1 ppb to 10 ppm (five orders of magnitude) can be distinguished in one reaction solution with NaBH₄ titration. Generally, chemosensor methods' dynamic range is two orders of magnitude, further showcasing the value of the stop-and-go approach described herein.



Figure 2-15. Visual quantification of palladium with RAE. (a) The appearance of a distinguishable color correlating to palladium concentration occurs in less than 1 min using a set of palladium standards. Conditions: 29 μ M RAE, 0 – 4.0 ppm, 200 μ M TFP, 800 mM NH₄OAc, 1.0 mM NaBH₄, EtOH, 25 °C. (b) Colorimetric plate showing dependence of color formation on palladium and NaBH₄ concentrations. Conditions: 29 μ M RAE, 200 μ M TFP, 0 – 50 ppm Pd, 0 – 100 mM NaBH₄, 800 mM NH₄OAc, 25 °C, EtOH, 10 min. *n* = 3.

2.3.4 Applying stop-and-go fluorogenic palladium detection to real world samples

To confirm that the stop-and-go assay approach is providing quantitative data, we analyzed realworld samples. We first tested intermediates used in the preparation of active pharmaceutical ingredients in collaboration with Merck & Co. In pharmaceutical synthesis, reactions may leave behind residual palladium in the products, which is often difficult to remove.¹⁴⁰ Various samples were tested from active projects in the Process and Analytical Chemistry Department at Merck Research Laboratories in which residual palladium removal has proven difficult. Quantification of palladium was initially performed by ICP-MS followed by analysis using RAE. Compared with the ICP-MS analysis, the stop-and-go approach with RAE provided accuracy from 70 to 120%, with residual palladium concentrations ranging from 62 to 800 ppm (Figure 2-16). These results were satisfactory for this assay approach to be used for screening dozens of routine palladium remediation protocols.



Figure 2-16. Correlation of palladium quantification of palladium to ICP-MS. Conditions: Colorimetric: 29 μ M RAE, 200 μ M TFP, 0 – 800 ppm Pd²⁺, 800 mM NH₄OAc, 1.0 mM NaBH₄, EtOH, 24 °C; ICP-MS: Samples for ICP-MS were suspended in concentrated HNO₃ prior to analysis. See Appendix A for further information.

Microscale screening of process adsorbents is often used to identify resins or activated carbons that can be used for selective adsorption of metal impurities in pharmaceutical process research and development.^{141,142} Traditionally, this approach requires close coordination with ICP-MS specialists to allow for quick turnaround time. However, often because of instrument

calibration, the vast number of samples, and preparation time, this can be time consuming. As such, the pharmaceutical industry has been interested in a faster technology for trace metal analysis.¹⁴³

The application of the colorimetric method enables rapid determination of palladium concentrations "on the spot", in the same laboratory where the process development studies are being carried out. Figure 2-17 shows the results of a high throughput screen of palladium impurity remediation treatments of a pharmaceutical intermediate with 48 metal-scavenging adsorbents, using the stop-and-go assay with RAE to visualize relative palladium levels. An aliquot from each well is treated with the reaction cocktail (Figure 2-17a), then with NaBH₄. In less than 5 min, gross differences in palladium concentration are readily apparent to the naked eye by distinguishable colors (Figure 2-17b). At this point, the reaction had stalled, and too many hits were identified. Accordingly, more NaBH4 was added to restart the reaction, accentuating the differences between wells and enabling rapid determination of the potential most-effective treatments for residual palladium remediation (Figure 2-17c). high-А throughput mapping of relative palladium concentration was obtained by plotting the ratio of absorbance at 580 and 460 nm using a ultraviolet-visible plate reader (read time for 48 samples <30 s; Figure 2-17d). These results quantitatively confirm the most effective palladium removal treatments to be wells A5, C4, E6, F3 and F5. Spot-checking several adsorbent treatment samples using conventional ICP-MS showed a good correlation with the colorimetric method, with the selection of the most-effective adsorbent treatments (A5, E6) being identical in both cases. These results demonstrate the utility of a stop-and-go approach in trace metal quantification, providing an important advance for process chemists dealing with remediation of Pd impurity problems using point-of-use high-throughput analysis.



Figure 2-17. Demonstration of the stop-and-go approach with pharmaceutical samples. (a) Screening kits containing 48 commercial adsorbents¹⁴¹ are exposed to a solution containing a Pd-containing intermediate. (b) Aliquots from screening kits are evaluated for palladium content using the colorimetric method, as described in Appendix A, 1 mM NaBH₄ (c) Finding the best potential hits visually by adding more NaBH₄ (final concentration 2 mM). (d) High-throughput mapping of a relative palladium concentration by measurement of ultraviolet-visible 570 nm/460 nm using ultraviolet-visible plate reader.

APE was previously applied for quantifying palladium in ore samples, provided by Stillwater Mining Company, without requiring acidic sample digestion,¹⁴⁴ a significant improvement over standard analytical methods such as ICP-MS, but still requiring the use of a blue light source to check fluorescence intensity. This requirement associated with the fluorometric method was not ideal for turbid samples such as ores. Therefore, we applied our colorimetric method for more convenient visualization. We found that the reaction was stalled before effective palladium extraction from the ores, leading to a need to add a large excess of NaBH₄. However, a 1-min pre-incubation in a DMSO solution of TFP with sonication, followed by the addition of RAE, NH₄OAc, EtOH and NaBH₄ afforded subpar semi-quantitative data. A second addition of NaBH₄ after the reaction had stalled provided good colorimetric agreement with previous semi-quantitative analysis¹⁴⁴ within 20 min (Figure 2-18).



Figure 2-18. Analysis of ore samples. Conditions: 29 μ M RAE, 200 μ M TFP, 800 mM NH₄OAc, 75 mM NaBH₄, 200 mg milled ore sample, EtOH, 25 °C, 20 min. Samples i, ii, and iii contain 0.030, 0.068, and 0.094% palladium by weight, respectively. Samples iv and v contain no palladium. Palladium concentrations were determined by aqua regia digestion and analysis.¹⁴⁴

Trace palladium is also a significant concern in materials science. Residual palladium can interfere with desired properties of polymers prepared by palladium catalysis.^{145,146} To further validate the fluorogenic/colorimetric method, we aimed to detect residual palladium in palladium-prepared polymers, graciously provided by the Krebs Group (Technical University of Denmark). Relative palladium amounts from preparation are shown in Table 2-2.

Polymer Sample	Polymerization method (scale)	Mol% Pd ₂ (dba) ₃	Reaction Time
Sample 1	Batch (0.3 g)	3	24 h
Sample 2	FSP (0.3 g)	1	30 min
Sample 3	FSP (0.3 g)	3	30 min
Sample 4	FSP (0.3 g)	5	30 min
Sample 5	FSP (0.3 g)	3	15 min
Sample 6	FSP (0.3 g)	3	45 min

Table 2-2. Polymer samples prepared by palladium catalysis analyzed by RAE

30 min

*FSP = flow synthesis polymerization

Initially, we tested digesting the polymers with aqua regia followed by analysis with RAE. However, this led to signals inconsistent with the projected amount of palladium shown in Table 2-2. Relative concentrations showed Sample 1 to contain the highest content instead of Sample 4 (Figure 2-19).



Figure 2-19. Analysis of polymers after digestion with aqua regia. Conditions: 29 μ M RAE, 5 ppm polymer, 200 μ M TFP, 800 mM NH₄OAc, 25 mM NaBH₄, 25 °C, EtOH, 30 min, *n* = 1. Relative palladium concentrations did not match Table 2-2.

To determine if the result observed in Figure 2-19 was due to polymer interference, we tested spiking aliquots of Sample 4 with palladium (final concentration = 1 ppb) and analyzing the resulting solutions with RAE. We observed that in the presence of the polymer, fluorescence decreased, indicating that the polymer is interfering with effective palladium detection (Figure 2-20a).

To alleviate the problem, we turned to acid digestion, removing aliquots of the polymer solutions, evaporating them to dryness, and re-suspending them in 5% HNO₃. Heating the samples affected a rapid extraction of palladium, and subsequent analysis using RAE revealed

relative palladium concentrations in each polymer. The highest palladium-containing polymer was subjected to further analysis, highlighting the use of RAE as a colorimetric chemodosimeter for analysis of digested polymers (Figure 2-20b).



Figure 2-20. Interference by polymers on analysis with RAE and determination of residual palladium in polymers. (a) Interference by polymers. Conditions: 29 μ M RAE, 0 – 8 ppm polymer, 0 – 1 ppm Pd²⁺, 200 μ M TFP, 25 mM NaBH₄, EtOH, 24 °C, 30 min, *n* = 3. (b) Analysis of residual palladium in digested polymer samples. Conditions: 29 μ M RAE, 16 ppm polymer, 200 μ M TFP, 50 mM NaBH₄, 800 mM NH₄OAc, EtOH, 24 °C, 30 min, *n* = 3. Relative palladium concentrations correlated to the amount of palladium from each polymer preparation.

2.3.5 Summary

In summary, the development of a new palladium-sensitive fluorogenic chemosensor enabled the high-throughput facile detection of trace palladium across 5 orders of magnitude, rivaling the range of low-throughput instrumentation, such as ICP-MS. The fluorescence signal was favorable over measuring absorption due to potential turbidity of solutions as observed with samples contaminated with Ag and Au. The stop-and-go approach enables detection of trace amounts of palladium without concerns for how long the catalytic reaction proceeds. These

results indicate the utility of fluorescence to detect residual palladium in complex mixtures such as pharmaceutical intermediates, ore samples, and palladium-contaminated polymers.

2.4 COMPETITIVE AND REVERSIBLE DEACTIVATION IN AN ENZYMATIC ASSAY

2.4.1 Stop-and-go approach in horseradish peroxidase

To illustrate the discontinuous catalysis approach in an enzymatic assay, we tested H_2O_2 mediated conversion of Amplex Red to resorufin with horseradish peroxidase (HRP) (Figure 2-21a). HRP is a common enzyme for detection and quantification in biological assays.^{147,148} The conversion of Amplex Red to resorufin proceeds through a 1:1 stoichiometry to H_2O_2 , producing acetic acid, H_2O and resorufin.¹⁴⁹ In the presence of excess H_2O_2 , reactivity is determined by concentration of HRP. As a competitive inhibitor, we tested PhB(OH)₂ to reduce H_2O_2 , while the H_2O_2 mediated oxidation of Amplex Red occurs. Figure 2-21b shows that PhB(OH)₂ was able to do so in a concentration-dependent manner, affording lower signals.

Reactions halted by consumption of H_2O_2 could be restarted by an addition of a fresh aliquot of H_2O_2 (Figure 2-21c,d). With a further addition of H_2O_2 , signal saturation occurred (Figure 2-21d). With the inclusion of the competitive scavenger, PhB(OH)₂ to remove H_2O_2 from the system, the discontinuous catalysis alleviated the problem of overshooting signals, as well as allowed us to restart the reaction without problematic increases in fluorescence (Figure 2-21c,d). Although the protocol has not been fully optimized in an HRP system, these data indicate a great potential for the applications of discontinuous catalysis in other enzyme assays.



Figure 2-21. Stop-and-go approach in a horseradish peroxidase system. (a) Conversion of Amplex Red to resorufin. (b) Effect of PhB(OH)₂ on horseradish peroxidase assay. Conditions: $50 \,\mu$ M Amplex Red, 0.1 U ml⁻¹ horseradish peroxidase, 25 μ M H₂O₂, 0, 25, 50, 250 mM PhB(OH)₂, PBS pH 7.4. (c) Restarting a stopped enzymatic reaction in the presence of an inhibitor. Conditions: $50 \,\mu$ M Amplex Red, 0.05 U ml⁻¹ horseradish peroxidase, $0 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ at 0 min, 20 μ M H₂O₂at 10 min, PBS pH 7.4. (d) Restarting a stopped enzymatic reaction in the presence of an inhibitor saturation. Conditions: $50 \,\mu$ M Amplex Red, 1 U ml⁻¹ horseradish peroxidase, $0 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $0 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ (0–20 min) for the circle and triangle. For others, $10 \,\mu$ M H₂O₂ at $0 \,m$ in, $30 \,\mu$ M H₂O₂ at $10 \,m$ in, PBS pH 7.4. After the addition of H₂O₂ at 10 min, the PhB(OH)₂-free sample (square) showed a signal above the upper limit of the instrument (above 2 × 10⁶ units).

2.4.2 Summary

In summary, we demonstrated the expansion of the "stop-and-go" approach when applied to enzymatic systems. Measuring the resultant fluorescence signal allowed us to monitor the reaction over time. PhB(OH)₂ as a competitive inhibitor to H_2O_2 lowered the signal overall. When the reaction was run without the inhibitor, the addition of a second aliquot of H_2O_2 led to signal saturation, whereas in the presence of PhB(OH)₂ as a competitive inhibitor, no saturation was observed. Although the protocol has not been fully optimized in an HRP system, these data indicate a great potential for the applications of discontinuous catalysis in other enzymatic assays.

3.0 USING A FLOROGENIC REACTION FOR KINETIC STUDIES OF A PALLADIUM-CATALYZED TSUJI-TROST-TYPE REACTION

A significant part of this chapter were *submitted for publication* as Tracey, M.P.; LeClaire, M.J.; Lu, G.; Liu, P.; Koide, K. "Isoinversion Principle and Inverse Temperature-Dependence in a Tsuji-Trost Reaction". In this work, both M.J. LeClaire and I carried out experiments and performed data analysis. Computational experiments were performed by G. Lu.

3.1 INTRODUCTION

The Tsuji-Trost reaction is the transposition of an electrophilic allylic group through palladium catalysis. In 1965, utilizing an allylpalladium chloride dimer, Tsuji and coworkers disclosed the allylation of diethyl malonate in basic conditions (Figure 3-1a).¹⁵⁰ In 1973, the Trost group reported the acceleration of the reaction to "near instantaneous rates" with the addition of triphenylphosphine as a ligand (Figure 3-1b).¹⁵¹ The inclusion of the phosphine expanded the substrate scope as well as the aforementioned acceleration. Furthermore, various phosphine ligands have been developed to expand scope and reactivity, including asymmetric ligands opening the use of allylic alkylation in total synthesis.¹⁵²



Figure 3-1. The Tsuji-Trost reaction

The activation of an electrophilic palladium-olefin complex was initially exploited in 1959 when palladium chloride was utilized to facilitate hydroxide attack on ethylene.¹⁵³ As such, the Tsuji-Trost reaction was designed from this reactivity, utilizing a carbon nucleophile on an activated palladium complex. Mechanistically, the reaction involves the association of a 14-electron Pd⁰ species to the allyl moiety, affording 16-electron species **Pd-2** (Figure 3-2, k_I). Subsequent oxidative insertion between the leaving group and the allyl moiety furnishes electrophilic **Pd-3** (Figure 3-2, k_2). Common leaving groups for this reaction include acetates, carbonates, carbamates, esters, phosphonates, and halides among others.¹⁵⁴ The activated π -allyl complex **Pd-3** is susceptible to nucleophilic attack (Figure 3-2; k_3) at either the carbon or metal center, depending on the nucleophile. Hard nucleophiles attack at the metal center, with carbon bond formation following reductive elimination, whereas soft nucleophiles attack the carbon center directly.¹⁵⁵ Following nucleophilic attack, the Pd⁰ dissociates from the allyl group, releasing the product and catalytically active palladium (Figure 3-2; k_4).



Figure 3-2. Accepted mechanism of the palladium-catalyzed Tsuji-Trost reaction

The palladium-catalyzed Tsuji-Trost reaction is useful not only in synthetic organic chemistry^{150,151,156} but also in the development of chemosensors,^{138,157,158} signal amplification,¹⁵⁹ and bioorthogonal organometallic (BOOM) chemistry through screening reaction conditions.¹⁶⁰ There are many examples of allylic alkylation in total synthesis.¹⁵² For example, the synthesis of callipeltoside A by Trost and coworkers demonstrates the need for screening reaction conditions for optimal reactivity (Figure 3-3).¹⁶¹ A chiral ligand was envisioned as a driving force to afford the appropriate diastereomer **3.8**. Of the six ligands screened, anthracene-like ligands displayed complete inhibition of the reactivity, whereas diphenyl ligands, notably ligand **L** (Figure 3-3) afforded the highest conversion and diasteromeric excess.¹⁶¹ This example highlights the need for rational design to a given target through understanding reactivity.



Figure 3-3. Synthesis of callipeltoside A, utilizing a palladium-catalyzed allylation as a key step.

In BOOM chemistry development, fluorogenic substrates have been used as model systems for intracellular compound activation; this enables a facile readout for activity for palladium catalysis. Notably, prodrugs of 5-fluorouracil¹⁶² and gemcitabine¹⁶³ have been developed utilizing fluorescent precursors to determine biological reactivity of the catalytic system. In both cases, whereas the propargyl derivative was nearly as active as the naked drug upon exposure to palladium resins, the allyl derivative did not perform as desirably, likely due to unoptimized reaction conditions.^{162,163} Rational design utilizing a fluorogenic precursor may allow for more active activation of allyl compounds under biological conditions.

In the development of chemosensors for palladium, the concentrations of a chemosensor have been chosen arbitrarily, with no obvious justification. This is problematic because the quantification of palladium by the reactivity-based chemosensor methods is based on the premise that the kinetic profile of a palladium-catalyzed deallylation remains the same regardless of chemosensor concentrations. For the accuracy and reproducibility of reaction-based sensing
methods, it is paramount to identify a turnover-limiting step under specific reaction conditions and ask this unanswered question.

To better understand the reaction and guide future optimization, the kinetics of the Tsuji-Trost reaction have been rigorously studied at millimolar concentrations. At these concentrations palladium species may aggregate and make it difficult to accurately study the system.¹⁶⁴ Nonetheless, at these concentrations, depending on the structure of the displaced R group, the smallest rate constant can be either k_2 or k_3 (Figure 3-2).¹⁶⁵ For example, with a linear substrate, the π -allyl palladium complex was the resting state of the catalytic cycle (i.e., $k_3 < k_1$, k_2 , k_4 ; Figure 3-2).^{166,167} With a cyclic substrate, the oxidative addition of Pd⁰ to an allylic C-O bond is the turnover-limiting step (i.e., $k_2 < k_1$, k_3 , k_4).¹⁶⁸ Taken together, this illustrates the turnoverliming step (TLS) of a Tsuji-Trost reaction depends on the substrate structure. However, it is unknown whether the identity of an RDS depends on substrate concentrations in a micromolar range or temperatures. Micromolar concentrations of substrate are highly relevant to chemosensor development, signal amplification, and BOOM chemistry, especially in aqueous conditions.

3.2 PREVIOUS KINETIC STUDIES OF THE TSUJI-TROST REACTION

The turnover-limiting step has been shown to change for the Tsuji-Trost reaction with varying substrates. In 2010, Shintani, Hayashi and coworkers reported that with a cyclic substrate, the oxidative insertion step is turnover limiting with lactone **3.9**. NMR studies showed that the rate of reaction is first order to **3.9** and to the palladium catalyst, $Pd(PPh_3)_4$ while zeroth order to the isocyanate nucleophile. The latter observation ruled out the possibility of nucleophilic attack as

the turnover limiting step (Figure 3-4).¹⁶⁸ It was concluded that the turnover-limiting step was oxidative insertion (analogously shown as **TS2** in Figure 3-2) due to the observed rate law, however this conclusion ignores the allyl association step, which would depend on both palladium and substrate concentration (analogously shown as **TS1** in Figure 3-2).¹⁶⁸



Figure 3-4. Kinetic study of palladium-catalyzed allylation by Shintani and Hayashi

In 2008, the Lloyd-Jones group disclosed the resting state of the Tsuji-Trost reaction with linear substrate **3.13**. Through studying trends in electrophilicity and ion-pairing, it was reported the resting state of the catalytic cycle is the L₂Pd(η^2 -allyl) complex (Figure 3-2, **Pd-2**). In contrast to the findings of Shintani and Hayashi, Lloyd-Jones reported that the rate of product formation as shown in Equation 3-1, indicating that the turnover-limiting step was the nucleophilic attack at the π -allylpalladium complex by NaC(Me)(CO₂Me)₂ instead of oxidative insertion, as previously hypothesized (Figure 3-2, **Pd-3**), affected heavily by counterion to the complex.¹⁶⁸ The conclusions of the Lloyd-Jones group assume a steady-state amount of the electrophilic π -allylpalladium complex formed from **3.13**, as further supported by titration of new ion pairings to create a more activated complex.¹⁶⁶



Figure 3-5. Linear substrate studied by Lloyd-Jones for Tsuji-Trost kinetics

$$\frac{d[3.14]}{dt} = k[Pd][NaC(CO_2Me)_2]$$

Equation 3-1. Observed rate reported by Lloyd-Jones and coworkers

Similarly in 2009, Stoltz and coworkers disclosed further validation of the resting state of the reaction. Utilizing ³¹P NMR, they identified a peak corresponding to a square planar 16-electron species with a σ -bound, η^1 -allyl ligand and were able to isolate and characterize it by crystallization (Figure 3-6, **3.18**). Impure samples of **3.18** were studied as well, and were found to proceed with decarboxylation through visible gas expulsion.¹⁶⁷ As **3.18** is formed following oxidative addition and is the only isolable intermediate from the catalytic system, it was concluded to be the resting state of this system, though conclusions of this kinetic study are limited to analogous substrates for decarboxylative allylic substitution. Analogous η^1 -allyl complex **3.19** was also synthesized, stemming from oxidative insertion to allyl acetate for characterization (Figure 3-6), which supported the idea of neutral intermediates in related catalytic systems.



Figure 3-6. Substrates utilized by Stoltz to study the Tsuji-Trost reaction.

3.2.1 Summary

The Tsuji-Trost is a widely useful reaction for a variety of chemical endeavors, including chemosensor development and total synthesis. The mechanism is widely accepted, as the reaction was discovered with mechanistic considerations in mind, and various kinetic studies to identify parts of it have been undertaken. Taken together, the results from the groups of Shintani, Hayashi, Lloyd-Jones, and Stoltz indicate a change in the TLS based on substrate (such as linear vs. cyclic substrates). In each case, only a single RDS was disclosed. The resting state of the reaction has been shown to be the π -allyl complex for the studied substrates. It is likely that the η^1 complex is the resting state, as it is not susceptible to nucleophilic attack prior to shifting to the η^3 conformation. It is important to note that all previous kinetic studies were performed in organic solvent, such as toluene or THF, whereas kinetic studies in H₂O are lacking.

3.3 STUDYING NANOPARTICLE ACTIVITY BY A FLUOROGENIC REACTION

Palladium nanoparticles (PdNPs) and various heterogenous catalysts have emerged as novel ways to elicit reactivity for various palladium-catalyzed reactions, including cross-coupling and allylic transposition.¹⁶⁹⁻¹⁷³ Notably, as reported by Gómez, Claver, Chaudret, and Philippot, nanoparticle ligands can influence reactivity and mechanism.¹⁷⁴ Additionally, heterogenous catalysts benefit greatly from the addition of a phosphine ligand in the case of allylic alkylation.¹⁶⁹

Much work has been done to study the reactivity of and nature therein of PdNPs. Curiously, with Pd/C as the heterogenous catalyst, ICP-MS indicated no leaching into solution, indicating no homogenous catalyst formation.¹⁷¹ Additionally, TEM imaging was insufficient to study changes in nanoparticle size with respect to reactivity.¹⁷⁰ With this unresolved need in 2011, Semigana reported the first study of PdNP activity, with respect to conformation and size utilizing the fluorogenic conversion of APE to fluorescent Pittsburgh Green.⁶²

In Semagina's pioneering work, the fluorogenic conversion of APE afforded a facile, high-throughput readout to determine activity of PdNPs: more reactive PdNPs would provide a higher signal. Smaller PdNPs (2.4 nm) were found to be twice as reactive as larger ones (3.8 nm). Similarly, larger PdNP cubes (18 nm) were nearly inactive even with significantly longer reaction scales. Previously, defects in the nanostructure were suspected as the active sites, which was supported by reactivity analysis of various PdNPs.^{62,175,176} TEM analysis of the reactions revealed Ostwald ripening: palladium was leached into solution with concurrent PdNP size increase, with a growth from 2.4 ± 0.7 nm to 3.4 ± 0.6 nm. Leaching was only observed in the presence of PPh₃, indicating its role in the process. Taken together, it was concluded that the palladium was leached, forming a catalytically active phosphine-bound palladium species, which

was subsequently re-deposited leading to the formation of larger PdNPs.⁶² These results support the atomic dissolution model for PdNP catalysis, in which leaching is required for formation of a catalytically active species, though Ostwald ripening may mask this phenomenon. Additionally, this work highlights the robust utility of fluorescence for measuring reactivity and reaction kinetics in a unique system (Figure 3-7).



Figure 3-7. Conversion of fluorogenic APE to Pittsburgh Green by PdNPs with Ostwald ripening

3.3.1 Summary

The unique study of palladium nanoparticle activity by Semigana, demonstrates the utility of fluorogenic palladium-catalyzed reactions in high throughput kinetic study. Semigana demonstrated that it can distinguish between active palladium sites in PdNPs based on size and used this as a lead to further study PdNP catalysis, leading to the confirmation of the leaching by oxidative addition model proposed previously.

3.4 ISOINVERSION IN A TSUJI-TROST REACTION

The Koide group previously reported the palladium-catalyzed deallylation of fluorogenic chemodosimeter APE in 5% DMSO/pH 7 buffer (Figure 2-6).²⁰ Pittsburgh Green can be an analog of a leaving group in Tsuji-Trost reactions because the pK_a value of the phenolic hydroxyl group is 4.27,¹¹⁷ similar to the acidity of carboxylic acids or electron deficient phenols, typical leaving groups in Tsuji-Trost reactions. As the PdNP studies by Semagina showed, the use of APE for kinetic studies can provide new insights because the reaction can be continuously monitored by means of fluorescence at low micromolar concentrations.⁶²

Since the first publication using APE with Ph₃P in 50 mM phosphate pH 7 buffer, the Koide group improved the reaction rate by changing the phosphine ligand from Ph₃P to tri(2-furyl)phosphine (TFP),³⁶ increasing the phosphate concentration from 0.05 M to 1.23 M, and adding NaBH₄.¹⁹ As shown below (Figure 3-13), NaBH₄ does not have an impact on the conclusion of this work, but facilitated kinetic studies: it eliminated the deactivation of Pd⁰ by air oxidation when high-throughput experiments were carried out under an air atmosphere.¹⁷⁷ Although PdNP formation complicates the interpretation of data, because the solvents in this

study are DMSO and water and palladium concentrations are in the 9 nM to 4 μ M range, palladium species are most likely monomeric.¹⁷⁸

Figure 3-8 shows the consumption of APE as a function of time. Fitting the consumption of APE to a one-phase decay model revealed that the reaction rapidly slowed as the substrate concentration approached 4 μ M (Table 3-1). When the reaction started with 5 μ M APE, the reaction progressed at a similar rate as when the above reaction slowed after the substrate concentration reached 4 μ M, excluding product inhibition and indicating that the RDS may change at this concentration (Figure 3-8, Table 3-1). If the RDS remains the same during the course of the reaction, a single kinetic profile should be observed from 50 to 0 μ M. As this was not the case, we turned to Eyring plots to quantify energetic contributions in the reaction.



Figure 3-8. Measuring consumption of APE as a function of time. Conditions: (a) 25, 50 (b) 5 μ M APE, 1 μ M Pd(NO₃)₂, 80 μ M TFP, 10 mM NaBH₄, 10% v/v DMSO/1.23 M phosphate pH 7 buffer, 298 K, 270 min, *n* = 3.

Table 3-1. Calculated plateau of APE from fitting to a one-phase decay as reaction continues indefinitely; data over time also shown in Figure 3-8. Conditions: 5, 25 or 50 μ M APE, 1 μ M Pd(NO₃)₂, 80 μ M TFP, 10 mM NaBH₄, 10% DMSO v/v 1.23 M phosphate pH 7 buffer.

Initial APE concentration, µM	Calculated plateau of APE (M) as reaction proceeds infinitely
5	8.0±4.0 x 10 ⁻⁷
25	4.1±0.7 x 10 ⁻⁶
50	4.1±1.3 x 10 ⁻⁶

Preparation of an Eyring plot involves plotting the natural log of the rate constant against the inverse temperature affords a line where the slope is $-\Delta H^{\ddagger}/R$ and the y-intercept is $\ln(k_b/T) + \Delta S^{\ddagger}/R$ (Figure 3-9). I chose APE as the substrate and measured fluorescence stemming from its product, Pittsburgh Green, after 30 min, at which point a vast majority of the starting material remained intact. This choice allowed for high-throughput and accurate data collection under analytically and biologically relevant highly diluted conditions in a 96-well format. Previously, only small amounts of data were accessible for Eyring plot analysis, as shown in Figure 3-9. ΔH^{\ddagger} values are intrinsically independent of concentration of reactants, while ΔS^{\ddagger} values are often dependent. Since it was not possible to measure concentrations of reactive species in the RDS, this work will not discuss experimental ΔS^{\ddagger} values. Furthermore, Eyring plots have been shown to govern selectivity between competing pathways (Figure 3-9),^{179,180} this system produces a single product, allowing me to compare different kinetic profiles and by extension, potentially different TLS.



Figure 3-9. Eyring Plot. Left: Cartoon representation of an Eyring plot illustrating enthalpic and entropic determinants. Right: Eyring plot for the selectivity factor *E* in the kinetic resolution of an azirinol catalyzed by Amano PS lipase.¹⁷⁹ At the inversion temperature (231 K), enantioselectivty is at a maximum. Reprinted from *Curr. Opin. Chem. Biol.*, 6, 2, Reetz, M.T., "Lipases as practical biocatalysts", p. 145-150, Copyright 2002, with permission from Elsevier.

3.4.1 Initial Investigation with Previously Optimized Conditions: Effect of Substrate Concentration.

We first established a benchmark with previously optimized conditions at 293–358 K.¹⁹ When the reaction started with 5 μ M APE at elevated temperatures, the reaction was too fast to afford viable data. With 12.5 μ M APE, we uncovered two inversion points (i.e., three regimes) (Figure 3-10). The A shape indicates a change in TLS.^{181,182} Table 3-2 summarizes the observed calculated Δ H[‡] values. These benchmark values for Δ H[‡] provided a standard against which we could evaluate effects on the TLS by changing conditions, using the generally accepted mechanism (Figure 3-2).

The Eyring plots with three concentrations of APE are shown in Figure 3-10, revealing three regimes, with $\Delta H^{\ddagger>0}$ (regime 1), $\Delta H^{\ddagger\sim0}$ (regime 2), and $\Delta H^{\ddagger<0}$ (regime 3). A notable trend is that as the substrate concentration decreases, so does the inversion temperature (T_{inv}) (Figure

3-10b). This observation can account for the rapid change in consumption of APE observed in Figure 3-10: as the reaction proceeds at a specific temperature, the kinetic profile at a specific reaction temperature shifts, initially from regime 1 to regime 2, finally to regime 3 as the substrate is consumed (Figure 3-10e). Each profile change would affect the rate. Isoinversion may occur in other Tsuji-Trost reactions.



Figure 3-10. Summary of regime changes with various concentrations of APE. (a) Eyring plot under the previously developed reaction conditions with various APE concentrations: 9.4 nM Pd(NO₃)₂, 120 µM TFP, 10 mM

NaBH₄, 5% v/v DMSO, 1.23 M phosphate pH 7 buffer, 30 min. (b) Expanded view of Regime 1. (c) Expanded view of Regime 2, (d) Expanded view of Regime 3. (e) Summary of changing regime at a single temperature.

 [APE], μM	Temperature (K)	ΔH^{\ddagger} (kJ/mol)
 12.5	293–326	55±2
	326–347	7.9±2
	347–358	-28±3
25	313–347	48±2
	347–353	6±3
50	313–353	42±1

 Table 3-2. Experimentally determined enthalpic contributions at various substrate concentrations in

 Figure 3-10.

For regime 1, we proposed that the TLS is the oxidative addition (i.e., **Pd-2** to **Pd-3**, Figure 3-2) as this step converts the stronger C-O bond (1076.5 kJ/mol) to the weaker Pd-O (234 kJ/mol) and Pd-C (436 kJ/mol) bonds.¹⁸³ For regime 2, the TLS barrier is entropically controlled; this may be attributed to either the nucleophilic attack toward **Pd-3** or an ion pair exchange between either a phosphate or DMSO and **Pd-3**, as will be discussed later. For regime 3, an inverse temperature-dependence is observed ($\Delta H^{\ddagger<0}$). As found in literature, this is not a readily explained, though often observed, phenomenon.¹⁸⁴⁻²¹⁶ One explanation is the exothermic formation of a reactive complex following rapid equilibrium.¹⁸⁵ We therefore propose that the RDS for regime 3 is the association of the TFP-ligated palladium species to the allyl group (i.e., **Pd-1** to **Pd-2**) as the coordinatively unsaturated 14-electron species **Pd-1** gains electrons to form a more stable 16-electron species **Pd-2**. As will be discussed later, increasing palladium

concentration causes this regime to disappear, further supporting the proposed identity. Another plausible, but less likely, cause for the inverse temperature-dependence is that at higher temperatures, the phosphine ligand may dissociate from the palladium to form a mono-ligated dormant species.

With 25 μ M APE (Figure 3-10a, red square), regime 3 was missing within the experimental temperature range. With 50 μ M APE, we only observed regime 1. At all the APE concentrations, ΔH^{\ddagger} was similar in regime 1 (42–55 kJ/mol). The inversion temperature (T_{inv}) is intertwined with the substrate concentration as well, as the three regimes of the 12.5 μ M APE profile shifted to the left (higher temperatures) with the 25 μ M APE with the regime 3 outside of the experimental temperature range. The 50 μ M APE experiment displayed only regime 1, possibly because the higher substrate concentration requires less entropic loss in the association step. Altogether, these results indicate that the identities of TLSs depend on both the temperatures and the substrate concentrations and that at elevated temperatures with very low substrate concentrations, the ΔH^{\ddagger} has a negative value. To better understand the energetic contributions of each reaction component, we began to systematically perturb the benchmark reaction conditions.

3.4.2 Effect of Phosphine Concentrations

Phosphine structures substantially impact palladium catalysis,²¹⁷⁻²¹⁹ but much less is known about the impact of phosphine concentrations. We studied the kinetics of the deallylation at 60, 120, and 180 μ M TFP. With 50 μ M APE (Figure 3-11a), the results did not differ significantly at the three TFP concentrations (Table 3-3). With 12.5 μ M APE (Figure 3-11b), we observed at least one isoinversion at all the TFP concentrations. Higher temperatures (>345K) led to

indistinguishable kinetics across all TFP concentrations. The correlation between the TFP concentration and deallylation rate showed sigmoidal curves at both 298 and 318 K (Figure 3-11c). At 298 K, the rate declined with >80 μ M TFP, which is consistent with the literature.²²⁰



Figure 3-11. Summary of effects of TFP concentration. (a)/(b) Eyring plots prepared with various TFP concentrations. Conditions: 9.4 nM Pd(NO₃)₂, 10 mM NaBH₄, 50 (a) or 12.5 (b) μ M APE, 5% v/v DMSO/1.23 M phosphate pH 7 buffer, 30 min. *n* = 3. (c) TFP concentration dependence. Conditions: 94 nM Pd(NO₃)₂, 2 mM NaBH₄, 10% v/v DMSO/1.23 M phosphate pH 7 buffer, 30 min. *n* = 3.

Table 3-3. Experimentally determined enthalpic contributions at various TFP concentrations with 9.4 nM Pd(NO₃)₂, 12.5 or 50 μM APE, 10 mM NaBH₄, 5% v/v DMSO/1.23 M phosphate pH 7 buffer, 30 min, 298 – 358 K.

[APE], μ M	[TFP], μM	Temperature (K)	$\Delta ext{H}^{\ddagger}$ (kJ/mol)
12.5	60	293-326	68±2
		326-347	16±3
		347-358	-9.3±5
	120	293–326	55.4±2
		326–347	-6.1±3
		347–358	-25±4
	180	293–326	98.1±2

		326–347	-9±3
		347–358	-4.9±1
50	60	293–358	37±0.5
	120	293–358	37±1
	180	293–358	46±1

Because the Pd-phosphine binding can be reversible,^{3,221,222} I asked whether a component in the reaction mixture could act as a competitive ligand against TFP toward palladium. Various concentrations of phosphate ions (Figure 3-12a) and DMSO (Figure 3-12b) did not change the optimal concentration of TFP, indicating that these two species are not competing.



Figure 3-12. Effects of DMSO and phosphate on TFP-palladium binding. (a) Conditions: 470 nM $Pd(NO_3)_2$, 10 mM NaBH₄, 20 μ M APE, 5% v/v DMSO/0.50-1.23 M phosphate pH 7 buffer, 298 K, 60 min. (b) 470 nM $Pd(NO_3)_2$, 10 mM NaBH₄, 20 μ M APE, 2.5-10% DMSO/1.23 M phosphate pH 7 buffer, 298 K, 60 min.

3.4.3 Effect of Phosphate Ions

 π -Allylpalladium-organic phosphate complexes are reactive intermediates in related reactions.^{223,224} Thus, we asked how inorganic phosphates affect the deallylation. It was previously reported that phosphate ions are not a nucleophile toward an electrophilic allyl species.¹⁹ Unexpectedly, at 0.3 and 0.6 M concentrations of phosphate ions, the corresponding Eyring plots consisted of one V-shaped isoinversion with 50 µM substrate (Figure 3-13a). The V-shape implies that the two different mechanisms operate above and below the T_{inv} .²²⁵ The V-shape also shows that the faster reaction above 335 K despite the higher ΔH^{\ddagger} likely indicating a larger entropic contribution to the TLS. One plausible explanation for the two mechanisms within the parameters of well-established mechanism (Figure 3-2) is different solvation clusters,²²⁶ as the π -allylpalladium complex benefits from solvation.

Higher concentrations of phosphate ions (1.2 M) eliminated the change in the mechanism (Table 3-4). This is possibly because an ion pair analogous to **Pd-3** is formed with phosphate ions. The ion pair effect may prevent reversibility of the oxidative addition, though under different conditions this step was reversible.²²⁷ It is not clear why the ΔH^{\ddagger} values are lower with less phosphate ions at lower temperatures, but it should be noted that the Baran and Gaunt groups independently reported an unspecified but positive role of phosphate ions in palladium catalyses.^{228,229} With 12.5 μ M substrate, no notable change was observed in the energetic profile of the reaction (Figure 3-13 and Table 3-4) compared to the benchmark (Figure 3-10).



Figure 3-13. Eyring plots with changes in benchmark conditions. Unless listed otherwise, conditions were as follows: 9.4 nM Pd(NO₃)₂, 120 μ M TFP, 10 mM NaBH₄, 5% v/v DMSO/1.23 M phosphate pH 7 buffer, 293 – 358 K. (a)-(b) Various phosphate concentrations, (c)-(d) Various NaBH₄ concentrations, (e)-(f) Various DMSO concentrations, n = 4.

Table 3-4. Experimentally determined enthalpic contributions at various phosphate concentrations with 9.4 nM Pd(NO₃)₂, 12.5 or 50 μM APE, 120 μM TFP, 30 min.

 [APE], μ Μ	[phosphate]	Temperature (K	ΔH [‡] (kJ/mol)
 12.5	0.3	308-326	51±2
		326-347	-4±8
		347-358	-32±2
	0.6	295-331	40±1
		331-346	-18±4
		346-358	-55±2
	1.2	293-326	55±2
		326-347	7.9±2
		347-358	-28±3
50	0.3	293-326	9.8±0.56
	0.3	326-358	28±1.1
	0.6	293-336	13±0.41
	0.6	336-358	50±1.0
	1.2	293-358	37±1

3.4.4 Effect of NaBH₄

The reduction of Pd^{2+} with >10 mM NaBH₄ is not the TLS because the deallylation rate was independent of NaBH₄ concentration.¹⁹ However, little was known about the energetic contributions of NaBH₄ at or below 10 mM. With 10 mM NaBH₄ and 50 μ M APE (Figure 3-13c

and Table 3-5), one steep descending linear line was observed. With 0, 0.1, and 1 mM NaBH₄ and 50 μ M APE, one similarly steep descending linear line appeared, followed by a less steep descending linear line with a T_{inv} at 343 K. The V-shape indicates two mechanisms. Energetic profiles appeared similar to those observed with lower phosphate concentrations shown in Figure 3-13. With 12.5 μ M APE (Figure 3-13d and Table 3-5), the overall profiles were similar in the NaBH₄ concentration range. We did not observe distinct Eyring plots under the NaBH₄-free conditions in Figure 3-13, broadening the implication of this study in synthetic organic chemistry, sensor studies, and BOOM chemistry that are not compatible with NaBH₄.

[APE], μM	[NaBH ₄] (mM)	Temperature (K)	ΔH [‡] (kJ/mol)
12.5	0	293-326	80±2
		326-347	-10±10
		347-358	-25±1
	0.1	293-326	89±2
		326-347	11±3
		347-358	-19±5
	1	293-326	99±2
		326-347	10±1
		347-358	-36±3
	10	293-326	55±2

Table 3-5. Experimentally determined enthalpic contributions at various NaBH₄ concentrations with 12.5 μ M or 50 μ M APE, 9.4 nM Pd(NO₃)₂, 120 μ M TFP, 5% v/v DMSO in 1.23 M phosphate pH buffer, 30 min.

7 7.9±2
8 -28±3
3 18±0.45
8 62±2.7
3 18±0.83
8 57±1.6
.3 15±0.83
8 58±0.97
8 37±1

3.4.5 Effect of DMSO

Water-miscible organic solvents have been used as co-solvents in aqueous palladium-catalyzed reactions to solubilize hydrophobic reactants.^{163,230} Although DMSO binds palladium,^{231,232} DMSO does not appear to do so in the system of interest, as discussed above (Figure 3-12b). We wondered if varying concentrations could impact the energetics of the deallylation through solvation. The results of changing the DMSO concentration with 12.5 and 50 μ M APE are shown in Figure 3-13e,f and summarized in Table 3-6. Greater than 10% DMSO quenched fluorescence, disrupting effective estimation of the deallylation rate, while less than 5% DMSO led to solubility problems of TFP and APE. With 50 μ M APE in 5% DMSO, we observed only a single line (Figure 3-13e). With 10% DMSO, the Evring plot showed a unique two-regime

profile with both 12.5 and 50 μ M, with a disappearance of the enthalpically neutral TLS (Figure 3-13f). The converse could be summarized as the appearance of the enthalpically neutral TLS when DMSO was reduced from 10% to 5% at appropriate temperatures at both 12.5 and 50 μ M.

Table 3-6. Experimentally determined enthalpic contributions at various DMSO concentrations with 12.5 μ M or 50 μ M APE, 9.4 nM Pd(NO₃)₂, 10 mM NaBH₄, 120 μ M TFP, 5 or 10% v/v DMSO in 1.23 M phosphate pH 7 buffer, 30 min.

[APE] µM	Temperature (K)	DMSO (%)	ΔH [‡] (kJ/mol)
12.5	293-326	5	55±2
	326-347	5	7.9±2
	347-358	5	-28±2
	297-326	10	34±2
	326-358	10	-32±2
50	313-353	5	42±1
	297-335	10	43±1
	335-353	10	-16±3

How does the lesser amount of DMSO slow the nucleophilic attack? The first explanation is that the enthalpically neutral step is not a nucleophilic attack but it is an ion pair exchange. Specifically, the aryloxy anion of **Pd-3** may be replaced by DMSO or a DMSO-solvated phosphate anion, which can be less favorable when the DMSO concentration is lower. As reported by Lloyd-Jones and Jutand, weaker ion pairs afford faster reactions.^{166,227} Thus, it is possible that the lower DMSO concentration favors a tighter ion pair, forcing the TLS to be the ion pair exchange. The second explanation is that DMSO is a palladium ligand at elevated

temperatures and the enthalpically neutral RDS is the ligand exchange between DMSO and TFP. This may not be turnover limiting when the DMSO concentration is higher (10%). A third explanation is that DMSO stabilizes the transition state of the nucleophilic attack, possibly through an interaction between the partially positively charged phosphorus atom of TFP as a nucleophile and the oxygen atom of DMSO. At this time, the exact reason is not known. The solvent effects have been vastly overlooked in the previous studies, and our study suggests that further studies on the solvent effects are warranted.



Equation 3-2. Ion pair switching between phenoxide and DMSO

3.4.6 Identification of the Nucleophile

To model the reaction with density functional theory experiments, we needed to identify the nucleophile of the reaction. Previous investigations revealed that the phosphate ions were not the nucleophile.¹⁹ Spurred on by this, a reaction mixture was analyzed by LC-MS. A peak corresponding to P-allylated TFP (Equation 3-3) was found, indicating that TFP may be the nucleophile. This is similar to the precedence in the literature, in which Ph₃P was allylated under Tsuji-Trost reaction conditions.²³³ As such, we further investigated TFP as the reaction nucleophile through decreasing both APE and TFP simultaneously to mimic reaction progress. The "same excess" models²³⁴⁻²³⁶ would afford

a single line if the TFP is acting as the nucleophile.ⁱ In effect, each decreasing concentration of APE was paired with the equally lower concentration of TFP to mimic the reaction progressing under the assumption that TFP is the only nucleophile. If TFP is not consumed, the reactions with 60, 50, and 40 μ M TFP would show different rates as indicated by Figure 3-11. As Figure 3-14b shows, further increasing TFP concentration (120, 110, or 100 μ M) at the same APE concentrations afforded the same effect as shown in Figure 3-14a.

Equation 3-3. Formation of allyl-phosphine species

In the "same excess" experiments, we found that unlike the kinetic data observed in Figure 3-8, the reaction continued at the same rate instead of slowing down over time (Figure 3-14). To investigate this seemingly contradictory result, we prepared an Eyring plot with the increased $Pd(NO_3)_2$ concentrations (3.6 µM instead of 9.4 nM; Figure 3-15, Table 3-7). With the higher $Pd(NO_3)_2$ concentration, the temperature dependence of the reaction changed, overwhelming reversibility of the oxidative addition and association to leave the nucleophilic attack as the RDS. It is also possible that at the higher palladium concentration, an equilibrium between dimeric and monomeric palladium species influences the RDS through the change in the concentration of catalytically active palladium species.

ⁱ We thank Professor Donna Blackmond (The Scripps Research Institute) for suggesting this experiment.



Figure 3-14. Same excess experiments with TFP. On right, graphs are adjusted to match concentration of APE at given time points. (a) and (b): Conditions: 3.6 μ M Pd(NO₃)₂, 10 mM NaBH₄, 5% v/v DMSO in 1.23 M phosphate pH 7 buffer, 293 K, n = 3.

Although this warrants further studies, it is noteworthy that the higher palladium concentration increased the ΔH^{\ddagger} value from 55±2 to 94±2 kJ/mol in the 293–326K range. Taken together, these results indicate that TFP is likely the nucleophile for the deallylation. This should not be surprising because the phosphorus atom is poorly solvated and thus remains nucleophilic in water.



Figure 3-15. Eyring plot with increased palladium. Conditions: 3.6 μ M Pd(NO₃)₂, 50 μ M APE, 120 μ M TFP, 10 mM NaBH₄, 5% v/v DMSO in 1.23 M phosphate pH 7 buffer, 295 – 357 K, 30 min, *n* = 3.

Table 3-7. Experimentally determined enthalpic contributions using increased Pd(NO₃)₂ with 50 μM APE,
3.6 μM Pd(NO₃)₂, 10 mM NaBH₄, 120 μM TFP, 5% v/v DMSO in 1.23 M phosphate buffer, 10 min.

$[Pd(NO_3)_2] (nM)$	Temperature (K)	[APE] (µM)	ΔH^{\ddagger} (kJ/mol)
9.4	293-326	12.5	55±2
	326-347	12.5	7.9±2
	347-358	12.5	-28±3
9.4	313-353	50	42±1
3600	297-324	50	94±2
	324-348	50	-14±3
	348-357	50	25±3

3.4.7 Density Functional Theory Calculations

In collaboration with the P. Liu group (University of Pittsburgh), density functional theory (DFT) calculations were performed by Gang Lu to provide further insights into the RDSs of the

Pd-catalyzed deallylation reaction (for previous computational studies of Tsuji-Trost-type reactions, see the cited references.^{237,238,239,240} The activation enthalpies and entropies of three key steps of the reaction of allyl phenyl ether with the Pd(TFP)₂ catalyst were calculated using Gaussian 09 (See Appendix A for details). The calculations were performed using B3LYP/LANL2DZ-6-31G(d) for geometry optimization and M06/SDD-6-311+G(d,p) for single point energy calculations. Thermal corrections to the enthalpies and entropies were calculated using the gas-phase harmonic vibrational frequencies at 298K. Solvation effects were taken into account in the single point energy calculations using the SMD solvation model²⁴¹ and water as the solvent. A few possible nucleophiles were considered in the DFT calculations, including TFP, H₂PO₄, and OH⁻. Among all the nucleophiles considered in the calculations, TFP has the lowest computed activation Gibbs free energies for the nucleophilic attack of the Pdallyl intermediate (Figure 3-16), in agreement with the "same excess" experiments. The computed gas-phase ΔS^{\ddagger} cannot be compared to experimental data.ⁱⁱ though the trend is consistent with the experimentally proposed RDSs at different temperatures. The DFT calculations indicated that the allyl association step (TS1) is the least favorable entropically with the most negative ΔS^{\ddagger} and the most favorable enthalpically with a negative ΔH^{\ddagger} (-30.4 kJ/mol), supporting the propose assignment for the TLS of regime 1.

ⁱⁱ To properly account for the solvation effects on entropy, molecular dynamics simulations using explicit solvent (water) are typically required. This is beyond the scope of this current study. See: (a) Levy, R. M.; Gallicchio, E. *Annu. Rev. Phys. Chem.* **1998**, *49*, 531. (b) Wana, S.; Stote, R. H.; Karplus, M. J. Chem. Phys. **2004**, *121*, 9539.



Figure 3-16. Optimized structures and activation energies of the transition states of allyl association (TS1), oxidative addition (TS2), and nucleophilic attack (TS3).

These data, shown in Table 3-8, are consistent with the experimental ΔH^{\ddagger} values at 347-358K. The oxidative addition (**TS2**) is the only unimolecular step, and thus is the most favorable entropically with the least negative ΔS^{\ddagger} . The computed ΔH^{\ddagger} of the oxidative addition step (40.9 kJ/mol) is also in reasonable agreement with the experimental value of 55±2 kJ/mol measured at 293-326K. Differences in these values may be due to solvation, as the DFT studies and the experiments were carried out in different media (H₂O vs. phosphate buffer). This suggests that the oxidative addition is the RDS at 293-326K. The nucleophilic attack of the Pd-allyl complex by TFP (**TS3**) occurs via the outersphere pathway (Figure 3-16). This bimolecular process also has a large negative ΔS^{\ddagger} , However, the DFT calculations significantly overestimated the ΔH^{\ddagger} of the nucleophilic attack (54.9 kJ/mol versus the experimental value of 7.9 kJ/mol determined at 326-347K), possibly due to the challenges of calculating solvation effects of the cationic Pd-allyl species.²⁴² Taken together, the computational results support the hypothesis that three different TLSs are operating at different temperatures , although quantitative prediction ΔS^{\ddagger} in aqueous solution remains challenging. Table 3-8. Computed activation enthalpies and entropies of the allyl association, oxidative insertion, andnucleophilicattacksteps.CalculationswereperformedattheMD(H₂O)/B3LYP/LANL2DZ-6-31G(d) level of theory at 298K.



	ΔH [‡] (kJ/mol)	ΔS [‡] (J/mol*K)
Allyl association (TS1)	-30.4	-168.7
Oxidative insertion (TS2)	40.9	-29.9
Nucleophilic attack (TS3)	54.9	-149.4

3.4.8 Summary

The sensitive and high throughput fluorometric method allowed for measuring the activation enthalpy and entropy energies at low concentrations of the substrate, revealing new kinetic insights. A surprising finding was that at the low substrate concentration, three regimes were observed. We have assigned as the oxidative addition (regime 1; lower temperatures), nucleophilic attack (regime 2; middle temperatures), and association (regime 3; high temperatures). This insight may aid in a rational approach to the acceleration of Tsuji-Trost reactions under diluted biological and analytical conditions. Furthermore, fluorogenic probes may find use in kinetic studies of other organic reactions.

4.0 DEVELOPMENT OF A STOCHATISTIC BLINKING DYE FOR USE IN SUPER-RESOLUTION IMAGING

In this work, I designed and carried out synthetic experiments. Blinking analysis was carried out at the Center for Biological Imaging at the University of Pittsburgh by Callen Wallace.

4.1 DIFFRACTION-LIMITED FLUORESCENCE MICROSCOPY

Microscopy enables for visualization at levels well-below unaided optical detection, utilizing reflective light to study samples. However, the resolution of microscopy is limited by the Abbe diffraction limit. : First reported in 1873, it describes the minimum distance between two points required for resolution (Equation 4-1), where where *d* is the minimum distance required between two points for resolution, *n* is the refractive index of the solvent, and θ is the incident angle of the light being used with emissive wavelength λ .⁸⁷ The value of *n*sin θ refers to the numerical aperture (NA) of the microscope, and can be simplified to 2NA. Increasing the NA of the system can be achieved by changing the incident angle (Figure 4-1), changing the solvent, or by replacing lenses of the microscope. In the absence of solvent, the refractive index is 1.0, for aqueous samples, the refractive index increases to 1.33. For imaging in biological samples, changing solvent to increase NA is not always possible.

$$d=\frac{\lambda}{2n\sin\theta}$$

Equation 4-1. Abbe diffraction limit



Figure 4-1. Increasing numerical aperture of a system by changing incident angle θ .

Beyond traditional light-based microscopy, in which an image of an entire sample is collected and is limited by depth of light penetration, fluorescence microscopy collects only the emission from fluorophores that are emitting. Wide-field fluorescence excites an entire sample, collecting all emissive fluorophores at a time (Figure 4-2).²⁴³ This limits resolution, as the whole sample is illuminated, creating a higher signal-to-noise ratio.



Figure 4-2. Schematic representation of wide-field fluorescence microscopy. The incident light passes through a filter to remove undesired wavelengths followed by reflection toward the sample. The emitted light is collected and measured, affording an image of the fluorophores in the sample.

Resolution can be further increased through the addition of a spatial pinhole, referred to as confocal microscopy, minimizing sample excitation. This increases the resolution, as the out-of-focus background is minimized. Scanning over a series of parallel areas allows for reconstruction of the image in 3-D through repeated scanning of small depths (Figure 4-3).^{127,244}



Figure 4-3. Schematic representation of a confocal microscope. The pinhole apertures enable smaller field of view and higher resolution than wide field excitation.

Despite these advances, fluorescence microscopy is still limited by the Abbe diffraction limit. For example, green fluorescent protein (GFP) has a limit of approximately 250 nm, based on an emission of 509 nm and an assumed NA of 1. Though useful for tracking localization of fluorophores in a cellular area, lower resolution is required for better spatiotemporal studies of intracellular and extracellular networks, indicating a need for lower resolution limits in fluorescence microscopy.

4.2 OVERVIEW OF SUPER-RESOLUTION MICROSCOPY

With resolution limited to approximately 200-250 nm with visible light, much work has been dedicated to superseding or "breaking" this diffraction limit. These techniques, referred to as "super-resolution microscopy" utilize the overlay of various frames collected by emitting fluorophores in a very small area at a time, similar to confocal microscopy. These small emission areas are controlled through patterned illumination, photobleaching and quenching of excited fluorophores. In 2008, *Nature Methods* honored super-resolution microscopy as "Method of the Year". Furthermore, in 2014, Eric Betzig (Howard Hughes Medical Institute Janelia Farm Campus/University of California at Berkley), William E. Moerner (Stanford University), and Stefan Hell (German Cancer Research Center/Max Planck Institute for Biophysical Chemistry) were awarded the Nobel Prize in Chemistry "for the development of super-resolved fluorescence microscopy". Representative examples of these super-resolution techniques will be discussed.

4.2.1 Structured illumination microscopy (SIM)

Structured illumination microscopy (SIM) is a super-resolution technique that relies on patterned excitation of a sample. The light source is passed through a known patterned filter and the unknown distribution of fluorophores in a sample affords a second pattern. This combination affords an optical phenomenon known as a Moiré fringe pattern, where bands wider than the patterning are observed. The widening of the pattern due to the Moiré fringe allows for collection of previously inaccessible data, measuring reciprocal space. Thus, using a known pattern, the structure of the fluorophore pattern can be calculated, affording spatial resolution below the

diffraction limit for the sample.⁷⁶ A representative Moiré fringe is shown in Figure 4-4, with the widened observable range represented as the apparent vertical lines between the two patterns.



Figure 4-4. Expansion of observable region by Moiré fringe patterning.

A major advantage to SIM is, whereas other super-resolution methods require laser intensities ranging from 10³ to 10⁸ W/cm², it requires less power laser intensities and therefore is more attractive for live cell imaging.⁸⁶ Measurements from SIM are rapid, requiring no non-linear manipulation to achieve increased resolution images, though further manipulation with non-linear constrained deconvolution algorithms is possible.^{72,73,76,77,86}

A remarkable example of SIM for imaging in live cells was reported by Betzig and coworkers in 2015. Utilizing SIM coupled with total internal reflection fluorescence (TIRF) microscopy, they were able to image with 84 nm resolution in live cells. With laser intensity as low as 30-100 W/cm², events such as initiation, growth, and internalization of clathrin-coated pits and interaction of actin with clathrin were directly observed in living cells. Nonlinear SIM with patterned activation enabled the observation of remodeling of the actin cytoskeleton, approaching a resolution limit of 60 nm (Figure 4-5).⁸⁶



Figure 4-5. Two approaches for improved live-cell imaging at sub-100-nm resolution. (Left) Association of cortical actin (purple) with clathrin-coated pits (green), the latter seen as rings (inset) at 84-nm resolution via a combination of total internal reflection fluorescence and structured illumination microscopy at ultrahigh numerical aperture (high-NA TIRF-SIM). (Right) Progression of resolution improvement across the actin cytoskeleton of a COS-7 cell, from conventional, diffraction-limited TIRF (220-nm resolution), to TIRF-SIM (97-nm resolution), and nonlinear SIM based on the patterned activation of a reversibly photoswitchable fluorescent protein (PA NL-SIM, 62 nm resolution). (Left and right represent single frames from time-lapse movies over 91 and 30 frames, respectively. Scale bars, 2 μm (left); 3 μm (right). From: D. Li *et al.*, *Science*, **2015**, *349*, aab3500. DOI: 10.1126/science.aab3500. Reprinted with permission from AAAS.

4.2.2 Stimulated emission depletion (STED)

Another representative super-resolution technique for super-resolution microscopy is stimulated emission depletion (STED). Whereas SIM relies on measuring a sample in the reciprocal space, STED relies directed inactivation of fluorophores in a spatially controlled area, exploiting the non-linear response of fluorophore inactivation. As such, only a fraction of the fluorophores in the desired area is emissive at a given time. The fluorophore deactivation allows for concurrent excitation of a single spot and deactivation of surrounding fluorophores to provide the small fraction of active fluorophores at a time (Figure 4-6).



Figure 4-6. Cartoon representation of STED microscopy. The whole area of the sample is excited followed by controlled inactivation by a second pulse, leaving a small subsection of fluorophores active for imaging.

From an energetic standpoint, the excitation of the fluorophores in the sample is similar to the previously outlined Jabłoński diagram, updated in Figure 4-7, in which the absorbed photon (blue arrow) leads to entering the excited S₁ state. In STED, the donut-shaped de-excitation (purple arrow) acts as stimulated emission, returning the excited electron to a different energy level than required for fluorescence emission, leading to a longer wavelength emission (red arrow). Due to this red-shift, the emitted photon is filtered out, providing a very focused region in which the remaining fluorophores remain in the "on" state. Furthermore, it is possible that the de-excitation laser may lead to photobleaching of the sample, due to the high intensity irradiation.^{75,90} Though Stefan W. Hell and Jan Wichmann, to whom STED is credited, first reported the method in 1994,⁷⁹ with the first application reported 5 years later,⁸² a patent by V.A. Okhonin (USSR Academy of Sciences) was filed in 1986, disclosing the idea for STED.


Figure 4-7. Representative Jabłoński diagram of STED microscopy. The blue arrow represents excitation of the sample. The green arrow represents fluorescent emission. The purple arrow is the second pulse for deactivation. The red arrow represents the stimulated non-fluorescent emission and subsequent relaxation.

Resolution from STED has ranged from approximately 80 nm to as low as 2.4 nm.⁹² Dyes for STED, initially limited to Rhodamine B⁷⁹ have greatly expanded for immunolabeled cells. For example, red-emitting MR-121SE⁷⁵ and yellow emitting Atto 590⁹³ have demonstrated utility upon conjugation to secondary antibodies for STED imaging. The major limitation of STED is the required second laser pulse, which may induce phototoxicity in live cell experiments.

4.2.3 Single molecule localization microscopy

The last representative examples of super-resolution microscopy I will discuss are single molecule localization (SLM) microscopy, specifically photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). Both methods utilize time-resolved activation and localization, overlaying numerous frames to prepare an image and minimize background noise.^{245,246} In the case of PALM, fluorophores are inactivated through

photobleaching, allowing for spatial resolution as the fluorophores bleach in a non-linear fashion. In STORM imaging, the fluorophores are inactivated by a second laser pulse, similar to STED (Figure 4-8). Though utilizing the similar principles, PALM was initially developed for photoswitchable fluorescent proteins, ²⁴⁵ whereas STORM was developed for donor-acceptor FRET pairs Cy3 and Cy5. ²⁴⁶



Figure 4-8. Cartoon representation of PALM and STORM. In either case, a small set of fluorophores is activated followed by inactivation either by a laser pulse (STORM) or photobleaching (PALM).

STORM has expanded to various photoswitchable fluorophores, including Cy5,⁹⁶ in which the formation of an adduct leads to the dark state. Furthermore, the addition of additives such as β -mercaptoethylamine enables STORM imaging to span the entire visible spectrum.⁷⁸ Whereas photobleaching in PALM affords a permanent dark state, the addition of additives for collisional quenching enables a reversible prolonged dark state which may not be permanent

(Figure 1-6). The downside of additives is that they may perturb the intracellular environment. Although the use of intracellular glutathione has enabled dSTORM imaging in live cells,^{83,89,94} laser intensities are potentially phototoxic at this level, leading to issues with widespread use.

SLM microscopy has shown some of the most impressive resolution limits for superresolution microscopy, with Selvin and coworkers utilizing SLM to measure myosin V walking on actin in a hand-over-hand method with 2.4 nm resolution.⁹⁵ Despite this remarkable limit measuring biological processes, applications of PALM and SLM in living cells proves to be a challenge, due to higher laser intensities which lead to phototoxicity or the addition of additives that disrupt the intracellular environment as previously described.

To address the issue of laser intensities, advances in SLM have focused on using membrane-permeable fluorophores that do not require high intensity irradiation for photoswitching. Whereas common photoswitchable dyes for PALM and STORM include Cy5 and Alexa 647,^{74,84} a new class of desirable fluorophore would stochastically "blink" to induce a prolonged dark state without relying on additives or photobleaching followed by unassisted reentry into the emissive state. Aside from the lower laser intensities, these fluorophores would not be irreversibly turned off, rather turning off and on repeatedly, and can therefore be measured several times for localization prior to inevitable bleaching (Figure 4-9).



Figure 4-9. SLM utilizing a stochastically blinking fluorophore. The repeated switching between on and off state enables the repeated measurement of the same fluorophores in varying localization.

In 2014, Urano and coworkers reported the "first-in-kind" of a stochastic blinking fluorophore for use in SLM. As described in Section 1.3.3, spirocyclic fluorophores induce an "off" state when the spirocycle is present; opening of the spirocycle restores fluorescence. To manipulate this, a library of compounds was prepared to possess favorable cyclization equilibrium with a prolonged dark state (Figure 1-10). An antibody handle enabled the immobilization of the compounds for direct measurement of blinking. Screening identified silicon-based rhodamine derivative **4.1** for favorable characteristics for super-resolution imaging, imaging β -tubulin-Halo fusion proteins in with Vero cells.⁹¹ **4.1** displayed a resolution limit of 47 nm with 40 W cm⁻¹ intensity and 29 nm with 100 W cm⁻¹, both significantly lower intensities than used in other methods (1 kW cm⁻¹).⁹¹ This pioneering result highlights the power of stochastically blinking fluorophores for super-resolution microscopy with significantly lower laser intensities.



Figure 4-10. Stochastically blinking fluorophore 4.1 reported by Urano.

4.2.4 Summary

Super-resolution microscopy has emerged as a method to image systems below the diffraction limit. By overlaying numerous frames, a clearer image can be obtained, minimizing background signal and maximizing signal-to-noise ratio. As demonstrated in the representative examples, the induction of a long-lived dark state affords higher resolution, often at the expense of highintensity lasers or disruptive additives. The "first-in-kind" blinking fluorophore reported by the Urano group has laid groundwork for the future development of blinking fluorophores as superresolution fluorophores.

4.3 DEVELOPMENT OF A STOCHASTIC BLINKING PITTSBURGH GREEN DERIVATIVE

Pittsburgh Green was previously reported to reversibly form a fluorescence-quenching spirocycle through intramolecular attack of a hydroxymethyl group,^{117,118} however blinking frequency was not measured at the time. Pittsburgh Green already demonstrated potential for imaging in biological samples, boasting good membrane retention when utilized in zebrafish embryos.¹¹⁷ The reported rhodamine derivatives by the Urano group contain an antibody "handle" to measure blinking, but the handle is in an electronically-biased position that may directly influence blinking frequency.⁹¹ To avoid this and utilize previously reported spirocycle formation, I designed a Pittsburgh Green derivative that placed the handle in the electronically-neutral 4' position; a similar approach was reported by the Koide group in 2008.¹¹⁷ Furthermore, as the Bertozzi group reported using AlexaFluor-488 alkyne to tag azide-functionalized glycan

membranes through click chemistry.²⁴⁷ I envisioned an alkyne-Pittsburgh Green derivative as the first "clickable" blinking fluorophore for super-resolution imaging, especially with an electronically neutral handle.



Figure 4-11. Left: Proposed blinking of Pittsburgh Green through spirocycle formation. Box: Pittsburgh Yellowgreen.

4.3.1 Synthesis of Pittsburgh Green derivatives

Retrosynthetically, I envisioned that Pittsburgh Yellowgreen (Figure 4-11, box) would be an amenable starting point to prepare a conjugation-ready Pittsburgh Green derivative. The alkene could be oxidized to a carboxylic acid, which provides a robust motif for other reactions, including amide formation for antibody or alkyne attachment.

Heating chemodosimeter APE in Ph_2O to 160 °C induces a thermal Claisen rearrangement affording Pittsburgh Yellowgreen.¹¹⁷ Initial attempts to use Pittsburgh Yellowgreen for subsequent oxidation directly were met with failure, forcing installation of protecting groups at the phenolic positions. Benzyl ethers were initially considered; however, the closed spirocycle is itself a benzyl ether, complicating the desired removal from the phenolic position. PMB ethers were thus selected for their facile removal with DDQ.^{248,249} To simplify and expedite synthesis, I isolated Pittsburgh Yellowgreen by chemically active extraction and utilized the crude intermediate fpr the subsequent protection. The residual Ph₂O would not affect the protection, and could be easily removed by silica gel flash chromatography. Treatment of the crude residue with PMBCl in DMF with Cs₂CO₃ afforded intermediate **4.1** on a gram scale. Attempts at direct oxidation of the alkene to the carboxylic acid by OsO₄ and OxoneTM were unsuccessful.²⁵⁰ Lemieux-Johnson oxidation, with 2,6-lutidine as an additive^{251,252} furnished **4.2** (Figure 4-12) en route to the desired carboxylic acid.



Figure 4-12. Preparation of aldehyde 4.2 by Lemieux-Johnson oxidation.

Pinnick oxidation of **4.2** afforded an intermediate carboxylic acid, which was used crude for subsequent succinimidyl ester formation and amide formation with propargylamine, affording the protected alkyne derivative in 55% yield over 3 steps. Removal of the PMB ethers with DDQ^{248,249} proved unsuccessful, affording inseparable mixtures of byproducts. Instead, the PMB ethers of **4.3** were cleaved dilute with HCl in fluoroalcohol,^{253,254} indicated by rapid appearance of green fluorescence in the reaction flask. The desired deprotected amide **4.4** was isolated in 79% yield following purification by prep TLC.

With **4.4** in hand, I sought to demonstrate the utility of the alkyne-Pittsburgh Green conjugate to attach biologically relevant molecules. Standard click conditions for Huisgen cycloaddition, CuSO₄, TBTA, and sodium ascorbate,²⁵⁵⁻²⁵⁷ combined with a biotin-azide conjugate (Figure 4-13, box) afforded **4.5** in 55% yield, with structure and relative purity confirmed by LC-MS and HRMS.



Figure 4-13. Preparation of clickable Pittsburgh Green derivative 4.4 and biotin conjugate 4.5.

Though initial attempts to measure blinking for biotin derivative **4.5**, in collaboration with Callen Wallace of the Watkins Group (University of Pittsburgh Center for Biological Imaging) were unsuccessful, the formation of the biotin-tagged Pittsburgh Green highlights the

utility of the alkynyl amide for click chemistry. To prepare a better handle for blinking studies, I opted to use an antibody conjugate, similar to that utilized by the Urano group.⁹¹ Oxidation of **4.2** to the carboxylic acid and subsequent HCl-mediated deprotection afforded **4.6** in 87% yield over 2 steps. The derivative was conjugated to donkey anti-mouse whole molecule IgG through formation of the succinimidyl ester and subsequent mixing with donkey anti-mouse whole molecule IgG in PBS. The activated ester was expected to react with free alcohols, amines, and thiols to conjugate to the antibody. Purification through a NAP-5 column afforded the IgG-bound Pittsburgh Green **4.7**.



Figure 4-14. Preparation of Pittsburgh Green-IgG conjugate.

4.3.2 Super-resolution applications of Pittsburgh Green conjugates

In collaboration with Dr. Simon Watkins and Callen Wallace of the Center for Biological Imaging (University of Pittsburgh), the IgG-bound Pittsburgh Green was studied as a blinking fluorophore. Samples of antibody-labeled microtubules were treated with **4.7** and measured by STORM analysis. Tracking of blink events revealed a non-linear blinking frequency with several events prior to photobleaching (Figure 4-15). See Appendix A for further details.



Figure 4-15. Plotting intensity against time for a tracked spot of immobilized IgG-Pittsburgh Green.

These preliminary results show Pittsburgh Green has potential as a blinking fluorophore for super-resolution imaging, as it exists predominantly in a dark state. The recurring blink events are consistent with the pattern observed by Urano,⁹¹ further indicating that Pittsburgh Green displays similar properties to previously reported fluorophores. Future work will be aimed at utilizing Pittsburgh Green derivatives for super-resolution imaging of various structures.

4.3.3 Summary

I have successfully prepared the clickable blinking fluorophore, Pittsburgh Green derivative **4.4**, for super-resolution imaging. Attachment to a biotin-azide conjugate affording **4.5** through click chemistry demonstrates the potential for this compound as a fluorophore tag for biologically relevant motifs. The alkyne handle in **4.4** affords a robust platform for immobilization to azide-containing molecules. A Pittsburgh Green-IgG conjugate **4.7** for antibody labeling was prepared

and was found to exist in a predominantly dark state with multiple blink event as found by tracking a single spot with STORM analysis.

APPENDIX A

EXPERIMENTAL PROCEDURES

A.1 A COMPETITIVE AND REVERSIBLE DEACTIVATION APPROACH TO CATALYSIS-BASED QUANTITATIVE ASSAYS

A.1.1 Instrumentation

Ultraviolet–visible spectroscopy. The ultraviolet–visible spectra of RAE and resorufin solutions were acquired using a diode array spectrophotometer (Agilent Technologies, Santa Clara, CA) in a quartz cuvette. Other absorbance measurements were recorded in either a 96-well plate using a Modulus II Microplate Multimode reader (Promega, Madison, WI) measuring absorbance at 560 nm or in a clear, round bottom 96-well plates on a Spectra Max M5 spectrometer (Molecular Devices, Sunnyvale, CA) under the control of a Windows-based PC running software pro V5. The samples were analysed at λ =580 nm for the resorufin, and at λ =525 nm for RAE.

Fluorescence measurement. Fluorescence measurements were read on a Modulus II Microplate Multimode Reader (excitation 525 nm, emission 580–640 nm) or using a HoribaMax Fluorometer (excitation 578 nm, emission 350–700 nm).

A.1.2 Experimental Procedures.

Metal analysis by ICP-MS. The samples were either diluted or suspended directly in concentrated nitric acid or evaporated with a rotary evaporator first and then re-dissolved in concentrated nitric acid for ICP-MS analysis. Depending on the concentration range of the element, either a Perkin-Elmer Elan 6000 quadrupole ICP-MS spectrometer (Perkin-Elmer, Norwalk, CT) or a Thermo Finnigan Element 2 high-resolution ICP-MS spectrometer (Finnigan, Bremen, Germany) was used for the analysis.

General protocol for deallylation of RAE. A reaction cocktail was prepared by mixing 800 mM NH₄OAc in EtOH (10 ml) with 800 μ M RAE in EtOH (400 μ l) and 3 mM TFP in DMSO, with 250 ppm. BHT (800 μ l). The reaction cocktail (1 ml) was added to individual 2-ml Eppendorf tubes. To half of the samples was added 5% TraceMetal HNO₃ (20 μ l) as a control. To the other half of the samples was added a Pd²⁺ solution in 5% TraceMetal HNO₃ (20 μ l). To all the samples was added NaBH₄ in 10 N NaOH (20 μ l). The samples were mixed and transferred (200 μ l) to a 96-well black fluorescence well plate. Fluorescence (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 60 min using a Modulus II Microplate Multimode Reader.

Synthesis of resorufin allyl ether, RAE. A solution of resorufin sodium salt (200 mg, 0.850 mmol) in DMF (5 mL) was treated with K₂CO₃ (356 mg, 2.55 mmol, 3.0 equiv) followed by allyl bromide (1.4 mL, 0.94 mmol, 1.1 equiv) in DMF (1 mL) at 24 °C and stirred at the same temperature for 12 h. The reaction mixture was then guenched with water (100 mL), and the resulting mixture was filtered through a coarse fritted funnel. The resulting solid was washed with water $(3 \times 100 \text{ mL})$ and cold hexanes (100 mL). The solid was recrystallized from ethyl acetate and hexanes, affording resorufin allyl ether (181 mg, 0.71 mmol, 85% yield) as a dark red-orange solid. Data for resorufin allyl ether: $R_f = 0.34$ (50% ethyl acetate in hexanes); m.p.: 210.3–211.6 °C (decomp); IR: 2960, 2873, 1733 (C=O), 1466, 1369, 1151, 1031, 955.45, 862, 785, 735 cm-1; 1 H NMR (400 MHz, CDCl₃, 293 K): δ 7.71 (d, J = 8.8 Hz, 1H, Ar), 7.42 (d, J = 10.2 Hz, 1H, Ar), 6.96 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 6.84 (dd, J = 10.2, 2.0 Hz, 1H, Ar), 6.82 (d, J = 2.4 Hz, 1 H, Ar), 6.32 (d, J = 2.0 Hz, 1H, Ar), 6.06 (ddt, J = 17.2, 10.2, 5.1 Hz, 1H, 2'-H), 5.46 (ddt, J = 17.2, 3.0, 1.5 Hz, 1H, 3'-H_{cis}), 5.37(ddt, J = 10.2, 3.0, 1.5 Hz 1 H, 3'-H_{trans}), 4.65 (ddt, J = 5.1, 1.5, 1.5 Hz, 2H, 1'-H). 13C NMR (75 MHz, CDCl3, 293 K): δ 186.3, 162.6, 149.8, 145.65, 145.62 134.7, 134.3, 131.8, 131.6, 128.4, 118.9, 114.2, 106.8, 100.9, 69.6; HRMS: (ESI+) calc'd for $C_{15}H_{11}NO_3$ [M+H] = 254.0814, found 254.0798.

Determination of optimal phosphine ligand for deallylation of RAE. To separate scintillation vials was added triphenylphosphine (**P1**, Figure 2-9) (26.2 mg, 0.100 mmol), 1,2-bis(diphenylphosphino)ethane (**P2**) (39.5 mg, 0.100 mmol), 1,3-bis(diphenylphosphino)propane (**P3**) (41.6 mg, 0.100 mmol), 1,1'-bis(diphenylphosphino)ferrocene (**P4**) (55.4 mg, 0.100 mmol), bis(2-diphenylphosphinophenyl)ether (**P5**) (53.8 mg, 0.100 mmol), racemic-2,2'-bis(diphenylphosphino-1,1'-binaphthalene (**P6**) (62.2 mg, 0.100 mmol), tris(2,4-ditert-

butylphenyl)phosphite (P7) (64.8 mg, 0.100 mmol), (2-biphenyl)-di-tert-butylphosphine (P8) (29.6 mg, 0.100 mmol, 8), tri(2-furyl)phosphine (TFP) (P9) (23.7 mg, 0.100 mmol), 1,3,5triazaphosphaadamantane (P10) (15.6 mg, 0.100 mmol), tris(4-methoxyphenyl)phosphine (P11) (35.2 mg, 0.100 mmol), tri-tert-butylphosphonium tetrafluoroborate (P12) (29.8 mg, 0.100 mmol), tri(o-tolyl)phosphine (P13) (30.3 mg, 0.100 mmol), tri(p-tolyl)phosphine (P14) (30.6 mg, 0.100 mmol), and tris(4-fluorophenyl)phosphine (P15) (31.2 mg, 0.100 mmol), and DMSO (5.0 mL, [phosphine ligand] = 20 mM). To a separate vial, an aliquot of previously prepared solutions (48 μ L) were diluted in DMSO (252 μ L, [phosphine] = 3.2 mM). Two-fold serial dilutions were performed to afford 3200, 1600, 800, 400, 200, and 100 µM phosphine ligand solutions in DMSO. The reaction cocktail was prepared by mixing EtOH (23.6 mL), 800 µM RAE in EtOH (683 µL), PhCH₂NH₂ (621 µL), and 2.5 M NaBH₄ in 10 N NaOH (99 µL) at 0 °C. The resulting solution (175 μ L) was distributed to each well of a black round-bottom 96-well plate. The phosphine solutions (25 μ L) described above were added to each well affording final concentrations of 400, 200, 100, 50, 25, and 12.5 µM phosphine. To each well was added 1 ppm palladium standard solution in 5% TraceMetal nitric acid (10 μ L, [Pd²⁺]_{final} = 40 ppb). Fluorescence (excitation 525 nm, emission 570–640 nm) was recorded after 1, 30, and 60 min. The ratio of fluorescence was compared to 400 µM triphenylphosphine as a control and is reported.

Rescreening of active phosphine ligand. The reaction cocktail was prepared by mixing EtOH (23.6 mL), 800 μ M RAE in EtOH (683 μ L), PhCH₂NH₂ (621 μ L), and 2.5 M NaBH₄ in 10 N NaOH (99 μ L) at 0 °C. The resulting solution (175 μ L) was distributed to each well of a black round-bottom 96-well plate. The phosphine solutions of **P1**, **P9**, and **P11** (25 μ L) described

above were added to each well affording final concentrations of 400, 200, 100, 50, 25, and 12.5 μ M phosphine. To each well was added 1 ppm palladium standard solution in 5% TraceMetal nitric acid (10 μ L, [Pd²⁺]_{final} = 40 ppb). Absorbance (560 nm) was measured after 60 min.

Determination of optimal tri-(2-furyl)phosphine (P9) concentration. In a scintillation vial was added TFP (**P9**) (23.2 mg, 0.100 mmol) and DMSO containing 250 ppm BHT (5.0 mL; [TFP] = 20 mM). To a white chemically resistant plate was added this solution (192 μ L) and DMSO (108 μ L, [TFP] = 12.8 mM). From this solution, 2x serial dilutions in DMSO were performed to concentrations of 0.400, 0.200, 0.100, 0.0500, 0.0250, and 0 mM. The reaction cocktail was prepared by mixing EtOH (7.6 mL), 800 μ M RAE in EtOH (220 μ L), PhCH₂NH₂ (200 μ L) and 2.5 M NaBH₄ in 10 N NaOH (32 μ L) at 0 °C. The solution (175 μ L) was transferred to each reaction well in a 96- well clear, flat-bottom absorbance plate. The TFP solutions (25 μ L) described above were added to each well for final concentrations of 400, 200, 100, 50, 25, and 12.5 μ M. A 20 ppb palldium standard solution in 5% TraceMetal HNO₃ (10 μ L) was measured after 1 and 60 min using a Modulus II Microplate Multimode Reader.

Preparation of stock metal solutions. To separate scintillation vials were added AgNO₃ (425 mg, 2.50 mmol), AuCl₃ (759 mg g, 2.50 mmol), CdCl₂ (459 mg, 2.50 mmol), CoCl₂ (325 mg, 2.50 mmol), CrCl₃ (396 mg, 2.50 mmol), FeCl₃ (406 mg, 2.50 mmol), HgCl₂ (679 g, 2.501 mmol), MnCl₂ (315 mg, 2.50 mmol), NiCl₂ (324 mg, 2.50 mmol), PtCl₂ (665 mg, 2.50 mmol), ZnCl₂ (341 mg, 2.50 mmol), Sr(NO₃)₂ (529 mg, 2.50 mmol), IrCl₃ (745 mg, 2.50 mmol) and Cu(NO₃)₂ (336 mg, 2.50 mmol) and ultrapure H₂O (2.5 mL; [metal] = 1.0 M). To separate

scintillation vials were added a metal solution (50 μ L) that was prepared above, and ultrapure H₂O (4.95 mL; [metal] = 10 mM. Two serial dilutions of the metal solution (20 μ L) with ultrapure water (180 μ L) were carried out to afford a final concentration of 100 μ M. In separate scintillation vials was added either RuCl₃ (10.4 mg, 50.1 μ mol) or RhCl(PPh₃)₃ (45 mg, 50 μ mol) and ultrapure H₂O (5.00 mL) To separate scintillation vials was added these solutions (100 μ L) and Ultrapure H₂O (9.9 mL, [metal] = 100 μ M). A 10 μ M solution of Pd(NO₃)₂ was prepared through the serial dilution of a 100 ppm palladium standard solution in 10% HNO₃ (10 μ L) with ultrapure water (990 μ L).

Determination of metal selectivity for RAE. The reaction cocktail was prepared by mixing 800 mM NH₄OAc in EtOH (15 mL), 800 μ M RAE in EtOH (600 μ L), 3.0 M TFP (**P9**) in DMSO, stabilized by 250 ppm BHT in DMSO (1.2 mL) and 0.1 M NaBH₄ in 10 N NaOH (450 μ L) at 0 °C. The solution (180 μ L) was distributed to each well in a clear flat-bottom 96-well plate. The deallylation reaction was performed in triplicate by transferring the 100- μ M metal solutions or 10 μ M in the case of Pd²⁺ (20 μ L) to the reaction cocktail. The well plates were incubated at 25 °C. Absorbance (560 nm) was measured 1 min and 1 h after the transfer using a Modulus II Microplate Multimode Reader. After 1 h, wells corresponding to Au, Ag, Hg, Pd, and Pt were transferred to a 96-well black fluorescence well plate and fluorescence (excitation 525 nm, emission 580–640 nm) was measured using a Modulus II Microplate Multimode Reader.

Determination of interference by other metals. Metal solutions were prepared as in "Preparation of stock metal solutions". On a black 96-well plate was added each 100 μ M solution of metals in ultrapure H₂O (990 μ L) that was prepared as described above and a 100 μ M solution of Pd(NO₃)₂ in ultrapure H₂O (10 μ L). The reaction cocktail was prepared by mixing 800 mM NH₄OAc in EtOH (15 mL), 800 μ M RAE in EtOH (600 μ L), 3.0 M TFP (P9) in DMSO

stabilized by 250 ppm BHT (1.2 mL) and 0.1 M NaBH₄ in 10 N NaOH (450 μ L) at 0 °C. The solution (180 μ L) was distributed to a clear flat-bottom 96-well plate. The deallylation reaction was performed in triplicate by transferring the metal solutions described above (20 μ L) to the reaction cocktail on the plate. The well plates were incubated at 25 °C. Absorbance (560 nm) was measured 1 min and 1 h after the transfer using a Modulus II Microplate Multimode Reader.

Testing allyl Pittsburgh Green ether (APE) for autonomous stalling with NH₄OAc. The acetate-containing reaction cocktail was prepared by mixing NH₄OAc (440 mg, 5.71 mmol) with EtOH (2 mL) and ultrapure H₂O (7.5 mL), 800 μ M APE in DMSO (250 μ L), and 3.2 mM TFP in DMSO stabilized with 250 ppm BHT (250 μ L). In a separate vial, the phosphate-containing reaction cocktail was prepared by mixing 1.2 M potassium phosphate pH 7 buffer (5 mL), EtOH (2 mL), ultrapure H₂O (2.5 mL), 800 μ M APE in DMSO (250 μ L), and 3.2 mM TFP (**P9**) in DMSO stabilized with 250 ppm BHT (250 μ L). The reaction cocktail (1 mL) was added to 2-mL Eppendorf tubes. To half of the samples for each buffer was added 5% TraceMetal HNO₃ (20 μ L). To all samples was added 0.5 M NaBH₄ in 10 N NaOH (20 μ L). The samples were mixed and added (200 μ L) to a 96-well black fluorescence well plate. Fluorescence (excitation 490 nm, emission 510–570 nm) was measured every 2 min for 60 min using a Modulus II Microplate Multimode Reader.

Deallylation under inert atmosphere. Three separate round-bottom flasks were vacuumed and sealed with a rubber stopper. Either a balloon of N₂, O₂, or air (78% N₂, 21% O₂) was then attached with a needle. A scintillation vial was treated with 800 mM NH₄OAc in EtOH (9 mL), 3 mM TFP in DMSO stabilized by 250 ppm BHT (720 μ L), and 800 μ M RAE in EtOH (360 μ L). Aliquots (2 mL) of this solution were added to each flask followed by 1 ppm Pd(NO₃)₂ in 5%

HNO₃ (100 μ L) and 40 mM NaBH₄ in 10 N NaOH (50 μ L) via syringe. After 15 min, aliquots (200 μ L) of each solution were transferred to a black 96-well plate, and fluorescence (excitation 525 nm, emission 570–640 nm) was measured using a Modulus II Microplate Multimode Reader.

Analysis of ores. Ore samples used were provided by Stillwater Mining Co. and were previously analyzed for palladium content using APE following aqua regia digestion and by solid-state palladium extraction Initial attempts to directly analyze the samples with RAE failed, likely due to difficulty in palladium extraction from the rock samples in the short reaction time. Whereas our previous method with APE could extract palladium and the reaction would run indefinitely, our new method stalls before a signal could be recovered and before significant palladium was extracted. To alleviate this with the single cocktail, I extracted palladium using TFP in DMSO followed by the addition of RAE and NH₄OAc-containing EtOH. NaBH₄ was subsequently added to the samples. To 2-mL Eppendorf tubes were added ore samples (75 mg, Note: These samples contained ranges of 0 to 0.068% Pd by weight as determined by previous analysis using APE) and 3 mM TFP in DMSO stabilized by 250 ppm BHT (145 µL). Samples were sonicated for 60 s. To a scintillation vial was added 800 mM NH₄OAc in EtOH (6.48 mL) and 800 μM RAE in EtOH (259 μ L). This solution (1.86 mL) was added to each Eppendorf tube containing ore samples. To each sample was added 2.5 M NaBH₄ in 1 N NaOH (20 µL). The samples were incubated at 25 °C for 5 min. An additional 2.5 M NaBH₄ in 1 N NaOH (20 µL) was added and again the samples were incubated for 5 min. To each sample was again added 2.5 M NaBH₄ in 1 N NaOH (20 µL, 75 mM NaBH₄ final) and the samples were incubated for 5 min, centrifuged using a Galaxy II Mini benchtop centrifuge for 20 s, and the resulting slurry was recorded with a photograph obtained under ambient light.

Preparation of polymer solutions. Solutions of polymers provided by the Krebs group (Technical University of Denmark), prepared by either batch or flow synthesis with varying amounts of palladium catalyst (Table 2-2) were dissolved in either PhMe ("Sample 2") or CHCl₃ ("Sample 1, 3, 4, 5, 6, 7") to afford concentrations of 1,000 μ g/mL (1,000 ppm) of polymer.

Analysis of polymers by aqua regia digestion. To separate 2-dram vials were added polymer solution (400 μ L), placed under a stream of air at 25 °C and evaporated to dryness. To these vials was added freshly prepared aqua regia (100 μ L) and the samples were allowed to digest for 16 h at 25 °C. To each vial was added ultrapure H₂O (900 μ L). Reaction cocktail described in "General protocol for deallylation of RAE" (1 mL) was added to 2-mL Eppendorf tubes and to this solution was added either 10% aqua regia or polymer suspension in 10% aqua regia (20 μ L, [polymer] = 5 ppm) and 2.5 M NaBH₄ in 10 N NaOH (20 μ L). Each solution (200 μ L) was transferred to a black 96-well fluorescence plate and the fluorescence (excitation 525 nm, emission 570–640 nm) was measured after 30 min. The observed result did not match the expected palladium concentration shown in Table 2-2.

Determination of interference by polymers. To separate 2-dram vials was added 1000 ppm "Sample 4" in CHCl₃ (400 μ L). Vials were placed under a stream of air at 25 °C and evaporated to dryness. To these vials was added freshly prepared aqua regia (100 μ L) and the samples were allowed to digest for 16 h at 25 °C. To each vial was added ultrapure H₂O (900 μ L). Reaction cocktail described in "General protocol for deallylation of RAE" (1 mL) was added to 2-mL Eppendorf tubes and to this solution was added either 10% aqua regia or polymer suspension in 10% aqua regia (20 μ L, [polymer] = 8 ppm), to half of the samples was added H₂O, to the other half was added 50 ppb Pd(NO₃)₂ in H₂O (20 μ L), and 2.5 M NaBH₄ in 10 N NaOH (20 μ L).

Each solution (200 μ L) was transferred to a black 96-well fluorescence plate and the fluorescence (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 30 min.

Heat-assisted polymer analysis. To separate 2-dram vials were added 1000 ppm polymer solutions in PhMe ("Sample 2") or CHCl₃ ("Samples 1, 3, 4, 5, 6, 7") (400 μ L). The vials were placed under a stream of air at 25 °C and evaporated to dryness. To each vial was added 5% TraceMetal HNO₃ (500 μ L) and the resulting suspensions (800 ppm polymer) were loosely sealed with threaded tape and screw caps and incubated in a 70 °C water bath for 3 h. Reaction cocktail described in "General protocol for deallylation of RAE" (1 mL) was added to 2-mL Eppendorf tubes and to each vial was added either 5% TraceMetal HNO₃ or polymer suspension in 5% HNO₃ (20 μ L, [polymer] = 16 ppm) and 2.5 M NaBH₄ in 10 N NaOH (20 μ L). Each solution (200 μ L) was transferred to a black 96-well fluorescence plate and the fluorescence (excitation 525 nm, emission 570–640 nm) was measured after 30 min.

Inhibition of horseradish peroxidase (HRP). Amplex Red (a.k.a. Ampliflu Red, 10-acetyl-3,7dihydroxyphenoxazine) was purchased from Life Technologies (Catalog no. A22188) and was stored at -20 °C in single use ampules as provided. To a scintillation vial was added horseradish peroxidase (3.3 mg 303 U/mg) and 1× PBS (10 mL, 10 U/mL final concentration). The solution was separated into single use 1 mL aliquots and stored at -20 °C. To a 2-dram vial was added PhB(OH)₂ (15, 30, 150 mg; 0.12, 0.14, 1.2 mmol) and 1× PBS (4.96 mL) to afford stock solutions. To each solution of PhB(OH)₂ was added either H₂O or 6.36 mM H₂O₂ in H₂O (40 μ L) and the solutions were incubated at 24 °C for 10 min. While the solutions were incubating, to a single ampule of Amplex Red (154 μ g) was added DMSO (60 μ L). The reaction cocktail was prepared by combining 10 mM Amplex Red in DMSO (50 μ L), 10 U/mL HRP in 1× PBS (100 μ L), and 1× PBS (4.85 mL). Aliquots of the reaction cocktail (50 μ L) were transferred to a 96-well black fluorescence plate. To each well was added either $1 \times PBS$ with or without 50 μ M H₂O₂ containing 50, 100, or 500 μ M PhB(OH)₂. The plate was incubated at 25 °C and fluorescence intensity (excitation 525 nm, emission 570–640 nm) was measured after 30 min.

Restarting a stopped HRP reaction. A 20 mM stock solution of PhB(OH)₂ was prepared by adding PhB(OH)₂ (24 mg, 0.20 mmol) to $1 \times$ PBS (10 mL). This solution was divided into aliquots (1.5 mL) and to each was added either H₂O or 1.05 M H₂O₂ in H₂O (30 μ L). A reaction cocktail was prepared by combining 10 mM Amplex Red in DMSO (25 µL), 10 U/mL horseradish peroxidase (25 μ L), and 1× PBS (2.5 mL). Aliquots of the reaction cocktail (50 μ L) were transferred to a 96-well black fluorescence plate and to each well was added either 1× PBS with or without 10 µM H₂O₂ containing 0 or 10 mM PhB(OH)₂ (50 µL). Fluorescence intensity (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 10 min. To each well was again added either $1 \times PBS$ with or without 10 $\mu M H_2O_2$ containing 0 or 10 mM PhB(OH)₂ (50 μ L). Fluorescence intensity (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 10 min. Data presented in Figure 2-21c are normalized to account for the dilution from adding the second aliquot (50 μ L) to the solutions on the plate (100 μ L) to afford a final volume of 150 μ L. Fluorescence intensities are scaled to 1.5x observed values to account for this. Observed saturation in restarting a stopped HRP reaction. A 40 mM stock solution of PhB(OH)2 was prepared by adding PhB(OH)₂ (48 mg, 0.40 mmol) to $1 \times$ PBS (10 mL). This solution was divided into aliquots (1.5 mL) and to each was added either H₂O or 1.05 M H₂O₂ in H₂O (30 µL). A reaction cocktail was prepared by combining 10 mM Amplex Red in DMSO (25 μ L), 10 U/mL horseradish peroxidase (250 μ L), and 1× PBS (2.25 mL). Aliquots of the reaction cocktail (50 μ L) were transferred to a 96-well black fluorescence plate and to each well was added either 1× PBS with or without 10 μ M H₂O₂ containing 0 or 20 mM PhB(OH)₂ (50 μ L).

Fluorescence intensity (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 10 min. To separate scintillation vials was added 0 or 10 mM PhB(OH)2 in 1× PBS (1.5 mL) and either H₂O (60 μ L) or 1.05 M H₂O₂ (60 μ L), affording new stock solutions. To each well was added the new stock solution, either 1× PBS with or without 20 μ M H₂O₂ containing 0 or 20 mM PhB(OH)₂ (50 μ L). Fluorescence intensity (excitation 525 nm, emission 570–640 nm) was measured every 2 min for 10 min. Data presented in Figure 2-21d are normalized to account for the dilution from adding the second aliquot (50 μ L) to the solutions on the plate (100 μ L) to afford a final volume of 150 μ L. Fluorescence intensities are scaled to 1.5x observed value to account for this.

A.2 FLUORESCENCE-BASED KINETICS OF A TSUJI-TROST REACTION IN AQUEOUS SOLUTION

A.2.1 Materials and Methods

Stock solutions. Solutions of APE in DMSO were prepared and stored in amber bottles at 25 °C. Solutions of TFP in DMSO, stabilized by 500 ppm BHT, were prepared and stored in amber bottles at 25 °C for >6 months without decomposition. Palladium solutions were prepared by diluting a high purity palladium standard (9.4 mM in 10% nitric acid) with 5% TraceMetal nitric acid in ultrapure H2O.

Instrumentation. All fluorescence measurements were carried out using a Turner Biosystems Modulus II Microplate Reader. Temperature gradients were prepared using an Eppendorf Mastercycler Gradient PCR thermocycler.

A.2.2 Experimental procedures.

Measuring consumption of APE. In a scintillation vial, 1.23 M phosphate pH 7 buffer (9.0 mL), 0.2, 1.0, 2.0 mM APE in DMSO (250 μ L; [APE]_{final} = 5.0, 25, or 50 μ M), DMSO (500 μ L), and 3.2 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 80 μ M) were combined. The resulting solution was cooled to 0 °C on ice. To this solution was added 500 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 1.0 μ M) and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting solution was warmed to room temperature. Aliquots (20 μ L) of the solution were transferred to a 96-well black fluorescence plate containing 1.2 M phosphate pH 7 buffer (180 μ L; final volume per well = 200 μ L) every 3 minutes for 20 min, followed by every 10 minutes for 200 min. Fluorescence (excitation 490 nm, emission 510-570 nm) was measured. Moles of Pittsburgh Green were calculated using a previously prepared standard curve and used to determine the quantities of the reaction product. Amount of remaining APE was calculated by subtracting the amount of Pittsburgh Green produced from the initial concentration of APE.

Deallylation under optimized conditions. In a scintillation vial, 1.23 M phosphate pH 7 buffer (9.5 mL), 800 μ M APE in DMSO (250 μ L; [APE]_{final} = 20 μ M), and 4.8 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 120 μ M) were combined. The resulting solution was cooled to 0 °C on ice. To this solution was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM) and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution was analyzed according to "General procedure for Eyring plot preparation" after incubation for 30 min.

General procedure for Eyring plot preparation. The reaction solution (200 μ L; preparation described below) was transferred to a 200- μ L thin-walled PCR tube, capped, and placed in the

thermocycler with 5 replicates per temperature. A 3 °C temperature gradient was applied, with median temperature as indicated, spanning a 20 °C range. After 30–180 min, where indicated, the vials were removed, cooled to 0 °C on ice, and each solution (180 μ L) was transferred to a black 96-well fluorescence plate. Fluorescence (excitation 490 nm, emission 510–570 nm) was measured. Moles of Pittsburgh Green were calculated using a previously prepared standard curve and used to determine the quantities of the reaction product. The natural log of the rate against the inverse of the temperature was plotted to afford an Eyring plot for each condition tested. Data were plotted and analyzed using Graphpad Prism 5.0c.

Effect of APE concentration. In separate scintillation vials, 1.23 M phosphate buffer (9.5 mL), 4.8 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 120 μ M) and either 0.5, 1.0, or 2.0 mM APE in DMSO (250 μ L; [APE]_{final} = 12.5, 25, 50 μ M), and were combined. The resulting solutions were cooled to 0 °C on ice. To these solutions was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO3 (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM) and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution was analyzed according to "General procedure for Eyring plot preparation". A temperature gradient was applied for 30 min, with median autocycler temperatures of 30, 45, 60, and 75 °C to span 20-85 °C.

Effect of phosphine ligand concentration. In separate scintillation vials, 1.23 M phosphate pH 7 buffer (9.5 mL), 0.5 or 2.0 mM APE in DMSO (250 μ L; [APE]_{final} = 12.5 or 50 μ M), and TFP (250 μ L of 2.4, 4.8, or 9.6 mM TFP in DMSO, stabilized by 500 ppm BHT; [TFP]_{final} = 60, 120, or 180 μ M) were combined. The resulting solutions were cooled to 0 °C on ice. To each solution was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM) and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution was analyzed according to General procedure for Eyring plot preparation. A temperature gradient

was applied for 30 min, with median autocycler temperatures of 30, 45, 60, and 75 °C to span 20-85 °C.

Determination of TFP Dependence. To separate Eppendorf tubes were added TFP in DMSO, stabilized by 500 ppm BHT (133 μ L of 0.23, 0.45, 0.68, 0.90, 1.1, 1.4, 1.6, 1.8, 2.0, 2.3, 2.5, 2.7, 3.0 mM stock in DMSO; [TFP]_{final} = 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 μ M) followed by 9.4 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 94 nM). To each vial was added 1.23 M phosphate pH 7 buffer (1.71 mL). The solutions were incubated at either 25 or 43 °C for 30 min. To each solution was added 60 mM NaBH₄ in 10 N NaOH (66 μ L, [NaBH₄]_{final} = 2 mM) followed by 1.5 mM ARE in DMSO (66 μ L; [APE]_{final} = 50 mM) in DMSO. The solutions were mixed and incubated at either 23 °C or 43 °C for 60 min. Aliquots (200 μ L) were transferred to a 96-well black fluorescence plate, and fluorescence (excitation 490 nm, emission 510–570 nm) was measured.

Effect of DMSO on TFP-palladium binding. To separate 2-mL Eppendorf tubes was added 1.23 M phosphate pH 7 buffer (937, 962, or 974 μ L), 1.6 mM APE in DMSO (13 μ L, [APE]_{final} = 20 μ M) and TFP in DMSO, stabilized by 500 ppm BHT (13 μ L of 16 mM, 25 μ L of 8 mM, 50 μ L of 4 mM TFP; [TFP]_{final} = 0, 50, 100, 200 μ M) to afford a final volume of 1 mL. To each solution was added 23.5 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L, [Pd(NO₃)₂]_{final} = 470 nM) and 500 mM NaBH₄ in 10 N NaOH (20 μ L). Each solution (200 μ L) was transferred to a black 96-well fluorescence plate, and fluorescence (excitation 490 nm, emission 510–570 nm) was recorded initially and after incubation at 25 °C for 60 min.

Effect of phosphate ions on TFP-palladium binding. To separate scintillation vials was added either 1.23 M phosphate pH 7 buffer (18 mL) or 1.23 M phosphate pH 7 buffer (7.32 mL 1.23 M phosphate buffer) and ultrapure H₂O (10.68 mL, [phosphate]_{final} = 500 mM). To each vial was added 800 μ M APE in DMSO (459 μ L; [APE]_{final} = 20 μ M). Each solution (975 μ L) was transferred to 2-mL Eppendorf tubes. To each solution, TFP solutions in DMSO (25 μ L of 0, 0.5, 1.0, 2.0, 4.0, 8.0 mM; [TFP]_{final} = 0, 12.5, 25, 50, 100, 200 μ M) were added. To each solution was added 23.5 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]final = 470 nM) and 500 mM NaBH₄ in 10 N NaOH (20 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution (200 μ L) was transferred to a black 96-well fluorescence plate, and fluorescence (excitation 490 nm, emission 510-570 nm) was recorded initially and after incubation at 25 °C for 60 min.

Effect of phosphate ion concentration. In separate scintillation vials, either 0.3, 0.6 or 1.23 M phosphate pH 7 buffer (9.5 mL), either 0.5 or 2.0 mM APE in DMSO (250 μ L; [APE]_{final} = 12.5 or 50 μ M), and 4.8 TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 120 μ M) were combined. Resulting solutions were cooled to 0 °C. To this cocktail was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution was analyzed according to "General procedure for Eyring plot preparation". A temperature gradient was applied for 30 min, with median autocycler temperatures of 30, 45, 60, and 75 °C to span 20-85 °C.

Effect of NaBH₄ **Concentration**. In separate scintillation vials, 1.23 M phosphate pH 7 buffer (9.5 mL), either 0.5 or 2.0 mM APE in DMSO (250 μ L; [APE]_{final} = 12.5 or 50 μ M), and 4.8 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 120 μ M) were combined. The resulting solutions was cooled to 0 °C. To each solution was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM) and NaBH₄ in 10 N NaOH (40 μ L 0, 25, 250, 2.5 M NaBH₄; [NaBH₄]_{final} = 0, 0.1, 1.0, 10 mM). The resulting reaction solution was analyzed according to "General procedure for Eyring plot preparation". A temperature gradient

was applied for 30 min, with median autocycler temperatures of 30, 45, 60, and 75 °C to span 20-85 °C.

Effect of DMSO concentration. In a scintillation vial, 1.23 M phosphate pH 7 buffer (9.5 mL), 500 or 2000 μ M APE in DMSO (250 μ L; [APE]_{final} = 12.5 or 50 μ M), and 4.8 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ M; [TFP]_{final} = 120 μ M, [DMSO]_{final} = 5%) were combined. In a separate scintillation vial, 1.23 M phosphate pH 7 buffer (9.0 mL), 500 or 2000 μ M APE in DMSO (250 μ L; [APE]_{final} = 12.5 or 50 μ M), and 4.8 mM TFP in DMSO, stabilized by 500 ppm BHT (250 μ L; [TFP]_{final} = 120 μ M), and DMSO (500 μ L; [DMSO]_{final} = 10%) were combined. All resulting solutions were cooled to 0 °C on ice. To each solution was added 4.7 μ M Pd(NO₃)₂ in 5% TraceMetal HNO₃ (20 μ L; [Pd(NO₃)₂]_{final} = 9.4 nM) and 2.5 M NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM). The resulting reaction solution was analyzed according to "General procedure for Eyring plot preparation". A temperature gradient was applied for 30 min, with median autocycler temperatures of 30, 45, 65, °C to span 20-75 °C.

Determination of TFP as nucleophile using "same excess" measurements with optimized TFP conditions. In a PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 2400 μ M APE in DMSO (50 μ L; [APE]_{final} = 60 μ M) and 3200 μ M TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; [TFP]_{final} = 80 μ M) were combined. In a separate PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 2000 μ M APE in DMSO (50 μ L; [APE]_{final} = 50 μ M) and 2800 μ M TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; [TFP]_{final} = 70 μ M) were combined. In a separate PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 2000 μ M APE in DMSO (50 μ L; [TFP]_{final} = 70 μ M) were combined. In a separate PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 1600 μ M APE in DMSO (50 μ L; [APE]_{final} = 40 μ M) and 2400 μ M TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; [TFP]_{final} = 40 μ M) and 2400 μ M TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; [TFP]_{final} = 60 μ M) were combined. All resulting solutions were cooled to 0 °C on ice. To each solution was added 180 μ M Pd(NO₃)₂ in 5% TraceMetal HNO3 (40 μ L; [Pd(NO₃)₂]_{final} = 3.6 μ M) and

500 mM NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM. The resulting solutions were warmed to 24 °C, and an aliquot (20 μ L) was transferred to 1.23 M phosphate pH 7 buffer (180 μ L) on a 96-well black fluorescence plate, and fluorescence (excitation 490 nm, emission 510-570 nm) of the resulting solution was measured. Aliquots (20 μ L) were removed and treated as previously described every 10 min for 120 min.

Determination of TFP as a nucleophile using "same excess" measurements with excess TFP conditions. In a PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 2400 µM APE in DMSO (50 μ L; [APE]_{final} = 60 μ M) and 4800 μ M TFP in DMSO, stabilized by 500 ppm BHT $(50 \ \mu\text{L}; [TFP]_{\text{final}} = 120 \ \mu\text{M})$ were combined. In a separate PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 2000 µM APE in DMSO (50 µL; [APE]_{final} = 50 µM) and 4400 µM TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; [TFP]_{final} = 110 μ M) were combined. In a separate PVC plastic tray, 1.23 M phosphate pH 7 buffer (1.9 mL), 1600 µM APE in DMSO (50 μ L; [APE]_{final} = 40 μ M) and 4000 μ M TFP in DMSO, stabilized by 500 ppm BHT (50 μ L; $[TFP]_{final} = 100 \ \mu M$) were combined. All resulting solutions were cooled to 0 °C on ice. To each solution was added 180 µM Pd(NO3)2 in 5% TraceMetal HNO3 (40 µL; [Pd(NO₃)₂]_{final} = 3.6 μ M) and 500 mM NaBH₄ in 10 N NaOH (40 μ L; [NaBH₄]_{final} = 10 mM. The resulting solutions were warmed to 24 $^{\circ}$ C, and an aliquot (20 μ L) was transferred to 1.23 M phosphate pH 7 buffer (180 μ L) on a 96-well black fluorescence plate, and fluorescence (excitation 490 nm, emission 510-570 nm) of the resulting solution was measured. Aliquots (20 μ L) were removed and treated as previously described every 10 min for 120 min.

A.2.3 Cartesian Coordinates (Å) and Energies of the Transition States TS1-TS3

TS1

B3LYP SCF energy: -2610.19661655 a.u.
B3LYP enthalpy: -2609.618326 a.u.
B3LYP free energy: -2609.749451 a.u.
M06 SCF energy in solution: -2610.64493225 a.u.
M06 enthalpy in solution: -2610.066642 a.u.
M06 free energy in solution: -2610.197767 a.u.
Three lowest frequencies (cm-1): -37.3737 8.7514 10.4212
Imaginary frequency: -37.3737 cm-1

Cartesian coordinates

ATO	M X	Y	Ζ
С	-0.474842	-2.208709	2.190637
Н	-0.108439	-3.120898	1.724911
Н	0.152507	-1.774422	2.963040
С	-1.628307	-1.644248	1.824811
Н	-1.973039	-0.731094	2.305904
С	-2.496767	-2.160833	0.717898
Н	-2.475969	-1.462453	-0.128662
Н	-2.145056	-3.143052	0.369770
0	-3.838310	-2.260917	1.211132
С	-4.836347	-2.532275	0.323978

С	-4.639266	-2.845637	-1.028569
С	-6.140637	-2.493079	0.843138
С	-5.746052	-3.108097	-1.844892
Н	-3.640966	-2.898106	-1.447691
С	-7.229010	-2.757575	0.019553
Н	-6.269760	-2.249826	1.893276
С	-7.041332	-3.065184	-1.333261
Н	-5.581567	-3.355163	-2.890930
Н	-8.232640	-2.721738	0.435639
Н	-7.893562	-3.271422	-1.974226
Pd	0.678965	0.153498	0.129322
Р	-0.757840	1.962544	-0.017261
Р	2.709815	-0.922198	-0.102060
0	-2.810481	0.726000	-1.294165
С	-3.777157	0.864625	-2.241650
С	-3.725960	2.108684	-2.793487
С	-2.655779	2.794076	-2.133225
С	-2.125287	1.916795	-1.224926
0	-1.113838	2.109777	2.685814
С	-1.680459	2.459085	1.480514
С	-1.948041	2.548185	3.665221
С	-3.040930	3.160282	3.128086
С	-2.867705	3.102101	1.709841

0	-0.599855	4.693235	-0.536316
С	0.085083	3.496016	-0.500730
С	0.292196	5.650694	-0.905037
С	1.526858	5.110173	-1.105638
С	1.393884	3.710256	-0.840767
С	1.606793	-3.431123	-1.010783
С	2.675967	-2.661613	-0.634235
0	3.822694	-3.423300	-0.692501
С	3.459354	-4.665999	-1.106528
С	2.114999	-4.732033	-1.319040
0	3.982360	1.142730	-1.347584
С	3.898942	-0.230085	-1.304000
С	4.895370	1.462392	-2.304111
С	5.397037	0.336760	-2.882758
С	4.753464	-0.763223	-2.231627
0	4.954105	-1.502718	1.446902
С	3.686316	-0.968511	1.431006
С	5.422630	-1.382347	2.715130
С	4.499094	-0.783887	3.520131
С	3.370983	-0.512210	2.684485
Н	-3.533509	3.486306	0.950388
Н	-3.868320	3.595479	3.671394
Н	-1.628470	2.346657	4.676707

Н	-4.407866	0.000791	-2.390575
Н	-4.368828	2.494813	-3.572377
Н	-2.325600	3.808854	-2.298579
Н	4.912989	-1.815390	-2.413131
Н	6.133920	0.290893	-3.672704
Н	5.070077	2.517805	-2.449302
Н	-0.101170	6.654364	-0.973389
Н	2.421210	5.641770	-1.399776
Н	2.157345	2.946224	-0.890250
Н	2.442799	-0.030258	2.958172
Н	4.608429	-0.560516	4.572229
Н	6.419235	-1.763284	2.883302
Н	4.262705	-5.382512	-1.194402
Н	1.556006	-5.596141	-1.649989
Н	0.581584	-3.092968	-1.058986

TS2

B3LYP SCF energy:	-2610.	14578595 a.u.
B3LYP enthalpy:	-2609.	570852 a.u.
B3LYP free energy:	-2609.	696249 a.u.
M06 SCF energy in so	lution:	-2610.64799040 a.u.
M06 enthalpy in soluti	on:	-2610.073056 a.u.
M06 free energy in sol	ution:	-2610.198453 a.u.

 Three lowest frequencies (cm-1):
 -82.8653
 10.7229
 17.9745

 Imaginary frequency:
 -82.8653 cm-1

Cartesian coordinates

ATO	M X	Y	Ζ
С	-0.573614	-2.674572	-1.975989
Н	-0.536289	-3.352410	-1.127213
Η	-1.433085	-2.788040	-2.630682
С	0.616146	-2.091100	-2.457601
Η	0.639059	-1.677360	-3.464827
С	1.664506	-1.807211	-1.563294
Η	2.503760	-1.199816	-1.879224
Η	1.816174	-2.377601	-0.650043
Pd	-0.285933	-0.718227	-0.923935
Р	0.927286	1.211897	-0.163778
Р	-2.483681	-0.558628	-0.022044
0	0.905883	-0.012490	2.258317
С	1.360729	0.073921	3.531769
С	2.130116	1.189042	3.696304
С	2.156311	1.839265	2.425513
С	1.399131	1.074317	1.577587
0	2.454979	2.265286	-2.140358
С	2.521623	1.582849	-0.947223

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- Н 5.708168 -2.617327 -1.784787
- C 6.238013 -0.922774 1.141537
- Н 4.882099 -0.944713 2.821861
- Н 7.367160 -1.133311 -0.688206
- Н 6.970068 -0.280123 1.624109

TS3

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B3LYP enthalpy:	-3332.08	83174	a.u.		
B3LYP free energy:	-3332.2	33989	a.u.		
M06 SCF energy in so	lution: -	-3333.	10718080) a.u.	
M06 enthalpy in soluti	on:	-3332	431310 a	.u.	
M06 free energy in solution: -3332.582125 a.u.					
Three lowest frequence	ies (cm-1): -2	01.2262	6.1534	9.5198
Imaginary frequency:	-201.22	262 cm	i-1		

Cartesian coordinates

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- C 6.304992 -3.895975 0.014208
- C 5.102980 -4.450972 -0.312626
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- Н 4.335539 0.529797 4.606868
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- Н 4.898970 -5.500205 -0.471490
- Н 3.126005 -3.419515 -0.637188

A.3 DEVELOPMENT OF A BLINKING FLUOROPHORE CONJUGATE FOR SUPER-RESOLUTION IMAGING APPLICATIONS

A.3.1 Synthetic Procedures



Preparation of bis-allyl-2',7'-dichlorofluorescein. To a solution of 2',7'-dichlorofluorescein (40 g; 100 mmol) in DMSO (200 mL) was added K₂CO₃ (34.6 g; 250 mmol) and allyl bromide (43.2 mL; 500 mmol). The resulting mixture was heated to 60 °C for 20 h. The mixture was cooled to 24 °C and poured into ice water (2.0 L). The resulting solid was collected by filtration, washed with H₂O (500 mL), cold hexanes (500 mL), and dried under air, affording bis-allyl-2',7'-dichlorofluorescein (23 g; 48% yield). Spectroscopic information matched those found in literature (Sparano, B.A.; Shahi, S.P.; Koide, K. *Org. Lett.* **2004**, *6*, 1947-1949).



Preparation of allyl Pittsburgh Green ether, APE. To a solution of bis-allyl-2',7'dichlorofluorescein (20 g; 41.6 mmol) in CH₂Cl₂ (200 mL) was added DIBALH (200 mL; 1.0 M in hexanes, 200 mmol) dropwise at -78 °C under an argon atmosphere. The mixture was stirred for 5 mins at the same temperature and then was warmed to 24 °C. After 6 h, Et₂O (200 mL) was added and the reaction mixture was quenched with saturated aqueous NH₄Cl (70 mL) at 0 °C. This mixture was warmed to 24 °C and stirred overnight. A second portion of Et₂O (1.2 L) was added to the mixture followed by DDQ (18.4 g; 81.0 mmol) at 0 °C. After stirring for 1 h at 24 °C, the mixture was filtered through Celite® and washed with EtOAc. The filtrate was dried over Na₂SO₄, and the solvents were evaporated under reduced pressure. The resulting residue was purified by silica gel flash chromatography (5 → 30% EtOAc in hexanes), affording allyl

Pittsburgh Green ether as a pale-yellow solid (12.5 g, 70%). Spectroscopic data matched that found in literature (F. Song; A.L. Garner; K. Koide *J. Am. Chem. Soc.* **2007**, *129*, 12354-12355).



Preparation of 4'-allyl-2',7'-dichloro-3',6'-bis((4-methoxybenzyl)oxy)-3H-spiro[isobenzofuran-1,9'-xanthene], **4.1.** A solution of APE (1.0 g, 2.3 mmol) in Ph₂O (3 mL) in a sealed tube was

heated with stirring in a sand bath at 165 °C (external temperature) for 16 h. After being cooled to 24 °C, the reaction mixture was diluted with CH₂Cl₂ (100 mL) and extracted 4 times with aqueous 4 N NaOH (50 mL each). The combined aqueous layers were cooled to 0 °C and acidified to pH < 1 with aqueous 12 M HCl. The acidified solution was extracted with CH_2Cl_2 (200 mL). The organic layer was separated and dried over Na₂SO₄ and concentrated. The crude residue was combined with Cs₂CO₃ (2.3 g, 7.0 mmol) under an argon atmosphere. DMF (25 mL) was added via syringe, and the resulting suspension was cooled to 0 °C. PMBCl (960 μ L, 7.1 mmol) was added at 0 °C, and the reaction was allowed to slowly warm up to 24 °C. After 16 h, the reaction was poured into H₂O (200 mL) and extracted with EtOAc (200 mL). The organic layer was washed with 1 N HCl (150 mL), H₂O (200 mL), brine (200 mL), dried over Na₂SO₄ and concentrated. The resulting residue was purified by flash chromatography ($0 \rightarrow 20\%$ EtOAc in hexanes) to afford 4.1 as a white solid (1.1 g, 72%). Rf = 0.48 (40% EtOAc in hexanes); mp =139.1-142.4 °C (decomp); IR: 3434, 1638, 1623, 1562, 1514, 1487, 1456, 1421, 1407, 1375, 1302, 1250, 1208, 1172, 1111, 1035, 1005, 920, 866, 822, 726 cm⁻¹, ¹H NMR (400 MHz, CDCl₃, 293K): δ 6.82 (s, 1H), 6.04 (app. ddt, J = 16.0, 10.0, 6.4 Hz, 1 H), 5.34 (s, 2H), 5.13 (s, 2H), 5.08 (s, 1H), 5.05 (s, 1H), 4.93 (dd, J = 10.2, 5.4 Hz, 2H), 3.83 (s, 3H), 3.82 (s, 3H), 3.65 (dd, J = 6.4, 1.6 Hz, 2 H); ¹³C NMR (100 MHz, CDCl₃, 293K) δ 159.7, 159.6, 154.8, 153.2, 149.6, 147.8, 143.7, 138.7, 136.0, 129.9, 129.4, 128.9, 128.7, 128.6, 127.9, 127.5, 123.7, 123.1, 123.0, 121.5, 121.01, 118.4, 117.4, 115.7, 114.1, 113.9, 101.8, 83.3, 75.2, 72.5, 70.9, 55.3, 55.3, 29.0; HRMS (ESI+) calcd for C₃₉H₃₂Cl₂O₆ [M+H] = 667.1648, found 667.1632.



Preparation of 2-(2',7'-dichloro-3',6'-bis((4-methoxybenzyl)oxy)-3H-spiro[isobenzofuran-1,9'xanthen]-4'-yl acetylaldehyde 4.2. To a solution of 4.1 (311 mg, 0.47 mmol) in 2:1 v/v THF/H₂O (13.5 mL) was added K₂OsO₄•2 H₂O (9.0 mg, 0.02 mmol), 2,6-lutidine (110 µL, 0.94 mmol) and NaIO₄ (400 mg, 1.9 mmol). The solution was stirred at 24 °C for 4 h, following which, NaIO₄ (200 mg, 0.95 mmol) was added, and the reaction mixture was allowed to stir at 24 °C for 1 h. The reaction was quenched by the addition of aqueous 1 N HCl (10 mL), and the product was extracted with EtOAc (20 mL). The organic layer was washed with H₂O (20 mL), brine (20 mL), dried over Na₂SO₄, and concentrated. The resulting residue was purified by flash chromatography ($10 \rightarrow 40\%$ EtOAc in hexanes) to afford 4.2 (190 mg, 61%) as a yellow-white solid. Rf = 0.53 (40% EtOAc in hexanes); mp = 76.4-78.0 °C (decomp); IR: 3399, 2094, 1641, 1514, 1488, 1457, 1407, 1303, 1250, 1210, 1173, 1110, 1084, 1034, 986 cm⁻¹, ¹H NMR (400 MHz, CDCl₃, 293K): δ 9.59 (t, J = 2.0 Hz, 1H), 7.51-7.301 (m, 8H, Ar), 6.76 (s, 1H, Ar), 5.34 (2H, s), 5.08 (2H, s), 4.93 (d, J = 4.0 Hz, 2H), 3.83 (s, 3H), 3.82 (s, 3H), 3.80 (t, J = 2.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, 293K) δ 199.0, 160.1, 159.7, 155.0, 153.7, 149.4, 148.0, 143.7, 138.8, 130.6, 129.6, 129.2, 129.1, 129.0 (two peaks), 128.4, 128.0, 123.9, 123.11, 121.9, 121.3,

118.9, 117.4, 116.8, 114.2, 101.8, 83.2, 75.6, 72.7, 71.0, 55.5, 55.4, 39.8, 31.7, 22.8, 14.3; HRMS (ESI+) calcd for $C_{38}H_{30}Cl_2O_7$ [M+H] = 669.1427, found 699.1428.



Preparation of 2-(2',7'-dichloro-3',6'-bis((4-methoxybenzyl)oxy-3H-spiro[isobenzufuran-1,9'xanthen]-4'-yl-N-prop-2-yn-1-yl)acetamide 4.3. To a solution of aldehyde 4.2 (88 mg, 0.13) mmol) in 1:1 v/v THF/tBuOH (7.6 mL) was added 2-methyl-2-butene (446 µL, 4.2 mmol). NaClO₂ (64 mg, 0.64 mmol) and NaH₂PO₄ (77 mg, 0.64 mmol) were dissolved in H₂O (2.6 mL), and the solution was added dropwise to the reaction flask. The solution was stirred at 24 °C for 12 h, after which the reaction solution was diluted with EtOAc (5 mL). The organic layer was washed with 1 N HCl (5 mL), H₂O (5 mL), brine (5 mL), dried over Na₂SO₄, and concentrated. To the crude residue was added HOSu (17 mg, 0.14 mmol) under an argon atmosphere. CH₂Cl₂ (4 mL) was added via syringe, followed by DCC (29 mg, 0.14 mmol) in CH₂Cl₂ (1 mL). The reaction mixture was stirred at 24 °C for 12 h, during which time a white precipitate formed. A solution of propargylamine hydrochloride (58 mg, 0.64 mmol) and NEt₃ (184 µL, 1.3 mmol) in CH₂Cl₂ (2 mL) were added to the flask, and the reaction mixture was stirred at 24 °C for 6 h. following which, the reaction mixture was filtered through a Celite pad and rinsed with CH₂Cl₂ (10 mL). The filtrate was washed with H₂O (10 mL), brine (10 mL), dried over Na₂SO₄, and concentrated. The resulting residue was purified by flash chromatography ($30 \rightarrow 60\%$ EtOAc in hexanes) to afford 4.3 (51 mg, 55%) as a yellow-white solid. Rf = 0.58 (60% EtOAc in hexanes);

mp = 75.3-77.2 °C (decomp); IR: 3373, 2100, 1641, 1515, 1457, 1430, 1408, 1303, 1250, 1211, 1173, 1029, 1008 cm⁻¹, ¹H NMR (400 MHz, CDCl₃, 293K): δ 7.42-7.32 (m, 7H, Ar), 7.33 (t, J = 6.4 Hz, 1H, Ar), 6.95-6.94 (m, 2H, Ar), 6.94-6.93 (m, 2H, Ar), 6.92-6.91 (m, 2H, Ar), 6.85 (s, 1H), 5.85 (t, J = 5.2 Hz, 1H), 5.35 (s, 2H), 5.30 (s, 1H), 5.03 (s, 2H), 5.00 (dd, J = 10.4, 15 Hz), 3.99 (dd, J = 2.0, 2.8 Hz, 2H), 3.97 (s, 3H), 3.97 (s, 3H), 3.77 (s, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃, 293K) δ 169.6, 159.9, 159.6, 154.8, 153.4, 149.2, 147.7, 143.6, 138.5, 130.4, 129.4, 129.0, 128.8, 128.8, 128.3, 127.8, 123.7, 123.1, 121.9, 121.1, 118.8, 118.3, 117.1, 114.1, 101.8, 83.1, 79.5, 75.6, 72.6, 71.5, 70.9, 55.3, 53.4, 32.6, 29.7, 29.3 ppm; HRMS (ESI+) calcd for C₄₁H₃₅Cl₂NO₇ [M+H] = 722.1707, found 722.1683.



Preparation of 2-(2',7'-dichloro-3',6'-dihydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-4'-yl)-N-(prop-2-yn-1-yl)acetamide **4.4**. To a solution of **4.3** (50 mg, 0.07 mmol) in 1:1 v/v CH₂Cl₂/TFE (2 mL) was added aqueous 1 M HCl (40 µL) and was stirred at 24 °C for 3 h. The reaction was quenched with sat. aq. NaHCO₃ (2 mL), and the organic layer was extracted with NaHCO₃ (2 mL) two more times. The combined aqueous layer was acidified at 0 °C with aq. 12 N HCl to pH < 1 and extracted with EtOAc (10 mL). The organic layer was washed with brine, dried over Na₂SO₄ and concentrated. The crude residue was purified by prep TLC (90% EtOAc in hexanes with 1% AcOH) followed by passing through a C18 column with a MeOH eluent, affording **4.4** (26 mg, 79%) as a red solid. Rf = 0.36 (90% EtOAc in hexanes with 1% AcOH); mp = > 200 °C; IR: 3400 (OH), 2049, 1640 (C=O), 1460, 1360, 1263, 1079, 1010 cm⁻¹; ¹H NMR

(400 MHz, d^6 -DMSO, 293K): 8.70 (t, J = 5.2 Hz, 1H, NH), 7.48 (d, J = 7.6 Hz, 1H, Ar), 7.40 (t, J = 7.2 Hz, 1H, Ar), 7.29 (t, J = 7.6 Hz, 1H, Ar), 6.82 (s, 2H), 6.80 (s, 1H), 6.74 (s, 1H), 5.30 (s, 1H), 3.92 (dd, J = 5.2, 2.4 Hz, 2H), 3.76 (s, 2H), 3.14 (t, 1H, J = 2.4 Hz); ¹³C NMR (d⁶-DMSO, 125 MHz, 293 K): 173.8, 170.8, 156.9, 153.7, 140.6, 131.5, 129.6, 129.3, 128.2, 127.6, 127.5, 127.0, 126.1, 110.6, 108.3, 108.2, 104.1, 82.0, 73.2, 60.7, 31.2, 29.5, 28.5 ppm; HRMS (ESI+) calcd for C₂₅H₁₇Cl₂NO₅ [M+H] = 428.056, found 428.056.



Preparation of N-(2-(2-(2-(4-((2-(2',7'-dichloro-6-hydroxy-9-(2-(hydroxymethylphenyl)-3-oxo-3H-xanthen-5-yl)acetamido)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aR, 4R, 6aS)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide 4.5. To a solution of 4-4 (20 mg, 0.04 mmol) in tBuOH (8 mL) and H₂O (4 mL) was added azido-PEG3-biotin (14 mg, 0.031 mmol), CuSO₄ (1.3 mg, 0.003 mmol), sodium ascorbate (0.6 mg, 0.003 mmol) and TBTA (0.8 mg, 0.0015 mmol) and was stirred at 24 °C for 1 h. The product was extracted with EtOAc (10 mL), washed with H₂O (10 mL), brine (10 mL), dried over Na₂SO₄, and concentrated. The resulting crude residue was purified by preparatory TLC (10% MeOH in CH₂Cl₂) affording 4.5 (22 mg, 79%) as a red solid. R_f = 0.2 (10% MeOH in CH₂Cl₂); HRMS (ESI+) calcd for

 $C_{43}H_{49}Cl_2N_7O_{10}S$ [M+H] = 924.2555 found 924.2585; LC-MS: $t_R = 6.5$ min. LC-MS trace can be found in Appendix B.



Preparation of 2-(2',7'-dichloro-3',6'-dihydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-4'yl]acetic acid 4-6. To a solution of 4-2 (140 mg, 0.21 mmol) in THF/tBuOH (1:1; 12 mL) was added 2-methyl-2-butene (712 μ L, 6.93 mmol). NaClO₂ (99.0 mg, 1.10 mmol) and NaH₂PO₄ (120 mg, 1.00 mmol) were dissolved in H₂O (4 mL) and added to the solution of 4-2 dropwise at 24 °C. The resulting solution was stirred at 24 °C for 16 h. The reaction was quenched with aqueous 1 N HCl (10 mL) and extracted with EtOAc (15 mL). The organic layer was washed with H₂O (15 mL) and brine (15 mL), dried over Na₂SO₄, and concentrated. The crude residue was dissolved in CH₂Cl₂ (1.2 mL). An aliquot (400 μ L) was transferred to a round-bottom flask and concentrated. The crude residue (47 mg) was dissolved in 1:1 ν/ν CH₂Cl₂/TFE (1.36 mL) and a single drop of 12 N HCl was added. The resulting solution was stirred at 24 °C for 2 h. The reaction was quenched with saturated aqueous NaHCO₃ (2 mL) and was diluted with CH₂Cl₂ (2 mL). The organic layer was isolated and treated with saturated aqueous NaHCO₃ (2 mL). The combined aqueous layers were acidified to pH < 1 with 12 N HCl and were extracted with EtOAc (10 mL). The organic layer was washed with brine (10 mL), dried over Na₂SO₄, and concentrated. The crude residue was purified by preparative TLC, eluting with 100% EtOAc. The desired band was extracted from the silica using MeOH and passed through a C18 column, affording **4.6** as a red solid (27 mg; 87%). $R_f = 0.1$ (5% MeOH in CH₂Cl₂); mp = 192.5-193.4 °C (decomp); IR: 3340 (OH), 2094, 1641 (C=O), 1417, 1341, 1261 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 293K) δ 7.79 (d, J = 7.0 Hz, 1H, Ar), 7.66 (t, J = 7.0 Hz, 1H, Ar), 7.55 (t, J = 7.0 Hz, Ar), 7.47-7.43 (m, 1H, Ar), 7.34 (t, J = 7.0 Hz, 0.5H, see note), 7.25-7.22 (m, 1H, Ar), 7.21-7.20 (m, 1H, Ar), 7.15 (d, J = 7.5 Hz, Ar), 7.10 (t, J = 7.0 Hz, 1H, Ar), 7.00 (s, 1H), 6.95 (s, 0.5H, see note), 6.88 (d, J = 2.5 Hz, Ar), 6.84 (s, 0.5H, Ar, see note), 6.73 (s, 0.5H, Ar, see note), 6.67 (s, 1H, Ar), 5.27 (s, 1H), 5.12 (s, 1H), 4.34 (d, 2H), *Note*: compound exists as a mixture of open spirocycle tautomers and a closed spirocycle leading to non-integer integrations; HRMS: calcd for C₂₂H₁₄Cl₂O₆ [M+H]: 445.0242, found 445.0255.

Prepration of IgG-bound 2',7'-dichloro-4'-igG-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3'6'-diol. To a round-bottom flask was added 4-6 (6 mg; 0.014 mmol), HOSu (3 mg; 0.028 under an argon atmosphere. CH₂Cl₂ (1 mL) was added followed by a solution of DCC (3 mg; 0.015 mmol) in CH₂Cl₂ (1 mL). The reaction was stirred at 24 °C protected from light for 20 h. The reaction was subsequently filtered through a Celite pad (rinsed with CH₂Cl₂) and concentrated. The resulting residue was dissolved in DMSO (3.65 mL) to prepare a 0.02 mg/10 μ L stock solution. When not in use, this stock solution was stored under argon at -20 °C. To a 2mL polypropylene Eppendorf tube was combined 0.25 mg/mL donkey anti-rabbit whole molecule IgG in MilliQ H₂O (50 μ L), 1 M aqueous NaHCO₃ (6 μ L), and 0.02 mg/10 μ L of the succinimidyl ester solution previously described. The resulting solution was shaken at 24 °C for 30 min wrapped in foil, protected from light. During this time, a NAP-5 column for protein purification was drained and flushed with 1x PBS (3 mL). The reaction solution was diluted with 1x PBS (140 μ L; final volume 200 μ L) and the tube was vortexed to mix. The resulting solution was loaded directly onto the NAP-5 column and eluted by gravity. After the last drip, 1x PBS (500 μ L) was added to the column and allowed to pass through. Subsequently, 1x PBS (300 μ L) was added to the column and the filtrate collected. The NAP-5 column was rinsed further with 1x PBS (500 μ L). The collected fraction was submitted to the University of Pittsburgh Center for Biological Imaging for blinking analysis. Callen Wallace performed the analysis.

Analysis of blink events of IgG-bound Pittsburgh Green

IgG-bound Pittsburgh Green was used to fluorescently label microtubules (Rockland Product ID: 200-301-880) in HeLa cells. Samples were imaged with an oblique angle of illumination utilizing a Nikon Ti microscope equipped with a TIRF system and Andor iXON EMCCD camera in the presence of an oxygen-scavenging buffer. Blinking events were defined utilizing a spot detection and autoregressive tracking algorithm in NIS Elements.

APPENDIX B

¹H AND ¹³C NMR SPECTRA



Spectrum 1. ¹H NMR spectrum of alkene **4.1** (400 MHz, CDCl₃, 293 K).



Spectrum 2. ¹³C NMR spectrum of alkene 4.1 (75 MHz, CDCl₃, 293 K).



Spectrum 3. ¹H NMR spectrum of aldehyde 4.2 (400 MHz, CDCl₃, 293 K).



Spectrum 4. ¹³C NMR spectrum of aldehyde **4.2** (75 MHz, CDCl₃, 293 K).



Spectrum 5. ¹H NMR spectrum of amide **4.3** (400 MHz, CDCl₃, 293 K).



Spectrum 6. ¹³C NMR spectrum of amide 4.3 (100 MHz, CDCl₃, 293 K)



Spectrum 7. ¹H NMR spectrum of amide **4.4** (400 MHz, *d*⁶-DMSO, 293 K)



Spectrum 8. ¹³C NMR spectrum of amide 4.4 (100 MHz, d⁶-DMSO, 293K)



Spectrum 9. ¹H NMR spectrum of carboxylic acid 4.6 (125 MHz, CD₃OD, 293K)



Spectrum 10. LC-MS trace of **4.5.** (a) Chromatogram. $t_R = 6.5$ for **4.5.** (b) Corresponding mass spectrum for peak at 6.5. LRMS (ESI-) calcd for $C_{43}H_{49}Cl_2N_7O_{10}S$ [M+O] = 940. Shift of +16 Da indicates oxidation.²⁵⁸ Method: C18 reverse phase Phenominex column; mobile phase: 10% MeCN with 0.1% formic acid/H₂O with 0.1% formic acid/H₂O with 0.1% formic acid/H₂O with 0.1% formic acid/H₂O with 0.1% formic acid (10 min).

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