Palladium Reagents for Bioconjugation

by

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Palladium Reagents for Bioconjugation

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Anthony J. Rojas

Submitted to the Department of Chemistry on July 23, 2018
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
at the Massachusetts Institute of Technology

Abstracts

Chapter 1: Macrocyclic peptides are important therapeutic candidates due to their improved physicochemical properties in comparison to their linear counterparts. Here we detail a method for a divergent macrocyclization of unprotected peptides by crosslinking two cysteine residues with bis-palladium organometallic reagents. These synthetic intermediates are prepared in a single step from commercially available aryl bis-halides. Two bioactive linear peptides with cysteine residues at i, $i + 4$ and i, $i + 7$ positions, respectively, were cyclised to introduce a diverse array of aryl and bi-aryl linkers. These two series of macrocyclic peptides displayed similar linker-dependent lipophilicity, phospholipid affinity, and unique volume of distributions. Additionally, one of the bioactive peptides showed target binding affinity that was predominantly affected by the length of the linker. Collectively, this divergent strategy allowed rapid and convenient access to various aryl linkers, enabling the systematic evaluation of the effect of appending unit on the medicinal properties of macrocyclic peptides.

Chapter 2: We report the use of a sulfonated biarylphosphine ligand (sSPhos) to promote the chemoselective modification of cysteine containing proteins and peptides with palladium reagents in aqueous medium. The use of sSPhos allowed for the isolation of several air-stable and water-soluble mono- and bis-palladium reagents, which were used in an improved protocol for the rapid *S*-arylation of cysteines under benign and physiologically relevant conditions. The cosolvent-free aqueous conditions were applied to the conjugation of a variety of biomolecules with affinity tags, heterocycles, fluorophores, and functional handles. Additionally, bis-palladium reagents were used to perform macrocyclization of peptides bearing two cysteine residues.

Chapter 3: The synthesis of palladium oxidative addition complexes of unprotected peptides is described. Incorporation of 4-halophenylalanine into a peptide during solid phase peptide synthesis allows for subsequent oxidative addition at this position of the unprotected peptide upon treatment with a palladium precursor and suitable ligand. The resulting palladium–peptide complexes are solid, storable, water-soluble, and easily purified via high-performance liquid chromatography. These complexes react rapidly with thiols at low micromolar concentrations in an aqueous buffer, offering an efficient method for bioconjugation. Using this strategy, peptides can be rapidly functionalized with small molecules to prepare modified aryl thioether side-chains. Additionally, peptide–peptide and peptide–protein ligations are demonstrated under dilute aqueous conditions.

Thesis Supervisors: Prof. Stephen L. Buchwald and Prof. Bradley L. Pentelute
Titles: Camille Dreyfus Professor of Chemistry and Associate Professor, respectively.

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I've always felt out of place at MIT. I'm an inorganic student, in an organic lab, working on a project that is almost wholly biological. I'm surrounded by the greatest minds I've ever seen; yet I 'failed' my first class here. I'm surrounded by people who live, breathe, dream, and fixate entirely on their work – their science. Yet, I can't honestly say that chemistry, on the whole, is in my top-10 list of life priorities. I've always felt that the people surrounding me were entirely encompassed by their work. Don't get me wrong. I love what I do, think it's fascinating, and truly enjoy waking up and coming to lab to try and discover something new every day. But, to be honest, I'm much more interested in dancing, discussing socioeconomic impacts on society, listening to music, reading a love story, traveling, learning about effective teaching skills, playing with my nephew, throwing a dinner party, hosting an event, advocating for minorities in STEM, coaching the cheerleading team, mentoring, socializing, etc. etc. etc. And yet, here I am, getting a Ph.D. from the best institution on the planet for chemistry. All of this is okay, I think. I don't want to 'fit in.' I wouldn't have wanted it any other way.

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Respective Contributions

This work is the result of extensive collaborations between the author and other researchers at Novartis and MIT. The specific contributions of the author are outlined below.

Chapter 1: Dr. Chi Zhang (Pentelute Lab, MIT) and Dr. Ekaterina Vinogradova (Buchwald Lab, MIT) uncovered the process for using bis-palladium oxidative addition complexes for forming peptide macrocycles and performed some of the initial experiments. Nathan Buchwald assisted with some experiments. Dr. John Reilly (Novartis) performed the pharmacokinetic studies. The author carried out the in-depth investigation of cross-linker structure, synthesized all of the materials, and, with the assistance of Dr. Chi Zhang, performed all binding studies.

Chapter 2: This work was carried out by the author. Dr. Ekaterina Vinogradova initially suggested the use of sSPhos as the supporting ligand for aqueous cysteine arylation.

Chapter 3: This work was carried out in collaboration with Dr. Justin Wolfe (Pentelute Lab, MIT). Dr. Wolfe performed most of the peptide purifications and syntheses of peptides containing 4-halophenylalanine. The author synthesized the oxidative addition complexes, some of the linear peptides, and performed all of the reactions and analysis with the palladium reagents.

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Preface

Parts of this thesis have been adapted from the following published articles co-written by the author. In all cases, the articles are adapted with permission from the appropriate journal.

Chapter 1:
Rojas, A. J.; Zhang, C.; Vinogradova, E. V.; Buchwald, N. H.; Reilly, J.; Pentelute, B. L.; Buchwald, S. L.; "Divergent Unprotected Peptide Macrocyclisation by Palladium-Mediated Cysteine Arylation." *Chem. Sci.* **2017**, *8*, 4257.

Chapter 2:
Rojas, A. J.; Pentelute, B. L.; Buchwald, S. L.; "Water-Soluble Palladium Reagents for Cysteine *S*-Arylation under Ambient Aqueous Conditions." *Org. Lett.* **2017**, *19*, 4263.

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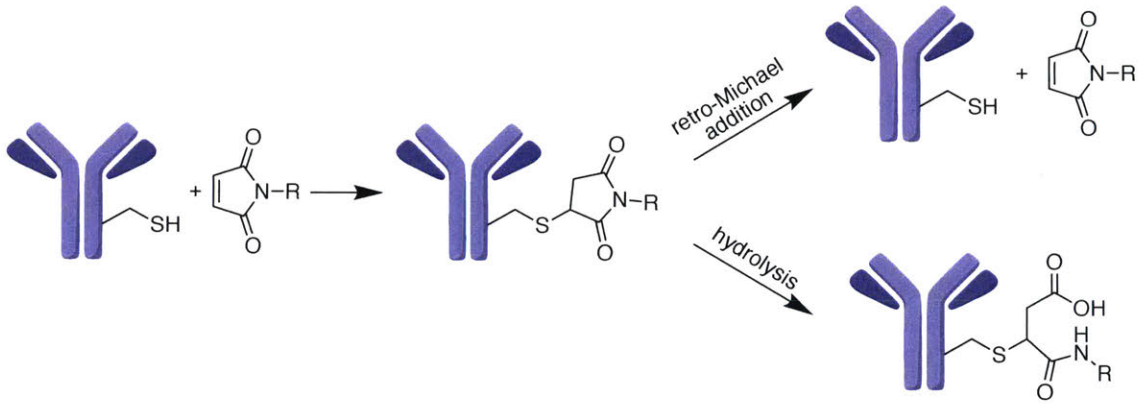
Introduction

The chemical modification of biomolecules, or bioconjugation,[1] is an important tool for generating therapeutic conjugates, probing the structure or function of natural proteins, designing novel constructs capable of performing unique functions, and diagnosing diseases.[2] The complexity and diversity of biomolecules presents many challenges for the design of new bioconjugation reactions. In particular, the development of site-selective reactions to modify biomolecules with high chemo- and regioselectivity is of utmost importance to ensure uniform construction of bioconjugates. Additionally, transformations that occur under ambient, aqueous conditions at low concentrations is highly valuable, as they avoid disrupting native structure and function.[3]

The tremendous advances in the application of transition metal catalysts in organic chemistry are well chronicled.[4] However, transition metal-mediated cross-coupling has only recently emerged as an effective tool for the selective transformation of complex biomolecules.[5] These biomolecules, such as peptides and proteins, are structurally diverse, and they tolerate only a narrow range of chemical environments that do not compromise the integrity of their structure or function.[6] Despite these challenges, recent innovations in organometallic chemistry have inspired researchers to explore transition metal reagents for the selective modification of biomolecules.[5] Specifically, the development of transition metal-mediated transformations that proceed in aqueous media, under mild conditions, exhibiting high selectivity, and excellent functional group tolerance have been developed in the context of organic synthesis. These qualities foreshadowed their usefulness for bioconjugation, for which exactly such benign conditions and refined selectivity are often required.

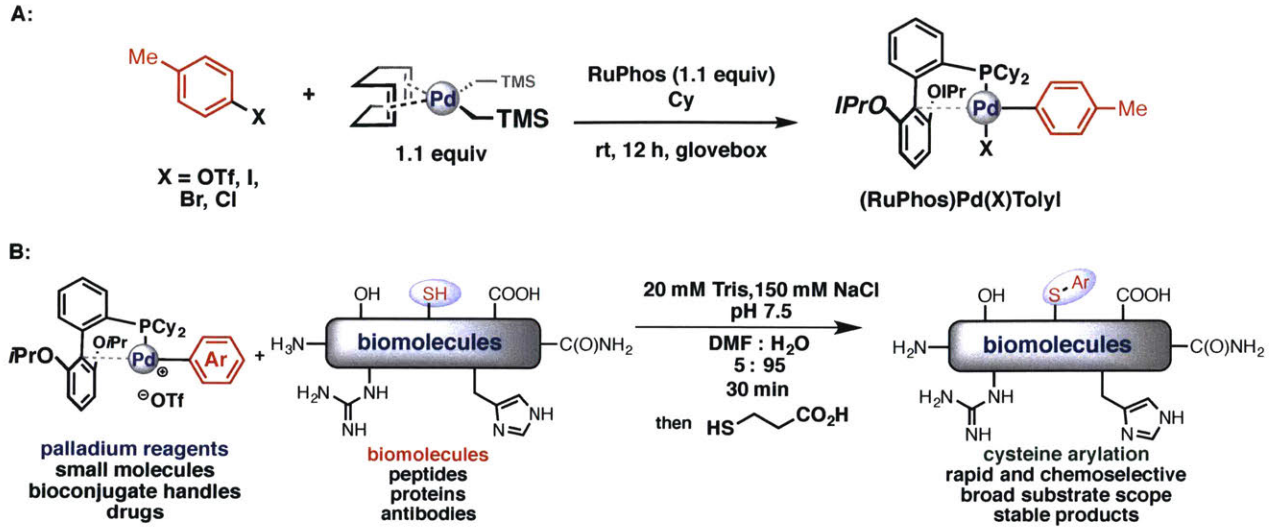
The cysteine amino acid residue, with its inherently low pK_a (~8.3),[7] low natural abundance (0.5% - 2.3% abundance),[8] and highly nucleophilic character,[9] provides a useful target for chemical modification. Cysteine-maleimide conjugation has seen widespread adoption as a method of choice due to the rapid rate of this Michael addition reaction. However, this reaction results in an unstable linkage prone to hydrolysis or retro-Michael addition (Figure 1).[10] Several less widely-used methods for the modification of cysteine have also been introduced, yet each retains significant drawbacks in either efficiency, ease of preparation, or linkage stability.[7]

Figure 1: Maleimide Linkage Instability. Maleimide conjugation to cysteine residues are prone to hydrolysis or retro-Michael addition.



Recently, the combined research efforts of the Pentelute and Buchwald laboratories have led to the development of palladium(II) oxidative addition complexes that are able to modify cysteine residues of several classes of biomolecules under mild conditions (Figure 2).[11] Notably, these cysteine arylation transformations have been shown to be highly chemoselective, rapid, and tolerant of the functional groups present in proteins, peptides, and antibodies. Additionally, the resulting C-S bonds demonstrated significantly increased linkage stability relative to cysteine-maleimide linkages. Our findings toward the improvement of this methodology and the discovery of new applications are described in this thesis.

Figure 2: Previous work from Pentelute, Buchwald, and Coworkers on Cysteine Arylation.[11] (A) Synthesis of palladium(II) oxidative addition complexes and (B) *S*-arylation of biomolecules under mild conditions.



One potential application of this newly discovered bioconjugation reaction is the production of macrocyclic, or "stapled," peptides. Macrocyclic peptides are a remarkably varied family of peptides found in a broad range of therapeutic applications due to their ability to potentially inhibit protein-protein interactions,[12] permeate through specific cells,[13] resist proteolytic degradation,[14] and act as drugs themselves.[14] Macrocyclic peptides can be produced by forging one of several types of bonds across a peptide backbone, such as head-to-tail, head-to-sidechain, sidechain-to-tail, and sidechain-to-sidechain linkages.[15] Due to the interest in the development of new therapeutic agents using these structures, several types of ring-forming reactions have been discovered, including lactam formation,[16] ring-closing metathesis,[17] disulfide formation,[18] and alkylation.[19] A notable limitation of all of these strategies is the inability to introduce structural diversity (e.g., incorporation of a variety of functional groups) in the linker at a late stage of macrocyclic peptide synthesis. Chapter 1 of this thesis focuses on the development of a divergent macrocyclisation strategy using bis-palladium oxidative addition complexes capable of installing distinct aryl, biaryl, and biaryl ether elements into peptides

containing two cysteine residues. This method allowed us to evaluate the effects of cross-linkers on important pharmaceutical properties such as lipophilicity, human serum albumin binding, target affinity binding, and volume of distribution.[20]

As mentioned previously, the development of bioconjugation reactions that adhere to biologically relevant conditions is of importance when seeking to modify sensitive biomolecules.[3] Chapter 2 focuses on the use of a modified supporting ligand for the preparation of the oxidative addition complexes that allows for the palladium-mediated cysteine arylation reaction to proceed under ambient, aqueous conditions without diminished reactivity.[21] These studies showcase an advantage of transition metal-mediated bioconjugation, namely, the ability for palladium complexes to be structurally tailored to produce the desired selectivity and reactivity.

Finally, Chapter 3 focuses on the development of a new class of palladium(II) oxidative addition complexes. Through the introduction, during solid-phase peptide synthesis, of an unnatural amino acid that contains an aryl halide, it is possible to form previously unreported palladium-biomolecule type oxidative addition complexes. Subsequent reactions between these complexes are possible with a variety of nucleophiles such as small molecules, peptides, and proteins, resulting in the direct functionalization of biomolecules and effectively reversing the reactivity of the previously described palladium-mediated *S*-arylation methods.

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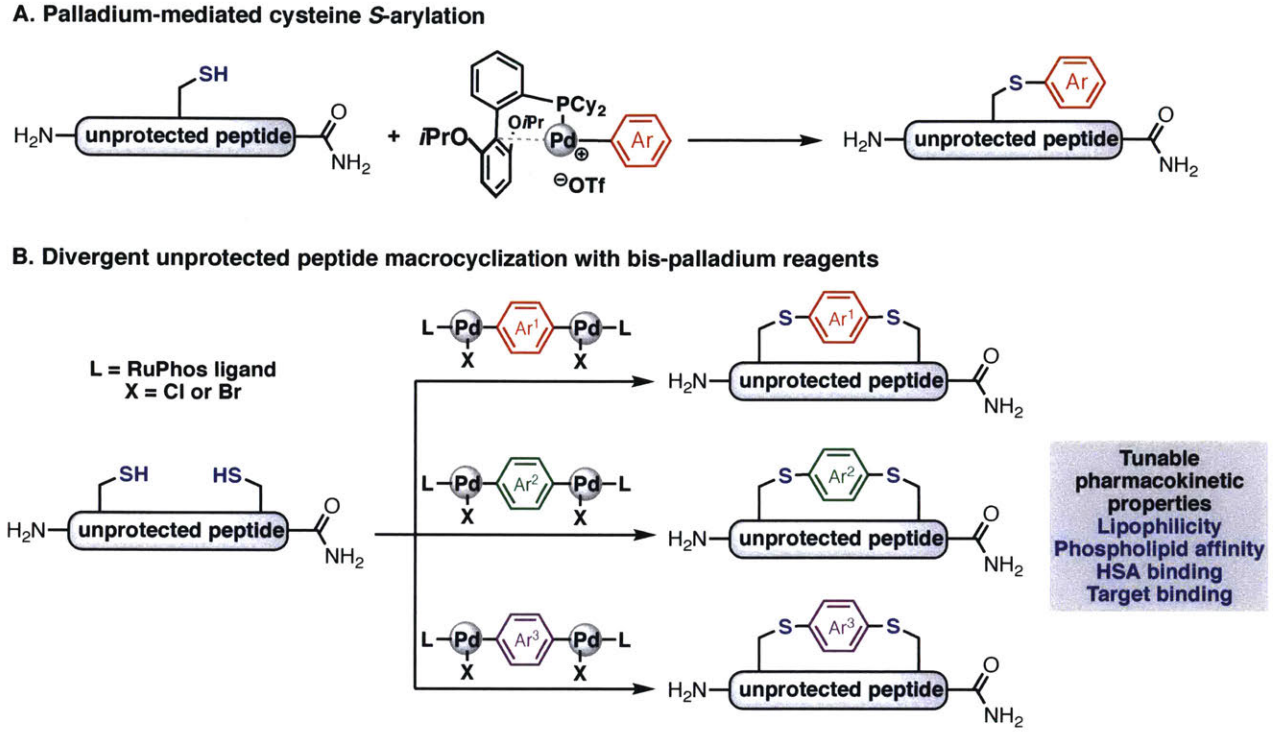
Chapter 1. Divergent Unprotected Peptide Macrocyclization by Palladium-Mediated Cysteine Arylation

1.1 – Introduction:

The selective inhibition of protein-protein interactions (PPIs) is a general tactic that may lead to the development of therapeutics for targets once thought "undruggable."[1] One approach to achieve this is the use of peptide inhibitors, which feature high potency, selectivity, and often have low toxicity.[2–4] Linear peptides, however, are readily degraded by proteases, exhibit low cell-permeability, and often adopt disordered conformations that affect their target binding affinity.[5] Peptide macrocyclization has emerged as a compelling strategy to overcome these issues.[6–9] Compared to their linear counterparts, macrocyclic peptides often display enhanced binding affinity, resistance to protease degradation, and in certain cases, readily enter cells.[4,10] However, few systematic studies of the pharmacokinetic and physicochemical properties (e.g. lipophilicity and phospholipid affinity) have been reported.

Numerous synthetic tools for peptide macrocyclization have been developed in recent years. One notable strategy is the crosslinking of two amino acid side chains. Classic techniques utilise lysine and glutamic acid/aspartic acid residues for lactam formation.[11] More recently, these techniques have been augmented by protocols involving ring-closing metathesis,[12] cycloaddtions,[13,14] oxime formation,[15] alkylation,[16] thiol-ene reaction,[17] arylation chemistry by nucleophilic aromatic substitution (S_NAr),[18,19] and metal-mediated reactions.[20] Many of these macrocyclization protocols rely on unnatural amino acids requiring multistep synthesis to diversify their structures. Few methods allow for modular tuning of the linker structure without altering the amino acid sequences.[21–24] Previous studies have indicated that the nature of the linker strongly influence the stability, structure, binding affinity, and cell permeability of macrocyclic peptides.[18,19,21] However, it is unclear how linkers influence the pharmacokinetic

Figure 1: S-arylation and macrocyclization of unprotected peptides. This work expanded the Palladium-mediated S-arylation chemistry (A) to peptide macrocyclization (B), providing a diverse array of aryl linkers with the capability to tune and manipulate the physiochemical properties of the resulting macrocyclic peptides.



properties such as lipophilicity, phospholipid affinity, volume of distribution, and Human Serum Albumin (HSA) binding profiles. It remains uncertain if these physicochemical properties can be modulated, generally, based on linker design or if their impact is peptide sequence dependent. Understanding the effect of linkers on these parameters will aid in assessing the "drug likeness" of macrocyclic peptides and facilitate the development of future therapeutics.

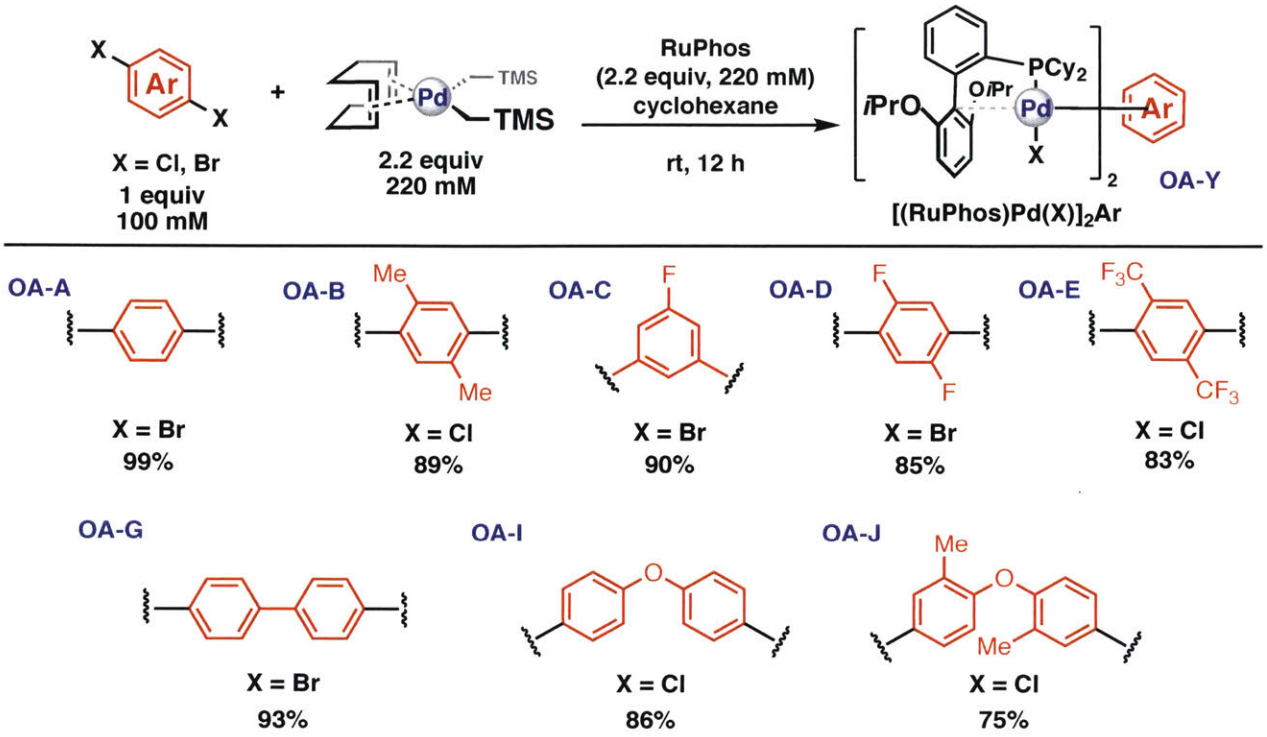
Recently, we have described a palladium-mediated method for cysteine arylation (**Fig. 1A**), using biaryldialkylphosphine-based complexes of type LPd(Ar)(X) (X = Cl, Br, OTf; L = ligand) as arylating reagents.[25] Preliminary application to peptide macrocyclization was demonstrated using a 4-4'-benzophenone-linked bis-palladium reagent along with a few others, resulting in effective crosslinking of two cysteine residues at the *i, i+4* positions of an unprotected peptide.[25] Here we report expanding this method to synthesis of aryl linkers of

varying length, rigidity, and electronic properties (**Fig. 1B**) and a systematic study of the effect of linkers on the lipophilicity, HSA binding, phospholipid affinity, and target binding affinity of these macrocyclic peptides. In particular, a series of bis- palladium reagents were readily prepared from commercially available aryl bis-halides. These air-stable palladium reagents were used to macrocyclize two bioactive peptides: a peptide (NYAD-1) that binds the HIV-1 *C*-terminal capsid protein (C-CA) with cysteine residues at the *i, i+4* positions and a p53/MDM2 inhibitor analogue based on the p53 protein with cysteine residues at the *i, i+7* positions.[10,26,27] These two model systems are employed in peptide macrocyclization studies and would let us determine if the resulting physicochemical trends were dependent on peptide sequences or crosslinking positions. The generality of this approach should allow for the further development of therapeutic reagents that can be finely tuned to meet the challenges during the development of peptide therapeutics.

1.2 – Results and Discussion:

To initiate our study, a series of bis-palladium complexes (**OA-Y,** Table 1) were synthesized as macrocyclization reagents. 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl (RuPhos) was selected as the ligand for palladium, in combination with a series of aryl, biaryl, and biaryl ether dihalides, eight oxidative addition complexes were synthesized (**OA-A** through **OA-I**). These were chosen to probe the effects of linker length, rigidity, and degree of fluorination on the physicochemical properties of the macrocyclized peptide.

Table 1. One-step synthesis of oxidative addition complexes as stapling reagents are synthesized in good yields.



Reported isolated yields are an average of two runs after purification. Oxidative addition complexes are air-stable and readily characterized by solution ^{1}H, ^{31}P, and ^{19}F NMR (where applicable), elemental analysis, and FT-IR (See Experimental for characterization).

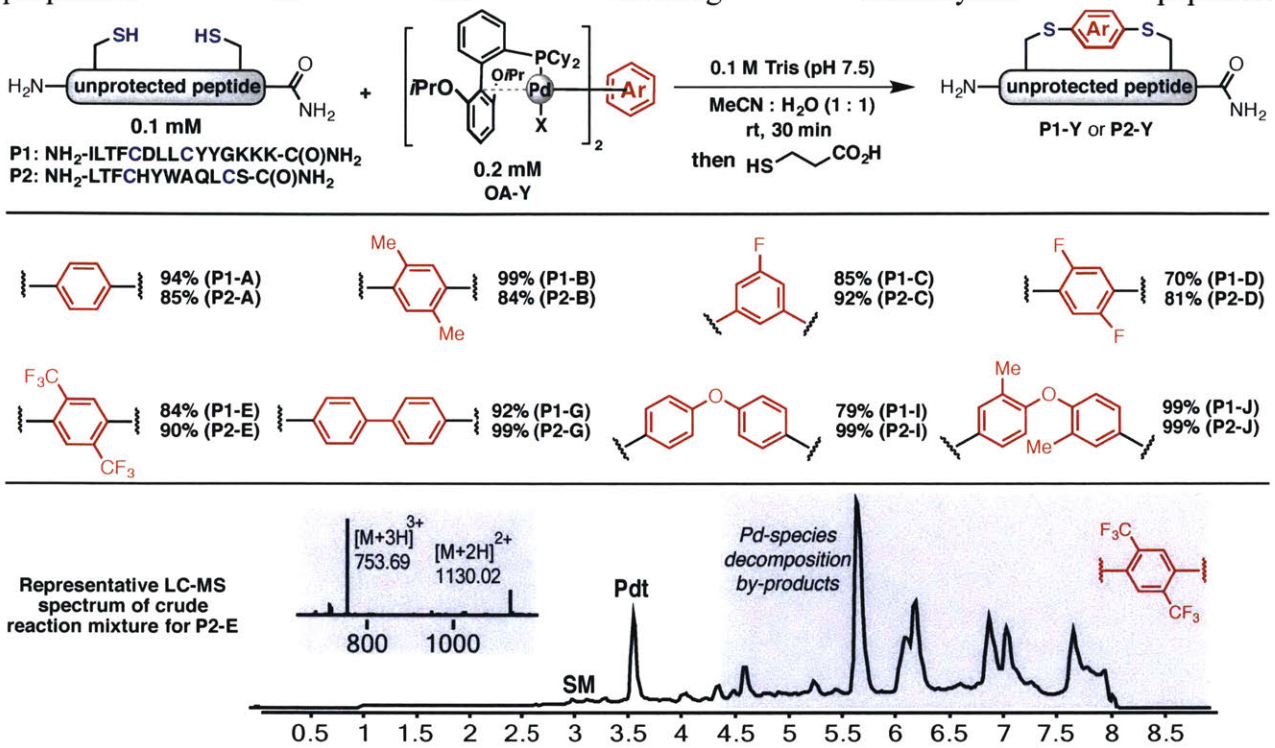
As shown in Table 1, the bis-palladium macrocyclization reagents were prepared in good to excellent yields, regardless of the substitution pattern of the aryl dihalide. These oxidative addition complexes could be stored under air at 4 °C for extended periods without loss of reactivity, as demonstrated by similar reaction profiles of a freshly prepared sample and a sample stored in a freezer at 4 °C for more than two years (see Experimental for details).

Using a combination of batch and rapid flow solid phase peptide synthesis (SPPS),[28] peptides **P1** and **P2** were synthesized on resin, cleaved, and later purified by reverse-phase HPLC to afford the starting material for the subsequent macrocyclization reactions (see Experimental for details). Peptide **P1** is a variant of a known inhibitor of the HIV-1 *C*-terminal capsid protein (C-CA)[27] and peptide **P2** is a mutant of a peptide that disrupts p53/MDM2

interaction based on the p53 protein.[10] Cysteine residues were placed at *i*, *i+4* and *i*, *i+7* positions for **P1** and **P2**, respectively. As such, these two systems will allow us to probe whether linkers will have the same effect when the peptide sequence and the position of the crosslinking is varied.

Fine-tuning of the reaction conditions led to an optimised protocol for peptide macroclisation (Fig. 2, top). Exposure of the purified peptides **P1** and **P2** to these conditions afforded macroclyclisation products in good to excellent yields (70-99%, Fig. 2) as determined by LC-MS analysis of the crude reaction mixtures (see Experimental for details). As shown by a representative LC- MS trace, the crude reaction mixtures generally contained only desired macrocyclic product along with a few by-products derived from the macrocyclization reagent (see Experimental for full characterization of each purified macrocyclic peptide). Upon completion of the reaction, the crude reaction mixture was treated with 3-thiopropionic acid,[25,29] subjected to centrifugation, and the supernatant liquid was lyophilized and subsequently purified by reverse-phase HPLC. ICP-MS analysis of the purified peptides showed that more than 99% of palladium was removed using this purification protocol. Importantly, dimerization of two peptides was not observed. Side reactions whereby two peptides form dimers are common complications for two-component macrocyclization reactions, but were not found here.[6] The perfluorinated macrocyclic peptides **P1**-F, P2-F, P1-H, and P2-H (Table 3) were synthesized by previously reported SNAr chemistry.[18]

Figure 2. S-arylation and macrocyclization of unprotected peptides. This work expanded the Palladium-mediated S-arylation chemistry (A) to peptide macrocyclization (B), providing a diverse array of aryl linkers with the capability to tune and manipulate the physiochemical properties of the resulting macrocyclic peptides.



After purifying the conjugates, we sought to investigate how the linking units affect the lipohilicity, human serum albumin (HSA) binding profiles, and if possible, the phospholipid affinity and volume of distribution for these macrocyclic peptides.

Lipophilicity is a critical parameter for drug formulation and which determines whether a compound will have the tendency to dissolve into lipid or aqueous environments.[18] This is an important consideration for pharmaceutical discovery and part of Lipinski's 'Rule of 5.'[30] For neutral small molecules, lipophilicity is often measured as Log *P* where *P* is the partitioning coefficient between octanol and water. For compounds potentially exhibiting zwitterionic character, a more appropriate measure is Log *D* where D is the distribution coefficient, which measures the overall distribution of both ionised and neutral forms of the compound when the aqueous phase is buffered to a particular pH.[20] We sought to determine whether this parameter

could be manipulated for macrocyclic peptides by varying just the aryl linkers. Being able to successfully modulate this parameter without disrupting other activity profiles could be a useful tactic for peptide drug development.

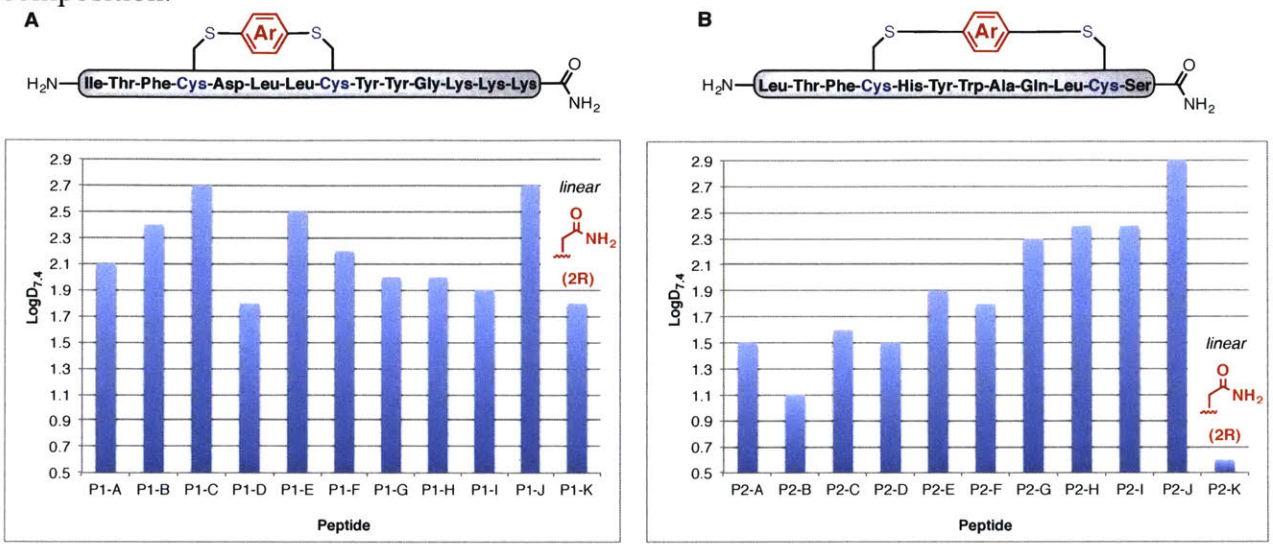
Using known methods,[31] retention time of the HPLC profiles of cyclised and linear variants of peptides **P1** and **P2** were measured and correlated to octanol-water distribution in solutions buffered to pH 7.4. Retention times, and mass spectrometric data were correlated to literature values for the Log *D* of other compounds and then used to assign numerical values of lipophilicity to these peptides.

Fig. 3A shows how these Log $D_{7.4}$ values can be manipulated between 1.8 for uncyclised **P1-K** to 2.7 for **P1-C** and **P1-J**. Interestingly, **P1-G** and **P1-H** exhibit the same Log $D_{7.4}$ value of 2.0, indicating that incorporation of fluorine substituents does not necessarily lead to increased lipophilicity. Peptides **P1-B** and **P1-E** (methyl substituted and *p*-trifluoromethyl phenyl linkers), however, do differ slightly with Log $D_{7.4}$ values of 2.3 and 2.4, respectively.

To determine whether the measured lipophilicity depends on peptide sequence and crosslinking positions, we also cyclised a variant of the P53/MDM2 inhibitor, **P2**, and measured lipophilicity values using the same methods (Fig. 3B).

Notably, the cyclisation of peptide **P2** significantly increased the lipophilicity values from 0.6 for uncyclised **P2-K** to 2.9 for **P2-J**. The methylated diaryl ether cross linker again yielded the highest Log *D* values for both peptides. However, the change in Log *D* does not solely depend on the linker, rather a combination of linker and peptide. For instance, **P1-C** has the same Log *D* as **P1-J**, but for **P2-C** the lipophilicity is intermediate. Another notable example is for linker **B**, peptides **P1-B** and **P2-B** display contrasting lipophilicities.

Figure 3. Lipophilicity measurements of two series of macrocyclic peptides show dependency on relationship between linker structure and amino acid sequence. Log D at pH 7.4, a measurement of lipophilicity for amphiphillic compounds, as measured by dissolving peptides in DMSO followed by rapid LC-UV-MS correlated to water/octanol partitioning for each macrocyclic peptide. Lipophilicity shows dependence on both crosslinker and amino acid composition.



Another parameter governing therapeutic utility is human serum albumin (HSA) binding. HSA, a prominent protein in plasma, is capable of binding many different classes of ligands. This binding is partially responsible for the pharmacokinetic behaviour of many drugs and can strongly influence their efficacy and delivery.[32] By using a stationary phase comprising immobilized HSA, the propensity of a molecule to bind to this protein can be determined by HPLC methods.[33] Although minor deviations in HSA binding were observed, all macrocyclized **P1** and **P2** variants were found to exhibit high HSA binding percentage, regardless of the aryl crosslinker (see Experimental for details).

Measurement of gradient HPLC retention times on immobilized artificial membrane columns allows for phospholipid binding analysis (CHI $IAM_{7.4}$). In conjunction with the lipophilicity results, modelling of volume of distribution (VD) can be performed on the modified peptides.[34] VD relates administered doses of drugs to their distribution between tissues and plasma *in vivo*. Knowledge of this parameter, along with clearance times, allows for the duration

of drug exposure to be estimated, making VD another key pharmacokinetic parameter in the development of new therapeutics.

Table 2: Phospholipid affinity and volume of distribution for the P2 peptides controlled by linker structure. CHI IAM, chromatographic hydrophobicity index obtained on an immobilized artificial membrane column were used along with HSA binding to calculate Log VD values.[34]

Peptide	CHI IAM pH 7.4	Log VD
P2-A	47.3	0.46
P2-B	36.7	-0.26
P2-C	48.2	0.49
P2-D	46.1	0.35
P2-E	49.9	0.59
P2-F	49.2	0.59
P2-G	64.5	2.37
P2-H	59.2	1.54
P2-I	61.8	1.90
P2-J	70.0	3.42
P2-K	32.4	0.13

The results presented in Table 2 are for peptides based on the p53/MDM2 inhibitor **P2**. Undesirable physicochemical properties can lead to a promiscuous profile and increased adverse effects. For a given lipophilicity, basic compounds tend to be less selective.[35] Phospholipid affinity, a measure of this property, was therefore determined for the macrocyclized peptides. Four **P2** peptides **P2-G-J** demonstrate high phospholipid affinity and considerably higher predicted volumes of distribution and would be expected to have a higher promiscuity index than

other **P2** peptides. This is an example of when the HPLC biomimetic methodologies can offer a perspective in structure activity relationships for pharmaceuticals. VD values could not be obtained for the **P1** variants due to the high levels of binding to the immobilized artificial membrane column, which could be indicative of large tissue to plasma ratios *in vivo*.

Tuning the physiochemical properties without diminishing the activity of a peptide is valuable for developing peptide therapeutics. We chose **P1** variants to study how the linkers affect the binding of these macrocyclic peptides to C-CA. Biotinylated **P1** variants were prepared for immobilization on the streptavidin biosensors and were sampled against serially diluted concentrations of C-CA using BioLayer Interferometer (see Experimental for details). Two control peptides with scrambled sequences, **P4-A** and **P4-K**, were also measured to ensure that the observed binding events were not due to nonspecific binding of the aryl linkers (**Table 3**).

The linear control peptide **(P1-K)** showed an equilibrium dissociation constant (K_D) of 5.5 ± 0.6 μM in line with previous literature reports.[27,36] All of the mono-aryl linkers with 1,4-substitutions (**P1-B**, **P1-D**, **P1-E**, and **P1-F**) had K_D comparable to the linear control. However, variant **P1-C** with 1,3-substitution showed slightly lower binding affinity (K_D = 21.0 ± 2.4 μM). Moreover, macrocyclic peptides with the biphenyl and biaryl ether crosslinkers all showed little to no binding affinity to C-CA. Taken together, these results indicated that the length of the crosslinker mpact binding to the target. It is worth noting that, although differing greatly in their lipophilicity (**Fig. 3**), the 1,4-substituted mono-aryl linkers (**P1-B**, **P1-D**, **P1-E**, and **P1-F**) did not exhibit significant differences in binding affinity C-CA.

Table 3. Target binding affinity for P1 macrocyclic peptides. Reported dissociation constants (K_D) for macrocyclic peptide variants of P1 and the scrambled P4 controls against the C-terminal domain of HIV-1 capsid protein (C-CA), as measured by BioLayer Interferometry.

Peptide	Cross-linker	K_D (μM)
P1-A		9.2± 0.4
P1-B	Me, Me	9.7 ± 0.4
P1-C	F	21.0 ± 2.4
P1-D	F, F	9.5 ± 0.5
P1-E	F_3C, CF_3	12.0 ± 0.8
P1-F	F, F, F, F	12.0 ± 0.6
P1-G		61 ± 35
P1-H	F, F, F, F, F, F, F, F	>100
P1-I	O	56 ± 21
P1-J	Me, O, Me	>100
P1-K	2 x O, NH_2	5.5 ± 0.6
P4-A		>100
P4-K	2 x O, NH_2	>100

1.3 – Conclusion:

We have shown that palladium-mediated *S*-arylation chemistry is a "shovel ready" technology for rapid and straightforward divergent macrocyclization of peptides; linkers, sequences, and crosslinking positions can be varied. Preparation of bis-palladium crosslinking reagents is straightforward and one-step from commercially available aryl dihalides. The linker had a significant affect on the physicochemical properties and in some cases the target binding affinity was maintained.

The design and eventual use of macrocyclic peptides in the clinic is uncharted, especially when compared to small molecules. Here we show by combining powerful chemistry, and simple chromatographic techniques to measure the physicochemical properties of each variant we could rank order peptides on basis of protein binding, lipohilicity, phospholipid affinity, and predicted volume of distribution. These measurements may find application in medicinal chemistry by providing a means to filter out peptides with undesirable physicochemical characteristics such as high phospholipid affinity at an early stage within the drug discovery process.

1.4 – Experimental:

General Reagent Information

1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5- b]pyridinium 3-oxid hexafluorophosphate (HATU), D-Biotin, Fmoc-Rink amide linker, Fmoc-L- Gly-OH, Fmoc-L-Leu-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Ala-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Glu(O*t*Bu)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Thr(*t*Bu)-OH, Fmoc-L-Tyr(*t*Bu)-OH, and Fmoc-L-His(Trt)-OH were purchased from Chem-Impex International (Wood Dale, IL). Peptide synthesis-grade

N,N-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethyl ether, HPLC-grade acetonitrile, and guanidine hydrochloride were obtained from VWR International (Philadelphia, PA). Aryl halides and aryl trifluoromethanesulfonates were purchased from Aldrich Chemical Co., Alfa Aesar, or Matrix Scientific and were used without additional purification. All deuterated solvents were purchased from Cambridge Isotopes and used without further purification. All other reagents were purchased from Sigma-Aldrich and used as received.

All reactions with peptides were set up on the bench top and carried out under ambient conditions. For procedures carried out in the nitrogen-filled glovebox, the dry degassed THF was obtained by passage through activated alumina columns followed by purging with argon. Anhydrous pentane, cyclohexane, and acetonitrile were purchased from Aldrich Chemical Company in Sureseal® bottles and were purged with argon before use.

All small-molecule organic and organometallic compounds were characterized by ^{1}H, ^{13}C NMR, and IR spectroscopy, as well as elemental analysis or high resolution mass spectrometry (unless otherwise noted). ^{19}F NMR spectroscopy was used for organometallic complexes containing fluorine atoms. ^{31}P NMR spectroscopy was used for characterization of palladium complexes. Copies of the ^{1}H, ^{13}C, ^{31}P, and ^{19}F NMR spectra can be found at the end of the Experimental Section. Nuclear Magnetic Resonance spectra were recorded on a Bruker 400 MHz instrument and a Varian 300 MHz instrument. Unless otherwise stated, all ^{1}H NMR experiments are reported in δ units, parts per million (ppm), and were measured relative to the signals of the residual proton resonances CH_2Cl_2 (5.32 ppm) or CH_3CN (1.94 ppm) in the deuterated solvents. All ^{13}C NMR spectra are measured decoupled from ^{1}H nuclei and are reported in δ units (ppm) relative to CD_2Cl_2 (54.00 ppm) or CD_3CN (118.69 ppm), unless otherwise stated. All ^{31}P NMR spectra are measured decoupled from ^{1}H nuclei and are reported relative to H_3PO_4 (0.00 ppm).

^{19}F NMR spectra are measured decoupled from ^{1}H nuclei and are reported in ppm relative to $CFCl_3$ (0.00 ppm) or α,α,α-trifluorotoluene (-63.72 ppm). All FT-IR spectra were recorded on a Thermo Scientific – Nicolet iS5 spectrometer (iD5 ATR – diamond). Elemental analyses were performed by Atlantic Microlabs Inc., Norcross, GA. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed using an Agilent 7900.

LC-MS chromatograms and associated mass spectra were acquired using Agilent 6520 ESI-Q-TOF mass spectrometer. Solvent compositions used in the LC-MS are 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC-MS method was used:

Method A LC conditions: Zorbax 300SB C3 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-3 min 5% B, 3-8 min 5-95% B, 8-9 min 95% B, flow rate: 0.8 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300 – 3000 *m/z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octapole rf voltages were set at 4000, 175, and 750, respectively.

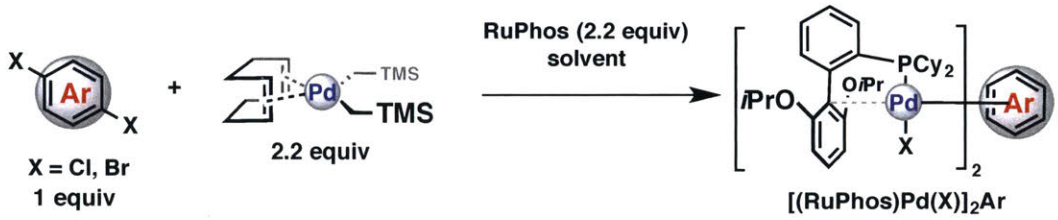
Determination of Macrocyclization Yields

Data were processed using Agilent MassHunter software package. All reported yields were determined by integrating total ion current (TIC) spectra. First, the peak areas for all relevant peptide-containing species on the chromatogram were integrated using Agilent MassHunter software package. Since no peptide-based side products were generated in the experiments, the yields shown in **Table 2** were determined as follows: %yield = S_{pr}/S_{total} where S_{pr} is the peak area of the product and S_{total} is the peak area of combined peptide-containing species (product and starting material).

Synthesis of Oxidative Addition Complexes

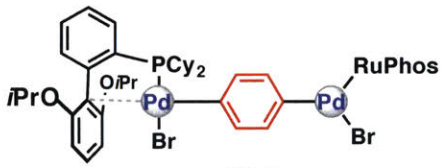
Synthesis of [(1,5-COD)Pd(CH_2TMS)$_2$]. A flame-dried Schlenk flask (100 mL), equipped with a magnetic stir bar, was filled with argon and charged with (1,5-COD)$PdCl_2$ (3.15g, 11.05 mmol) made according to literature reports.[1] The flask was put under vacuum and backfilled with argon (the procedure was repeated 3 consecutive times). Diethyl ether (49.3 mL) was added via syringe, the reaction was cooled to –40 °C (acetonitrile/dry ice bath) and $TMSCH_2MgCl$ (23.4 mL, 1.0 M) purchased from Sigma-Aldrich was added dropwise over 10–20 min. The reaction mixture was stirred at –40 °C for 1 h and then at 0 °C (ice/water bath) for an additional 20 min. Acetone (1.3 mL) was added at 0 °C, the reaction mixture was stirred for 5 min, after which time the solvent was removed under vacuum using an external trap (the flask was kept at 0 °C). The flask was then opened to air, pentane (100 mL) was added and the crude material was filtered through a pad of Celite into a new round-bottom flask (500 mL) at 0 °C. The filter cake was washed with pentane (50 mL × 2). Pentane from the combined washes was removed with the aid of a rotary evaporator at 0 °C (ice/water bath). The resulting white solid was dried under vacuum for 2 h at 0 °C, and transferred into a 20 mL scintillation vial in the glovebox (3.00 g, 70%). The ^1H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature.[1] The title compound was stored in the glovebox at –20 °C.

General Procedure for the Synthesis of bis-Palladium Oxidative Addition Complexes.



In a nitrogen-filled glovebox, an oven-dried scintillation vial (10 mL), equipped with a magnetic stir bar, was charged with RuPhos (2.2 equiv), Ar–X_2 (1 equiv), and cyclohexane.

Solid (1,5-COD)Pd(CH_2SiMe_3)$_2$ (2.2 equiv) was added rapidly in one portion and the resulting solution was stirred for 16 h at rt. After this time, pentane (3 mL) was added and the resulting mixture was placed into a –20 °C freezer for 3 h. The vial was removed from the glovebox and, in the air, the resulting precipitate was filtered, washed with pentane (5 × 3 mL), and dried under reduced pressure to afford the oxidative addition complex.

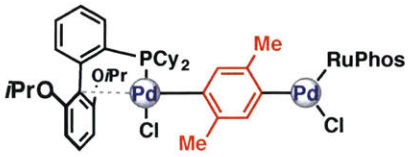


OA-A

Following the general procedure, a mixture containing 1,4-dibromobenzene (29.4 mg, 0.125 mmol), RuPhos (128 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (107 mg, 0.275 mmol) was stirred at rt in cyclohexane (1.5 mL) for 12 h. General work-up afforded **OA-A** as a grey solid (172 mg, 99%).

^{1}H NMR (400 MHz, CD_2Cl_2) d 7.72 – 7.59 (m, 1H), 7.54 – 7.37 (m, 1H), 7.11 (d, *J* = 8.0 Hz, 1H), 7.02 (dd, *J* = 8.5, 1.9 Hz, 1H), 6.92 (dd, *J* = 7.0, 2.5 Hz, 1H), 6.70 (d, *J* = 8.5 Hz, 1H), 6.64 (t, *J* = 1.6 Hz, 1H), 4.66 (dt, *J* = 12.1, 6.1 Hz, 1H), 2.23 – 2.12 (m, 1H), 1.80 (br, 4H), 1.59 (br, 1H), 1.48 (s, 1H), 1.42 (d, *J* = 6.0 Hz, 3H), 1.32 – 1.12 (m, 2H), 1.05 (dd, *J* = 6.0, 3.7 Hz, 4H), 0.93 (t, *J* = 7.1 Hz, 1H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d 159.08, 144.66, 144.48, 138.97, 138.93, 135.89, 135.12, 134.64, 134.15, 133.01, 132.66, 132.62, 132.51, 131.09, 130.96, 130.62, 130.60, 130.20, 129.17, 129.15, 126.40, 126.34, 126.06, 117.33, 107.47, 106.97, 71.09, 70.64, 34.12, 33.90, 33.63, 33.26, 28.23, 28.02, 27.71, 27.69, 27.35, 27.16, 27.03, 26.91, 26.84, 26.81, 26.72, 26.69, 26.03, 26.02, 22.33, 21.87, 21.38, 21.30, 13.81. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 31.99. **FT-IR (neat, cm^{-1}):** 2929.76, 2851.34,

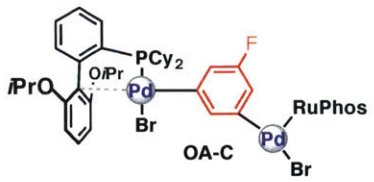
1453.14, 1383.05, 1373.23, 1269.20, 1227.14, 1105.13, 1066.00, 1037.87, 997.86, 850.63, 816.23, 742.05, 575.3. **Anal. Calcd. for $C_{66}H_{90}Br_2O_4P_2Pd_2$:** C, 57.36; H, 6.56. Found: C, 57.27; H, 6.61.



OA-B

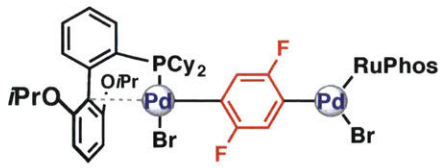
Following the general procedure, a mixture containing 1,4-dichloro-2,5-dimethylbenzene (20.0 mg, 0.125 mmol), RuPhos (133.3 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (111.0 mg, 0.275 mmol) was stirred at rt in THF (1.0 mL) for 12 h. General work-up afforded **OA-B** as a light grey solid (133.5 mg, 89 %).

^{1}H NMR (400 MHz, CD_2Cl_2) d 7.71 (t, J = 8.4 Hz, 2H), 7.64 (t, J = 7.3 Hz, 2H), 7.45 (m, 2H), 7.40 (m, 2H), 6.87 (ddd, J = 7.5, 2.9, 1.5 Hz, 3H), 6.65 (d, J = 8.5 Hz, 2H), 6.60 (d, J = 8.4 Hz, 2H), 6.27 (br, 2H), 4.61 (m, 4H), 2.54 (s, 6H), 2.27 (m, 4H), 1.83 (m, 18H), 1.43 (m, 18H), 1.16 (m, 17H), 1.05 (m, 5H), 0.91 (d, J = 6.1 Hz, 6H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d 160.66, 159.65, 146.01, 145.83, 140.48, 135.81, 135.75, 135.38, 134.08, 134.03, 133.89, 132.78, 132.67, 131.14, 130.94, 126.78, 126.72, 111.18, 111.14, 107.07, 106.80, 71.69, 70.45, 35.57, 35.29, 32.47, 32.21, 31.12, 31.07, 29.61, 29.56, 27.95, 27.88, 27.80, 27.70, 27.53, 27.45, 27.10, 26.99, 26.77, 26.30, 22.77, 22.35, 22.25, 22.04 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 32.03. **FT-IR (neat, cm^{-1}):** 2972.34, 2927.52, 2851.98, 1590.82, 1450.73, 1382.13, 1370.98, 1264.81, 1249.18, 1135.64, 1104.19, 1064.08, 1041.81, 1002.1, 898.23, 865.21, 848.18, 812.89, 767.71, 757.24, 744.88, 732.59, 667.8. **HRMS (ESI) m/z calcd. for $C_{68}H_{94}Cl_2O_4P_2Pd_2$ [M-Cl]$^+$:** 1285.4386. Found: 1285.4389.



Following the general procedure, a mixture containing 1,3-Dibromo-5-fluorobenzene (32 mg, 0.125 mmol), RuPhos (128 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (107 mg, 0.275 mmol) was stirred at rt in cyclohexane (1.5 mL) for 12 h. General work-up afforded **OA-C** as a grey solid (157 mg, 90%).

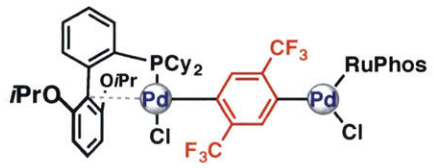
^{1}H NMR (400 MHz, CD_2Cl_2) d 7.54 (t, *J* = 6.9 Hz, 1H), 7.48 (t, *J* = 8.4 Hz, 1H), 7.29 (dt, *J* = 21.5, 7.4 Hz, 2H), 6.77 (ddd, *J* = 7.6, 3.0, 1.5 Hz, 1H), 6.54 (dd, *J* = 41.6, 8.4 Hz, 2H), 6.35 (d, *J* = 9.4 Hz, 1H), 6.27 (s, 1H), 4.48 (ddt, *J* = 35.1, 12.2, 6.2 Hz, 2H), 2.36 (s, 0H), 2.06 (s, 1H), 1.44 (s, 5H), 1.36 (d, *J* = 6.2 Hz, 3H), 1.18 (d, *J* = 6.0 Hz, 2H), 0.96 (d, *J* = 6.0 Hz, 2H), 0.85 (d, *J* = 6.1 Hz, 2H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d 159.48, 158.46, 156.64, 144.44, 140.63, 135.28, 135.12, 132.74, 132.65, 132.54, 132.37, 130.96, 130.81, 130.79, 128.84, 126.52, 126.46, 123.09, 122.87, 113.35, 113.11, 110.48, 107.55, 107.07, 71.28, 71.04, 34.40, 29.10, 28.19, 27.21, 27.08, 26.91, 26.80, 26.68, 26.28, 26.03, 21.85, 21.78, 21.39, 21.26, 13.81. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 31.10. **^{19}F NMR (376 MHz, CD_2Cl_2)** δ -119.28. **FT-IR (neat, cm^{-1}):** 2927.02, 2849.68, 1590.32, 1569.92, 1536.95, 1464.97, 1448.21, 1387.36, 1376.81, 1276.85, 1250.56, 1184.18, 1112.72, 1062.63, 861.73, 831.04, 766.54, 748.1, 737.28, 709.96, 576.55. **HRMS (ESI) m/z calcd. for $C_{66}H_{89}Cl_2FO_4P_2Pd_2$ [M-Cl]$^+$:** 1319.3496. Found: 1319.3490.



OA-D

Following the general procedure, a mixture containing 1,4-Dibromo-2,5-difluorobenzene (36 mg, 0.125 mmol), RuPhos (128 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (107 mg, 0.275 mmol) was stirred at rt in cyclohexane (1.5 mL) for 12 h. General work-up afforded **OA-D** as a grey solid (150 mg, 85%).

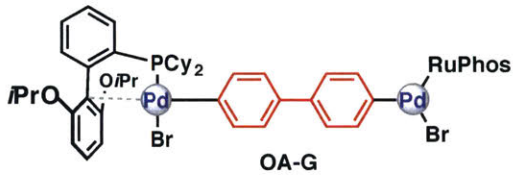
^{1}H NMR (400 MHz, CD_2Cl_2) d 7.74 (q, *J* = 8.1 Hz, 1H), 7.66 (t, *J* = 7.5 Hz, 1H), 7.48 (dd, *J* = 18.1, 8.6 Hz, 2H), 7.25 (t, *J* = 8.3 Hz, 1H), 7.08 (dd, *J* = 6.5, 2.3 Hz, 1H), 6.94 – 6.83 (m, 1H), 6.76 (d, *J* = 9.8 Hz, 1H), 6.69 (d, *J* = 8.4 Hz, 1H), 6.62 (t, *J* = 8.9 Hz, 1H), 4.64 (dt, *J* = 12.2, 5.8 Hz, 2H), 4.45 (dt, *J* = 12.1, 6.0 Hz, 1H), 2.32 – 2.18 (m, 1H), 1.83 (br, 1H), 1.63 (s, 4H), 1.47 (br, 8H), 1.37 (d, *J* = 6.0 Hz, 3H), 1.13 (d, *J* = 6.0 Hz, 3H), 1.05 (d, *J* = 6.0 Hz, 2H), 0.98 (d, *J* = 6.0 Hz, 2H), 0.13 (s, 1H).**^{13}C NMR (101 MHz, CD_2Cl_2)** d 156.71, 136.37, 130.79, 128.21, 71.88, 71.12, 70.58, 37.54, 36.88, 26.91, 26.65, 26.52, 26.24, 25.95, 22.28, 21.82, 21.62, 21.43, 21.24, 0.75 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 31.81. **^{19}F NMR (376 MHz, CD_2Cl_2)** δ -117.91. **FT-IR (neat, cm^{-1}):** 2927.34, 2850.02, 1590.07, 1451.45, 1385.02, 1374.65, 1277.87, 1251.54, 1135.22, 1110.91, 1061.23, 866.25, 850.62, 765.31, 753.05, 737.00, 609.04. **Anal. Calcd. for $C_{66}H_{88}Br_2F_2O_4P_2Pd_2$:** C, 55.90; H, 6.26. Found: C, 55.90; H, 6.37.



OA-E

Following the general procedure, a mixture containing 1,4-Bis(trifluoromethyl)-2,5-dichlorobenzene (32 mg, 0.125 mmol), RuPhos (128 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (107 mg, 0.275 mmol) was stirred at rt in cyclohexane (1.5 mL) for 12 h. General work-up afforded **OA-E** as a grey solid (132 mg, 83%).

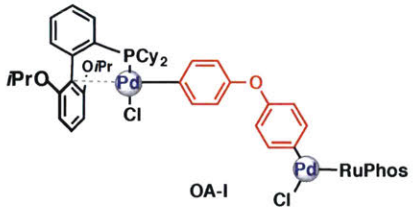
^{1}H NMR (400 MHz, CD_2Cl_2) d 7.84 – 7.71 (m, 1H), 7.62 (q, *J* = 7.6 Hz, 1H), 7.45 (dq, *J* = 21.5, 7.1 Hz, 3H), 7.09 (s, 1H), 7.00 (s, 1H), 6.84 (dd, *J* = 16.4, 7.6 Hz, 1H), 6.65 (dd, *J* = 23.7, 8.5 Hz, 2H), 4.63 (dp, *J* = 18.1, 6.0 Hz, 3H), 1.82 (br, 8H), 1.48 (br 3H), 1.35 (s, 2H), 1.17 (s, 2H), 0.91 (dd, *J* = 17.4, 6.0 Hz, 4H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d δ 161.59, 161.35, 161.00, 160.94, 145.63, 145.45, 136.82, 136.30, 134.86, 134.46, 133.90, 132.23, 132.11, 131.90, 130.76, 130.73, 130.58, 126.43, 126.37, 107.85, 106.75, 106.65, 106.52, 106.36, 72.20, 71.63, 70.25, 70.16, 35.06, 34.78, 31.80, 31.54, 30.18, 27.41, 27.05, 26.91, 26.79, 26.72, 26.55, 26.50, 26.43, 26.34, 26.00, 25.86, 25.53, 25.45, 21.95, 21.81, 21.74, 21.35, 21.27, 21.23, 21.10. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 32.03. **^{19}F NMR (376 MHz, CD_2Cl_2)** δ -54.53. **FT-IR (neat, cm^{-1}):** 3855.14, 3745.16, 2923.80, 2856.75, 1589.64, 1560.57, 1444.39, 1334.44, 1252.70, 1154.35, 1093.12, 1067.17, 1047.83, 1020.47, 766.65. **HRMS (ESI) m/z calcd. for $C_{68}H_{89}Cl_2O_4P_2F_6Pd_2$ [M-Cl]$^+$:** 1391.3820. Found: 1393.3881.



OA-G

Following the general procedure, a mixture containing 4,4-Dibromobiphenyl (39 mg, 0.125 mmol), RuPhos (128 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (107 mg, 0.275 mmol) was stirred at rt in cyclohexane (1.5 mL) for 12 h. General work-up afforded **OA-G** as a grey solid (209 mg, 98%).

^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.63 (t, J = 8.4 Hz, 1H), 7.41 (dt, J = 23.5, 7.4 Hz, 1H), 7.16 (d, J = 8.1 Hz, 1H), 7.09 (dd, J = 8.4, 2.0 Hz, 1H), 6.87 (dd, J = 7.8, 2.9 Hz, 0H), 6.65 (d, J = 8.5 Hz, 1H), 4.62 (p, J = 6.0 Hz, 1H), 2.18 (q, J = 12.3, 11.8 Hz, 1H), 1.71 (d, J = 52.0 Hz, 1H), 1.52 (s, 1H), 1.40 (d, J = 6.0 Hz, 3H), 1.21 (b, 1H), 1.03 (d, J = 6.0 Hz, 3H), 0.78 (q, J = 13.4 Hz, 1H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d 159.02, 144.84, 144.67, 137.58, 137.55, 136.27, 134.46, 134.39, 133.71, 133.36, 132.48, 132.38, 131.04, 130.47, 130.44, 126.26, 126.21, 124.85, 124.83, 111.52, 111.48, 107.25, 70.86, 33.85, 33.59, 28.14, 27.99, 27.61, 27.59, 27.23, 27.10, 26.92, 26.88, 26.77, 26.02, 21.89, 21.38 bserved complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 31.80. **FT-IR (neat, cm^{-1}):** 2976.53, 2920.64, 2849.62, 2354.67, 1588.59, 1455.66, 1242.77, 1110.89, 1059.83, 996.47, 797.70, 731.52, 667.91, 610.43, 577.71. **HRMS (ESI) m/z calcd. for $C_{72}H_{94}Br_2O_4P_2Pd_2$ [M-2Br]$^{2+}$:** 649.2364. Found: 649.2390.

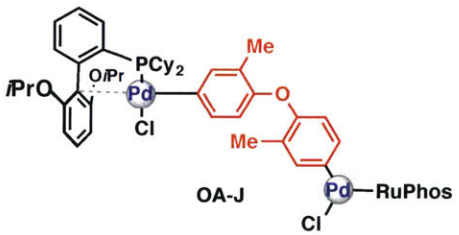


OA-I

Following the general procedure, a mixture containing 4-chlorophenyl ether (60 mg, 0.125 mmol), RuPhos (292.8 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (244 mg, 0.275 mmol) was stirred at rt in cyclohexane (2.4 mL) for 12 h. General work-up afforded **OA-I** as a light brown solid (304.3 mg, 88 %).

^{1}H NMR (400 MHz, CD_2Cl_2) d 7.67 (q, J = 7.7, 7.0 Hz, 1H), 7.45 (dt, J = 23.0, 7.4 Hz, 1H), 7.01 (dd, J = 8.7, 2.0 Hz, 1H), 6.90 (ddd, J = 7.6, 3.0, 1.4 Hz, 1H), 6.67 (dd, J = 8.5, 3.7 Hz, 3H), 4.66 (p, J = 6.0 Hz, 1H), 2.20 (q, J = 12.1 Hz, 2H), 1.81 (br, 3H), 1.61 (br, 1H), 1.42 (d, J = 6.0 Hz, 3H), 1.27 (b, 2H), 1.05 (d, J = 6.0 Hz, 3H), 0.78 (q, J = 12.9 Hz, 1H). **^{13}C NMR (101 MHz,**

CD_2Cl_2) d 159.16, 154.65 145.05, 144.88, 137.00, 136.96, 134.81, 133.90, 133.54, 132.37, 132.26, 130.95, 130.55, 130.52, 129.45, 126.28, 126.22, 117.46, 117.43, 110.90, 110.87, 106.91, 70.75, 33.67, 33.40, 28.12, 27.71, 27.69, 27.24, 27.11, 26.84, 26.72, 26.00, 21.83, 21.34 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 33.45. **FT-IR (neat, cm^{-1}):** 2923.66, 2849.80, 1591.49, 1471.28, 1456.00, 1435.99, 1383.09, 1371.62, 1225.85, 1164.77, 1111.35, 1050.67, 1004.19, 848.86, 809.35, 784.21, 758.68, 731.48, 609.16, 575.02, 564.82. **Anal. Calcd. for $C_{72}H_{94}Cl_2O_5P_2Pd_2$:** C, 62.43; H, 6.84. Found: C, 62.33; H, 6.82.



OA-J

Following the general procedure, a mixture containing 4,4'-oxybis(1-chloro-3-methylbenzene) (21.9 mg, 0.125 mmol), RuPhos (96.0 mg, 0.275 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (80.0 mg, 0.275 mmol) was stirred at rt in cyclohexane (0.75 mL) for 12 h. General work-up afforded **OA-J** as a brown solid (86.6 mg, 75 %).

^{1}H NMR (400 MHz, CD_2Cl_2) d 7.67 (q, *J* = 7.6, 6.7 Hz, 1H), 7.45 (dt, *J* = 23.0, 7.4 Hz, 1H), 6.95 (s, 1H), 6.90 (ddd, *J* = 7.6, 2.9, 1.3 Hz, 1H), 6.78 (d, *J* = 8.3 Hz, 1H), 6.67 (d, *J* = 8.5 Hz, 1H), 6.37 (d, *J* = 8.4 Hz, 1H), 4.66 (p, *J* = 6.0 Hz, 1H), 2.18 (s, 2H), 1.78 (s, 4H), 1.47 (s, 1H), 1.25 (s, 1H), 1.05 (d, *J* = 6.0 Hz, 3H), 0.78 (br, 1H). **^{13}C NMR (101 MHz, CD_2Cl_2)** d 159.61, 153.10, 145.62, 145.45, 139.16, 135.24, 134.91, 134.88, 134.86, 134.55, 134.19, 132.94, 132.84, 131.51, 131.06, 131.04, 129.47, 127.70, 126.82, 126.77, 116.76, 111.74, 107.51, 71.32, 34.22, 33.93, 28.69, 27.90, 27.77, 27.48, 27.45, 27.34, 26.64, 22.43, 21.91, 16.36 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, CD_2Cl_2)** d 33.23. **FT-IR (neat, cm^{-1}):** 2972.95, 2927.54, 2852.92, 1592.71, 1453.1, 1382.83, 1371.1, 1267.8, 1230.18, 1173.8, 1112.72, 1054.44,

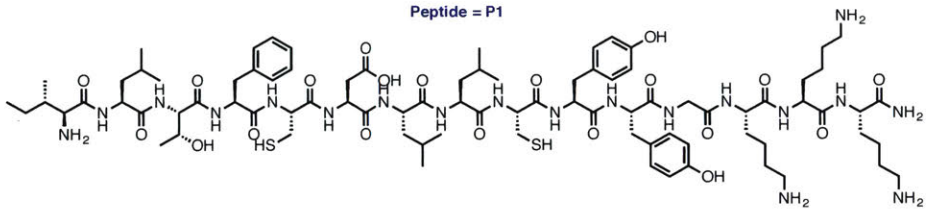
1002.37, 898.56, 848.98, 803.64, 785.06, 756.54, 735.1, 667.84. **Anal. Calcd. for $C_{74}H_{98}Cl_2O_5P_2Pd_2$:** C, 62.89; H, 6.99. Found: C, 62.77; H, 6.94.

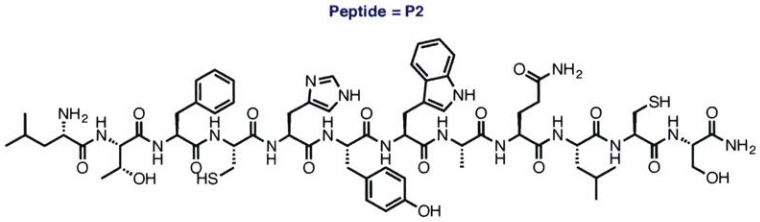
Linear Peptide Synthesis

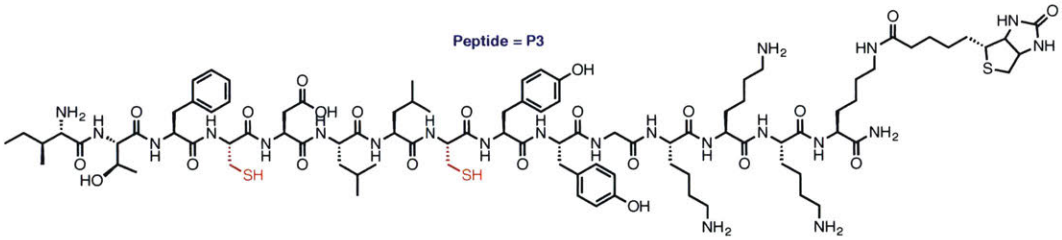
General Linear Peptide Synthesis Procedure:

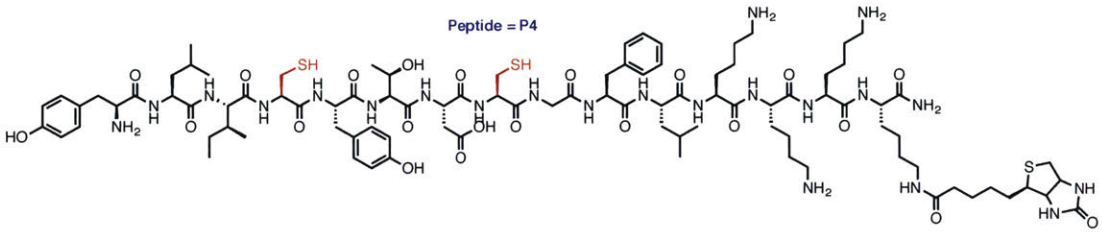
The peptides (**P1-P4**) were synthesized on a 0.9 mmol scale using manual Fmoc-SPPS (Solid Phase Peptide Synthesis) chemistry. Specifically, the resin was pre-wetted with DMF in a peptide synthesis vessel equipped with a T-bore stopcock connected to a vacuum pump for faster drainage. Each amino acid (5 mmol) was pre-dissolved in HBTU (0.2 M, 25 mL). The procedure for each amino acid coupling cycle included: 1) pre-activation of the amino acid with DIEA (2.5 mL; note, 0.9 mL was used in the case of cysteine to prevent racemization); 2) 10 min coupling time; 3) Wash with DMF (4 × 30 mL; or enough DMF to cover the resin); 4) Deprotection with 20% (v/v) piperidine in DMF (2 × 3 min; 30 mL each time, or enough of the solution to cover the resin); 5) Wash with DMF (4 × 30 mL; or enough DMF to cover the resin). After completion of the stepwise SPPS, the resin was washed thoroughly with CH_2Cl_2 and dried under vacuum. Peptides **P1, P2, P3,** and **P4** were cleaved from the resin and deprotected on the side-chains by treatment with 2.5% (v/v) water, 2.5% (v/v) 1,2-ethanedithiol (EDT), and 1% (v/v) triisopropylsilane in neat trifluoroacetic acid (TFA) for 7 min at 60 °C (water bath, 5 mL cleavage solution/250 mg of final resin). Cold (–80 °C) diethyl ether was added to the solution and the resulting precipitate was centrifuged at 4000 rpm for 5 min. The residue was washed with cold ether (2x). The obtained solid was dissolved in 50% H_2O : 50% acetonitrile containing 0.1% TFA, the resin was removed via filtration and the final solution was lyophilized to provide the crude peptide material. For peptides containing biotin, the unnatural amino acid Fmoc-

Lys(biotin)-OH was used as the coupling amino acid. The following peptides were all synthesized using this procedure:









Peptide Purification

Solvent compositions for RP-HPLC purification are water with 0.1% TFA (solvent C) and acetonitrile with 0.1% TFA (solvent D). The crude peptide was dissolved in 50% C : 50% D and purified by semi-preparative RP-HPLC (Agilent Zorbax 300SB C_{18} column: 21.2 x 250 mm, 7 μm, linear gradient: 5-50% B over 65 min, flow rate: 5 mL/min). Each HPLC fraction was analyzed by mass-directed preparative LC-MS. HPLC fractions containing pure product were

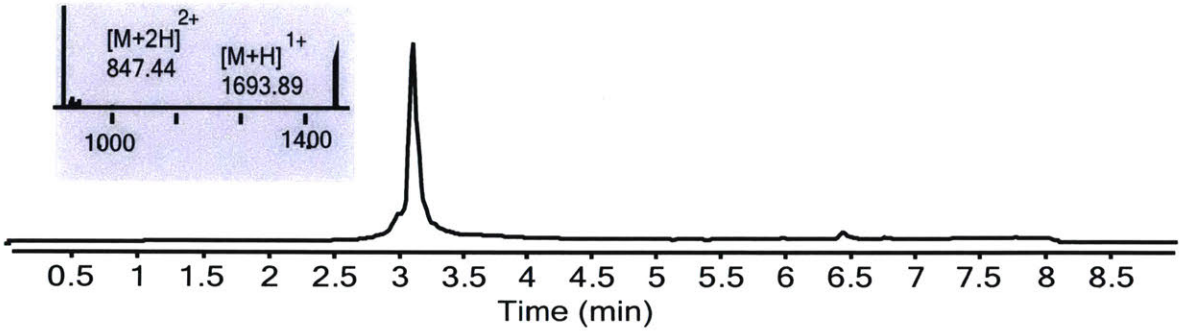
further confirmed by LC-MS, combined, and lyophilized. Peptides synthesized using manual SPPS and purified by RP-HPLC are listed in Table S1.

Table S1. Sequences and masses of peptides synthesized by manual fast flow SPPS.

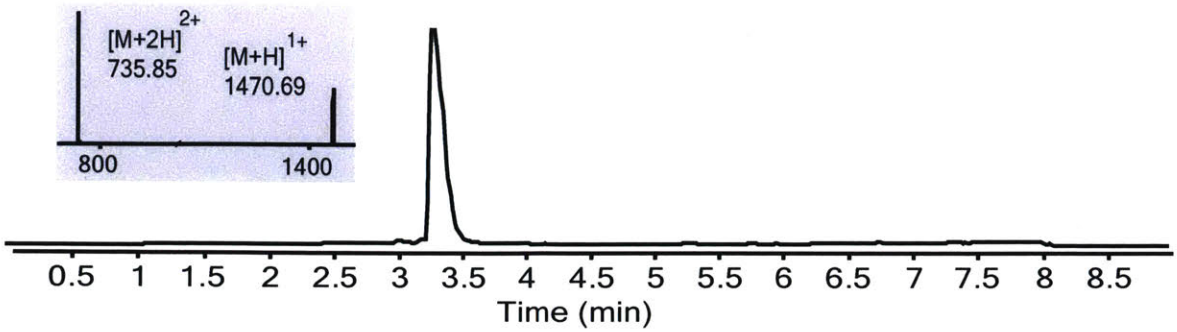
Peptide	Sequence[a]	Calculated mass	Observed Mass $[M+H]^+$ or $[M+H]^{2+}$
P1	NH_2-ITF**C**DLL**C**YYGKKK-$CONH_2$	1692.87	1693.89
P2	NH_2-LTF**C**HYWAQL**C**S-$CONH_2$	1469.66	1470.69
P3	NH_2-ITF**C**DLL**C**YYGKKKK(Biotin)-$CONH_2$	2047.05	1025.04
P4	NH_2-YLI**C**YTD**C**GFLKKKK(Biotin)-$CONH_2$	2047.05	1025.05

[a] **Cysteine residues are highlighted in red.**

P1:



P2:



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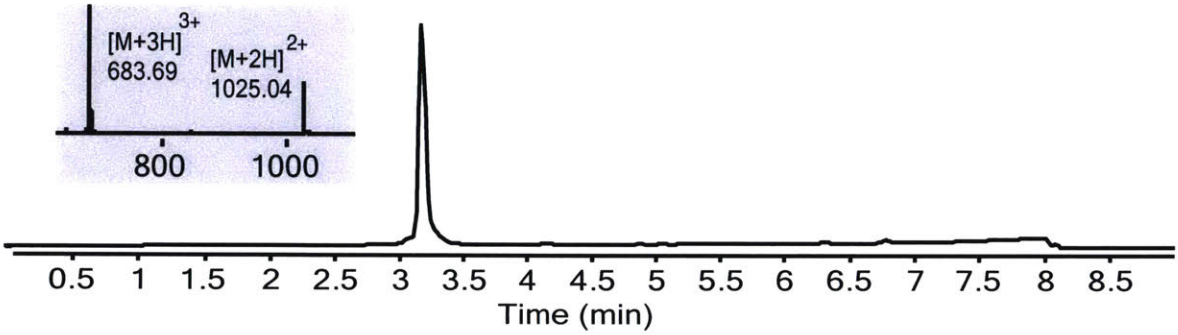
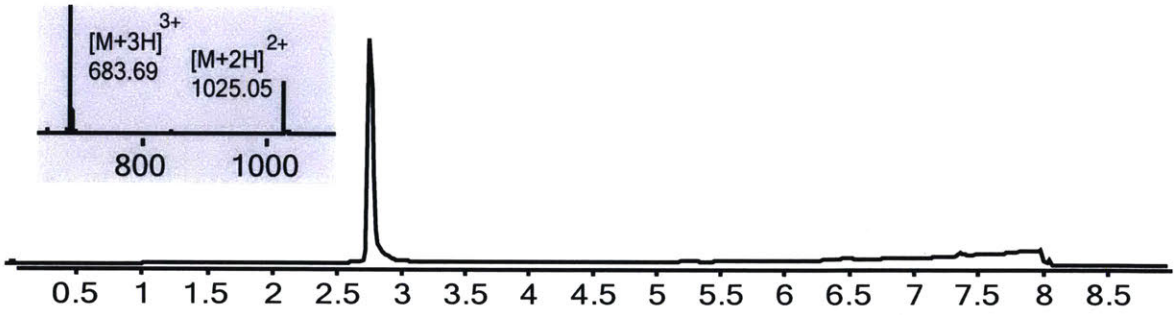
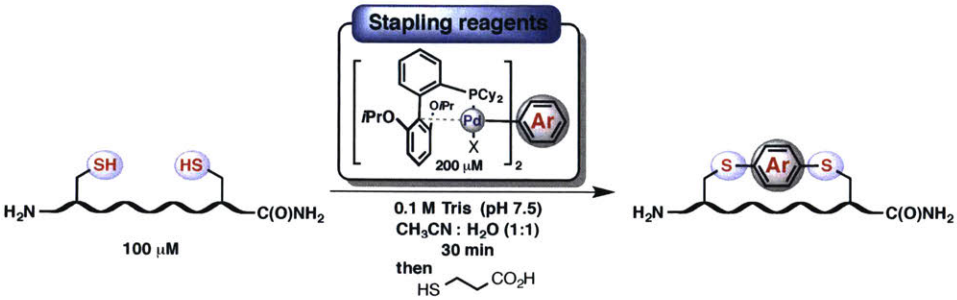


P4:

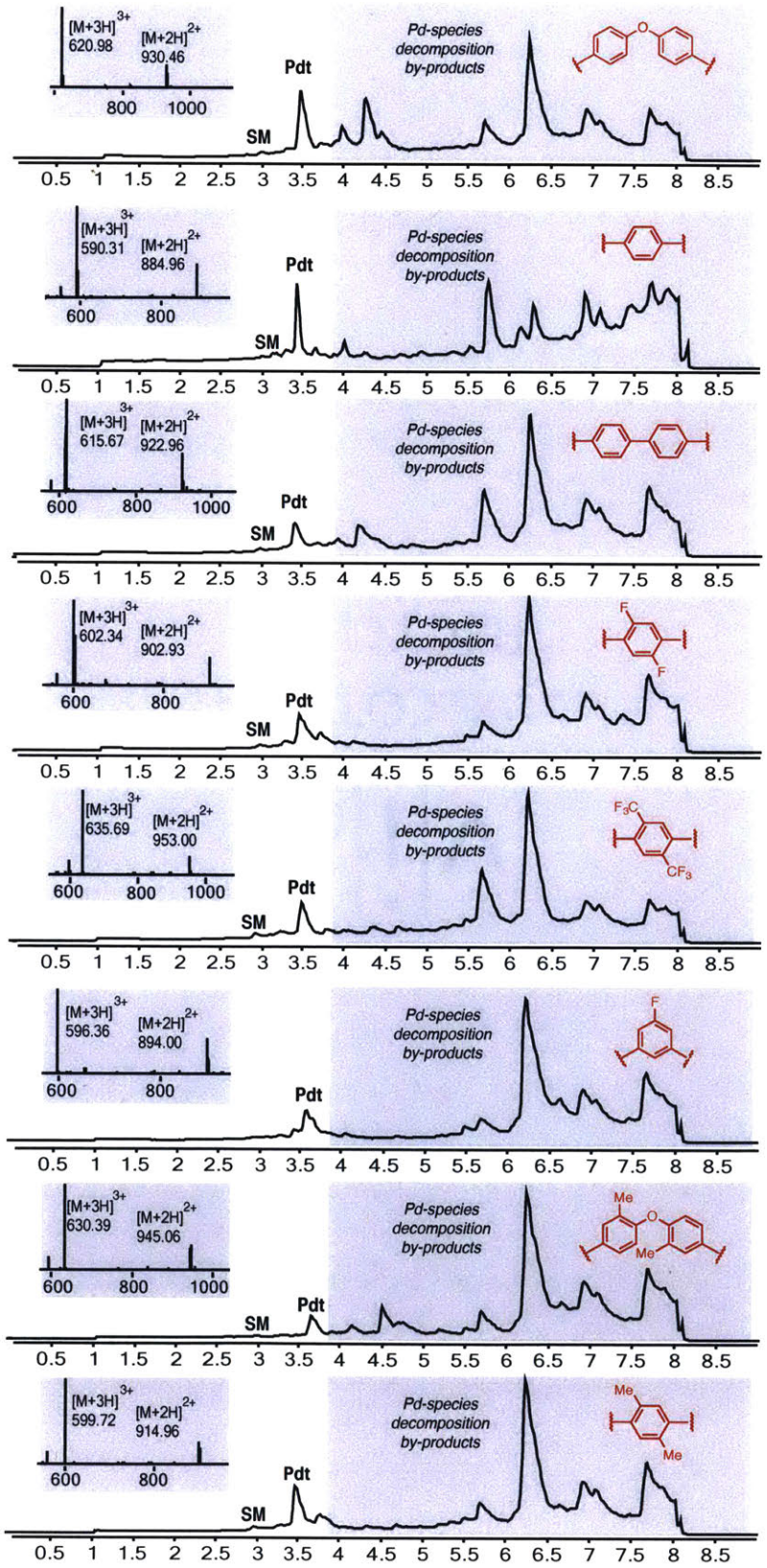


General Macrocyclization Procedure

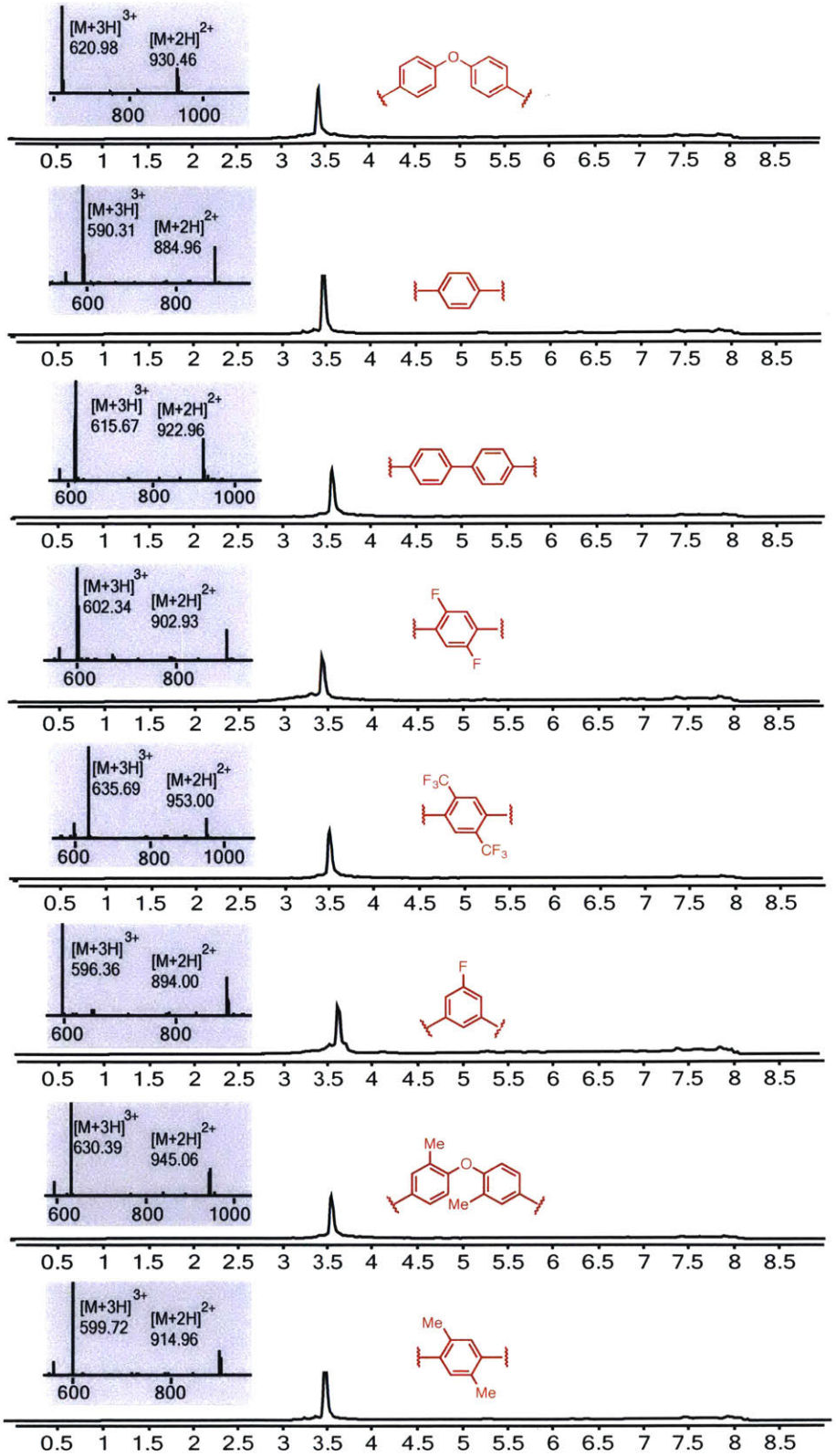


A solution of the Pd-stapling reagent in acetonitrile (400 mM) was added to a solution of peptide (200 mM) in Tris buffer (0.1 M, pH 7.5). Note: if the palladium reagent was not readily soluble in CH_3CN, the slurry was sonicated for 30 s to facilitate this process. Final conditions: [Pd] = 200 mM; [peptide] = 100 mM; H_2O : CH_3CN = 1 : 1. After 30 min stirring at rt, 3-mercaptopropionic acid (3 equiv to the palladium complex, solution in 1 mL of H_2O) was added to the reaction mixture to quench and remove some of the remaining palladium species. The reaction was allowed to stand for 1 h for precipitate formation, the solids were centrifuged at 4000 rpm for 5 min followed by washing with an additional 5 mL of 50 % : 50 % CH_3CN : H_2O (v/v). The supernatant was collected via decantation and the acetonitrile was removed via rotary evaporation and the resulting crude material was lyophilized. The crude peptide was purified using preparative HPLC as described above.

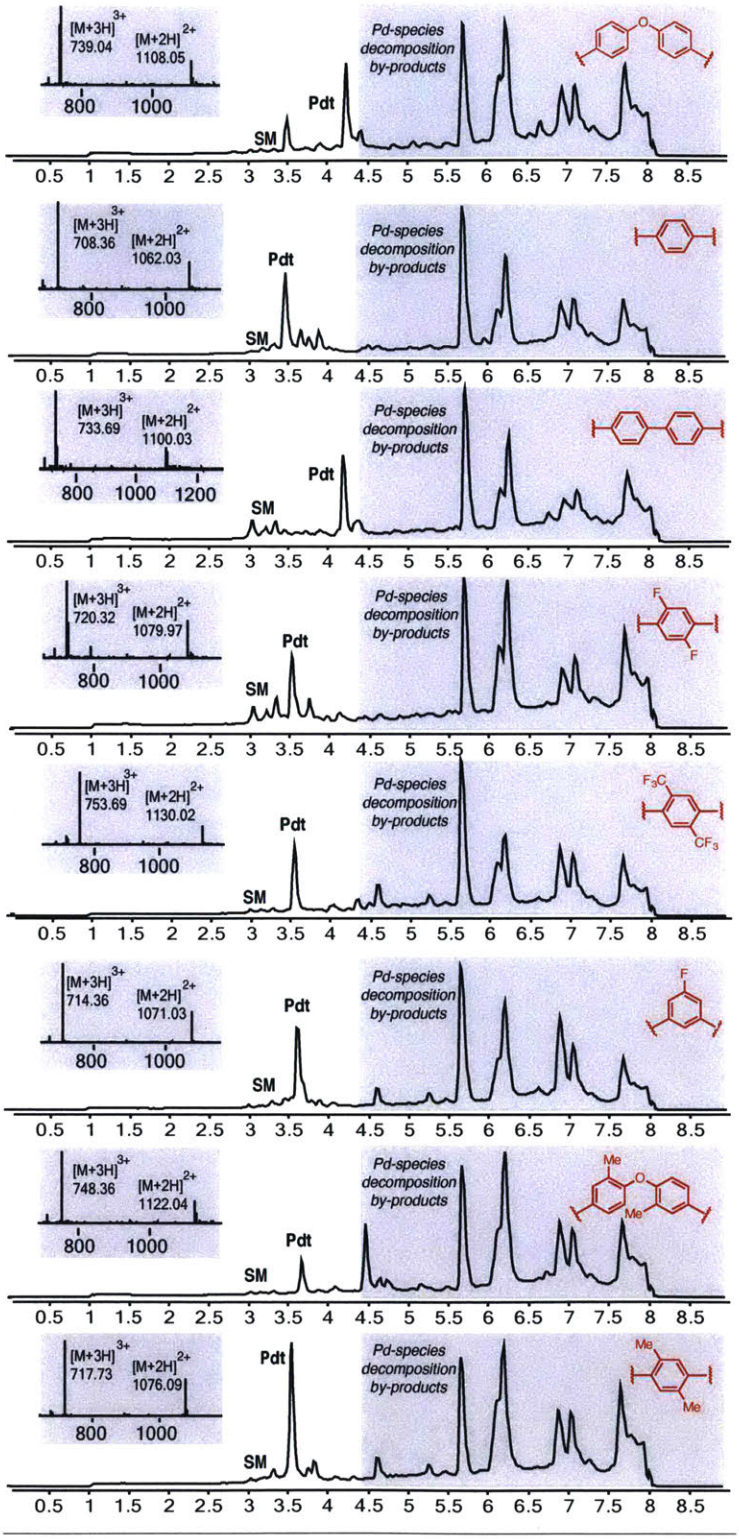
LC-MS Traces of Crude Reaction Mixtures of Macrocyclization Experiments (for P1)



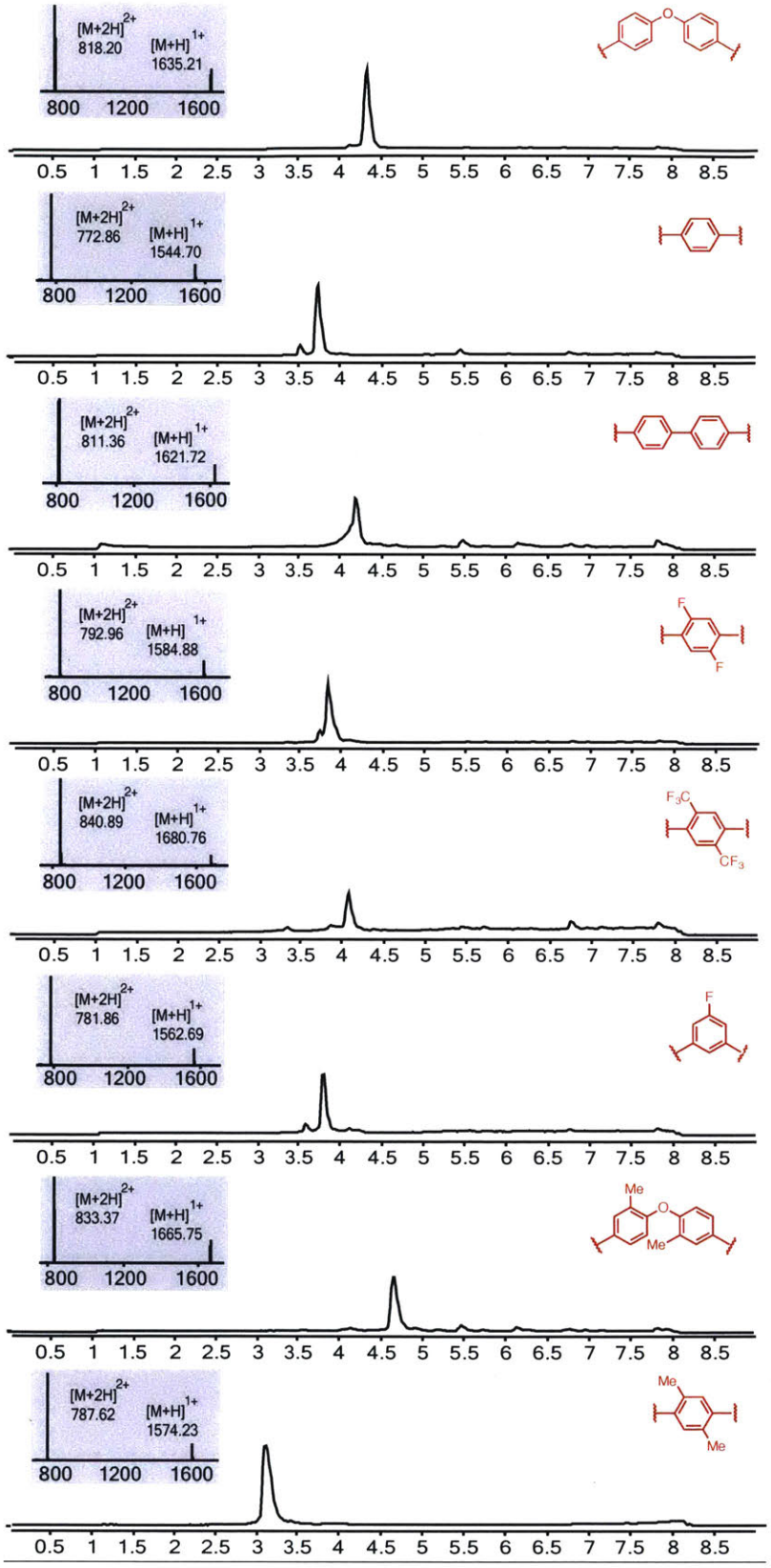
LC-MS Traces for Isolated Pure Macrocyclic Peptides (for P1)



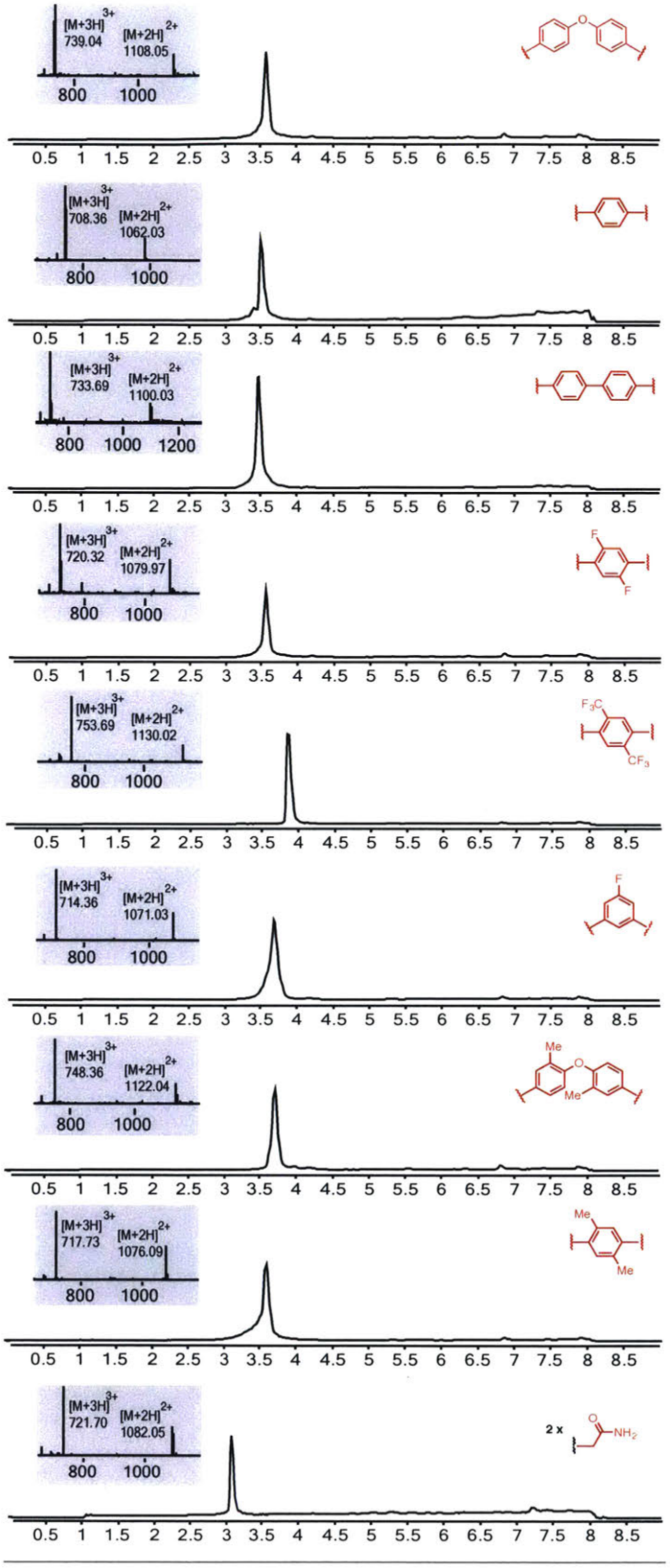
LC-MS Traces of Crude Reaction Mixtures of Macrocyclization Experiments (for P2)



LC-MS Traces for Isolated Pure Macrocyclic Peptides (for P2)

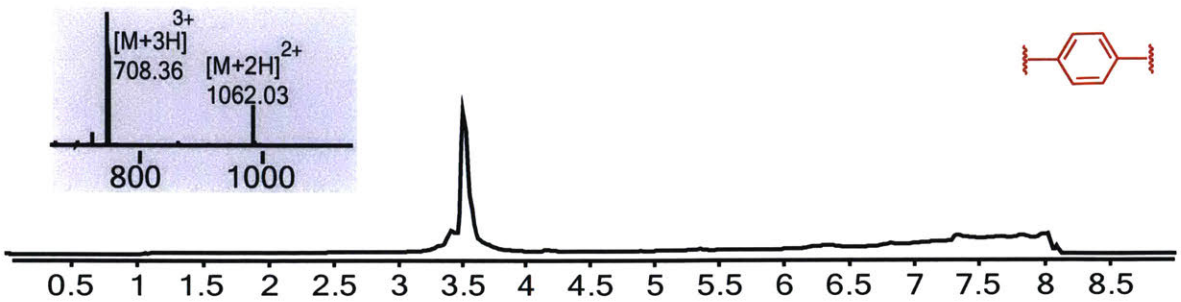


LC-MS Traces for Isolated Pure Macrocyclic Peptides (for P3)

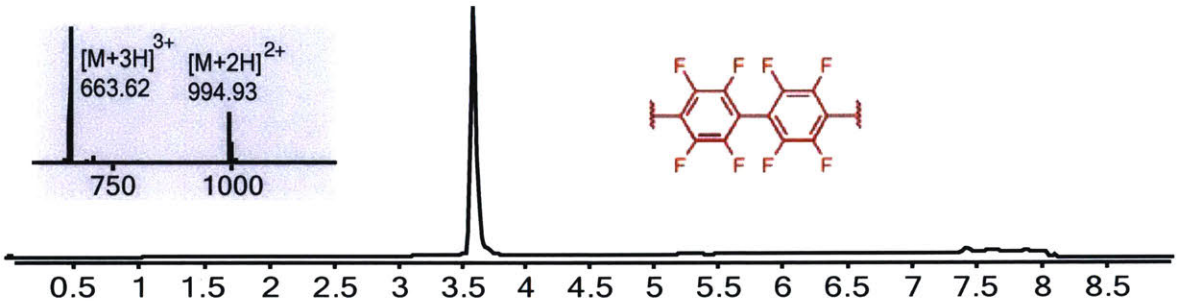


LC-MS Traces for Isolated Pure Macrocyclic Scrambled Peptides (for P4)

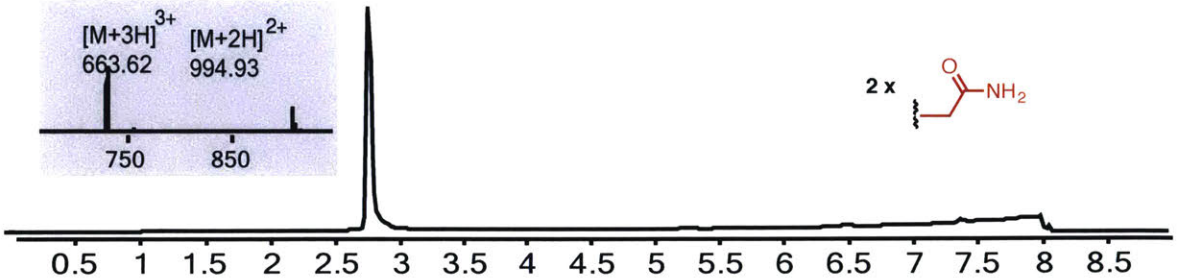
P4-A



P4-H



P4-K



C-CA Protein Expression

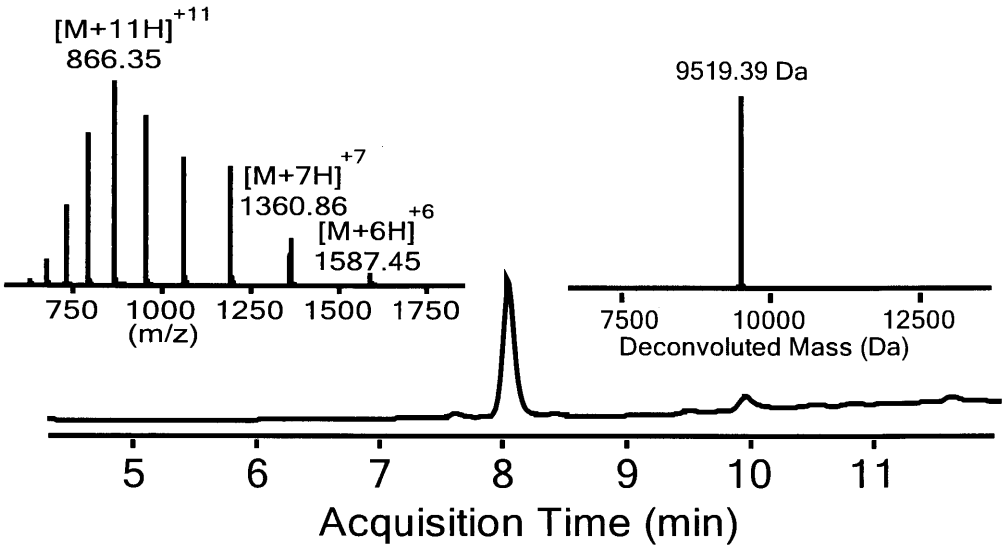
This procedure and vector was obtained based on a previously reported protein expression.[3] Full-length HIV1 Core Antigen was obtained from Invitrogen. C-CA sequence was cloned into pET-SUMO plasmid and ligated with T4 ligase overnight at 4 °C. The resulting construct was transformed into DH5α cells and then purified with the QIAprep Spin Miniprep Kit. BL21 DE3

competent cells were transformed via heat shock with 100 ng of plasmid containing properly oriented C-CA inserts. Single transformed colonies were grown in 1L LB broth containing 30 μg/mL kanamycin. The cultures were induced with 1 mL 0.4 M IPTG upon reaching OD600 = 0.6.

Following overnight expression, cells were pelleted and resuspended in 50 mL Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4) containing 20 mg lysozyme, 2 mg DNAse I, and a half tablet of cOmplete Mini protease inhibitor cocktail (Roche Life Science). Following sonication, the suspension was pelleted (25 min, 17000 rpm) and supernatant loaded onto a Ni-NTA column (HisTrap FF crude, GE Healthcare). The column was washed with 30 mL of Tris buffer (pH 8.0) and 40 mM imidazole in Tris buffer (pH 8.0) each and the SUMO-CCA fusion protein was eluted in 10 mL 150 mM imidazole in Tris buffer (pH 8.0). The protein was then subject to buffer exchange on Akta size exclusion column FPLC into Tris buffer at pH 7.4.

Protein concentration was determined by measuring absorbance at 280 nm (MW = 22916.9 Da, ε = 9970 as found on ExPASy ProtParam (http://web.expasy.org/protparam/)); SUMO cleavage was subsequently performed by incubating SUMO-CCA overnight at 4 °C with 1 ng SUMO protease per 1 mg protein. The resulting mixture was loaded onto a Ni-NTA column and washed with 10 mL 40 mM imidazole in Tris buffer (pH 8.0) to isolate C-CA. The protein was subjected to buffer exchange into Tris buffer at pH 7.4, concentrated over a 3000 Da Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore), analyzed by HPLC-MS, and flash frozen in liquid nitrogen for long-term storage at -80 °C.

Figure S2: Deconvoluted spectrum of CCA protein after purification



Calculated molecular weight: 9518.9 Da. Found: 9519.39 Da.

BioLayer Interferometry Binding

Experimental Design

Binding kinetics for the **C-CA/P3-A – P3-K** interaction were measured on Octet RED96 instrument from ForteBio. All measurements were performed at 30° C in a 96-well plate shaking at 1000 rpm. In general, streptavidin-coated tips (from ForteBio) were dipped in binding buffer (1x phosphate buffered saline, 0.1% w/v bovine serum albumin, 0.02% tween-20, pH 7.4) for 10 min to remove sucrose coating. A typical experiment consisted of five steps:

· Base line reading. Tips were dipped into a well containing fresh binding buffer for 60 sec.

· Ligand loading. ~45 μM solutions of **P3,4-A – P3,4-K** in binding buffer were associated onto the tips for 90-300 sec.

· Base line reading #2. Tips were dipped into a well containing fresh binding buffer for 90-120 sec.

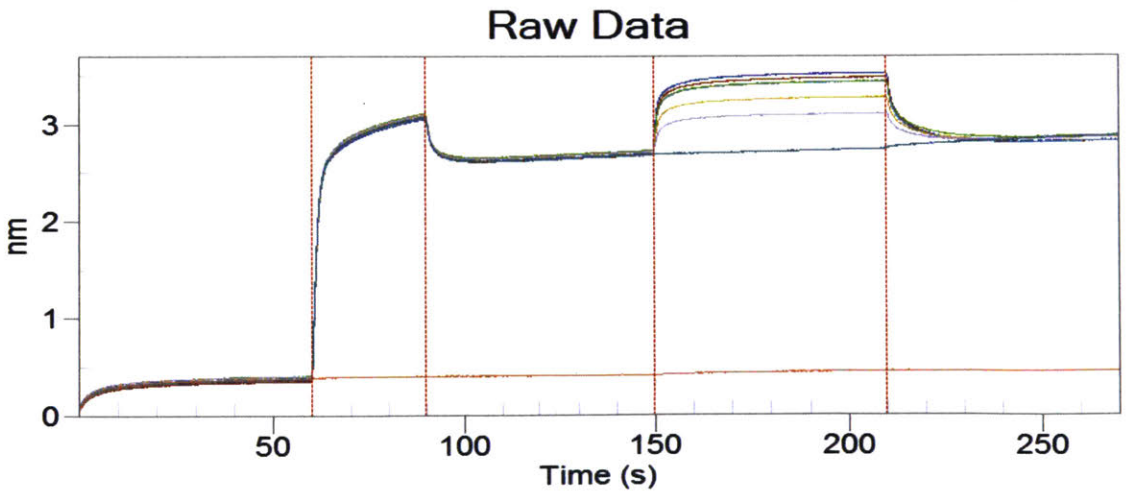
· Association. **P3 and P4** loaded tips were dipped into wells containing variable concentrations of **C- CA** (5 μM – 100 μM) in binding buffer for 90-300 sec.

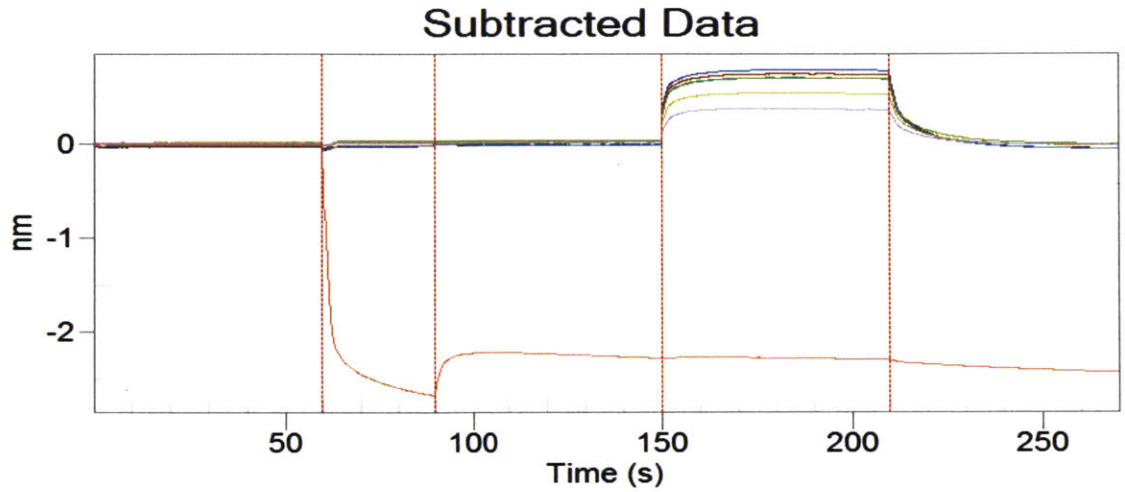
· Dissociation. The tips were dipped back into wells containing binding buffer only for 300 sec or until the response decreased back to the base line level. Double referencing using wells containing no **C-CA** and tips loaded with no ligands was used to subtract the background.

Data Analysis

Binding data collected for peptides **P3-A-K and P4-A, K** according to the procedure described in the section above was processed as follows. Reference readings were subtracted universally from both association and dissociation curves. Association curves were aligned to the last five sec of the base line, and dissociation curves were aligned to the last value of association curves. No curve-smoothing filtering was performed. The K_D values reported were calculated using the automated software for the Forte Bio-Layer Interferrometry software manual. The curves and subsequent fittings have been provided below for each peptide, as well as a table with the calculated binding values.

P1-A

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-2
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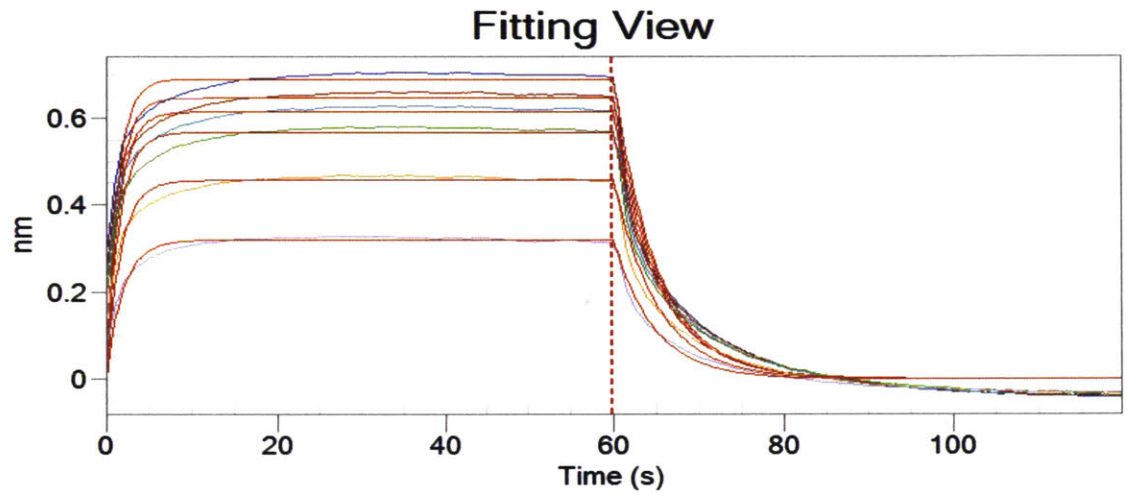
100
150
200
250
Time (s)


Fitting View
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80
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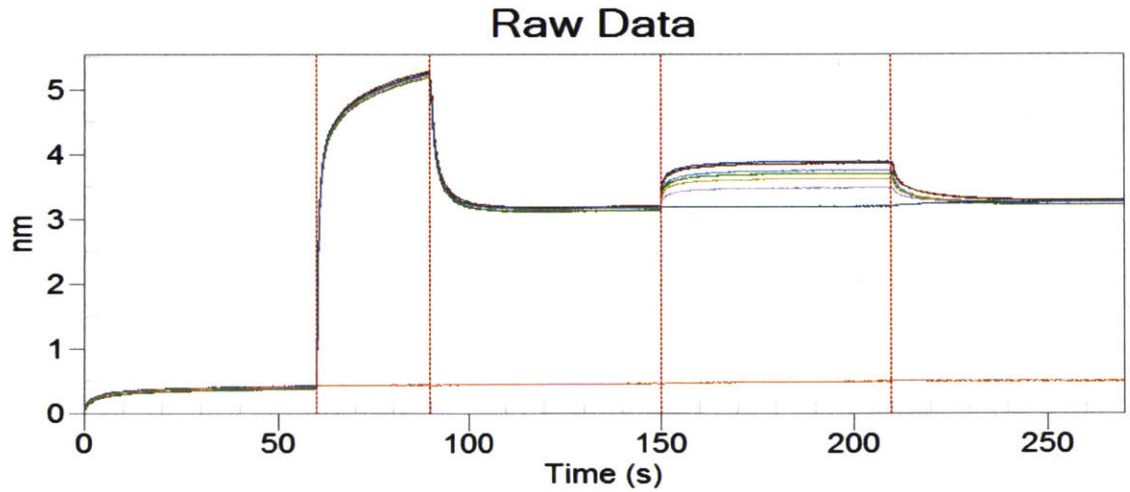
Time (s)

P1-B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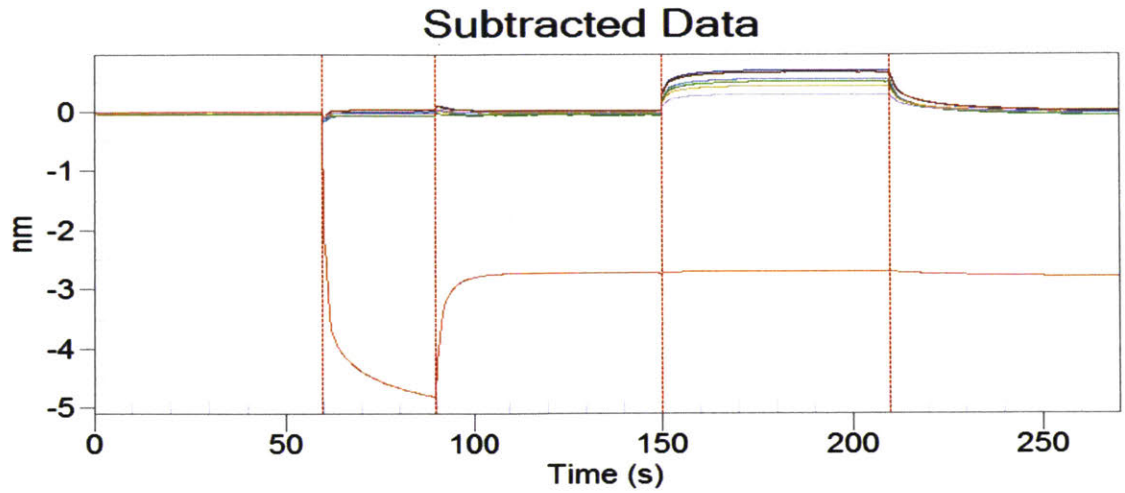
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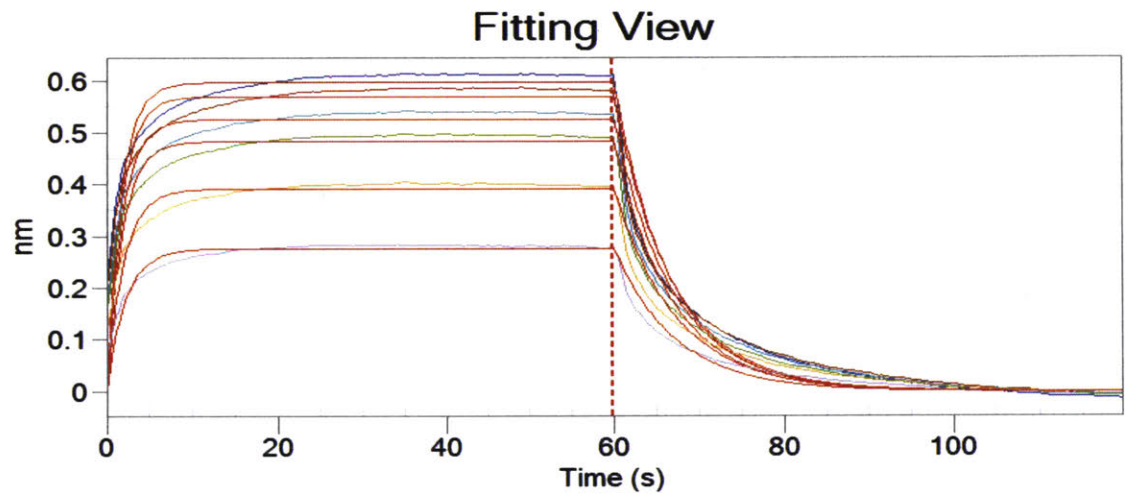
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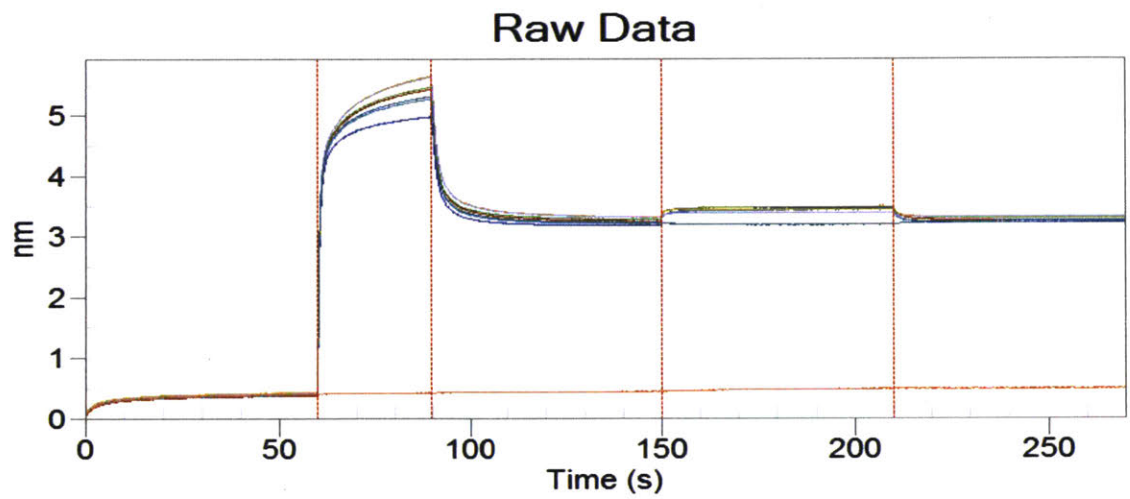
Time (s)

P1-C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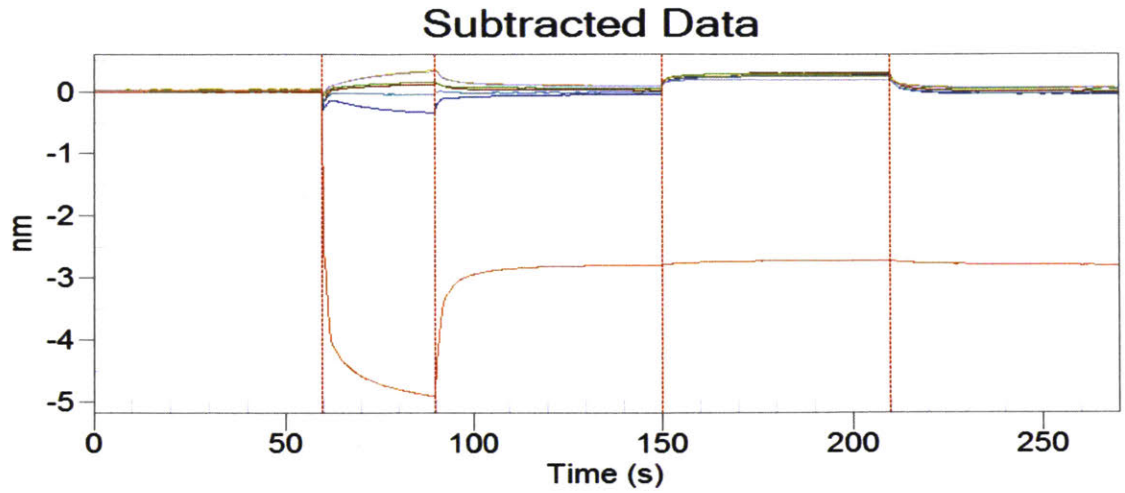
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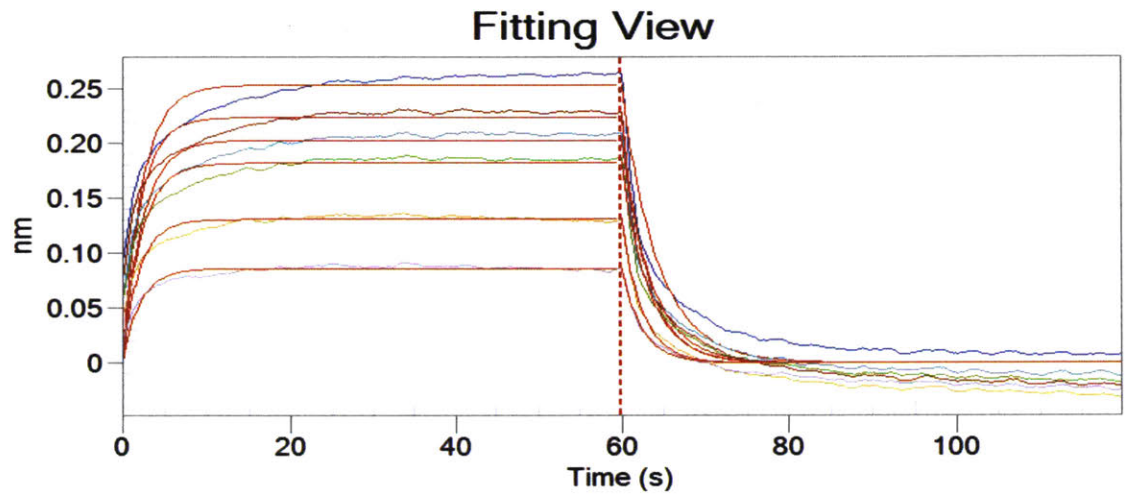
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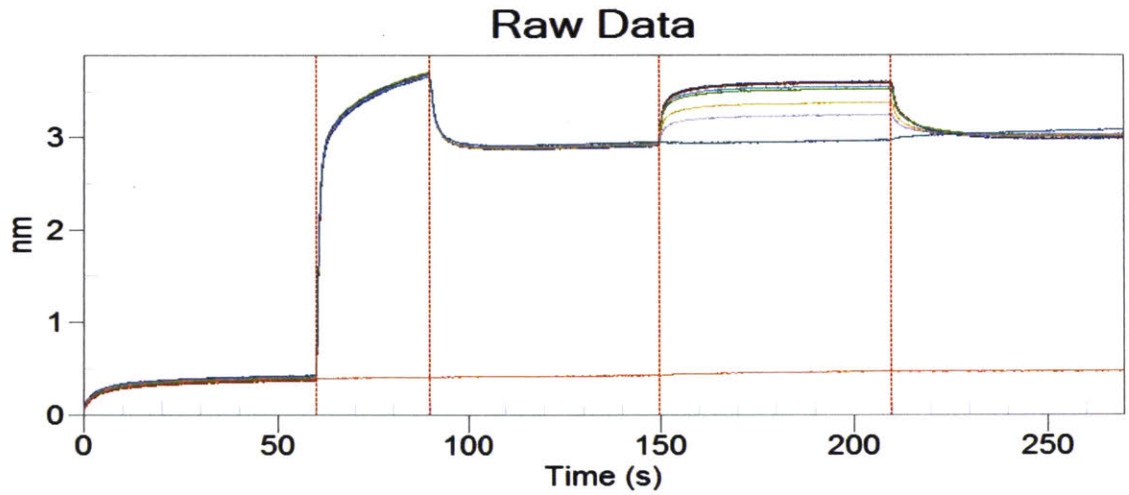
Time (s)

P1-D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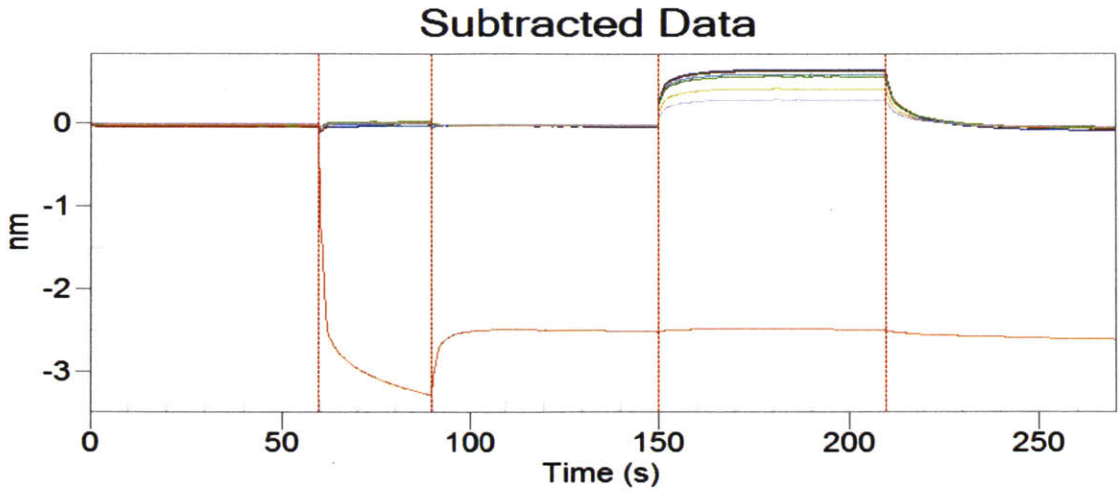
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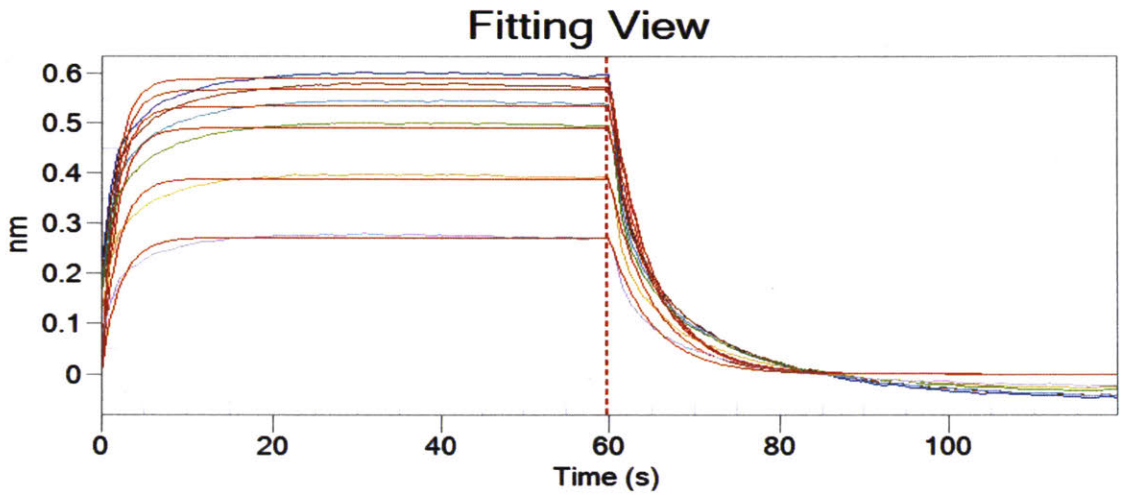
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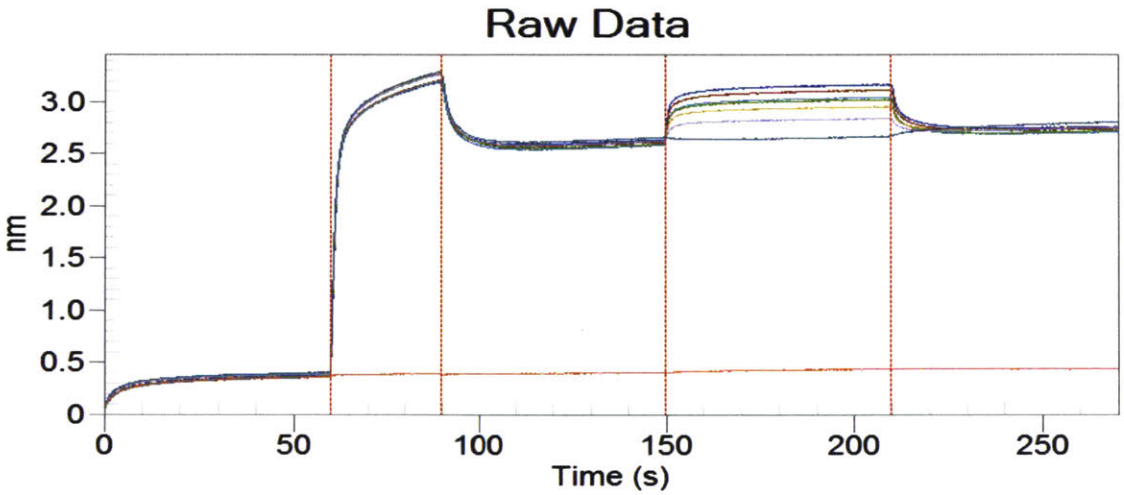
Time (s)

P1-E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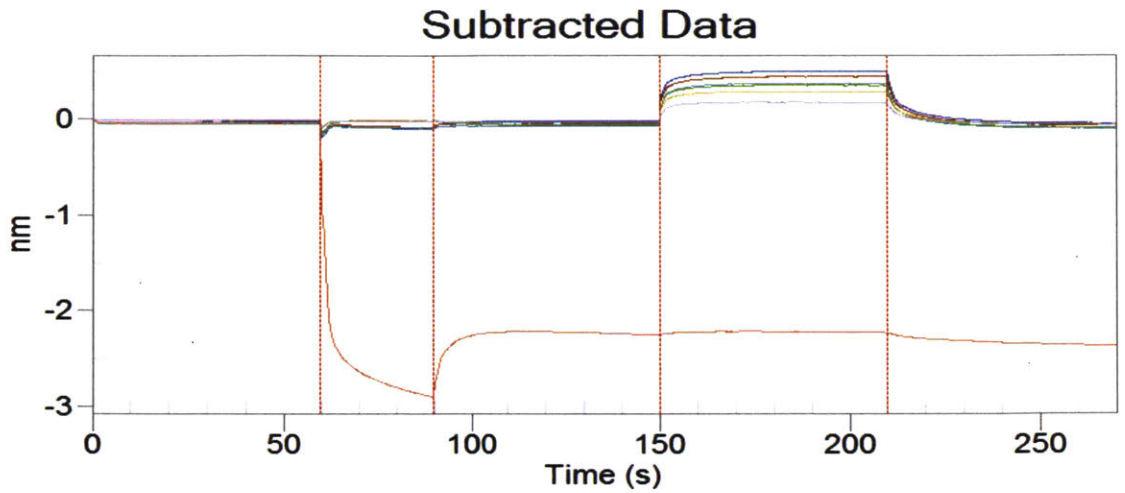
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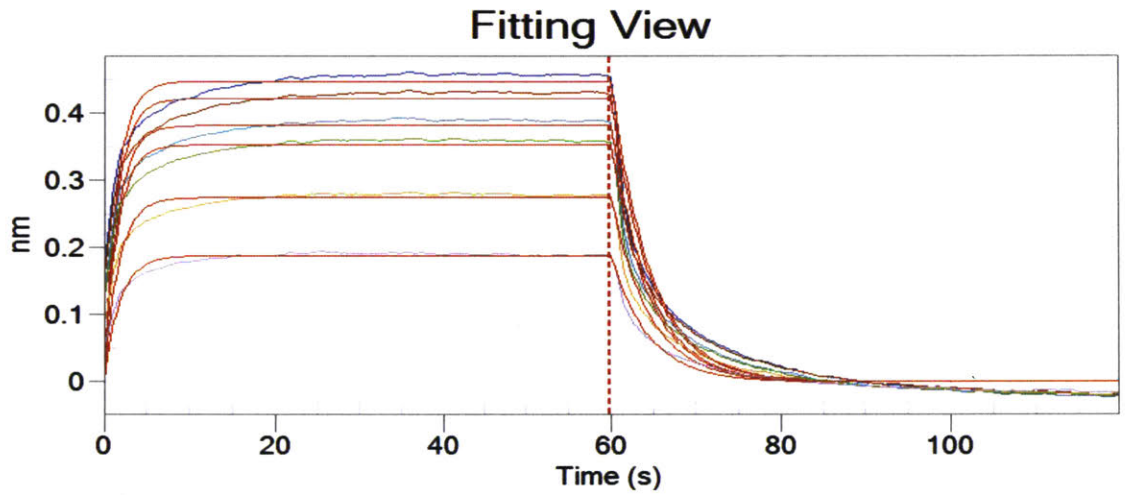
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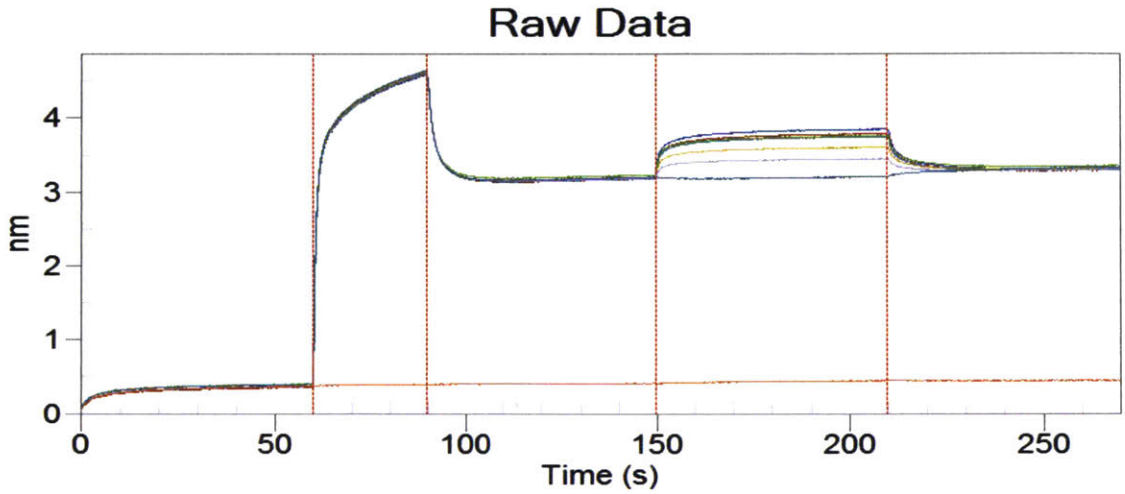
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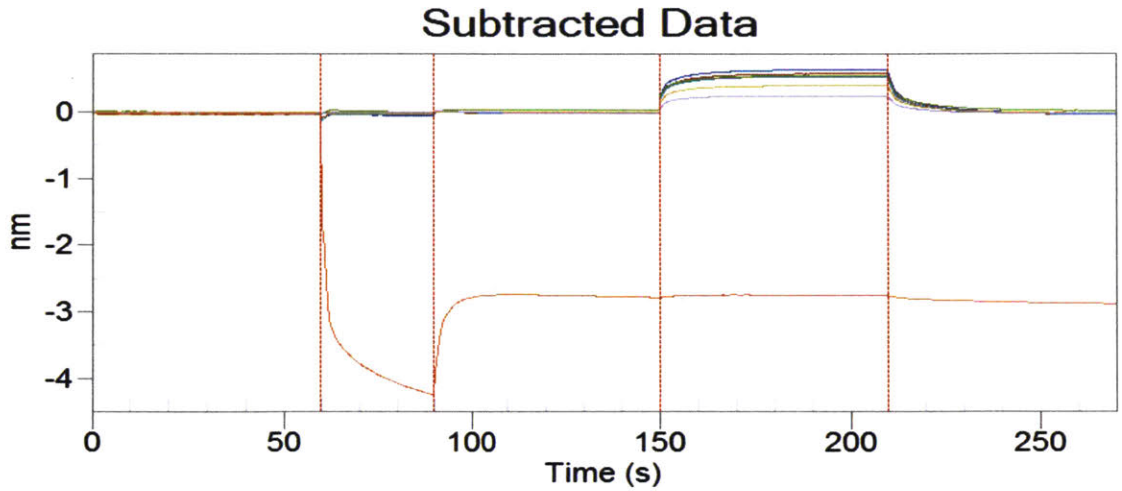
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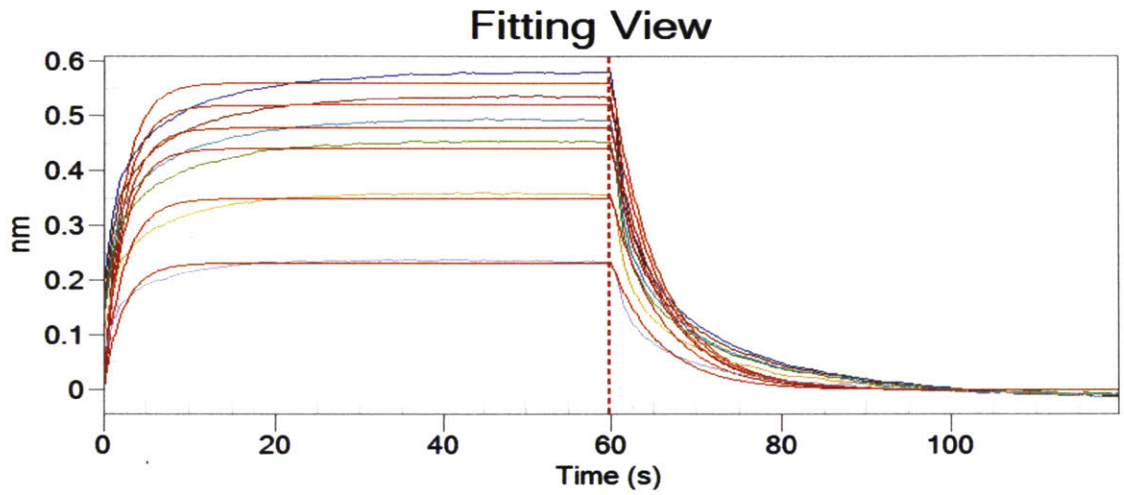
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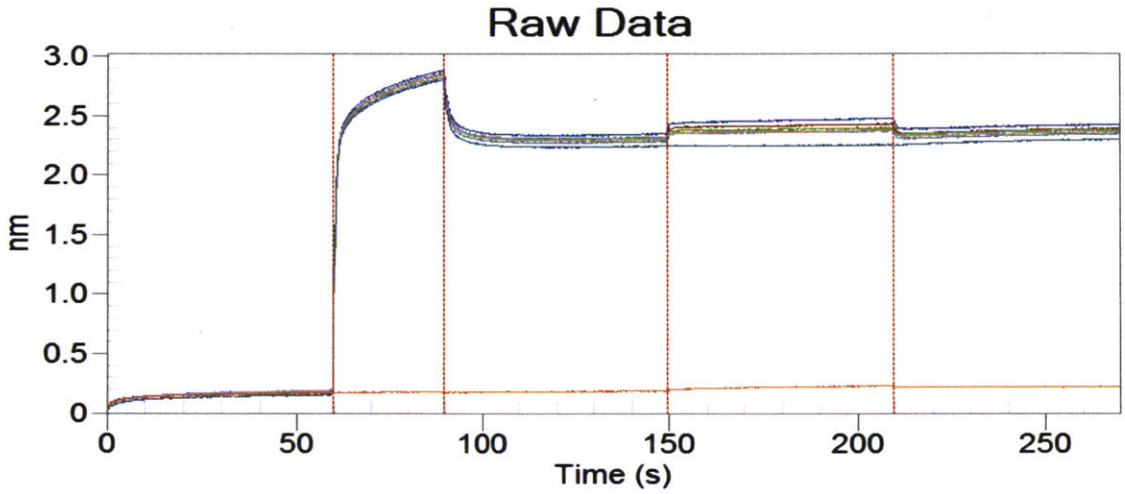
Time (s)

P1-G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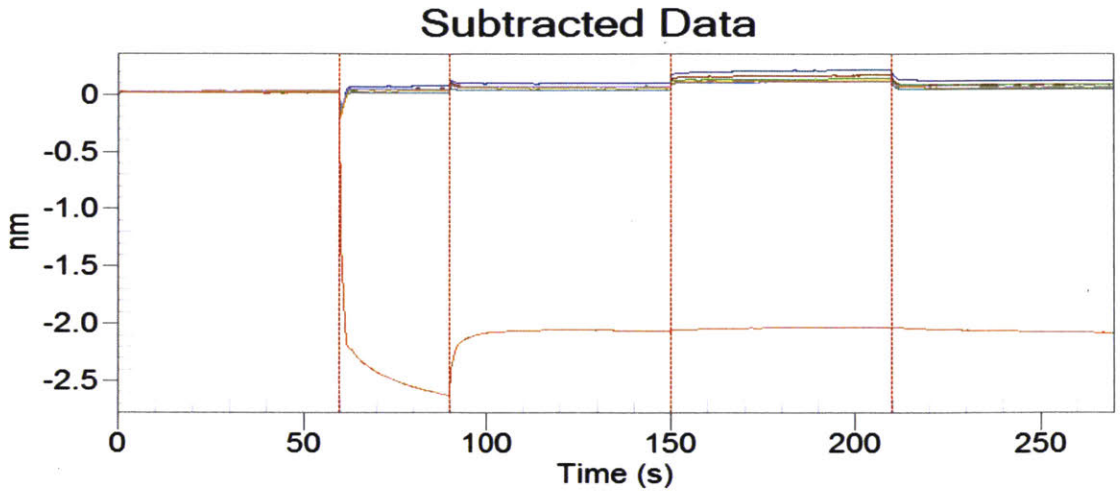
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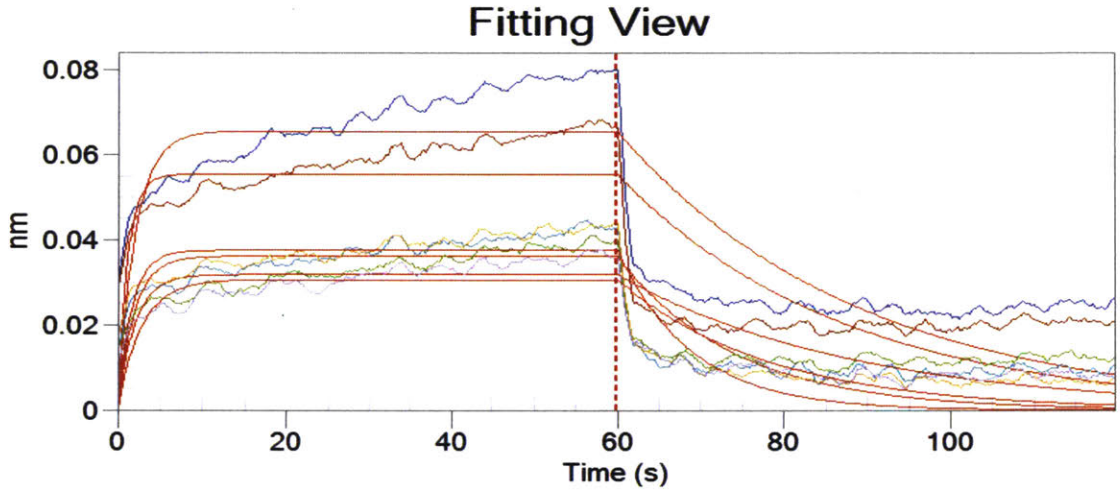
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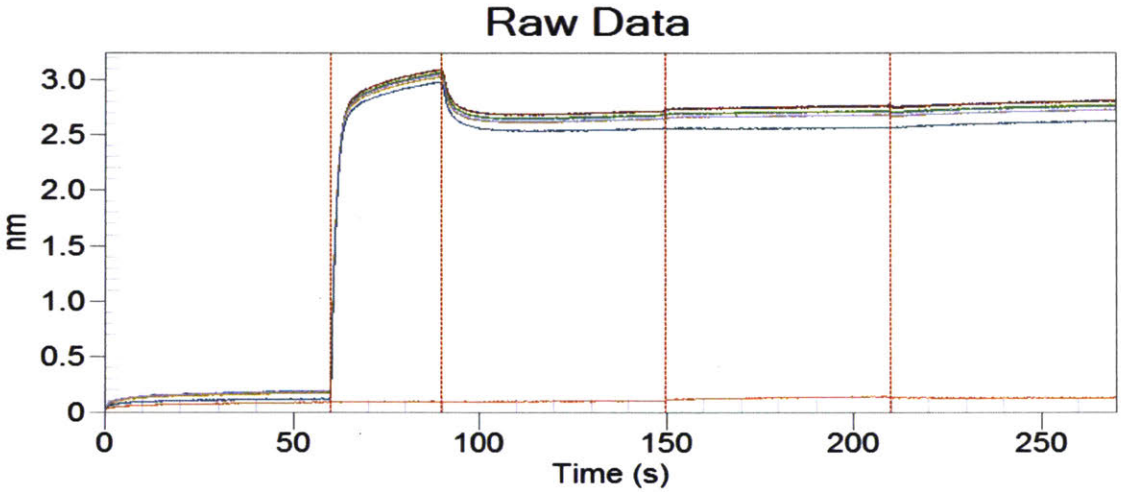
P1-H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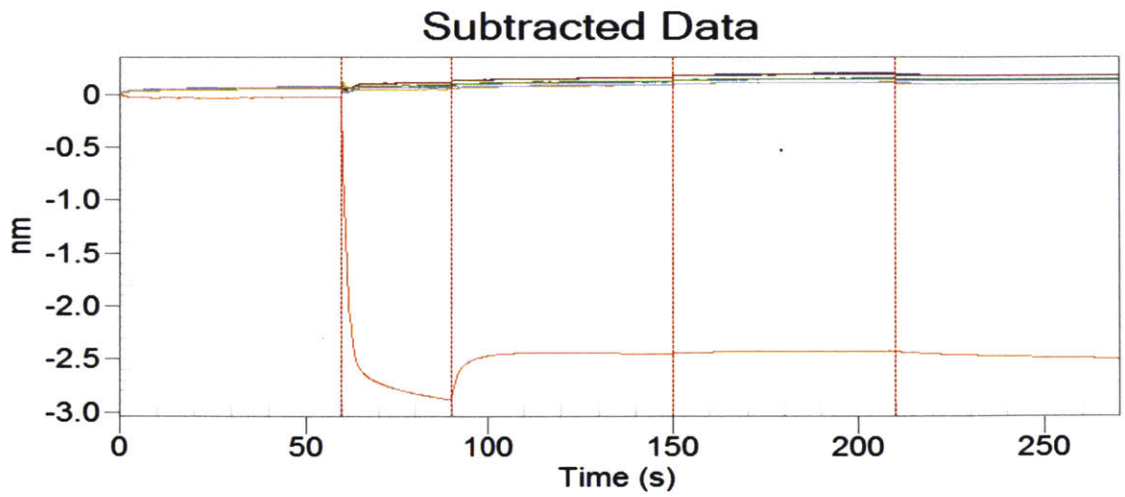
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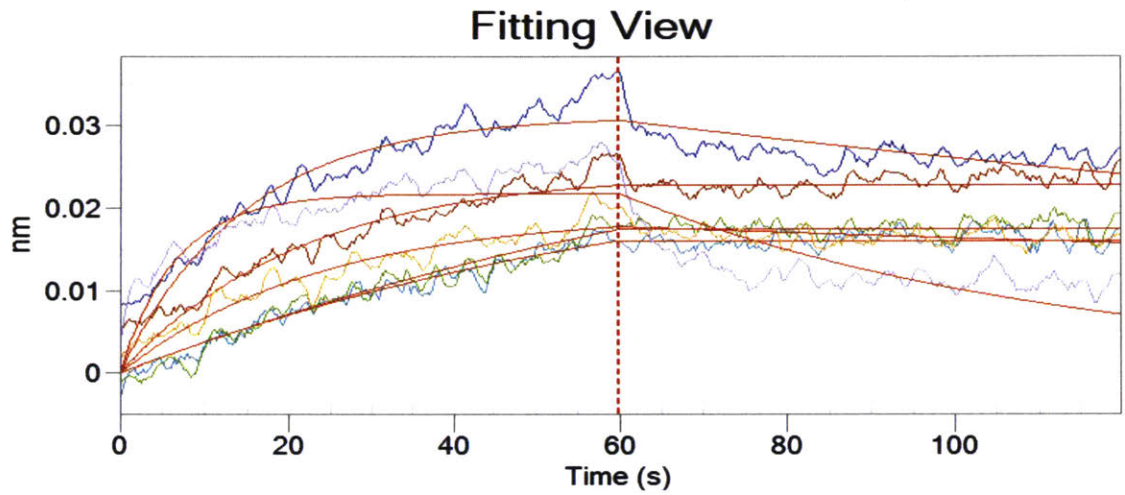
Time (s)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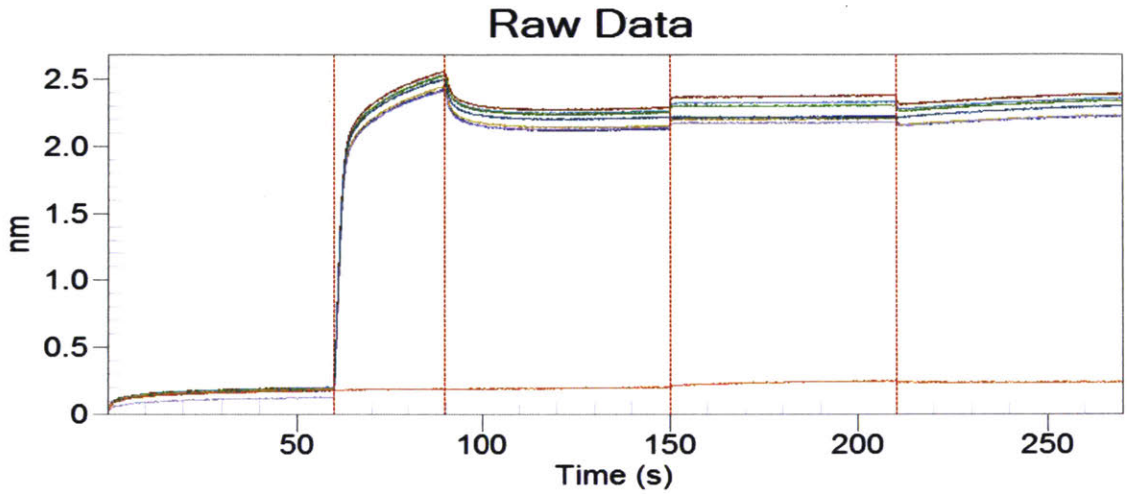
Time (s)

P1-I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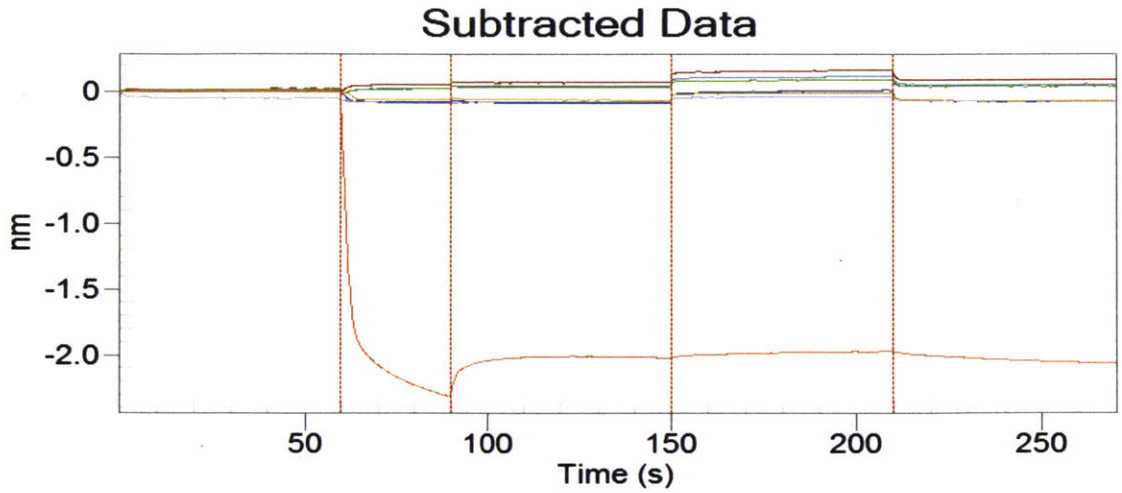
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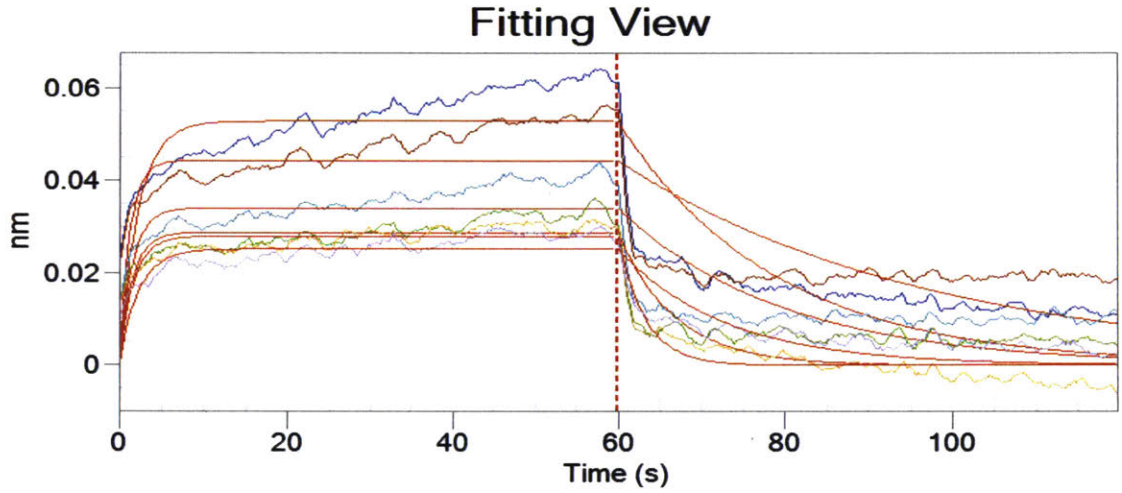
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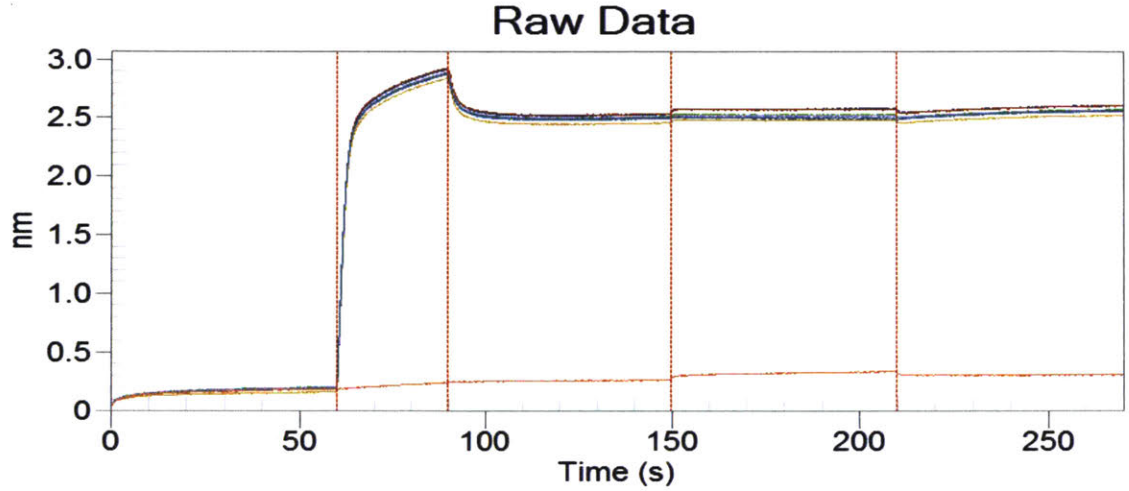
Time (s)

P1-J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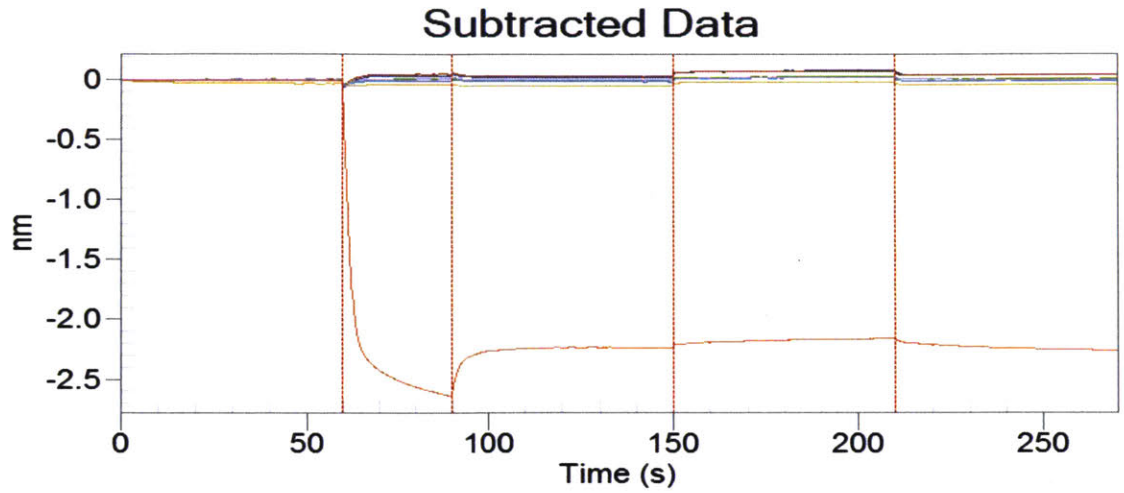
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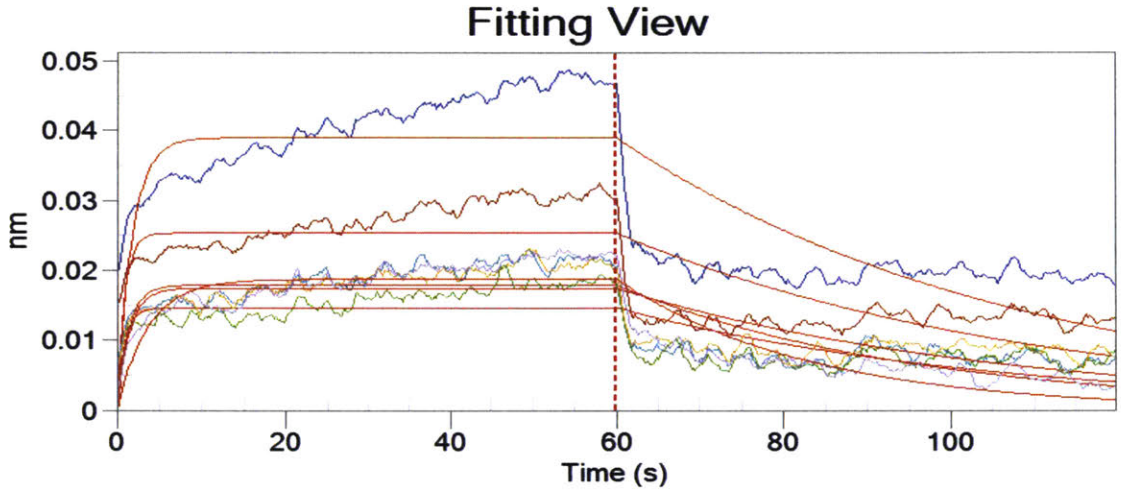
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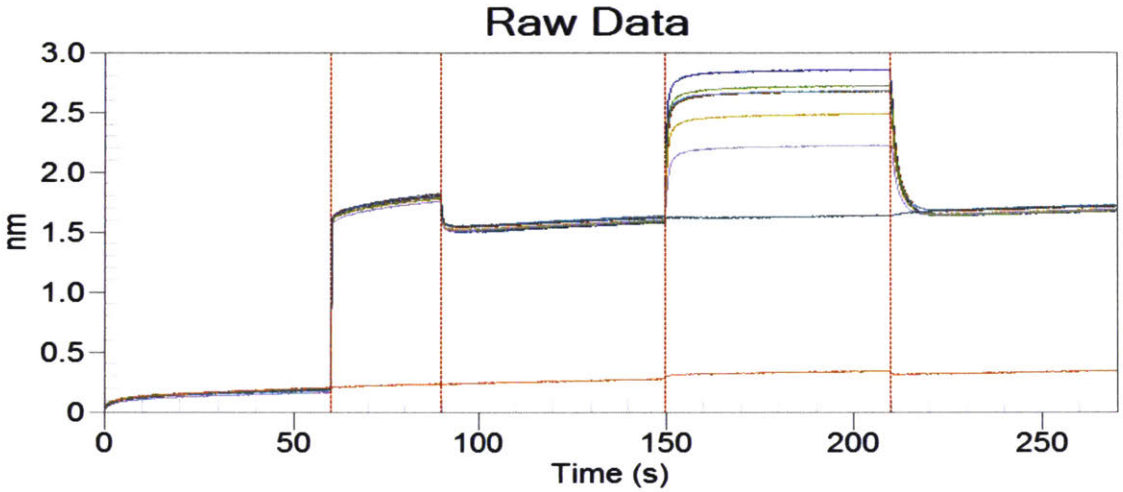
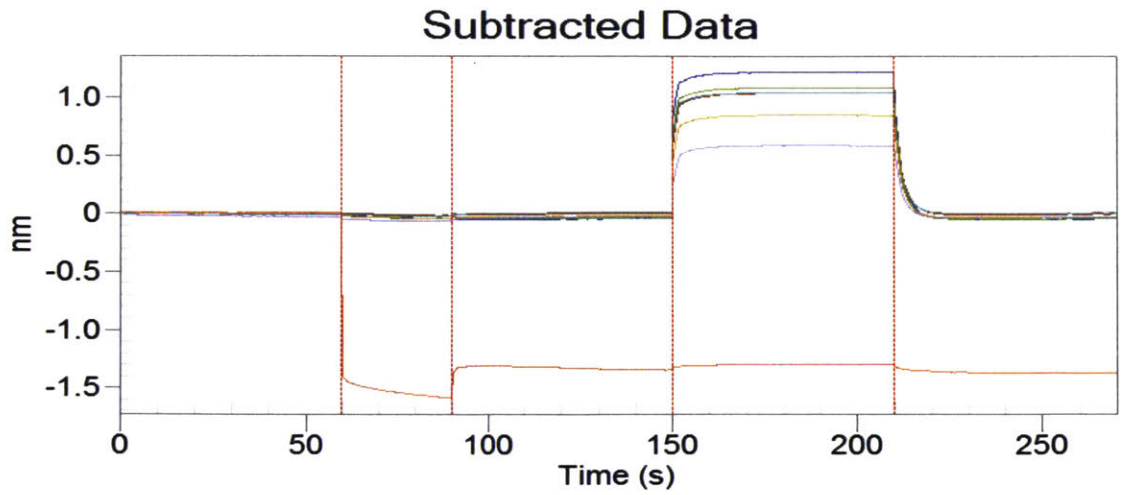
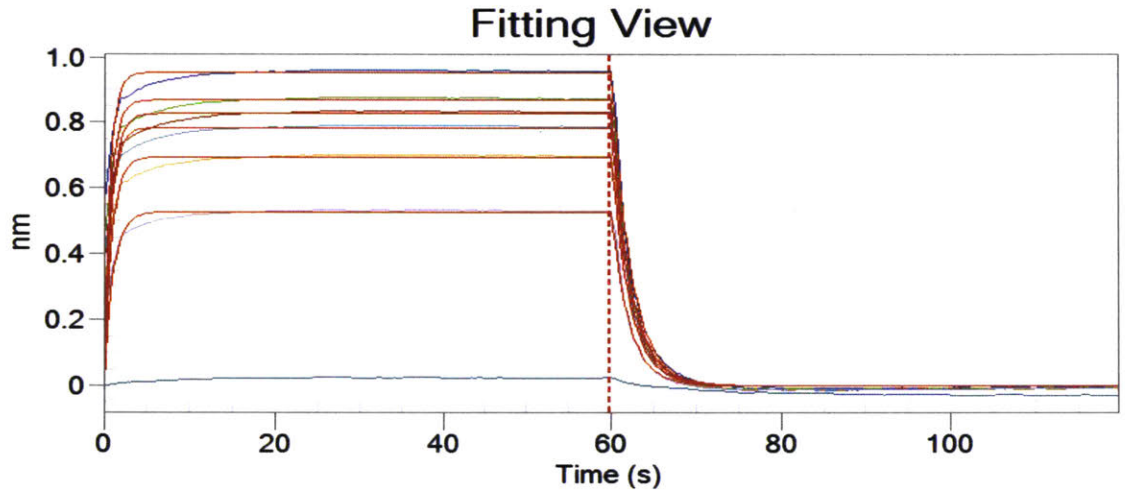
nm
0
20
40
60
80
100
Time (s)

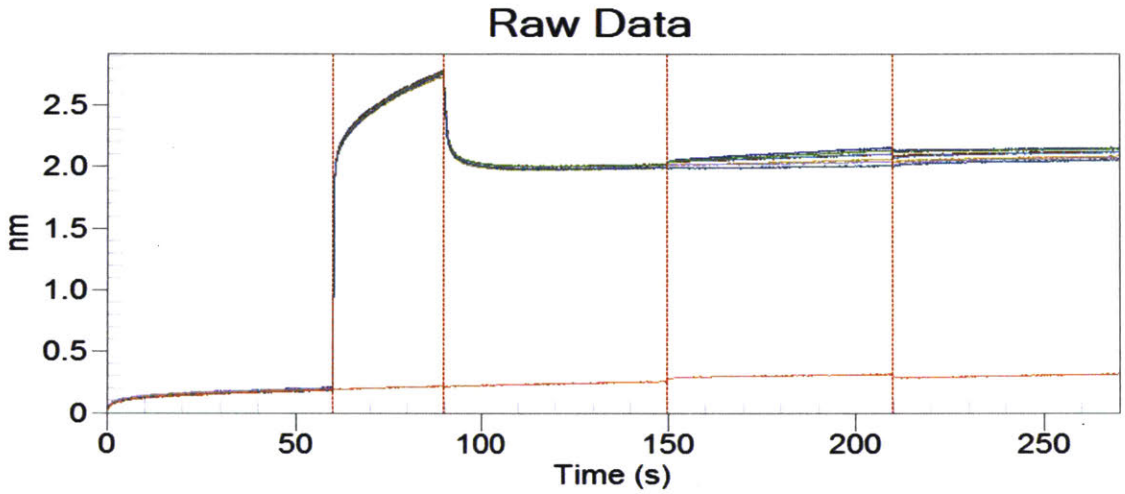
P1-K

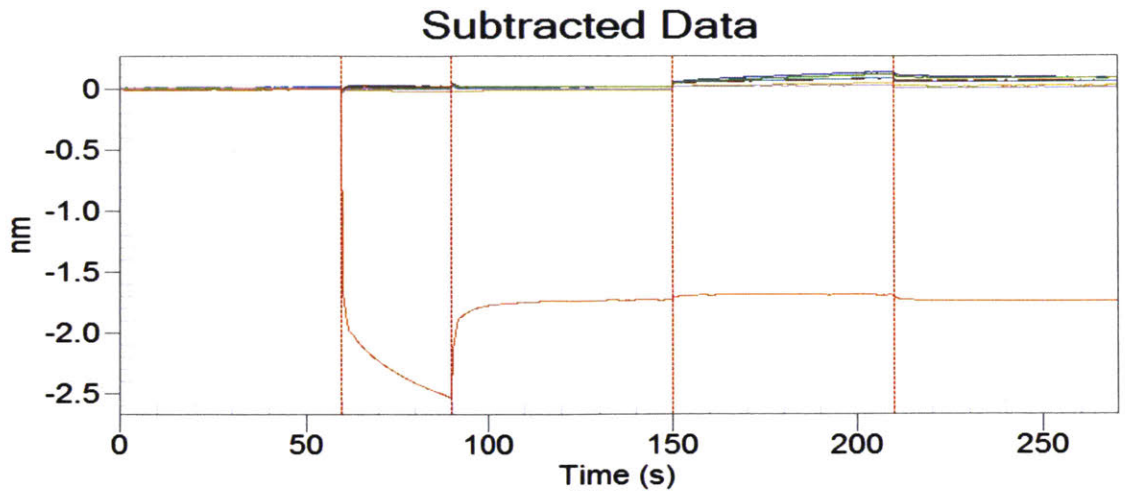

Raw Data
3.0
2.5
2.0
1.5
1.0
0.5
0
nm
0
50
100
150
200
250
Time (s)

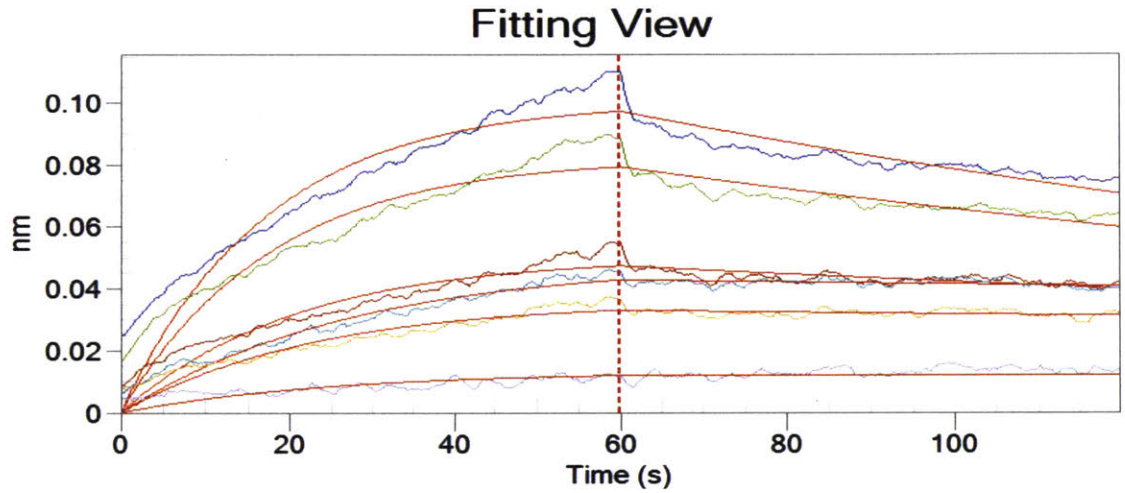

Subtracted Data
1.0
0.5
0
-0.5
-1.0
-1.5
nm
0
50
100
150
200
250
Time (s)


Fitting View
1.0
0.8
0.6
0.4
0.2
0
nm
0
20
40
60
80
100
Time (s)

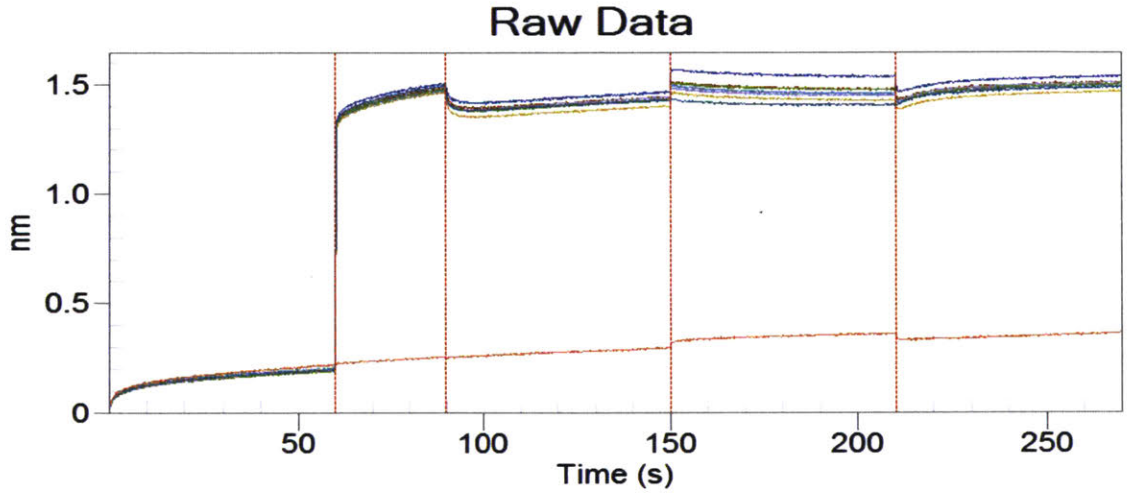
P4-A

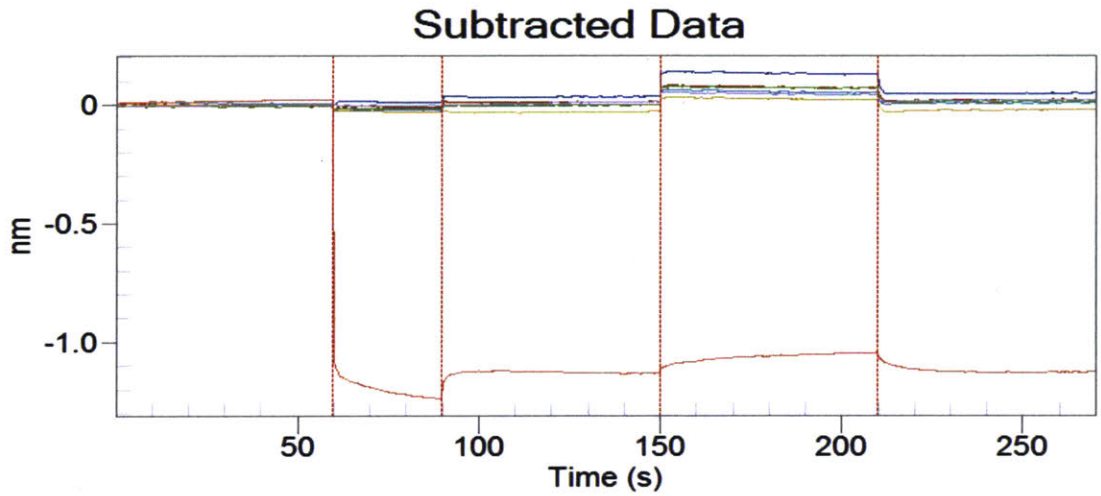

Raw Data
2.5
2.0
1.5
1.0
0.5
0
nm
0
50
100
150
200
250
Time (s)

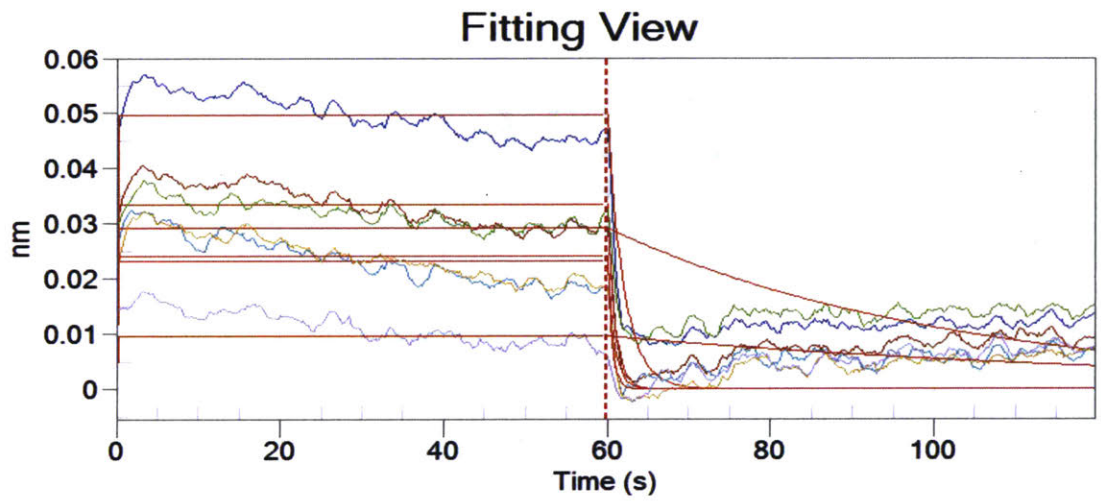

Subtracted Data
nm
0
-0.5
-1.0
-1.5
-2.0
-2.5
0
50
100
150
200
250
Time (s)


Fitting View
nm
0.10
0.08
0.06
0.04
0.02
0
0
20
40
60
80
100
Time (s)

P4-K


Raw Data
nm
1.5
1.0
0.5
0
50
100
150
200
250
Time (s)





Lipophilicity (Log D), Phospholipid Affinity (CHI IAM), Predicted Volume of Distribution (Log VD), and Human Serum Albumin Binding (HSA%) Data

The peptides (~1 mg) were dissolved in 1 mL of DMSO and injected onto various HPLCs for biomimetic analysis of the physicochemical properties presented below. Relevant information regarding each method has been described below.

Lipophilicity measurements: A method has been described by Kerns *et al*[4] for a quick HPLC method to estimate the logarithm of the octanol-water partition coefficient at pH 7.4.

Instrument

Agilent HP1000 HPLC

Materials:

HPLC grade acetonitrile

HPLC grade Water

Ammonium Acetate

pH 7.0 Buffer solution

50 mM Ammonium acetate pH 7.4

1 M NaOH solution

Diluent:

70% MeCN / 30% water

Analytical Column:

5cm x 4.6mm Polaris C18 5μm HPLC column

Detection:

UV -230nm

Experimental:

Mobile Phase

Line A : 100% MeCN

Line B : 50mM ammonium acetate adjusted to pH 7.4 using 1M NaOH solution.

HPLC Gradient

Time	% Mobile Phase A	% Mobile Phase B	Flow Rate (mL min^{-1})
0	0	100	1.0
2.5	95	5	1.0
4.0	95	5	1.0
4.1	0	100	1.0
6.1	0	100	1.0

Column Temperature: 40°C

Injection volume: 2 μL

Phospholipid affinity measurements: A method has been described by Valko et al[5] for a quick HPLC method to characterize the interactions of drugs with immobilized artificial membrane (IAM). With a set of standards, the gradient retention times can be converted to Chromatographic Hydrophobicity Index values referring to IAM chromatography (CHIIAM) that approximates an acetonitrile concentration with which an equal distribution of compound can be achieved between the mobile phase and IAM.

Calibration standards:

Acetophenone, Propriopheneone, Valerophenone and Octanophenone made up at concentration of ~1 mg mL^{-1} using the diluent.

Diluent:

70% MeCN / 30% water

Analytical Column:

Regis 10 cm x 4.6mm IAM PC 10 μm HPLC column

Detection:

UV -230 nm

Experimental:

Mobile Phase

Line A : 100% MeCN

Line B : 50 mM ammonium acetate adjusted to pH 7.4 using 1M NaOH solution.

HPLC Gradient

Time	% Mobile Phase A	% Mobile Phase B	Flow Rate (mL min^{-1})
0	0	100	1.0
6.0	100	0	1.0
6.5	100	0	1.0
7.0	0	100	1.0
9.0	0	100	1.0

Column Temperature: 40°C

Injection volume: 2 μL

Human Serum Albumin (HSA) binding measurements: A fast gradient HPLC method (cycle time 15 min) is described by Valko et al[6] to determine Human Serum Albumin (HSA) binding of discovery compounds using chemically bonded protein stationary phases. The HSA binding values were derived from the gradient retention times that were converted to the logarithm of the

equilibrium constants (logKHSA) using data from a calibration set of molecules. A calibration graph is plotted with logarithm of the retention time vs logKHSA (Valko et al[6]).

Calibration standards:

Carbamazapine, Nicardipine, Warfarin, Diclofenec and Ketoprofen sample solutions made up at concentration of ~1 mg mL^{-1} using the diluent, respectively.

Diluent:

70% MeCN / 30% water

Analytical Column:

5 cm x 3.0 mm HSA 5μm HPLC column

Detection:

UV -280 nm or 230 nm

Experimental:

Mobile Phase

Line A : 50 mM ammonium acetate adjusted to pH 7.4 using 1M NaOH solution.

Line B : 100% IPA

HPLC Gradient

Time	% Mobile Phase A	% Mobile Phase B	Flow Rate (mL min^{-1})
0	100	0	1.2
4.5	70	30	1.2
9.0	70	30	1.2
9.5	100	0	1.2
14	100	0	1.2

Column Temperature: 40°C

Injection volume: 2 μL

The predicted volume of distribution was calculated from the following equation based on HSA binding and phospholipid affinity values corresponding to values reported by Valko et al[6]:

Log VD = 0.44(±0.02) log *K* (IAM) - 0.22(±0.02) log *K* (HSA) - 0.66

ICP-MS Analysis

Two distinct macrocyclic peptides, chosen at random (**P2-C** and **P1-A**), were dissolved in 0.4 mL of concentrated nitric acid. This solution was sonicated and diluted with MilliQ pure water to 0.2% nitric acid concentration. ICP-MS was performed on the resulting mixtures. Calibration curves were generated using Pd ICP-MS standards for a range between 1000 ppm and 100 ppb. There was no palladium found to be remaining in the peptide after purification. Initial palladium content was ~300 ppm used to perform the macrocyclization reactions indicating over 99% palladium removal.

1.5 – References:

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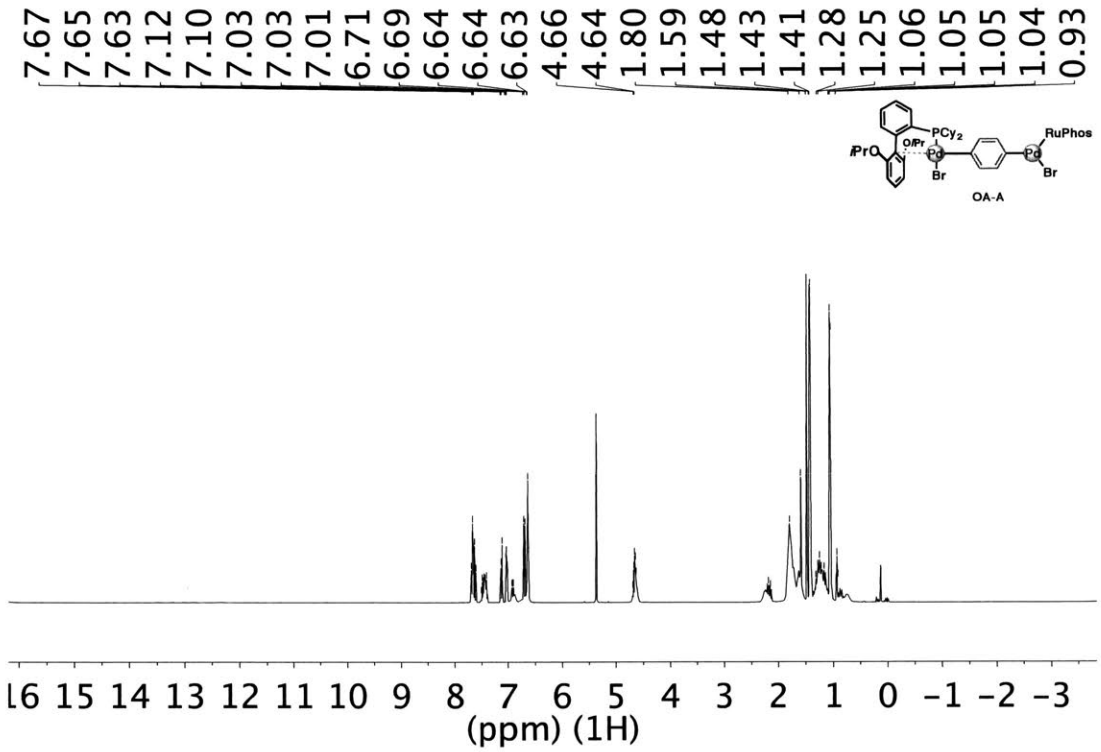
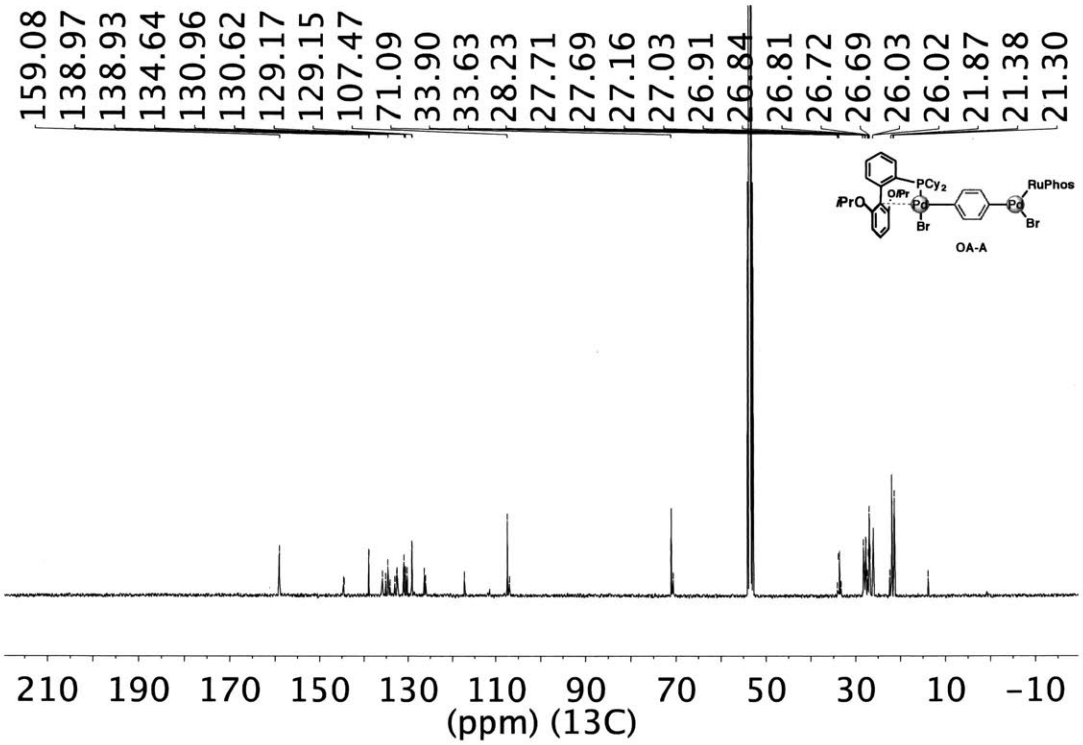
1.6 – Spectra:

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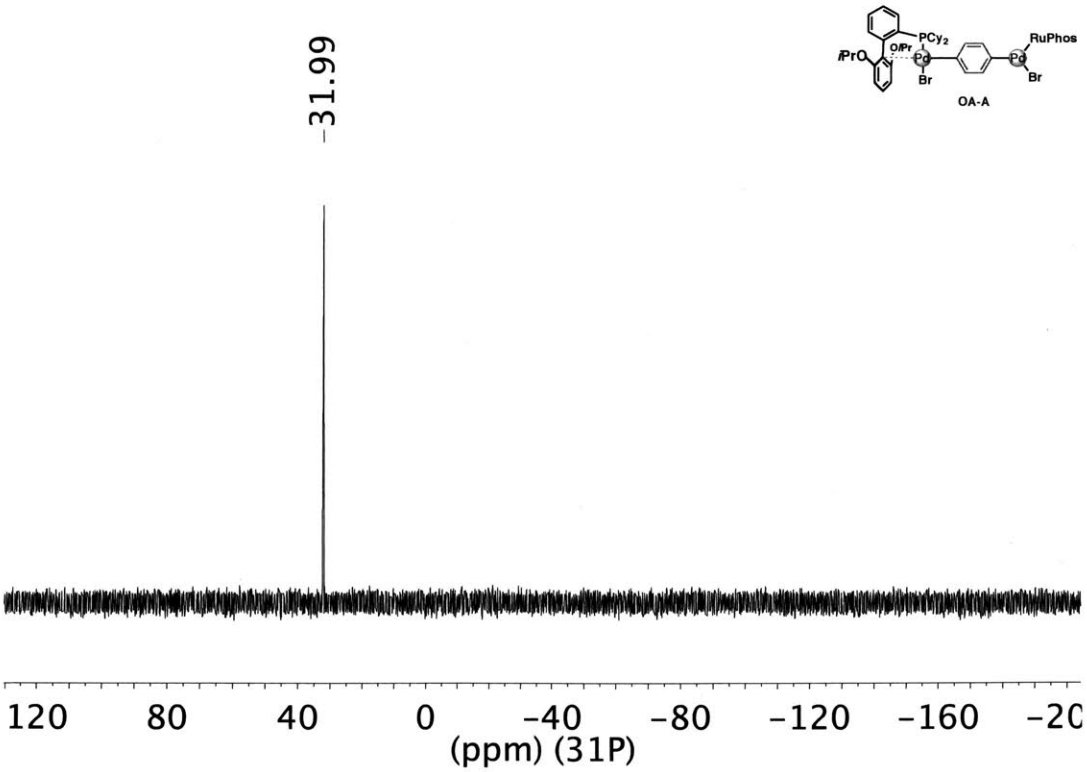
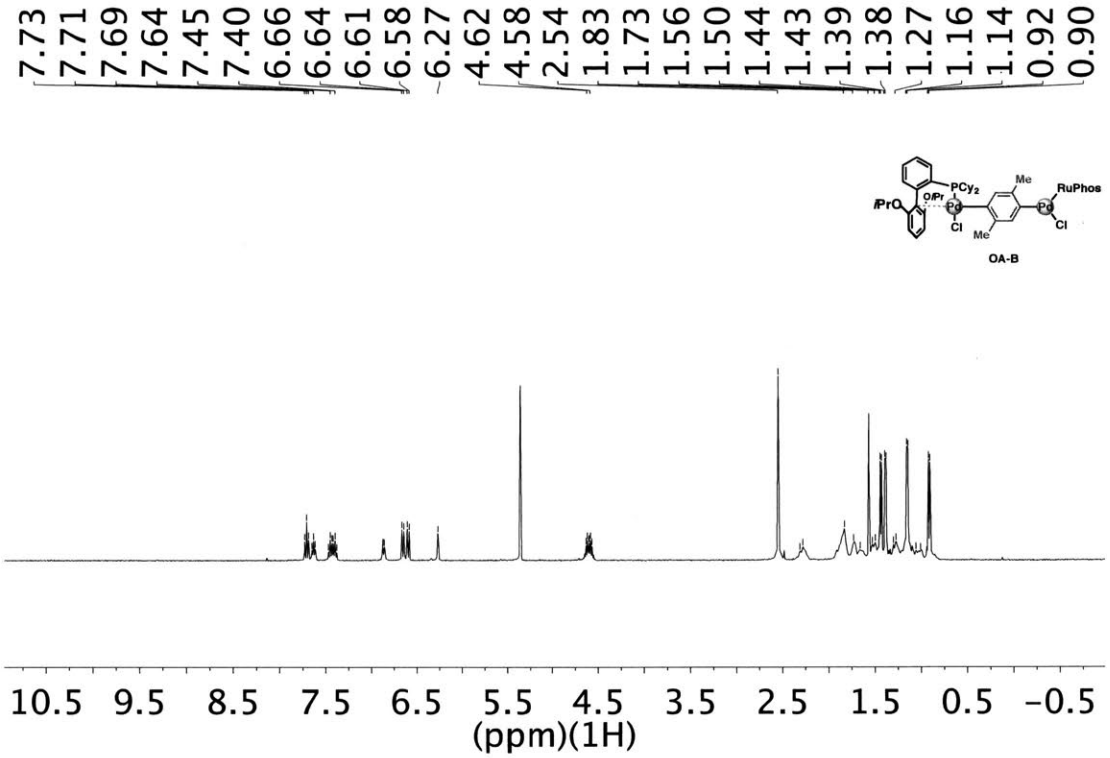
138.97
138.93
134.64
130.96
130.62
129.17
129.15
107.47
71.09
33.90
33.63
28.23
27.71
27.69
27.16
27.03
26.91
26.84
26.81
26.72
26.69
26.03
26.02
21.87
21.38
21.30
PCy2
iPrO
OiPr
Pd
Br
RuPhos
OA-A
210 190 170 150 130 110 90 70 50 30 10 -10
(ppm) (13C)


-31.99
PCy2
iPrO
OiPr
Pd
Br
RuPhos
OA-A
120 80 40 0 -40 -80 -120 -160 -20
(ppm) (31P)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7.71
7.69
7.64
7.45
7.40
6.66
6.64
6.61
6.58
6.27
4.62
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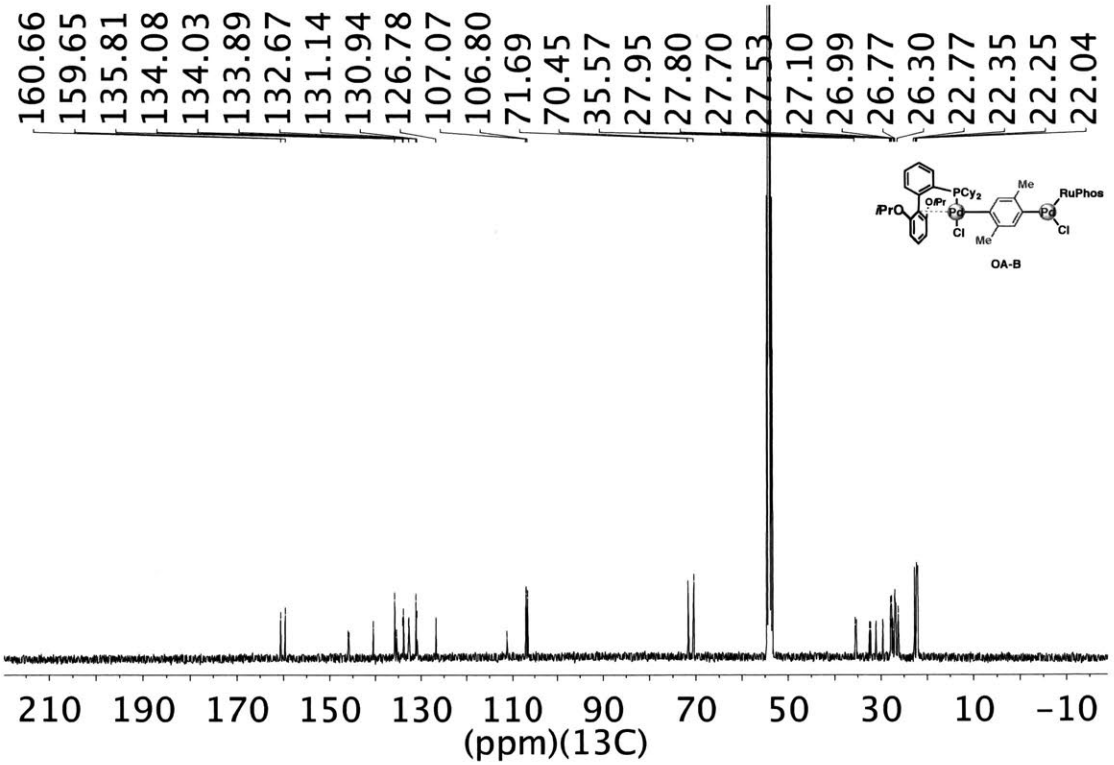
2.54
1.83
1.73
1.56
1.50
1.44
1.43
1.39
1.38
1.27
1.16
1.14
0.92
0.90
OA-B
10.5 9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 -0.5
(ppm)(1H)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134.03
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131.14
130.94
126.78
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71.69
70.45
35.57
27.95
27.80
27.70
27.53
27.10
26.99
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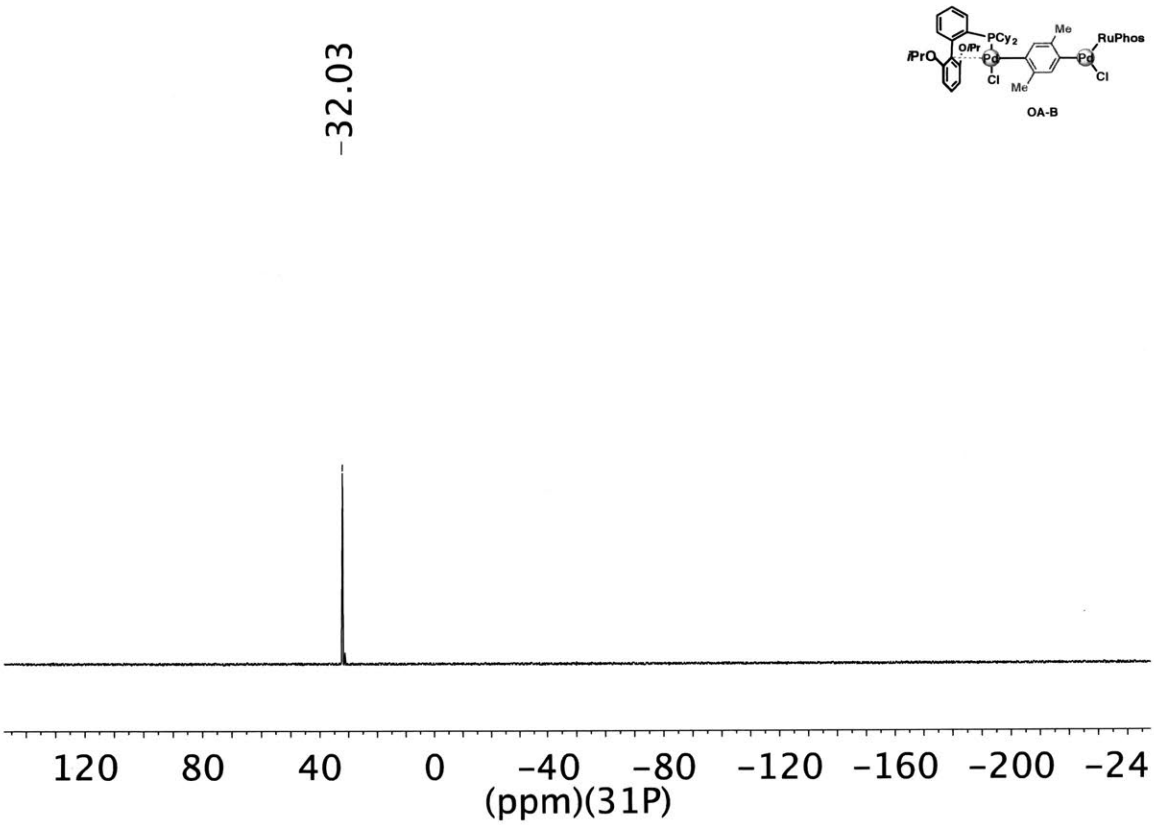
26.30
22.77
22.35
22.25
22.04
OA-B
210 190 170 150 130 110 90 70 50 30 10 -10
(ppm)(13C)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OA-B
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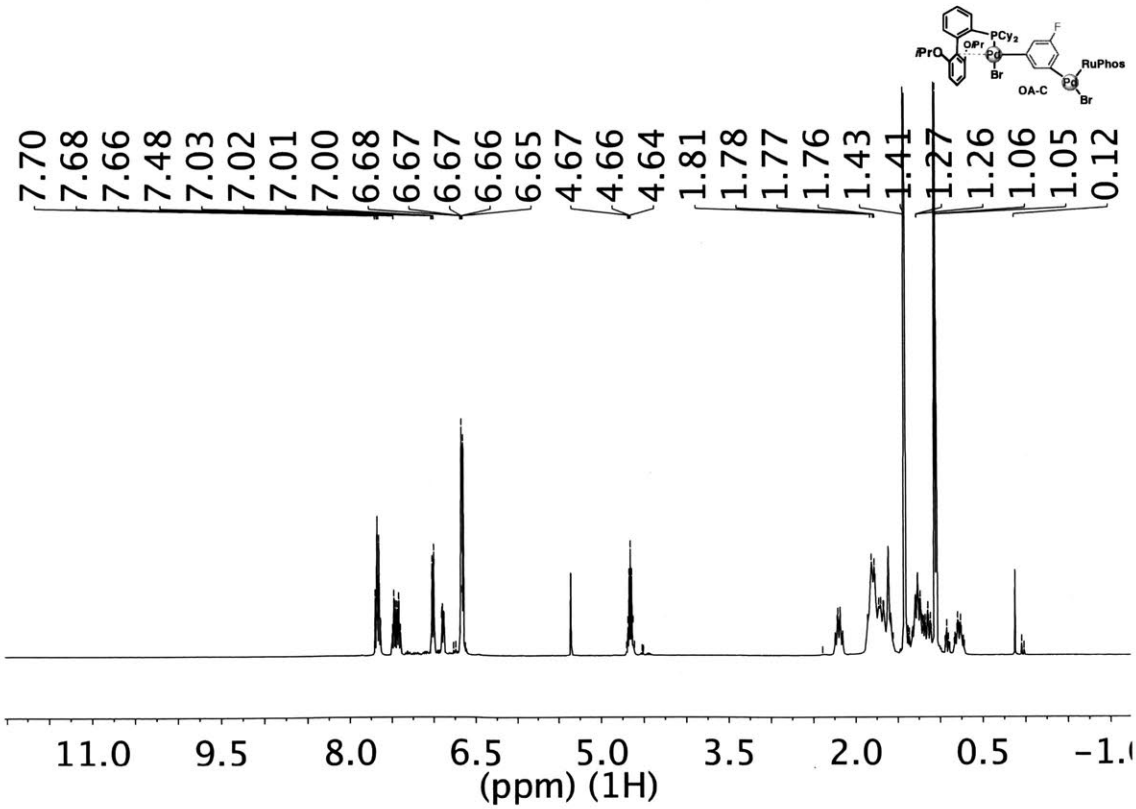
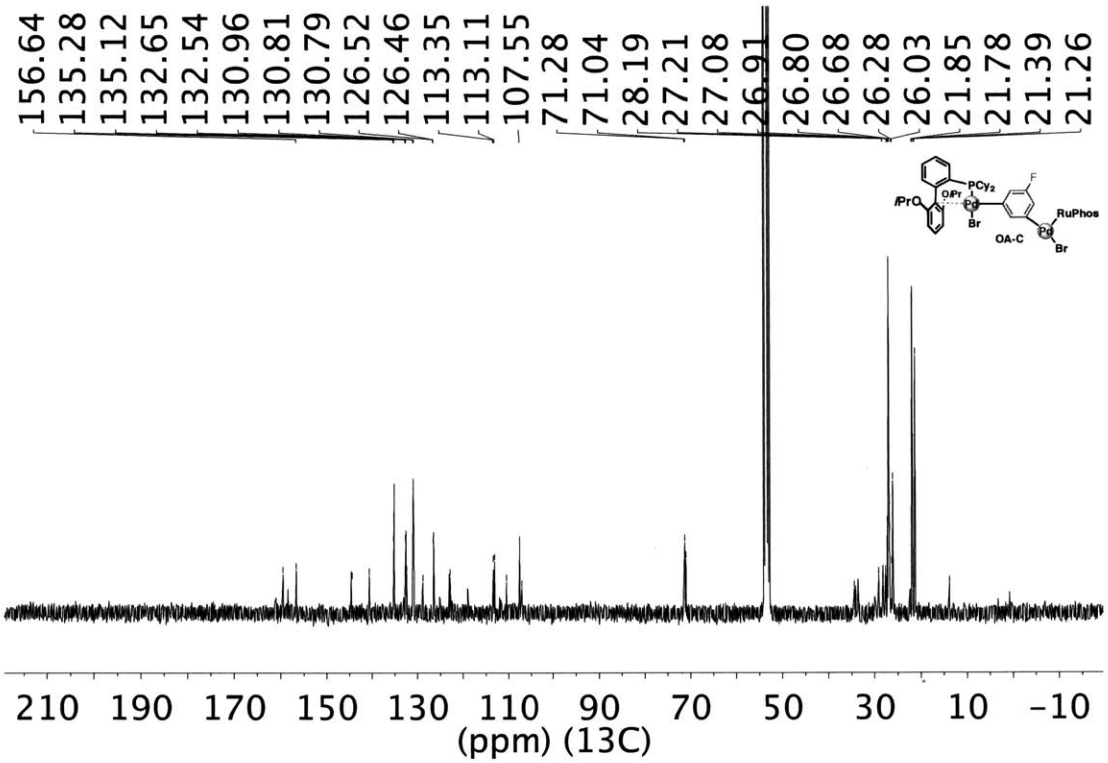

OA-C
7.70 7.68 7.66 7.48 7.03 7.02 7.01 7.00 6.68 6.67 6.67 6.66 6.65 4.67 4.66 4.64 1.81 1.78 1.77 1.76 1.43 1.41 1.27 1.26 1.06 1.05 0.12
11.0 9.5 8.0 6.5 5.0 3.5 2.0 0.5
(ppm) (1H)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135.12
132.65
132.54
130.96
130.81
130.79
126.52
126.46
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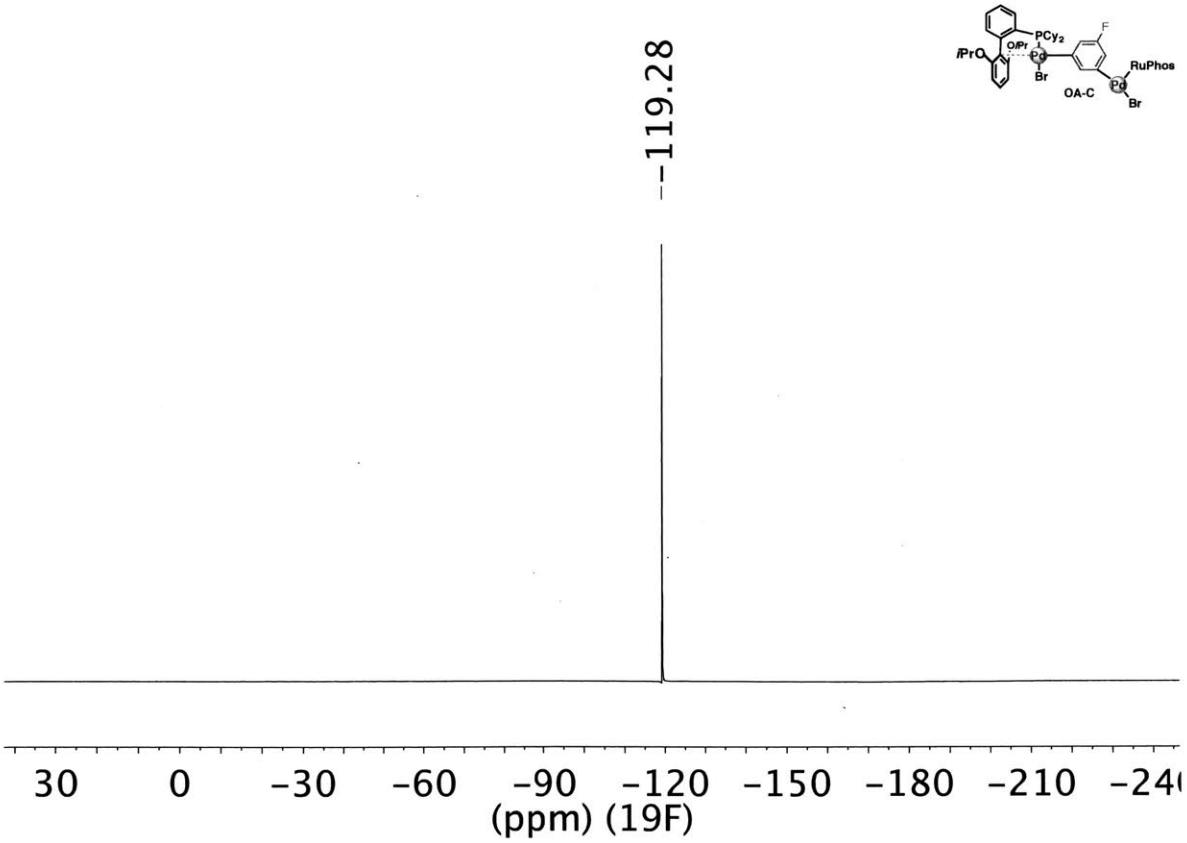
113.11
107.55
71.28
71.04
28.19
27.21
27.08
26.91
26.80
26.68
26.28
26.03
21.85
21.78
21.39
21.26
PCy2
iPrO
OiPr
Pd
Br
F
RuPhos
OA-C
210 190 170 150 130 110 90 70 50 30 10 -10
(ppm) (13C)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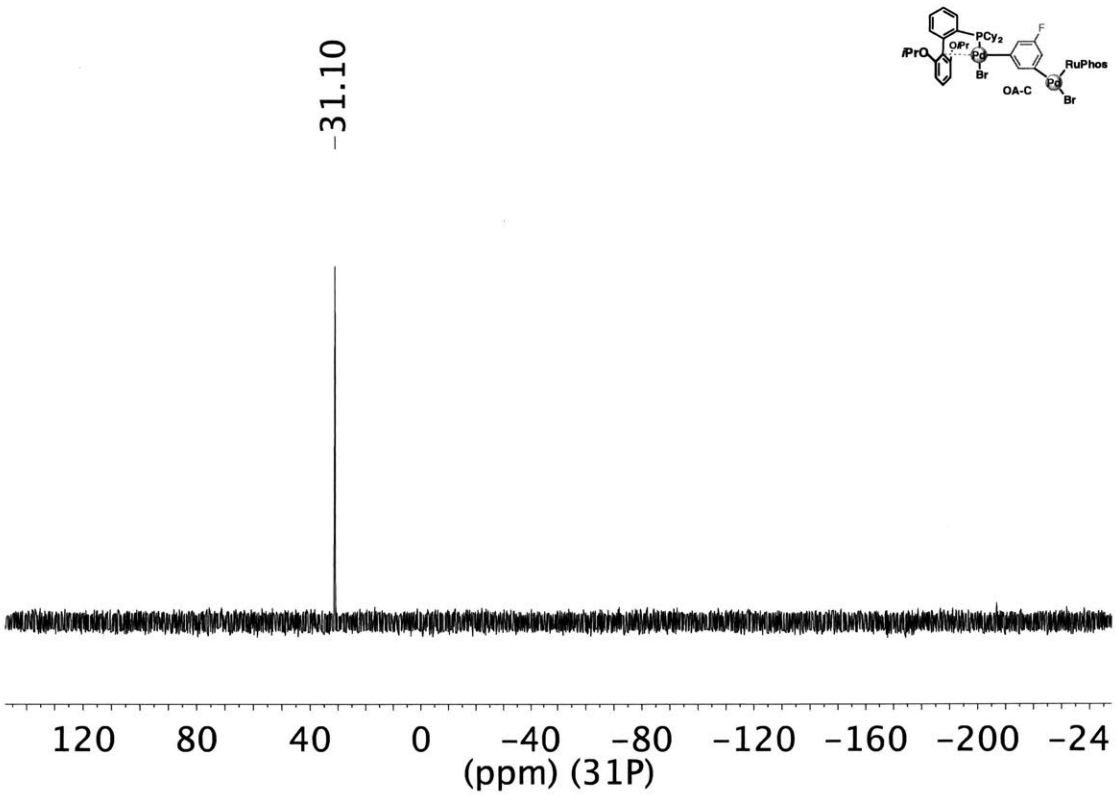
PCy2
iPrO
OiPr
Pd
Br
F
RuPhos
OA-C
30 0 -30 -60 -90 -120 -150 -180 -210
(ppm) (19F)


-31.10
120 80 40 0 -40 -80 -120 -160 -200 -24
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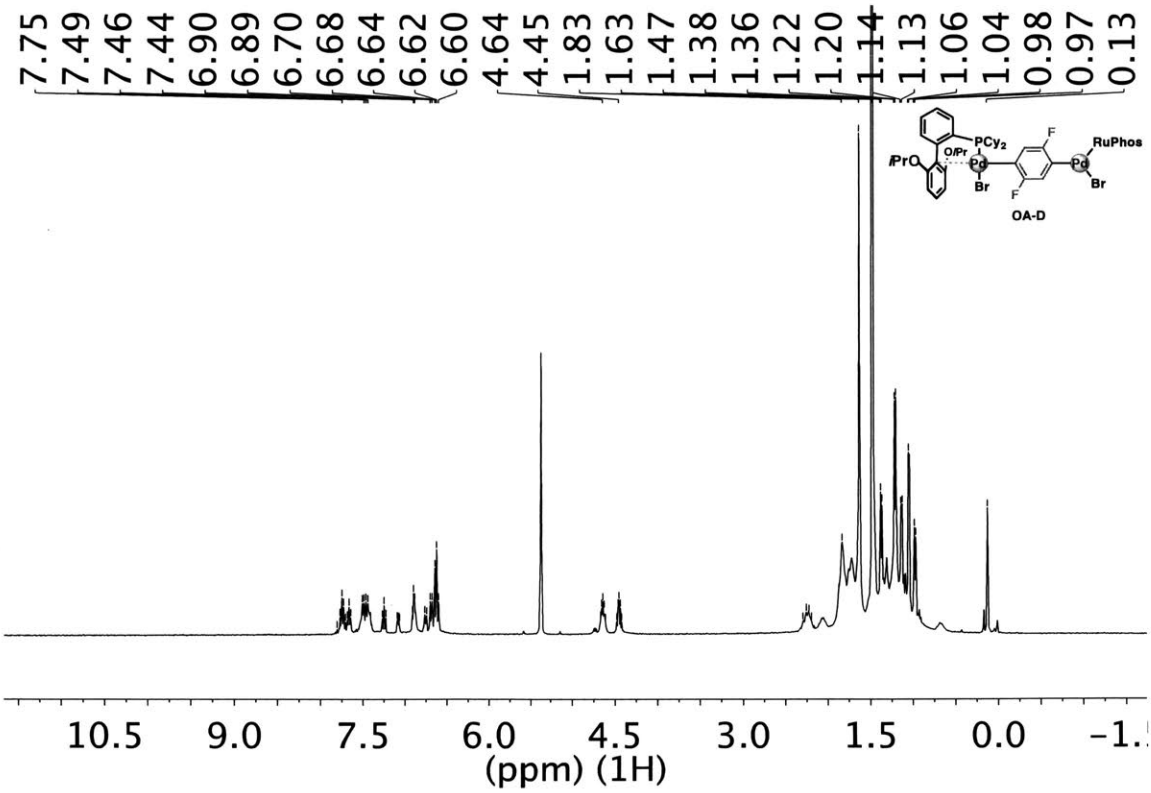
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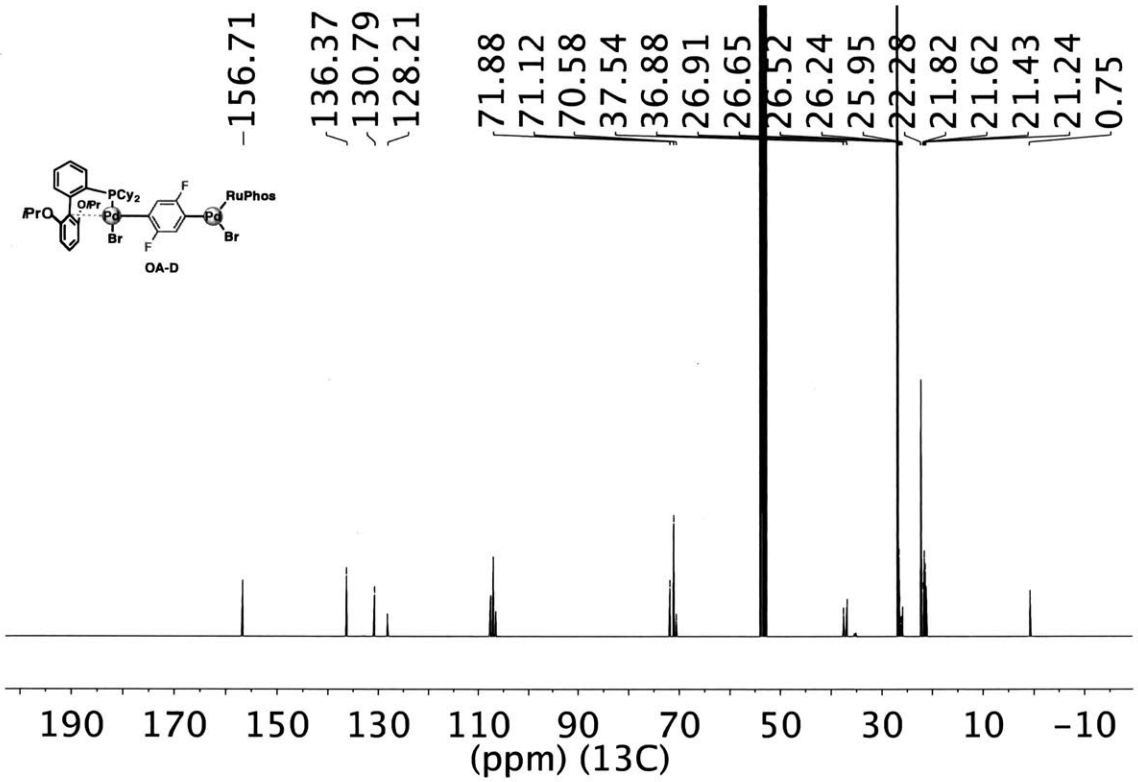
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26.24
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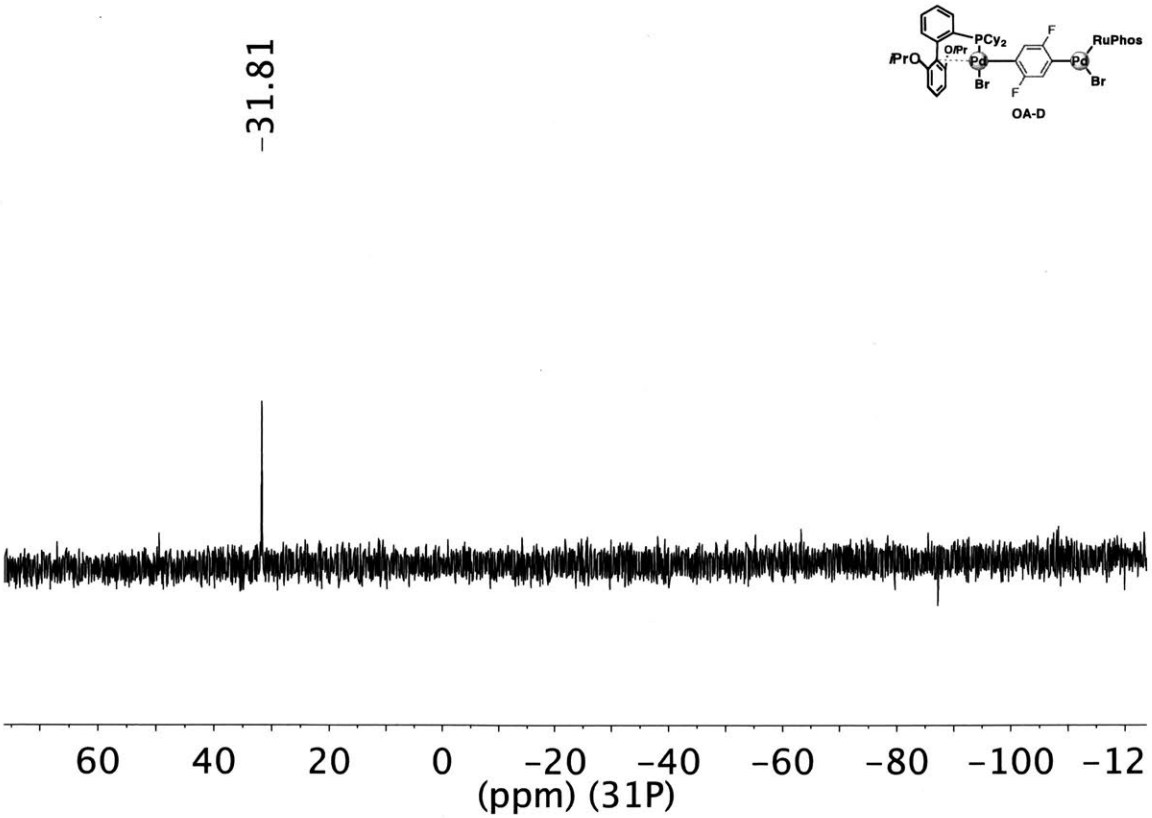
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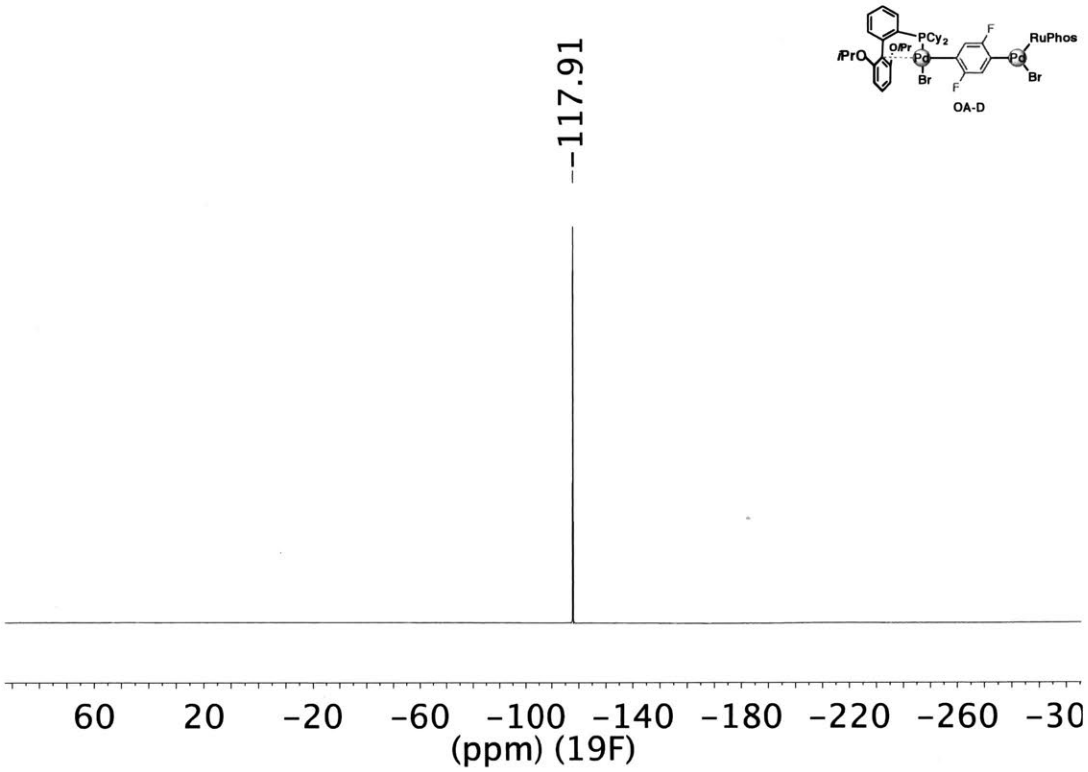
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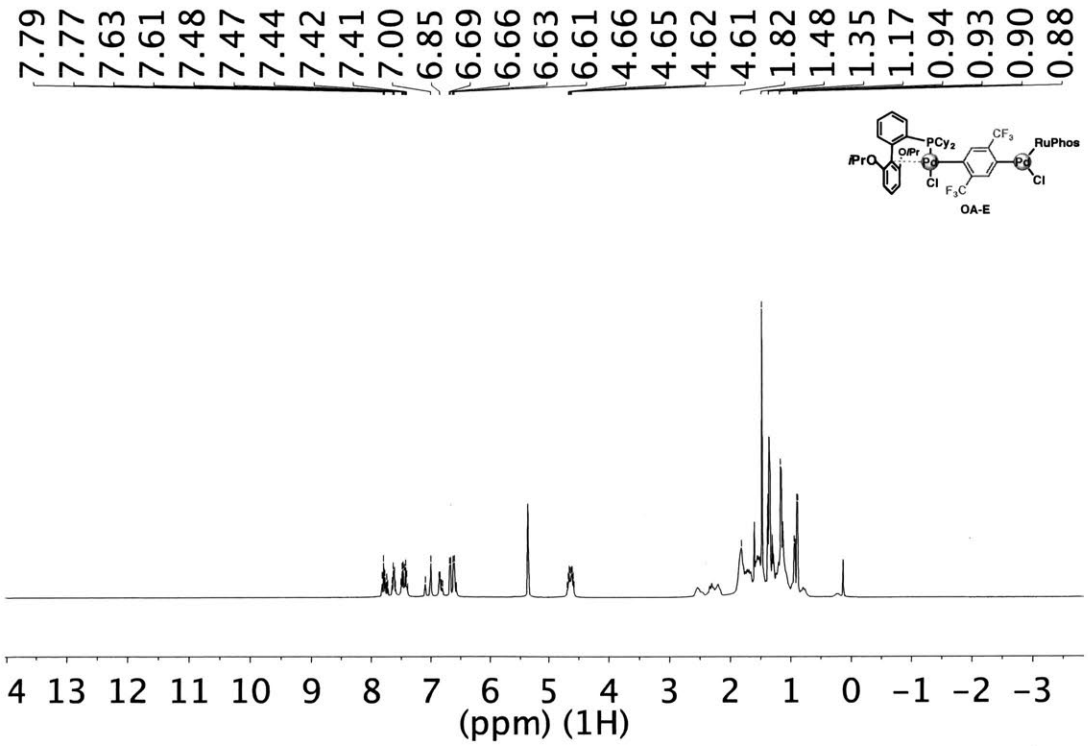
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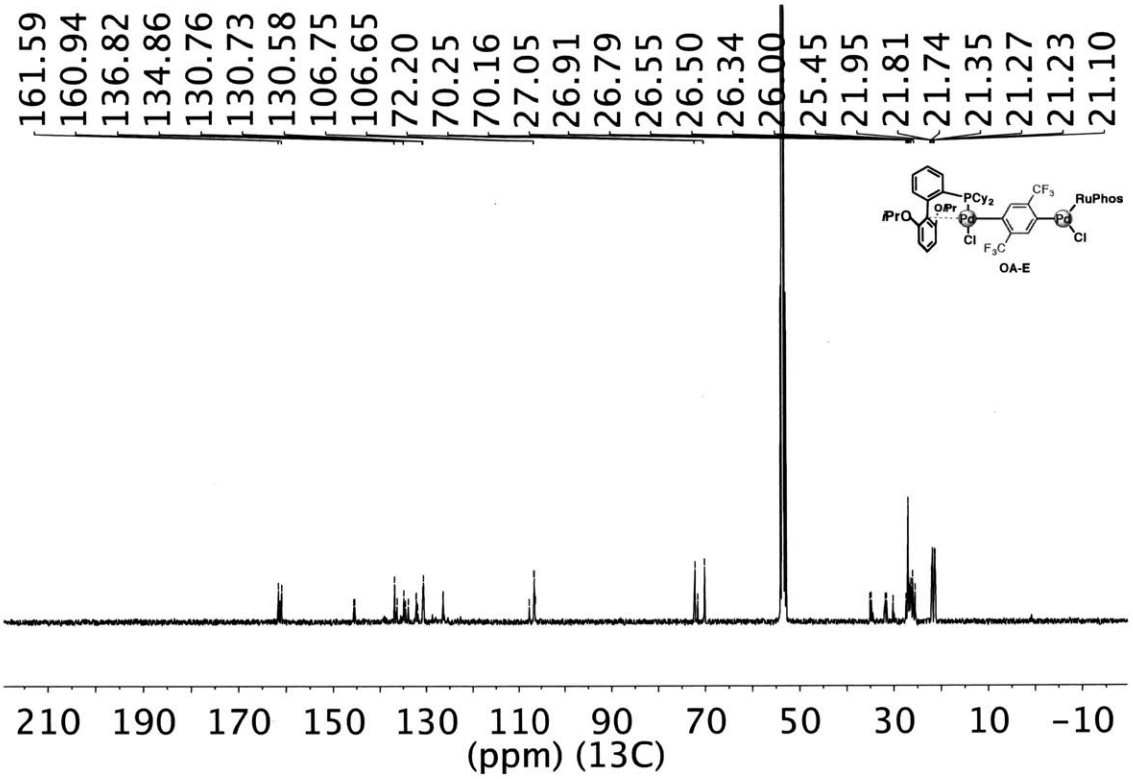
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26.50
26.34
26.00
25.45
21.95
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21.23
21.10
OA-E
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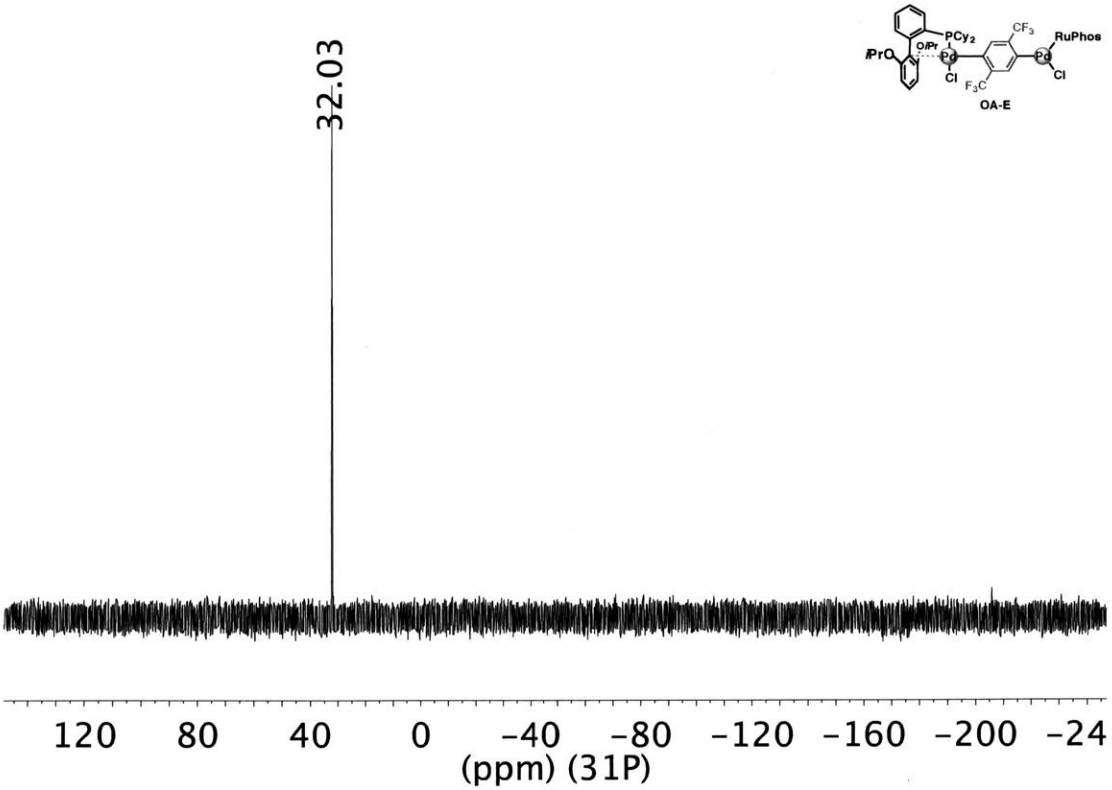
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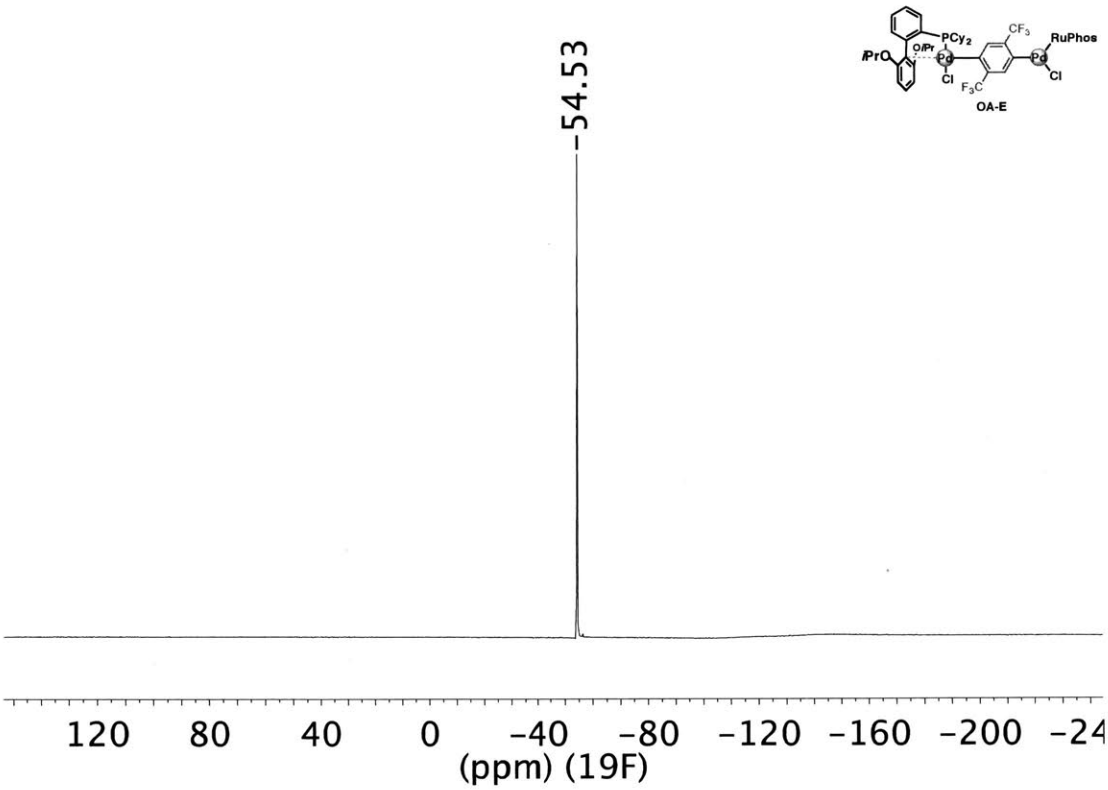
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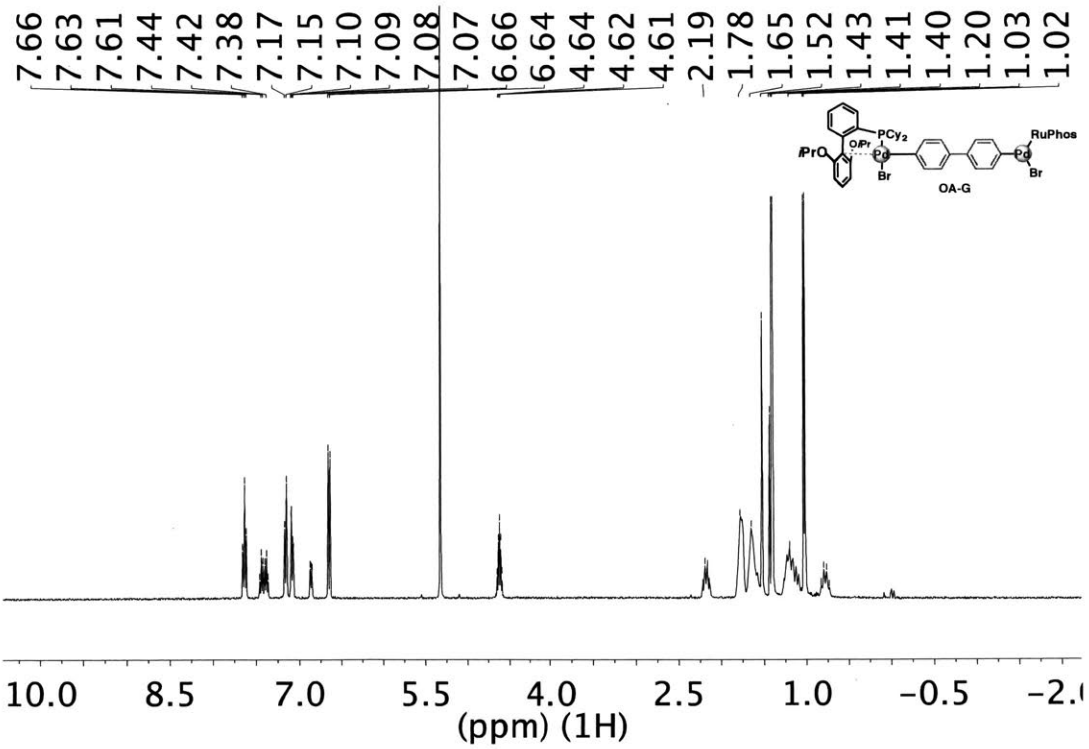
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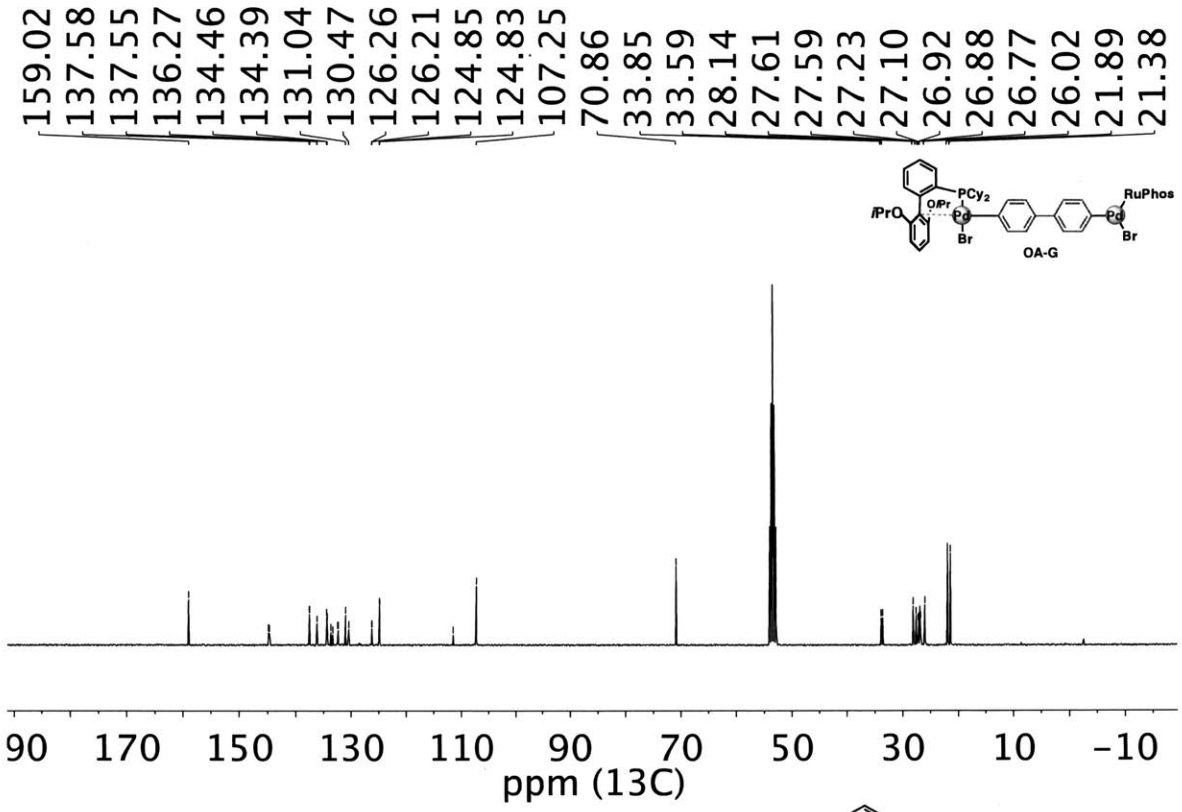
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33.85
33.59
28.14
27.61
27.59
27.23
27.10
26.92
26.88
26.77
26.02
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PCy2
OiPr
iPrO
Pd
Br
RuPhos
OA-G
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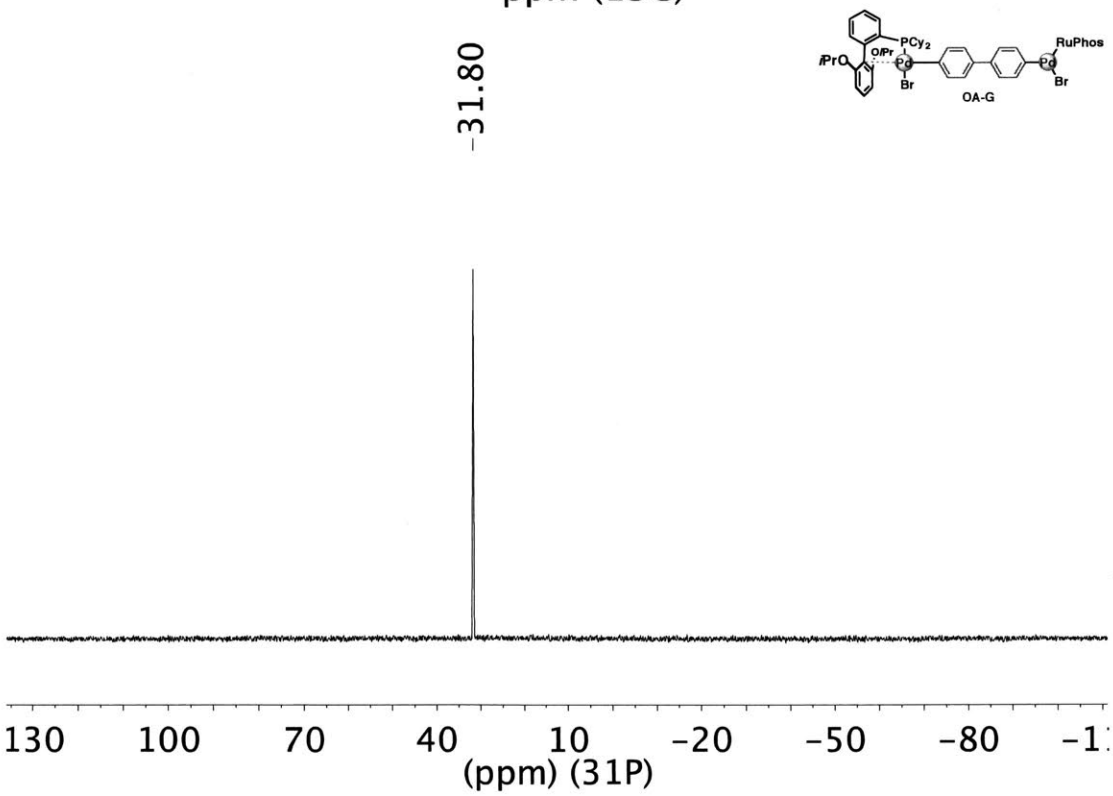
ppm (13C)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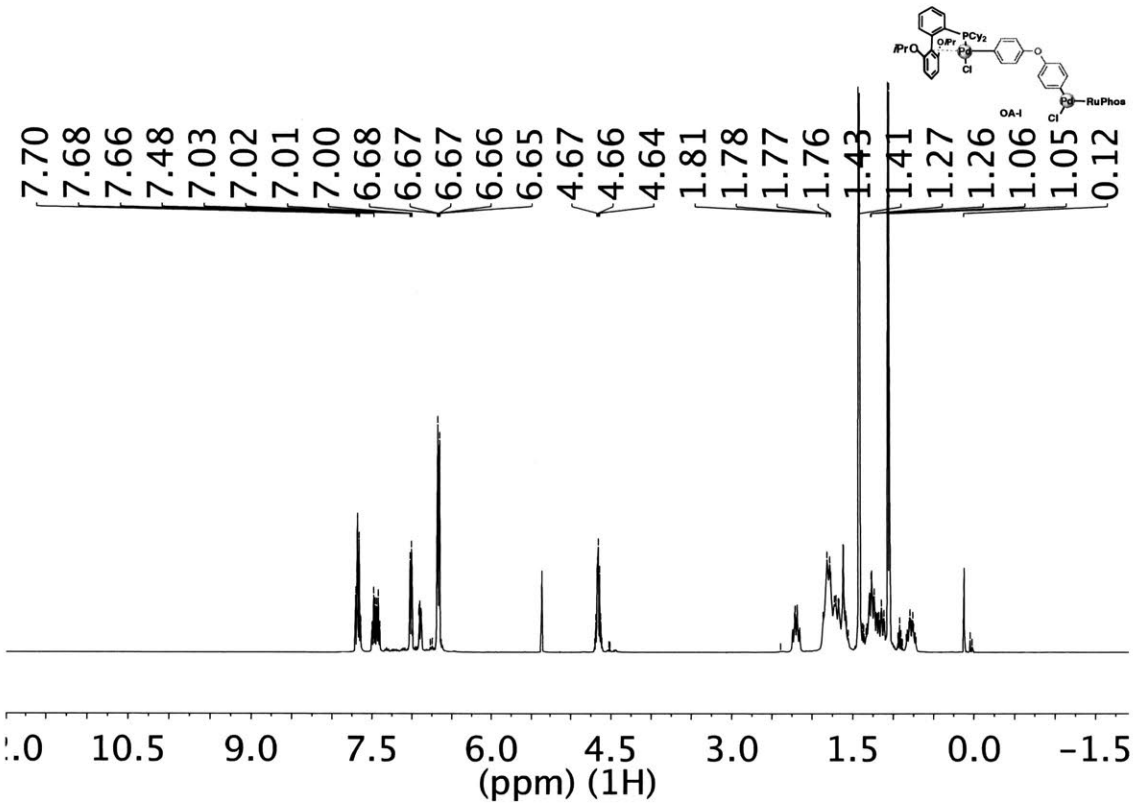
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4.64
1.81
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1.77
1.76
1.43
1.41
1.27
1.26
1.06
1.05
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4.5
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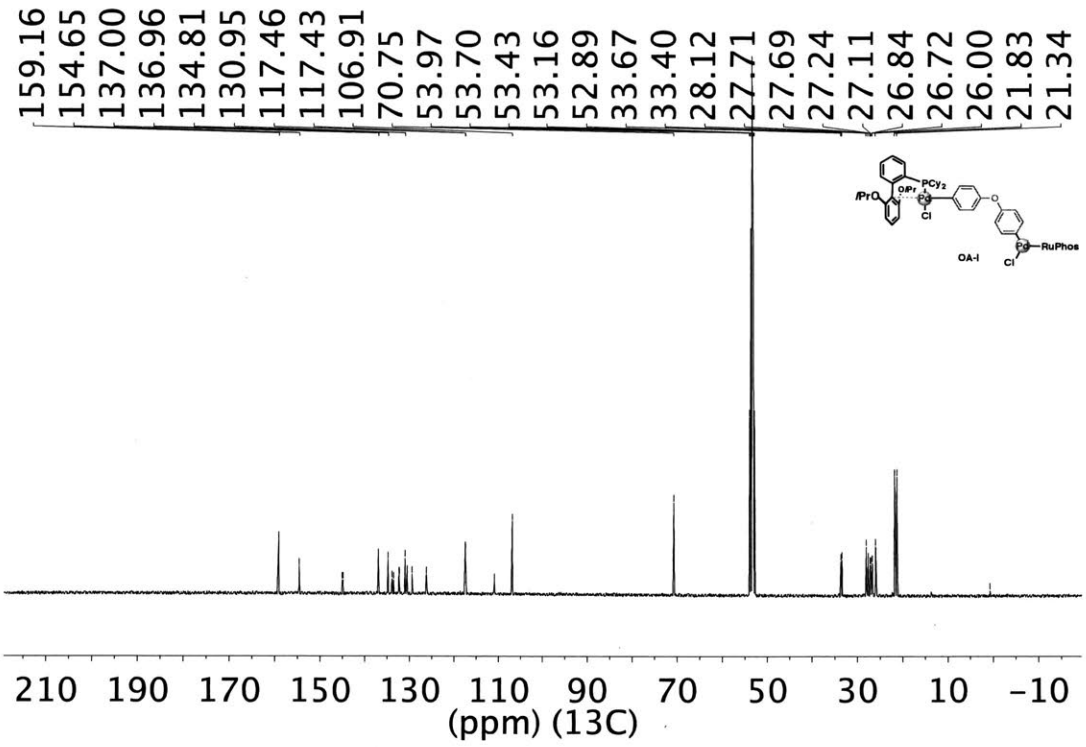
(ppm) (1H)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26.84
26.72
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130
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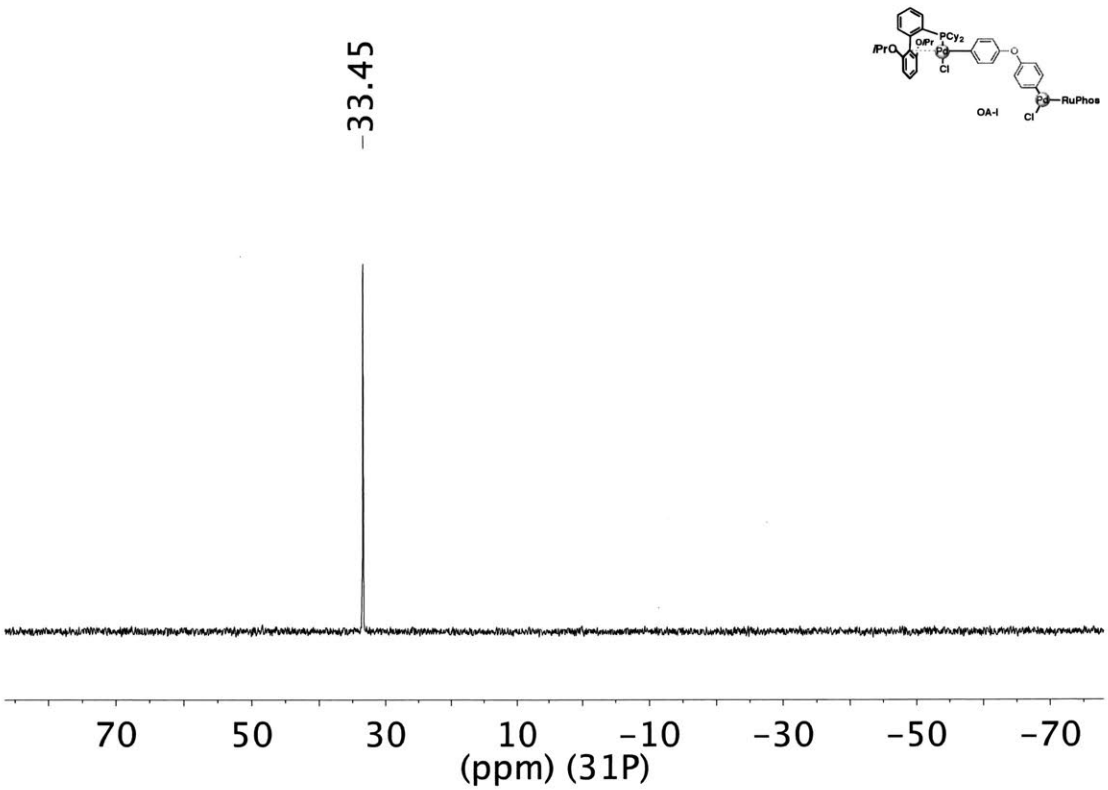
10
−10
(ppm) (13C)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-10
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-50
-70
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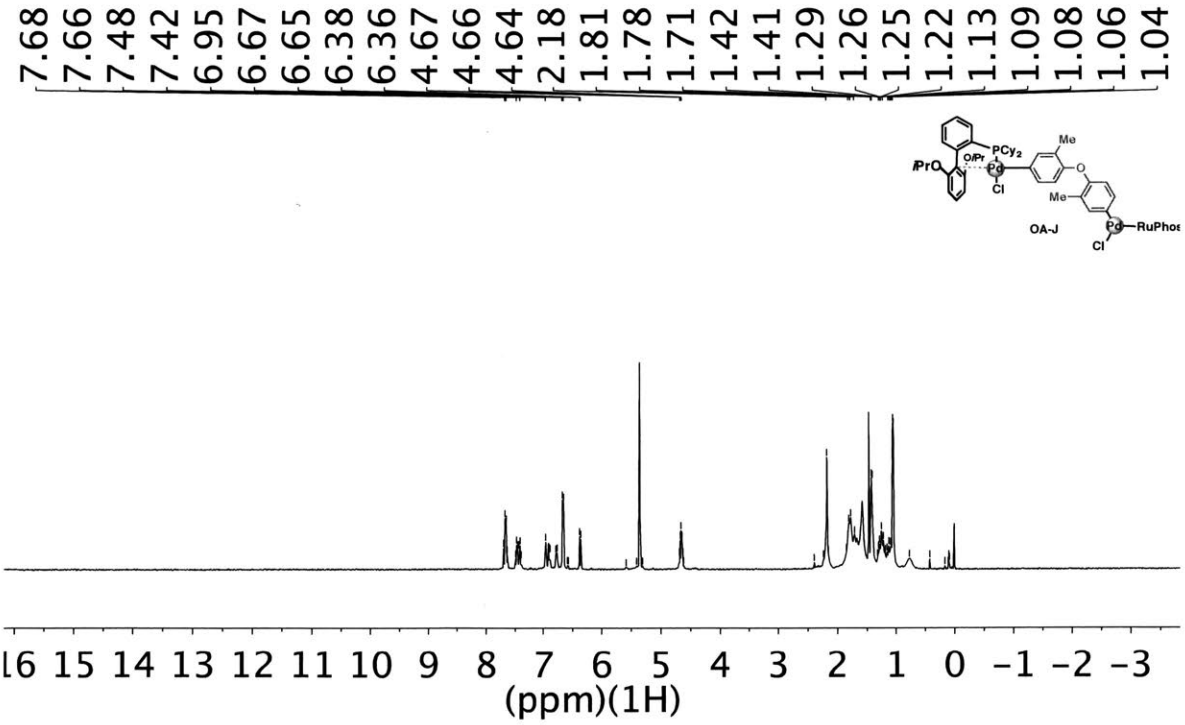
OA-I
RuPhos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1.78
1.71
1.42
1.41
1.29
1.26
1.25
1.22
1.13
1.09
1.08
1.06
1.04
OA-J
RuPhos
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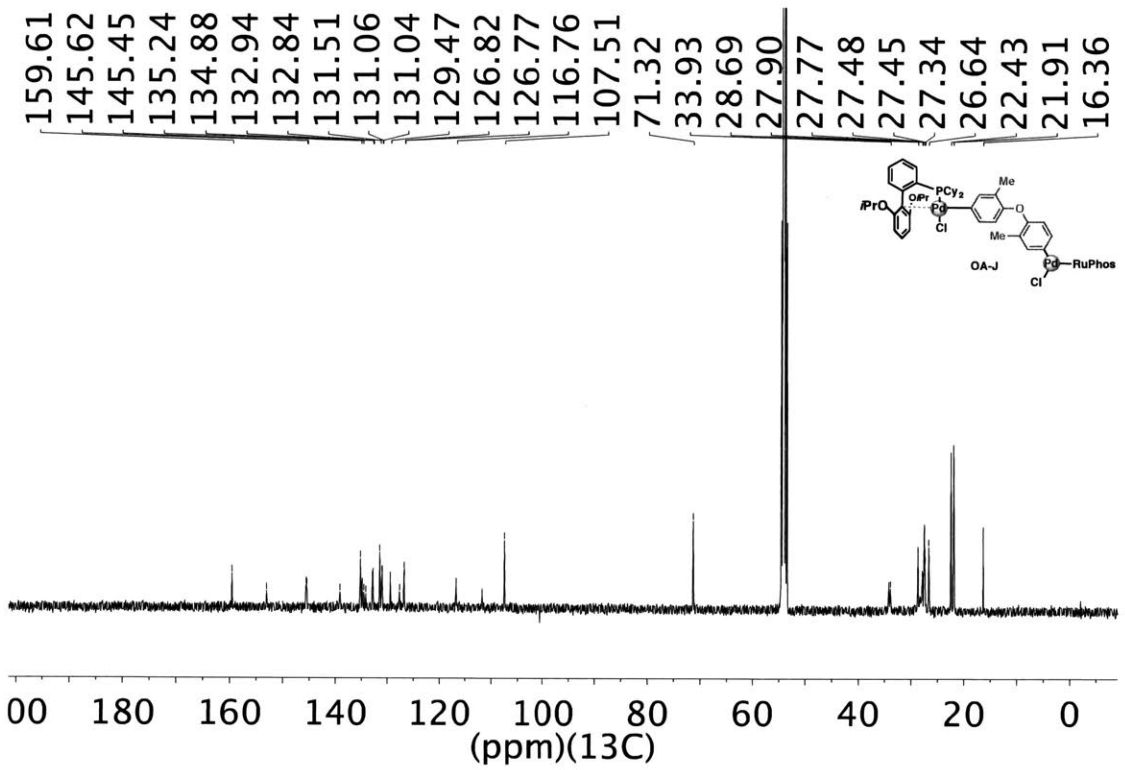
(ppm)(1H)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107.51
71.32
33.93
28.69
27.90
27.77
27.48
27.45
27.34
26.64
22.43
21.91
16.36
OA-J
RuPhos
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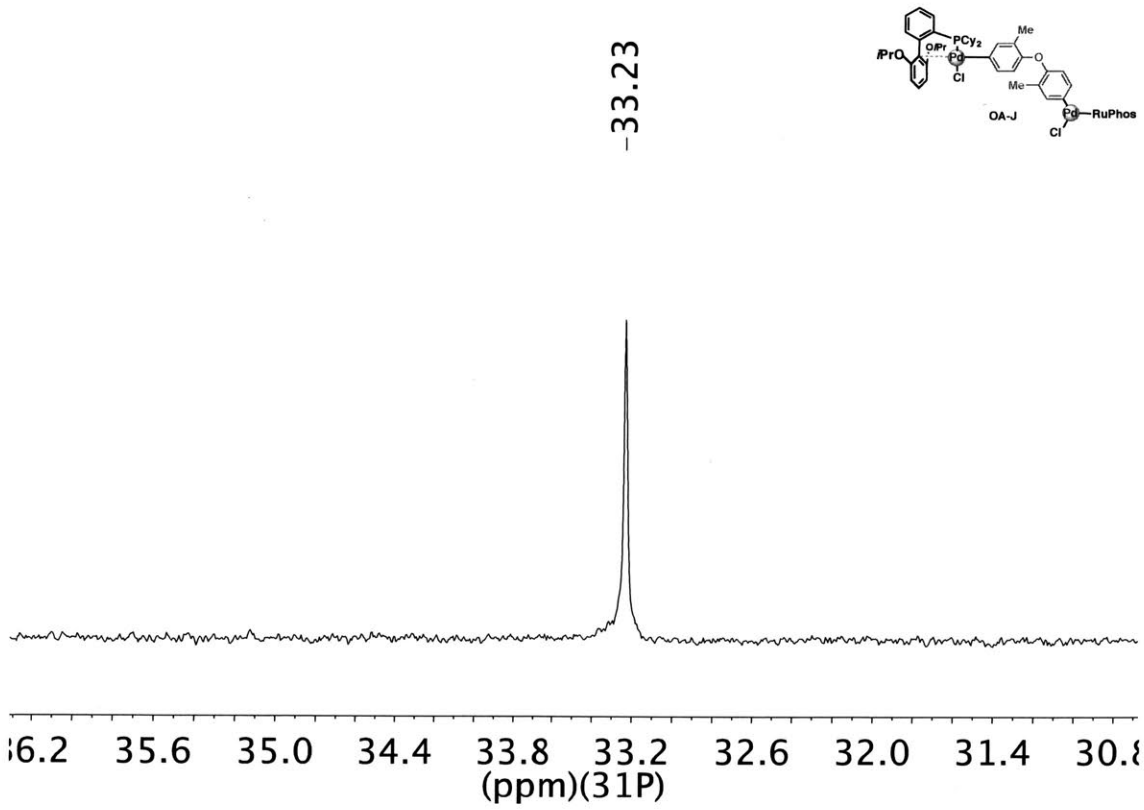
(ppm)(13C)


-33.23
OA-J
RuPhos
6.2 35.6 35.0 34.4 33.8 33.2 32.6 32.0 31.4 30.8
(ppm)(31P)

Chapter 2. Water-Soluble Palladium Reagents for Cysteine S-Arylation under Ambient Aqueous Conditions

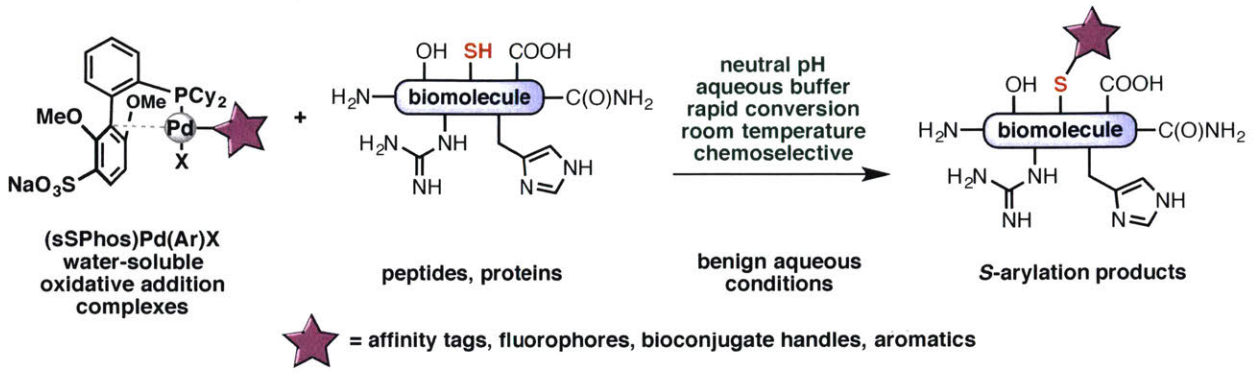
2.1 – Introduction:

Methods for the chemical modification of amino acids serve as fundamental tools for research at the interface of chemistry and biology and play an important role as enabling technologies for the pharmaceutical and materials sciences. Important applications include the preparation of probe molecules for the study of protein function,[1] the development of new protein-drug conjugates,[2] nanomedicines with unique therapeutic properties,[3] and the incorporation of biological macromolecules in functional hybrid materials.[4] Ideally, broadly applicable protocols to modify amino acids for bioconjugation are processes that are chemoselective, proceed at a high rate under mild reaction conditions (i.e. aqueous solvents/buffers, pH 6-8, temperature < 37 °C), and are tolerant to the functional group diversity present in complex biomolecules. In addition, such a process should forge stable chemical bonds in a modular manner that provides access to structural diversity.[5,6] The preparation of protein bioconjugates dates back over a century,[7] however, few reports exist which meet all of these specifications.

With these criteria in mind, we previously reported a palladium-mediated method that can be used for the reliable and rapid *S*-arylation of cysteine residues in peptides and proteins.[8] This method is highly chemoselective for cysteine among other nucleophilic residues, as confirmed by MS/MS analysis, and can be used without incorporation of unnatural amino acids. One notable limitation of this procedure, however, was the necessary addition of an organic cosolvent (N,N-dimethylformamide, dimethyl sulfoxide, or acetonitrile) to dissolve the organometallic reagents. These organic cosolvents have the potential to denature sensitive biomolecules, limiting the scope and utility of our

approach. To address this limitation, we sought an improved, cosolvent-free protocol for bioconjugation employing modified, water-soluble palladium complexes.

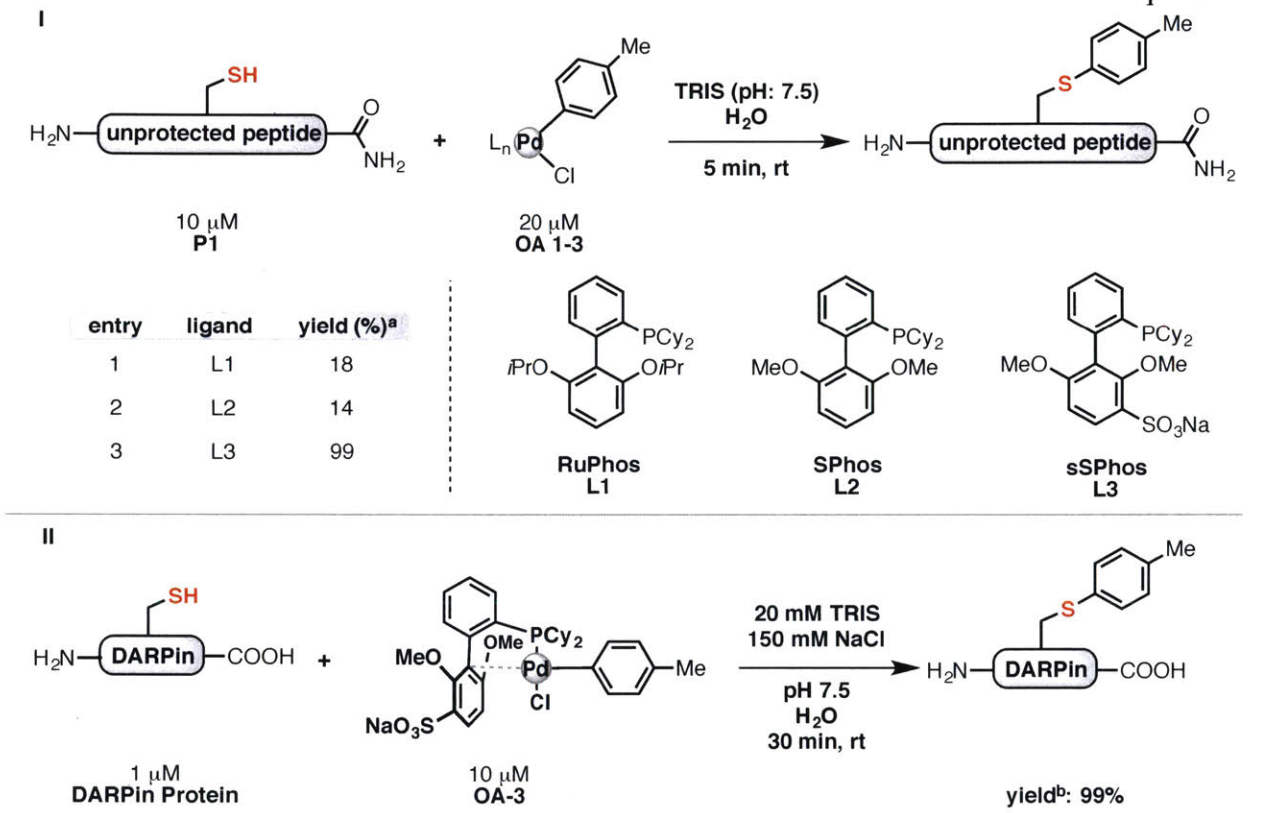
Figure 2: S-arylation of biomolecules under ambient aqueous conditions. This work expanded on previous reports of Palladium-mediated S-arylation chemistry by using a water-soluble ligand to facilitate bioconjugation under benign and fully aqueous conditions.



Our group reported the synthesis of a sulfonated ligand, sodium 2′-dicyclohexylphosphino-2,6-dimethoxy-1,1′-biphenyl-3-sulfonate hydrate (sSPhos), which has been utilized in the context of Suzuki-Miyaura couplings in water.[9] We hypothesized that palladium(II) complexes supported by this ligand might be sufficiently water-soluble to serve as arylation reagents for cysteine-containing biomolecules under completely aqueous conditions. To test this hypothesis, both a model peptide, and a designed ankyrin repeat protein (DARPin), each containing a single cysteine residue, were exposed to an sSPhos supported palladium reagent in a completely aqueous environment (Figure 2). We chose the DARPin protein to ensure the comparison in ligand-enhanced reactivity directly paralelled our previous study.[8] In both cases, the product of cysteine arylation was formed rapidly at room temperature with high chemoselectivity. In contrast, use of the complexes supported by RuPhos and SPhos ligands, under these cosolvent-free conditions, provided <20% yield. These results are consistent with the idea that the sulfonate group of sSPhos

allows the palladium reagent to be more soluble in water and, therefore, able to react with cysteine residues more efficiently.

Figure 3: Initial ligand screen. Examining effect ligand imposes on efficiency of palladium-mediated unprotected cysteine bioconjugation under ambient aqueous conditions. [a]Yield determined by LC-MS analysis. [b]Yield determined by deconvolution of mass spectrum.

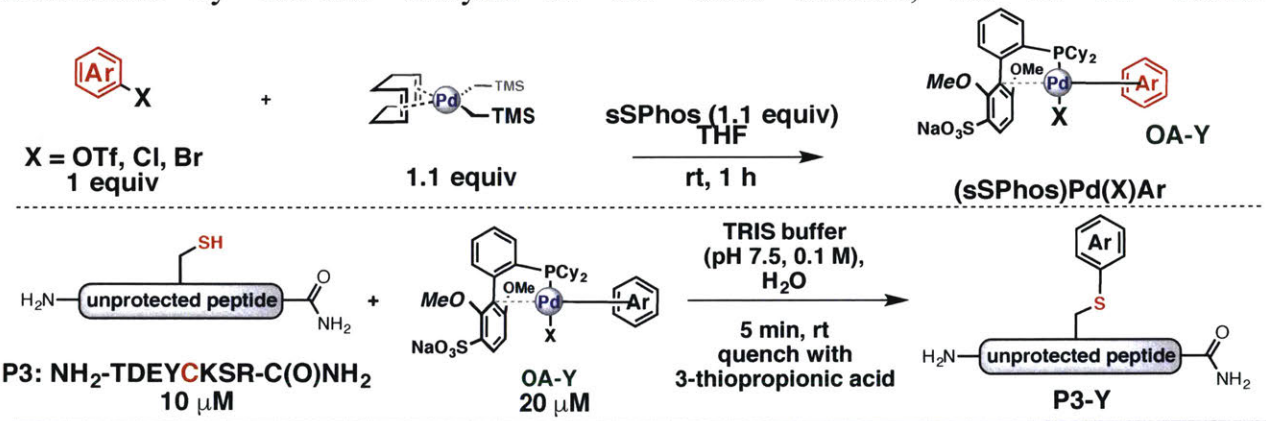


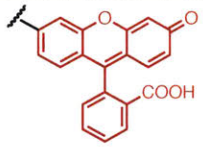
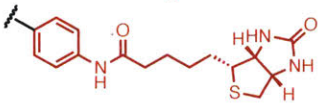
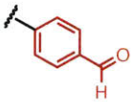
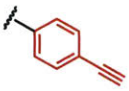
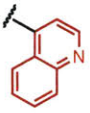
2.2 – Results and Discussion:

In our previous report, the palladium reagents were prepared in a nitrogen-filled glovebox, presenting a potential barrier for adoption by laboratories without routine access to a glovebox. To ameliorate this situation, we investigated the preparation of several sSPhos-ligated palladium complexes employing a bench top protocol, without the exclusion of moisture or oxygen from the reaction mixture (see Experimental for details). Under these conditions, it was found that a mixture of aryl halide (ArX), ligand, and

[(1,5-COD)Pd($CH_2TMS)_2$] dissolved in tetrahydrofuran (THF) afforded the desired palladium complexes in excellent yields after an hour of stirring (Table 1) and subsequent filtration. These easily purified arylation reagents, sSPhosPd(X)Ar, are air-stable and be stored under ambient atmosphere and temperature for over 12 months without displaying noticeably diminished reactivity.

Table 1: Preparation of Air-Stable, Storable Palladium Reagents of Interest in Chemical Biology[a] and subsequent *S*-arylation of an unprotected peptide[b]. [a]Reported isolated yields are an average of two runs after purification. [b]*S*-arylation conversions determined by LC-MS analysis of the crude mixture, see SI for details.

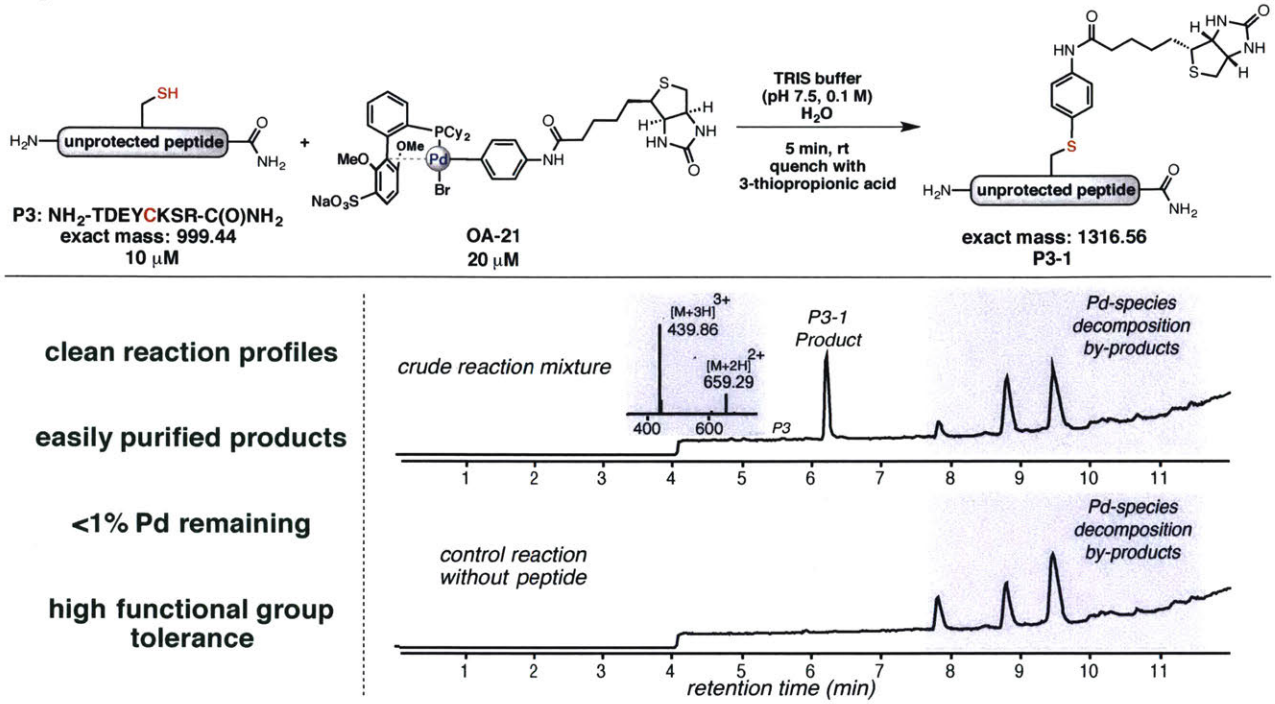


name	Ar	X	isolated yield of OA (%)	*S*-arylation (%)
OA-20		OTf	94	99
OA-21		Br	99	99
OA-22		Cl	86	99
OA-23		Br	82	99
OA-24		Cl	94	99
OA-25		Br	99	99

In order to showcase the utility of this process for application in chemical biology, several types of biologically relevant arylation reagents were isolated in high yield using this procedure (Table 1) including examples that contain a fluorescein dye (**OA-20**), a biotin labeling reagent (**OA-21**), and an aldehyde or an alkyne as bioconjugation handles for further diversification (**OA-22,23**). Additionally, a heterocycle-containing reagent (**OA-24**) and one containing a fluoroaromatic group (**OA-25**) were prepared in good to excellent yield. Each of these reagents, regardless of complexity, displayed similar reaction profiles with biomolecules containing cysteine (Table 1, Figure 3), highlighting the generality of this palladium-mediated *S*-arylation approach. Additionally, **P3-1** was generated on a larger scale, 8 mg of starting peptide, and subsequent purification by reverse-phase HPLC provided an isolated yield of 81% (see Experimental for details) indicating that these modified peptides can be obtained in measurable quantities with high efficiency.

Peptide macrocyclization is a burgeoning area in chemical biology and in the development of new therapeutics.[10,11] Compared to their linear counterparts, peptide macrocycles have been shown to exhibit enhanced cell permeability,[12] increased target binding affinity,[13] and resistance to proteolytic degradation.[14] As such, several methods have been developed to bridge natural and unnatural amino acid residues along a peptide chain.[15] Notably, many of these methods utilize the same cross-linker structures with few examples reported that introduce structural diversity at a late stage.[16] Methods that allow a divergent array of cross-linker structures to be used would better equip researchers to investigate structure activity relationships and manipulate the biological activity of peptide macrocycles. Towards this end, we recently published a report detailing the

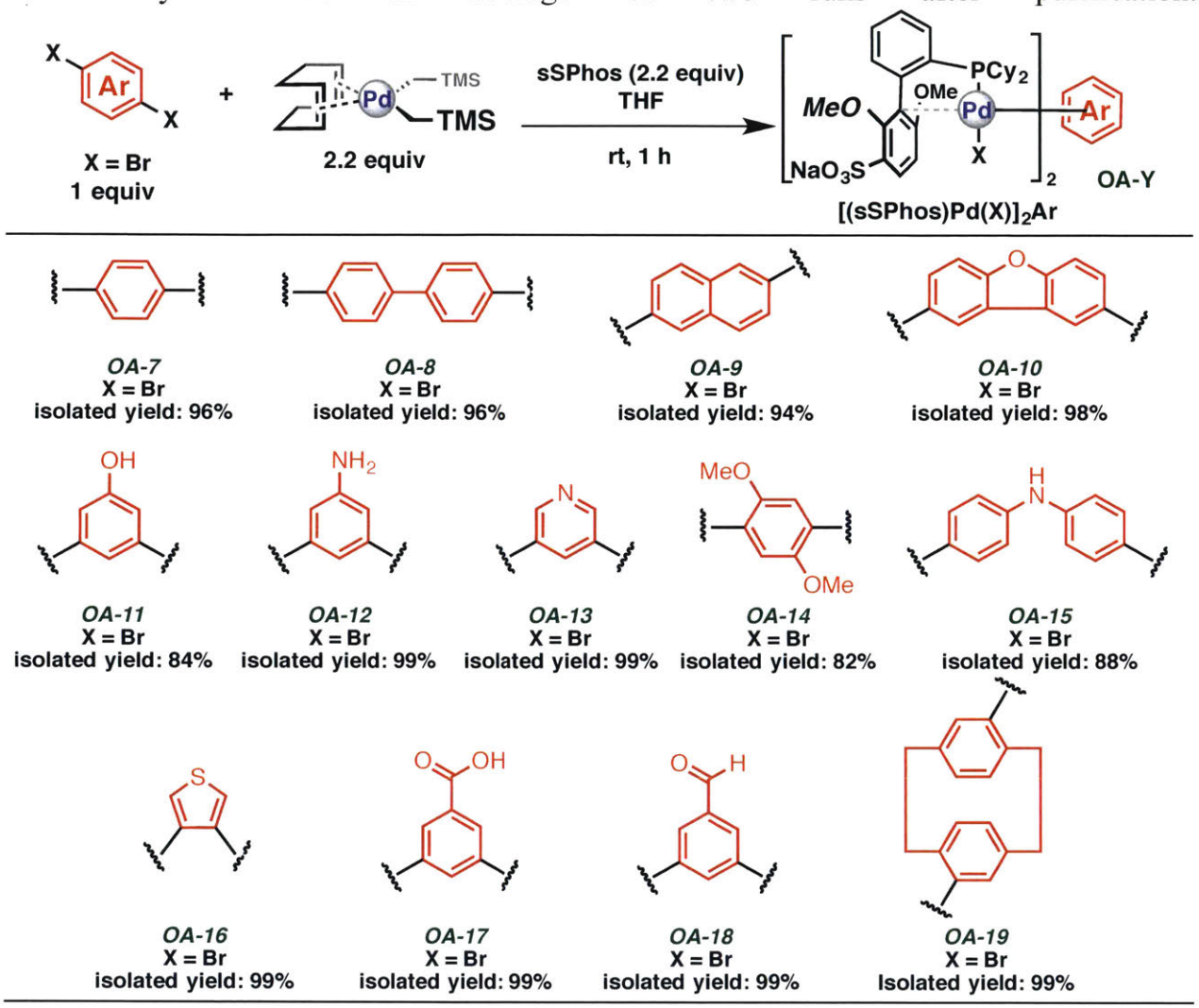
Figure 4: Reaction Characterization. LC-MS spectra depicting the reaction of an unprotected peptide with an sSPhos-ligated palladium biotin complex (OA-21) and the control reaction mixture without addition of peptide. All palladium reagents allowed for rapid transformation of biomolecules exhibiting similar reaction profiles and near-quantitative conversion. [a]Yield determined by LC-MS analysis of the crude mixture, see Experimental for details.



binding and pharmacokinetic differences that result from cross-linker manipulation utilizing bis-palladium reagents for macrocycle diversification.[17] It was found that a solution of 50% acetonitrile was required in order to dissolve the palladium reagents and obtain high conversion efficiencies.

We set out to determine whether our previously described method could be carried out without the aid of an organic cosolvent using sSPhos-derived oxidative addition complexes. Thus, we prepared 13 bis-palladium macrocyclization reagents ($[(sSPhos)Pd(X)]_2Ar$) of varying structure, functional group presence, substitution pattern, and conformation (Scheme 1) from commercially available aryl dihalides. We

Scheme 1: Preparation of bis-Palladium Macrocyclization Reagents[a]. [a]Reported isolated yields are an average of two runs after purification.

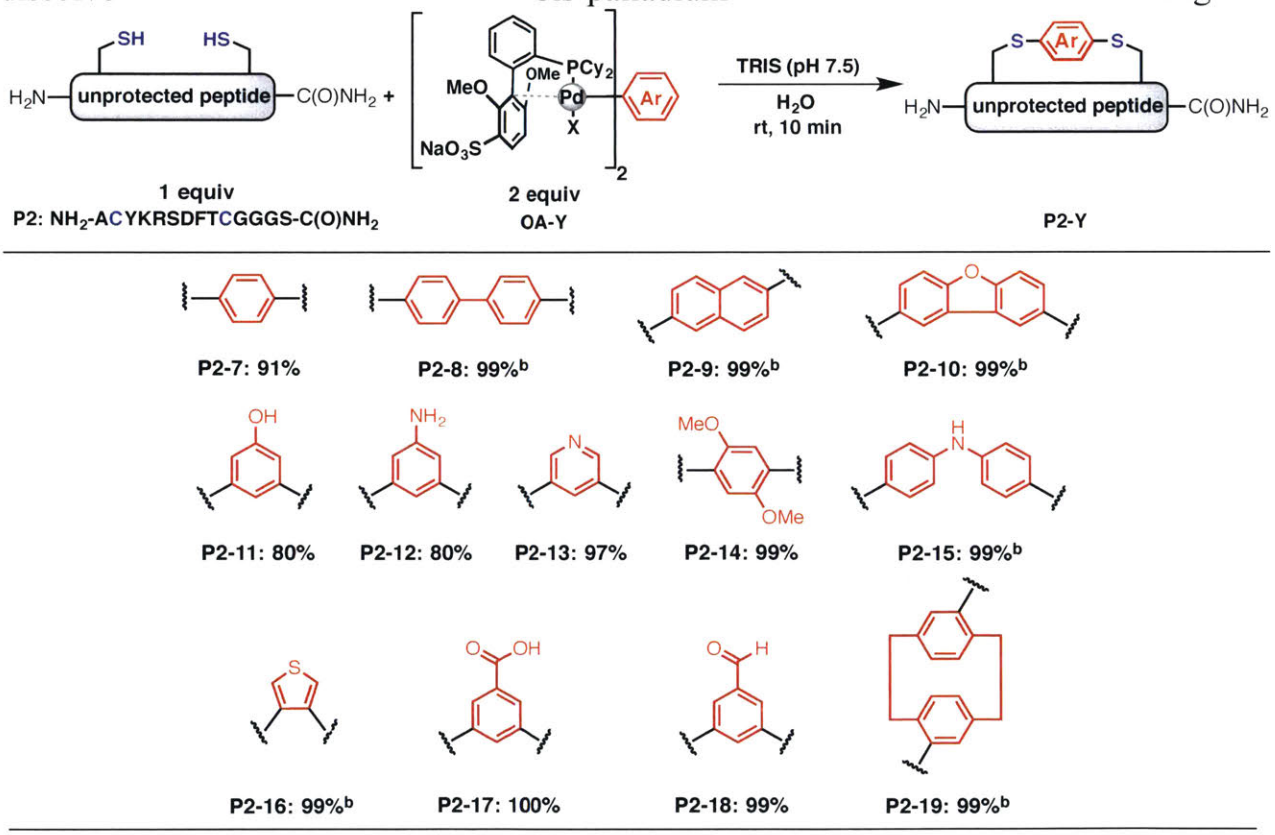


chose these, in most instances, to be structurally different than the examples we previously published. We note that regardless of these structural features, each of the bis-palladium macrocyclization reagents could be isolated in good to excellent yield (82-99% yield).

The high functional group tolerance of the cysteine arylation method may provide a general method for the tuning of macrocyclic peptides. To test the efficiency of peptide macrocyclization using sSPhos supported reagents, a model peptide (**P2**) was prepared

with two cysteines separated in an *i, i+7* position. After dissolving the peptide in water and *tris*(hydroxymethyl)aminomethane (TRIS, pH 7.5), the palladium reagents were introduced in an aqueous solution, and the mixture was gently vortexed for five seconds. The reactions were allowed to proceed for ten minutes at room temperature, followed by addition of 3-thiopropionic acid to quench the reaction, and LC-MS analysis indicated that most of the macrocyclization reactions were high yielding under these standard conditions (Scheme 2).

Scheme 2: Macrocyclization of Peptides Containing Two Unprotected Cysteines[a]. [a]Yield determined by LC-MS analysis. [b]Required 15% acetonitrile solution to fully dissolve bis-palladium reagents.



In approximately half of the examples, full conversion was not observed under our standard conditions (**P2-8**, **9**, **10**, **15**, **16**, and **19**). In these cases, the addition of a small amount of acetonitrile (15% by volume) was found to be beneficial, allowing reactions

previously capped at 60% yield to reach full conversion (see Experimental). We believe this is necessitated by the enhanced hydrophobicity of some of these macrocyclization reagents. The macrocyclic peptide products are readily purified through reverse-phase high performance liquid chromatography. ICP-MS analysis of the peptides showed that more than 99% of palladium was removed during the purification process. Moreover, no side reactions forming peptide oligomers through intermolecular cross-linking were observed (see Experimental).

2.3 – Conclusion:

In summary, we have developed a general method for the *S*-arylation of cysteine on biomolecules using bench-stable and water-soluble palladium reagents, prepared without the exclusion of air or ambient moisture. In most instances, no organic cosolvent was necessary. Key to the improvement of this method was the application of the commercially available sulfonated biaryl dialkyl phosphine ligand sSPhos to the synthesis of palladium arylation reagents. Given the importance of developing bioconjugation methods that adhere to physiological reaction conditions, we anticipate that the availability of a water soluble Pd arylation reagent will facilitate the adoption of this methodology by chemical biologists and medicinal chemists for the synthesis of peptide macrocycles, antibody-drug conjugates, modified proteins, and other biochemical probes.

2.4 – Experimental:

General Reagent Information

1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), D-Biotin, Fmoc-Rink amide linker, Fmoc-L-Gly-OH, Fmoc-L-Leu-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Ala-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Gln(Trt)-OH,

Fmoc-L-Asn(Trt)-OH, Fmoc-L-Glu(O*t*Bu)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Thr(*t*Bu)-OH, Fmoc-L-Tyr(*t*Bu)-OH, and Fmoc-L-His(Trt)-OH were purchased from CreoSalus, Inc (Louisville, KY). Peptide synthesis-grade *N,N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethyl ether, HPLC-grade acetonitrile, and guanidine hydrochloride were obtained from VWR International (Philadelphia, PA). Aryl halides and aryl trifluoromethanesulfonates were purchased from Aldrich Chemical Co., Alfa Aesar, or Matrix Scientific and were used without additional purification. All deuterated solvents were purchased from Cambridge Isotopes and used without further purification. All other reagents were purchased from Sigma-Aldrich and used as received.

All reactions with peptides were set up on the bench top and carried out under ambient conditions. Anhydrous THF, pentane, cyclohexane, and acetonitrile were purchased from Aldrich Chemical Company in SureSeal® bottles and were purged with argon before use.

All small-molecule organic and organometallic compounds were characterized by ^{1}H, ^{13}C NMR, and IR spectroscopy, as well as elemental analysis or high-resolution mass spectrometry (unless otherwise noted). ^{31}P NMR spectroscopy was used for characterization of palladium complexes. Copies of the ^{1}H, ^{13}C, and ^{31}P NMR spectra can be found at the end of the Supporting Information. Nuclear Magnetic Resonance spectra were recorded on a Bruker 400 MHz instrument and a Varian 300 MHz instrument. Unless otherwise stated, all ^{1}H NMR experiments are reported in δ units, parts per million (ppm), and were measured relative to the signals of the residual proton resonances methanol-d_4 (4.78 and 3.31 ppm) in the deuterated solvents. All ^{13}C NMR spectra are measured decoupled from ^{1}H nuclei and are reported in δ units (ppm) relative to methanol-d_4 (49.30 ppm), unless otherwise stated. All ^{31}P NMR spectra are measured decoupled from ^{1}H nuclei and are reported relative to H_3PO_4 (0.00 ppm). All FT-IR spectra were

recorded on a Thermo Scientific – Nicolet iS5 spectrometer (iD5 ATR – diamond). High resolution mass spectra were obtained on a Bruker Daltonics APEXIV 4.7 Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS).

LC-MS chromatograms and associated mass spectra were acquired using Agilent 6520 ESI-Q-TOF mass spectrometer. Solvent compositions used in the LC-MS are 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC-MS method was used:

Method A LC conditions: Zorbax 300SB C3 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-3 min 5% B, 3-8 min 5-95% B, 8-9 min 95% B, flow rate: 0.8 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300 – 3000 *m/z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octapole rf voltages were set at 4000, 175, and 750, respectively.

Determination of Bioconjugation and Macrocyclisation Yields

Data were processed using Agilent MassHunter software package. All reported yields were determined by integrating total ion current (TIC) spectra. First, the peak areas for all relevant peptide-containing species on the chromatogram were integrated using Agilent MassHunter software package. Since no peptide-based side products were generated in the experiments, the yields shown in **Table 2** were determined as follows: %yield = S_{pr}/S_{total} where S_{pr} is the peak area of the product and S_{total} is the peak area of combined peptide-containing species (product and starting material). For protein bioconjugation, deconvoluted masses of proteins were obtained using maximum entropy algorithm. LC-MS data shown were acquired

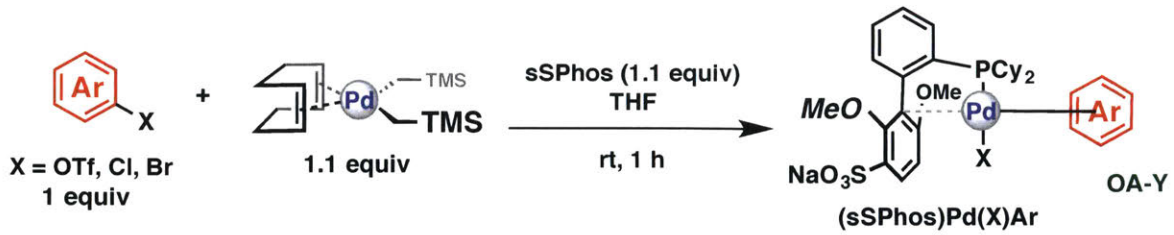
using Method A, unless otherwise noted. Mass spectrum insets correspond to the integration of the TIC peak unless otherwise noted.

In addition to this method, reaction **P3-1** was scaled up and subsequently purified via mass-directed preparative HPLC and in order to determine isolated yield. The results of this isolation can be found on page S24 under the subheading Large Scale Bioconjugation.

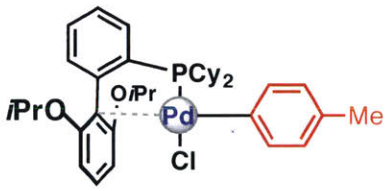
Synthesis of Oxidative Addition Complexes

Synthesis of [(1,5-COD)Pd(CH_2TMS)$_2$]. A flame-dried Schlenk flask (100 mL), equipped with a magnetic stir bar, was filled with argon and charged with (1,5-COD)$PdCl_2$ (3.15g, 11.05 mmol) made according to literature reports.[1] The flask was put under vacuum and backfilled with argon (the procedure was repeated 3 consecutive times). Diethyl ether (49.3 mL) was added via syringe, the reaction was cooled to –40 °C (acetonitrile/dry ice bath) and $TMSCH_2MgCl$ (23.4 mL, 1.0 M) purchased from Sigma-Aldrich was added dropwise over 10–20 min. The reaction mixture was stirred at –40 °C for 1 h and then at 0 °C (ice/water bath) for an additional 20 min. Acetone (1.3 mL) was added at 0 °C, the reaction mixture was stirred for 5 min, after which time the solvent was removed under vacuum using an external trap (the flask was kept at 0 °C). The flask was then opened to air, pentane (100 mL) was added and the crude material was filtered through a pad of Celite into a new round-bottom flask (500 mL) at 0 °C. The filter cake was washed with pentane (50 mL × 2). Pentane from the combined washes was removed with the aid of a rotary evaporator at 0 °C (ice/water bath). The resulting white solid was dried under vacuum for 2 h at 0 °C, and transferred into a 20 mL scintillation vial in the glovebox (3.00 g, 70%). The ^{1}H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature.[1] The title compound was stored in the glovebox at –20 °C.

General Procedure for the Synthesis of mono-Palladium Oxidative Addition Complexes.

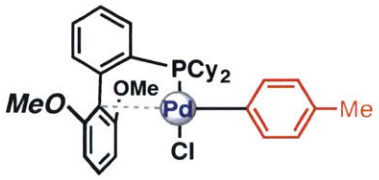


In a scintillation vial (10 mL) open to the air and equipped with a magnetic stir bar, the vial was charged with ligand (1.1 equiv), Ar–X (1 equiv), and THF (1 mL). Solid (1,5-COD)Pd(CH_2SiMe_3)$_2$ (1.1 equiv) was added rapidly in one portion and the resulting solution was stirred for 1 h at rt. After this time, pentane (3 mL) was added and the resulting mixture was capped and placed into a –20 °C freezer for 2 h. The vial was removed from the freezer and the resulting precipitate was filtered, washed with pentane (5 × 3 mL), and dried under reduced pressure to afford the oxidative addition complex.



OA-1

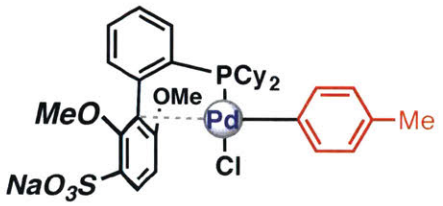
Following the general procedure, a mixture containing 4-chlorotoluene (6.4 μL, 0.054 mmol), RuPhos (28 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in a nitrogen-filled glovebox in cyclohexane (1.5 mL) for 18 h. The vial was removed from the glovebox, opened to the air, and general work-up afforded **OA-1** as a grey solid (37 mg, 96%). The ^{1}H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature.[1]



OA-2

Following the general procedure, a mixture containing 4-chlorotoluene (6.4 μL, 0.054 mmol), SPhos (24.6 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in a nitrogen-filled glovebox in cyclohexane (1.5 mL) for 18 h. General work-up afforded **OA-2** as a white solid (33 mg, 93%). The ^{1}H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature.[1]

HRMS electrospray (m/z): [M – Cl]$^+$ calcd for $C_{33}H_{42}ClO_2PPd$: 607.1969, found 607.1962.

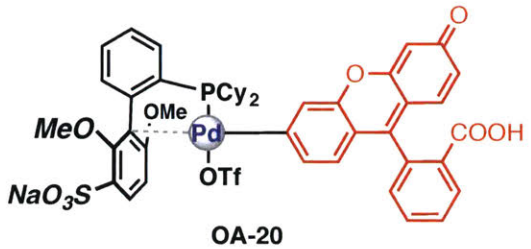


OA-3

Following the general procedure, a mixture containing 4-chlorotoluene (6.4 μL, 0.054 mmol), sSPhos (33 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-3** as a yellow solid (41 mg, 99%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.39, 161.35, 157.73, 157.61, 139.18, 138.45, 138.38, 138.05, 135.46, 135.37, 135.19, 134.96, 134.89, 134.04, 133.08, 132.63, 132.49, 132.25, 132.02, 131.99, 131.80, 131.22, 130.20, 130.01, 129.50, 129.39, 129.09, 128.98, 128.54, 128.43, 126.96, 126.92, 126.90, 107.08, 107.02, 69.14, 64.04, 62.36, 61.71, 57.04, 56.68, 49.94, 49.73, 49.51, 49.30, 49.09, 48.88, 48.66, 39.00, 38.55, 38.35, 37.90, 35.49, 31.49, 29.24, 28.73, 28.69, 28.00, 27.96, 27.91, 27.83, 27.78, 27.70, 27.51, 27.44, 27.39, 27.34, 27.31, 27.14, 26.85, 26.82, 26.78, 26.58, 26.39, 25.92, 21.79, 20.98. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.85. **FT-IR (neat, cm^{-1}):** 3438.12, 2929.53, 2849.99, 1570.73, 1438.17, 1395.08, 1275.77, 1229.37, 1176.35, 1106.75, 1083.55, 1053.72, 1017.27, 914.53, 894.65, 851.56, 815.11,

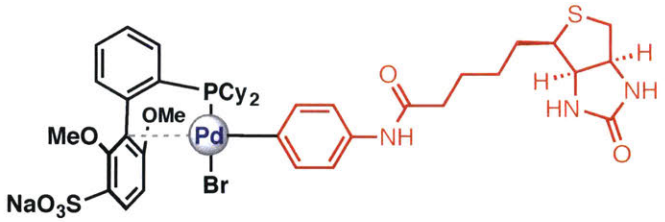
762.08, 702.43, 665.97, 652.71. **HRMS electrospray (m/z): [M – Cl]$^+$ calcd for $C_{33}H_{41}ClNaO_5PPdS$:** 709.1345, found 709.1337.



OA-20

Following the general procedure, a mixture containing fluorescein monotrifluoromethanesulfonate (30 mg, 0.054 mmol), sSPhos (33 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-20** as a red solid (54 mg, 94%).

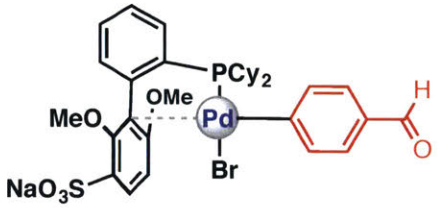
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, CD_2Cl_2)** d 171.22, 161.31, 157.55, 153.75, 153.62, 151.81, 150.75, 138.53, 138.46, 137.21, 135.48, 135.38, 134.85, 134.78, 134.18, 132.94, 132.46, 132.01, 131.99, 131.83, 131.72, 131.63, 131.16, 130.42, 128.53, 128.43, 127.93, 126.88, 126.86, 125.50, 123.61, 121.96, 121.68, 120.45, 118.78, 118.27, 111.88, 110.86, 108.61, 107.06, 104.01, 62.35, 57.39, 57.01, 56.66, 49.94, 49.73, 49.51, 49.30, 49.09, 48.88, 48.66, 39.02, 38.39, 38.36, 37.73, 35.86, 28.64, 28.61, 28.13, 27.86, 27.83, 27.78, 27.73, 27.66, 27.52, 27.48, 27.46, 27.39, 27.31, 27.26, 27.23, 27.11, 26.81, 26.77. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 55.32. **FT-IR (neat, cm^{-1}):** 2939.44, 2365.12, 2334.10, 2229.64, 2193.02, 2171.53, 2162.71, 2154.39, 2030.18, 2020.55, 1768.04, 1578.01, 1451.18, 1425.24, 1400.41, 1295.66, 1257.24, 1224.09, 1172.18, 1100.84, 1052.69, 1019.40, 990.73, 848.30, 759.20, 652.11, 596.38. **HRMS electrospray (m/z): [M – OTf]$^+$ calcd for $C_{47}H_{45}F_3NaO_{12}PPdS_2$:** 933.1468, found 933.1496.



OA-21

Following the general procedure, a mixture containing aryl bromide (biotin) (30 mg, 0.054 mmol), sSPhos (33 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-21** as a pink solid (54 mg, 99%).

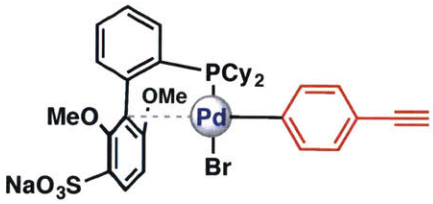
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, CD_2Cl_2)** d 161.36, 161.36, 157.63, 138.35, 135.44, 134.98, 133.14, 133.02, 132.73, 131.99, 131.25, 128.54, 128.44, 126.93, 123.13, 107.07, 62.37, 56.68, 49.94, 49.72, 49.51, 49.30, 49.09, 48.88, 48.85, 48.66, 38.96, 38.69, 38.31, 28.75, 28.07, 27.70, 27.50, 27.37, 27.12, 26.85. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 53.89. **FT-IR (neat, cm^{-1}):** 3282.15, 2919.10, 2851.48, 1688.19, 1586.10, 1521.18, 1586.10, 1488.30, 1451.98, 1395.17, 1275.99, 1226.01, 1185.10, 1099.39, 1049.20, 1007.70, 853.33, 815.64, 764.68, 688.85, 677.29, 667.72, 648.10, 595.07. **HRMS electrospray (m/z): [M – Br]$^+$ calcd for $C_{42}H_{54}BrN_3NaO_7PPdS_2$:** 936.2085, found 936.2087.



OA-22

Following the general procedure, a mixture containing 4-chlorobenzaldehyde (8.4 mg, 0.054 mmol), sSPhos (31 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-22** as a white solid (36 mg, 89%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, CD_2Cl_2)** d 161.29, 157.50, 135.52, 135.42, 134.71, 132.77, 132.07, 131.10, 128.57, 128.47, 126.83, 107.07, 69.12, 62.34, 57.51, 56.69, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.06, 38.41, 38.18, 37.52, 35.84, 35.44, 29.23, 27.84, 27.70, 27.66, 27.46, 27.35, 27.13, 26.76, 23.64, 14.68, -1.12. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 55.14. **FT-IR (neat, cm^{-1}):** 3501.12, 2928.70, 2849.99, 2361.49, 2344.31, 1690.19, 1571.43, 1550.27, 1450.50, 1398.96, 1211.92, 1098.84, 1050.83, 1008.78, 834.27, 808.78, 759.26, 693.54, 667.90, 662.51, 639.86, 590.44. **HRMS electrospray (m/z): [M – Br]$^+$ calcd for $C_{33}H_{39}BrNaO_6PPdS$:** 723.1148, found 723.1158.

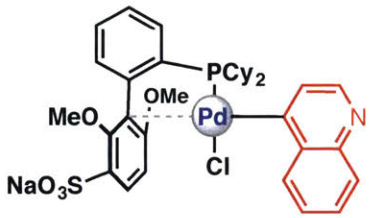


OA-23

Following the general procedure, a mixture containing 4-bromophenylacetylene (9.7 mg, 0.054 mmol), sSPhos (31 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-23** as a yellow solid (36 mg, 89%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD** d 161.54, 157.79, 138.72, 138.65, 135.69, 135.60, 135.11, 135.04,

133.22, 132.74, 132.24, 132.22, 131.91, 131.40, 128.76, 128.66, 127.10, 107.29, 69.35, 62.57, 56.89, 50.15, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 43.15, 39.23, 38.66, 38.58, 38.01, 28.90, 28.71, 28.10, 28.03, 27.97, 27.90, 27.71, 27.67, 27.61, 27.35, 27.05, 27.01, 26.99. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 58.96. **FT-IR (neat, cm^{-1}):** 3430.11, 3200.11, 2929.69, 2852.02, 1247071, 2339.26, 1572.29, 1450.85, 1398.15, 1228.42, 1098.54, 1050.80, 1009.71, 916.01, 849.38, 812.33, 759.15, 693.48, 667.86, 656.56, 585.72 **HRMS electrospray (m/z): [M – Br]$^+$ calcd for $C_{34}H_{39}BrNaO_5PPdS$:** 719.1199, found 719.1217.

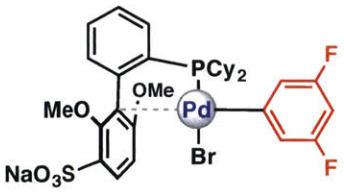


OA-24

Following the general procedure, a mixture containing 4-chloroquinoline (9.8 mg, 0.054 mmol), sSPhos (31 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-24** as a light brown solid (40 mg, 94%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.56, 157.82, 138.59, 135.68, 135.58, 135.18, 135.11, 133.28, 132.83, 132.21, 132.00, 131.43, 128.76, 128.65, 127.13, 107.29, 69.35, 62.58, 56.89, 50.15, 49.94, 49.73, 49.51, 49.30, 49.09, 48.88, 39.21, 38.76, 38.56, 38.10, 36.10, 35.81, 28.91, 28.17, 28.11, 28.04, 27.98, 27.90, 27.72, 27.52, 27.35, 27.03, 26.99, 23.88, 14.88 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.73. **FT-IR (neat, cm^{-1}):** 3418.60, 2928.65, 2849.95, 2360.04, 2339.28, 1572.13, 1495.74, 1450.01, 1398.16, 1367.97, 1280.36,

1189.32, 1097.91, 1049.60, 915.43, 813.53, 693.38, 635.12, 594.93. **HRMS electrospray (m/z): [M – Cl]$^+$ calcd for $C_{35}H_{40}ClNNaO_5PPdS$:** 746.1308, found 746.1310.

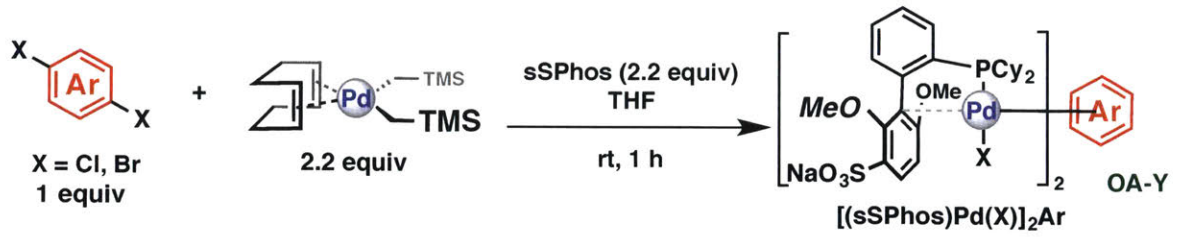


OA-25

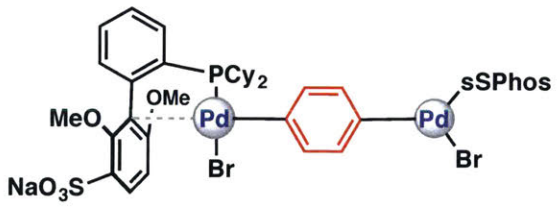
Following the general procedure, a mixture containing 1-Bromo-3,5-difluorobenzene (9.8 mg, 0.054 mmol), sSPhos (31 mg, 0.06 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (25 mg, 0.06 mmol) was stirred at rt in THF (1 mL) for 1 h. General work-up afforded **OA-25** as a yellow solid (45 mg, 99%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 163.75, 161.83, 161.35, 160.11, 158.07, 157.60, 155.19, 138.44, 138.36, 136.21, 135.48, 135.38, 134.96, 134.88, 133.03, 132.89, 132.57, 132.04, 132.01, 131.91, 131.74, 131.21, 130.32, 129.10, 128.56, 128.46, 127.24, 126.91, 126.89, 112.56, 112.49, 112.38, 112.31, 108.10, 107.07, 105.09, 104.83, 104.58, 69.15, 62.43, 62.36, 61.71, 56.86, 56.68, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 40.13, 38.99, 38.52, 38.34, 37.87, 32.17, 28.72, 27.98, 27.90, 27.82, 27.77, 27.69, 27.51, 27.44, 27.39, 27.31, 27.13, 26.84, 26.79, 9.92, 0.26, -1.91 (observed complexity is due to *C-P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.68. **FT-IR (neat, cm^{-1}):** 3390.01, 2931.31, 2849.99, 2362.81, 2344.29, 1597.25, 1577.36, 1448.11, 1398.96, 1227.38, 1182.98, 1100.89, 1023.90, 997.38, 968.81, 924.47, 897.96, 840.21, 808.48, 759.58, 699.11, 662.66, 590.27. **HRMS electrospray (m/z): [M – Br]$^+$ calcd for $C_{32}H_{37}BrF_2NaO_5PPdS$:** 731.1010, found 731.1020.

General Procedure for the Synthesis of mono-Palladium Oxidative Addition Complexes.



A scintillation vial (10 mL), equipped with a magnetic stir bar, was charged with sSPhos (2.2 equiv), Ar–X (1 equiv), and THF (1.5 mL). Solid (1,5-COD)Pd(CH_2SiMe_3)$_2$ (2.2 equiv) was added rapidly in one portion and the resulting solution was stirred for 1 h at rt. After this time, pentane (3 mL) was added and the resulting mixture was placed into a –20 °C freezer for 2 h. The vial was removed from the freezer and, in the air, the resulting precipitate was filtered, washed with pentane (5 × 3 mL), and dried under reduced pressure to afford the oxidative addition complex.

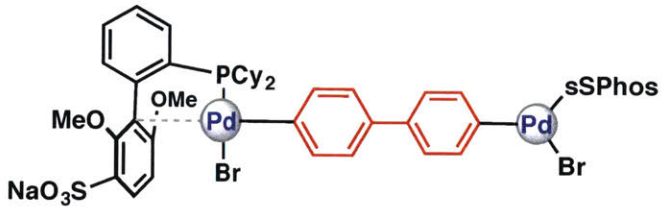


OA-7

Following the general procedure, a mixture containing 1,4-dibromobenzene (12.7 mg, 0.054 mmol), sSPhos (61 mg, 0.119 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (46 mg, 0.119 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-7** as a brown solid (76 mg, 96%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.33, 157.58, 135.48, 134.89, 134.82, 133.01, 132.02, 132.00, 131.18, 128.54, 128.44, 107.07, 69.14, 62.35, 56.68, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.02, 38.45, 38.37, 37.79, 27.89, 27.81, 27.76, 27.69, 27.50, 27.30, 27.14, 26.78, 2.88, -1.91. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 50.93. **FT-**

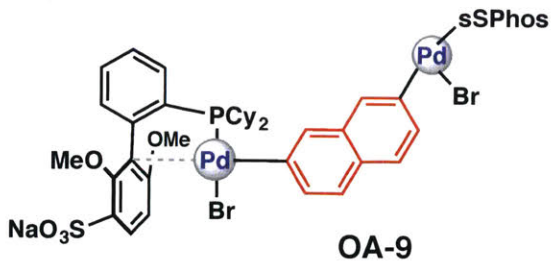
IR (neat, cm^{-1}): 3440.34, 2919.59, 2849.99, 2372.76, 2154.02, 1646.96, 1574.05, 1458.05, 1441.48, 1395.08, 1275.77, 1226.06, 1176.35, 1159.78, 1100.12, 1053.72, 1010.64, 944.07, 914.53, 891.33, 851.66, 798.54, 758.77, 732.25, 692.48, 646.08. **HRMS electrospray (m/z): $[M - NaBr_2]^+$ calcd for $C_{58}H_{72}Br_2Na_2O_{10}P_2Pd_2S_2$:** 1291.2038, found 1291.2011.



OA-8

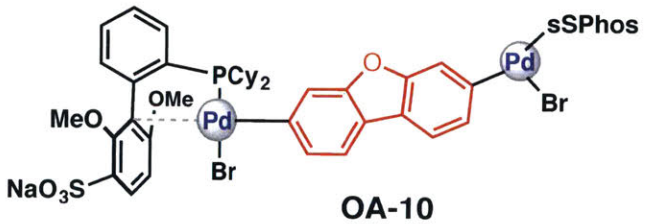
Following the general procedure, a mixture containing 4,4-Dibromobiphenyl (16.8 mg, 0.054 mmol), sSPhos (61 mg, 0.119 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (46 mg, 0.119 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-8** as a brown solid (81 mg, 96%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.31, 134.81, 132.90, 132.04, 131.59, 131.15, 129.59, 128.55, 128.45, 127.51, 107.07, 62.35, 57.08, 56.68, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.04, 38.39, 37.68, 35.58, 27.80, 27.74, 27.68, 27.48, 27.23, 27.13, 26.77, 23.66, 14.68, 2.95, -1.43. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 50.65. **FT-IR (neat, cm^{-1}):** 3461.21, 2929.43, 2846.68, 2361.39, 2163.96, 1576.27, 1450.80, 1141.65, 1272.46, 1232.69, 1179.17, 1096.81, 1051.15, 998.40, 924.47, 888.02, 851.56, 802.14, 759.54, 662.66, 656.69, 639.46. **HRMS electrospray (m/z): $[M - NaBr_2]^+$ calcd for $C_{64}H_{76}Br_2Na_2O_{10}P_2Pd_2S_2$:** 1367.2354, found 1367.2304.



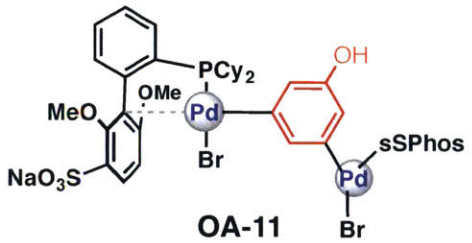
Following the general procedure, a mixture containing 2,6-Dibromonaphthalene (12.7 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-9** as a light red solid (63 mg, 94%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d ^{13}C NMR (101 MHz, MeOD) δ 159.73, 159.65, 155.98, 155.88, 137.04, 136.97, 133.88, 133.79, 133.47, 133.08, 133.00, 131.18, 130.75, 130.64, 130.57, 130.41, 129.81, 129.47, 128.35, 127.45, 127.34, 126.92, 126.80, 125.28, 125.22, 125.20, 105.43, 105.40, 67.49, 60.71, 60.11, 55.87, 55.06, 55.05, 48.31, 48.09, 47.88, 47.67, 47.45, 47.24, 47.03, 37.44, 36.78, 36.54, 35.88, 34.21, 33.93, 26.91, 26.21, 26.15, 26.08, 26.03, 25.92, 25.83, 25.73, 25.50, 25.13, 22.01, 13.05, -1.36, -2.95, -3.05. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.23. **FT-IR (neat, cm^{-1}):** 3438.11, 2928.54, 2850.71, 2361.41, 2339.24, 2151.04, 1637.01, 1572.72, 1450.76, 1398.53, 1275.60, 1180.17, 1098.57, 1017.90, 917.07, 872.32, 845.66, 805.86, 758.91, 693.63, 667.85, 640.04, 554.21. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{62}H_{74}Br_2Na_2O_{10}P_2Pd_2S_2$:** 1397.1481, found 1397.1495.



Following the general procedure, a mixture containing 2,8-dibromodibenzofuran (14.3 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-10** as a brown solid (67.3 mg, 98%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 159.65, 155.90, 136.90, 136.83, 133.49, 131.28, 130.78, 130.44, 129.95, 129.62, 129.38, 128.46, 128.21, 125.22, 105.89, 104.94, 68.44, 67.97, 67.48, 55.77, 55.28, 54.77, 48.28, 48.18, 48.07, 47.86, 47.65, 47.43, 47.22, 47.01, 36.42, 25.76, 25.12, 22.01, 12.91. (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.13. **FT-IR (neat, cm^{-1}):** 3431.09 2928.88, 2846.68, 2361.40 2339.29, 1576.61, 1450.75, 1398.27, 1275.76, 1184.90, 1098.21, 1184.90, 924.47, 891.33, 854.88, 805.64, 693.56, 618.24, 590.59. **HRMS electrospray (m/z): [M – NaBr$_2$]$^+$ calcd for $C_{64}H_{74}Br_2Na_2O_{11}P_2Pd_2S_2$:** 1381.2146, found 1381.2172.

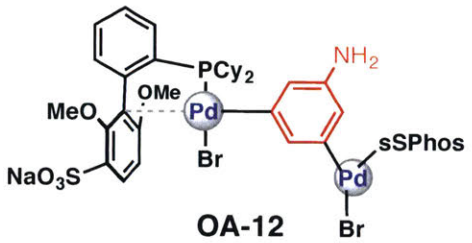


OA-11

Following the general procedure, a mixture containing 3,5-Dibromophenol (11.2 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-11** as a light red solid (55 mg, 86%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.14, 157.39, 138.12, 135.25, 135.16, 134.77, 134.70, 132.84, 132.39, 131.82, 131.56, 131.00, 128.35, 128.25, 126.69, 123.78, 106.86, 68.93, 62.15, 56.47,

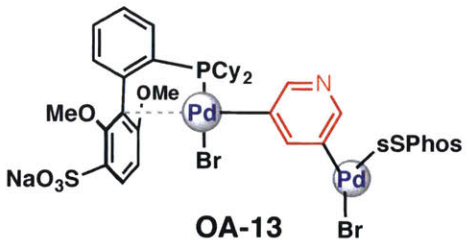
49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 48.45, 38.76, 38.35, 38.11, 37.69, 28.50, 27.77, 27.69, 27.61, 27.56, 27.48, 27.29, 27.09, 26.92, 26.63, 26.58, 23.48, 14.48 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 54.69. **FT-IR (neat, cm^{-1}):** 3439.91, 2929.07, 2851.92, 2360.02, 2339.23, 1572.55, 1495.89, 1449.24, 1398.44, 1275.67, 1180.53, 1097.678, 1049.39, 1017.65, 915.80, 890.18, 813.09, 758.97, 731.45, 693.45, 667.85, 639.68, 599.38. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{58}H_{72}Br_2Na_2O_{11}P_2Pd_2S_2$:** 1363.1273, found 1363.1279.



OA-12

Following the general procedure, a mixture containing 3,5-Dibromoaniline (11.2 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-12** as a brown solid (68 mg, 99%).

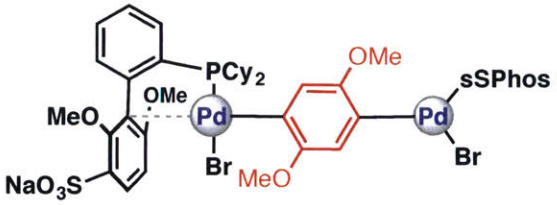
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 159.68, 155.91, 133.82, 133.26, 133.18, 131.32, 130.37, 129.52, 126.89, 126.79, 125.23, 105.41, 67.47, 60.69, 55.01, 48.27, 48.06, 47.84, 47.63, 47.42, 47.20, 46.99, 37.32, 36.79, 36.67, 36.14, 33.93, 27.04, 27.00, 26.14, 26.09, 26.01, 25.77, 25.72, 25.63, 25.46, 25.11, 22.01, 13.02 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 54.64. **FT-IR (neat, cm^{-1}):** 3438.02, 2929.17, 2850.03, 2360.11, 2344.27, 2156.91, 1576.54, 1450.55, 1400.19, 1275.83, 1226.92, 1179.84, 1098.40, 1051.05, 1017.72, 916.95, 889.51, 852.34, 815.45, 763.84, 693.63, 630.54, 575.96. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{58}H_{73}Br_2NNa_2O_{10}P_2Pd_2S_2$:** 1362.1432, found 1362.1460.



OA-13

Following the general procedure, a mixture containing 3,5-Dibromopyridine (10.5 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-13** as a brown solid (65 mg, 99%).

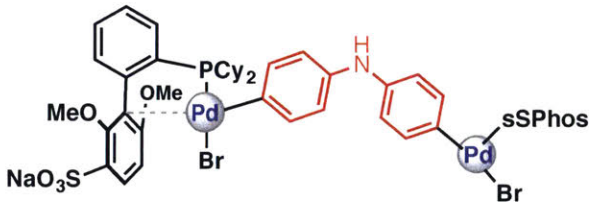
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.30, 160.89, 157.93, 157.52, 138.70, 135.53, 135.44, 134.73, 134.65, 132.83, 132.30, 132.05, 131.11, 128.55, 128.45, 126.87, 119.94, 107.78, 107.08, 72.31, 69.13, 62.34, 62.15, 57.47, 56.68, 49.94, 49.73, 49.51, 49.30, 49.09, 48.88, 48.66, 39.08, 38.42, 38.18, 37.52, 35.86, 35.57, 35.45, 31.55, 28.54, 27.71, 27.56, 27.47, 27.36, 26.77, 26.74, 26.58, 25.28, 23.65, 14.67 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.54. **FT-IR (neat, cm^{-1}):** 3431.02, 2928.78, 2849.99, 2361.41, 2339.26, 1575.57, 1514.39, 1450.71, 1398.62, 1275.14, 1179.98, 1098.18, 1049.74, 1017.55, 916.89, 808.43, 748.89, 693.58, 667.83, 656.40, 629.29, 623.44, 618.19, 613.58, 608.38, 585.53. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{57}H_{71}Br_2NNa_2O_{10}P_2Pd_2S_2$:** 1348.1276, found 1348.1303.



OA-14

Following the general procedure, a mixture containing 1,4-Dibromo-2,5-dimethoxybenzene (13.1 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-14** as a brown solid (56 mg, 84%).

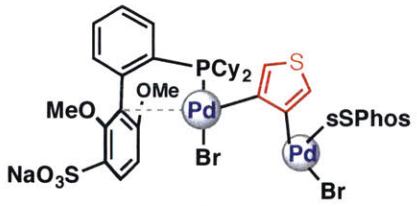
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.28, 157.50, 155.77, 151.98, 138.70, 138.63, 135.53, 135.43, 134.70, 133.38, 132.79, 132.23, 132.07, 131.40, 131.08, 128.56, 128.46, 126.84, 120.35, 115.84, 114.53, 113.05, 107.07, 106.50, 69.12, 62.33, 57.59, 57.52, 56.70, 56.66, 56.42, 56.15, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.07, 38.41, 38.11, 37.46, 35.84, 35.56, 35.44, 27.84, 27.77, 27.65, 27.45, 27.35, 27.13, 26.76, 26.73, 26.56, 25.27, 23.64, 14.67, -1.17 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.47. **FT-IR (neat, cm^{-1}):** 3438.09, 2928.62, 2850.33, 2362.65, 2342.57, 1572.78, 1450.63, 1398.55, 1274.73, 1179.23, 1097.97, 1050.91, 1018.53, 917.05, 889.44, 845.20, 805.62, 758.86, 693.43, 667.84, 656.47, 630.17, 623.71, 618.06, 580.82. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{60}H_{76}Br_2Na_2O_{12}P_2Pd_2S_2$:** 1407.1536, found 1407.1493.



OA-15

Following the general procedure, a mixture containing Bis(4-bromophenyl)amine (14.5 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in tetrahydrofuran (1.5 mL) for 1 h. General work-up afforded **OA-15** as a brown solid (61 mg, 90%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.58, 161.51, 157.74, 145.69, 138.81, 138.73, 135.72, 135.62, 135.00, 134.92, 133.08, 132.54, 132.43, 132.27, 131.71, 131.34, 130.86, 130.46, 130.19, 129.31, 129.19, 128.77, 128.67, 127.07, 127.04, 122.28, 121.54, 120.15, 118.77, 107.28, 107.23, 69.34, 62.55, 61.93, 56.90, 50.15, 49.94, 49.72, 49.51, 49.30, 49.08, 48.87, 39.24, 38.59, 38.46, 37.80, 36.05, 35.79, 28.07, 27.99, 27.94, 27.87, 27.75, 27.68, 27.62, 27.55, 27.34, 26.99, 26.95, 26.78, 23.88, 14.90, -0.62, -1.26. **^{31}P NMR (121 MHz, MeOD)** d 51.29. **FT-IR (neat, cm^{-1}):** 3433.28, 2929.21, 2849.91, 2361.32, 2339.25, 1576.44, 1549.56, 1527.65, 1449.33, 1398.11, 1275.60, 1180.69, 1098.13, 1049.70, 1017.93, 915.75, 888.02, 848.40, 758.90, 693.55, 559.72. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{64}H_{77}Br_2NNa_2O_{10}P_2Pd_2S_2$:** 1438.1717, found 1438.1705.

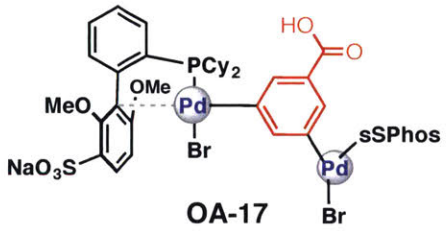


OA-16

Following the general procedure, a mixture containing 3,4-Dibromothiophene (5 μL, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-16** as a brown solid (57 mg, 89%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d ^{13}C NMR (101 MHz, MeOD) δ 161.67, 161.44, 161.28, 157.49, 135.54, 135.44, 134.67, 134.59, 133.84, 132.78, 132.06, 132.04, 131.39, 131.08, 129.79, 128.56, 128.45, 126.85, 124.69, 107.07, 104.95, 69.12, 63.94, 62.70, 62.33, 57.23, 56.69, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.08, 38.43, 38.10, 37.44, 32.32, 29.30, 29.18, 28.53, 27.84,

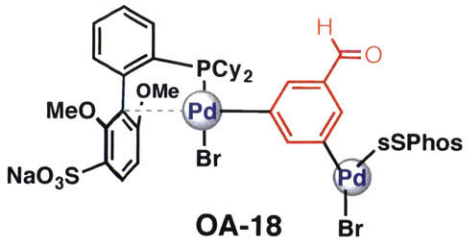
27.78, 27.71, 27.65, 27.46, 27.13, 26.77, 26.73, 2.91, 2.50, 2.45, 0.50 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 52.49. **FT-IR (neat, cm^{-1}):** 3391.02, 2928.68, 2852.01, 2360.05, 2339.30, 1576.87, 1450.45, 1399.99, 1274.77, 1226.06, 1179.26, 1098.10, 1051.00, 1017.61, 917.42, 889.34, 852.10, 763.72, 693.50, 667.84, 590.39. **HRMS electrospray (m/z): [M – Na]$^+$ calcd for $C_{56}H_{70}Br_2Na_2O_{10}P_2Pd_2S_3$:** 1456.9946, found 1456.9976.



OA-17

Following the general procedure, a mixture containing 3,5-Dibromobenzoic acid (12.3 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-17** as a light brown solid (72 mg, 99%).

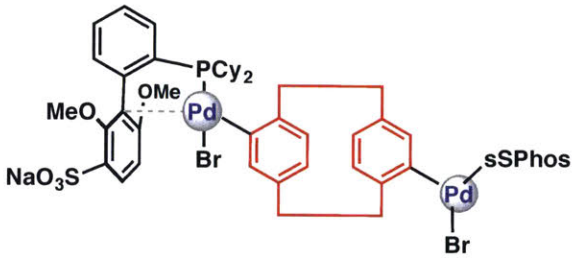
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.49, 157.72, 143.63, 138.87, 138.80, 136.63, 135.72, 135.62, 134.94, 134.86, 133.51, 133.04, 132.86, 132.51, 132.24, 132.22, 131.68, 131.31, 130.11, 128.76, 128.65, 128.20, 127.06, 127.04, 123.76, 121.57, 108.72, 107.27, 69.33, 63.76, 62.54, 57.67, 57.27, 57.12, 56.88, 56.58, 39.27, 38.62, 38.40, 37.74, 36.06, 35.77, 29.43, 28.94, 28.78, 28.53, 28.41, 28.05, 27.99, 27.93, 27.85, 27.76, 27.68, 27.56, 27.40, 27.34, 26.97, 26.94, 23.85, 14.89, 3.26, -0.62. **^{31}P NMR (121 MHz, MeOD)** d 31.99. **FT-IR (neat, cm^{-1}):** 3436.31, 2928.88, 2846.68, 2361.40, 2339.29, 1576.61, 1450.75, 1398.27, 1275.76, 1184.90, 1098.21, 1049.48, 1018.42, 924.47, 891.33, 854.88, 805.64, 749.56, 693.56, 580.79. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{59}H_{72}Br_2Na_2O_{12}P_2Pd_2S_2$:** 1391.1222, found 1391.1283.



OA-18

Following the general procedure, a mixture containing 3,5-Dibromobenzaldehyde (11.6 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-18** as a light brown solid (66 mg, 99%).

^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d 161.29, 157.50, 135.52, 135.42, 134.71, 132.77, 132.07, 131.10, 128.57, 128.47, 126.83, 107.07, 69.12, 62.34, 57.51, 56.69, 39.06, 38.41, 38.18, 37.52, 35.84, 35.44, 29.23, 27.84, 27.70, 27.66, 27.46, 27.35, 27.13, 26.76, 23.64, 14.68, -1.12 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 54.11. **FT-IR (neat, cm^{-1}):** 3430.23, 2931.05, 2852.56, 2361.33, 2342.60, 1573.87, 1450.86, 1398.46, 1275.65, 1179.90, 1098.08, 1050.93, 1018.24, 917.08, 845.59, 805.57, 758.75, 693.59, 667.86, 657.88, 629.64, 623.64, 595.12. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{59}H_{72}Br_2Na_2O_{11}P_2Pd_2S_2$:** 1375.1273, found 1375.1321.



OA-19

Following the general procedure, a mixture containing 5,11-dibromotricyclo[8.2.2.2~4,7~]hexadeca-1(12),4,6,10,13,15-hexaene (16.1 mg, 0.044 mmol), sSPhos (50 mg, 0.01 mmol), and (1,5-COD)Pd($CH_2TMS)_2$ (38 mg, 0.01 mmol) was stirred at rt in THF (1.5 mL) for 1 h. General work-up afforded **OA-19** as a yellow solid (70 mg, 99%).

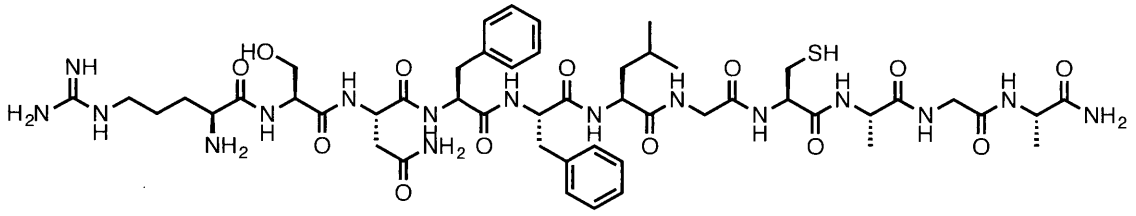
^{1}H NMR (400 MHz, MeOD) Complicated spectrum; please see the attached ^{1}H NMR spectrum. **^{13}C NMR (101 MHz, MeOD)** d ^{13}C NMR (101 MHz, MeOD) δ 161.28, 157.50, 138.66, 137.19, 135.54, 135.44, 134.67, 134.60, 132.79, 132.05, 131.08, 128.57, 128.47, 126.84, 107.07, 69.13, 62.34, 57.21, 56.69, 49.94, 49.73, 49.51, 49.30, 49.09, 48.87, 48.66, 39.09, 38.44, 38.11, 37.45, 36.89, 27.85, 27.79, 27.67, 27.57, 27.46, 27.38, 27.14, 26.77, 23.65, 14.68 (observed complexity is due to *C–P* coupling). **^{31}P NMR (121 MHz, MeOD)** d 51.64. **FT-IR (neat, cm^{-1}):** 3300.18, 2918.84, 2851.36, 2358.93, 1684.54, 1577.43, 1525.12, 1450.92, 1274.75, 1226.24, 1185.07, 1098.68, 1050.30, 1017.95, 914.53, 852.15, 818.16, 759.53, 707.02, 693.31, 667.99, 596.57. **HRMS electrospray (m/z): [M – Na_2Br]$^-$ calcd for $C_{68}H_{82}Br_2Na_2O_{10}P_2Pd_2S_2$:** 1477.2109, found 1477.2068.

Linear Peptide Synthesis

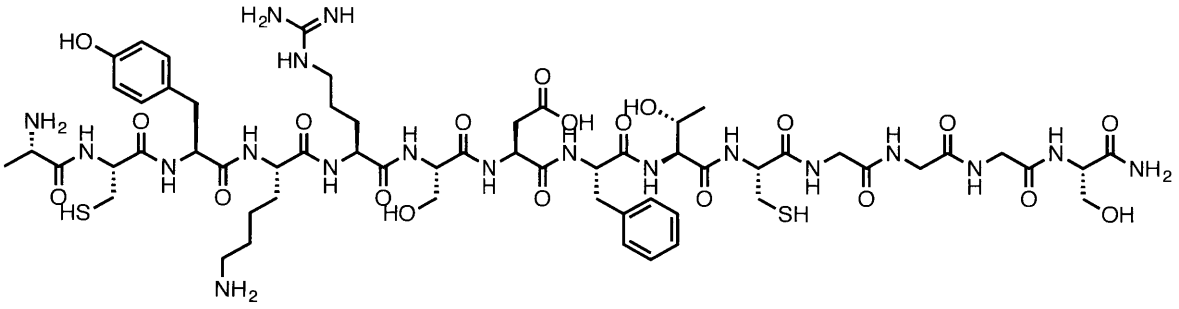
General Linear Peptide Synthesis Procedure:

All peptides were synthesized on a 0.2 mmol scale using manual Fmoc-SPPS chemistry under flow with a 3 min cycle for each amino acid.[2] Specifically, all reagents and solvents were delivered to a stainless steel reactor containing resins at a constant flow rate using HPLC pump; the temperature of the reactor was maintained at 60 °C during the synthesis using a water bath. The procedure for each amino acid coupling cycle included: 1) a 30 s coupling with 1 mmol of the corresponding Fmoc-protected amino acid, 1 mmol HBTU, and 500 μL of diisopropyl ethyl amine (DIPEA) in 2.5 mL of DMF at a flow rate of 6 mL/min (note that for the coupling of

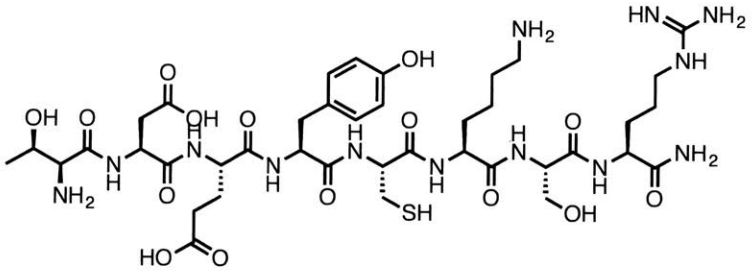
cysteine and tryptophan, 190 μL of DIPEA was used to prevent racemization); 2) 1 min wash with DMF at a flow rate of 20 mL/min; 3) 20 s deprotection with 50% (v/v) piperidine in DMF at a flow rate of 20 mL/min; and 4) 1 min wash with DMF at a flow rate of 20 mL/min. After completion of the stepwise SPPS, the resin was washed thoroughly with CH_2Cl_2 and dried under vacuum. The peptide was simultaneously cleaved from the resin and deprotected on the side-chains by treatment with 2.5% (v/v) water, 2.5% (v/v) 1,2-ethanedithiol (EDT), and 2.5% (v/v) triisoproprylsilane in neat trifluoroacetic acid (TFA) for 7 min at 60 °C. The resulting solution was then triturated and washed with cold diethyl ether (3x). The obtained solid was dissolved in 50% H2O : 50% acetonitrile containing 0.1% TFA and lyophilized. The following peptides were synthesized following this procedure:



P1
Arg Ser Asn Phe Phe Leu Gly Cys Ala Gly Ala
Exact Mass: 1140.55



P2
Ala Cys Tyr Lys Arg Ser Asp Phe Thr Cys Gly Gly Gly Ser
Exact Mass: 1449.61



P3
Thr Asp Glu Tyr Cys Lys Ser Arg
Exact Mass: 999.44

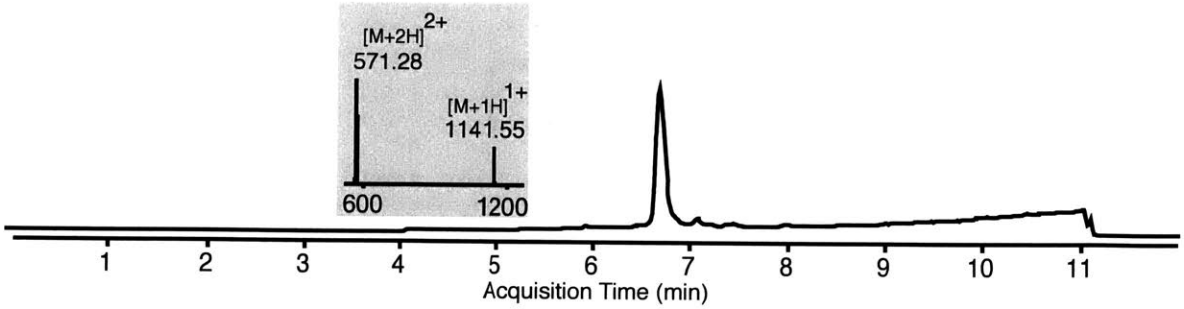
Peptide Purification

Table S1. Sequences and masses of peptides synthesized by manual fast flow SPPS.

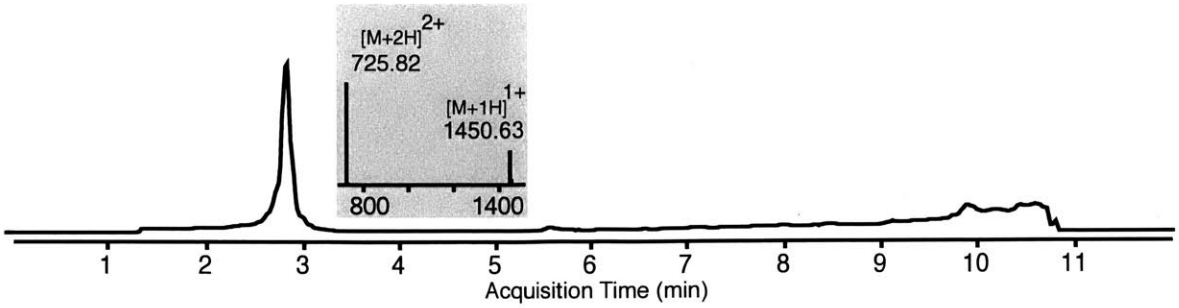
Peptide	Sequence[a]	Calculated mass	Observed Mass $[M+H]^+$
P1	NH_2-RSNFFLGCAGA-C(O)NH_2	1140.55	1141.55
P2	NH_2-ACYKRSDFTCGGGS-C(O)NH_2	1449.61	1450.63
P3	NH_2-TDGYCKSR-C(O)NH_2	999.44	1000.42

[a] Cysteine residues are highlighted in red.

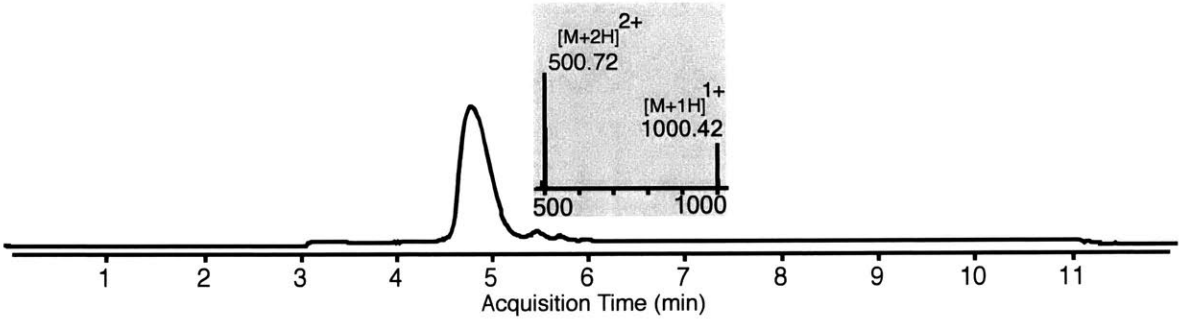
P1:



P2:

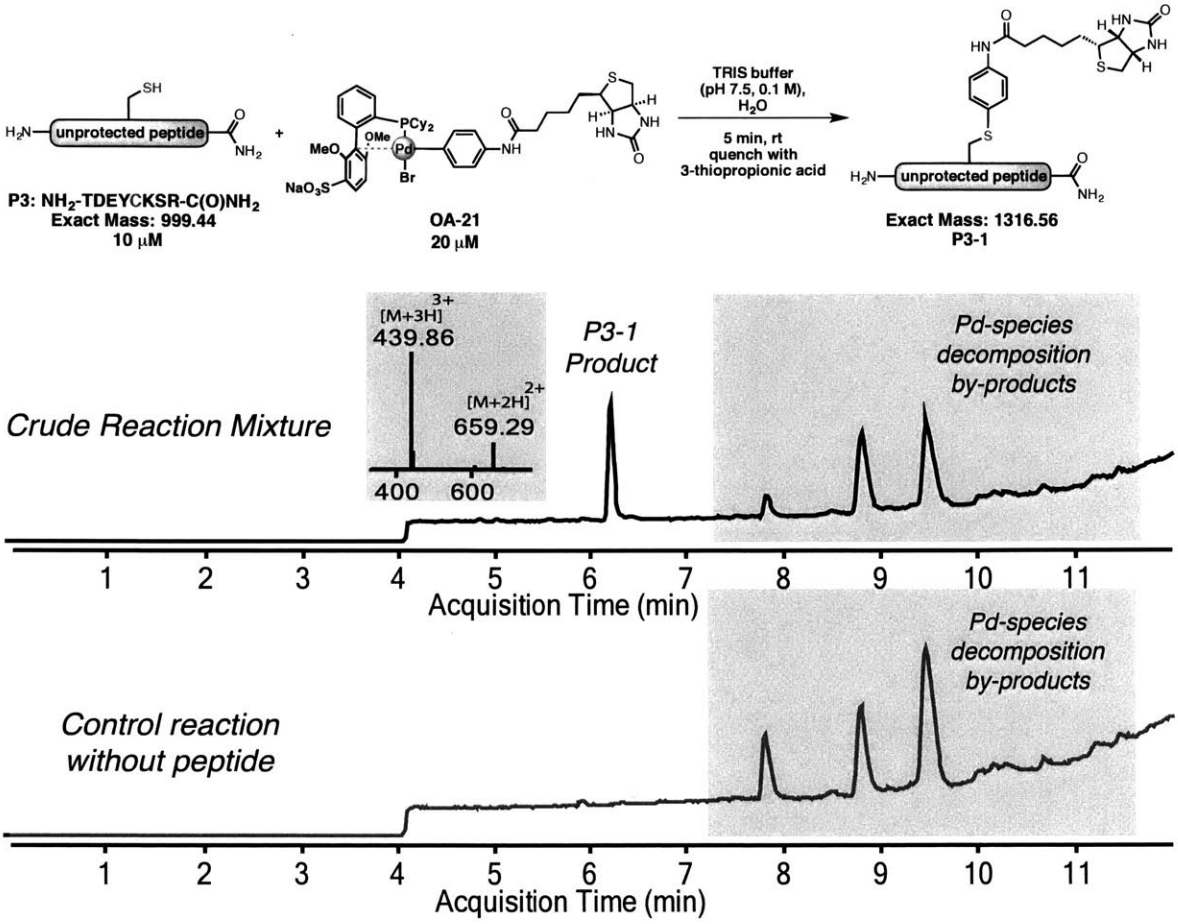


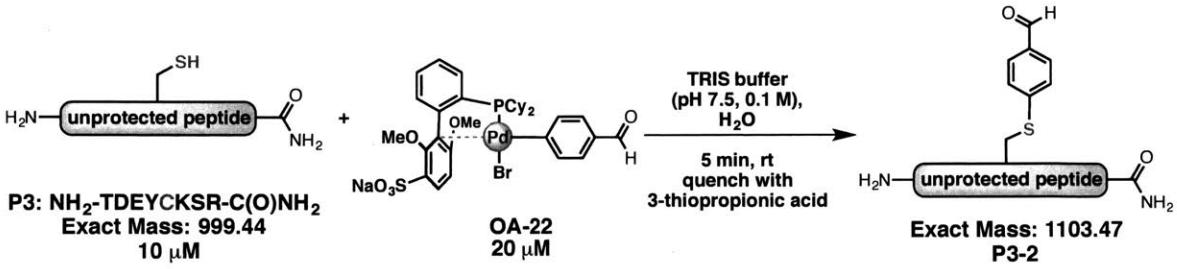
P3:

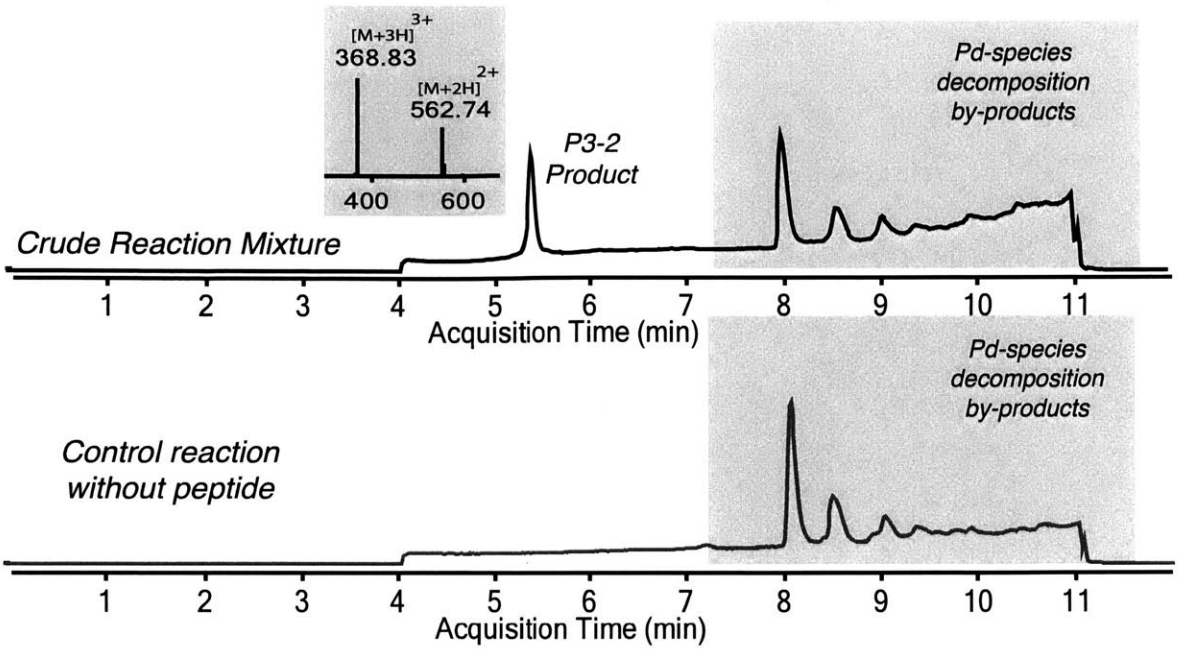


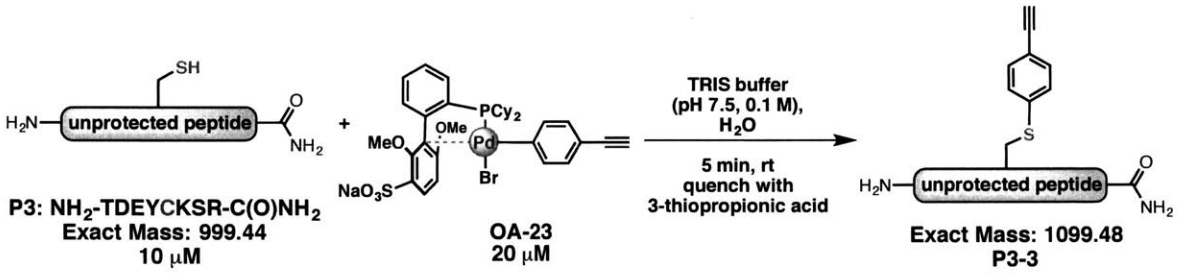
General Bioconjugation Procedure

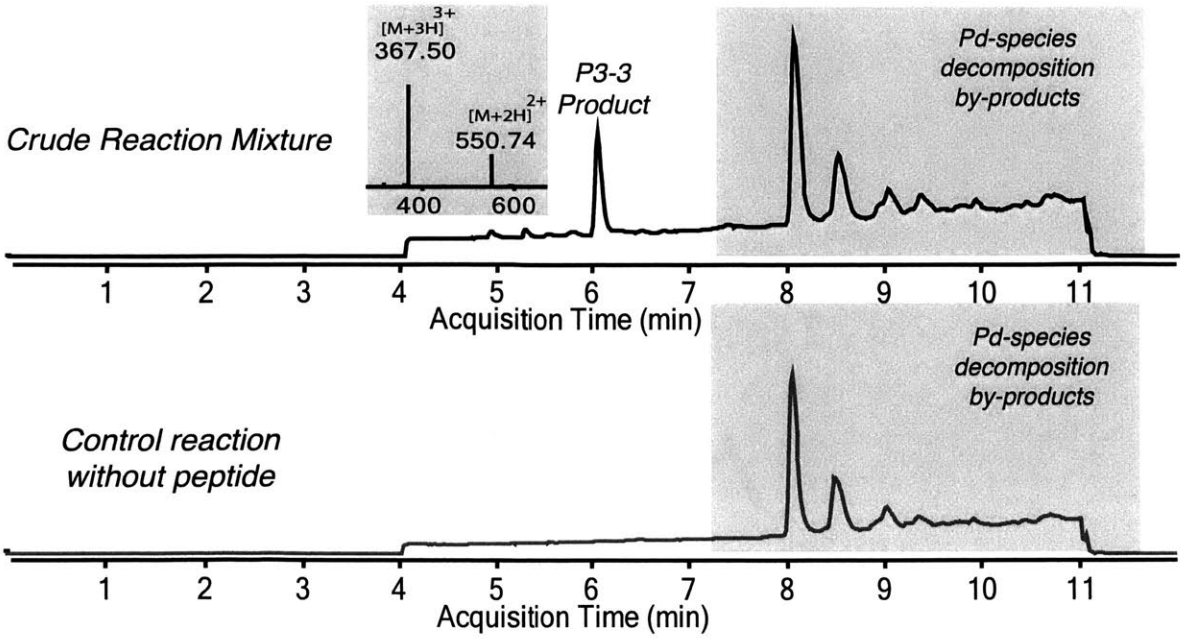
A solution of the Pd reagent (**OA-Y**) reagent in water (40 mM) was added to a solution of peptide **P3** (20 mM) in Tris buffer (0.1 M, pH 7.5). Note: if the palladium reagent was not readily soluble in H_2O, the slurry was sonicated for 10 s to facilitate this process and become fully solubilized. Final conditions: [Pd] = 20 mM; [peptide] = 10 mM; After 5 min at rt, 3-mercaptopropionic acid (3 equiv to the palladium complex, solution in 6 mL of H_2O) was added to the reaction mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min and subsequently characterized by LC-MS.

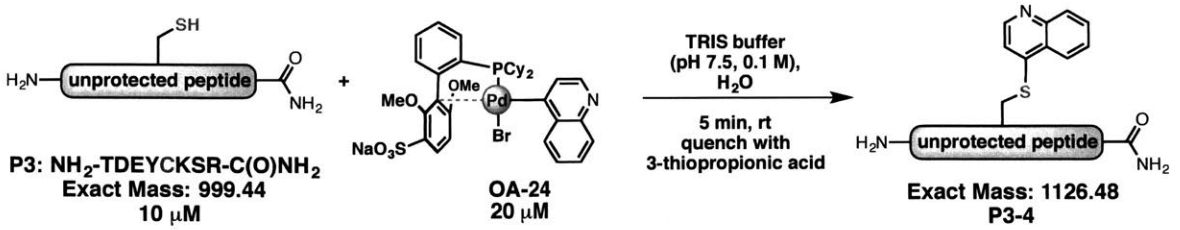

unprotected peptide
P3: NH2-TDEYCKSR-C(O)NH2
Exact Mass: 999.44
10 μM
OA-21
20 μM
TRIS buffer
(pH 7.5, 0.1 M),
H2O
5 min, rt
quench with
3-thiopropionic acid
Exact Mass: 1316.56
P3-1
Crude Reaction Mixture
[M+3H]3+
439.86
[M+2H]2+
659.29
400
600
P3-1
Product
Pd-species
decomposition
by-products
Acquisition Time (min)
Control reaction
without peptide
Pd-species
decomposition
by-products
Acquisition Time (min)

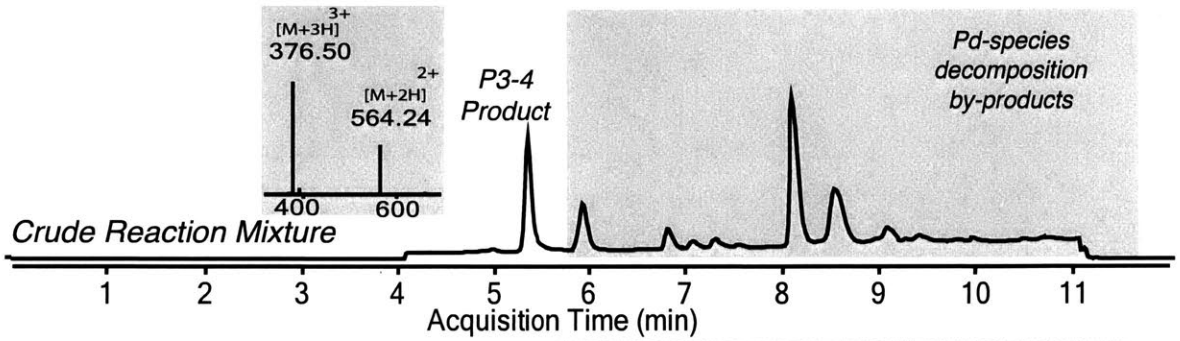

unprotected peptide
P3: NH2-TDEYCKSR-C(O)NH2
Exact Mass: 999.44
10 μM
OA-22
20 μM
TRIS buffer
(pH 7.5, 0.1 M),
H2O
5 min, rt
quench with
3-thiopropionic acid
Exact Mass: 1103.47
P3-2

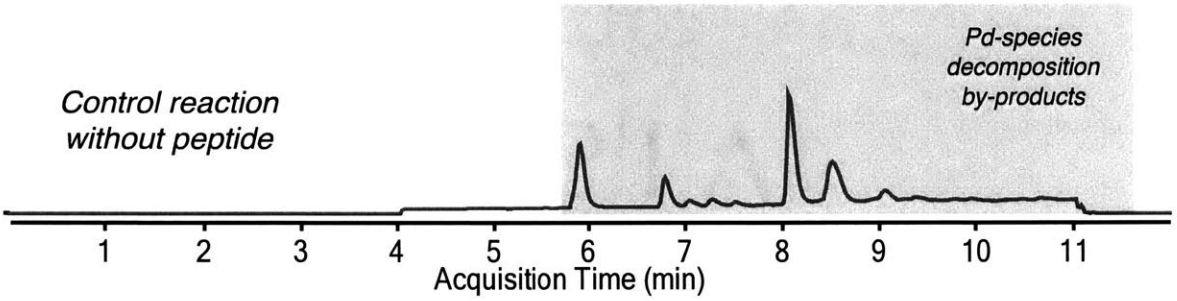

[M+3H]3+
368.83
[M+2H]2+
562.74
400
600
P3-2
Product
Pd-species
decomposition
by-products
Crude Reaction Mixture
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)
Pd-species
decomposition
by-products
Control reaction
without peptide
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)

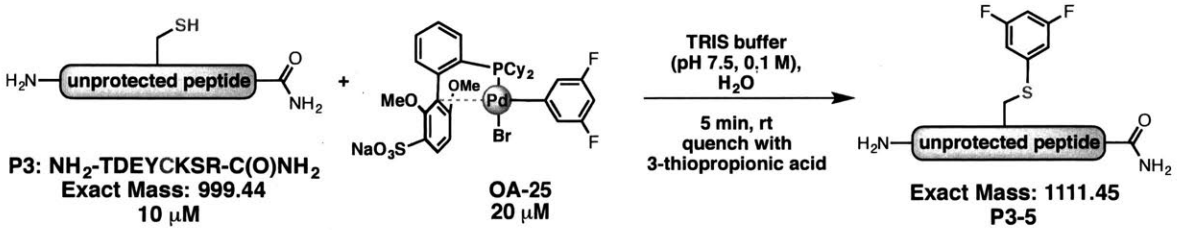

SH
H2N
unprotected peptide
O
NH2
+
PCy2
OMe
MeO
Pd
Br
NaO3S
TRIS buffer
(pH 7.5, 0.1 M),
H2O
5 min, rt
quench with
3-thiopropionic acid
S
H2N
unprotected peptide
O
NH2
P3: NH2-TDEYCKSR-C(O)NH2
Exact Mass: 999.44
10 μM
OA-23
20 μM
Exact Mass: 1099.48
P3-3

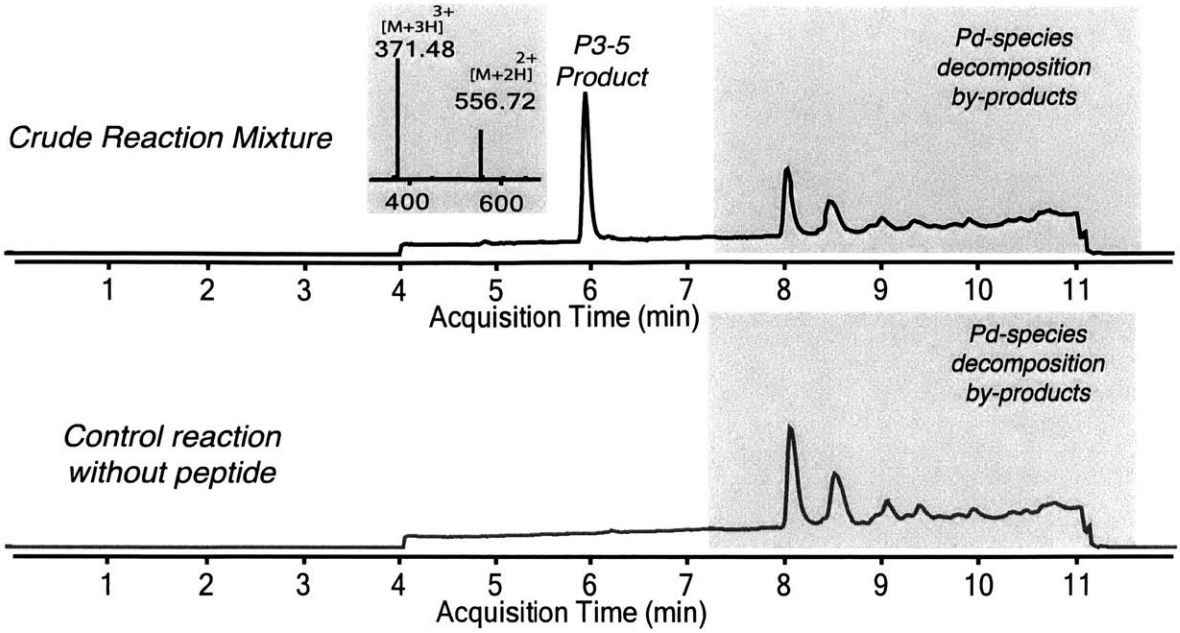

[M+3H]3+
367.50
[M+2H]2+
550.74
400
600
P3-3
Product
Pd-species
decomposition
by-products
Crude Reaction Mixture
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)
Pd-species
decomposition
by-products
Control reaction
without peptide
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)


SH
H2N
unprotected peptide
O
NH2
+
PCy2
OMe
MeO
Pd
Br
NaO3S
N
TRIS buffer
(pH 7.5, 0.1 M),
H2O
5 min, rt
quench with
3-thiopropionic acid
S
P3: NH2-TDEYCKSR-C(O)NH2
Exact Mass: 999.44
10 μM
OA-24
20 μM
Exact Mass: 1126.48
P3-4

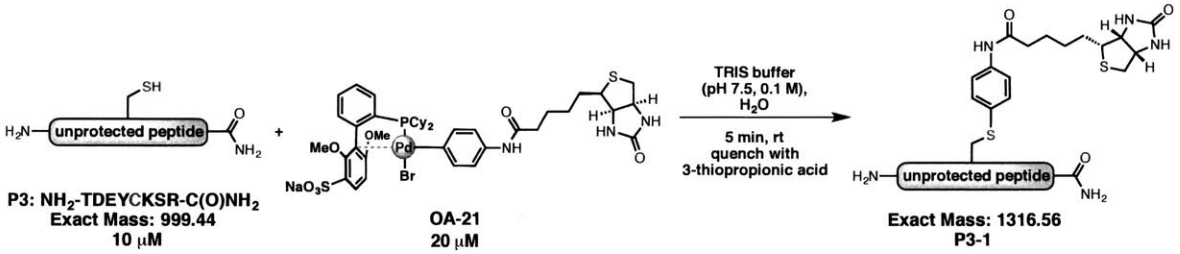

3+
[M+3H]
376.50
2+
[M+2H]
564.24
400
600
P3-4
Product
Pd-species
decomposition
by-products
Crude Reaction Mixture
1
2
3
4
5
6
7
8
9
10
11
Acquisition Time (min)


Pd-species
decomposition
by-products
Control reaction
without peptide
1
2
3
4
5
6
7
8
9
10
11
Acquisition Time (min)

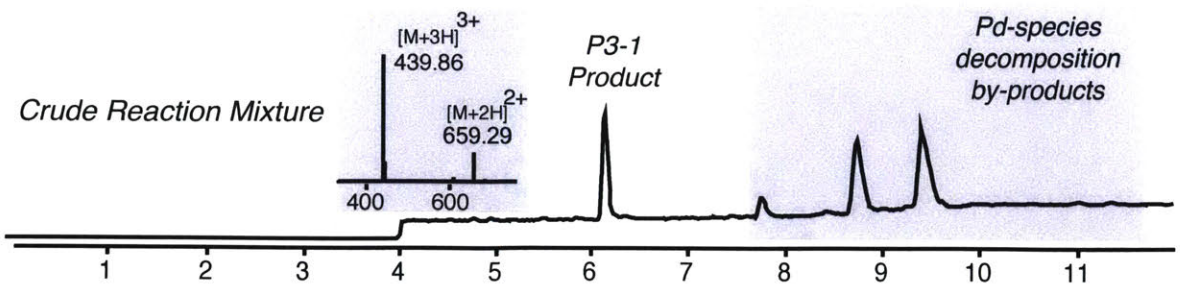

SH
H2N
unprotected peptide
O
NH2
+
PCy2
OMe
MeO
Pd
Br
NaO3S
F
F
F
TRIS buffer
(pH 7.5, 0.1 M),
H2O
5 min, rt
quench with
3-thiopropionic acid
S
P3: NH2-TDEYCKSR-C(O)NH2
Exact Mass: 999.44
10 μM
OA-25
20 μM
Exact Mass: 1111.45
P3-5

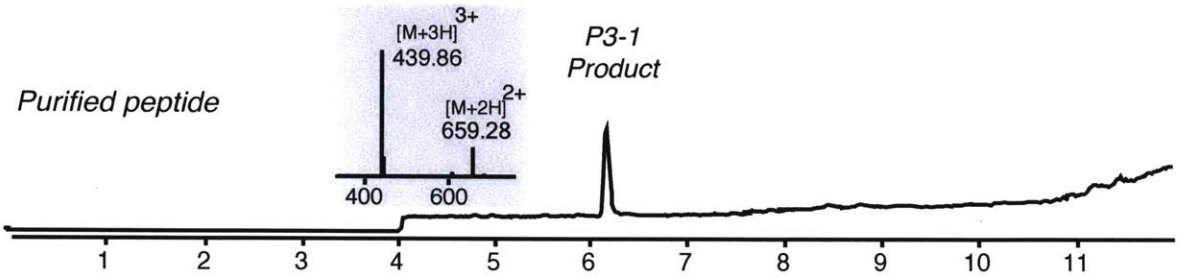


Large Scale Bioconjugation:

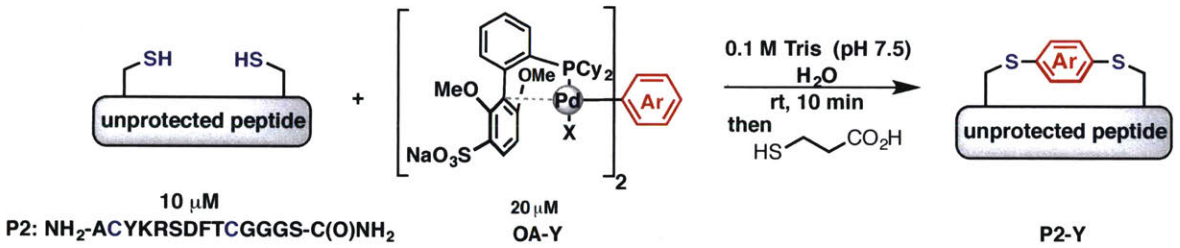


Into a 250 mL round bottomed flask equipped with a magnetic stir bar, 8.0 mg of peptide **P3** was dissolved in water (20 mM) and Tris buffer (0.1 M, pH 7.5). A solution of the Pd reagent (**OA-21**) reagent in water (40 mM) was added to the peptide solution and allowed to stir. Final conditions: [**OA-21**] = 20 mM; [**P3**] = 10 mM; After 5 min at rt, 3-mercaptopropionic acid (3 equiv to the palladium complex, solution in 1 mL of H_2O) was added to the reaction mixture to quench the remaining palladium species. The reaction mixture was allowed to stand for 5 min and subsequently purified via mass-directed preparative HP-LC yielding 8.54 mg of pure biotinylated peptide (**P3-1**, >99% conversion, 81% isolated yield).





General Macrocyclization Procedure



A solution of the Pd reagent (**OA-Y**) reagent in water (40 mM) was added to a solution of peptide (20 mM) in Tris buffer (0.1 M, pH 7.5). Note: if the palladium reagent was not readily soluble in H_2O, the slurry was sonicated for 10 s to facilitate this process. Final reaction concentrations: [Pd] = 20 mM; [peptide] = 10 mM; After 10 min stirring at rt, 3-mercaptopropionic acid (3 equiv to the palladium complex, solution in 6 mL of H_2O) was added to the reaction mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min. The crude peptide was purified using preparative HPLC as described above or immediately analysed by LC-MS (below).

Figure S-1: Macrocyclization P2-7

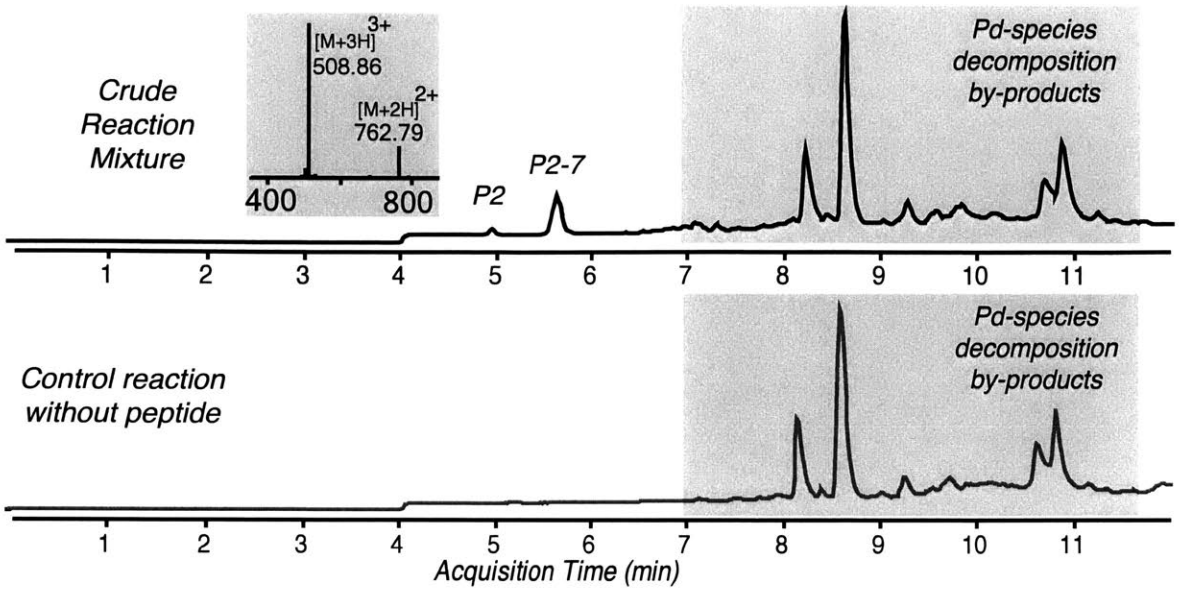


Figure S-2: Macrocyclization P2-8

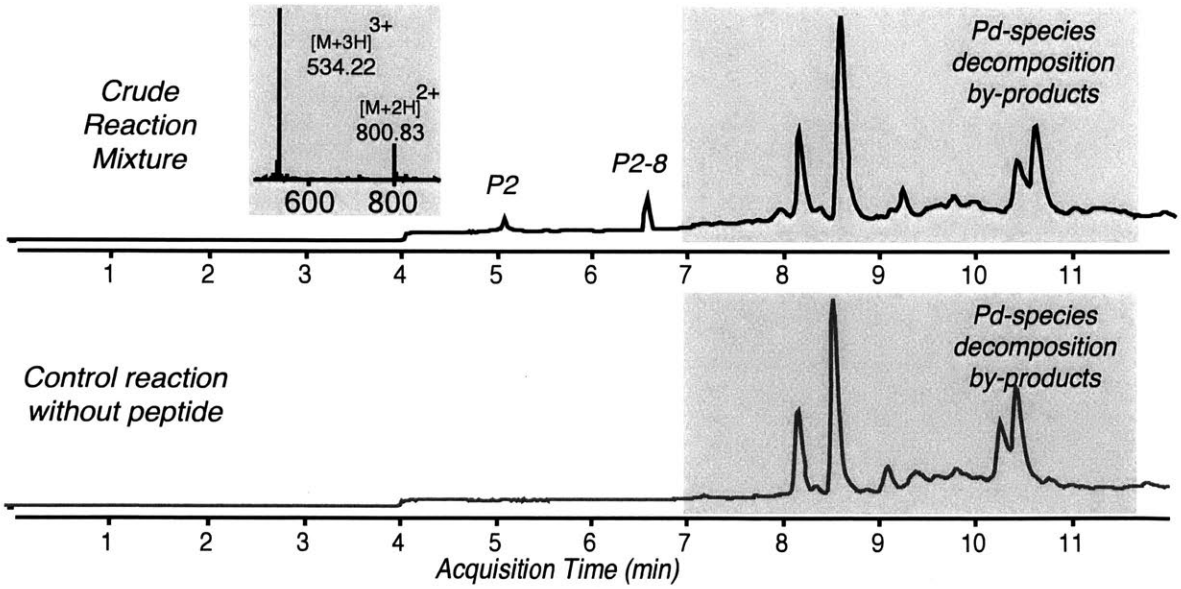


Figure S-3: Macrocyclization P2-9

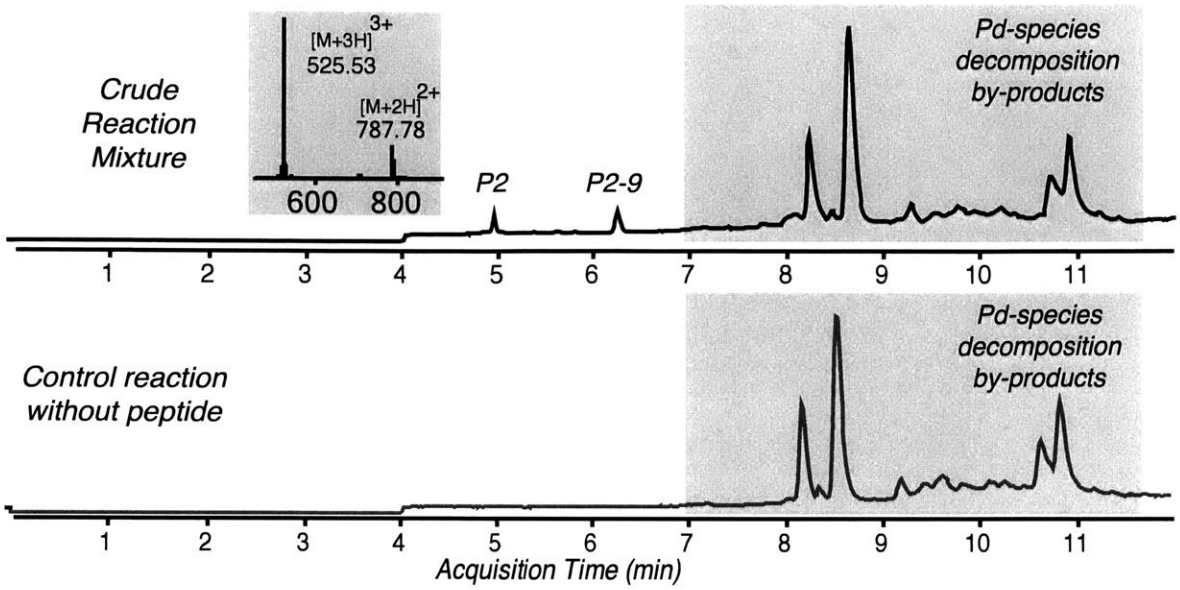


Figure S-4: Macrocyclization P2-9 with 15% MeCN

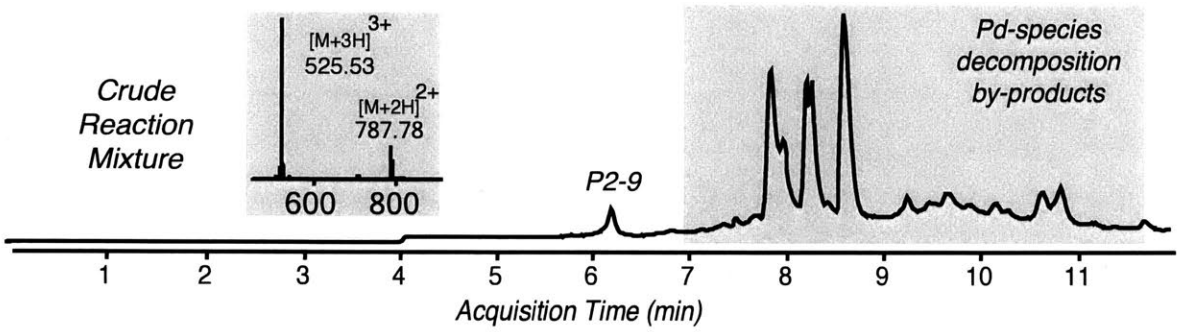


Figure S-5: Macrocyclization P2-10 with 15% MeCN

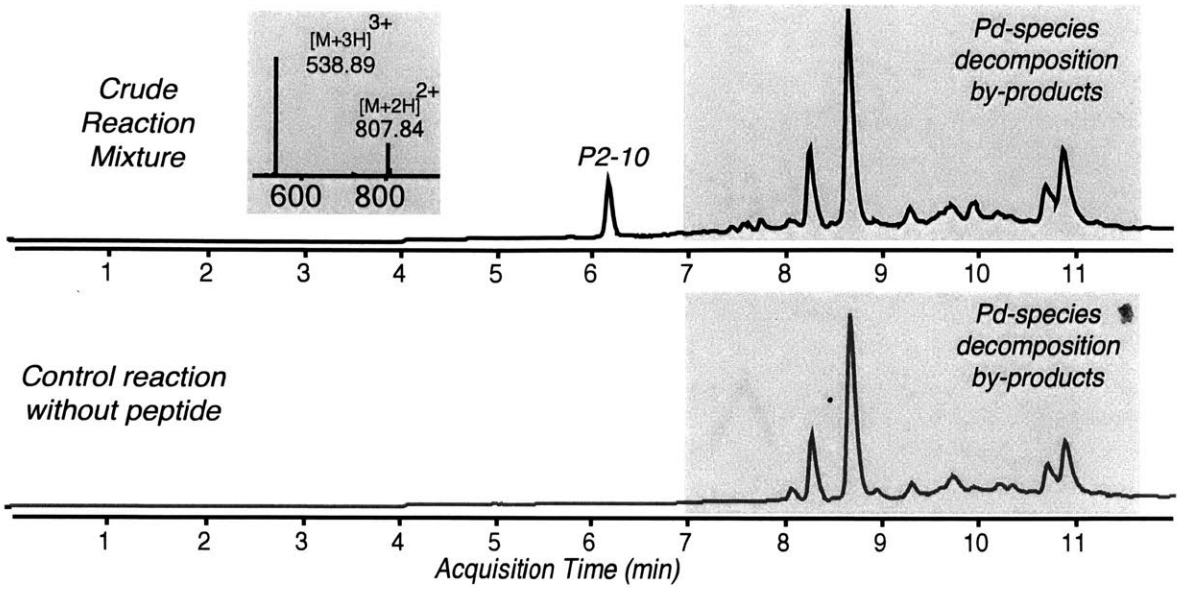


Figure S-6: Macrocyclization P2-11

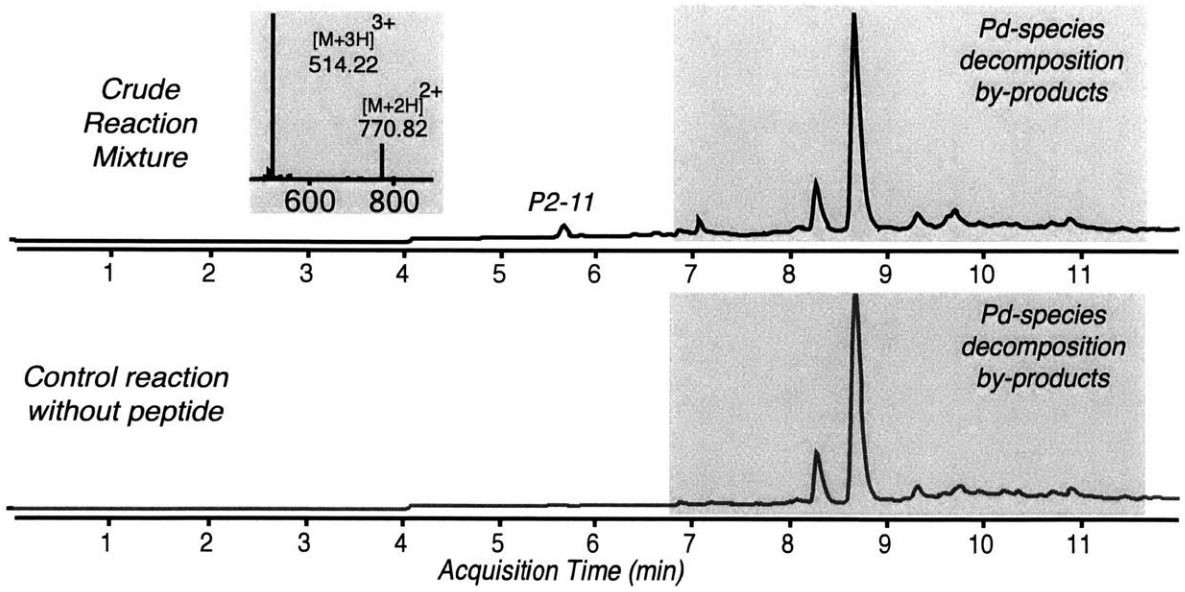


Figure S-7: Macrocyclization P2-12

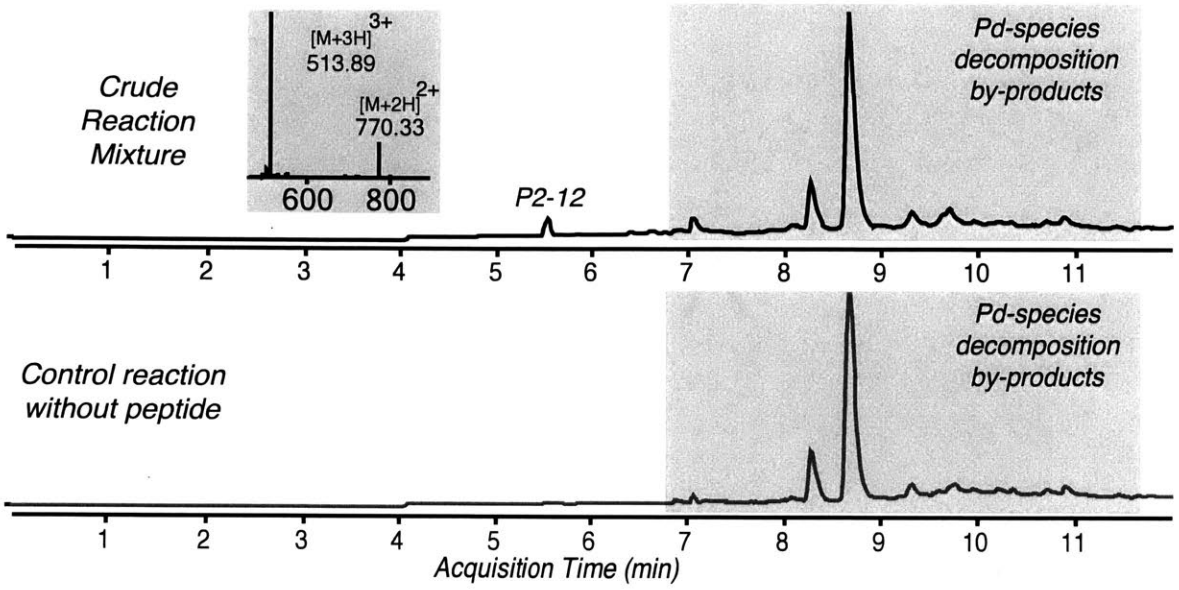


Figure S-8: Macrocyclization P2-13

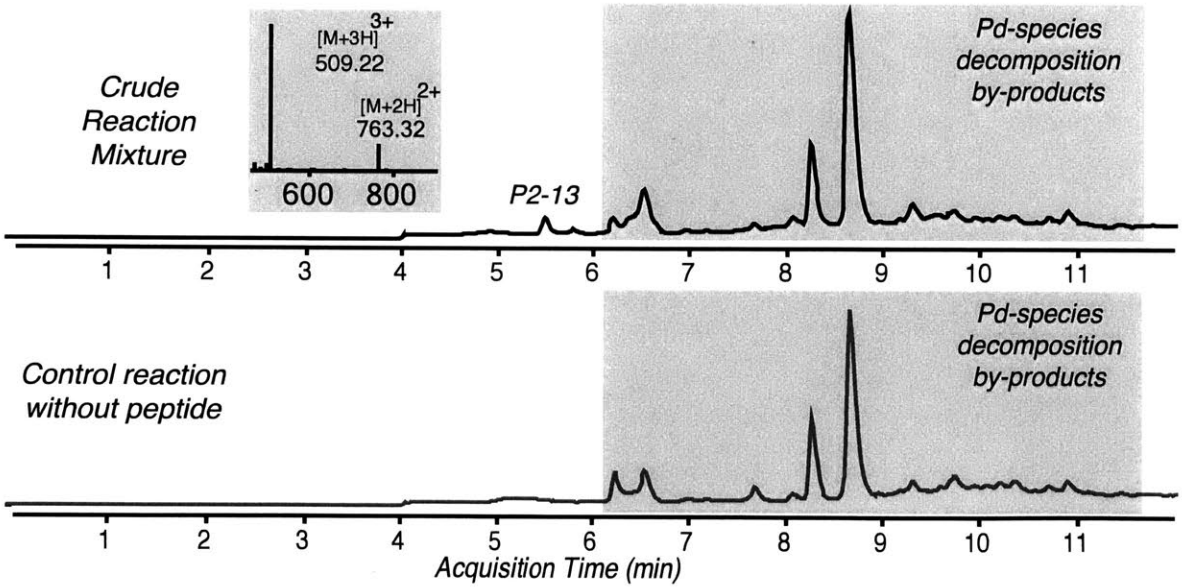


Figure S-9: Macrocyclization P2-14

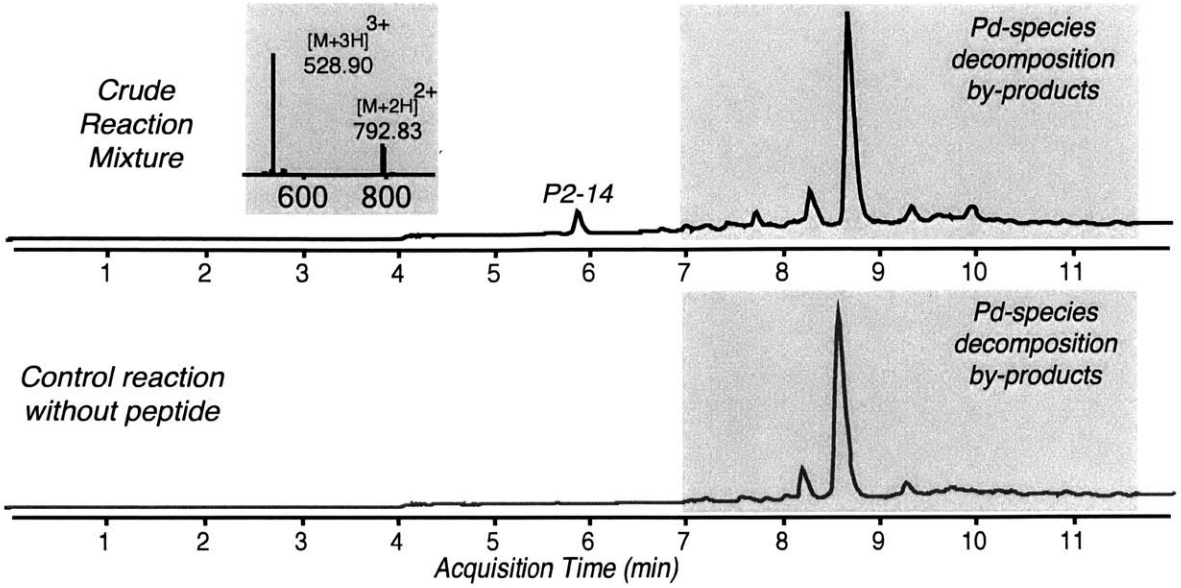


Figure S-10: Macrocyclization P2-15

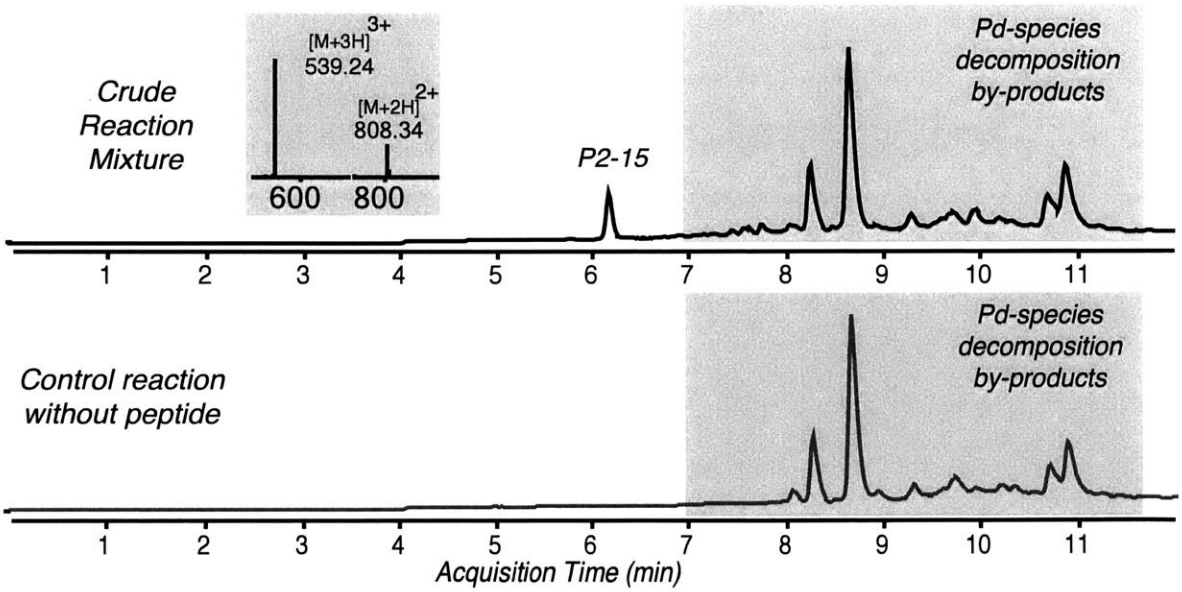


Figure S-11: Macrocyclization P2-16

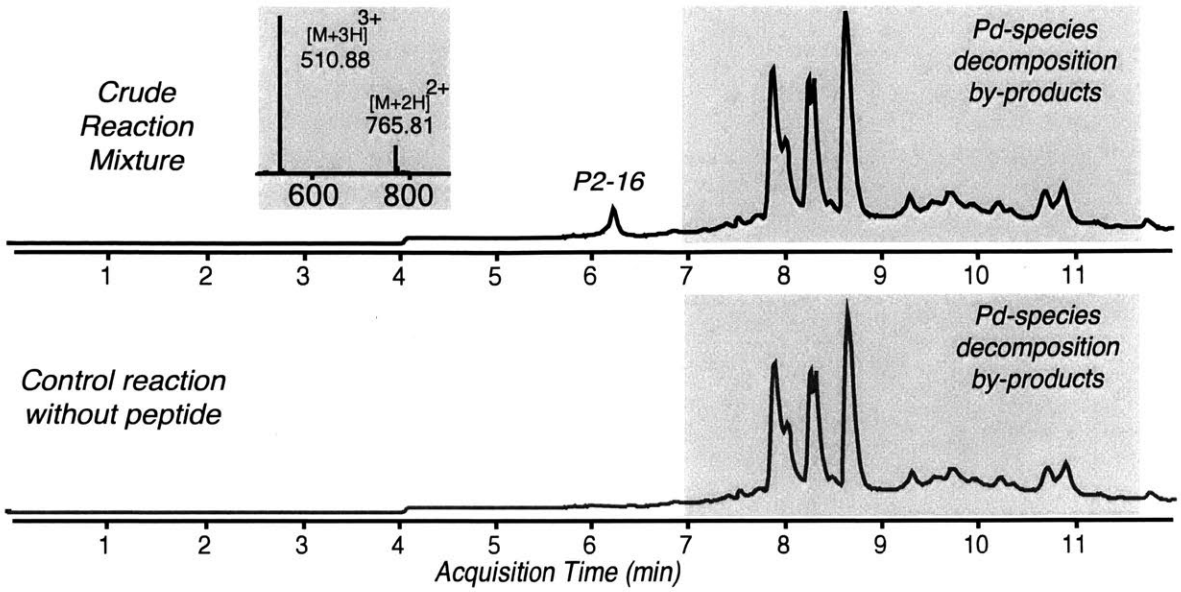


Figure S-12: Macrocyclization P2-17

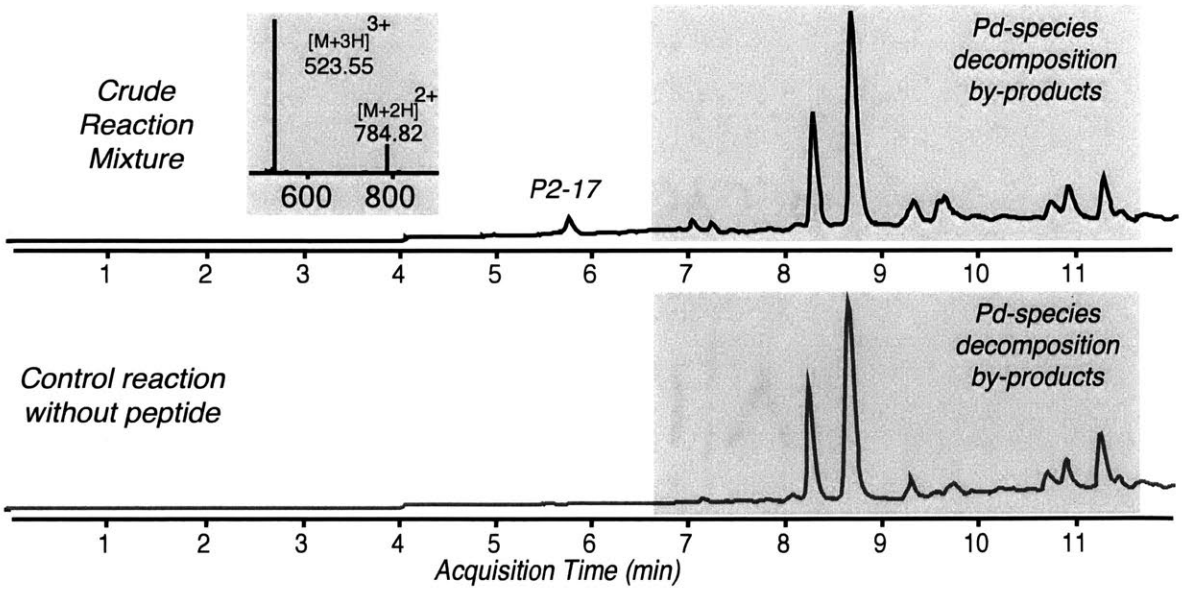


Figure S-13: Macrocyclization P2-18

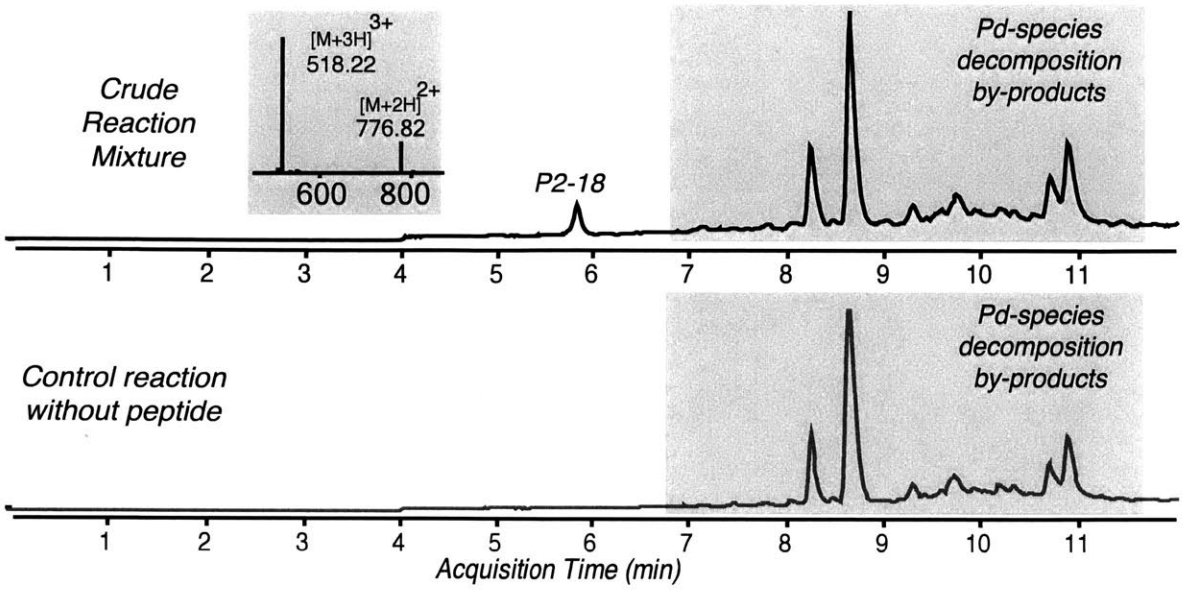
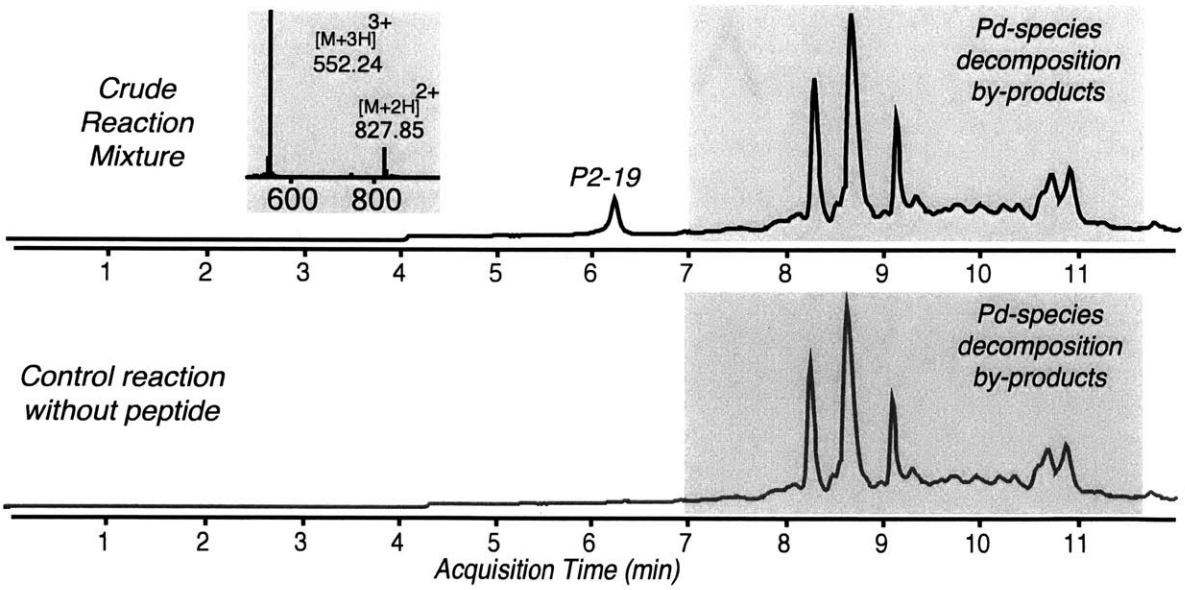


Figure S-14: Macrocyclization P2-19 with 15% MeCN



Protein Expression and Purification

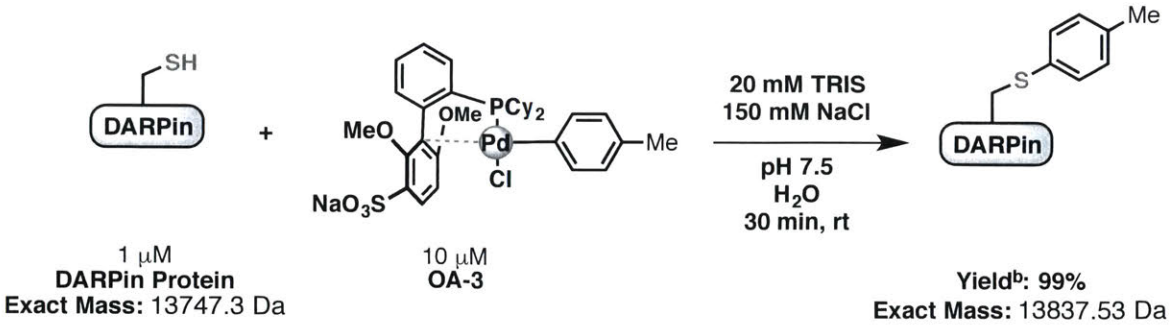
pET-SUMO-DARPin plasmids were constructed as reported previously.[3] Cysteine mutations were introduced by site-directed mutagenesis using QuickChange Lightning Single Site-directed

Mutagenesis Kit (Agilent) following manufacturer's instructions. Sequences of generated protein constructs are summarized in Table S6.

E. coli BL21(DE3) cells transformed with pET-SUMO-Protein plasmid were grown in 1 L of LB medium containing kanamycin (30 μg/mL) at 37 °C until OD600 = 0.6. Then, expression was induced by the addition of 0.5 mM IPTG overnight at 30 °C. After harvesting the cells by centrifugation (6,000 rpm for 10 min), the cell pellet was lysed by sonication in 25 mL of 50 mM Tris and 150 mM NaCl (pH 7.5) buffer containing 15 mg lysozyme (Calbiochem), 1 mg DNase I (Sigma-Aldrich), and 0.5 tablet of protease inhibitor cocktail (Roche Diagnostics, Germany). The resulting suspension was centrifuged at 17,000 rpm for 30 min to remove cell debris. The supernatant was loaded onto a 5 mL HisTrap FF crude Ni-NTA column (GE Healthcare, UK), first washed with 40 mL of 20 mM Tris and 150 mM NaCl (pH 8.5), and then washed with 40 mL of 40 mM imidazole in 20 mM Tris and 150 mM NaCl (pH 8.5). The protein was eluted from the column with buffer containing 500 mM imidazole in 20 mM Tris and 150 mM NaCl (pH 8.5). Imidazole was removed from protein using a HiPrep 26/10 Desalting column (GE Healthcare, UK), the protein was eluted into 20 mM Tris and 150 mM NaCl (pH 7.5) buffer. The protein was analyzed by LC-MS to confirm its purity and molecular weight.

SUMO group on SUMO-Protein was cleaved by incubating 1 μg of SUMO protease per mg of protein at room temperature for 60 min. The crude reaction mixture was loaded onto a 5 mL HisTrap FF crude Ni-NTA column (GE Healthcare, UK) and the flow through containing the desired protein was collected. The protein was analyzed by LC-MS confirming sample purity and molecular weight. Purified proteins were concentrated using Amicon 3K concentrator (50 mL, EMD Millipore); protein aliquots were flash frozen and stored in –80 °C freezer.

Protein Labeling Experiments



To a solution of protein (500 pmoles) in 475 μL of 20 mM Tris and 150 mM NaCl buffer (pH 7.5) was added palladium-tolyl complex **OA-3** (25 μL, 200 μM) in water. The solution was pipetted in and out of the pipette (10x) to ensure proper reagent mixing. The reaction mixture was left at room temperature for 30 min. After this time, the reaction was quenched by the addition of 3-thiopropionic acid (25 μL, 2 mM) dissolved in 20 mM Tris and 150 mM NaCl buffer (pH 7.5). After an additional 5 min at rt, 500 μL of 1 : 1 CH_3CN/H_2O (v/v) containing 0.2% TFA was added and the resulting mixture was analyzed by LC-MS.

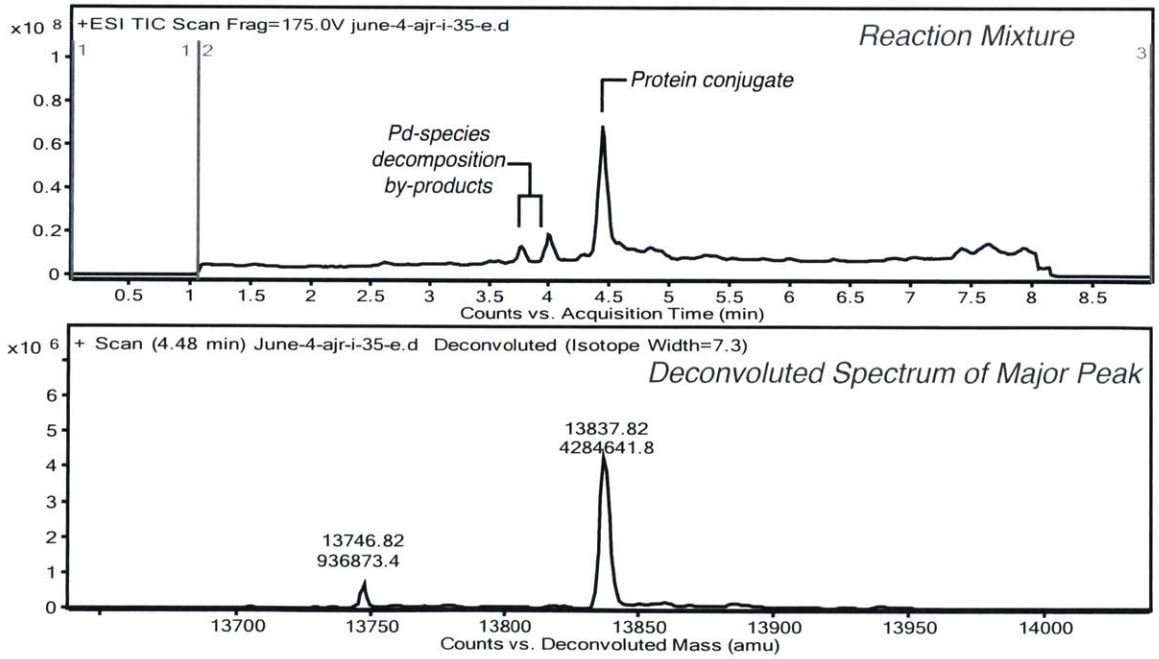


Table S2. **DARPin-Cys protein sequence and calculated mass**

Sequence[a]	Calculated mass
GGCGGSDLGKKLLEAARAGQDDEVRILMANGADVN AYDDNGVTPLHLAAFLGHLEIVEVLLKYGADVNAAD SWGTTPLHLAATWGHLEIVEVLLKHGADVNAQDKF GKTAF DISIDNGNEDLAEILQKLN	13747.3 Da

[a]Cysteine residues highlighted in red.

ICP-MS Analysis

Two distinct macrocyclic peptides, chosen at random (**P2-10** and **P2-14**), were dissolved in 0.4 mL of concentrated nitric acid. This solution was sonicated and diluted with MilliQ pure water to 0.2% nitric acid concentration. ICP-MS was performed on the resulting mixtures. Calibration curves were generated using Pd ICP-MS standards for a range between 1000 ppm and 100 ppb. There was no palladium found to be remaining in the peptide after purification. Initial palladium content was ~300 ppm used to perform the macrocyclization reactions indicating over 99% palladium removal.

LC-MS Characterization of Oxidative Addition Complexes

Each Pd reagent (**OA-Y**) reagent was dissolved in a 50:50 mixture of water:acetonitrile with 0.1% TFA and injected directly into the LC-MS and analysed via Method A in order to confirm purity of starting materials. Due to the complexity of the NMR spectra for each oxidative addition complex, we felt it was necessary to supply an additional measure of characterization of each complex.

Figure S-15: LC-MS Chromatogram of pure OA-3

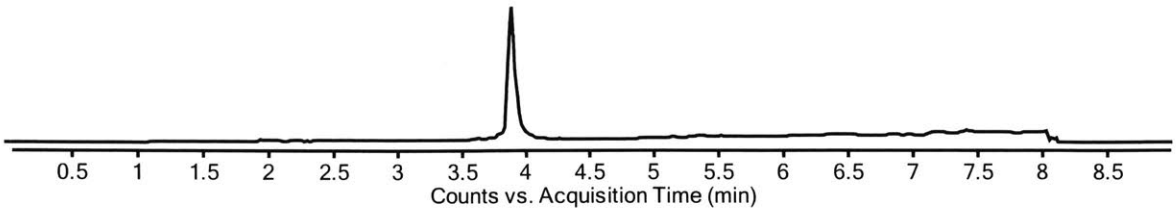


Figure S-16: LC-MS Chromatogram of pure OA-7

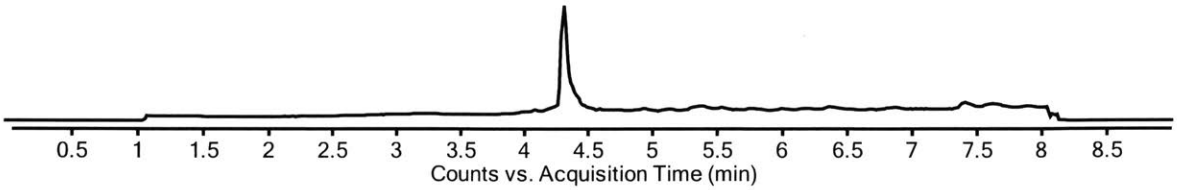


Figure S-17: LC-MS Chromatogram of pure OA-8

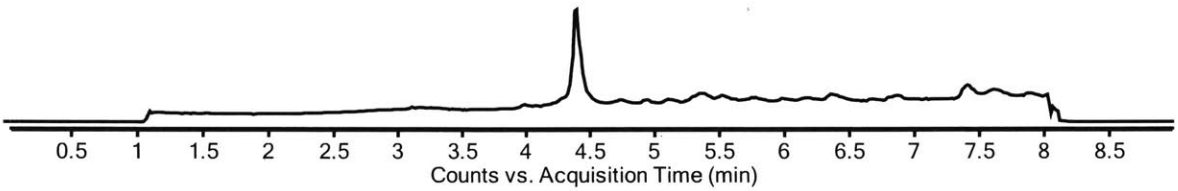


Figure S-18: LC-MS Chromatogram of pure OA-9

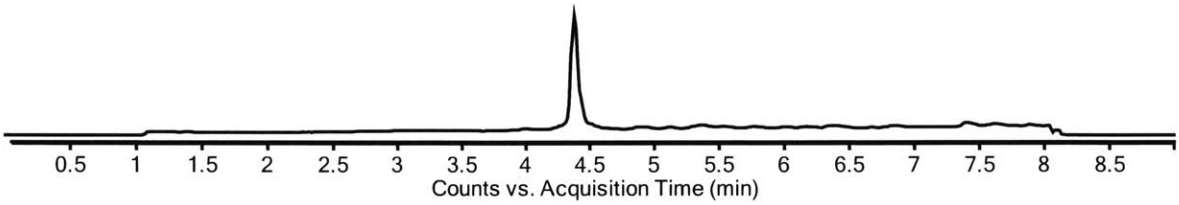


Figure S-19: LC-MS Chromatogram of pure OA-10

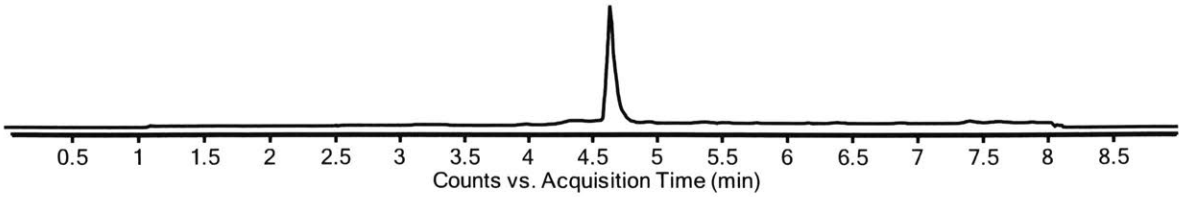


Figure S-20: LC-MS Chromatogram of pure OA-11

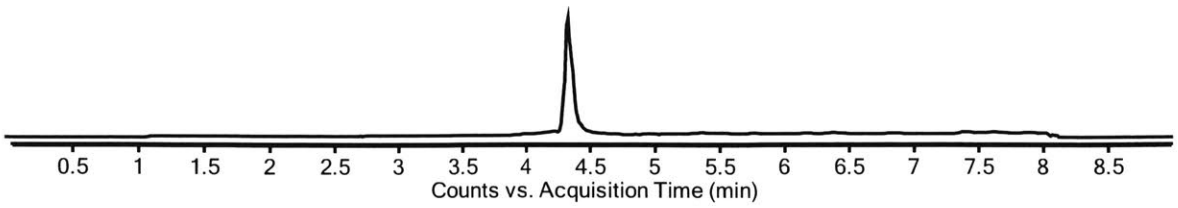


Figure S-21: LC-MS Chromatogram of pure OA-12

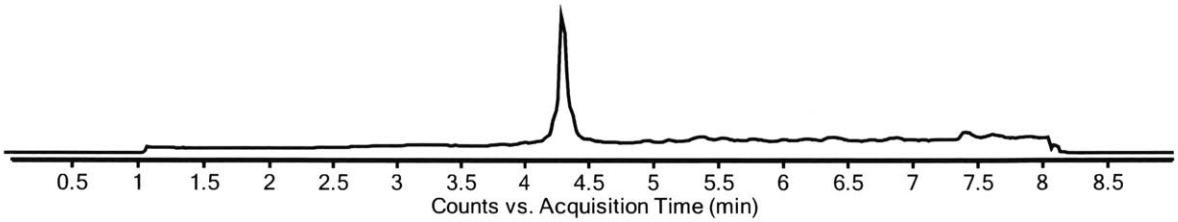


Figure S-22: LC-MS Chromatogram of pure OA-13

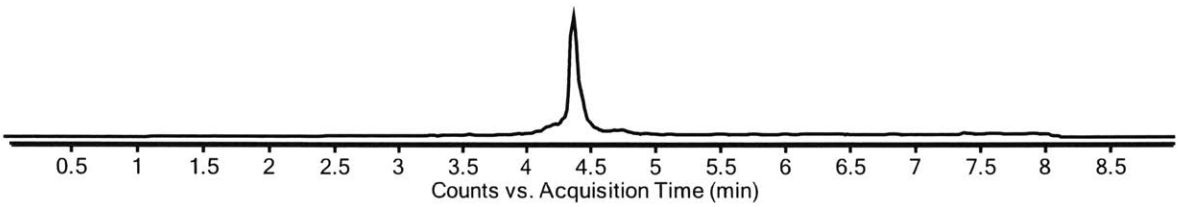


Figure S-23: LC-MS Chromatogram of pure OA-14

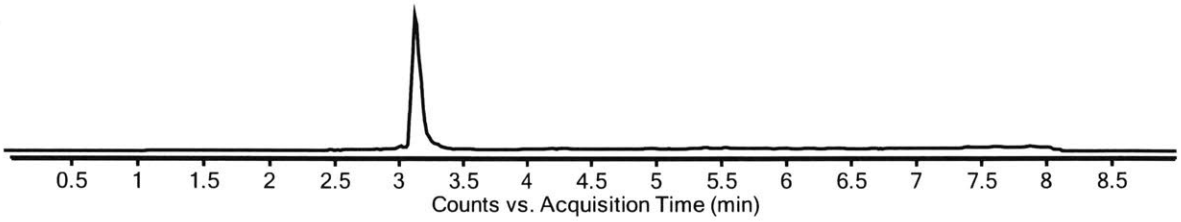


Figure S-24: LC-MS Chromatogram of pure OA-15

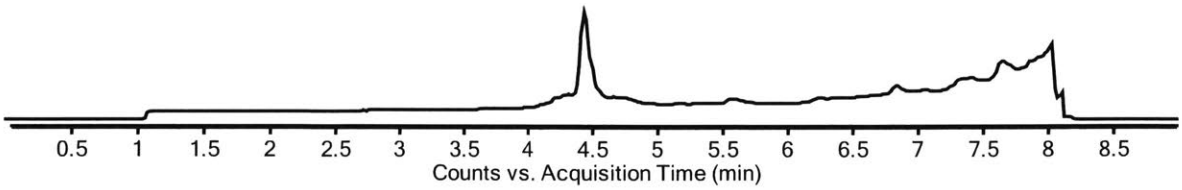


Figure S-25: LC-MS Chromatogram of pure OA-16

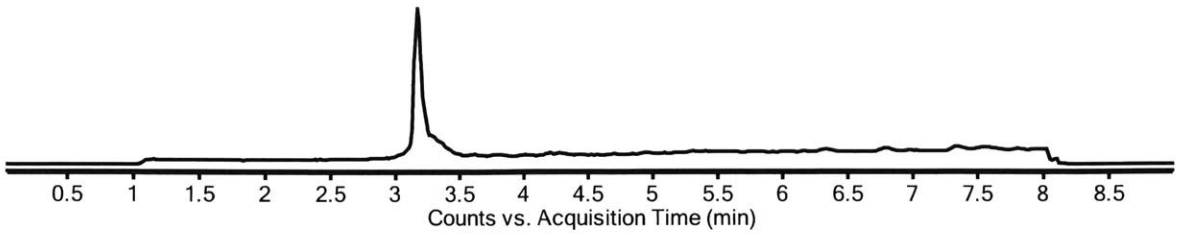


Figure S-26: LC-MS Chromatogram of pure OA-17

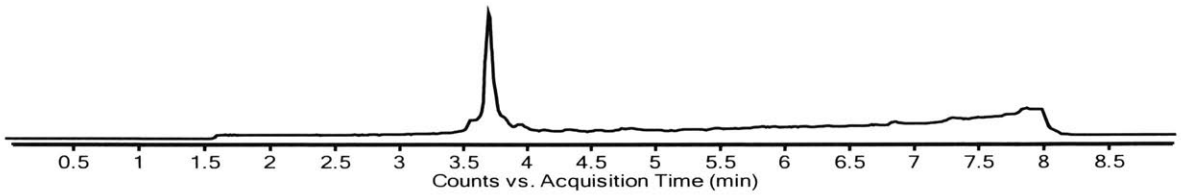


Figure S-27: LC-MS Chromatogram of pure OA-18

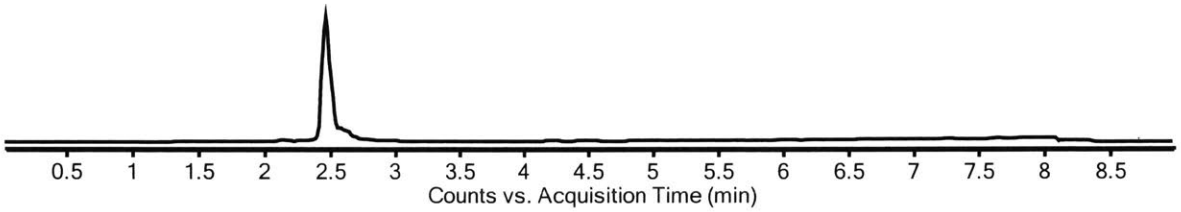


Figure S-28: LC-MS Chromatogram of pure OA-19

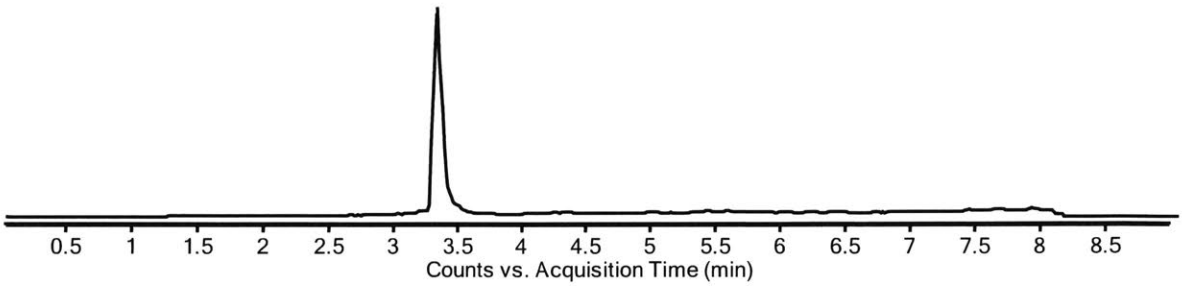


Figure S-29: LC-MS Chromatogram of pure OA-20

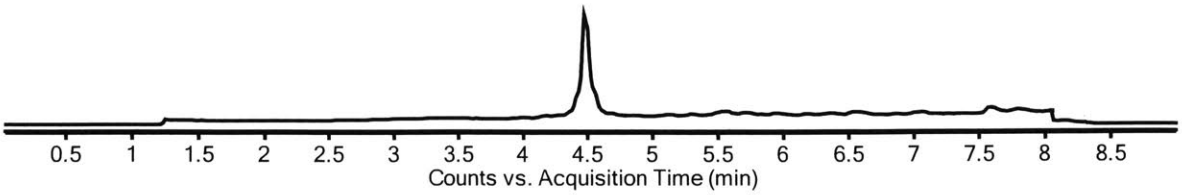


Figure S-30: LC-MS Chromatogram of pure OA-21

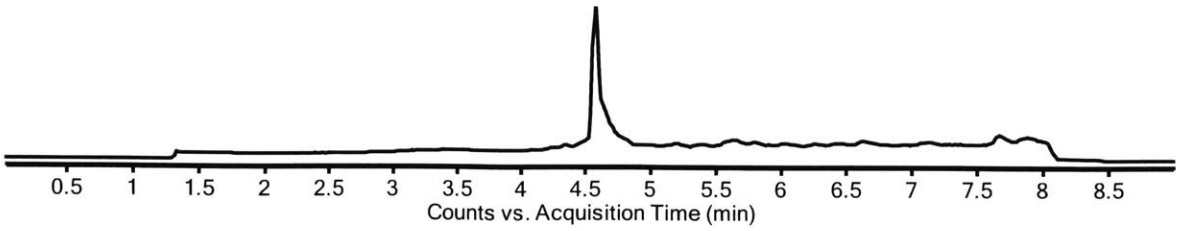


Figure S-31: LC-MS Chromatogram of pure OA-22

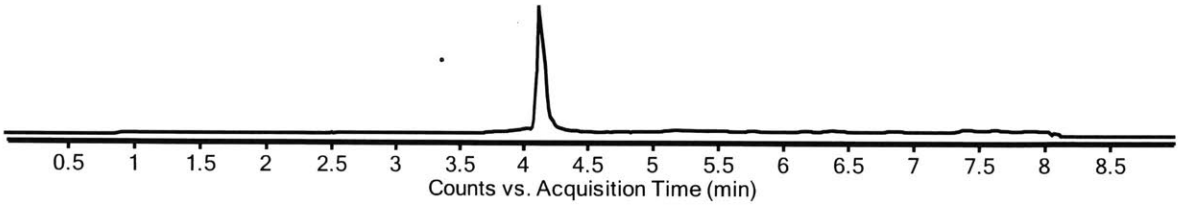


Figure S-32: LC-MS Chromatogram of pure OA-23

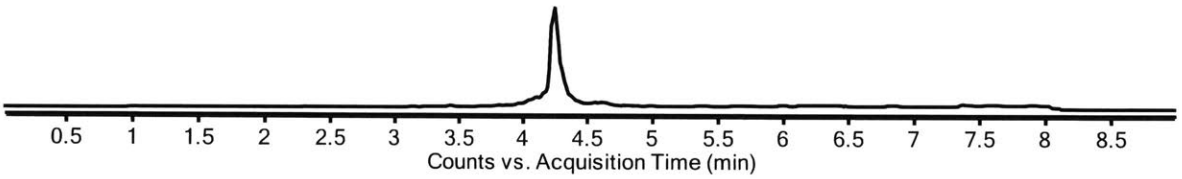


Figure S-33: LC-MS Chromatogram of pure OA-24

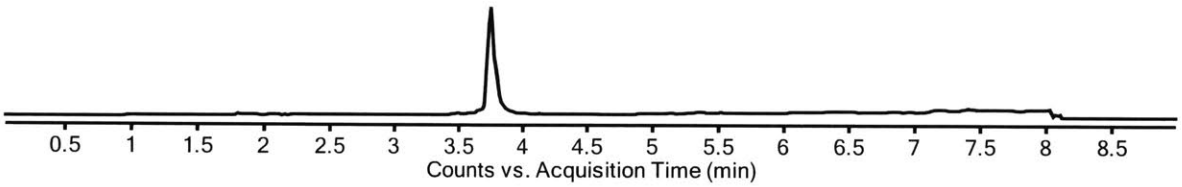
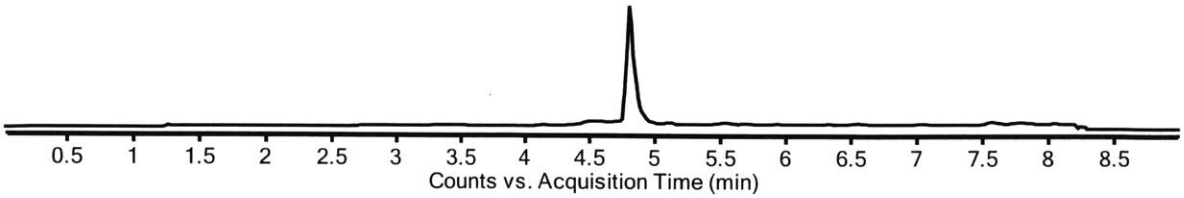


Figure S-34: LC-MS Chromatogram of pure OA-25



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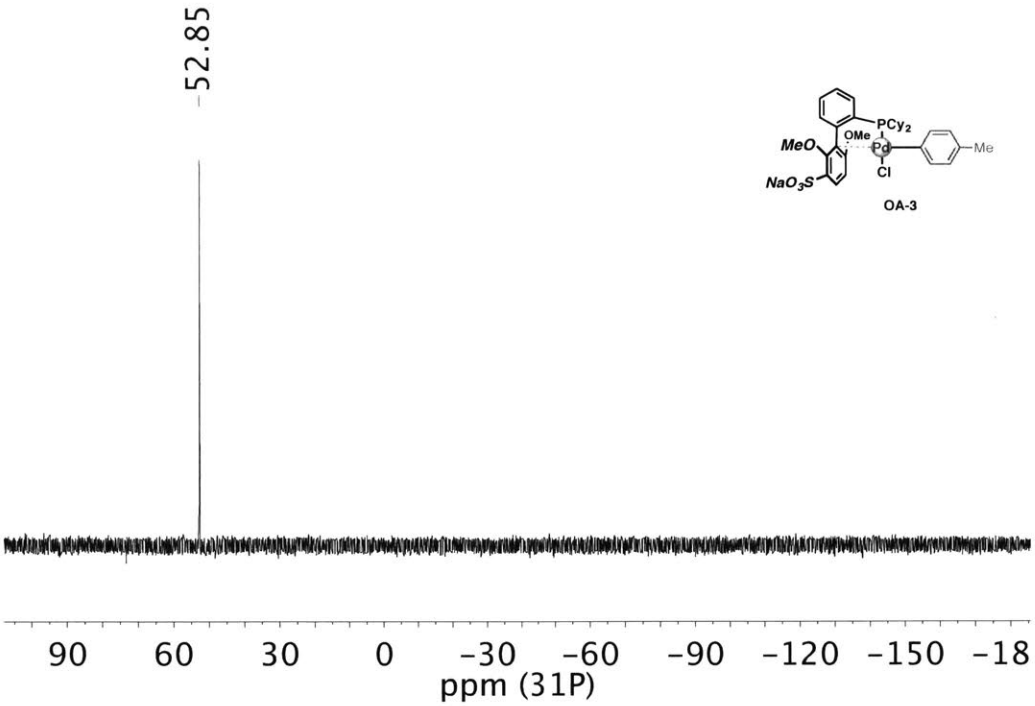
2.6 – Spectra: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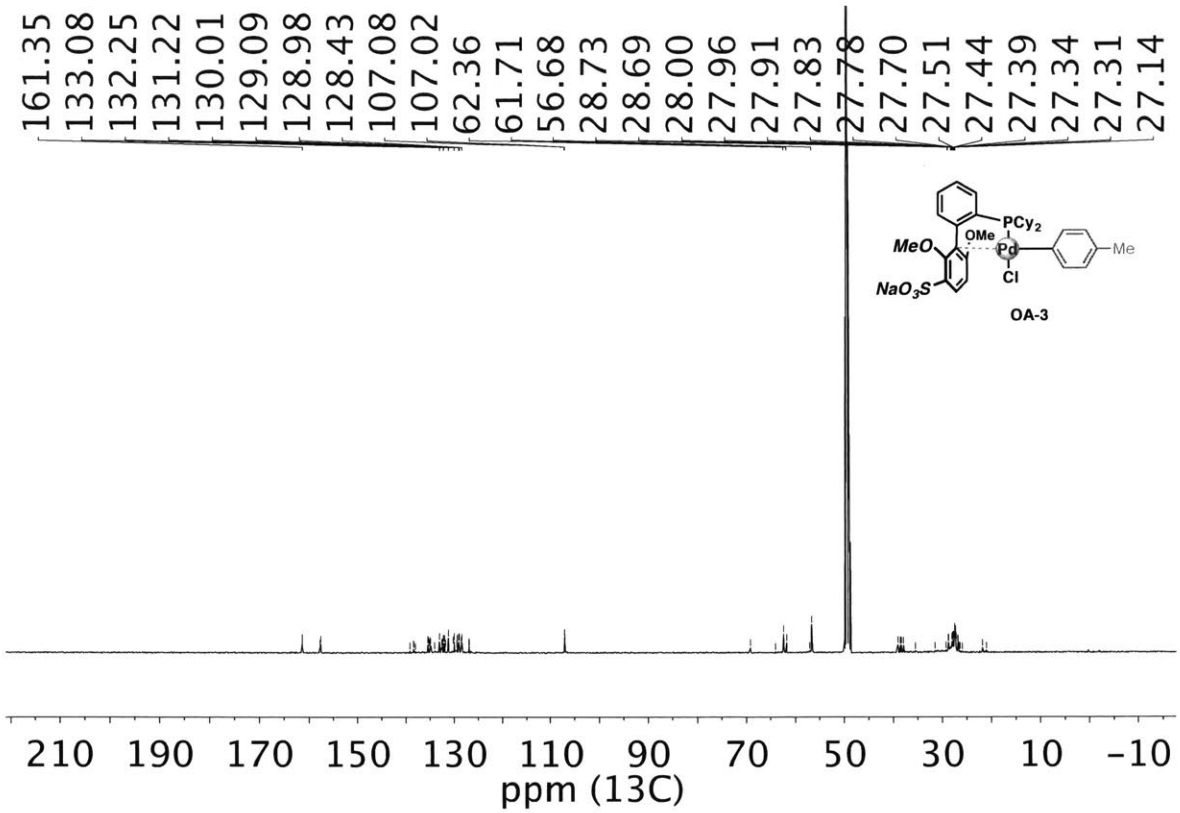
ppm (31P)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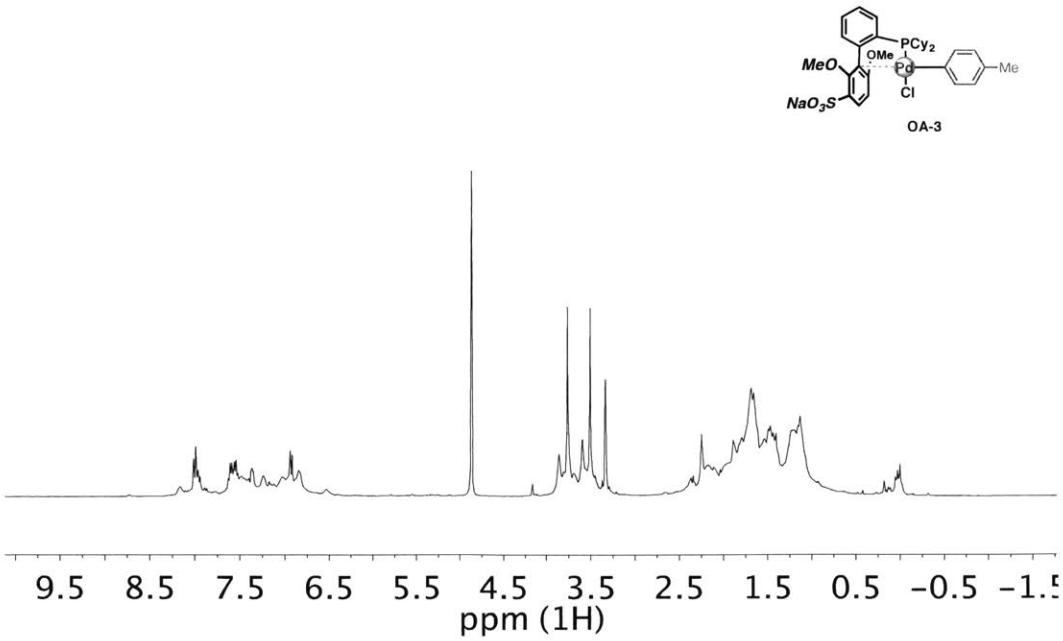
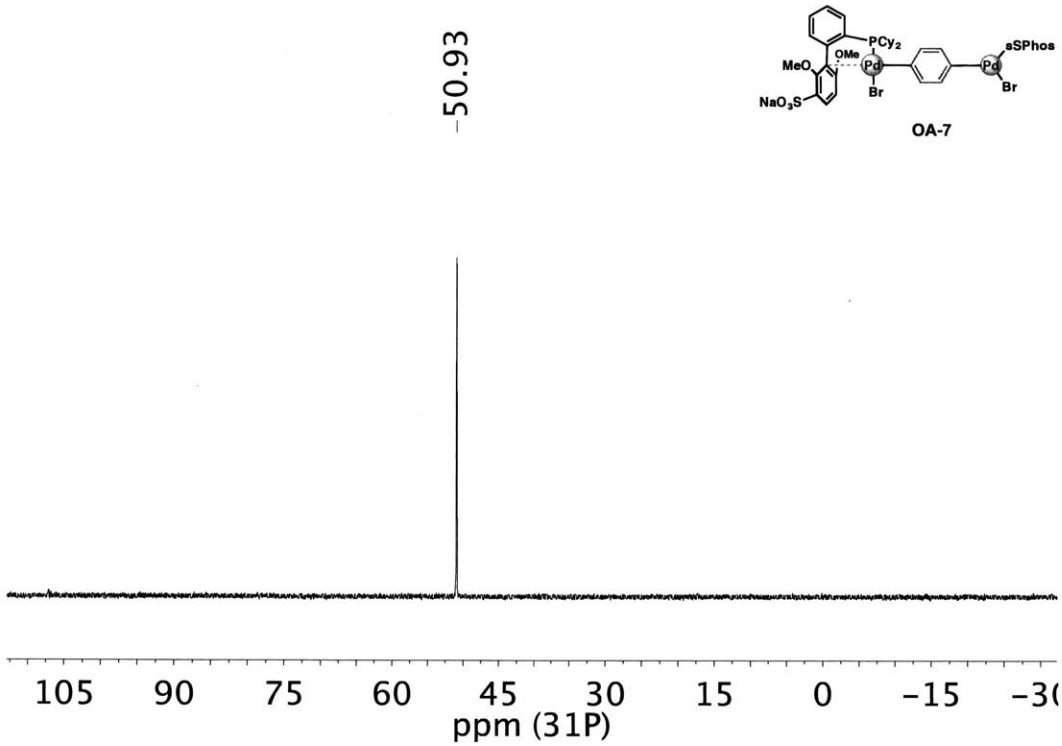
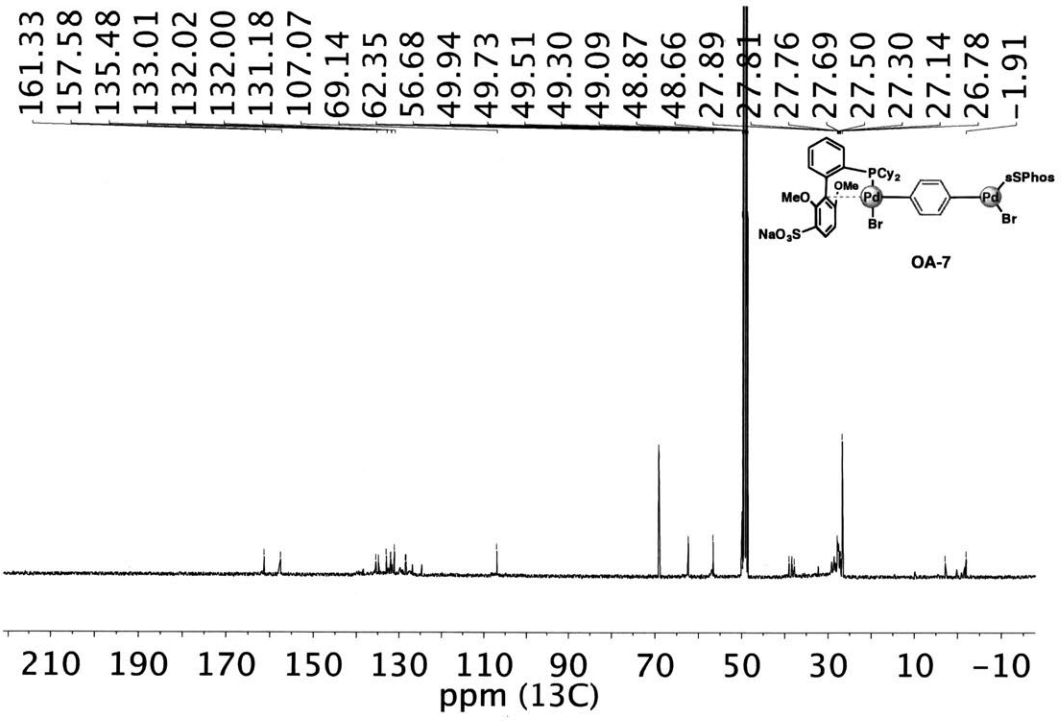
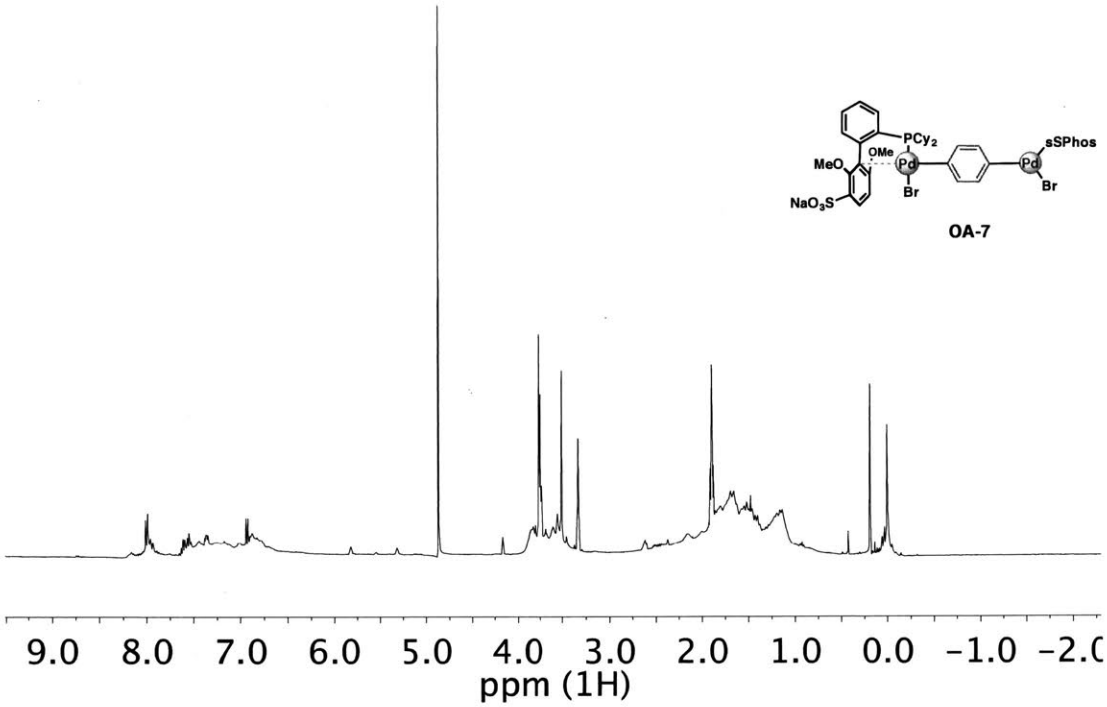
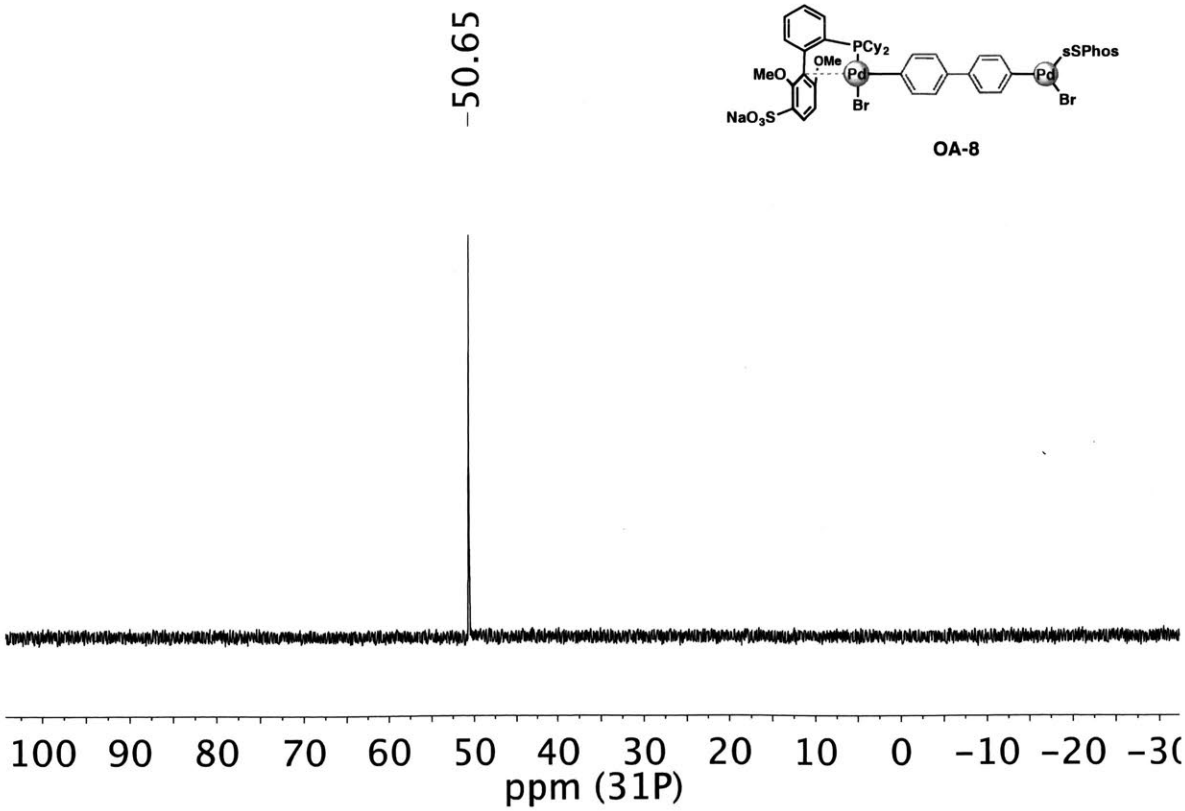
28.73
28.69
28.00
27.96
27.91
27.83
27.78
27.70
27.51
27.44
27.39
27.34
27.31
27.14
PCy2
OMe
MeO
Pd
Me
Cl
NaO3S
OA-3
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

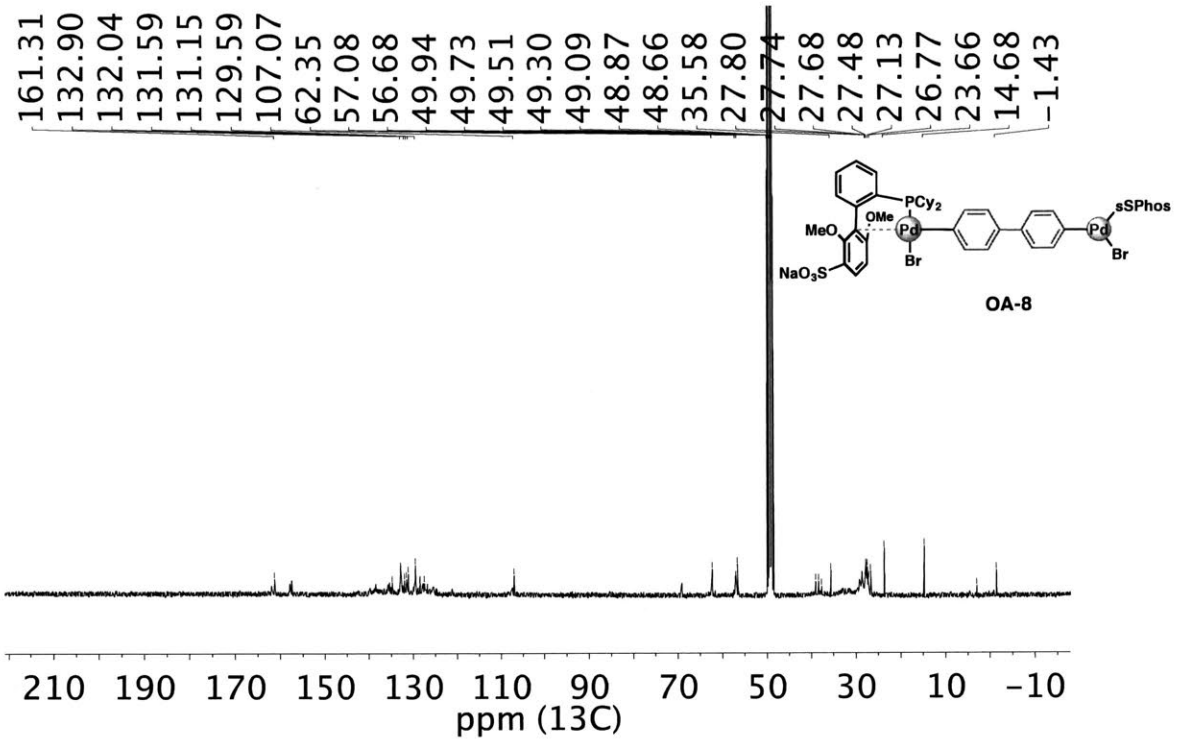

PCy2
OMe
MeO
Pd
Me
Cl
NaO3S
OA-3
9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 -0.5 -1.5
ppm (1H)

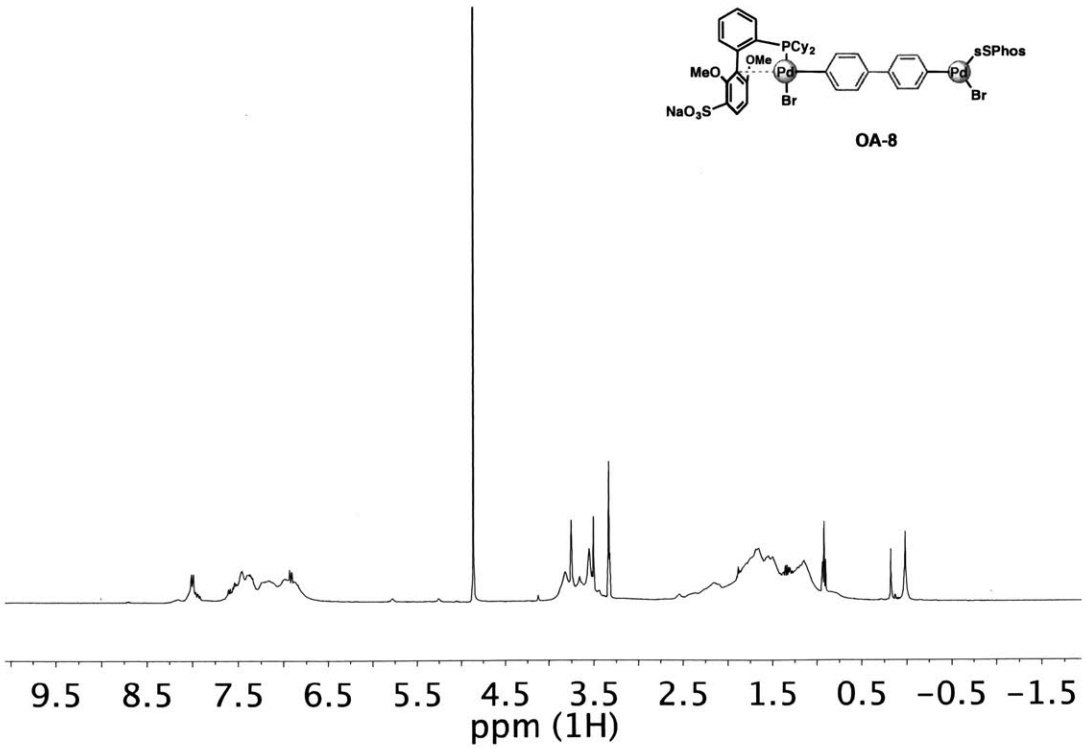

-50.93
PCy2
OMe
MeO
Pd
Br
NaO3S
sSPhos
Pd
Br
OA-7
105 90 75 60 45 30 15 0 -15 -30
ppm (31P)

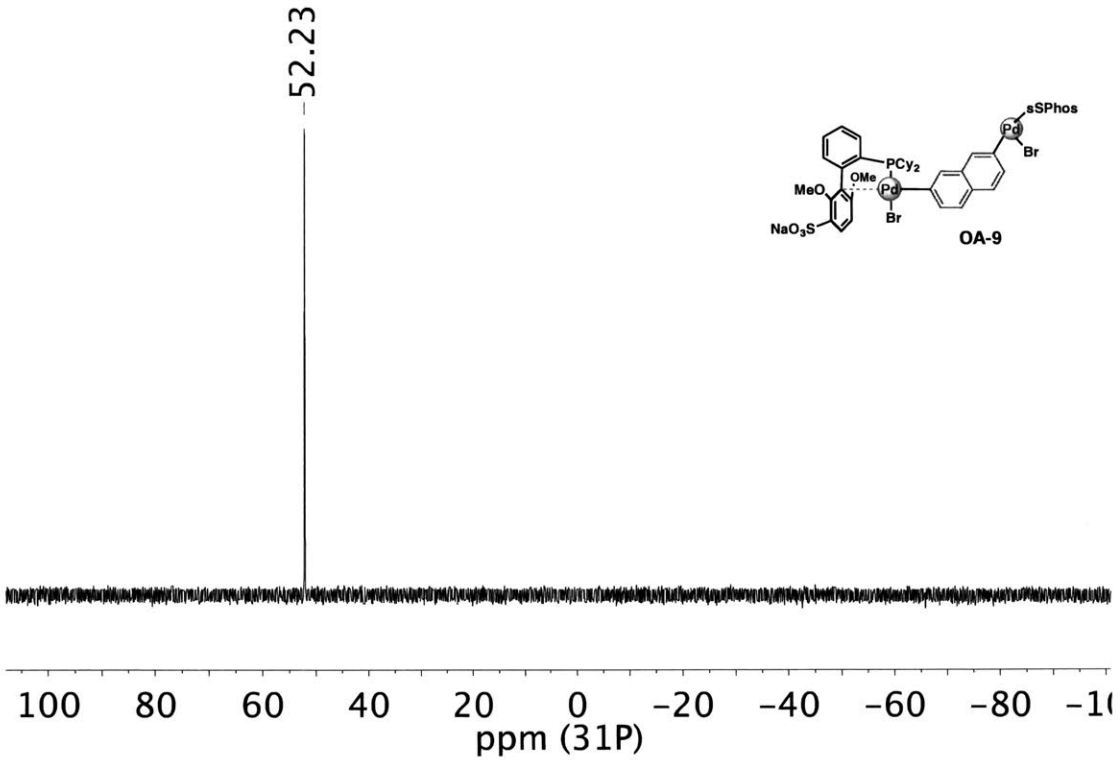

161.33
157.58
135.48
133.01
132.02
132.00
131.18
107.07
69.14
62.35
56.68
49.94
49.73
49.51
49.30
49.09
48.87
48.66
27.89
27.81
27.76
27.69
27.50
27.30
27.14
26.78
-1.91
PCy2
OMe
MeO
Pd
Br
NaO3S
sSPhos
Pd
Br
OA-7
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

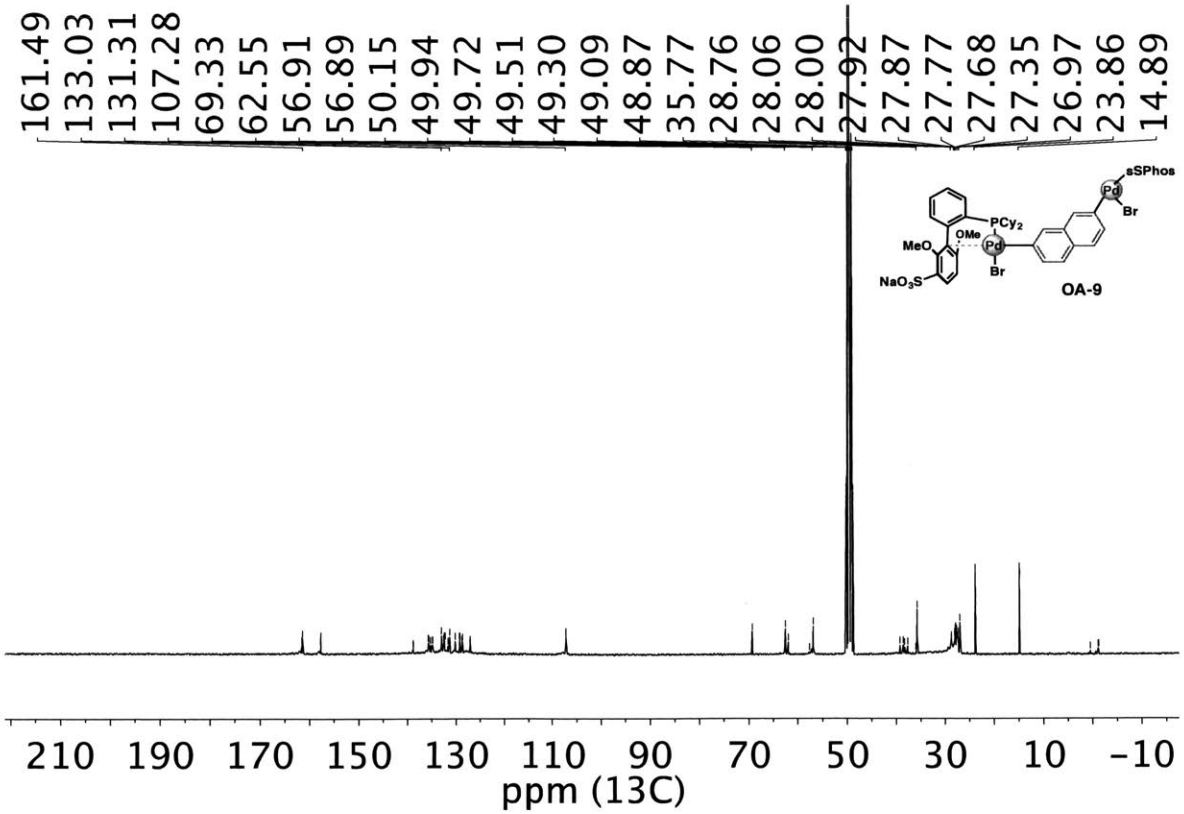

PCy2
OMe
MeO
Pd
Br
sSPhos
Pd
Br
NaO3S
OA-7
9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0 -1.0 -2.0
ppm (1H)

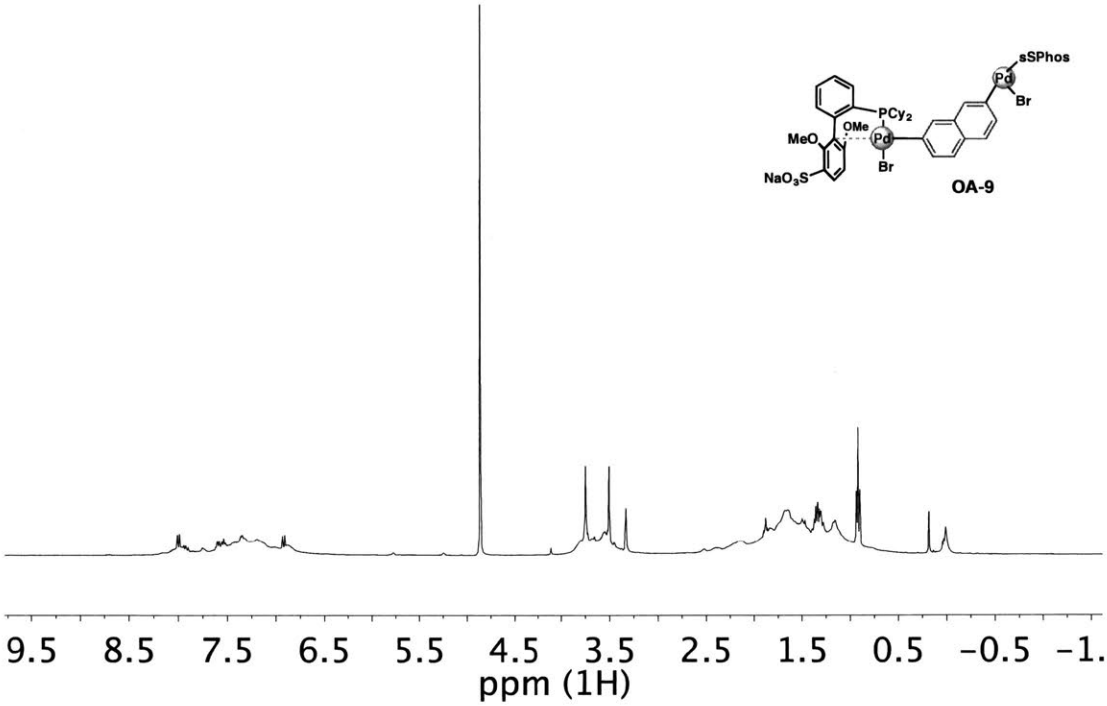

-50.65
PCy2
OMe
MeO
Pd
Br
sSPhos
Pd
Br
NaO3S
OA-8
100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30
ppm (31P)

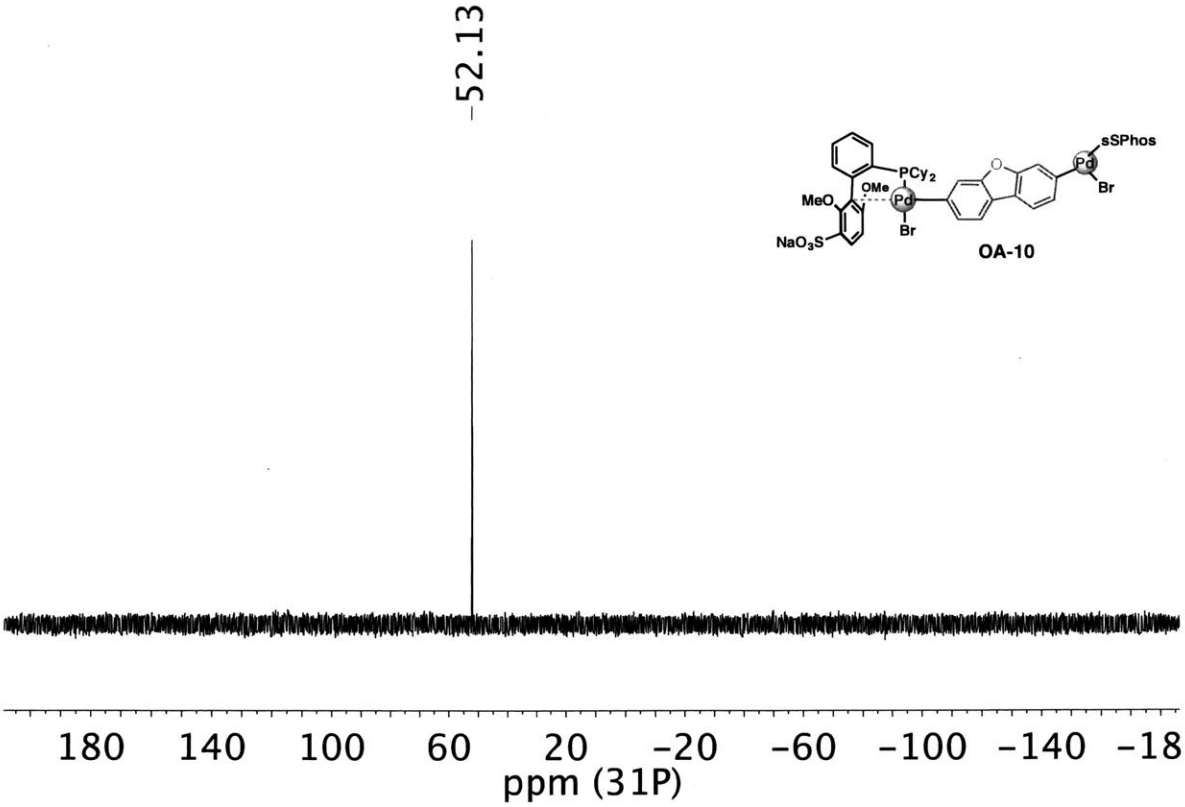

161.31
132.90
132.04
131.59
131.15
129.59
107.07
62.35
57.08
56.68
49.94
49.73
49.51
49.30
49.09
48.87
48.66
35.58
27.80
27.74
27.68
27.48
27.13
26.77
23.66
14.68
-1.43
PCy2
OMe
MeO
Pd
Br
NaO3S
sSPhos
OA-8
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

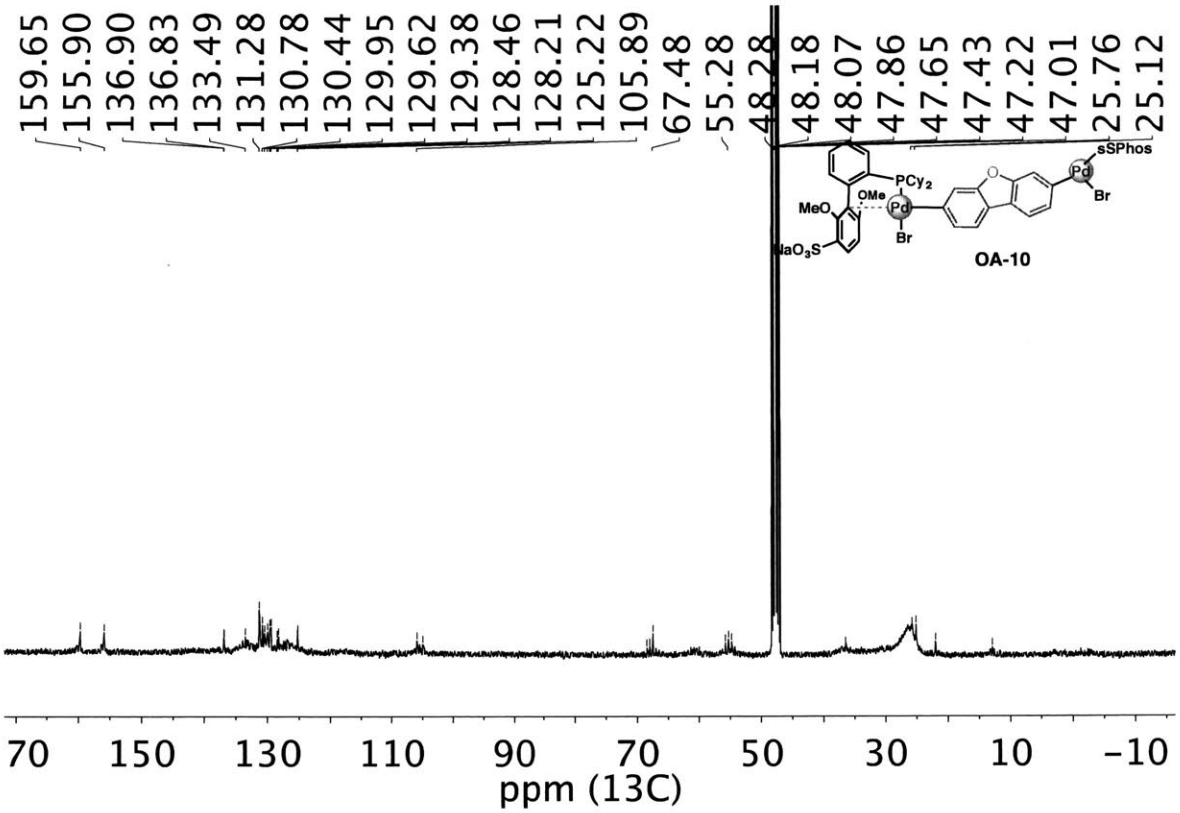

PCy2
OMe
MeO
Pd
Br
NaO3S
sSPhos
OA-8
9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 -0.5 -1.5
ppm (1H)

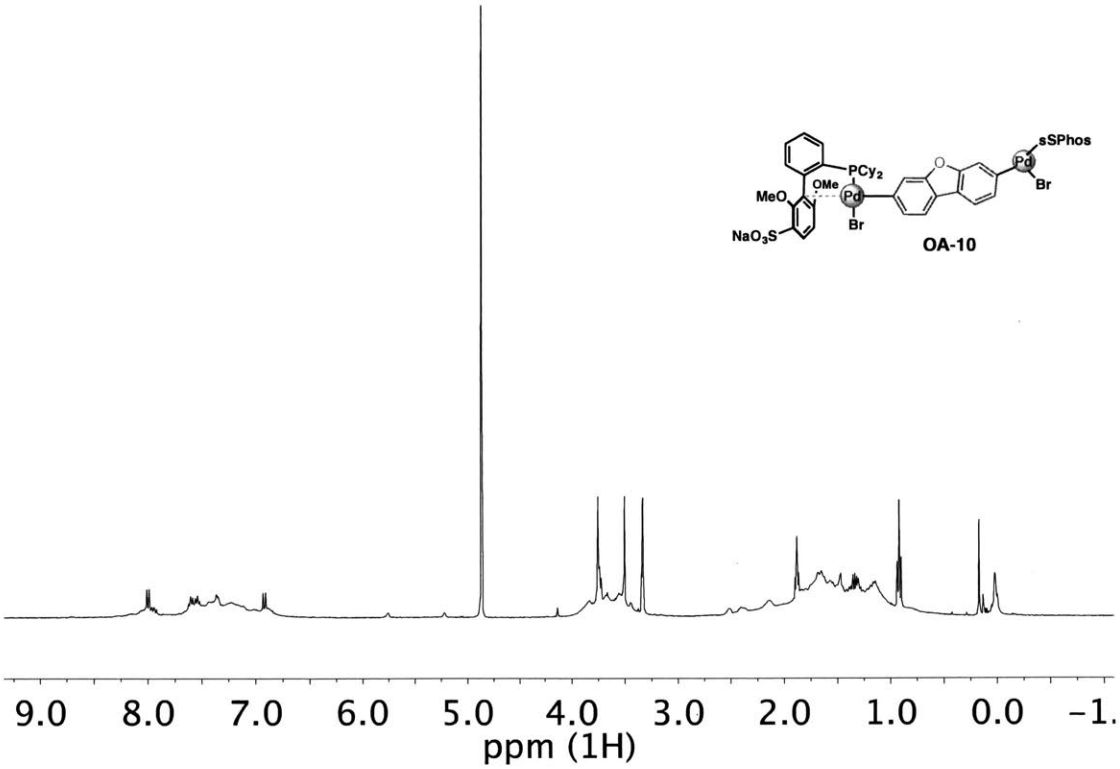

52.23
sSPhos
Pd
Br
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-9
100 80 60 40 20 0 -20 -40 -60 -80 -1(
ppm (31P)

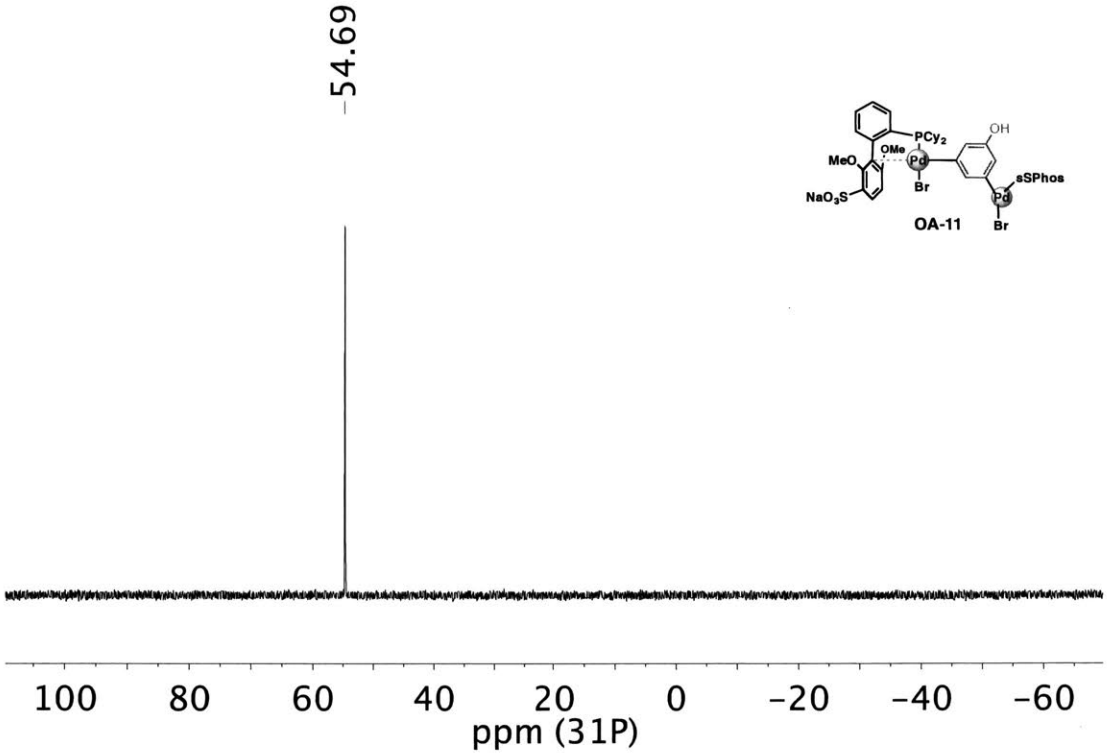

161.49
133.03
131.31
107.28
69.33
62.55
56.91
56.89
50.15
49.94
49.72
49.51
49.30
49.09
48.87
35.77
28.76
28.06
28.00
27.92
27.87
27.77
27.68
27.35
26.97
23.86
14.89
sSPhos
Pd
Br
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-9
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

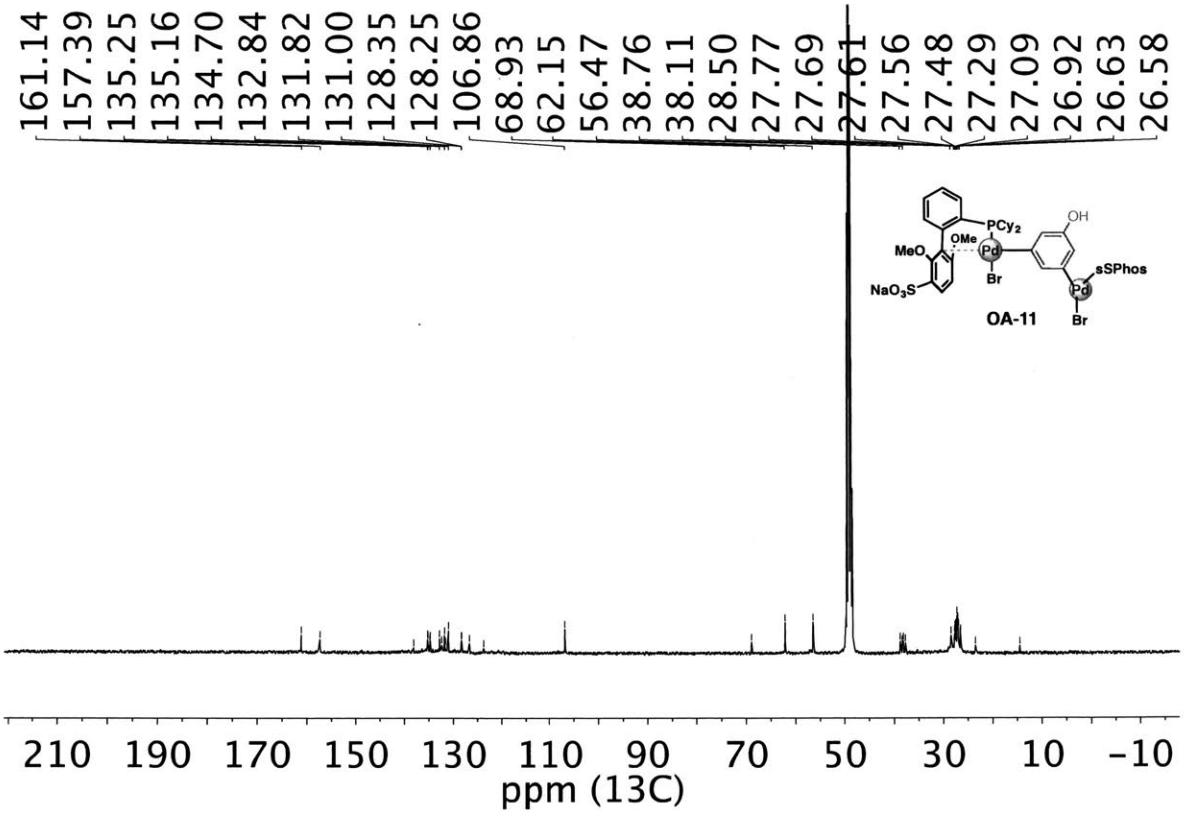

sSPhos
Pd
Br
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-9
9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 -0.5 -1.
ppm (1H)

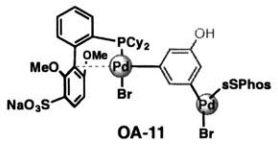

-52.13
sSPhos
Pd
Br
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-10
180 140 100 60 20 -20 -60 -100 -140 -18
ppm (31P)


159.65
155.90
136.90
136.83
133.49
131.28
130.78
130.44
129.95
129.62
129.38
128.46
128.21
125.22
105.89
67.48
55.28
48.28
48.18
48.07
47.86
47.65
47.43
47.22
47.01
25.76
25.12
sSPhos
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-10
70
150
130
110
90
70
50
30
10
-10
ppm (13C)

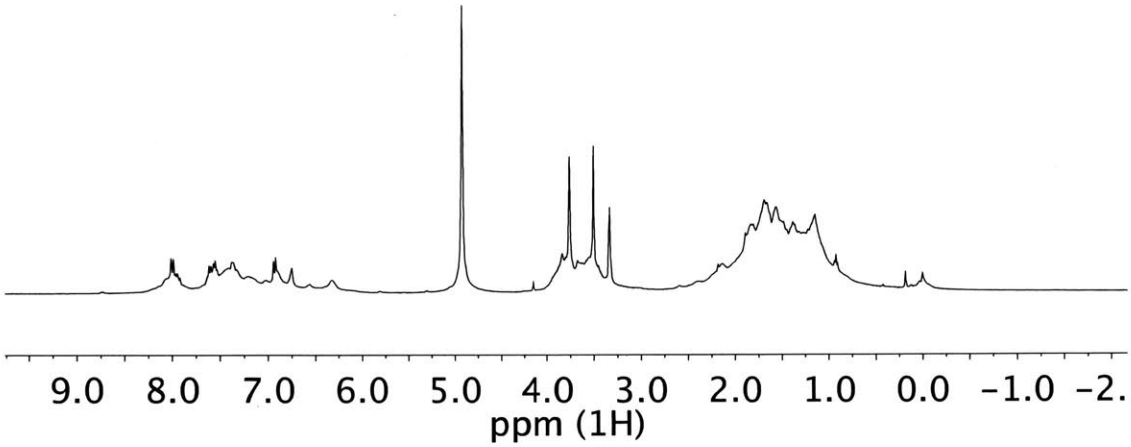

sSPhos
PCy2
OMe
MeO
Pd
Br
NaO3S
OA-10
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.
ppm (1H)

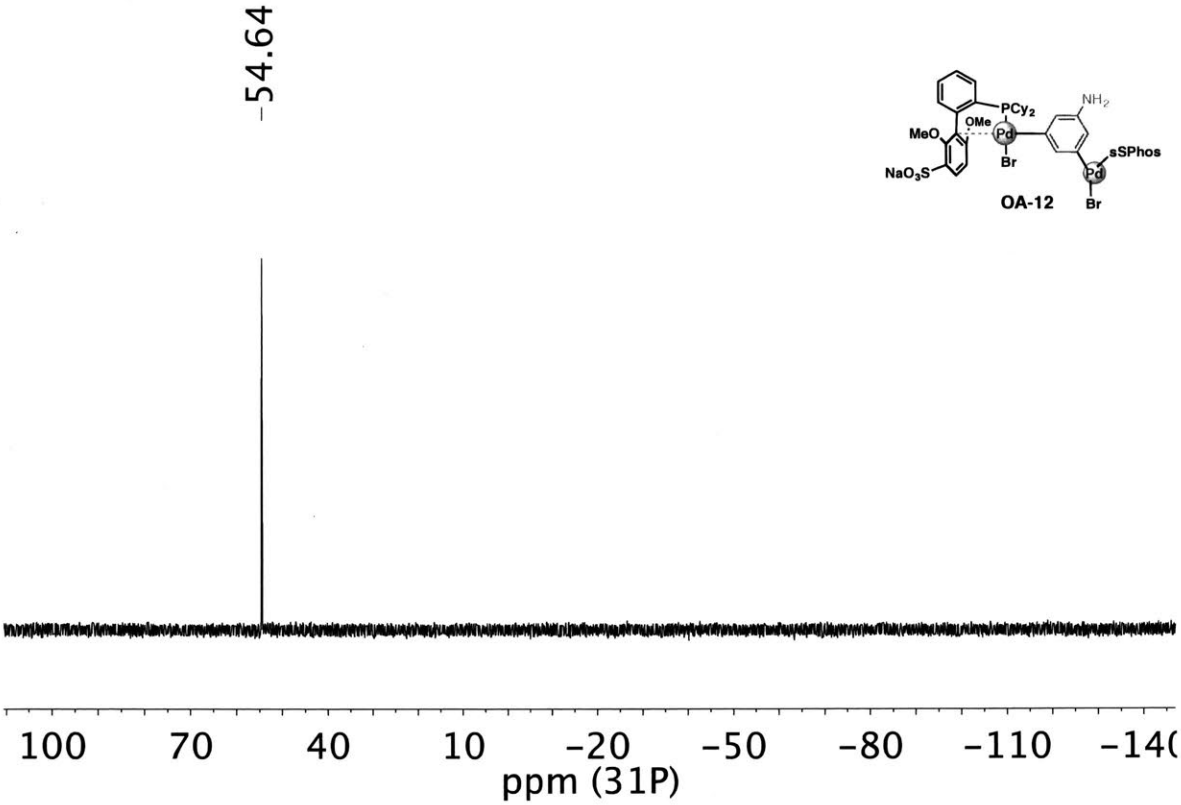

-54.69
OA-11
100 80 60 40 20 0 -20 -40 -60
ppm (31P)

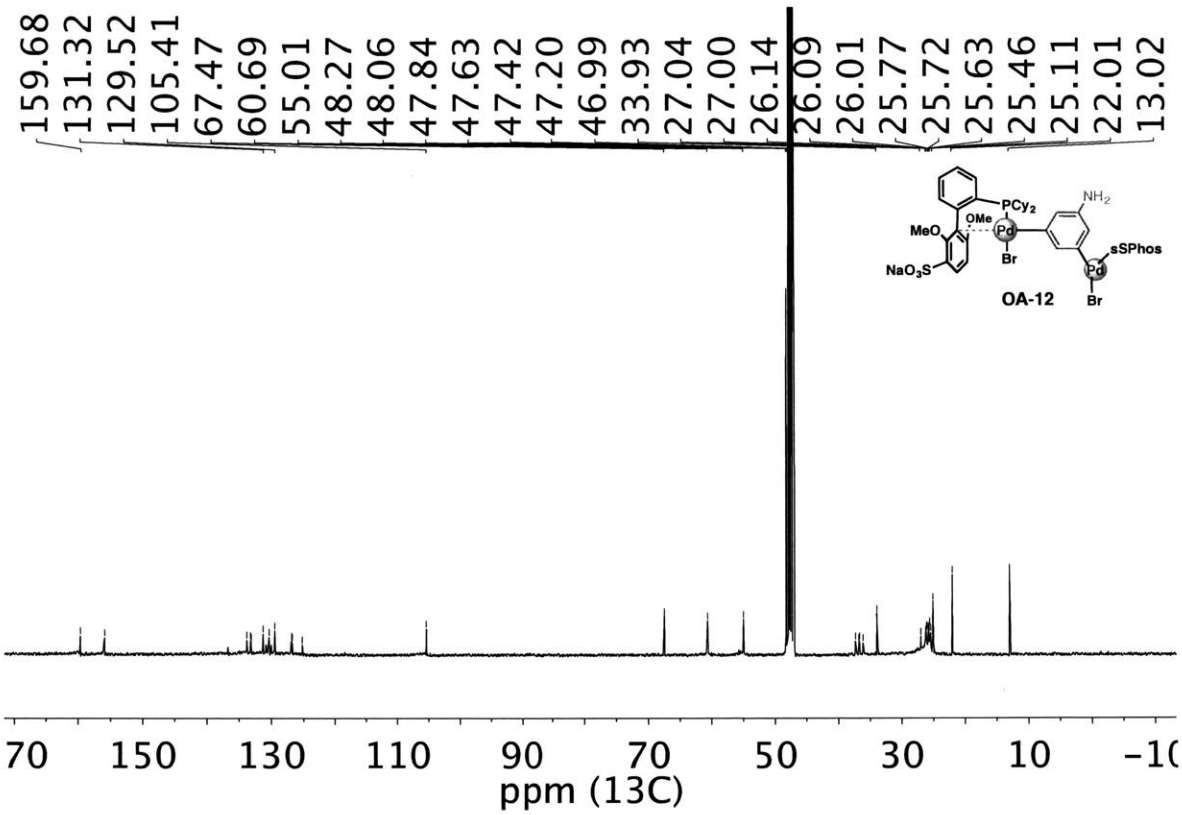

161.14
157.39
135.25
135.16
134.70
132.84
131.82
131.00
128.35
128.25
106.86
68.93
62.15
56.47
38.76
38.11
28.50
27.77
27.69
27.61
27.56
27.48
27.29
27.09
26.92
26.63
26.58
OA-11
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

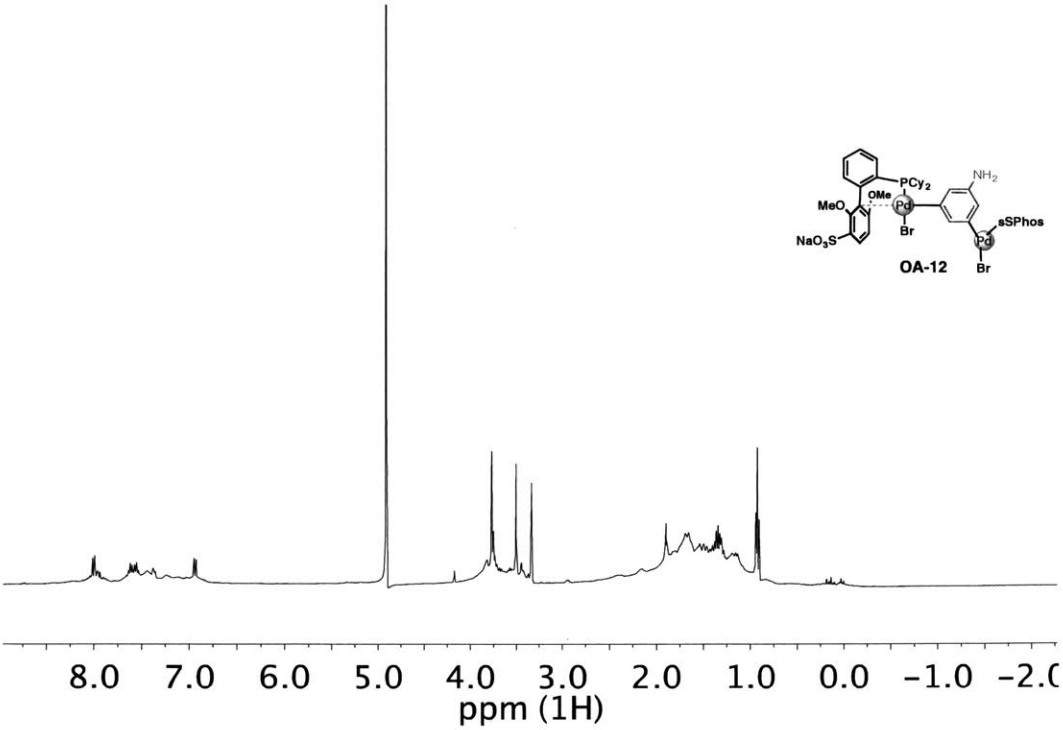

PCy2
OH
OMe
MeO
Pd
Br
sSPhos
NaO3S
Pd
Br

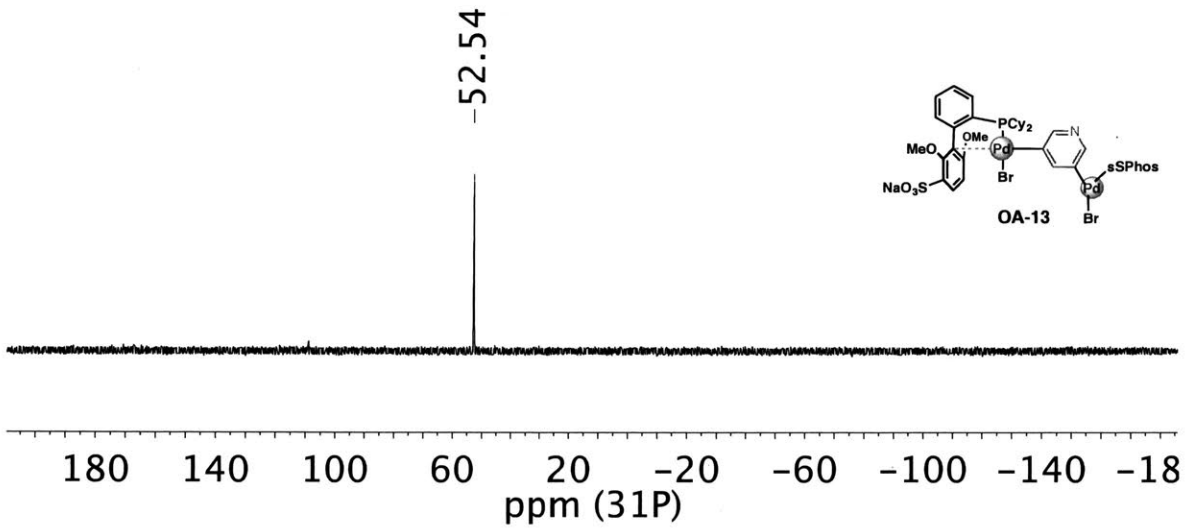
OA-11

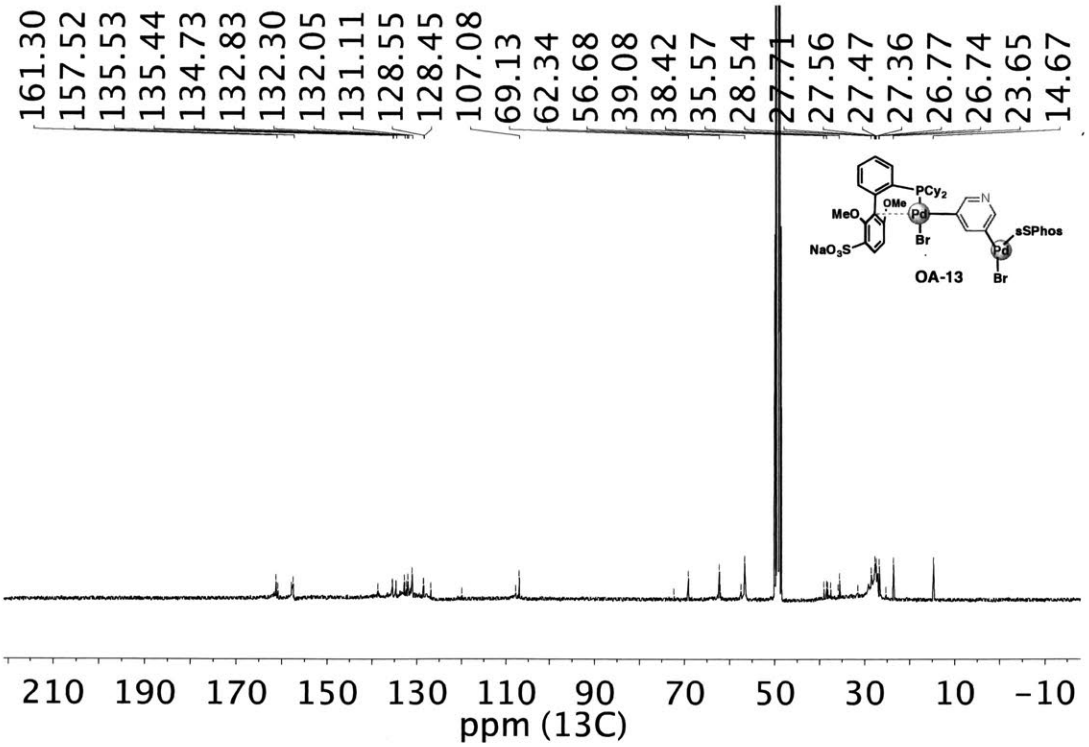

9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0 -1.0 -2.
ppm (1H)

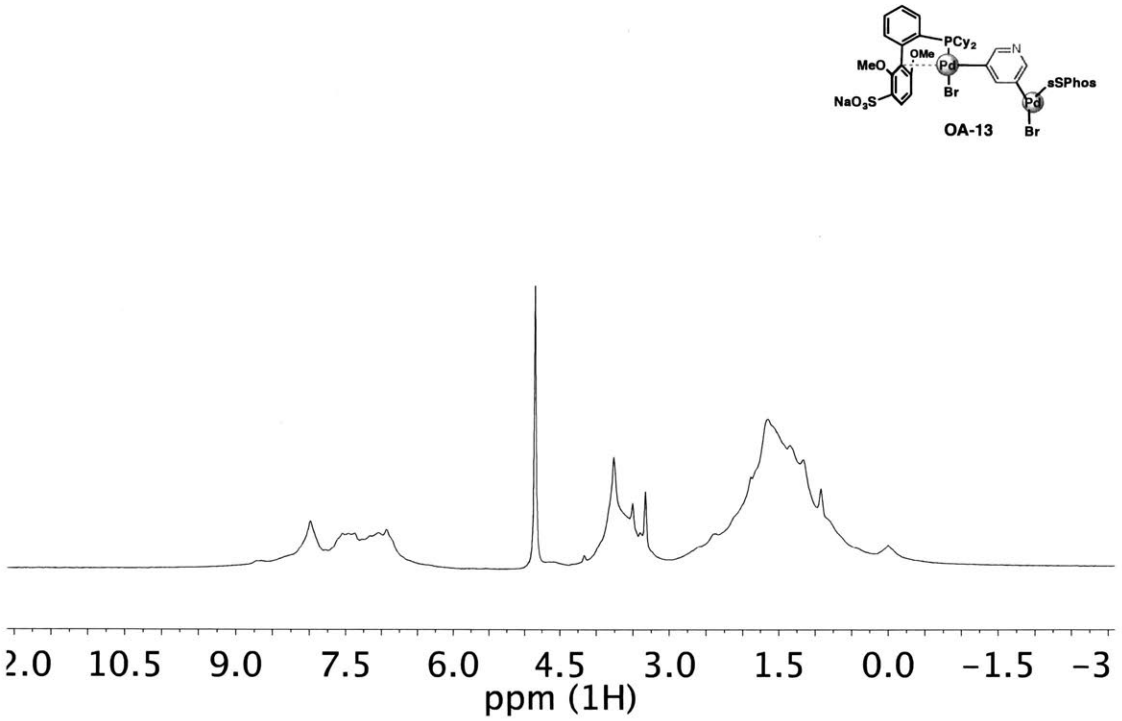

-54.64
PCy2
NH2
OMe
MeO
Pd
Br
sSPhos
NaO3S
Pd
Br
OA-12
100
70
40
10
-20
-50
-80
-110
-140
ppm (31P)

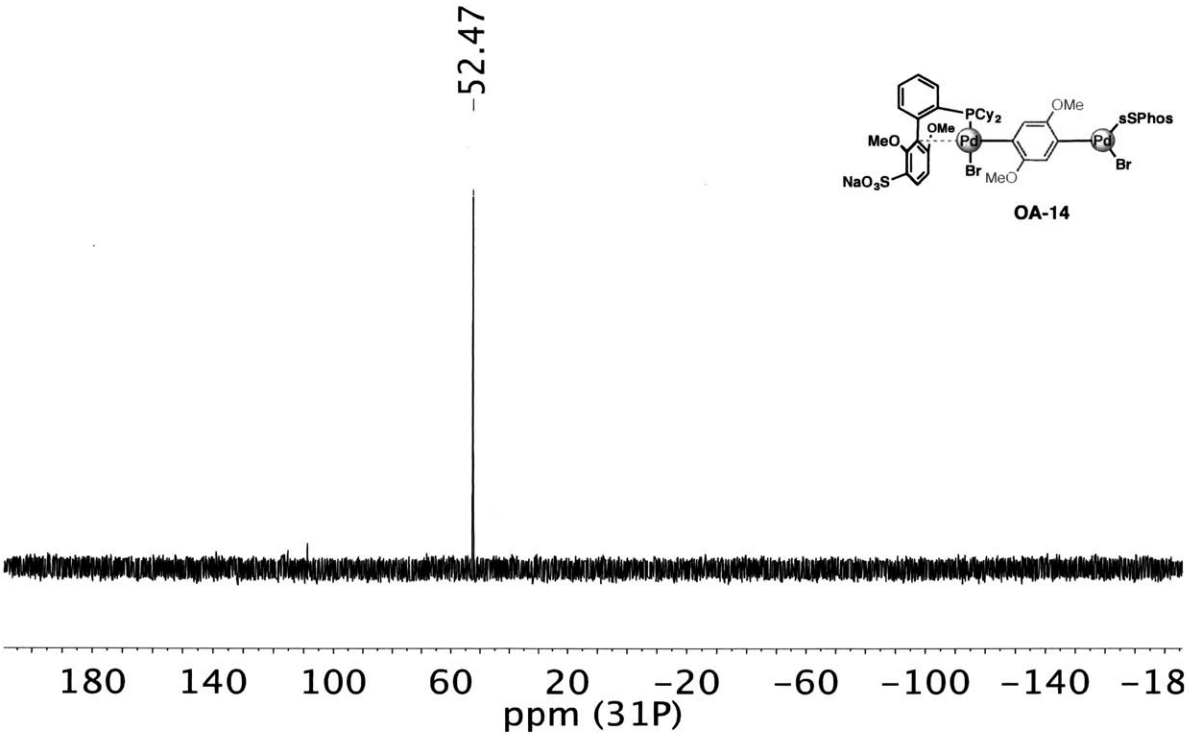

159.68
131.32
129.52
105.41
67.47
60.69
55.01
48.27
48.06
47.84
47.63
47.42
47.20
46.99
33.93
27.04
27.00
26.14
26.09
26.01
25.77
25.72
25.63
25.46
25.11
22.01
13.02
PCy2
NH2
OMe
MeO
Pd
Br
sSPhos
NaO3S
OA-12
70
150
130
110
90
70
50
30
10
-10
ppm (13C)

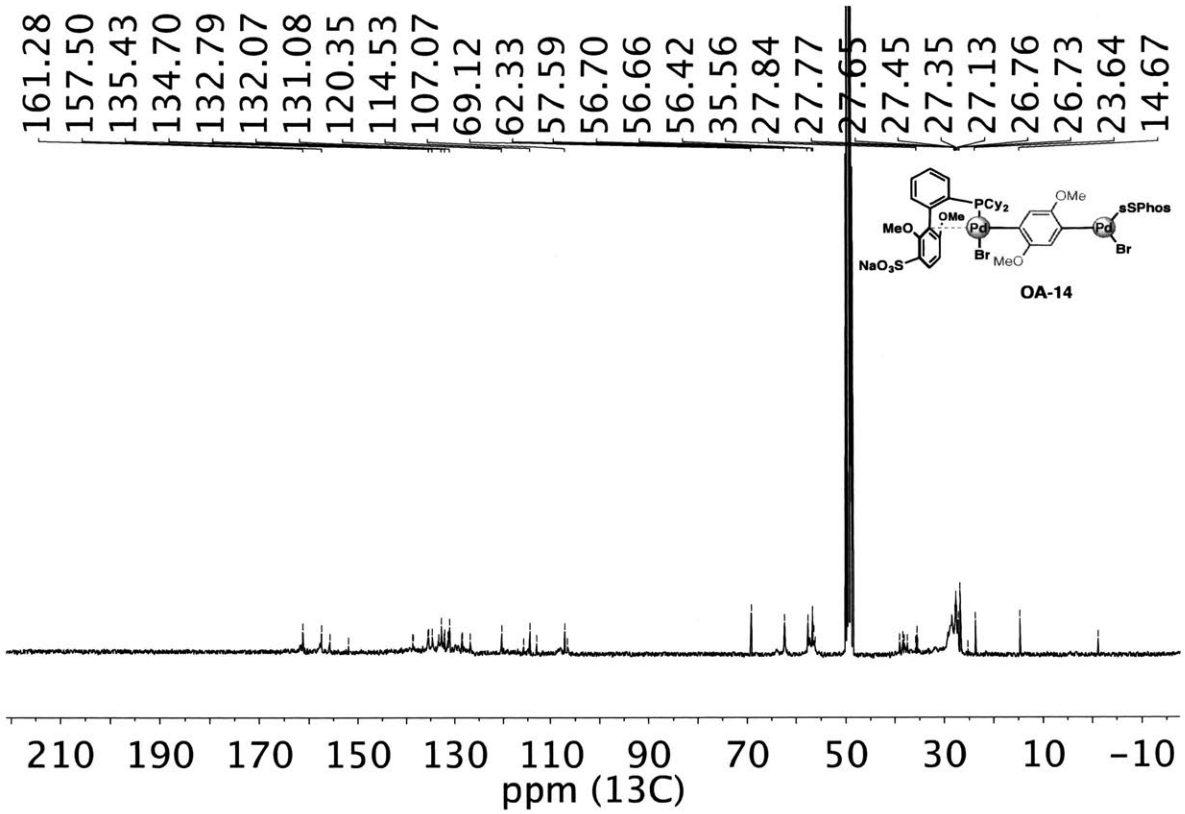

PCy2
NH2
OMe
MeO
Pd
Br
sSPhos
NaO3S
OA-12
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
ppm (1H)

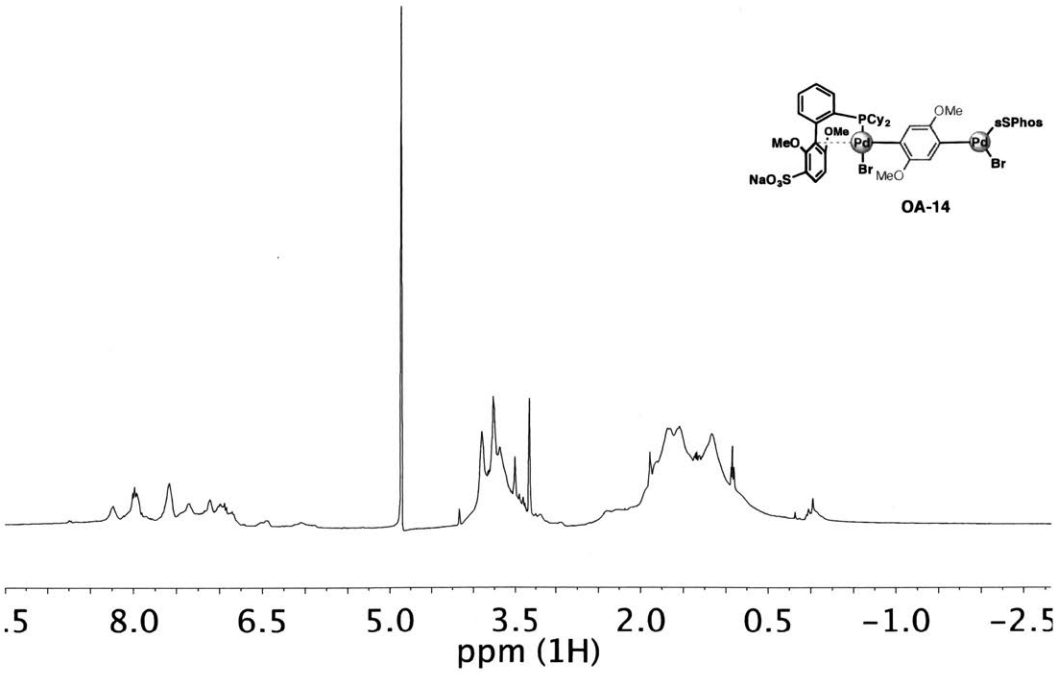

-52.54
PCy2
OMe
MeO
Pd
Br
N
sSPhos
NaO3S
OA-13
180 140 100 60 20 -20 -60 -100 -140 -18
ppm (31P)

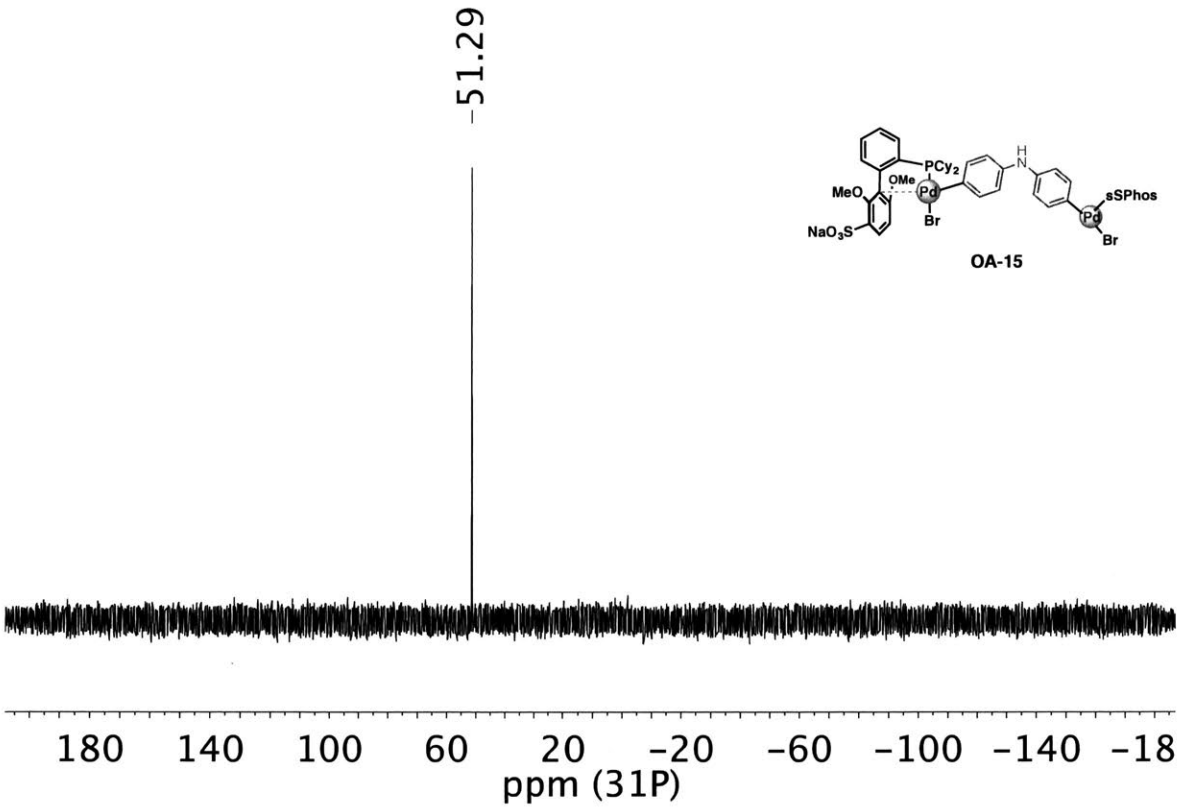

161.30
157.52
135.53
135.44
134.73
132.83
132.30
132.05
131.11
128.55
128.45
107.08
69.13
62.34
56.68
39.08
38.42
35.57
28.54
27.71
27.56
27.47
27.36
26.77
26.74
23.65
14.67
PCy2
OMe
MeO
Pd
Br
N
sSPhos
NaO3S
OA-13
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

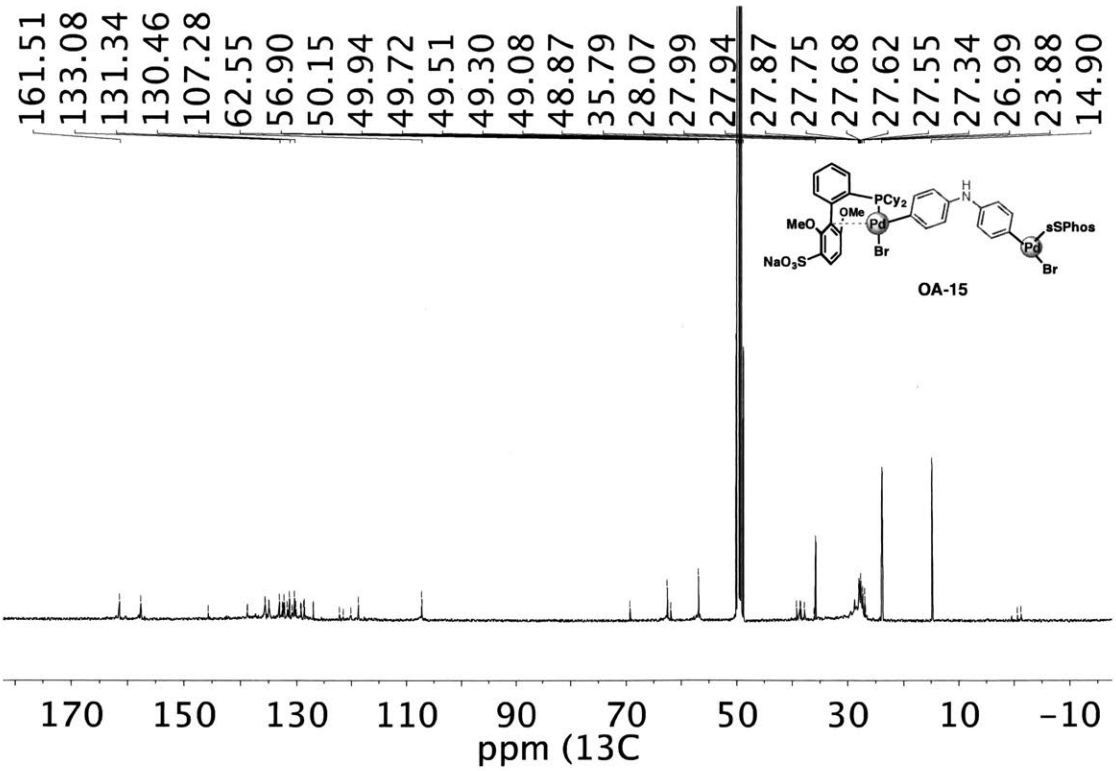

PCy2
OMe
MeO
Pd
Br
NaO3S
N
sSPhos
Pd
Br
OA-13
2.0 10.5 9.0 7.5 6.0 4.5 3.0 1.5 0.0 -1.5 -3
ppm (1H)

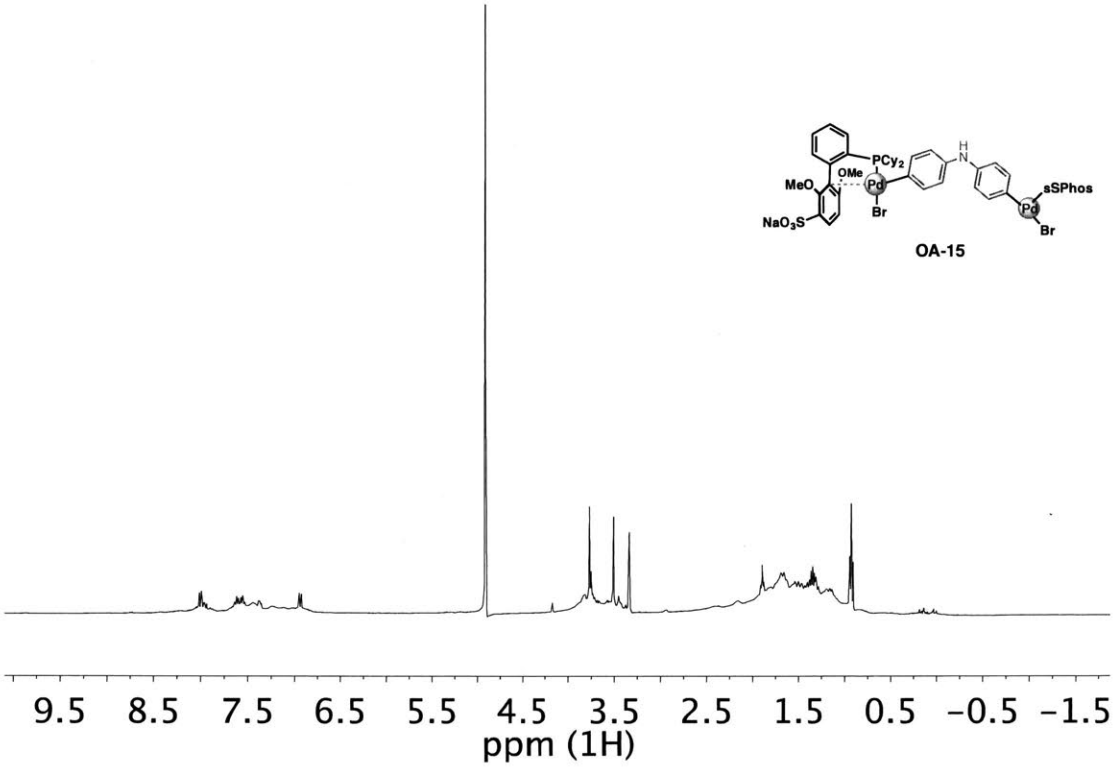

-52.47
PCy2
OMe
MeO
Pd
Br
NaO3S
OMe
MeO
sSPhos
Pd
Br
OA-14
180 140 100 60 20 -20 -60 -100 -140 -18
ppm (31P)

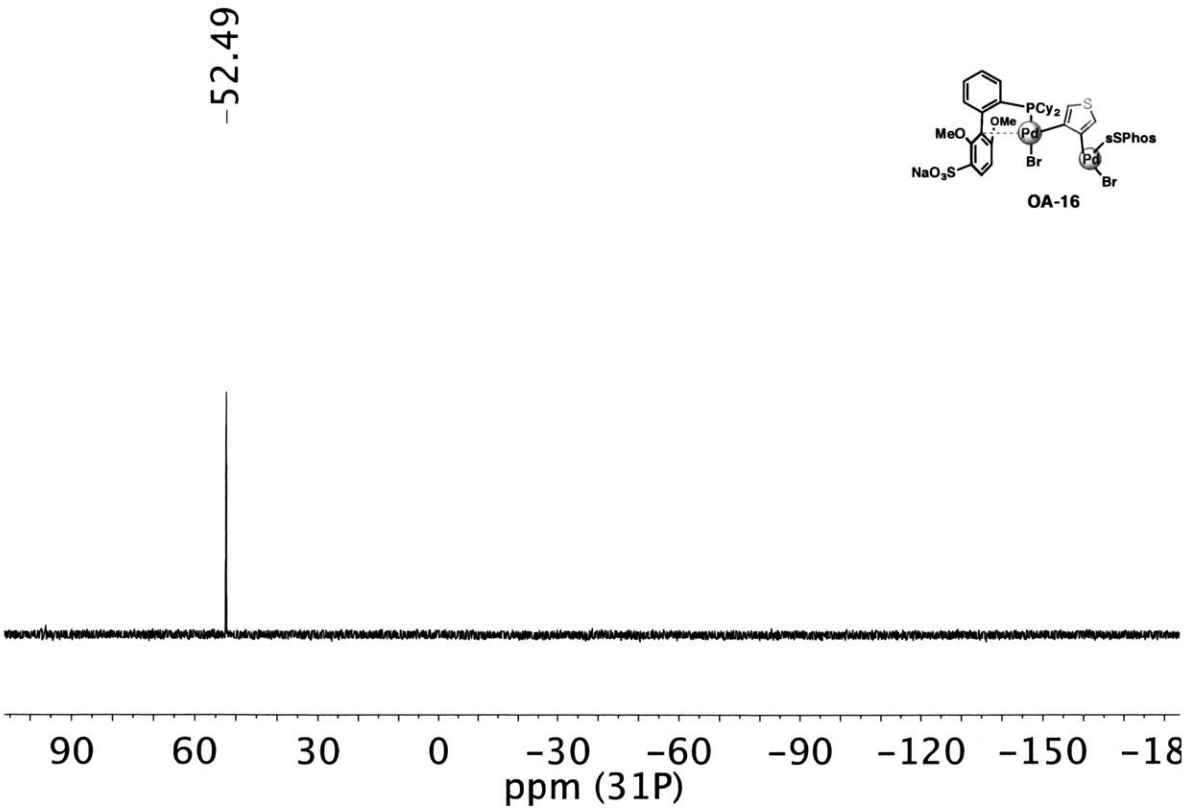

161.28
157.50
135.43
134.70
132.79
132.07
131.08
120.35
114.53
107.07
69.12
62.33
57.59
56.70
56.66
56.42
35.56
27.84
27.77
27.65
27.45
27.35
27.13
26.76
26.73
23.64
14.67
PCy2
OMe
sSPhos
MeO
Pd
Br
NaO3S
OA-14
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)

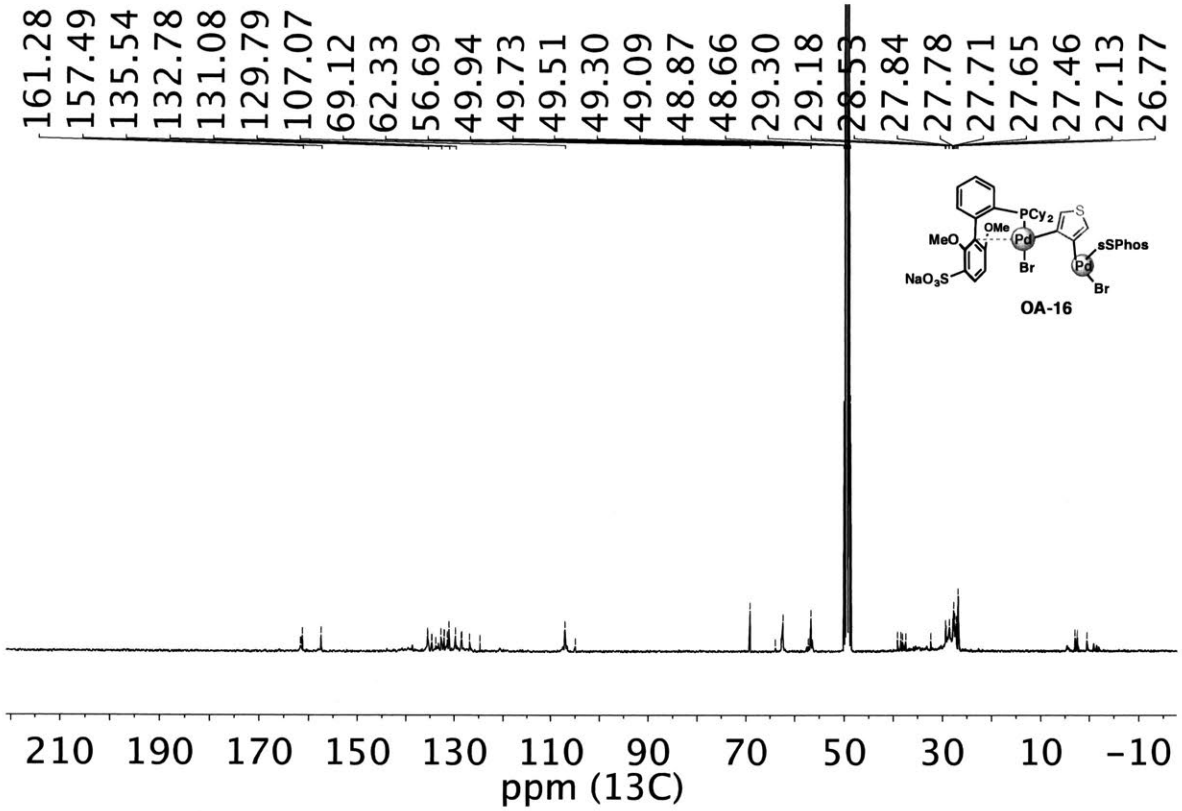

PCy2
OMe
sSPhos
MeO
Pd
Br
NaO3S
OA-14
8.0 6.5 5.0 3.5 2.0 0.5 -1.0 -2.5
ppm (1H)

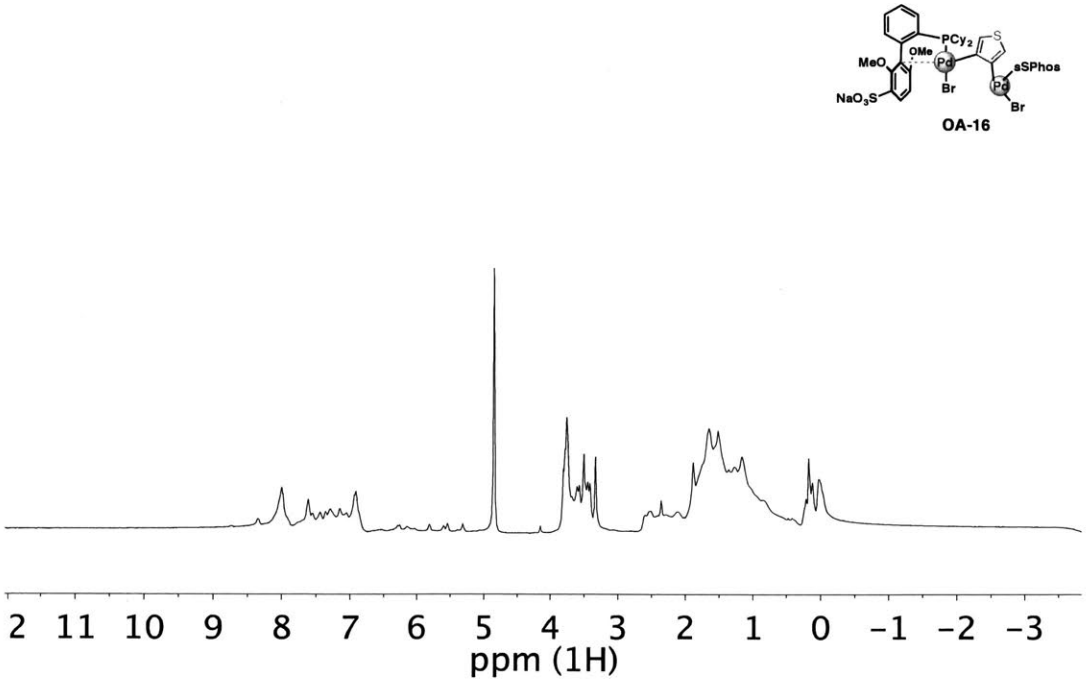

-51.29
OA-15
180 140 100 60 20 -20 -60 -100 -140 -18
ppm (31P)

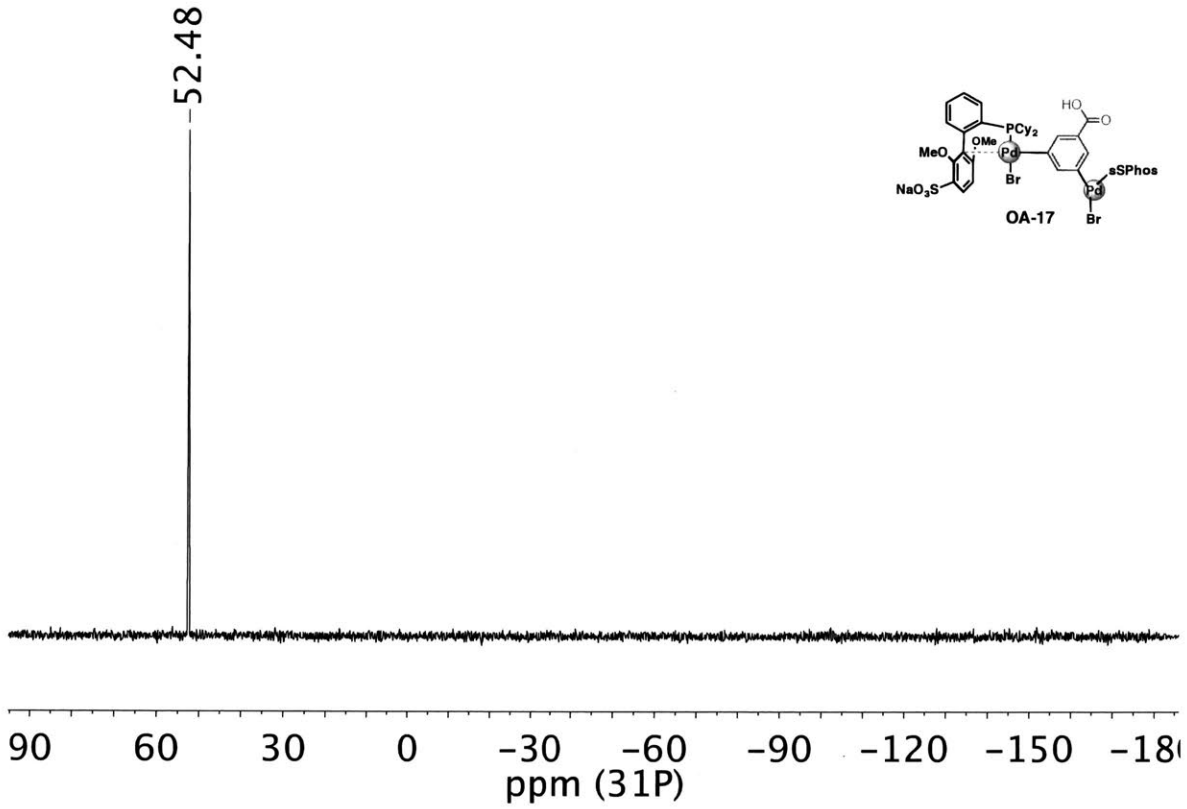

161.51
133.08
131.34
130.46
107.28
62.55
56.90
50.15
49.94
49.72
49.51
49.30
49.08
48.87
35.79
28.07
27.99
27.94
27.87
27.75
27.68
27.62
27.55
27.34
26.99
23.88
14.90
OA-15
170 150 130 110 90 70 50 30 10 -10
ppm (13C


OA-15
9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5 1.5 0.5 −0.5 −1.5
ppm (1H)


−52.49
OA-16
90 60 30 0 −30 −60 −90 −120 −150 −18
ppm (31P)


161.28
157.49
135.54
132.78
131.08
129.79
107.07
69.12
62.33
56.69
49.94
49.73
49.51
49.30
49.09
48.87
48.66
29.30
29.18
28.53
27.84
27.78
27.71
27.65
27.46
27.13
26.77
PCy2
S
OMe
MeO
Pd
Br
sSPhos
Pd
Br
NaO3S
OA-16
210 190 170 150 130 110 90 70 50 30 10 -10
ppm (13C)


PCy2
S
OMe
MeO
Pd
Br
sSPhos
Pd
Br
NaO3S
OA-16
2 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3
ppm (1H)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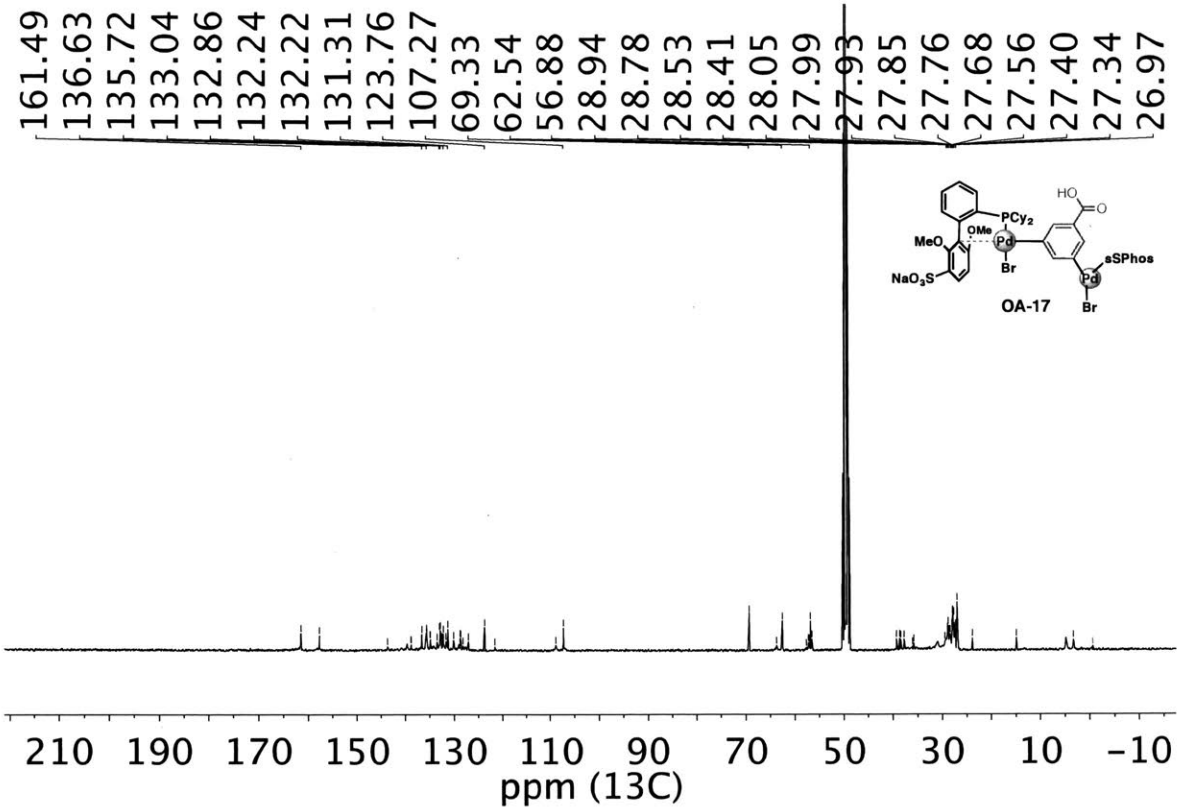
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PCy2
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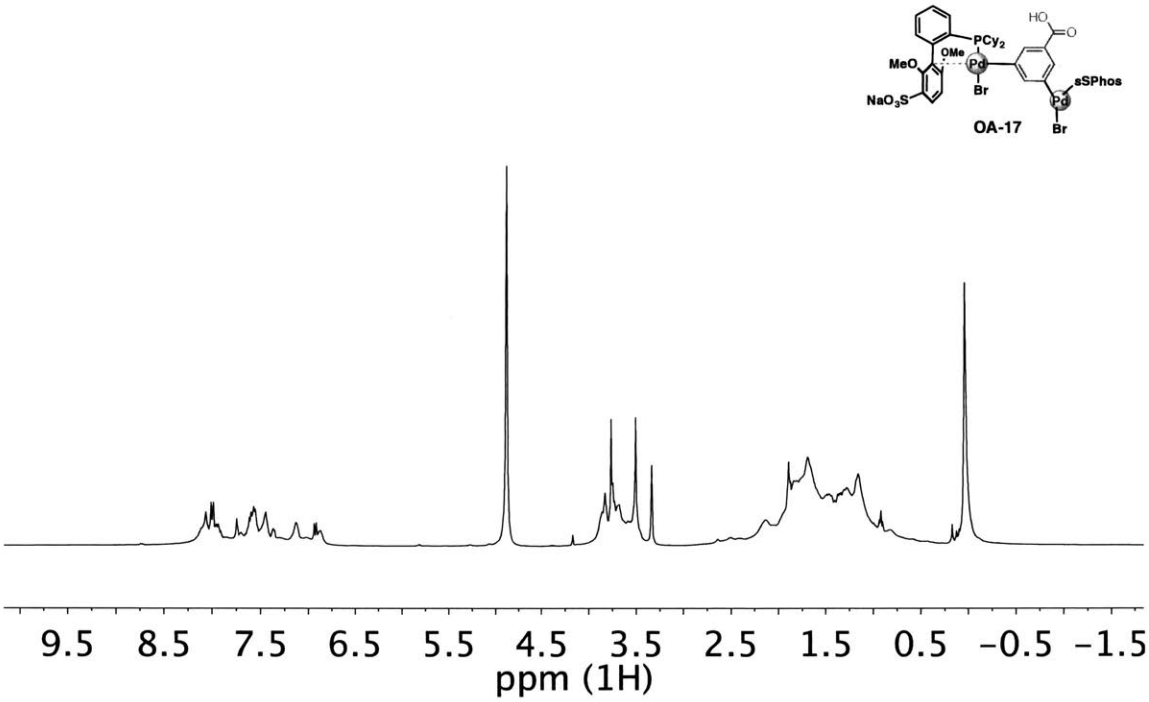
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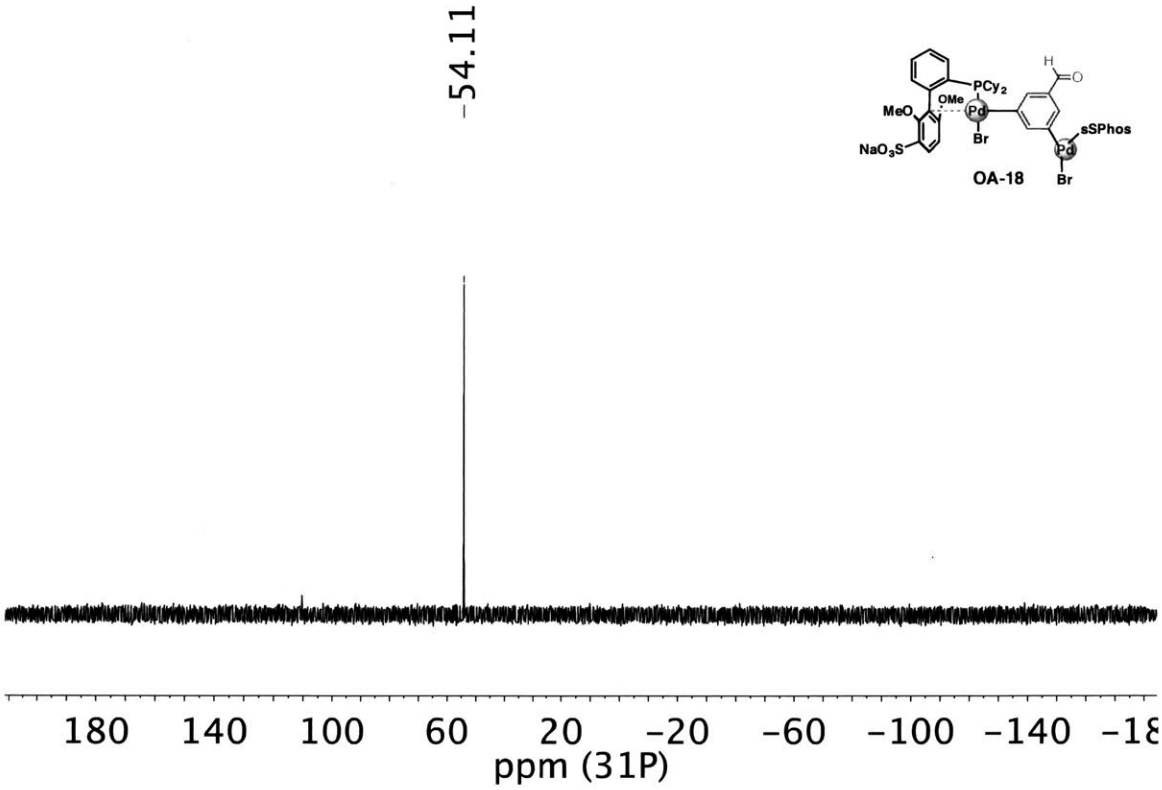
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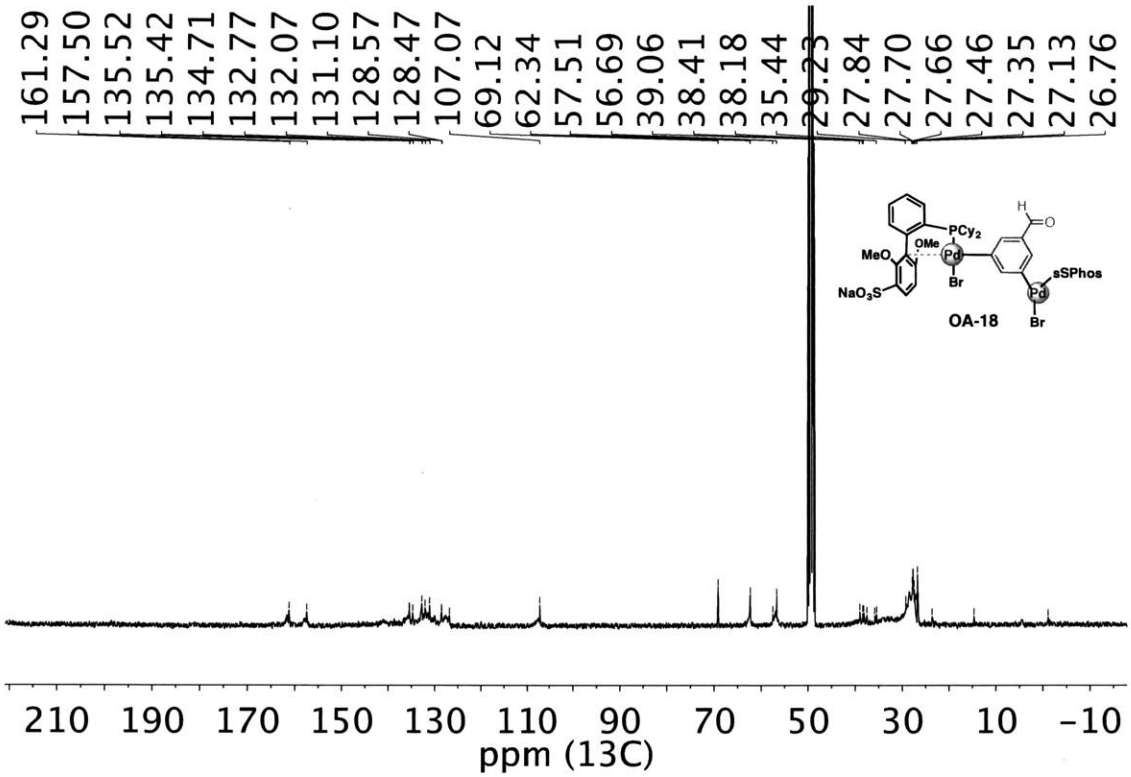
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27.66
27.46
27.35
27.13
26.76
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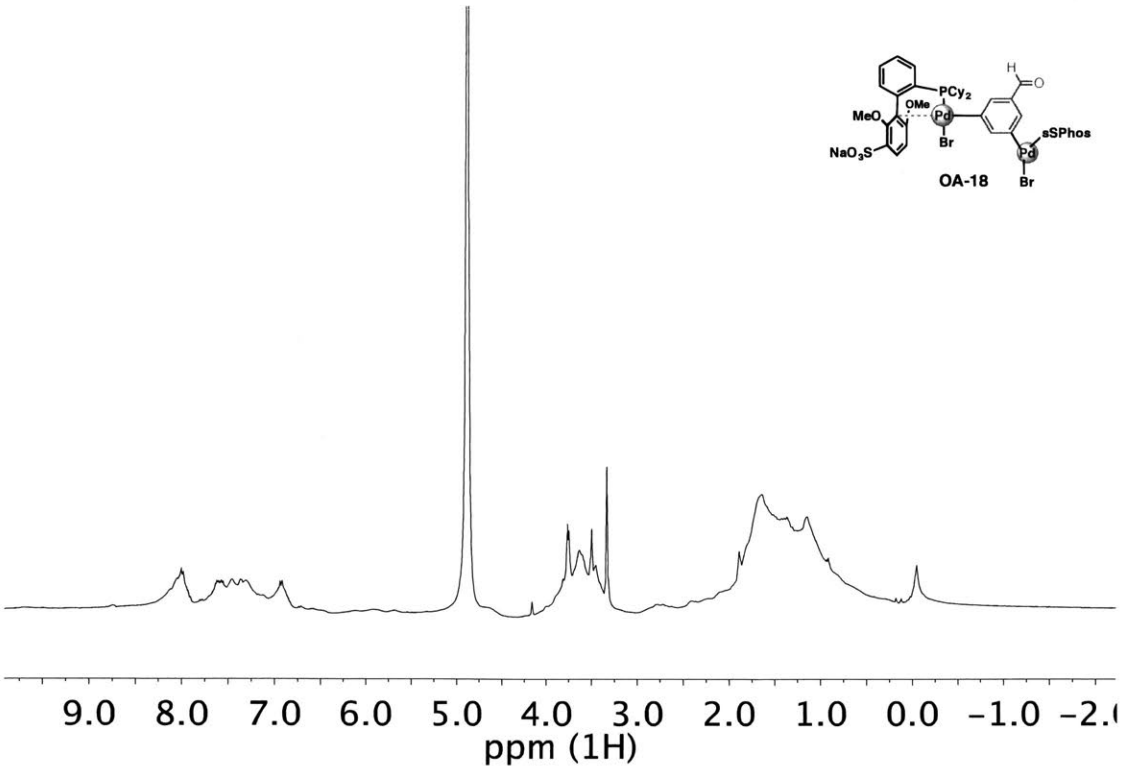
ppm (13C)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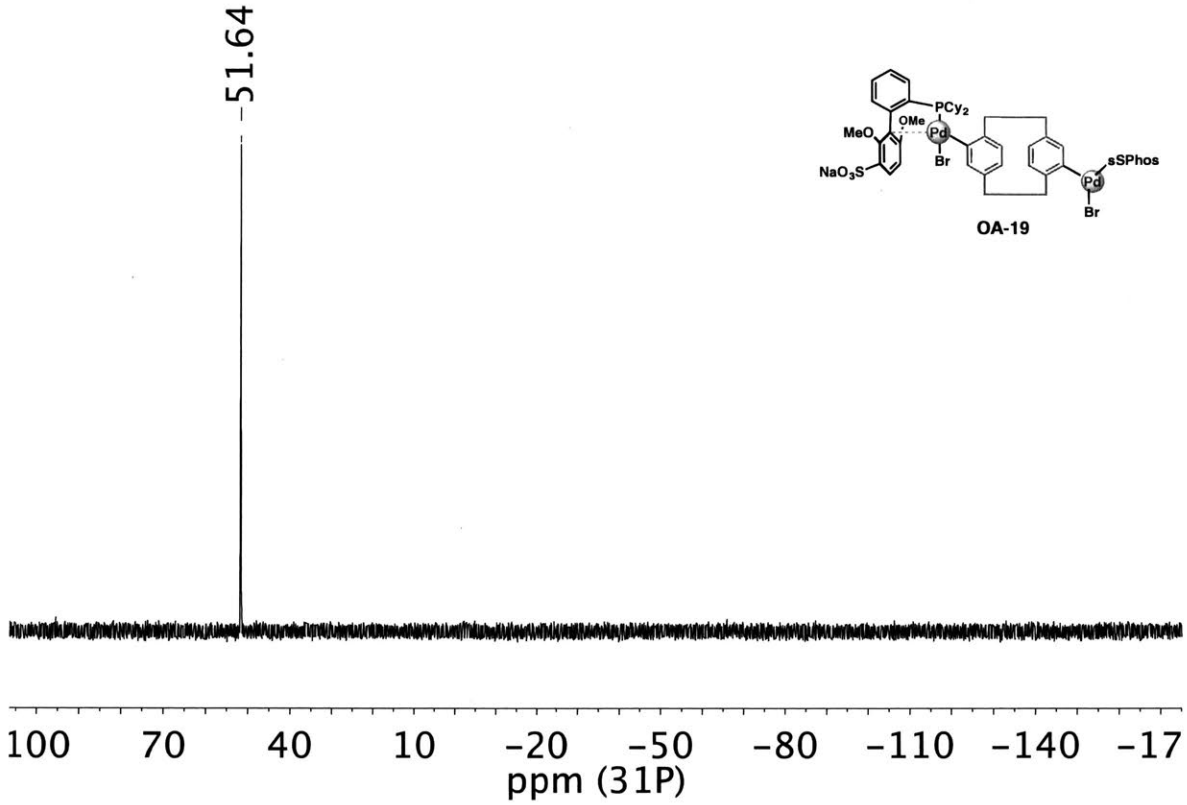
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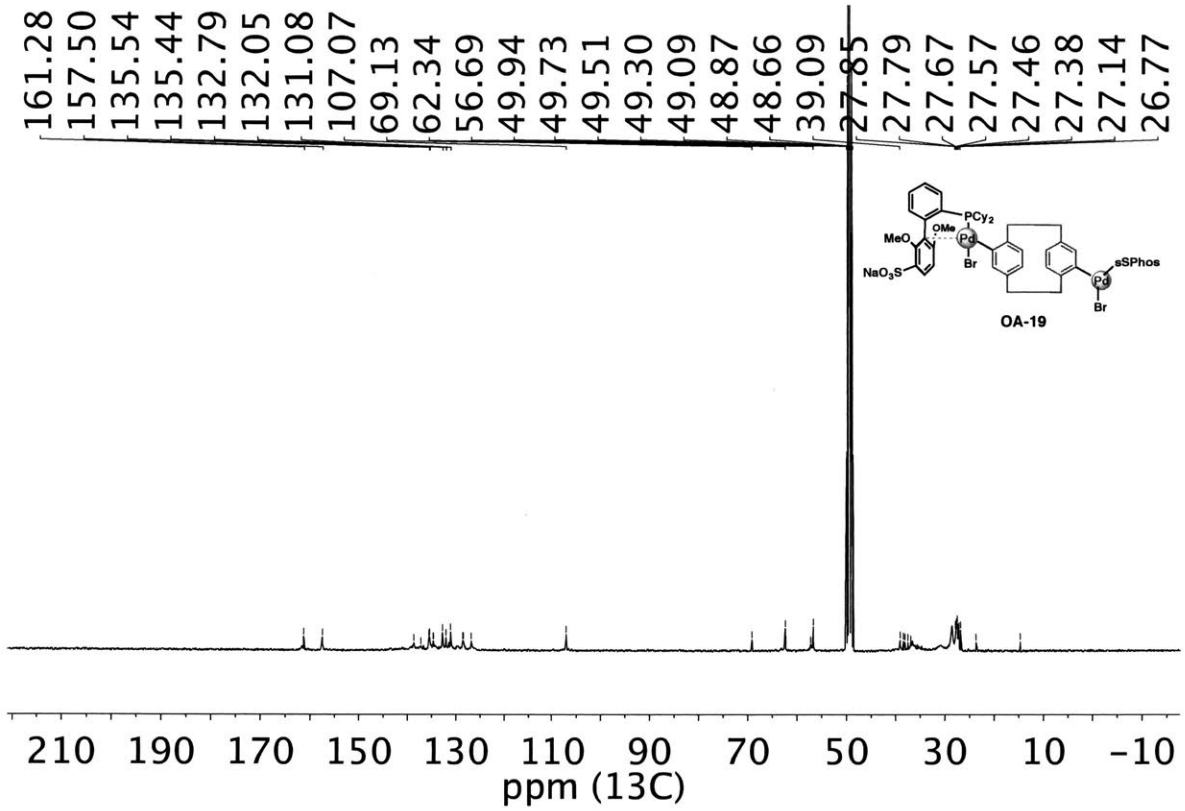
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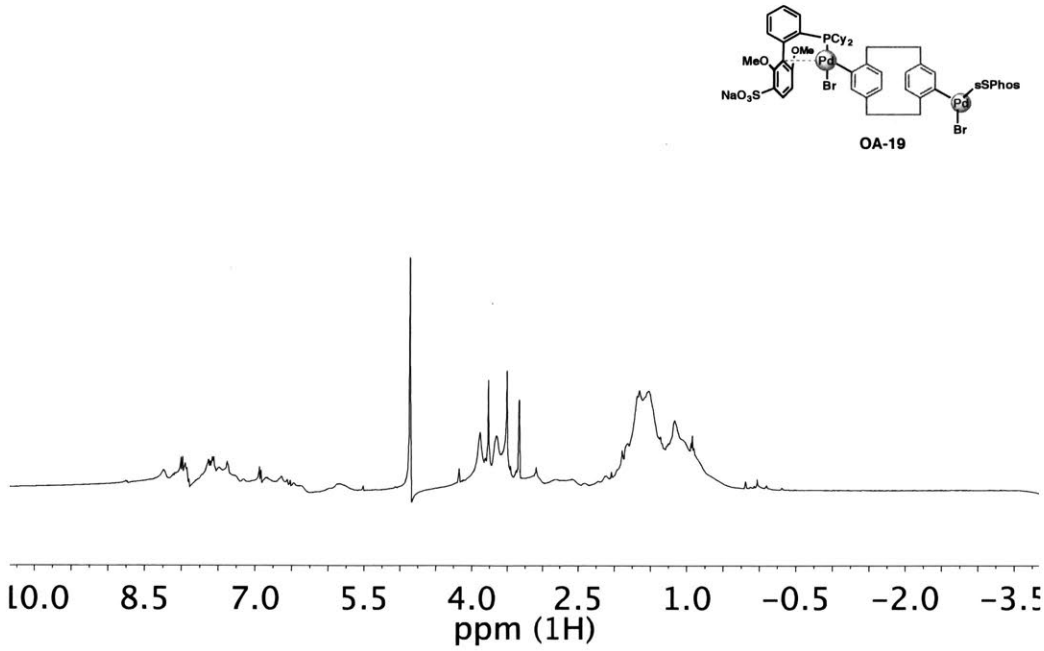
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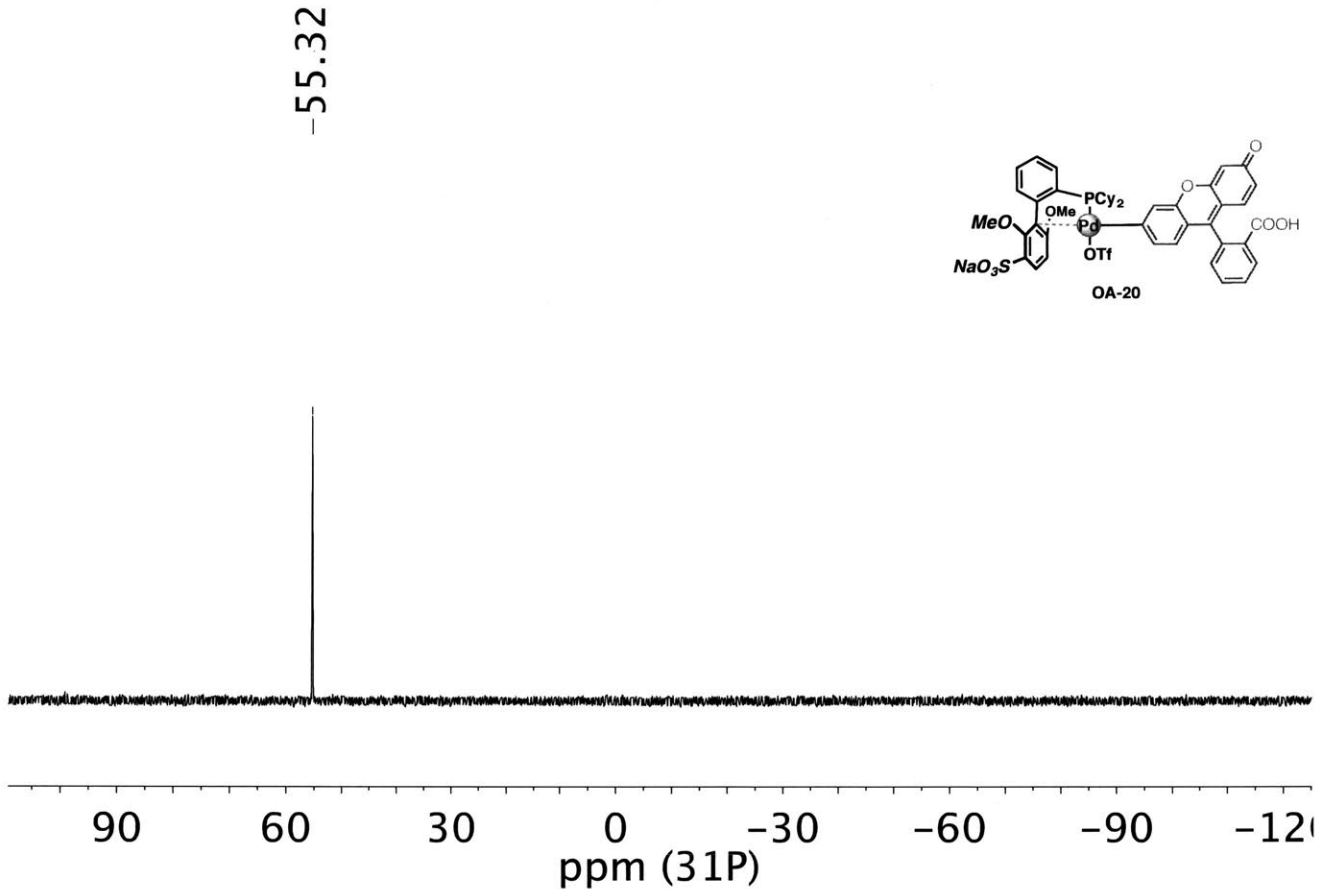
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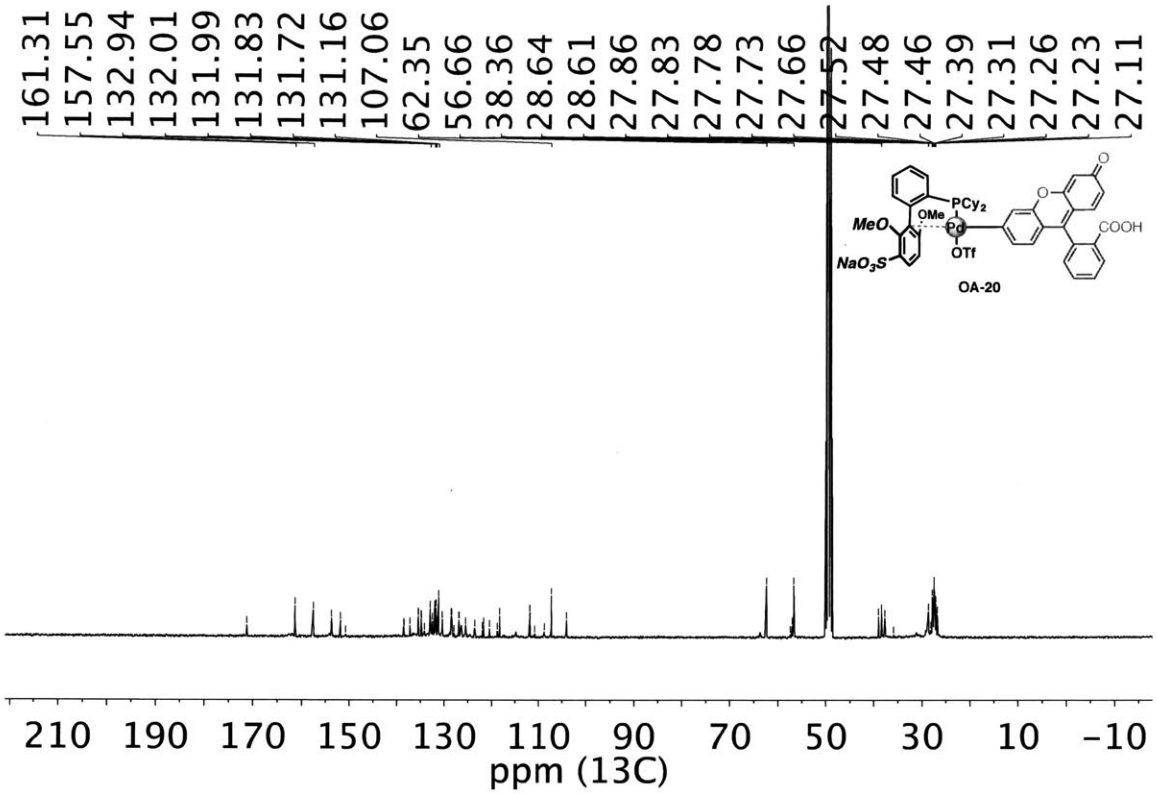
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27.39
27.31
27.26
27.23
27.11
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OTf
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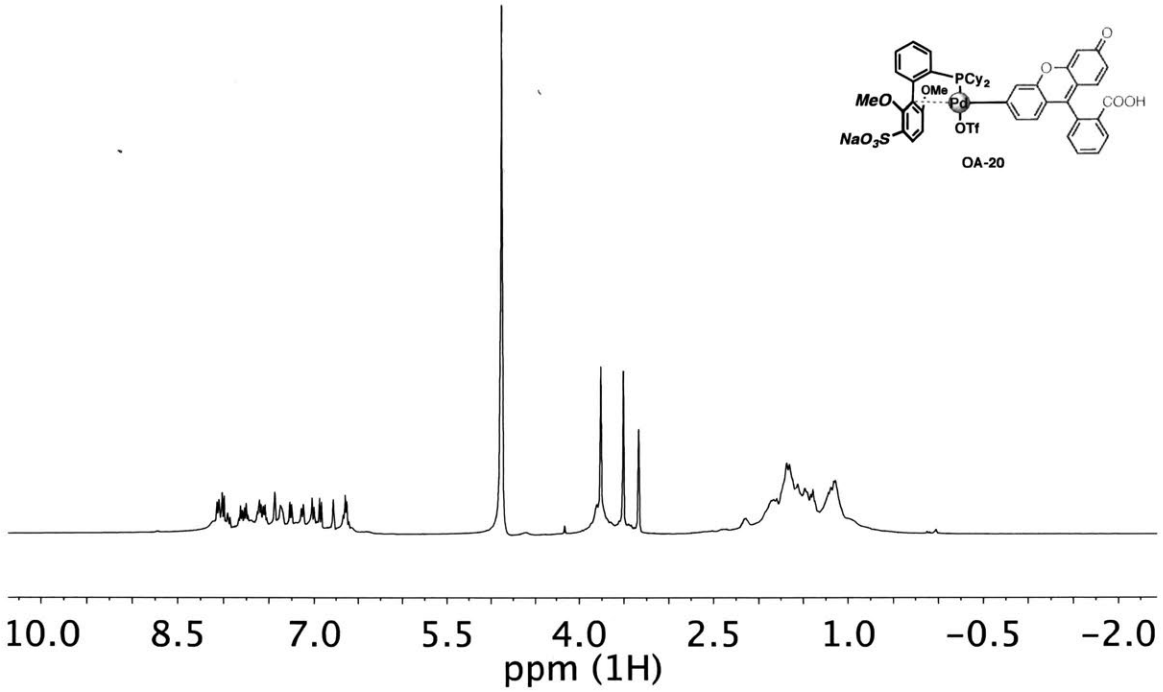
ppm (13C)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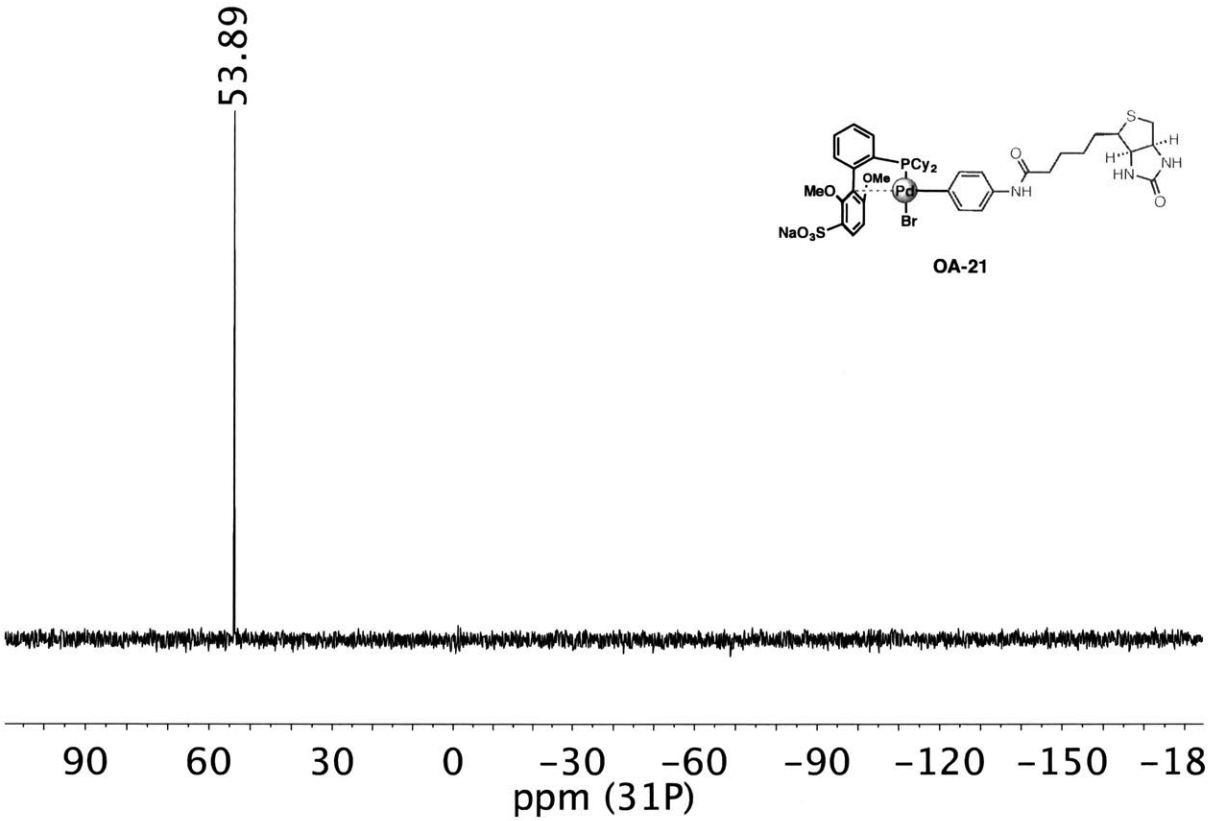
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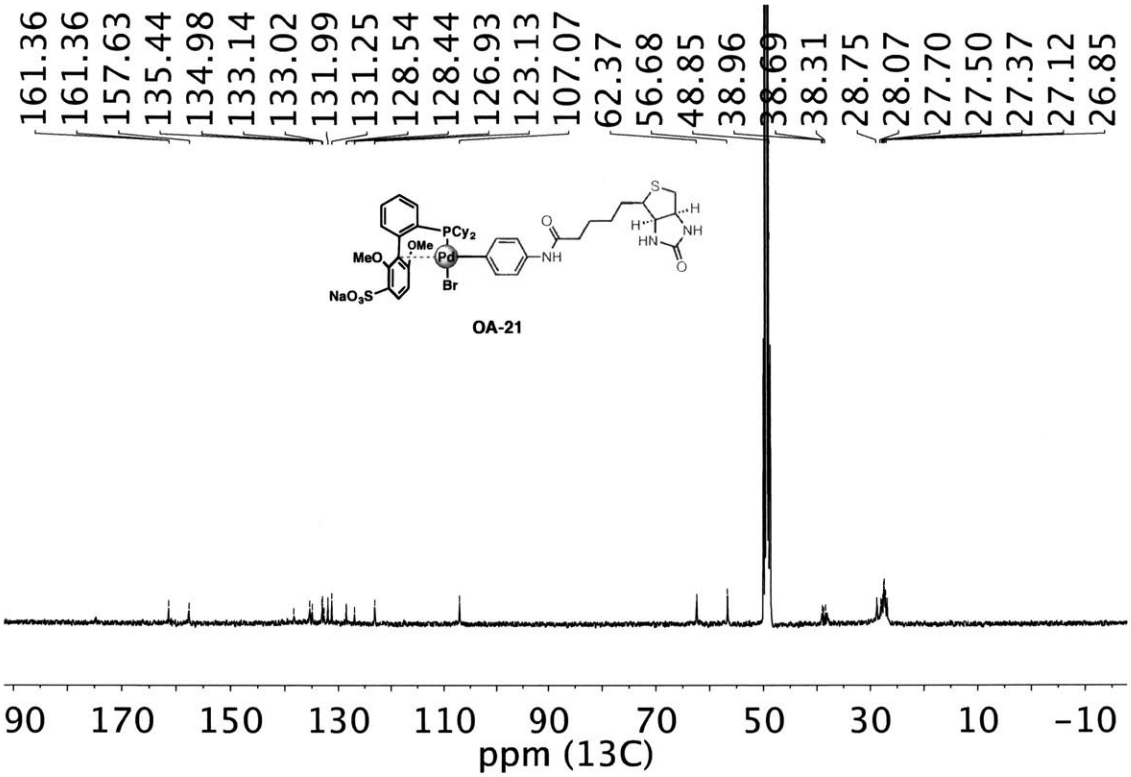
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PCy2
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Pd
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NaO3S
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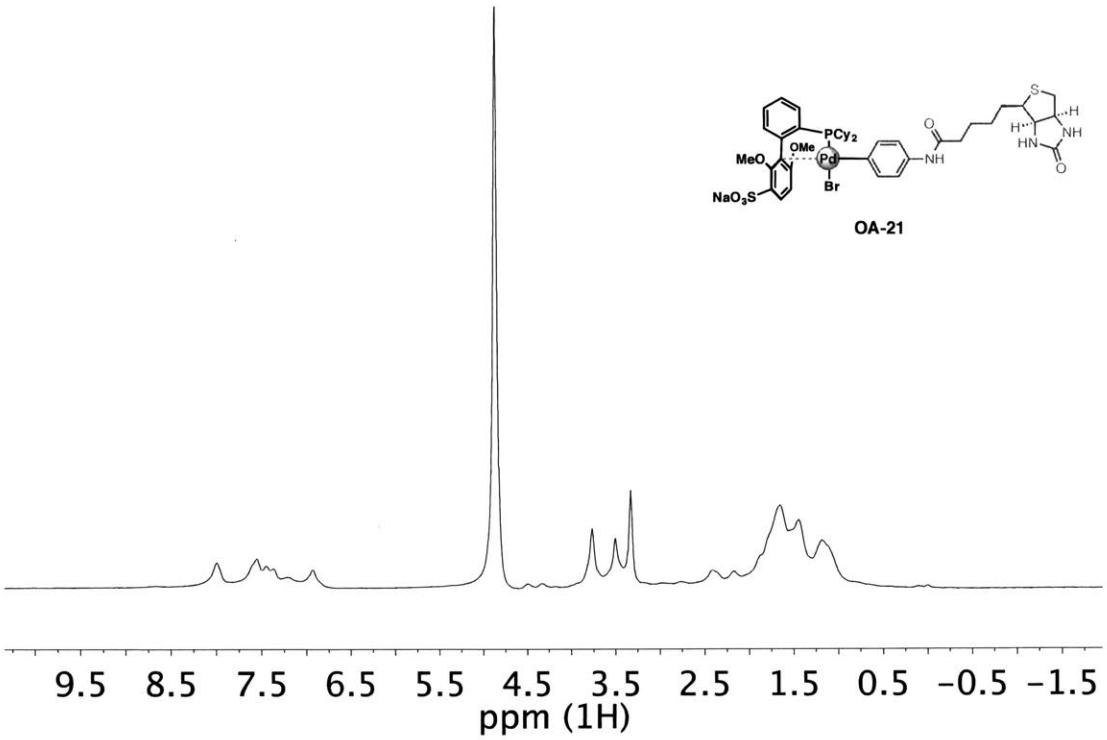
ppm (13C)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Pd
Br
NaO3S
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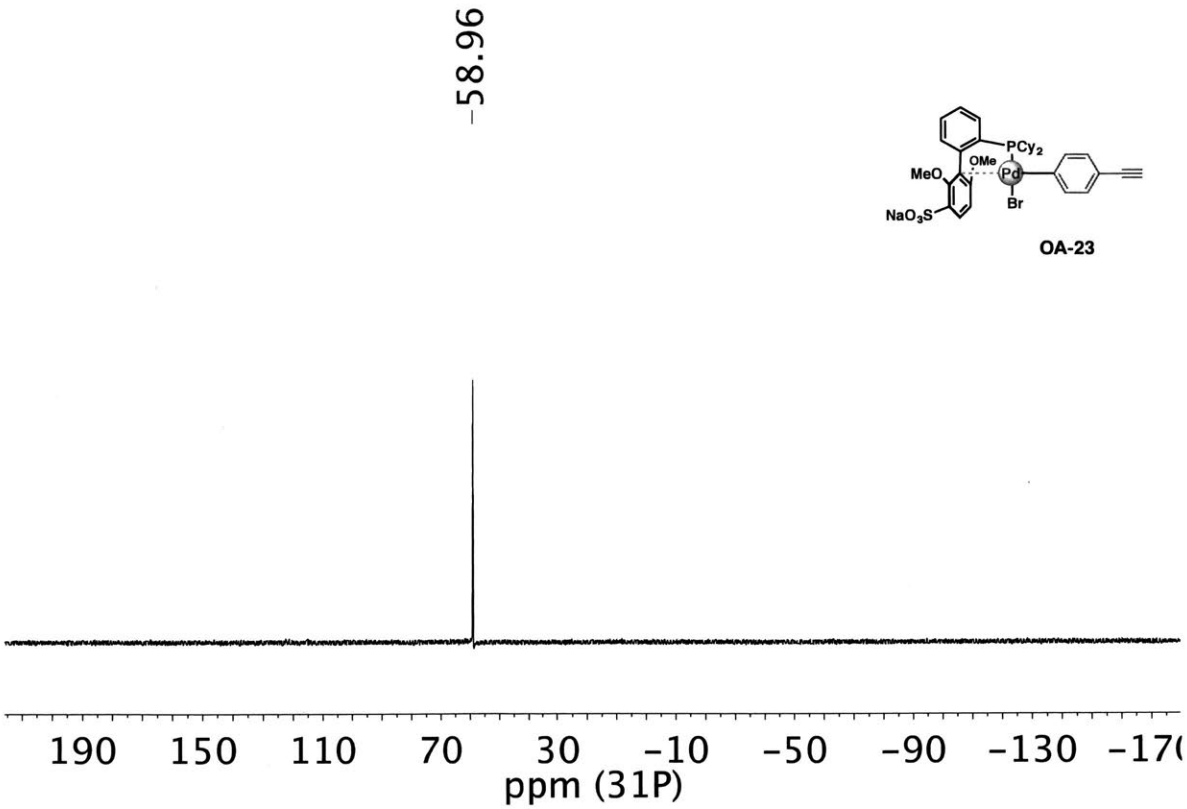
HN
O

OA-21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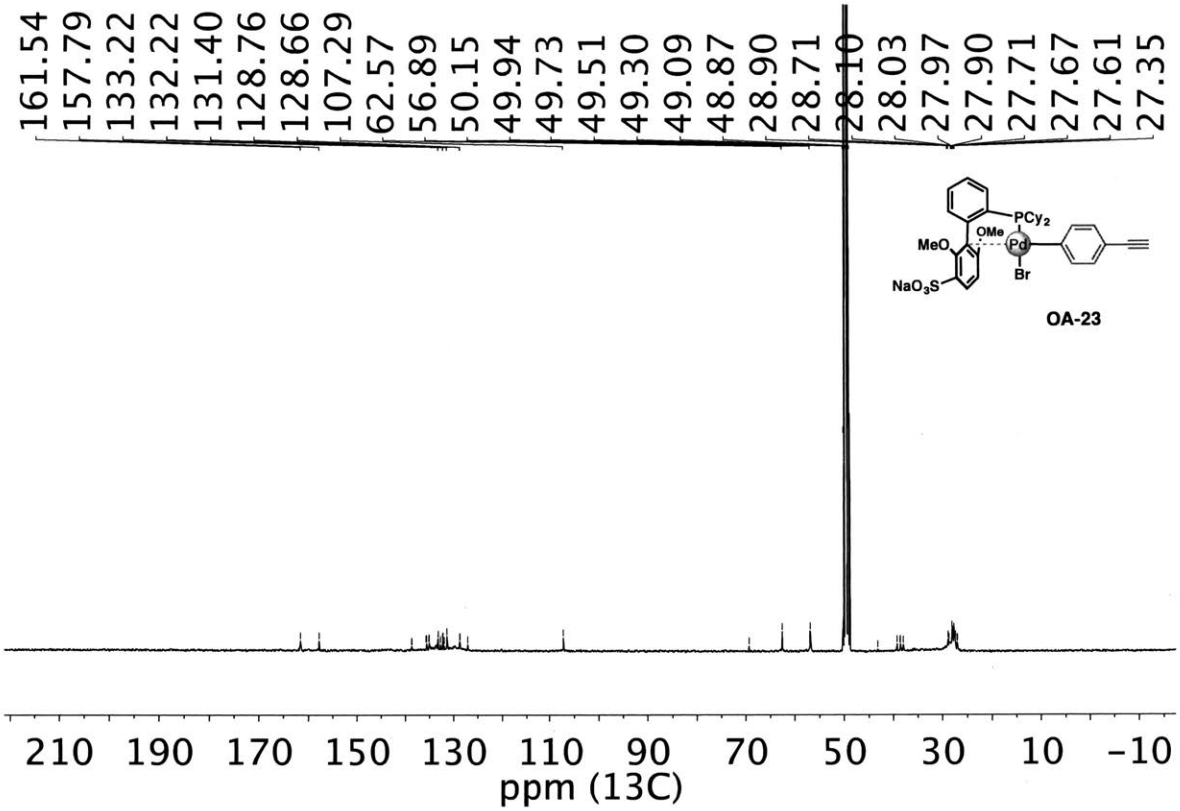
Pd
Br
NaO3S

OA-23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27.71
27.67
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MeO
Pd
Br
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OA-23
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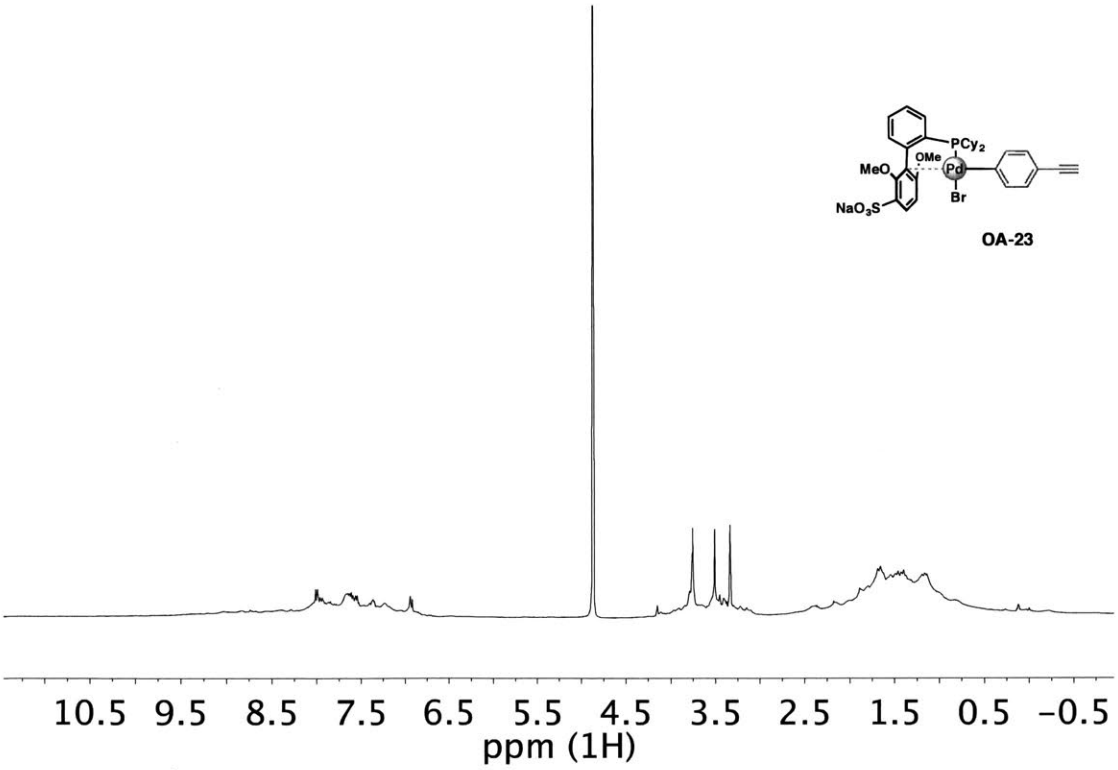
ppm (13C)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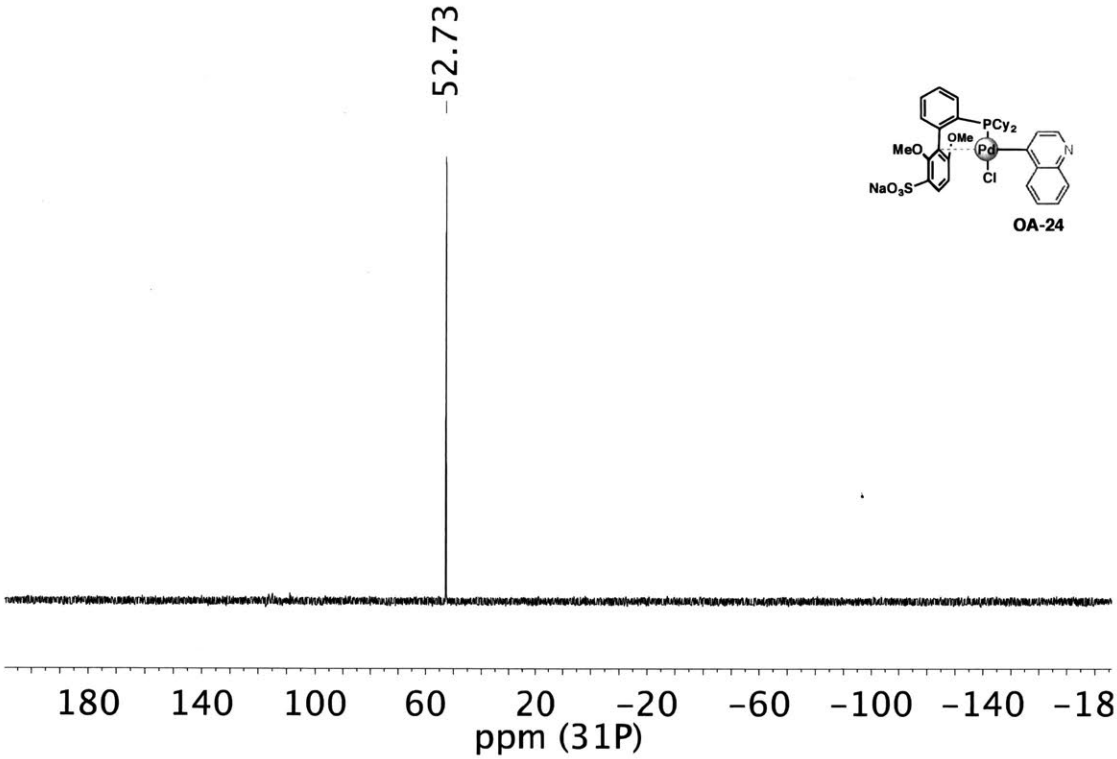
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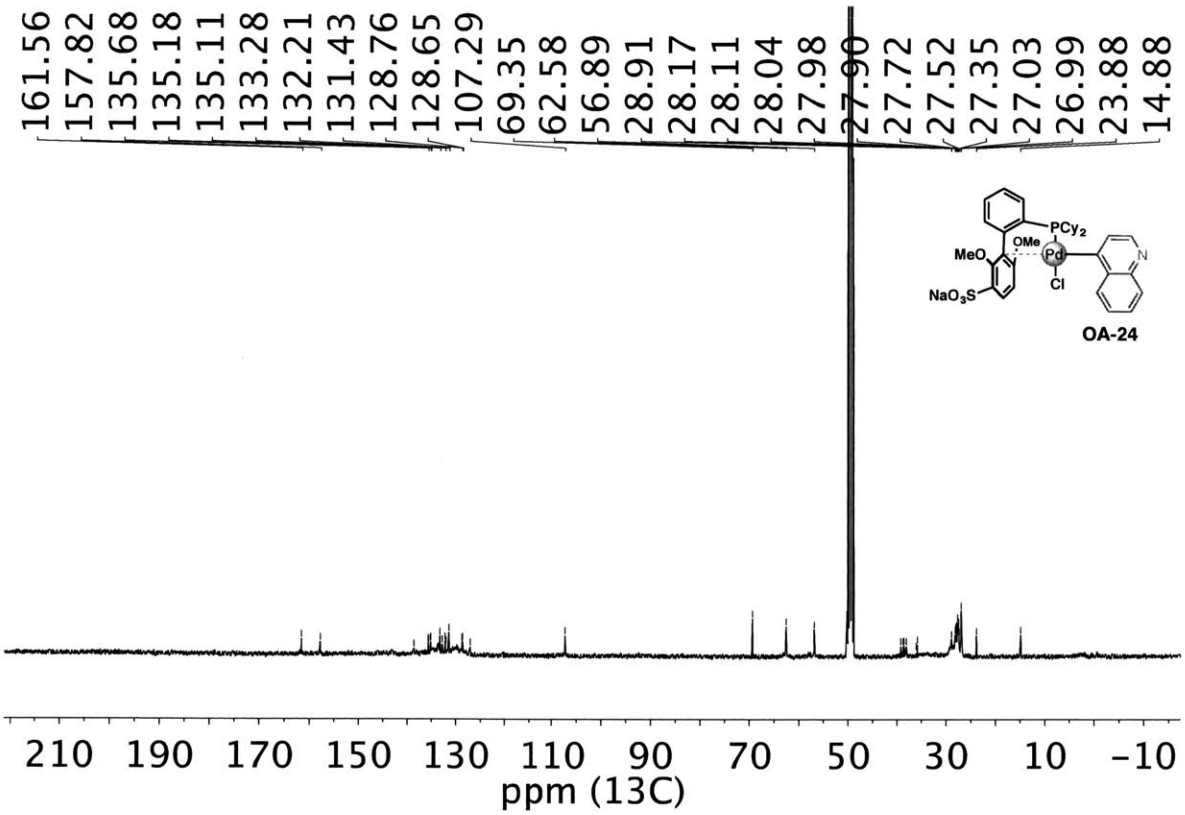
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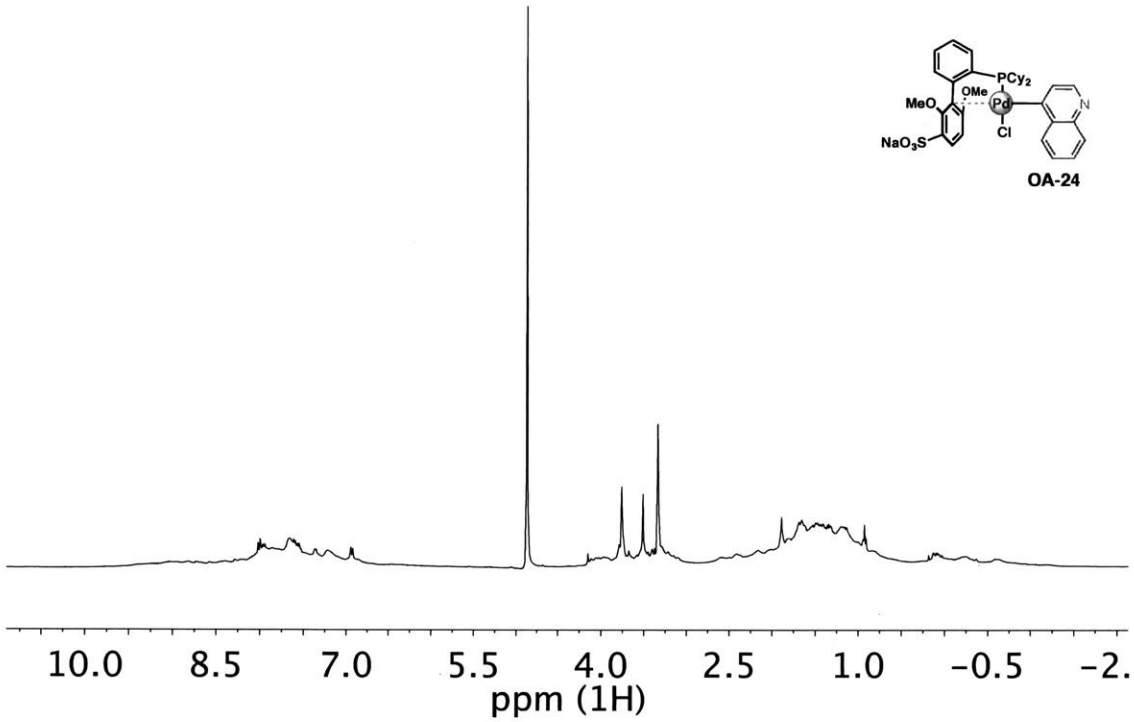
ppm (13C)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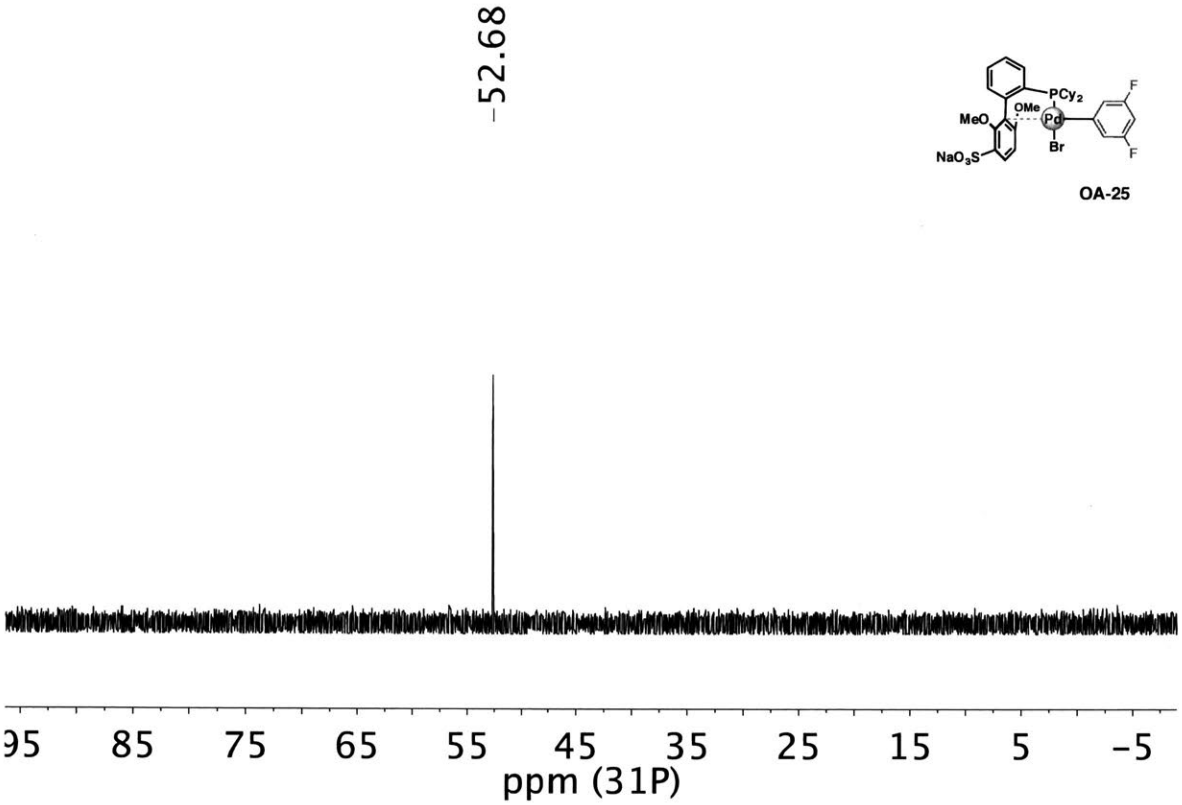
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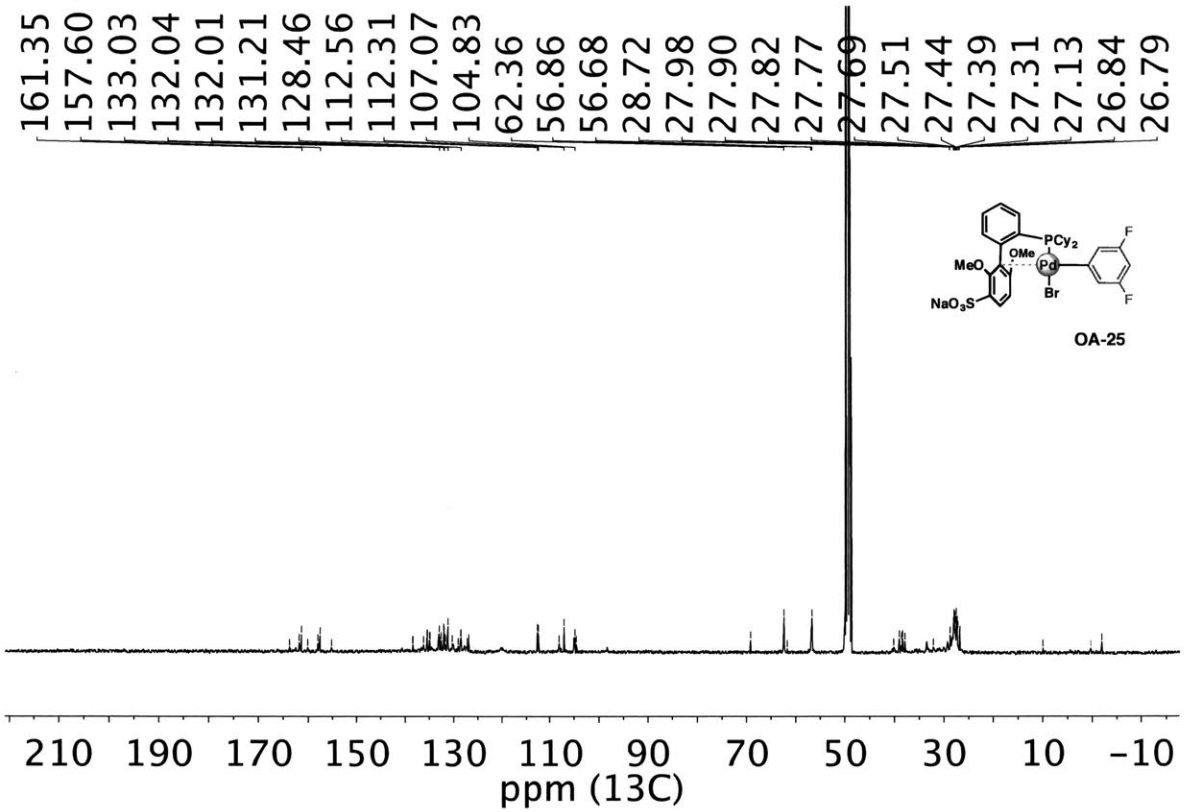
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27.31
27.13
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26.79
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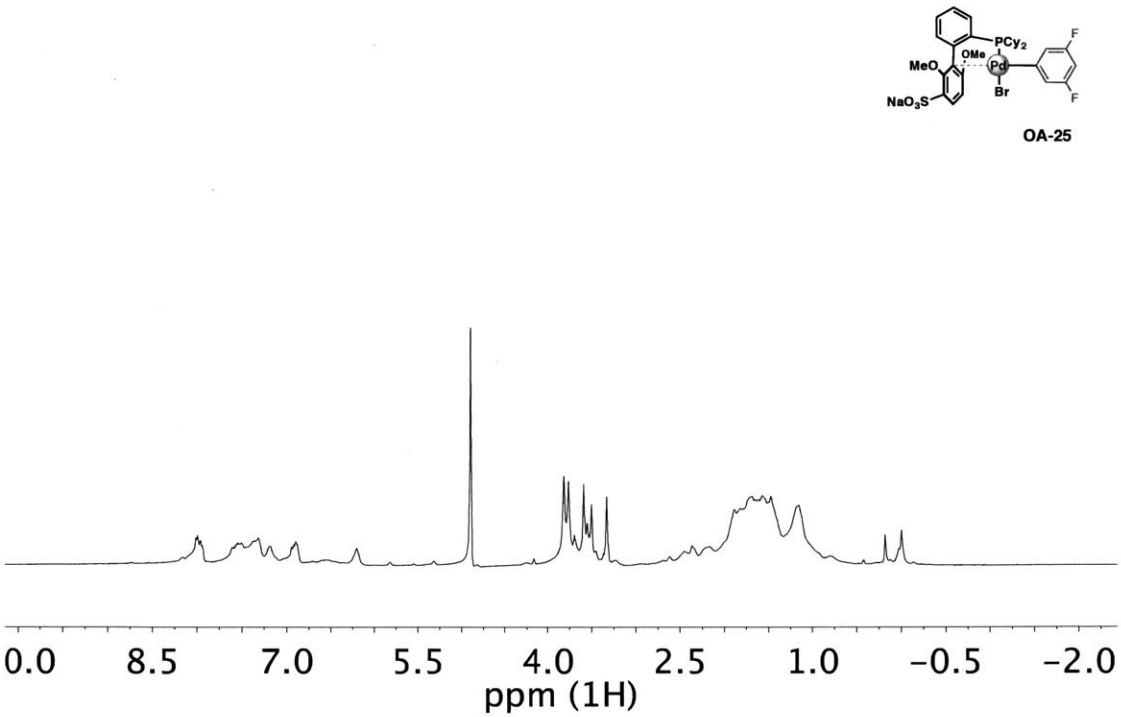
ppm (13C)


PCy2
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MeO
Pd
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OA-25
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ppm (1H)

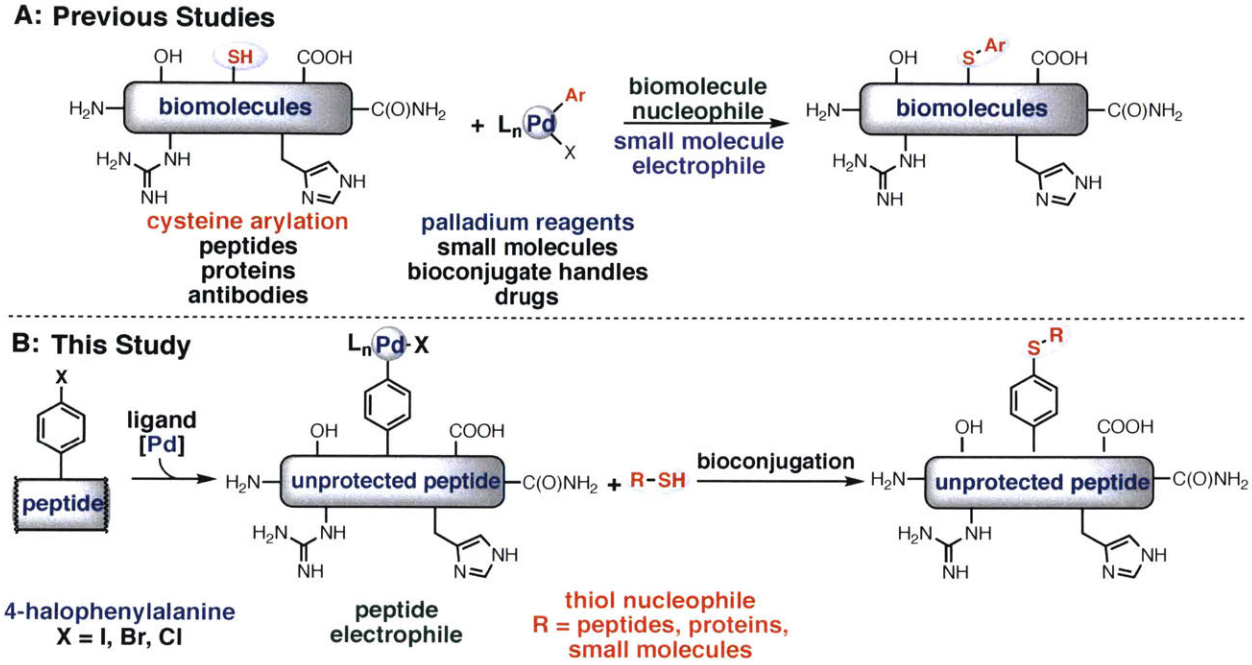
Chapter 3. Palladium−Peptide Oxidative Addition Complexes for Bioconjugation

3.1 – Introduction:

Reactions that modify complex biomolecules enable researchers to synthesize molecularly defined conjugates, probe structure–function relationships, and develop new therapeutics.[1,2] In particular, direct chemoselective bioconjugation methods are desirable because their ability to target specific functional groups in the presence of many others obviates the need for protecting groups or extraneous synthetic manipulations during the synthesis of large biomolecules.[3] Bioconjugation reactions must also satisfy a number of strict criteria to be considered practical: the reaction should be effective at low concentrations, proceed in aqueous solutions, generate kinetically stable linkages, and produce, at most, minor side products.[4,5] In particular, ligations between two large biomolecules are typically conducted at low micromolar concentrations, therefore, bioconjugation reactions must have kinetic rate constants large enough to achieve near-quantitative conversions on experimental timescales.[6]

Unfortunately, many of the widely used bioconjugation reactions are still lacking in one or more of these regards.[1,7] For instance, thiol–maleimide conjugation reactions, while effective, are known to generate chemically unstable linkages.[8] Moreover, even relatively fast reactions such as Staudinger reactions,[9] oxime ligations,[10] and copper-catalyzed azide–alkyne cycloadditions,[11] generally fail to be effective at low micromolar concentrations.[6] One noteworthy approach, which achieves the exceedingly high rates required, is the pericylic reaction between tetrazines and strained alkenes (e.g., *trans*-cyclooctenes). While impressive, the performance of the reaction is highly dependent on the nature of the tetrazine component.[12]

Figure 5: Strategies for bioconjugation mediated by palladium oxidative addition complexes. (A) Previous reports of cysteine *S*-arylation with palladium–small molecule oxidative addition complexes.[13] (B) This report of peptide bioconjugation achieved through the generation of palladium–peptide oxidative addition complexes.



Recently, transition metal-mediated transformations have emerged as effective tools in bioconjugation.[14–17] Transition metals are widely used in organic synthesis for highly selective and efficient transformations. Drawing from extensive research in this context, we have been able to develop bioconjugation reactions that occur rapidly, exhibit excellent chemoselectivity and proceed under the mild conditions necessary for use on biomolecules.[18] Recently, our laboratories reported palladium oxidative addition complexes that rapidly and chemoselectively modify cysteine,[13] a naturally occurring amino acid (Figure 1A). With appropriate ligand modifications, this reaction was found to take place under fully aqueous conditions while maintaining exclusive selectivity for reaction with cysteine over other nucleophilic amino acids such as lysine.[19]

As an alternative to small molecule-bearing palladium reagents that functionalize nucleophilic peptides, a complementary approach was envisioned wherein a peptide-

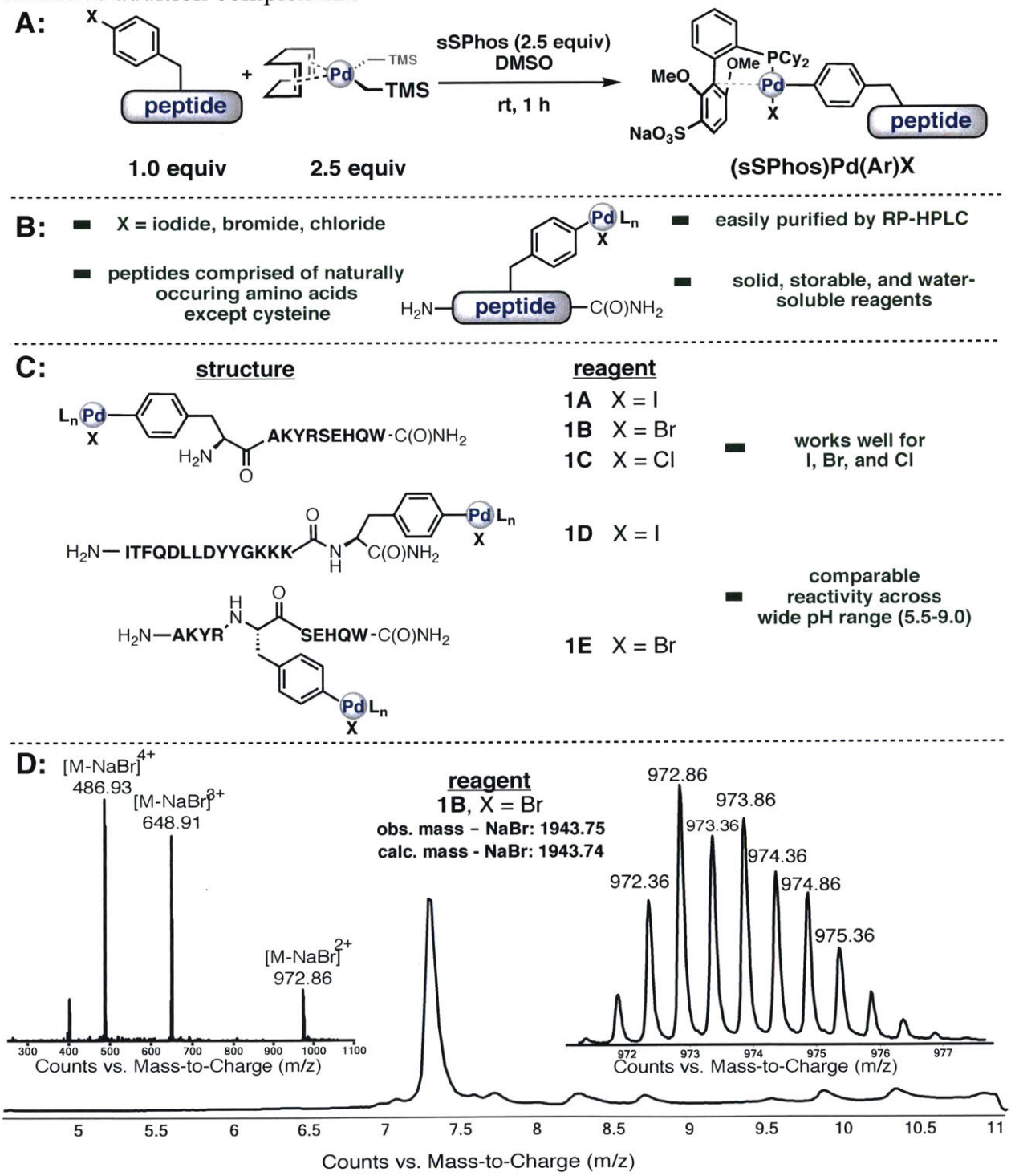
bound palladium reagent could engage a diverse array of external nucleophiles (Figure 1B). We reasoned that reversing the roles of the components would enable a peptide to be conjugated to a wide array of partners, including small molecules, peptides, or proteins, via the synthesis of a single palladium–peptide complex.

3.2 – Results and Discussion:

To synthesize a palladium-peptide reagent, we first incorporated 4-bromophenylalanine into the primary sequence of a peptide during solid-phase peptide synthesis. Next, the purified unprotected peptide was treated with a water-soluble ligand (sSPhos) and (1,5-COD)Pd($CH_2TMS)_2$ in dimethylsulfoxide (DMSO) (Figure 2A). Surprisingly, our first attempt afforded the corresponding palladium–peptide oxidative addition complex in good yield. Moreover, the synthesis did not require specialized air-free Schlenk techniques or the use of an inert-atmosphere glovebox. The reagents are all commercially available and easily handled on the bench. Additional experiments revealed that the reaction could proceed using peptides containing 4-iodophenylalanine and 4-chlorophenylalanine.

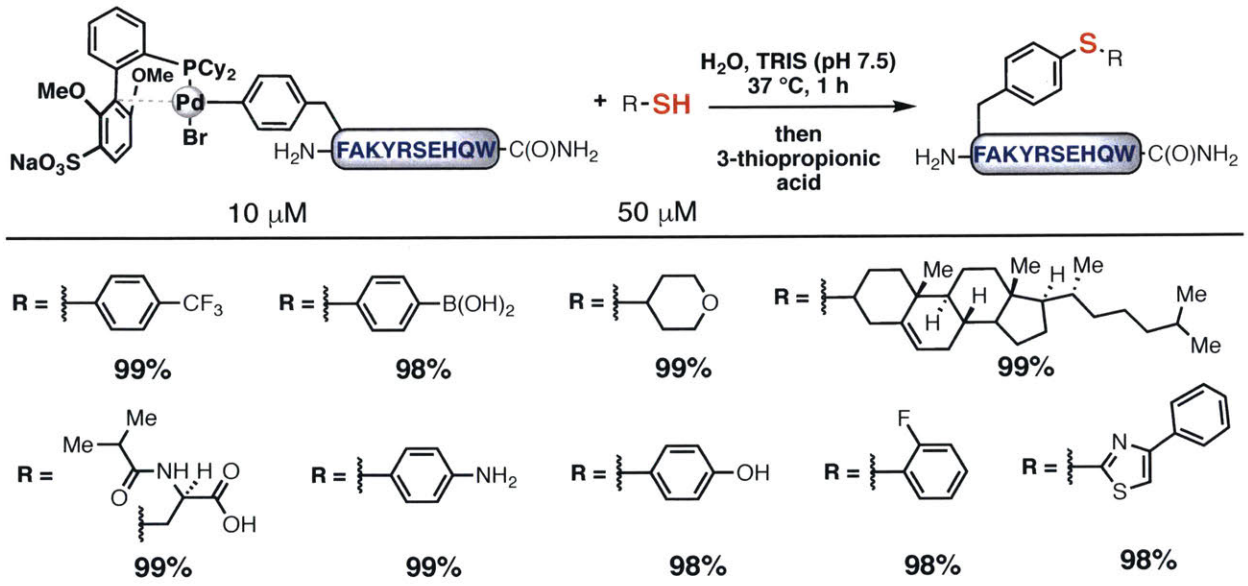
The resulting palladium oxidative addition complexes are exceptionally stable. When left under ambient conditions, they do not degrade for up to 12 months (Figure 2D, Experimental). The complexes can be purified via reverse-phase high-performance liquid chromatography (RP-HPLC) using water/acetronitrile mobile phases containing 0.1% (v/v) trifluoroacetic acid. Regardless of the position of the 4-halophenylalanine in the peptide, the palladium–peptide complexes can be prepared efficiently (Figure 2C). Most importantly, the presence of other nucleophilic or basic residues (except cysteine) do not hinder the oxidative addition process.

Figure 6: Synthesis and characterization of palladium–peptide oxidative addition complexes is facile and robust. (A) General procedure for the generation of palladium–peptide complexes. (B) Palladium–peptide complex formation is efficient for a variety of peptides containing 4-halophenylalanines. (C) Palladium–peptide oxidative addition complexes are successfully synthesized regardless of the position of the 4-halophenylalanine along the peptide backbone. (D) LC-MS analysis of palladium–peptide oxidative addition complex **1B**.



Initially, we sought to apply these complexes toward late-stage side-chain functionalization, a potentially powerful strategy for the rapid modification of unprotected peptides. To evaluate the feasibility of this method, the oxidative addition complex, **1B**, was mixed with 5.0 equivalents of a small-molecule thiol in low micromolar concentration in aqueous tris(hydroxymethyl)aminomethane (TRIS, pH 7.5) buffer at 37 °C. After only 30 min, we observed the expected site-selective modification of the aryl side-chain in excellent conversions using 9 distinct thiols (Scheme 1). Moreover, the reactivity was maintained across a wide range of aqueous buffered conditions (pH 5.5–9.0, see Experimental for details).

Scheme 1: Palladium–peptide reagents react efficiently with small molecule thiols[a]. [a]Yields determined by LC-MS analysis of the crude mixture as an average of two runs, see SI for details.



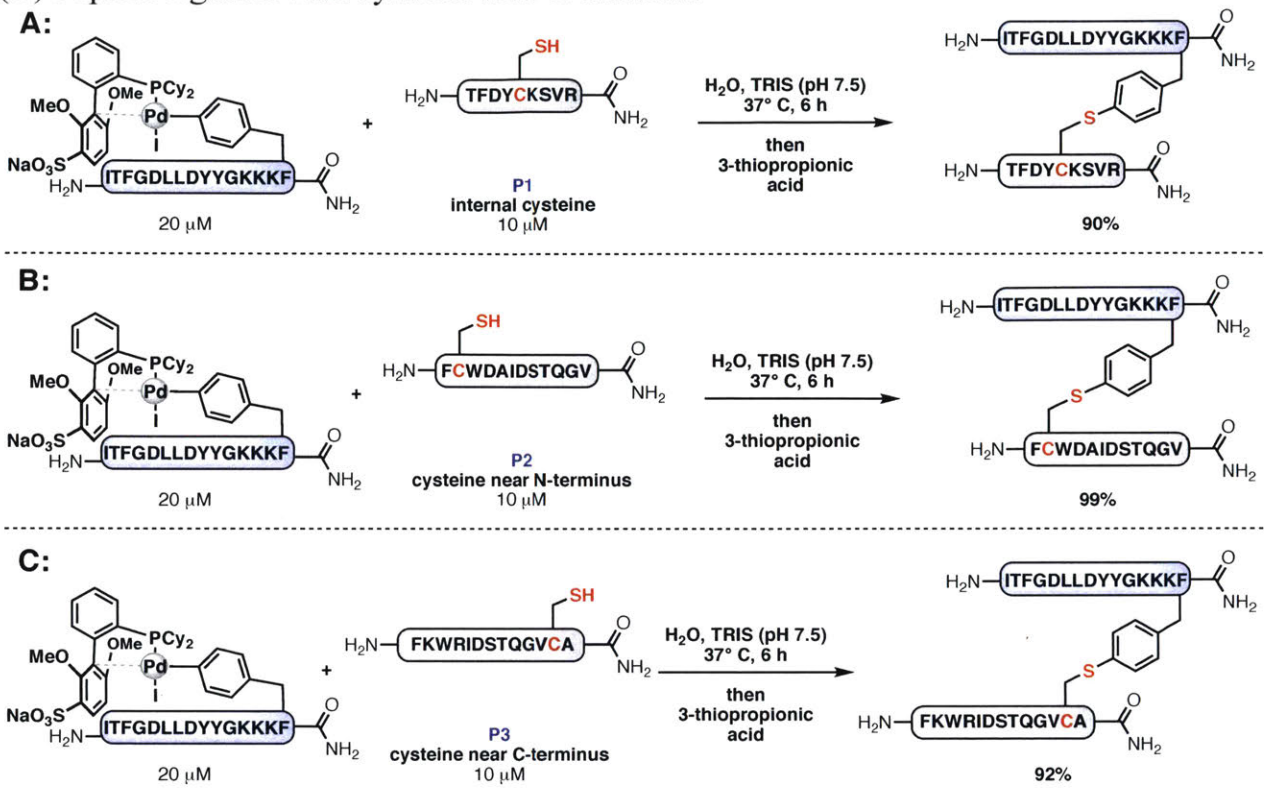
In addition to ligation with small molecules, we envisioned that these palladium–peptide complexes might be useful in ligation with other peptides. Peptide–peptide conjugates are important both for studying the function of biomolecules and developing new biotherapies for disease treatment.[20] Among others, leading chemical methods for peptide ligation include native chemical ligation,[21] enzyme-mediated ligation,[22] and

protein-templated ligation.[23] However, efficient conjugation of two large biomolecules remains an outstanding challenge, with many ligation reactions requiring organic co-solvents or failing at low micromolar concentrations. We reasoned that our palladium–peptide reagents might allow for rapid, irreversible ligation with a variety of cysteine-containing peptides. While the resulting linkages will not be native to proteins that occur in nature, proteins have been shown to retain their function even with the introduction of non-native backbones.[24]

Using automated flow-based peptide synthesis,[25] three peptides (**P1**, **P2**, and **P3**) were prepared containing a single cysteine residue at different locations along the primary amino acid sequence. The peptides were dissolved independently in aqueous TRIS (pH 7.5) buffer containing oxidative addition complex **1D**. After 6 h at 37 °C, 3-thiopropionic acid was added to the mixture to quench the reaction. Analysis of the reaction mixtures by LC-MS indicated that all of the peptide ligations proceeded with both high conversions and selectivity under these conditions (Scheme 2).

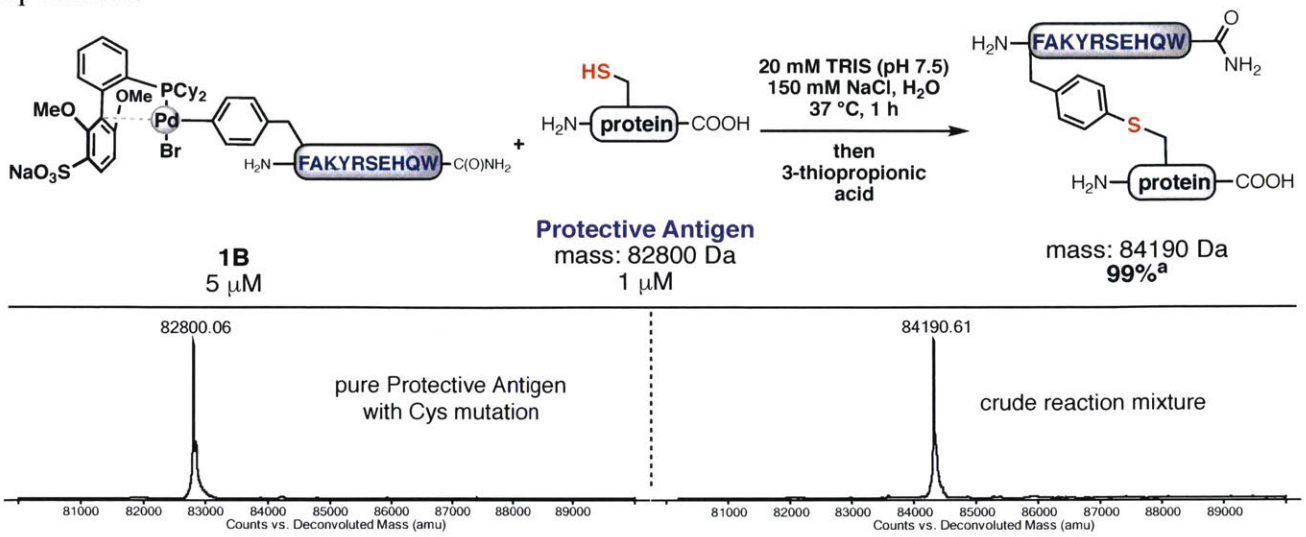
Given these results, we envisioned that another class of suitable nucleophiles would be cysteine-containing proteins. This kind of peptide–protein conjugation could potentially allow site-specific attachment of therapeutic peptides to antibodies or other large proteins containing cysteine, a strategy of significant interest for the development of targeted therapeutics or vaccines.[26,27] Furthermore, the ability to generate bioconjugates of natural proteins under physiologically relevant conditions in dilute aqueous solutions would be a unique application of metal-mediated cross coupling. An additional advantage of this method is that the aryl thioether bond is known to exhibit high chemical stability.[13]

Scheme 2: Palladium–peptide reagent enables peptide–peptide ligation. (A) Peptide ligation with internal cysteine, (B) Peptide ligation with cysteine near N-terminus, and (C) Peptide ligation with cysteine near C-terminus[a]



As a model substrate, the protein Protective Antigen (PA)[28,29] from *Bacillis anthacis* was modified to contain a single internal cysteine (see Experimental for details). In aqueous buffer containing TRIS (pH 7.5) and sodium chloride, PA (1 μM) was incubated with 5 equivalents of palladium–peptide complex **1B**. After only 1 hour at 37 °C, quantitative conjugation of the peptide to the protein was observed (Scheme 3).

Scheme 3: Ligation of a peptide to anthrax toxin Protective Antigen proceeds in one hour at low micromolar concentrations[a]. [a]Yield determined by deconvolution of mass spectrum.



3.3 – Conclusion:

In conclusion, palladium–peptide oxidative addition complexes were synthesized through the introduction of 4-halophenylalanine into peptides during SPPS. These complexes react efficiently with small molecule thiols, as well as cysteine-containing peptides and proteins. The ligation reactions proceed at low concentrations under physiologically relevant conditions. These transformations represent a new bioconjugation approach, complementary to current methods. We anticipate applications in the development of targeted therapeutics, the investigation of structure–function relationships, and the design of unconventional, branched polypeptide structures.

3.4 – Experimental:

General Reagent Information

1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), Fmoc-L-Gly-OH, Fmoc-L-Leu-OH, Fmoc-L-Ile-OH, Fmoc-L-

Lys(Boc)-OH, Fmoc-L-Ala-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Glu(O*t*Bu)-OH, Fmoc-L-Asp-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Phe-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Trp-OH, Fmoc-L-Thr(*t*Bu)-OH, Fmoc-L-Tyr(*t*Bu)-OH, and Fmoc-L-His(Trt)-OH were purchased from CreoSalus, Inc (Louisville, KY). Fmoc-4-iodo-L-phenylalanine, Fmoc-4-bromo-L-phenylalanine, and Fmoc-4-chloro-L-phenylalanine were purchased from Chem-Impex International (Wood Dale, IL). H-Rink Amide-ChemMatrix resin was obtained from PCAS BioMatrix Inc. (St-Jean-sur-Richelieu, Quebec, Canada). PyAOP was purchased from P3 BioSystems (Louisville, KY). Peptide synthesis-grade *N,N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethyl ether, and HPLC-grade acetonitrile were obtained from VWR International (Philadelphia, PA). Small molecule thiols were purchased from Aldrich Chemical Co., Alfa Aesar, or Matrix Scientific and were used without additional purification. All deuterated solvents were purchased from Cambridge Isotopes and used without further purification. All other reagents were purchased from Sigma-Aldrich and used as received.

All reactions with peptides were set up on the bench top and carried out under ambient conditions. Dimethylsulfoxide (DMSO), pentane, and cyclohexane were purchased from Aldrich Chemical Company in SureSeal™ bottles and were purged with argon before use. The ligand used during this study, sSPhos, was generously gifted from Aldrich Chemical Company.

All small-molecule organic and organometallic compounds were characterized by ^{1}H, ^{13}C NMR, and IR spectroscopy, as well as high-resolution mass spectrometry (unless otherwise noted). ^{31}P NMR spectroscopy was used for characterization of palladium complexes. Copies of the ^{1}H, ^{13}C, and ^{31}P NMR spectra can be found at the end of the Supporting Information. Nuclear Magnetic Resonance spectra were recorded on a Bruker 400 MHz instrument and a Varian 300

MHz instrument. Unless otherwise stated, all ^{1}H NMR experiments are reported in δ units, parts per million (ppm), and were measured relative to the signals of the residual proton resonances methanol-d_4 (4.78 and 3.31 ppm) in the deuterated solvents. High resolution mass spectra were obtained on a Bruker Daltonics APEXIV 4.7 Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis was performed on a Bruker MicroFlex using positive reflector mode.

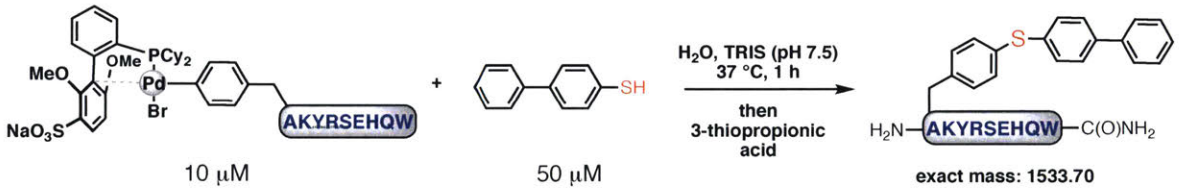
LC-MS chromatograms and associated mass spectra were acquired using Agilent 6520 ESI-Q-TOF mass spectrometer. Solvent compositions used in the LC-MS are 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC-MS method was used:

Method A LC conditions: Zorbax 300SB C3 column: 2.1 x 150 mm, 5 μm, column temperature: 40 °C, gradient: 0-2 min 1% B, 2-11 min 1-61% B, flow rate: 0.8 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300 – 3000 *m/z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octapole rf voltages were set at 4000, 175, and 750, respectively.

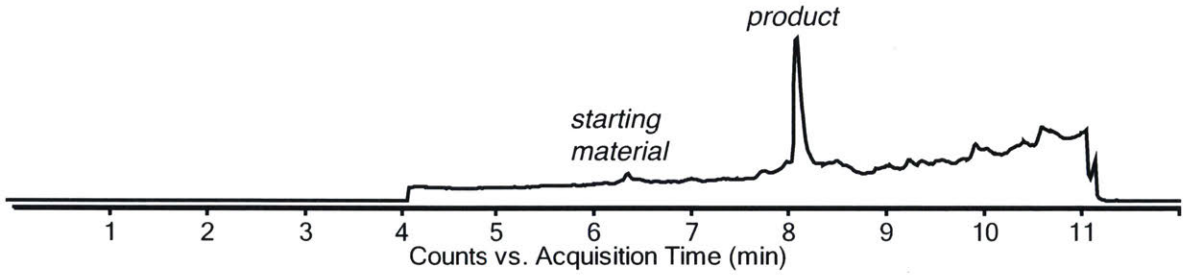
Determination of Bioconjugation Conversions

Data were processed using Agilent MassHunter software package. All reported yields were determined by integrating total ion current (TIC) spectra. First, the peak areas for all relevant peptide-containing species on the chromatogram were integrated using Agilent MassHunter software package. Since no peptide-based side products were generated in the experiments, the yields shown in **Schemes 1, 2, and 3** were determined as follows: %yield =

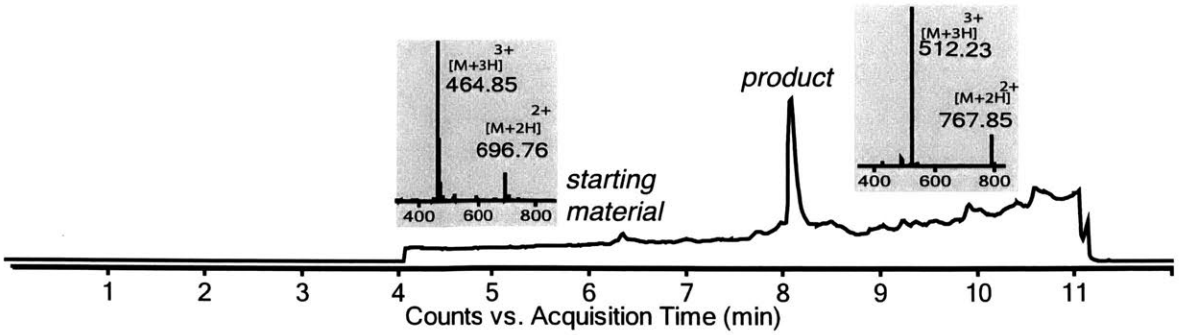
S_{pr}/S_{total} where S_{pr} is the peak area of the product and S_{total} is the peak area of combined peptide-containing species (product and starting material). For protein bioconjugation, deconvoluted masses of proteins were obtained using maximum entropy algorithm. LC-MS data shown were acquired using Method A, unless otherwise noted. Mass spectrum insets correspond to the integration of the TIC peak unless otherwise noted. In order to provide clarity, an example of how bioconjugation conversion is detailed below:



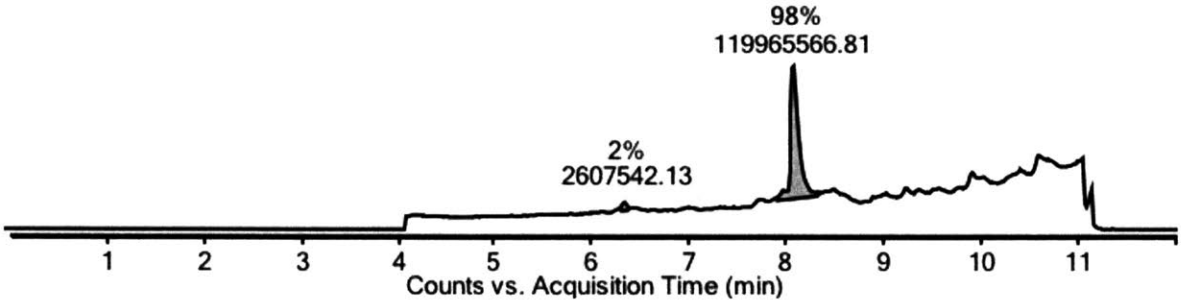
The crude reaction mixture is depicted below:



This mixture was analyzed by LC-MS using Agilent MassHunter software package. The peptide-containing peaks that correspond to the starting material and product were identified by mass as can be seen from the inlets depicted below.



Finally, using the calculation described above, the Agilent MassHunter software package was used to integrate below the starting material and product peaks to determine the percent of conversion to the product.



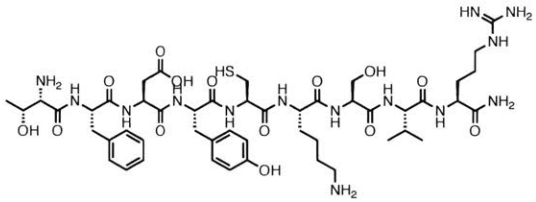
In some cases, a small non-peptidic impurity near the starting material was found, resulting in an additional peak. This peak was omitted from conversion calculations because it does not correspond to total peptide-containing species.

Linear Peptide Synthesis

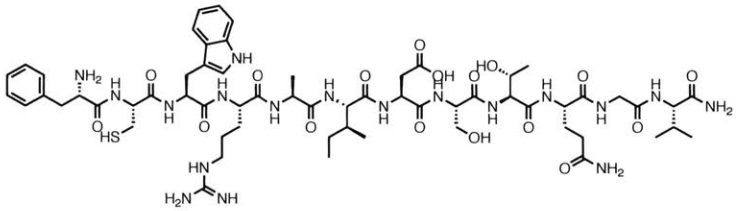
All peptides were synthesized on a 0.2 mmol scale using automated Fmoc-SPPS chemistry under flow as previously described.[2] Specifically, ChemMatrix Rink Amide HYR resin (200 mg) was loaded into a reactor maintained at 90 °C. All reagents were flowed at 80 mL/min with HPLC pumps through a stainless-steel loop maintained at 90 °C before introduction into the reactor. For each coupling, 10 mL of a solution containing 0.2 M amino acid and 0.17 M HATU in DMF were mixed with diisopropylethylamine (200 μL) and delivered to the reactor. Fmoc removal was accomplished using 20% (v/v) piperidine (10.4 mL). Between each step, DMF (15 mL) was

used to wash out the reactor. Special coupling conditions were used for arginine, in which the flow rate was reduced to 40 mL/min and 10 mL of a solution containing 0.2 M Fmoc-L-Arg(Pbf)-OH and 0.17 M PyAOP in DMF were mixed with diisopropylethylamine (200 μL) and delivered to the reactor.

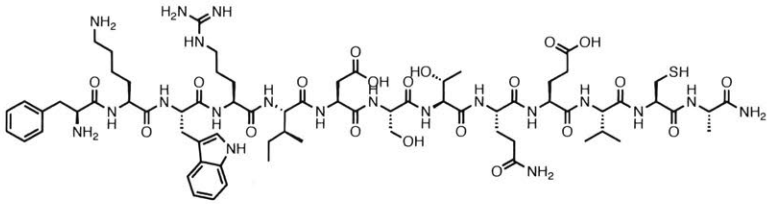
After completion of the stepwise, automated SPPS, the resin was washed thoroughly with CH_2Cl_2 and dried under vacuum. The peptide was simultaneously cleaved from the resin and the side-chains were deprotected by treatment with 5mL of the cleavage cocktail containing 82.5% TFA, 5% phenol, 5% thioanisole, 5% water, and 2.5% EDT (v/v) at 60 °C for 8 min. The resulting solution was then triturated and washed with cold diethyl ether (3x). The obtained solid was dissolved in a solution of 50% H_2O and 50% acetonitrile containing 0.1% TFA and lyophilized. The following peptides were synthesized following this procedure:



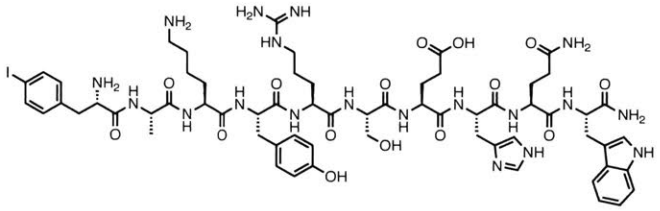
P1
Thr Phe Asp Tyr Cys Lys Ser Val Arg
mass: 1116.54



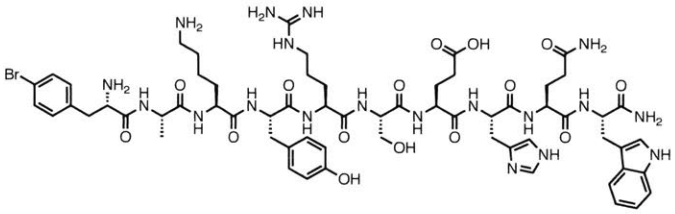
P2
Phe Cys Trp Arg Ala ile Asp Ser Thr Gln Gly Val
mass: 1380.66



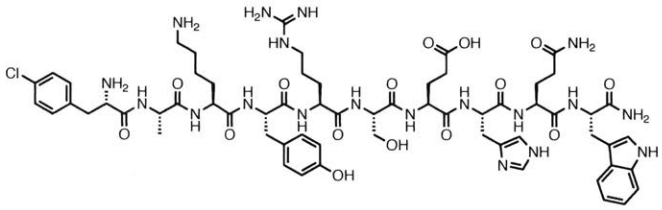
P3
Phe Lys Trp Arg Ile Asp Ser Thr Gln Glu Val Cys Ala
mass: 1580.78



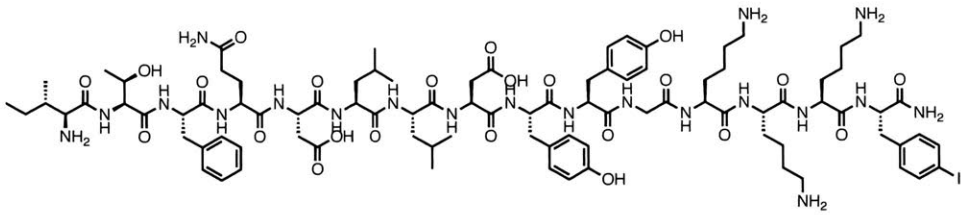
P4
4-iodoPhe Ala Lys Tyr Arg Ser Glu His Gln Trp
mass: 1475.56



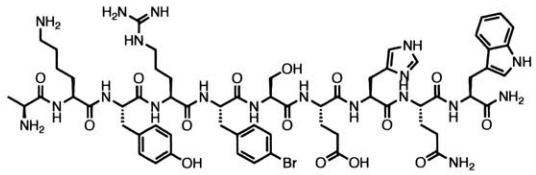
P5
4-bromoPhe Ala Lys Tyr Arg Ser Glu His Gln Trp
mass: 1427.52



P6
4-chloroPhe Ala Lys Tyr Arg Ser Glu His Gln Trp
mass: 1383.62



P7
Ile Thr Phe Gln Asp Leu Leu Asp Tyr Tyr Gly Lys Lys Lys 4-iodoPhe
mass: 2002.91

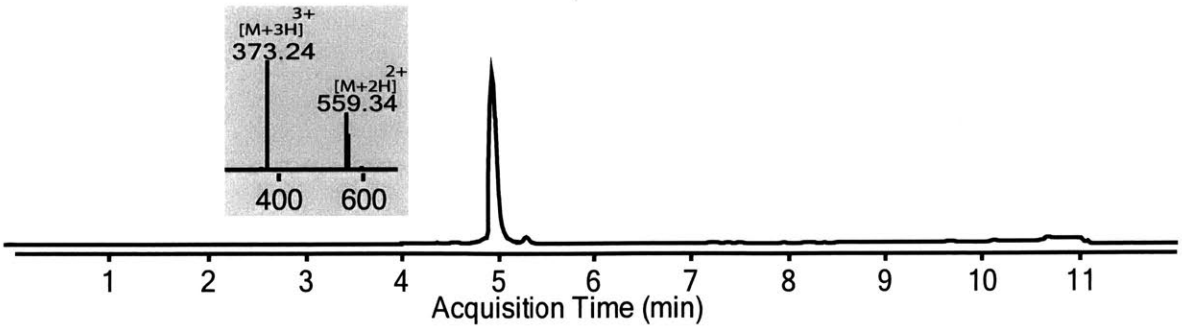


P8
Ala Lys Tyr Arg 4-bromoPhe Ser Glu His Gln Trp
mass: 1428.56

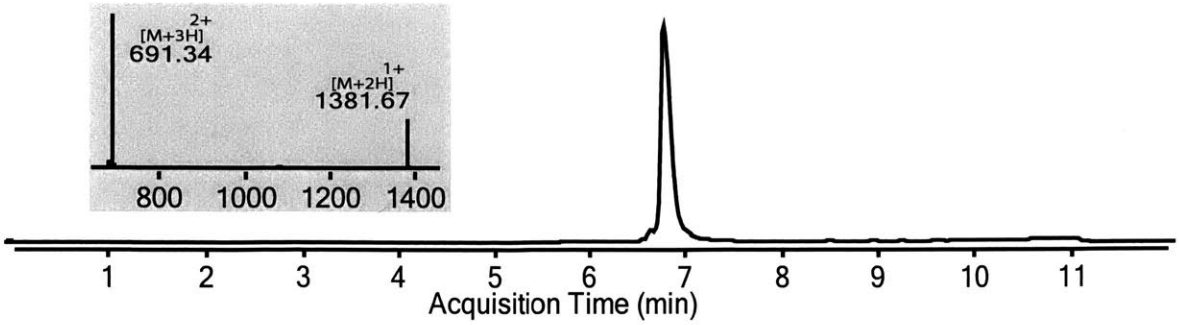
Peptide Purification

Peptides were dissolved in water and acetonitrile containing 0.1% TFA, filtered through a 0.22 μm nylon filter and purified by mass-directed semi-preparative reversed-phase HPLC (RP-HPLC). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient from 5-45% B changing a rate of 0.5% B/min was used. The peptides were purified on an Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm. Using the mass data collected for each fraction from the instrument, only pure fractions were combined and lyophilized. The purity of the fraction pool was confirmed by LC-MS. Total-ion current chromatograms for each peptide is shown below.

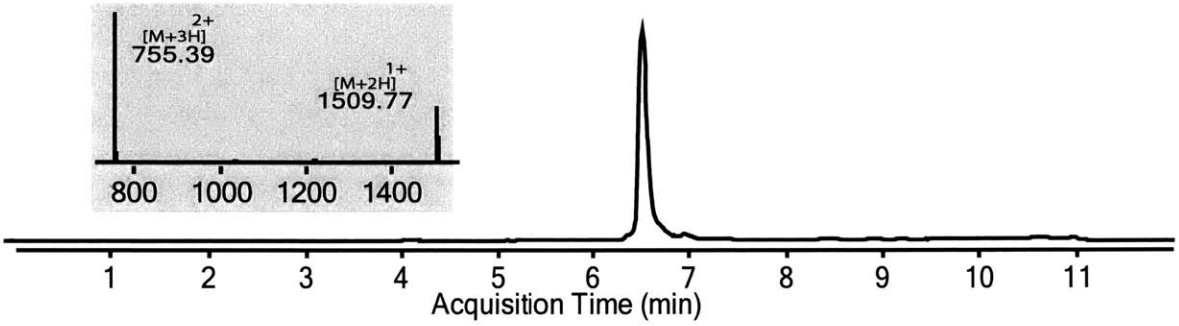
P1:



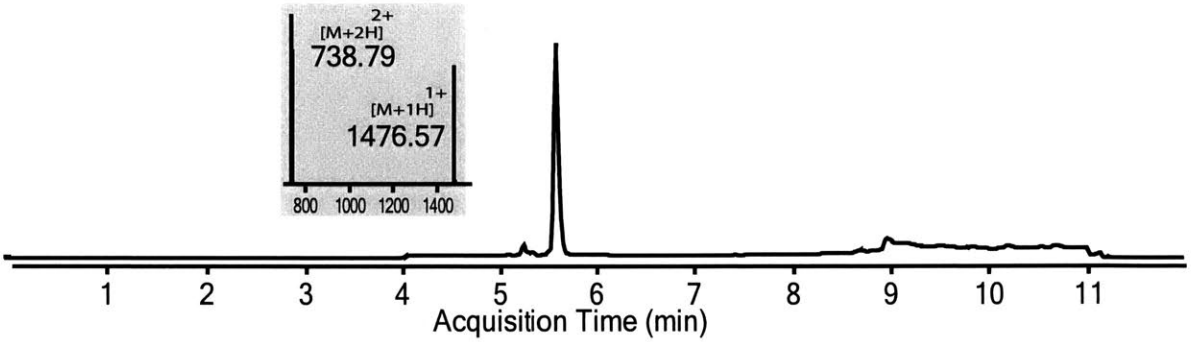
P2:


2+
[M+3H]
691.34
1+
[M+2H]
1381.67
800 1000 1200 1400
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)

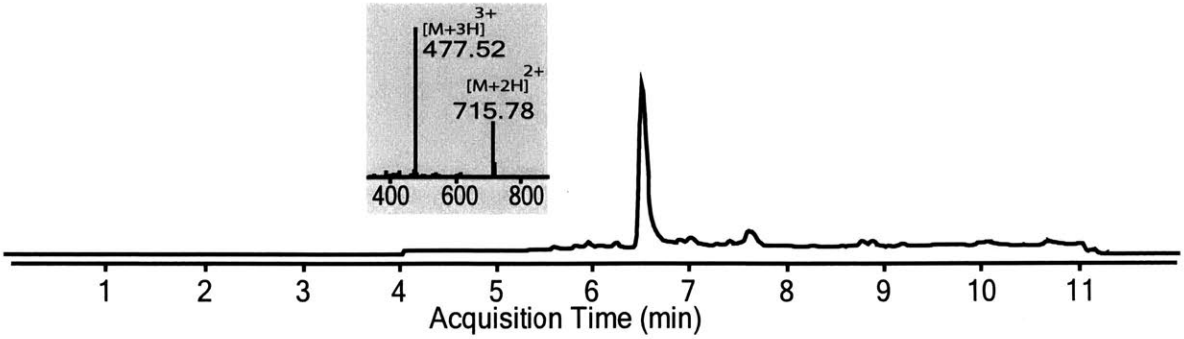
P3:


2+
[M+3H]
755.39
1+
[M+2H]
1509.77
800 1000 1200 1400
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)

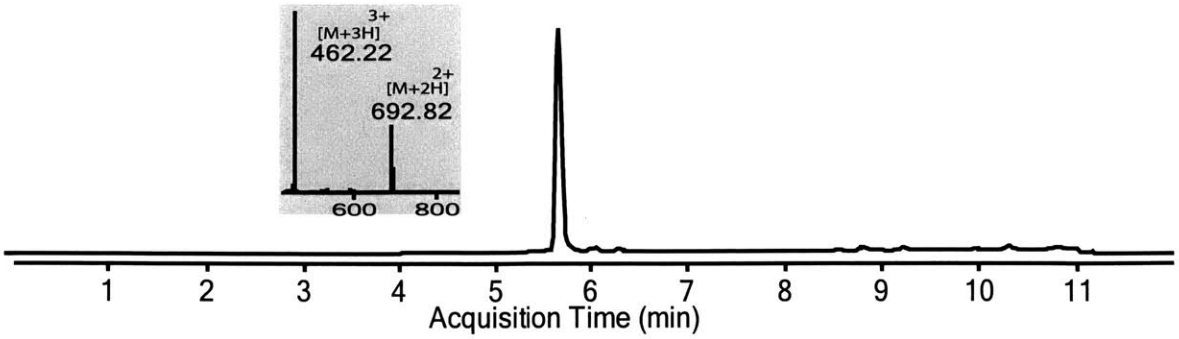
P4:


2+
[M+2H]
738.79
1+
[M+1H]
1476.57
800 1000 1200 1400
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)

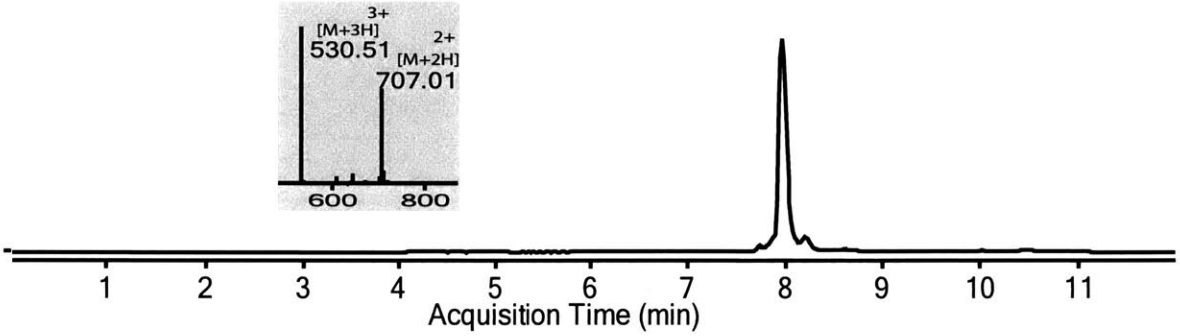
P5:


3+
[M+3H]
477.52
2+
[M+2H]
715.78
400 600 800
1 2 3 4 5 6 7 8 9 10 11
Acquisition Time (min)

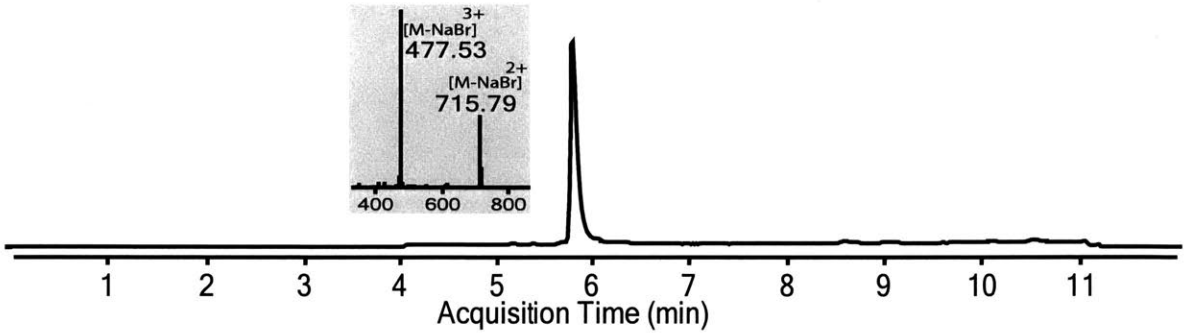
P6:



P7:



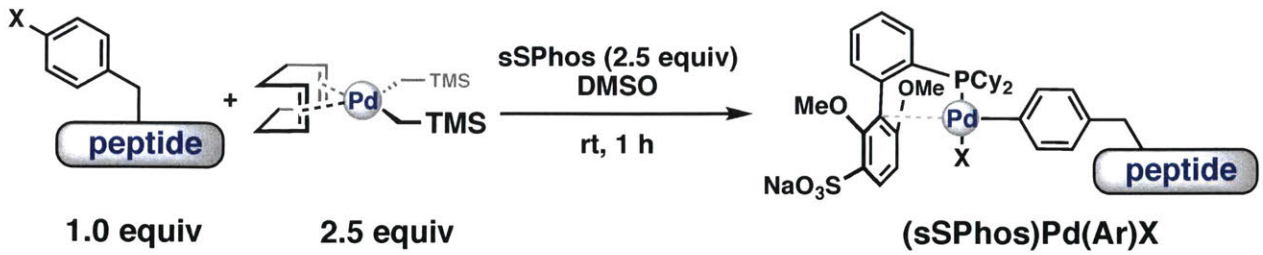
P8:



Synthesis of Pd-Peptide Oxidative Addition Complexes

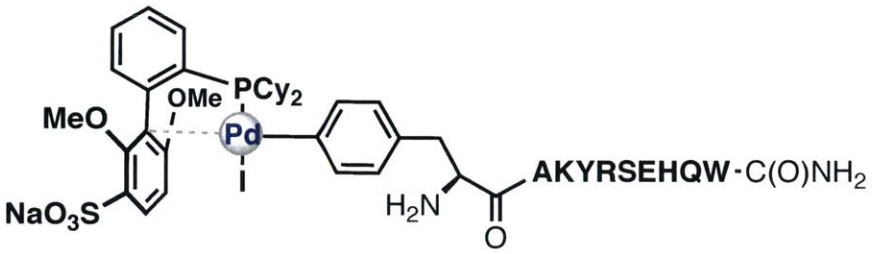
Synthesis of [(1,5-COD)Pd(CH_2TMS)$_2$]. This compound was synthesized according to the procedure reported in the literature.[1] The ^{1}H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature.[1] The title compound was stored in a freezer at –20 °C.

General Procedure for the Synthesis of Palladium-Peptide Oxidative Addition Complexes.



In a scintillation vial (10 mL) open to the air and equipped with a magnetic stir bar, the vial was charged with sSPhos ligand (2.5 equiv), peptide-Ar–X (1 equiv), and DMSO (1 mL). Solid (1,5-COD)Pd(CH_2SiMe_3)$_2$ (2.5 equiv) was added rapidly in one portion and the resulting solution was stirred for 1 h at rt.* After this time, the solution was frozen by placing the capped vial into a liquid nitrogen bath and subsequently lyophilized to remove the DMSO. The Pd-Peptide oxidative addition complexes were purified via mass-directed preparative RP-HPLC.

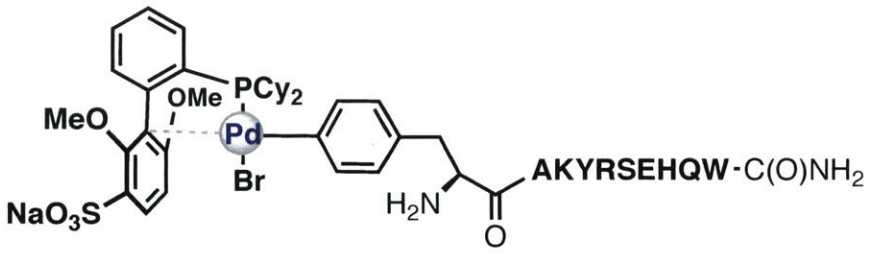
*The synthesis of the OA complex containing the ArCl (**1C**) requires 5 equiv of (1,5-COD)Pd(CH_2SiMe_3)$_2$ to reach full conversion in 1 h.



1A

Following the general procedure, a mixture containing peptide **P4** (10 mg, 0.006 mmol), sSPhos (8.7 mg, 0.017 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (6.5 mg, 0.017 mmol) was stirred at rt in DMSO (1.5 mL) for 1 h. General work-up afforded **1A** as a white solid (7.60 mg, 65%).

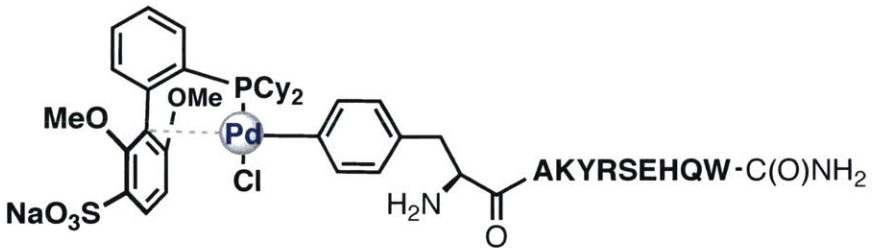
HRMS electrospray (m/z): $[M - NaI]^+$ calcd for $C_{89}H_{120}IN_{19}NaO_{20}PPdS$: 1943.75, found 1943.77.



1B

Following the general procedure, a mixture containing peptide **P5** (10 mg, 0.006 mmol), sSPhos (8.7 mg, 0.017 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (6.5 mg, 0.017 mmol) was stirred at rt in DMSO (1.5 mL) for 1 h. General work-up afforded **1B** as a white solid (5.60 mg, 48%).

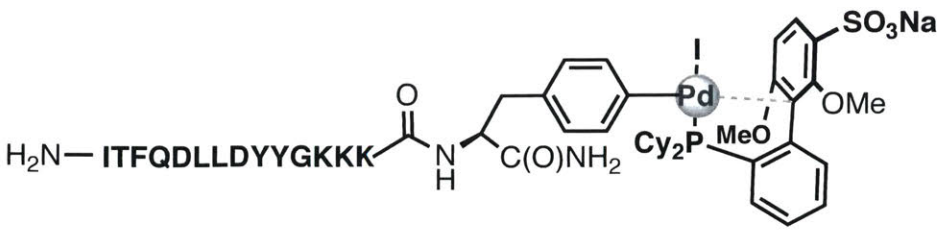
HRMS electrospray (m/z): [M – NaBr]$^+$ calcd for $C_{89}H_{120}BrN_{19}NaO_{20}PPdS$: 1943.75, found 1943.77.



1C

Following the general procedure, a mixture containing peptide **P6** (10 mg, 0.006 mmol), sSPhos (8.7 mg, 0.017 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (13 mg, 0.034 mmol) was stirred at rt in DMSO (1.5 mL) for 1 h. General work-up afforded **1C** as a white solid (1.60 mg, 33%).

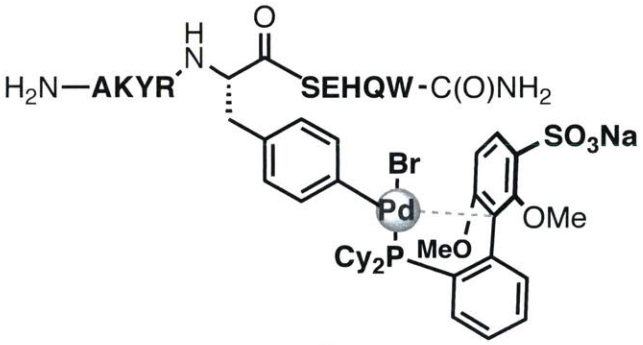
HRMS electrospray (m/z): [M – NaCl]$^+$ calcd for $C_{89}H_{120}ClN_{19}NaO_{20}PPdS$: 1943.75, found 1943.76.



1D

Following the general procedure, a mixture containing peptide **P7** (10 mg, 0.0038 mmol), sSPhos (4.8 mg, 0.0095 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (3.7 mg, 0.0095 mmol) was stirred at rt in DMSO (1.5 mL) for 1 h. General work-up afforded **1D** as a white solid (7.72 mg, 77%).

HRMS electrospray (m/z): [M – NaI]$^+$ calcd for $C_{117}H_{169}IN_{20}NaO_{28}PPdS$: 2620.99, found 2621.00.



1E

Following the general procedure, a mixture containing peptide **P8** (10 mg, 0.006 mmol), sSPhos (8.7 mg, 0.017 mmol), and (1,5-COD)Pd(CH_2TMS)$_2$ (6.5 mg, 0.017 mmol) was stirred at rt in DMSO (1.5 mL) for 1 h. General work-up afforded **1E** as a white solid (3.80 mg, 33%).

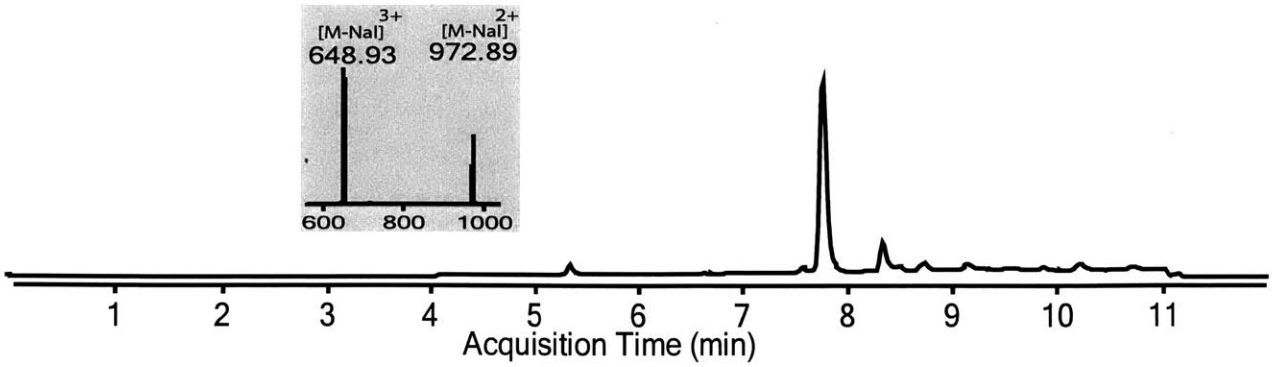
HRMS electrospray (m/z): [M – NaBr]$^+$ calcd for $C_{91}H_{122}N_{19}O_{21}PPdS$: 1943.75, found 1943.74.

<u>Palladium-Peptide Oxidative Addition Complex Purification</u>

Although the purified palladium-peptide oxidative addition complexes are readily soluble in aqueous solutions, we found that the crude palladium-peptide oxidative complexes exhibited limited solubility, likely due to the excess ligand. Therefore, after lyophization, the palladium-peptide oxidative addition complexes were dissolved in 2 mL of a 50:50 (v/v) mix of water and acetonitrile containing 0.1% TFA. The solution was diluted with 1 mL of water containing 0.1% TFA, filtered through a 0.22 μm nylon filter, and purified by mass-directed semi-preparative reversed-phase HPLC (RP-HPLC). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient from 15-50% B changing at a rate of 0.5% B/min was used. The peptides were purified on an Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm.

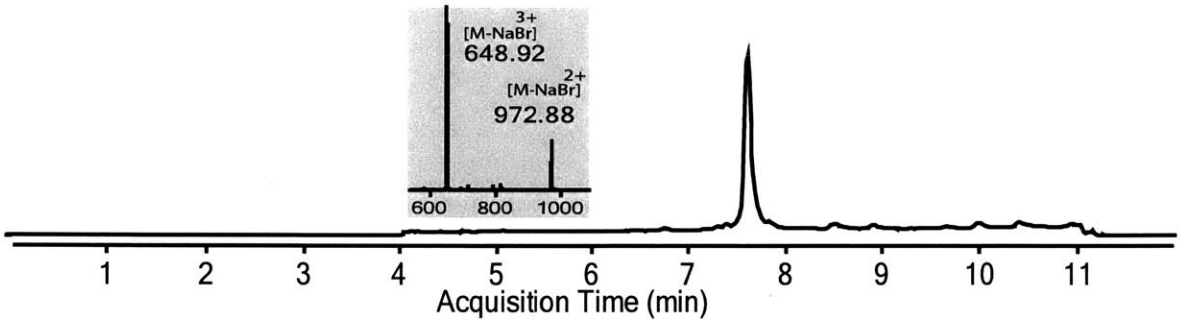
Using mass data from the instrument about each fraction, pure fractions were pooled and lyophilized. Despite different retention times, peptide OA complexes and ligand often co-elute, which can reduce overall yield (particularly for 1C). The purity of the fraction pool was confirmed by LC-MS. Total-ion current chromatograms for each complex is shown below. With electrospray ionization, we observe ions corresponding to each palladium-peptide oxidative addition complex without sodium and halide. An additional characterization of OA complex 1B using MALDI-TOF-MS is also presented, and we also observe the loss of sodium and halide.

1A:

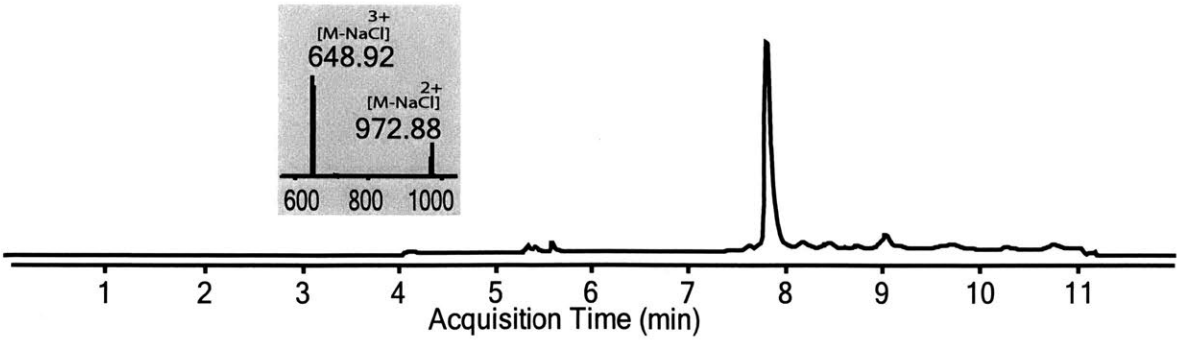


The LC-MS chromatogram of OA complex **1A** has a peak at 8.4 min that corresponds to free ligand. HPLC purification fails to fully resolve the complex from the ligand, and techniques to improve the purification have been unsuccessful but the subsequent reactivity of the complex is identical to that of the other Pd-peptide complexes.

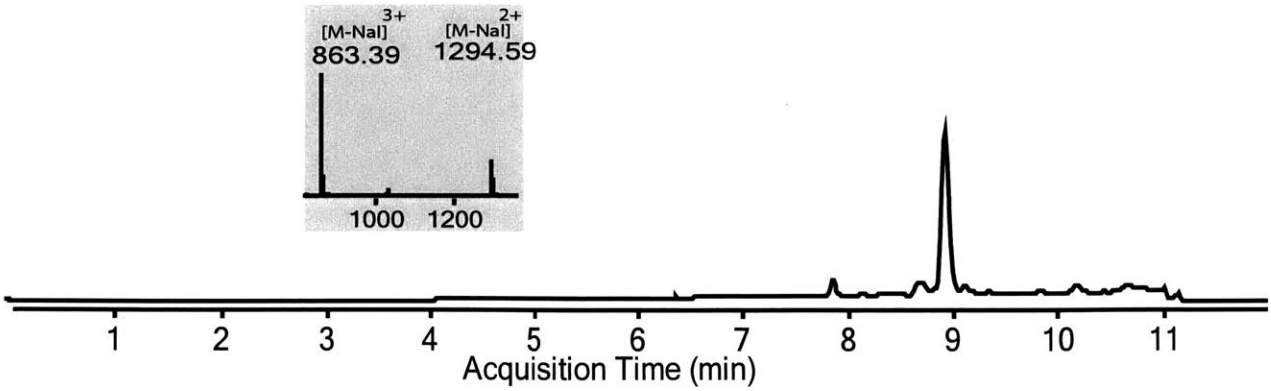
1B:



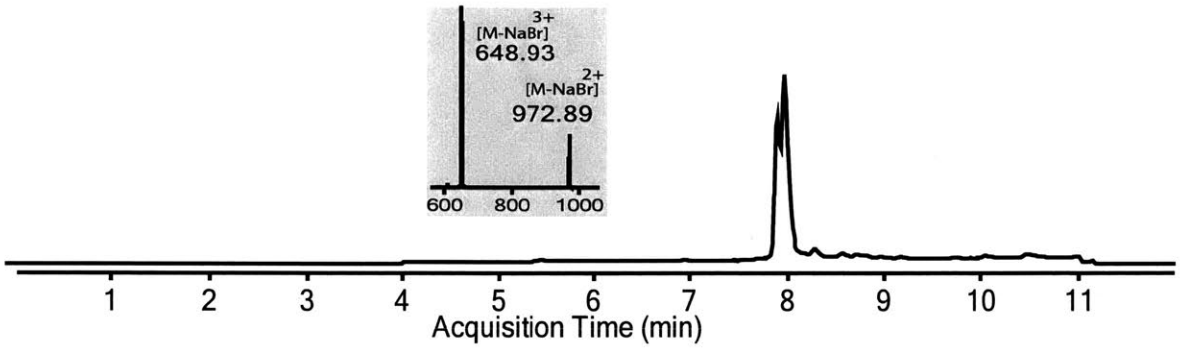
1C:



1D:



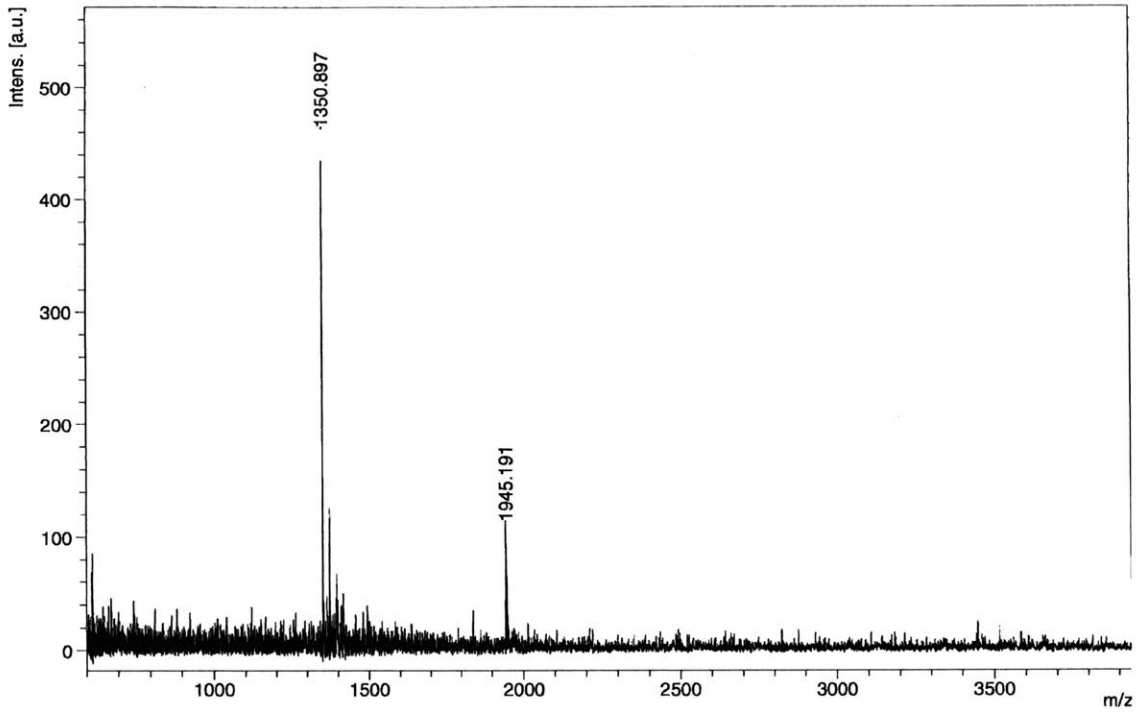
1E:



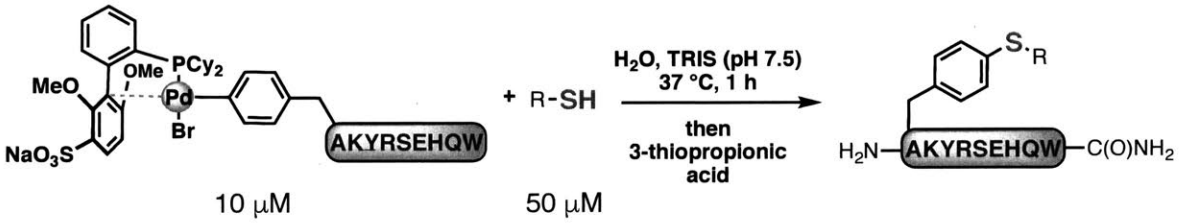
For the LC-MS chromatogram of OA complex **1E**, there are two peaks at 8 min. These peaks are isobaric, and presumably arise due to two distinct confirmations of the ligand when the bromophenylalanine is in the middle of the peptide (i.e. rotomers).

MALDI-TOF-MS Analysis

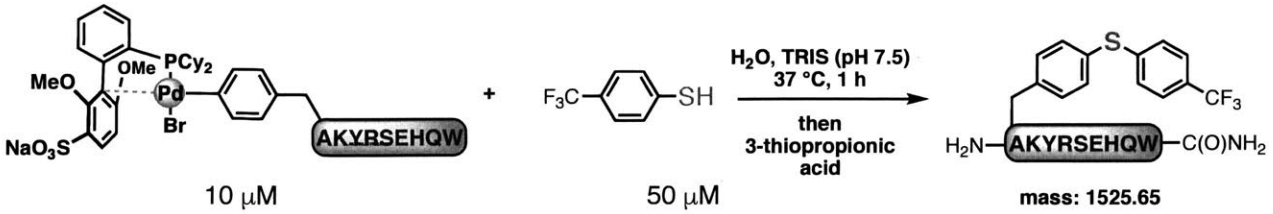
For purified OA complex **1B**, the compound was also analyzed using MALDI-TOF-MS. Shown below is the spectrum obtained after preparing the sample in α-cyano-4-hydroxycinnamic acid matrix. The spectrum was acquired in positive reflector mode, with PIE delay set to 240 ns, laser repetition rate set to 60 Hz, reflector detector voltage set to 1.854 kV, ion source voltage 1 set to 19 kV, ion source voltage 2 set to 15.9 kV, ion source lens voltage set to 9.1 kV, and the number of shots set to 400. Mass expected [M -Na, -Br, +H]: 1943.7. Mass observed: 1944.1. The additional peak at 1350.9 corresponds to M -Na, -Br, -Pd, -ligand, +H.



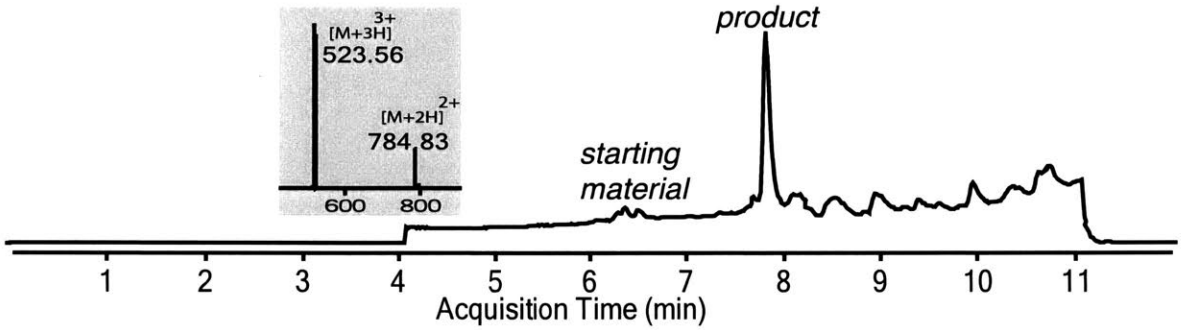
General Bioconjugation Procedure

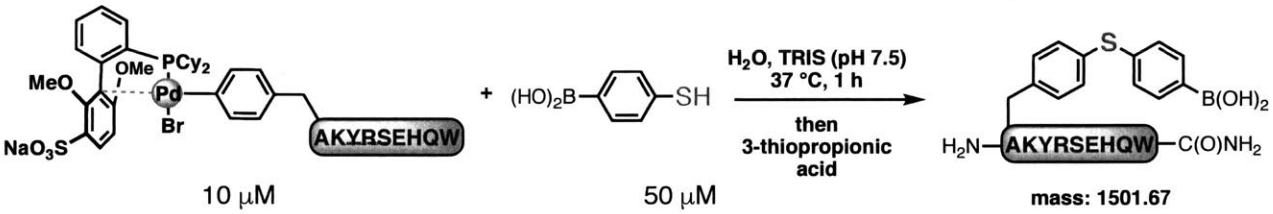


A solution of the palladium-peptide reagent **1B** (20 μM) in water was added to a equal volume of a solution containing the small molecule thiol (100 μM) in 0.1 M TRIS buffer at pH 7.5. The palladium reagents were sonicated for 10 s to ensure the reagents were fully dissolved. Final conditions: [Pd] = 10 μM; [R-SH] = 50 μM; After 1 h at 37 °C, of 3-mercaptopropionic acid (3 equiv) dissolved in H_2O was added to the reaction mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min and subsequently characterized by LC-MS.

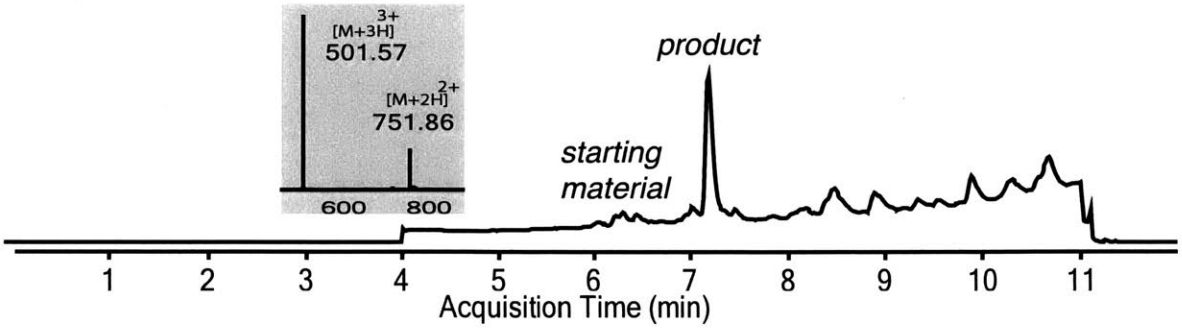


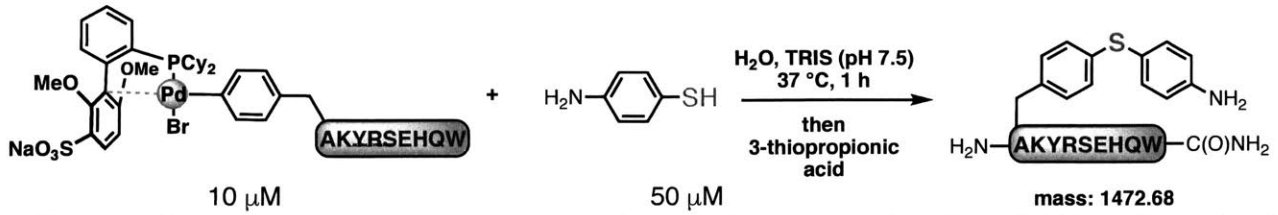
Following the general bioconjugation procedure with 4-(trifluoromethyl)benzenethiol (50 uM) resulted in a 99% conversion to the desired product as determined by LC-MS analysis.



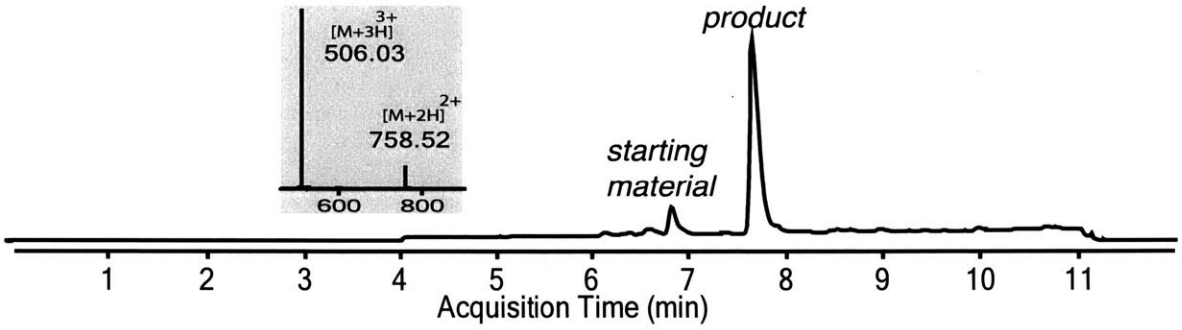


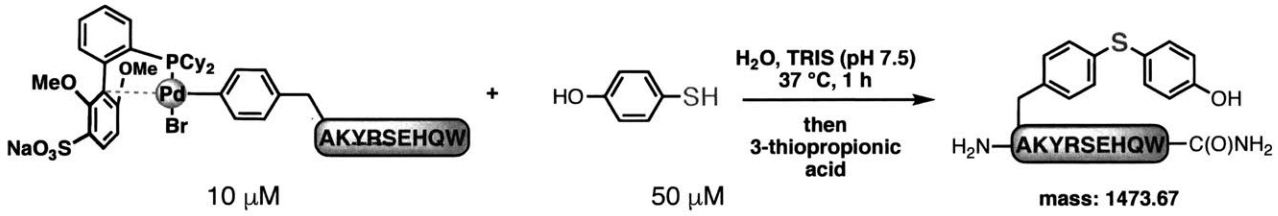
Following the general bioconjugation procedure with 4-mercaptophenylboronic acid (50 uM) resulted in a 98% conversion to the desired product as determined by LC-MS analysis.



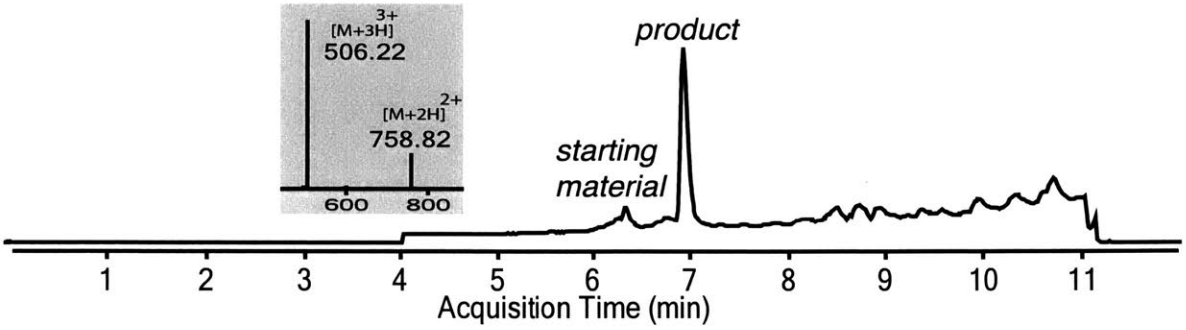


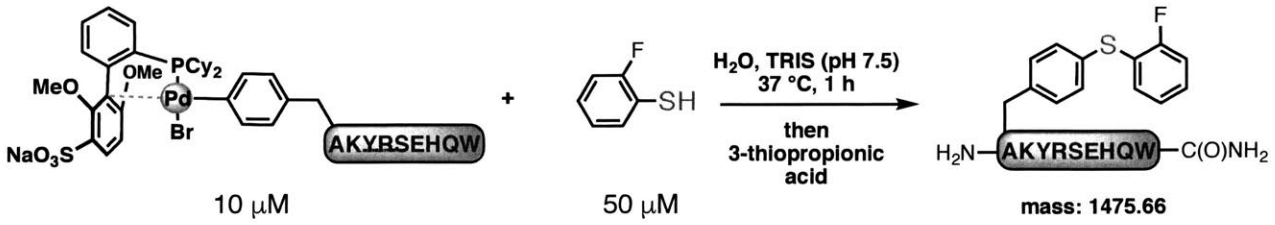
Following the general bioconjugation procedure with 4-aminothiophenol (50 uM) resulted in a 99% conversion to the desired product as determined by LC-MS analysis.



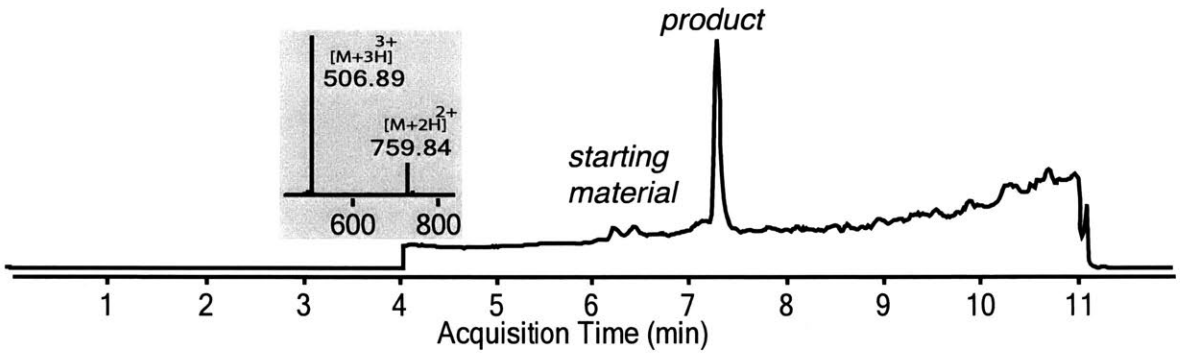


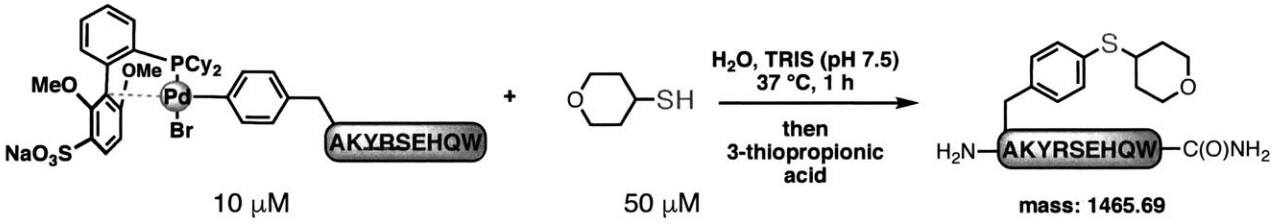
Following the general bioconjugation procedure with 4-mercaptophenol (50 uM) resulted in a 98% conversion to the desired product as determined by LC-MS analysis.



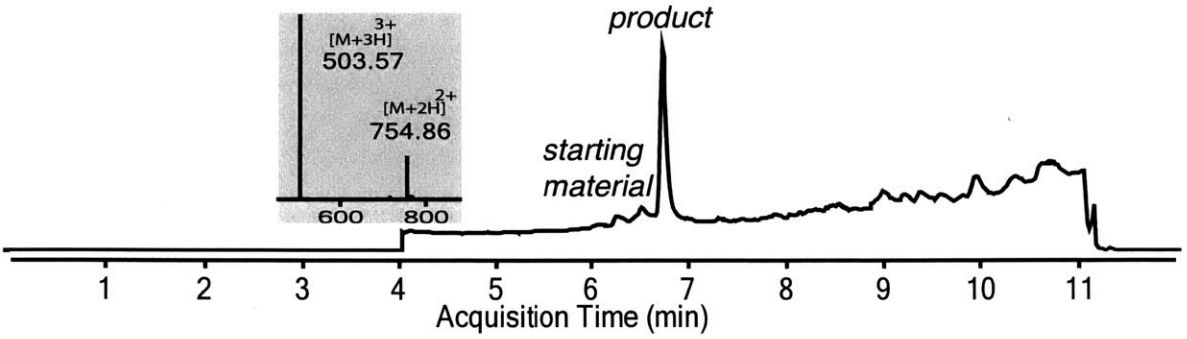


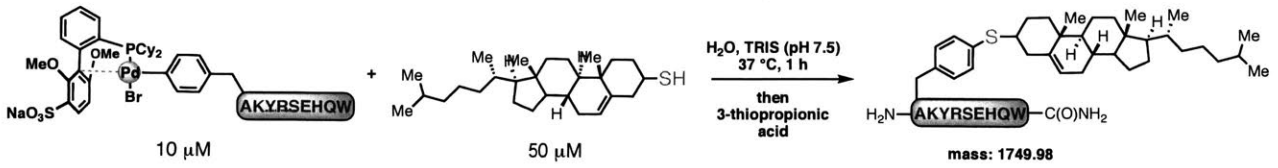
Following the general bioconjugation procedure with 2-fluorothiophenol (50 uM) resulted in a 98% conversion to the desired product as determined by LC-MS analysis.



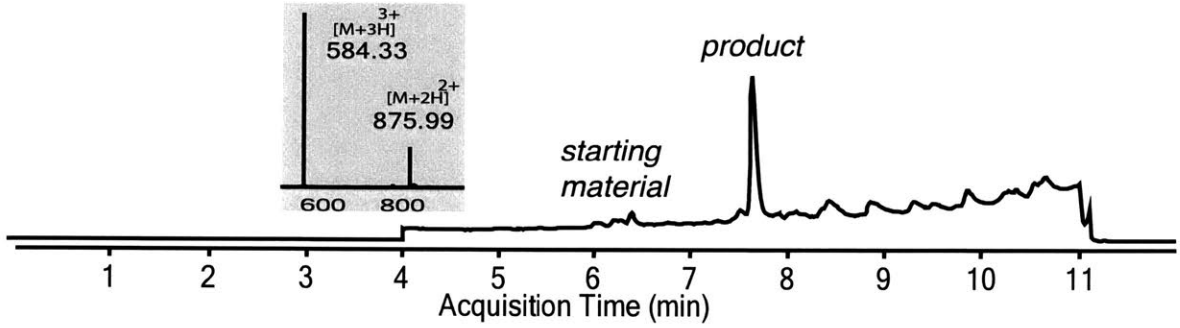


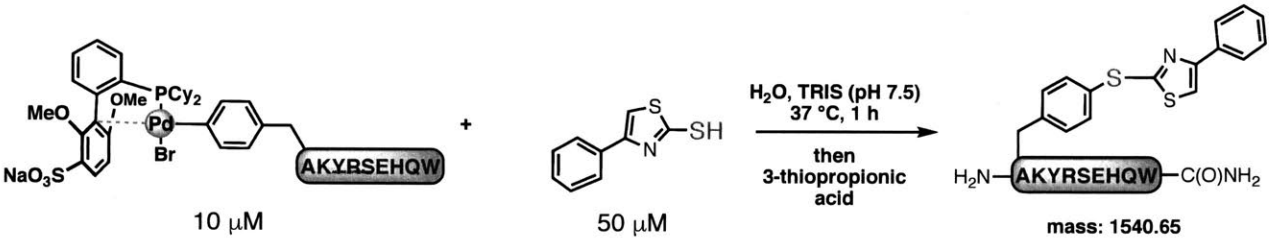
Following the general bioconjugation procedure with 4-mercaptotetrahydropyran (50 uM) resulted in a 99% conversion to the desired product as determined by LC-MS analysis.



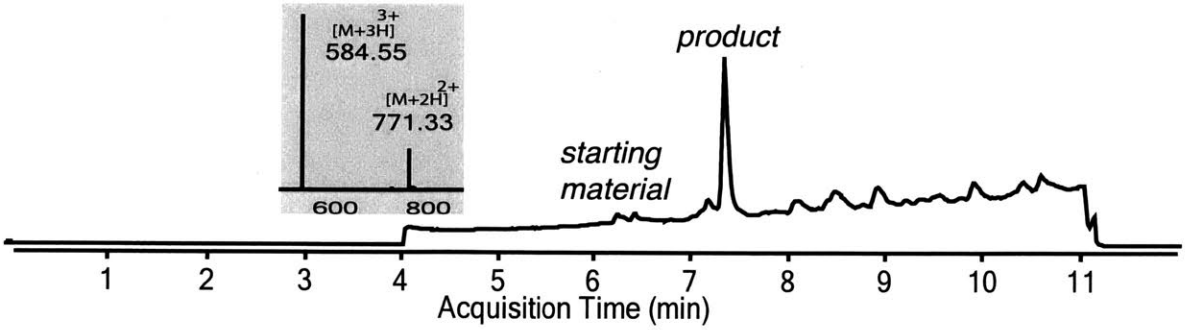


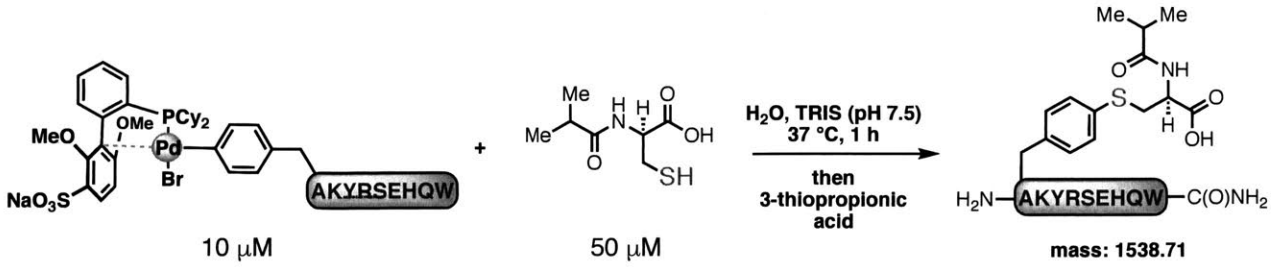
Following the general bioconjugation procedure with thiocholesterol (50 uM) resulted in a 99% conversion to the desired product as determined by LC-MS analysis.



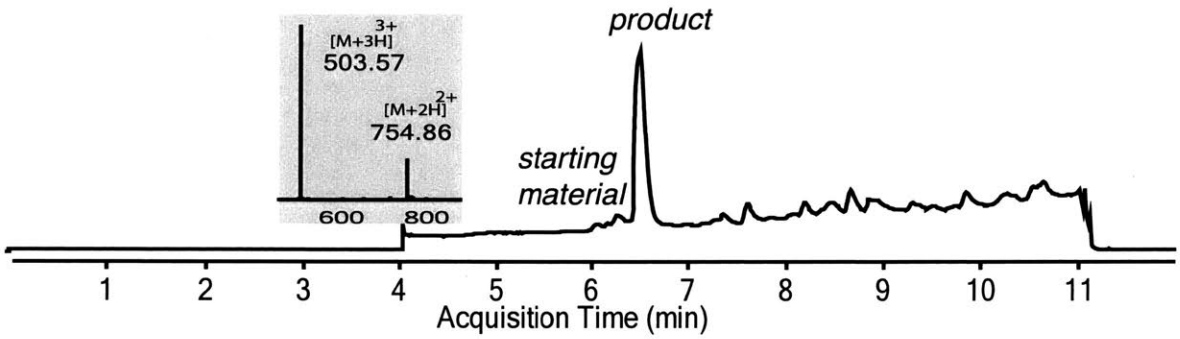


Following the general bioconjugation procedure with 4-phenylthiazole-2-thiol (50 uM) resulted in a 98% conversion to the desired product as determined by LC-MS analysis.

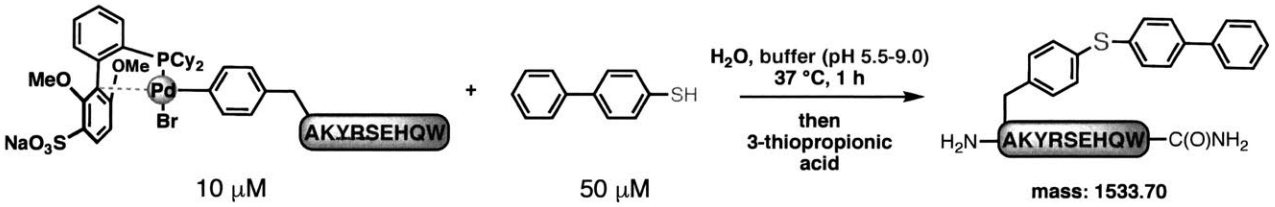




Following the general bioconjugation procedure with N-isobutyryl-D-cysteine (50 uM) resulted in a 99% conversion to the desired product as determined by LC-MS analysis.

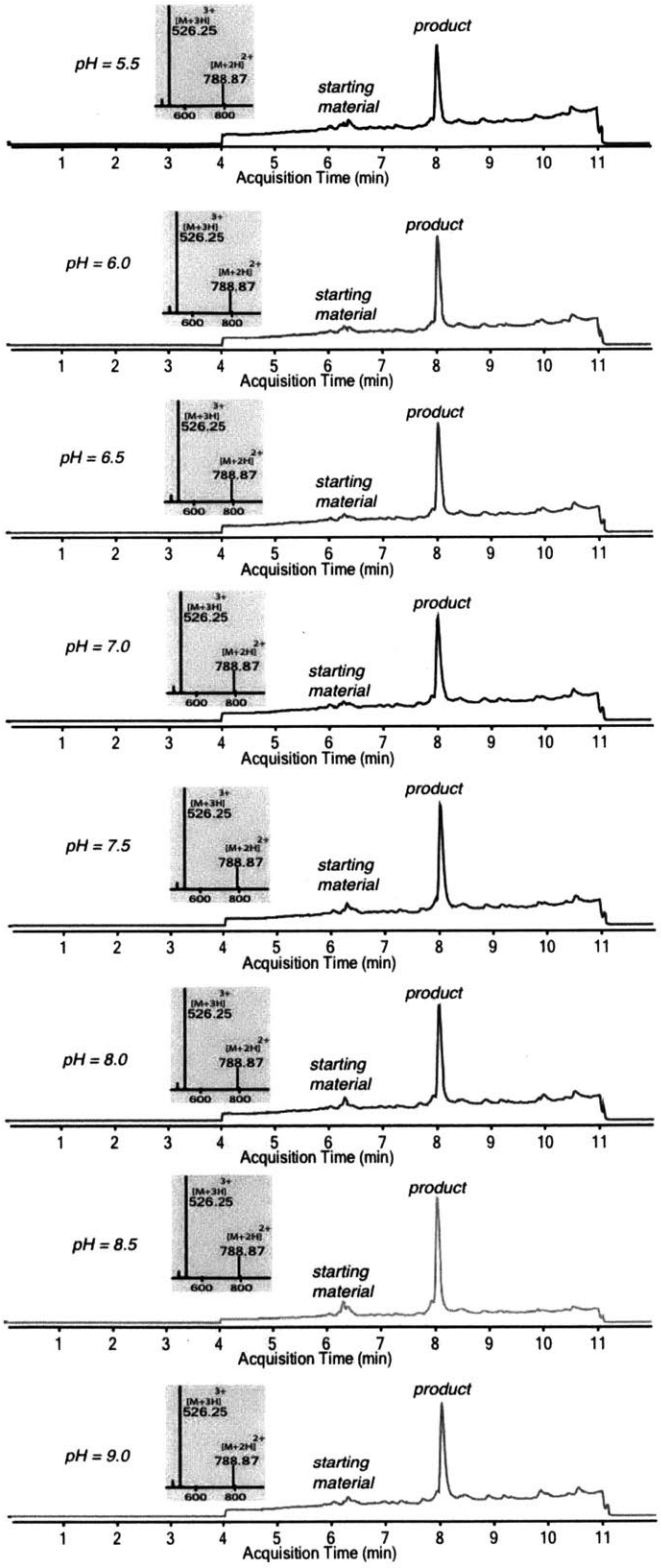


General pH Study Procedure – (Buffer Experiments)

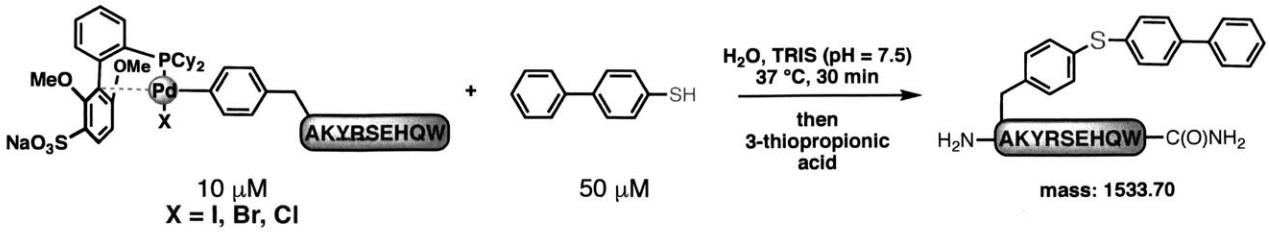


A solution of the palladium-peptide reagent **1B** (20 μM) in water was added to a equal volume of a solution containing biphenyl-4-thiol (100 μM) in buffer (0.1 M, Phosphate buffer = pH 5.5-7.0, TRIS buffer = pH 7.5-9.0). The palladium reagents were sonicated for 10 s to ensure the reagents were fully dissolved. Final conditions: [Pd] = 10 μM; [biphenyl-SH] = 50 μM; After 1 h at 37 °C, 3-mercaptopropionic acid (3 equiv) dissolved in H_2O was added to the reaction

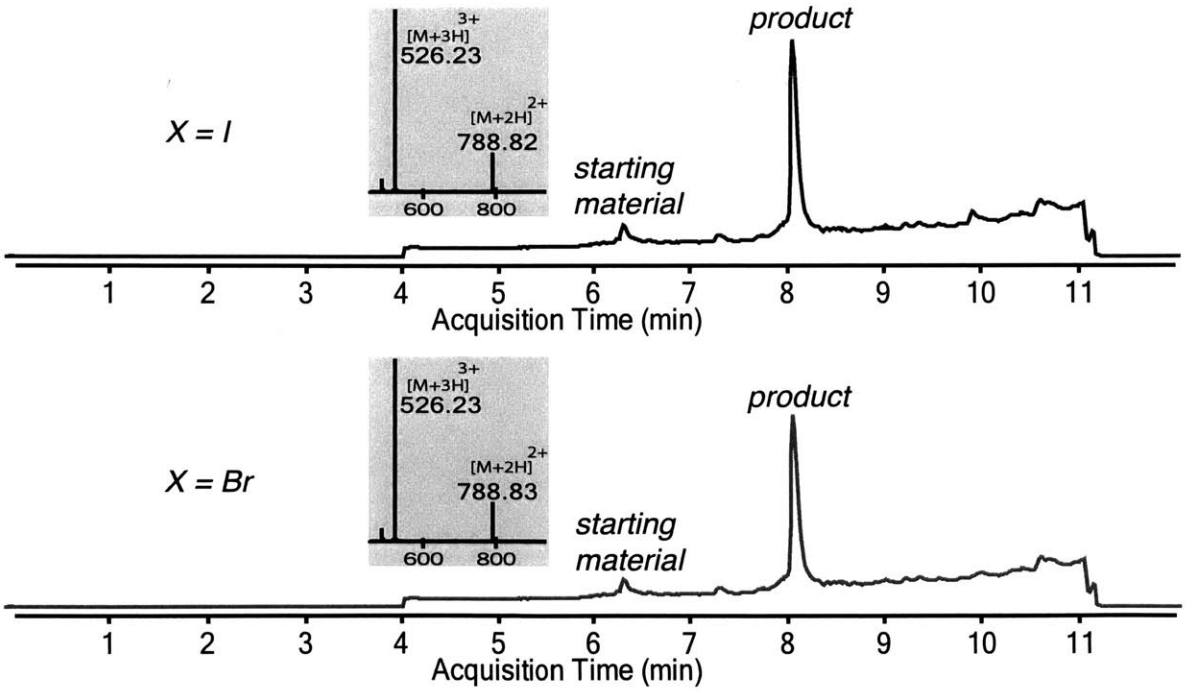
mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min and subsequently characterized by LC-MS.

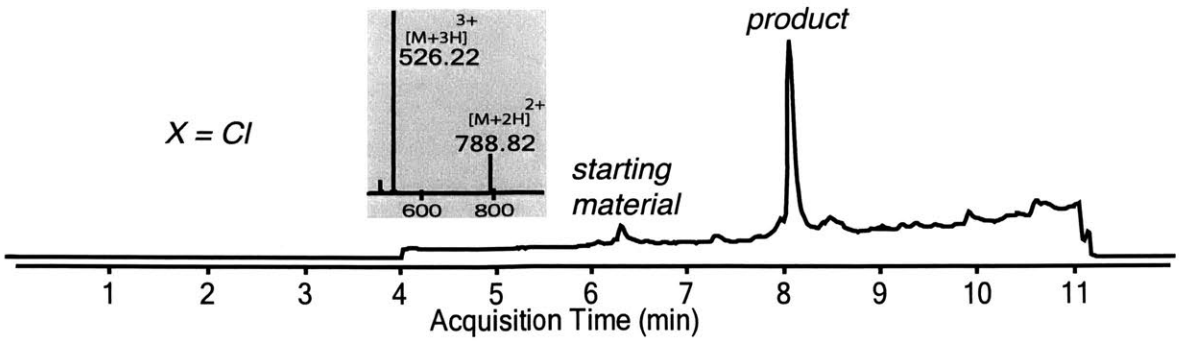


General Halide Study Procedure – (Halide Effect Experiments)



A solution of the palladium-peptide reagent **1A, 1B,** or **1C** (20 μM) in water was added to an equal volume of a solution containing biphenyl-4-thiol (100 μM) in in buffer (0.1 M TRIS buffer = pH 7.5). The palladium reagents were sonicated for 10 s to ensure the reagents were fully dissolved. Final conditions: [Pd] = 10 μM; [biphenyl-SH] = 50 μM; After 1 h at 37 °C, 3-mercaptopropionic acid (3 equiv) dissolved in H_2O was added to the reaction mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min and subsequently characterized by LC-MS.





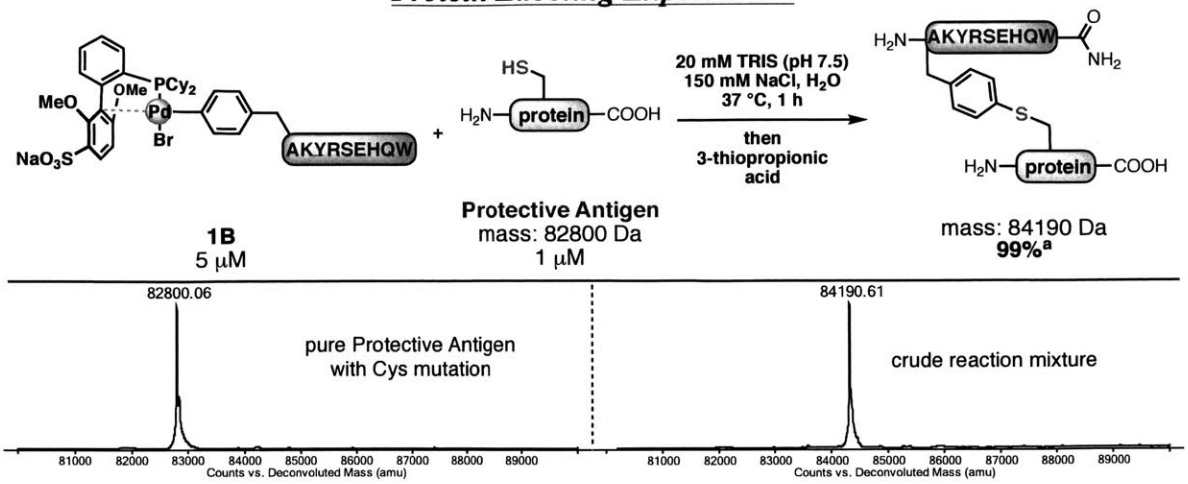
Protein Expression and Purification

pD444 PAC-CO plasmids were constructed as previously reported.[3] Cysteine mutations were introduced by site-directed mutagenesis using QuickChange Lightning Single Site-directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. Sequences of the generated protein construct are summarized in Table S1.

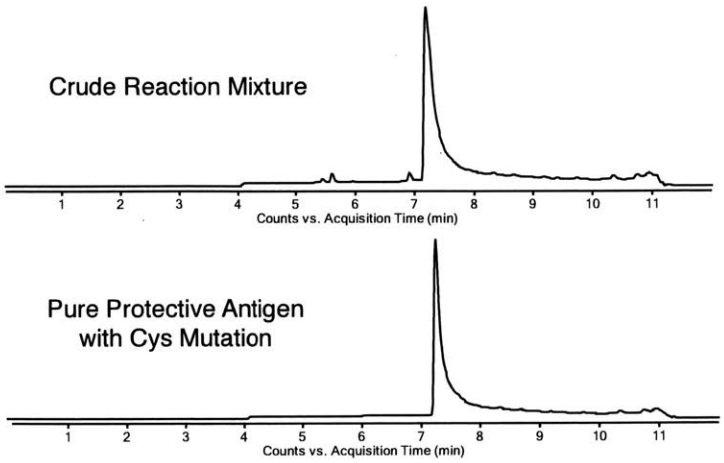
PA[K653C] was expressed in the periplasm of E. coli BL21(DE3) cells. Cells were grown in LB medium containing ampicillin (100 μg/mL) at 37 °C to an A_{600} of 0.8. Expression was induced by addition of IPTG to final concentration of 0.4 mM, and the culture was grown overnight at 18 °C. The protein was purified by anion exchange chromatography followed by size exclusion chromatography.

The protein was analyzed by LC-MS confirming sample purity and molecular weight. Protein aliquots were flash frozen and stored in –80 °C freezer.

Protein Labeling Experiments



To a solution of protein (500 pmoles) in 475 μL of 20 mM Tris and 150 mM NaCl buffer (pH 7.5) was added palladium-peptide complex **1B** (25 μL, 100 μM) in water. The solution was pipetted up and down (10x) to ensure proper reagent mixing. The reaction mixture was left at 37 °C for 1 h. After this time, the reaction was quenched by the addition of 3-thiopropionic acid (25 μL, 2 mM) dissolved in 20 mM Tris and 150 mM NaCl buffer (pH 7.5). After an additional 5 min at rt, 500 μL of 1:1 CH_3CN/H_2O (v/v) containing 0.1% TFA was added and the resulting mixture was analyzed by LC-MS.



Deconvoluted mass spectrum:

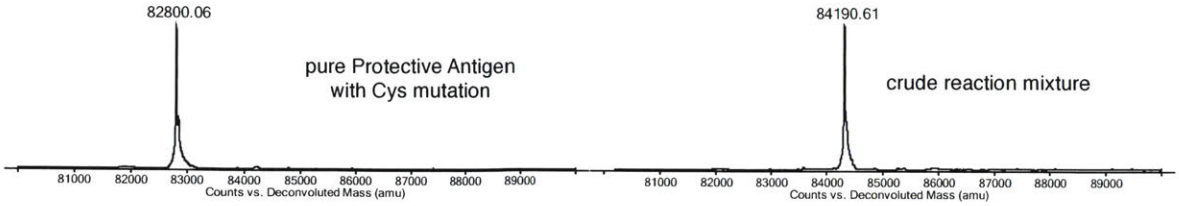


Table S1. **Protective Antigen-Cys protein sequence and calculated mass**

Sequence[a]	Calculated mass
MEVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVV TSSTTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKS DEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEK GRLYQIKIQYQRENPTEKGLDFKLYWTDSQNKKEVI SSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGI PDSLEVEGYTVDVKNKRTFLSPWISNIHEKKGLTKY KSSPEKWSTASDPYSDFEKVTGRIDKNVSPEARHPLV AAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTS TSRTHTSEVHGNAEVHASFFDIGGSVSAGFSNSNSSTV AIDHSLSLAGERTWAETMGLNTADTARLNANIRYVN TGTAPIYNVLPTTSLVLGKNQTLATIKAKENQLSQIL APNNYYPSKNLAPIALNAQDDFSSTPITMNYNQFLEL EKTKQLRLDTDQVYGNIATYNFENGRVRVDTGSNW SEVLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLE TTKPDMTLKEALKIAFGFNEPNGNLQYQGKDITEFD FNFDQQTSQNICNQLAELNATNIYTVLDKIKLNAKM NILIRDKRFHYDRNNIAVGADESVVKEAHREVINSST EGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYDM LNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVY AVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG	82800.04 Da

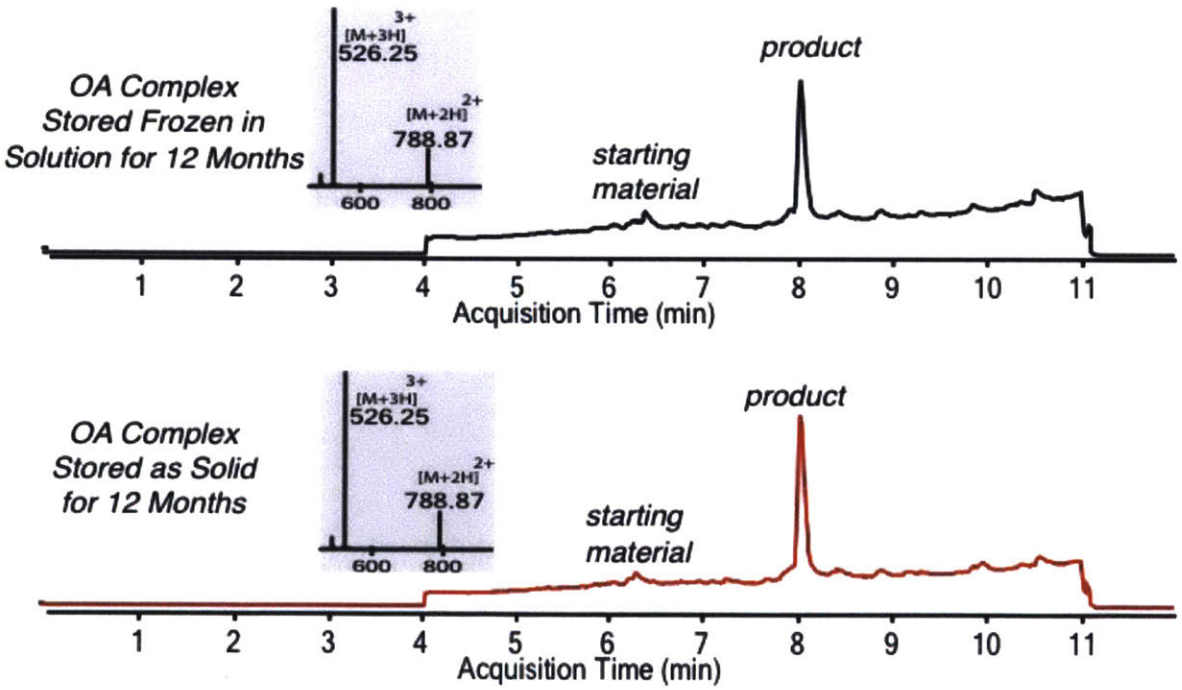
[a]Cysteine residue highlighted in red.

Oxidative Addition Complex Stability

When stored as a solid or kept frozen in solution, the palladium-peptide oxidative addition complexes display remarkable stability for upwards of 12 months and show no diminished reactivity. This is confirmed via a set of experiments wherein the oxidative addition complexes were stored as either a frozen solution or solid and then, after 12 months, allowed to react with a

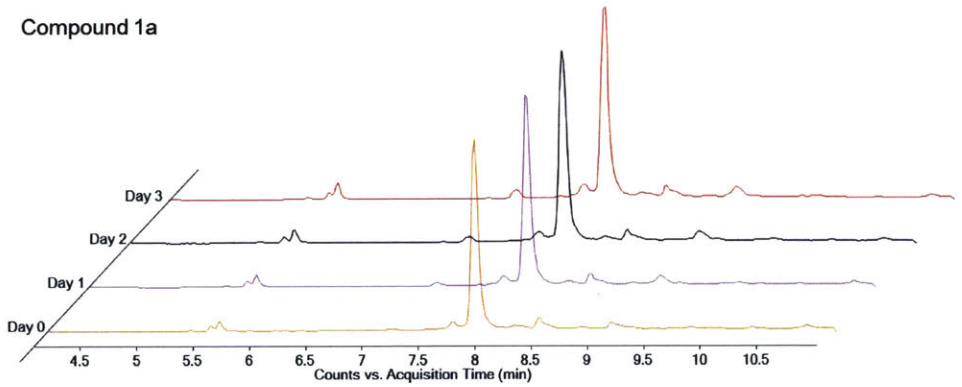
small molecule thiol. The reaction conversions are comparable to that of a freshly prepared palladium-peptide oxidative addition complex.

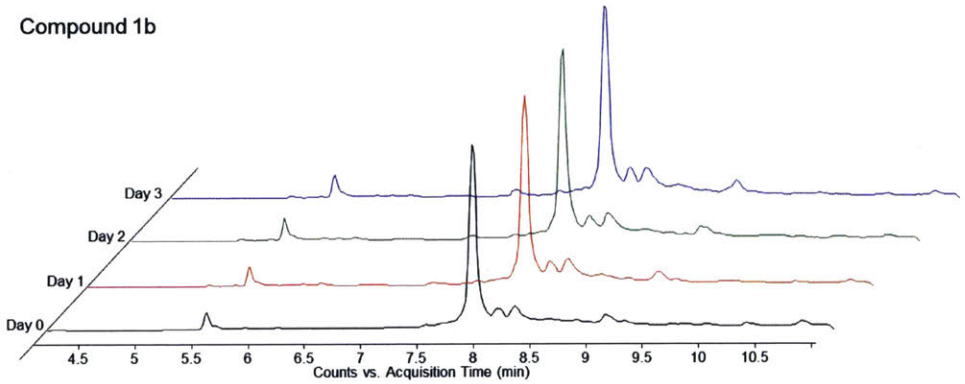
A solution of the palladium-peptide reagent **1B** (20 μM) in water was added to a equal volume of a solution containing biphenyl-4-thiol (100 μM) in TRIS buffer (0.1 M, pH 7.5). The palladium reagents were sonicated for 10 s to ensure the reagents were fully dissolved. Final conditions: [Pd] = 10 μM; [biphenyl-SH] = 50 μM; After 1 h at 37 °C, 3-mercaptopropionic acid (3 equiv) dissolved in H_2O was added to the reaction mixture to quench the remaining palladium species. The reaction was allowed to stand for 5 min and subsequently characterized by LC-MS.

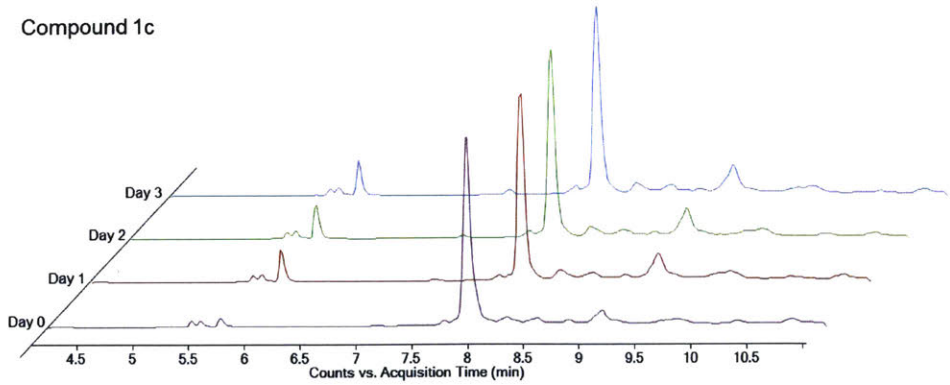


Stability in Solution

The three palladium-peptide oxidative addition complexes (**1A**, **1B**, and **1C**) were dissolved in 50% water / 50% acetonitrile containing 0.1% TFA at a concentration of 300 nanograms / μL. The solutions were analysed by LC-MS using method A after 1, 2, and 3 days. For compounds **1A**, **1B**, and **1C**, the peaks ~5.6 minutes have mass 1349.7 Da and 1374.7 Da, which corresponds with replacement of Pd with either a proton (H^+) or nitrile group (-CN). For compound **1C**, the peak ~5.7 minutes corresponds to peptide **P6**.

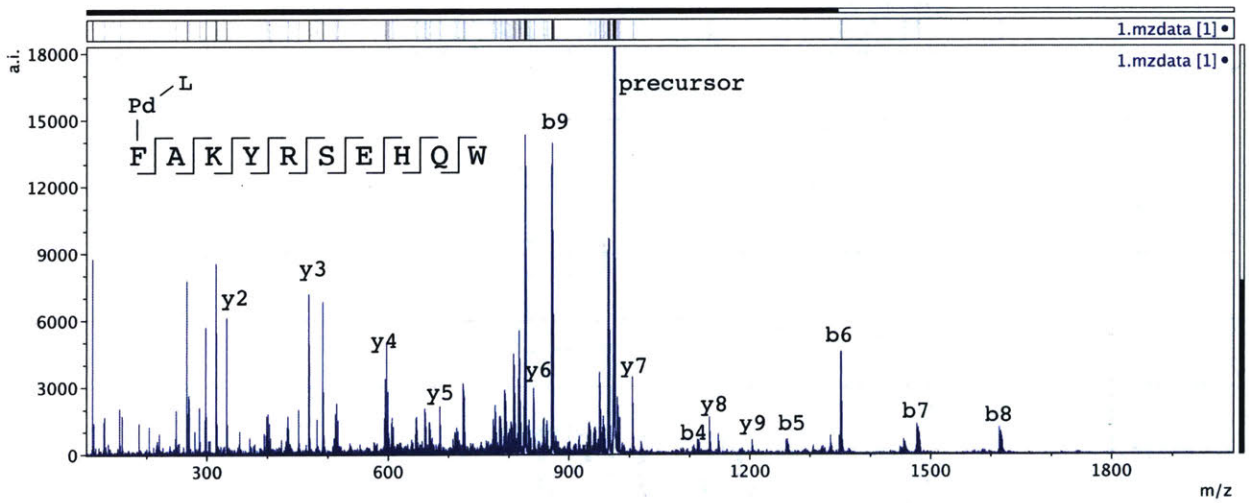






Complex 1B MS/MS Characterization

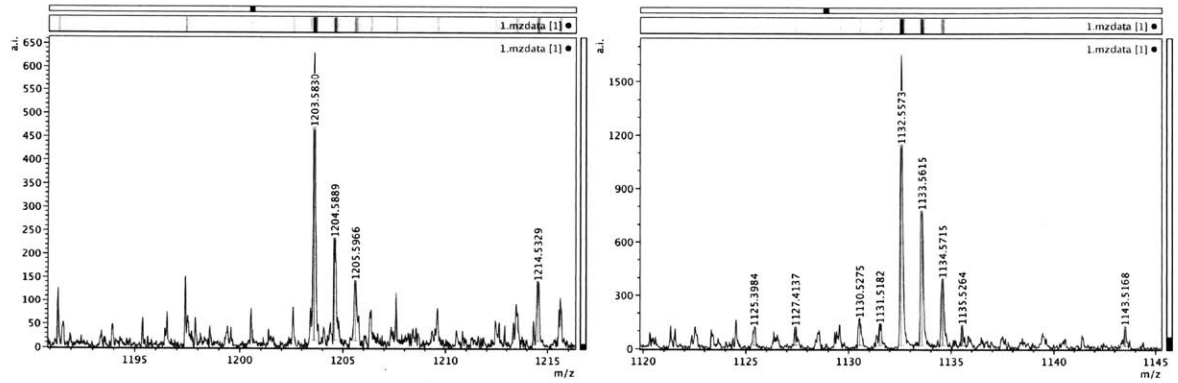
To confirm that the palladium is located at the N-terminal 4-bromophenylalanine residue, and not elsewhere on the peptide, we subjected oxidative addition complex **1B** to LC-MS/MS analysis. Method A was modified to acquire secondary MS spectra targeting specific ions. Ions with z = +2 and m/z = 973.87 or 973.37 Da were selected for secondary MS. Shown below is the secondary MS spectrum at 7.95 minutes for the 973.37 Da ion, with CID at 37.3.



The data were analysed using mMass software. The presence of palladium makes comprehensive analysis of the MS/MS data challenging due its isotopic abundance (deisotoping alogrithms do not account for the multiple Pd isotopes). Therefore, a few selected ions are shown below. Critically, no y-ions exhibited an isotope pattern suggestive of palladium, as would be expected

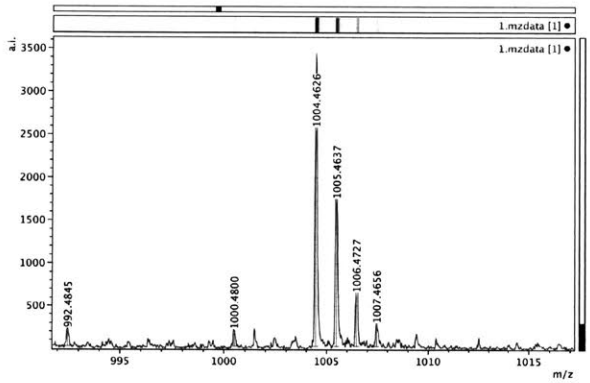
for a palladium complex on the N-terminal residue. Additionally, the b-ions exhibited the characteristic palladium isotope pattern.

Ion	Fragment	Expected Monoisotopic Mass (z = +1)
y_9	-AKYRSEHQW	1203.60
y_8	-KYRSEHQW	1132.56
y_7	-YRSEHQW	1004.47
y_6	-RSEHQW	841.41
b_9	[Pd]FAKYRSEHQ-	871.33 (z = +2)
b_5	[Pd]FAKYR-	1260.45

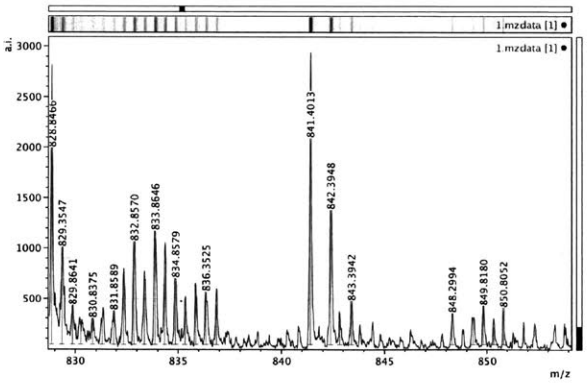


y_9

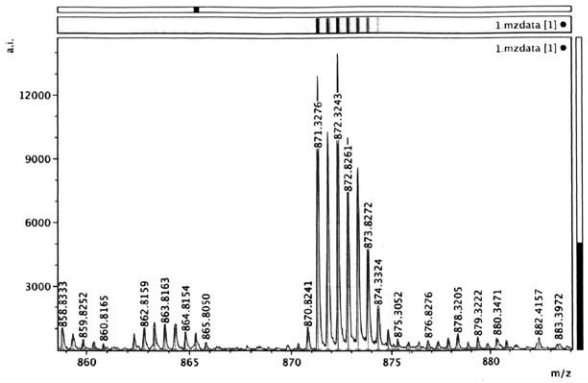
y_8



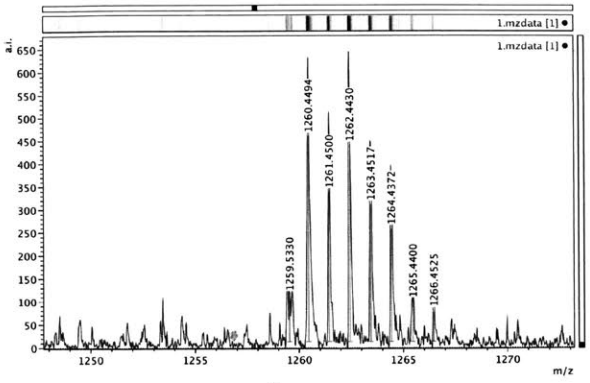
y_7



y_6



b9



b5

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