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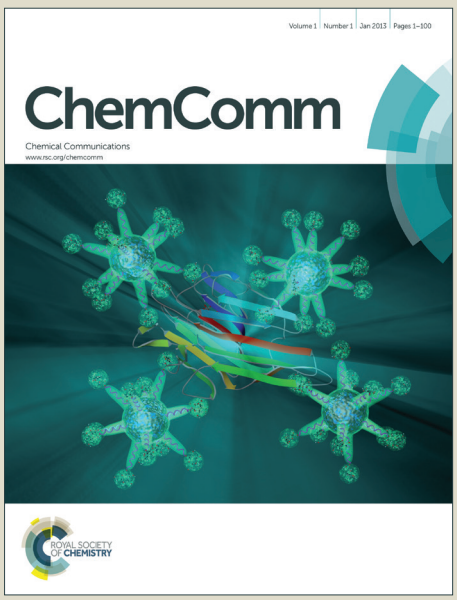

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Synthesis of High Contrast Fluorescein-Diethers for Rapid Bench-Top Sensing of Palladium†

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A new series of palladium sensors based on fluorescein bis(allyl ether) compounds has been designed. We utilized reduced fluoresceins as key synthetic intermediates. These probes exhibit negligible background fluorescence and rapid reaction with palladium, allowing a concentration of <100 ppt to be detected in minutes using a handheld UV lamp.

Reactions involving palladium catalysis have found wide use in modern synthetic chemistry due to their versatility, selectivity, and relatively mild conditions.[1] Although their ability to build molecular complexity has led to increased utilization in the synthesis of Active Pharmaceutical Ingredients (APIs), palladium byproducts are often difficult to remove and toxic to ingest.[2] Therefore, it is important to be able to efficiently and accurately identify residual palladium in synthetic API targets destined for use in biological studies, especially human consumption.

An attractive and innovative method to sense palladium involves the installation of allyl groups onto phenolic fluorophores.[3] Removal of these groups through a Pd(0)-catalyzed Tsuji-Trost deallylation[4] releases a fluorescent molecule, giving a large increase in fluorescence. This was first demonstrated by Koide and coworkers using the mono allyl ether Pittsburgh Green (**1**) (Figure 1).[5] Compound **1** has been shown to be useful and selective for measuring low concentrations of palladium using a fluorometer[6] and is being developed for pharmaceutical applications by Koide and Welch.[6d] Nevertheless, the current assay requires long reaction times (~hours) and the probe exhibits significant fluorescence background, which precludes facile bench-top use of this probe.[6]

The relatively high background fluorescence of probe **1** can be explained by its structure. Fluorescein dyes exist in equilibrium between a fluorescent (open) carboxylate form and a nonfluorescent (closed) lactone form. Although the open form of fluorescein predominates in polar media, acylation or alkylation of the phenolic oxygens can shift the equilibrium to the closed form, rendering the molecule less fluorescent until removal of these groups by a chemical reaction.[7] For compound **1**, the reduction of the key carboxyl group involved in the equilibrium and the presence of one allyl ether causes a significant decrease in fluorescence. Although the open–closed equilibrium shifts dramatically, the alcohol–cyclic ether transition is still present in xanthene dye derivatives[8] and some background signal exists due to the lingering presence of the open state. Upon specific reaction with palladium(0) and the resulting cleavage of the allyl ether, Pittsburgh Green fluoresces strongly.

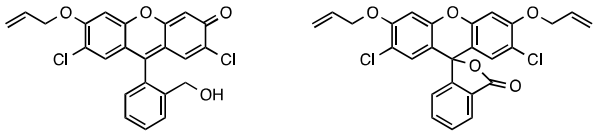


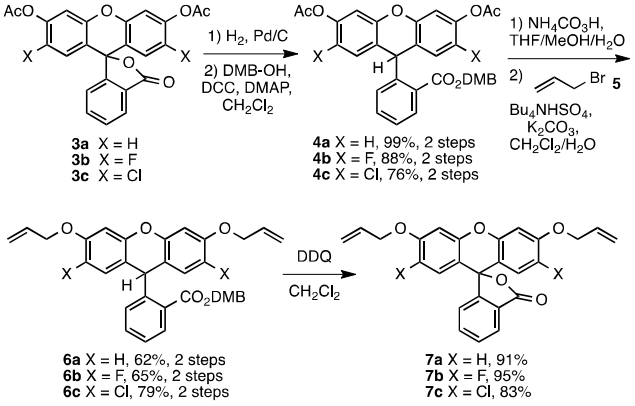
Figure 1. The structure of the palladium sensor allyl Pittsburgh Green (**1**) developed by Koide et al[5] and our proposed bis(allyl ether) 2',7'-dichlorofluorescein palladium sensor **2**.

To overcome the issue of background fluorescence, we planned to take advantage of the robust properties inherent in the open–closed equilibrium of fluorescein to achieve greater contrast and sensitivity to palladium(0). We proposed several bis(allyl ether) fluorescein derivatives, based on the Pd(0) catalyzed Tsuji-Trost deallylation reaction that has demonstrated selectivity with previous sensors. Koide and coworkers have shown that this unmasking event is selective for palladium over other metals and it takes place efficiently in the presence of several organic APIs.[6b] We also incorporated substitution at the 2'- and 7'- positions of the core dye, as in **2**, anticipating an effect on the efficiency of the unmasking reaction to reveal the fluorescent core. An electron-withdrawing group at this position should increase the ability of the xanthene core to act as a leaving group in the key ether cleavage, thereby accelerating the unmasking event. For practical reasons, a faster rate of reaction results in a more useful palladium sensor in most applications.

To synthesize fluorescein diether derivatives with the carboxyl group intact, a protection strategy was employed,[9] as shown below in Scheme 1. For the synthesis of the first generation palladium sensor

7a, reduction of the central carbon of fluorescein diacetate (**3a**) under atmospheric pressure isolates the carboxylic acid for further reaction. Subsequent protection via esterification using 2,4-dimethoxybenzyl (DMB) alcohol gives leuco-dye **4a** in 99% yield over two steps. Selective hydrolysis of the acetate esters reveals two phenolic groups that serve as nucleophiles in a phase transfer reaction using allyl bromide (**5**) as the alkylating agent. This cleanly provides diether **6a** in 62% yield over two steps. Finally, mild oxidation using DDQ simultaneously removes the DMB group[10] and returns the core xanthene dye to the proper oxidation state in 91% yield.

Scheme 1. Synthesis of Bis(allyl ether) fluorescein derivatives **7a-c**.



The synthetic approach described here results in selective alkylation to form the desired diether derivative without the unwanted ether-ester byproduct. Additionally, this route features several benefits when considering the issues commonly encountered in the synthesis of xanthene derivatives.[9b] Early reduction of fluorescein interrupts the extended conjugation of the xanthene core. This isolated phenolate moiety is a stronger nucleophile than the delocalized fluorescein anion, allowing higher yields under mild alkylation conditions. Each intermediate is soluble in common organic solvents and purification can be done with silica gel column chromatography. This compares favorably to a typical dye synthesis, which can be limited in versatility and accessibility because it requires high-boiling solvents like DMF and HPLC purification of complex mixtures to obtain the final product.

The synthesis of the 2',7'-dihalogenated bis(allyl ether) fluorescein derivatives **7b** and **7c** followed the same straightforward strategy, shown in Scheme 1. Unsurprisingly, the hydrolysis of the acetate esters was more facile with the incorporation of electron-withdrawing halogens, but each synthetic route proved to be direct and high-yielding.

With fluorogenic probes **7a**–**c** in hand, their ability to sense palladium was evaluated. Based on the optimized conditions used by Koide and coworkers,[6b] we carried out the palladium assay in the presence of the ligand tri(2-furyl)phosphine (TFP) and sodium borohydride as a reducing agent to ensure conversion of palladium(II) to the reactive palladium(0). Because the fluorescence of the unmasked fluorescein is pH dependent, basic conditions were used to optimize the fluorescence intensity of the probe after deallylation.[11] Although previous studies required elevated temperatures and aqueous phosphate buffer, our probes worked efficiently at room temperature with methanol as the solvent and $Pd(allyl)_2Cl_2$[6e] as the palladium source. These mild conditions would be better suited for the rapid testing of trace amounts of palladium in organic samples, like those that might be found in the pharmaceutical industry, because of the solubility of a wide variety of organic compounds in methanol.

The performance of the sensors synthesized here is summarized in Figure 2, which plots the palladium concentration tested in each sample in ppb against the rate of fluorescence increase, measured as the slope of the linear portion of the kinetic fluorescence trace. This allows a direct comparison over a wide range of concentrations among sensors that have very different rates of reaction. Kinetic traces and intensity measurements at a given time point for **7a-c** can be found in the Supporting Information. The fluorescence changes observed upon addition of varying concentrations of palladium to a sample solution of the sensor reveal a striking difference in rate of allyl ether cleavage when there is an electron-withdrawing substituent at the 2'- and 7'-positions of the xanthene ring core. As expected, this substitution makes the fluorescent dye a better leaving group, increasing the sensitivity of the fluorogenic probe with significantly shorter reaction times.

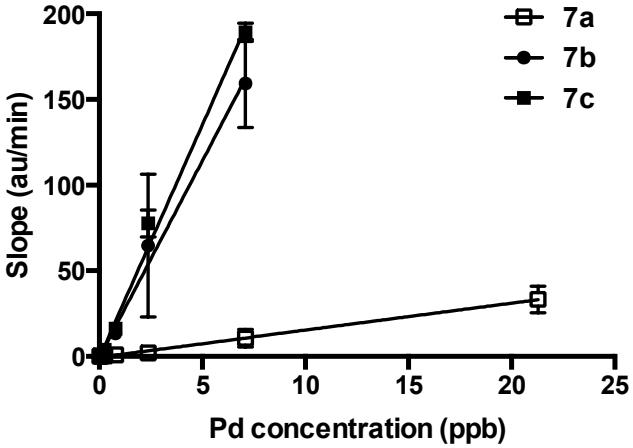


Figure 2. Comparison of fluorogenic bis(allyl ether) fluorescein derivatives **7a-c**. The slope is calculated from the linear portion of the kinetic trace resulting from the addition of varying concentrations of palladium to a solution of 125 μM of the probe, 500 μM TFP, and 1.25 mM $NaBH_4$ in MeOH. Kinetic data is available in the supporting information.

An interesting trend was observed when comparing compounds **7b** and **7c**. An explanation centered solely on electron-withdrawing ability affecting rate of reaction might suggest that the 2',7'-difluorofluorescein-based diether **7b** would have the fastest deallylation due to fluorine's electronegativity. However, **7b** and **7c** are very close in their reaction rates, with 2',7'-dichlorofluorescein-based diether **7c** exhibiting slightly faster kinetics. This experimental result matches the calculated substituent constants (σ) used for Hammett plots. Although fluorine is stronger in its inductive effect compared to chlorine, it is also more effective at electron-donation to the ring through resonance. When both factors are taken into account, the overall σ value for chlorine is slightly higher.[12] This observation is important for the practical design of fluorogenic substrates because the dichlorofluorescein derivative is more readily available, but less frequently employed than the difluorofluorescein, or Oregon Green, counterpart. Our results demonstrate that either derivative can improve the efficiency of reaction dramatically over the unsubstituted fluorescein core.

After evaluating the efficiency of our palladium sensors, a direct comparison can be made with the previously described Pittsburgh Green sensor **1**, as shown below in Figure 3. Probe **1** was prepared by the published procedure[5] and examined using Koide's optimized conditions for **1**,[6b] which include stirring at 45°C in a 1.25 M phosphate buffered aqueous solution. Compound **7c** was evaluated

using the conditions described here. Figure 3a shows the difference in fluorescence after stirring all samples for 5 min, in vials containing **1** with no palladium (i), **1** with 1 ppb palladium (ii), **7c** with no palladium (iii), and **7c** with 1 ppb palladium (iv). It is clear that the background fluorescence of **1** in the absence of the analyte makes a visual comparison of the difference in fluorescence difficult (Figure 3a, i and ii). However, there is a marked difference in fluorescence using **7c** due to the darkness of the sample without palladium (Figure 3a, iii and iv). Figure 3b shows a quantitative measurement of what is seen in the photo, with **7c** demonstrating a 314-fold increase in fluorescence after 5 min. After 1 h of stirring all samples, a moderate increase in fluorescence of **1** occurs with 1 ppb palladium as reported previously,[6] but background fluorescence would make visual detection of lower concentrations inconclusive. Importantly, after 1 h, the sample containing **9b** without palladium added is still nonfluorescent due to the stability of the ether functionality (data in Supporting Information).

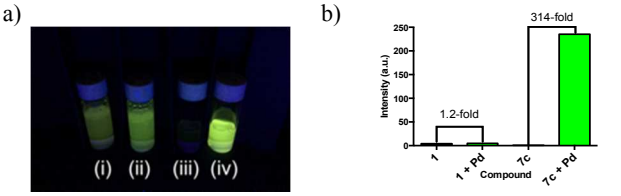


Figure 3. Direct comparison between the assay described by Koide et al with sensor **1** and the reported sensor **7c**. (a) after 5 min stirring times, the vials contain: (i) no palladium with **1**, (ii) 1 ppb palladium with **1**, (iii) no palladium with **7c**, and (iv) 1 ppb palladium with **7c**. (b) Fluorescence measurements of each experiment in (a) at 5 min time point.

This experiment highlights the importance of low background fluorescence in the implementation of useful fluorogenic probes. The conditions reported herein do not require elevated temperatures, high concentrations of buffers, or extended reaction times. Instead, a chemist could dissolve an organic sample in MeOH, add aliquots of the ligand, reducing agent, and **7c**, and be confident that palladium impurities are present if any fluorescence is observed after a short reaction time in ambient conditions. If multiple samples needed to be analyzed, this could easily be adapted to a microplate assay for rapid determination of palladium concentration.[6d]

The utility of this fluorescein diether design can be seen in Figure 4, which shows a photograph of vials containing low concentrations of palladium that have been stirred for 5 min and then illuminated by a handheld UV lamp. The vial on the right (vi) has no palladium added, and therefore represents background fluorescence. Palladium in concentrations as low as 67.5 parts per trillion (vial v) can be easily distinguished in this simple experiment. Although the calculated limits of detection for these probes are 0.673 ppb for **7a**, 0.455 ppb for **7b**, and 0.488 ppb for **7c** (see Supporting Information), we found that the presence of lower concentrations can be visually identified. In a practical sense, this represents an assay that could be performed quickly and reliably by a chemist working at the bench interested in testing for trace palladium impurities in an organic sample.

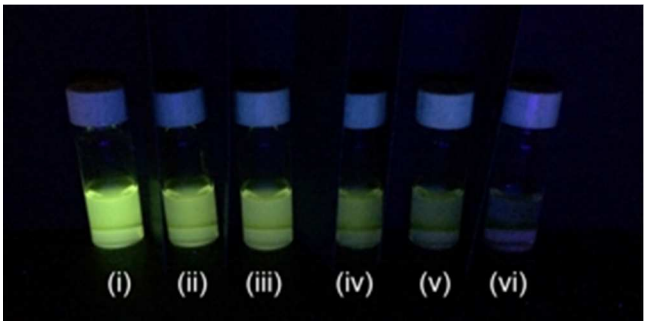


Figure 4. Sensor **7c** in the presence of varying palladium concentrations: (i) 1 ppb, (ii) 0.5 ppb, (iii) 0.25 ppb, (iv) 125 ppt, (v) 67.5 ppt, and (vi) no palladium. After the palladium was added to a solution of 125 μM **7c**, 500 μM TFP, and 1.25 mM $NaBH_4$ in 2 mL MeOH, the mixture was stirred for 5 min and illuminated by a handheld UV lamp for the photo.

Harnessing the critical open–closed equilibrium of fluorescein in the design of these molecules results in an easy-to-use and powerful sensor for palladium. Utilization of the reduced dye intermediate **4** allows for an efficient, versatile synthetic strategy to obtain fluorescein diether compounds with low background fluorescence, such as **7a-c**. For the detection of trace amounts of palladium, electron-withdrawing substituents speed up the unmasking reaction, but the slower kinetics of the unsubstituted probe **7a** may be ideal for quantitatively monitoring higher concentrations of palladium during the course of a reaction. With a colorless fluorogenic probe in the absence of palladium, the presence of the analyte is easier to detect in contrast. The diether structure of the derivatives reported here traps the molecule in the nonfluorescent form and these groups exhibit very slow hydrolysis, leading to a sustained dark background, allowing for a larger dynamic range of use. Future work may be done to anchor these probes to a solid support, thereby providing a further-simplified test for the presence of palladium in practical applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Reagents and instruments, experimental procedures, additional spectroscopic data, ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/c000000x/

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