Chemistry of Bifunctional Photoprobes. 1. Perfluoroaryl Azido Functionalized Phosphorus Hydrazides as Novel Photoreactive Heterobifunctional Chelating Agents: High Efficiency Nitrene Insertion on Model Solvents and Proteins

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Received October 2, 1996[⊗]

Synthesis and evaluation of a new class of photochemically activated heterobifunctional chelating agents for protein modification is described. Selective functionalization of perfluoroaryl azides by versatile phosphorus hydrazide ligating systems **2** and **3** for the complexation of transition metals and analogous radiometals form the basis for these new agents. The utility of the photogenerated precursors from these bifunctional agents to form covalent attachments is demonstrated through examination of C−H bond insertion on cyclohexane. Representative amide-coupled phosphorus hydrazides **5** and **6** provide >78% insertion of the probe into unactivated C−H bonds of cyclohexane with short photolysis times. Photoconjugation of the photoactivable heterobifunctional chelating agent **6** and its Pd metalated analog **7** with HSA is also evaluated. The uncomplexed chelate appears to add to HSA with high efficiency, consistent with the observed 82% bond insertion into model solvents. Covalent attachment of **7**, evaluated through the use of ^{109}Pd, was estimated to be between 49% and 74% with the uncertainty arising because of prephotolysis association of the ^{109}Pd complex with HSA. The application of *in situ* ^{19}F NMR to distinguish between bond insertion and noninsertion processes is demonstrated. These results suggest that functionalized perfluoroaryl azido phosphorus hydrazides may find utility as heterobifunctional photolabeling agents for attaching radionuclides to proteins and antibodies.

Introduction

The development of tumor-specific radiopharmaceuticals continues to be an important area of research because of their applications in the diagnosis and therapy of human cancer.[1−3] Attachment of radiolabels to biomolecules provides a viable approach for detection and subsequent therapy of certain cancers.[4,5] Conventional methods for radiolabeling of biomolecules require the intermediacy of two or more synthetic steps and the use of substrates which may be antagonistic to the biomolecule. These factors have frequently contributed to low radiolabeling yields and denaturation of the biomolecules,[6,7] suggesting the need for development of alternative techniques to conventional chemical affinity labeling.[8,9]

We are currently investigating the efficacy of radiolabeling of biomolecules via photochemical methods using bifunctional labels containing a photoprobe and a chelated radionuclide at the termini. Aromatic azides have been widely used for photoaffinity labeling,[10,11] but unproductive side reactions competing with the formation and insertion reactions of reactive singlet nitrene intermediates lead to low efficiencies. To achieve the high yields and optimum *in vivo* stability required for radiolabeling, it is crucial that the photoprobe exhibit a very high efficiency for covalent binding to the biomolecule. A high efficiency of conjugation also facilitates the isolation of labeled radioimmunoconjugates in pure form.[12]

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[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1997.

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S0022-3263(96)01867-1 CCC: $14.00

The groups of Platz[13] and Keana[14] have pioneered the development of polyfluorinated aromatic azides as singlet nitrene precursors which minimize the competitive pathways and enhance the insertion efficiency. Subsequently, we demonstrated that 4-azidotetrafluorobenzonitrile, upon photolysis, gave the highest yield of insertion into unactivated C−H bonds yet reported.[15] The photochemical attachment of polyfluorinated aryl azides to proteins is also characterized by high insertion efficiencies[16] and retention of immunoreactivity (>99%) in the postlabeling stage.[17] In fact, for a model photoprobe, the extent of bond insertion into proteins (HSA, IgG) exceeds the C−H insertion efficiency in cyclohexane.[16] This procedure does not involve external chemical activating agents, therefore minimizing physical and chemical manipulations of labeled biomolecules. While tritiated aryl azides have been extensively used to study the binding site of receptors, the functionalization of ligands with photolabels and their subsequent use in radiolabeling studies are mostly limited to the pioneering work of Katzenellenbogen *et al*.[18] The next logical step is establishment of a link between the photolabel and the ligating moiety. The photolabel-functionalized ligand can then be labeled with γ- or β-emitting radionuclides (^{99m}Tc, ^{109}Pd) for diagnostic or therapeutic applications, respectively. The favorable therapeutic values of ^{109}Pd (β-emitting radionuclide, 1.02 MeV, $\sigma = 12$ barn, medium range β source for therapy[19]) make it an ideal candidate for use in conjunction with chelating agents for potential radioimmunoconjugates.[20]

Herein we describe the synthesis and chemistry of the photoprobe−ligand complex utilizing a perfluoroaryl azide photoprobe and a versatile phosphorus hydrazide ligating system for the complexation of radiometals. The light-induced insertion of the photoprobe−ligand complexes into unactivated C−H bonds in cyclohexane is demonstrated by a quantitative comparison of the photoadduct with the independently synthesized cyclohexane adduct. The photoconjugation of this probe with the model protein human serum albumin (HSA) with and without chelation to ^{109}Pd is used to evaluate the potential of this technique for the labeling of biomolecules. A convenient screening technique, based on ^{19}F NMR, for evaluation of bond insertion reactions of the modified photoprobes is also described. This report forms the first quantitative analysis of the efficiency of photoconjugation by a radioanalytical method corroborated by spectroscopy (UV/vis and ^{19}F NMR). The current system is an example for future generations of heterobifunctional photoprobe chelating systems carrying diagnostic and therapeutic radionuclei.

Results and Discussion

Introduction of Perfluoroaryl Azido Moieties on Phosphorus Hydrazides. The choice of phosphorus hydrazides as the chelating moiety is based on the following characteristics: (a) these compounds are multifunctional so that the incorporation of the photolabel still leaves ligating moieties for chelating metallic radioisotopes, (b) phosphorus hydrazides are inert under photolytic conditions, allowing only the attached photolabel to undergo photolytic transformations, and (c) radiolabeling with metallic radioisotopes is versatile because phosphorus hydrazides offer a combination of electron donor centers in the form of hard-nitrogen bases and phosphorus chalcogenides.[21]

The bis(hydrazido)phosphine sulfide **1** is highly reactive toward nucleophilic substitution reactions at the NH_2 centers. In order to selectively direct a photolabel to a single NH_2 group of **1**, it was necessary to protect the other NH_2 as described in Scheme 1. The reaction of *m*-nitrobenzaldehyde with **1** in 1:1 stoichiometry at 0 °C is clean and highly selective and gave the mono Schiff

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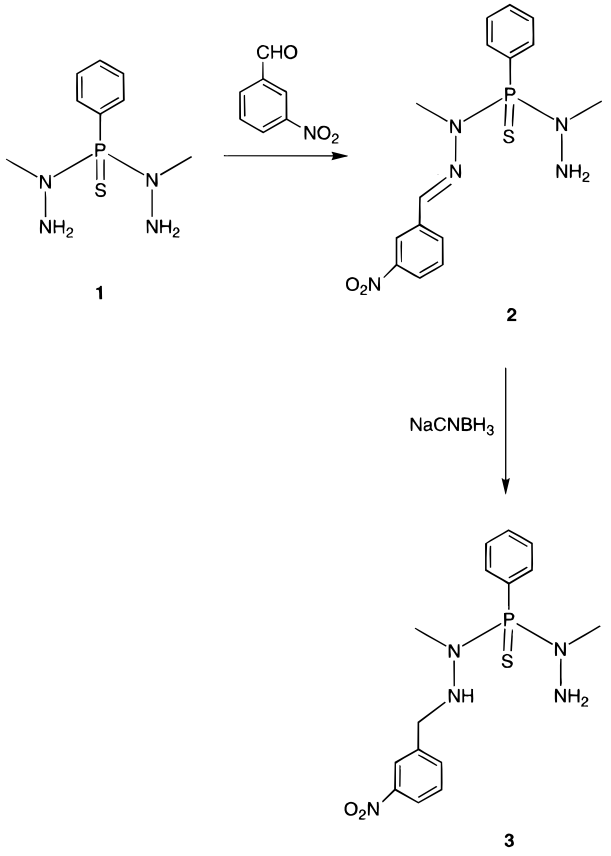
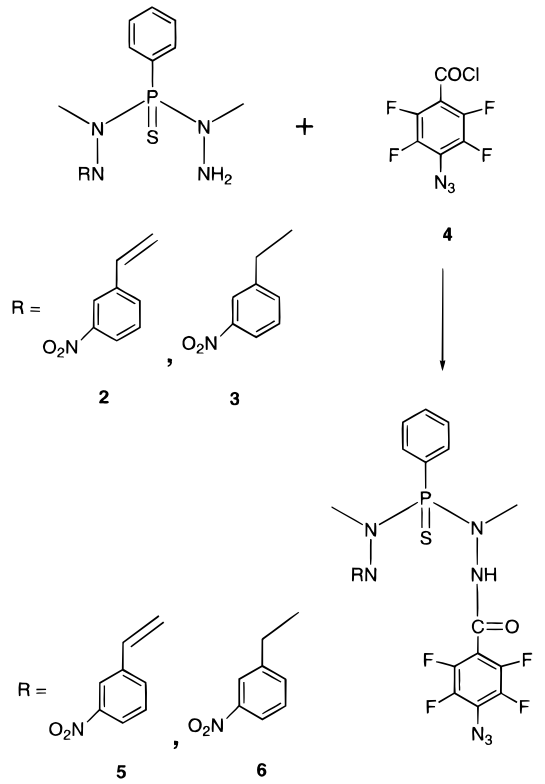
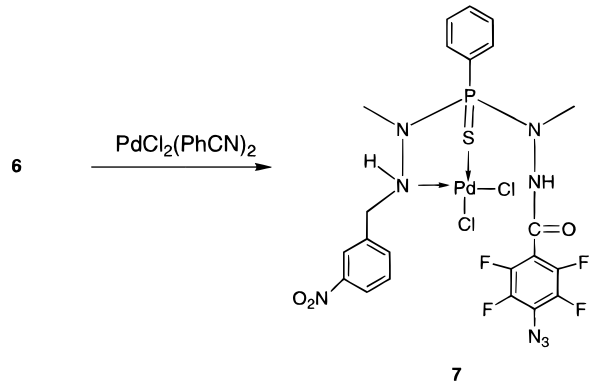
Scheme 1



Scheme 2



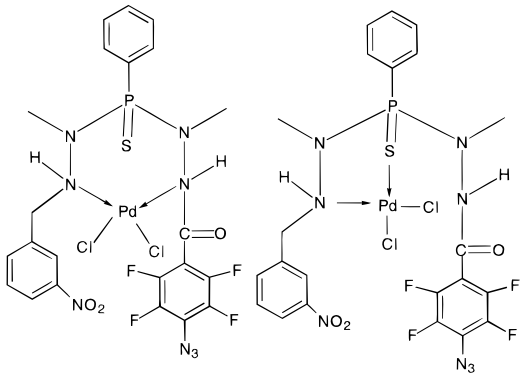
Scheme 3



base adduct **2** in good yields. The imine functionality in **2** was reduced to **3** in high yields with $NaBH_3CN$. The transformation of **2** to **3** is intended to increase the *in vitro* and *in vivo* stability of the hydrazido framework. In addition, the introduction of an NH functionality in **3** is expected to offer suitable electron donor characteristics for interactions with Pd(II) and other metallic radioisotopes. The monofunctionalized organophosphorus hydrazides **2** and **3** undergo coupling with **4** at the other terminal hydrazido center to produce the azidoperfluoroaryl amides **5** and **6**, respectively, as described in Scheme 2. The perfluoroaryl azido-functionalized phosphorus hydrazides **5** and **6** represent the first examples of main group ligating systems capable of undergoing photochemically induced CH (or NH) insertion reactions. New compounds **3**, **5**, and **6** were characterized by ^1H, ^{31}P, and ^{19}F NMR spectroscopy and C, H, and N elemental analysis. ^1H NMR spectra of the monosubstituted Schiff base adducts **2** and its reduced form **3** consisted of two doublets for the NMe protons (J_{P-H} = 9−12 Hz), indicating the presence of two dissimilar hydrazine backbones. The IR spectrum of **6** shows bands at 1689 cm^{-1} and 3267 cm^{-1} assigned to ν C═O stretching and ν N−H stretching modes, respectively, confirming the formation of the amide bond. The ^{31}P NMR spectroscopic data indicate modest upfield shifts (3−4 ppm) for **2**, **3**, **5**, **6** as compared to the parent **1**. It is also of note that the magnitude of J_{P-H} values (due to $H_3CNP(S)$) are 2−3 Hz lower as compared to **1**. This observation may be rationalized in terms of an electronic effect which, presumably, decreases the s-character across the $PNCH_3$ skeleton upon introduction of aryl or the perfluoroaryl substituents at the terminal nitrogens as outlined in Schemes 1 and 2. The IR spectroscopic data in conjunction with elemental analysis data further confirm the chemical constitutions of these compounds.

Metalation Reaction of 6 with $PdCl_2(PhCN)_2$. The reaction of **6** with $PdCl_2(PhCN)_2$ was carried out in CH_2Cl_2 at 25 °C to produce **7** (Scheme 3). Complex **7** is a dark-brown, air-stable, microcrystalline solid. The interaction of Pd(II) with **6** can occur in two possible ways: (i) one that involves the coordination of the two hydrazines (structure A), and (ii) via the coordination of the reduced amino (CH_2NH_2) arm of the hydrazine in **6** in conjunction with the P(S) functionality (structure B) as shown below.

The ^1H NMR spectrum of **7** consisted of features diagnostic of structure B. For example, the CH_2 functionality of the reduced nitroaromatic arm of **6** resonated as two inequivalent nuclei (H_A and H_B) centered at δ 3.76 and 4.04, respectively. These two resonances underwent a downfield shift to δ 4.12 and 4.85 in **7** suggesting that NH nitrogen attached to CH_2 functionality is involved in the complexation to Pd(II). The ν C═O stretching band at 1685 cm^{-1} observed for **7** is nearly identical to that found for **6**, indicating the lack of interaction of



A B

carbonyl moiety with the Pd(II) center. The ν P═S in **6** was observed at 622 cm^{-1}. This frequency was shifted to 598 cm^{-1} in **7**, indicating the coordination of the phosphorus chalcogenide with the Pd(II) center. Similar shifts in P═S stretching frequencies for a number of metal complexes of phosphorus hydrazides where N, S coordination with Pd(II) as depicted in structure B have been noted.[21] In fact, the N, S coordination as depicted for **7** has been demonstrated for a number of Pd(II) complexes and also confirmed by X-ray crystallography.[21,22] The intense absorption around 2120−2130 cm^{-1} in compounds **5**, **6**, and **7** is assigned to N_3 and indicates that the azido group is not perturbed or involved in the functionalization process. C, H, and N analysis of **7** reveals only one phosphorus hydrazide ligand per metal center.

Insertion Reactions of Perfluoroaryl Azido Phosphorus Hydrazides. The insertion efficiency of nitrenes generated by the photolysis of tetrafluoroaryl azides is known to be very sensitive to the substitution in the position para to the azide.[23] For example, electron-withdrawing groups (e.g., NO_2, CN, COCl, COOMe, etc.) are known to enhance nitrene insertion into cyclohexane as compared to electron-donating groups (e.g., NH_2, OH, etc.). Hence, azides used for labeling experiments should be evaluated for singlet nitrene insertion reactions with secondary amines to form substituted hydrazines (NH insertion) or cyclohexane (CH insertion) to give cyclohexyl derivatives. Photolysis of **5** and **6** in a mixture of CH_2Cl_2 and cyclohexane (1:1, v/v) produces an insertion adduct through the activation of a C−H bond as monitored by HPLC (Scheme 4). The insertion products elute at t_R = 4.7 and 4.9 min as compared to t_R = 2.1 and 2.2 min for the parent azides **5** and **6**, respectively (Table 1). In order to confirm the structure of the adduct and estimate the efficiency of insertion of the azides **5** and **6** onto the cyclohexane, a route was developed to synthesize the cyclohexane adduct of the perfluoroaryl azido phosphorus hydrazide as described in Scheme 5. HPLC retention time of the synthetic product was used to identify the inserted adduct among the photolyzed products and, subsequently, as a standard for quantitative estimation. Methyl 4-azido-2,3,5,6-tetrafluorobenzoate which is known for insertion onto cyclohexane was photolyzed in cyclohexane for 5 h and chromatographically separated to yield the CH insertion adduct **8** as reported earlier.[12a] Hydrolysis of **8** using 30% NaOH

Scheme 4

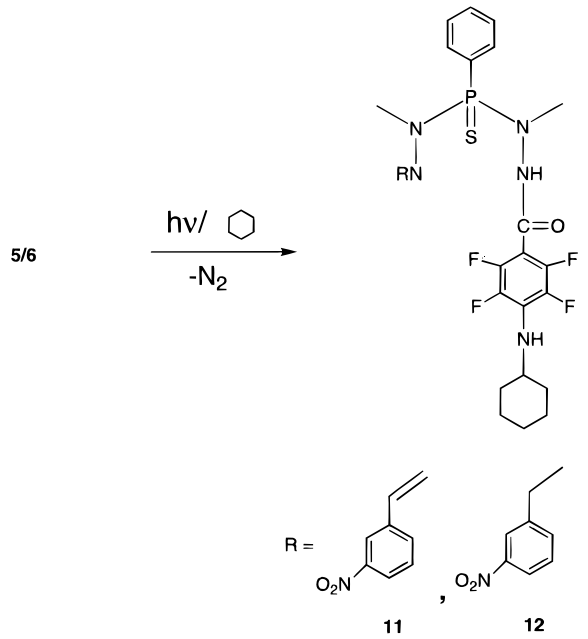


Table 1. Absorption Maxima and Retention Times for Perfluoroaryl Azido Phosphorus Hydrazides in Cyclohexane

compound	λ_{max} (nm)	retention time[a] (t_R/min) before photolysis	retention time[a] (t_R/min) after photolysis (C−H insertion adduct)
4-N_3-C_6H_4-$COOCH_3$	260	0.8	2.1
5	265	2.1	4.7
6	266	2.2	4.9
7	267	1.2	1.9

[a] CH_3CN:H_2O in the ratio 2:1 at a flow rate of 1 mL/min.

yielded **9** which upon refluxing with $SOCl_2$ yielded the acid chloride−cyclohexane adduct **10**. Treatment of **10** with **2** and **3** in the presence of Et_3N resulted in the desired cyclohexane adducts **11** and **12**. ^{1}H NMR of **12** shows characteristic peaks at δ 4.04 and 3.78 which have been assigned to NH and CH protons of the nitrene adduct, respectively, from previous studies on similiar systems.[15] The assignment was based on deuterium exchange where the δ 4.04 peak disappeared on addition of D_2O. Assignment of the methine carbon was further confirmed by ^{13}C NMR using the DEPT sequence which distinguished the methine carbon from the methylene carbons of cyclohexane. The synthesized **12** shows the same NMR spectral features and elutes at the same time (t_R = 4.9) as the major photolyzed product, identifying the photolytically produced cyclohexane adduct.

A significant outcome of this investigation is the determination of CH insertion efficiency of the nitrenes produced by photolysis of the perfluoroaryl azido phosphorous hydrazides in cyclohexane. Photolysis of dilute solutions (2×10^{-4} M) of **5** and **6** in cyclohexane and CH_2Cl_2 mixtures gave >90% destruction of the parent azide in 2 min photolysis with insertion product yields of 78 ± 3% for **5** and 81 ± 5% for **6** as determined by HPLC analysis. The short exposure times, consistent with a high quantum efficiency for photoprobe destruction,[15,16a] are of significance for potential photolabeling of proteins. Long exposure could directly damage the proteins and increases the possibility of secondary photolysis leading to decomposition of photoproducts or sensitized damage to the protein. Hence, these two azides provide the first

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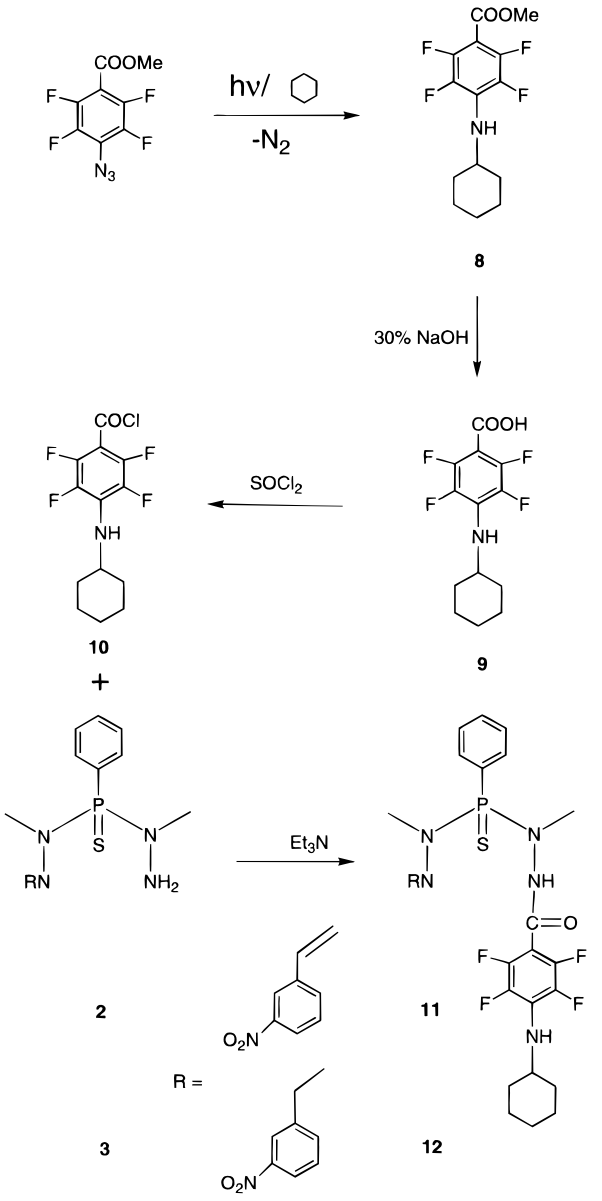
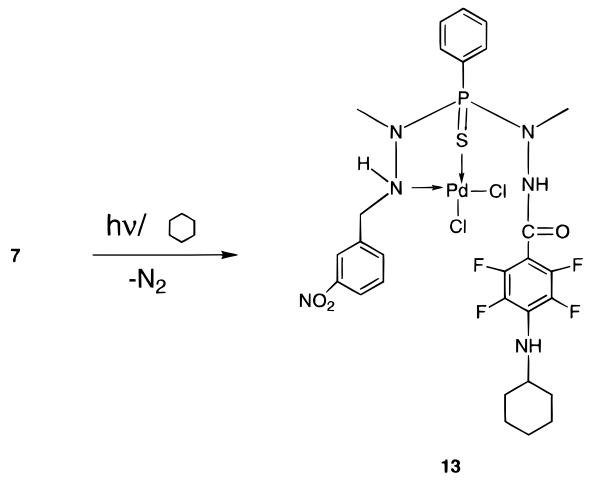
Scheme 5



example of a system in which a large molecular framework involving a complexing moiety shows high efficiency insertion reactions provided it is suitably tuned. This superior yield has a high impact on the utility of such chromophores for efficient photoattachment to different macromolecular probes. Similarly, the photolysis of **7** in a cyclohexane and CH_2Cl_2 mixture (1:1, v/v) resulted in a 45 ± 3% CH insertion adduct yield (Scheme 6) as determined by ^{19}F NMR[15] and HPLC analysis based on an authentic standard obtained by independent synthesis. Although the introduction of Pd metal into the hydrazine framework reduced the CH insertion yield, the complex still may conjugate to proteins with high efficiency by virtue of the close proximity of the ligand and the protein induced by hydrophobic interactions between the probe and hydrophobic pockets prevailing in proteins.[16a]

***In Situ* ^{19}F NMR Spectroscopy of Photoinsertion vs Noninsertion Products.** The advantage of ^{19}F NMR spectroscopy for the study of fluorinated biomolecules originates from the high sensitivity, wide chemical shift range and high abundance of the ^{19}F nucleus. In particular, the chemical shifts of fluorines flanking the

Scheme 6



azide moiety are useful for identifying different photoproducts. Typical photochemical reactions of perfluoroaryl azides involve generation of singlet products (bond insertion) and triplet products (noninsertion products including amines and azo compounds).[24] The symmetrical AA′XX′ pattern for ^{19}F NMR of perfluoroaryl azides could be useful in the identification of ring-expanded products which often plague the photochemistry of aryl azides.[10a] Compound **6** in cyclohexane shows two multiplets δ −139.4 and −151.9 corresponding to F-2, F-6 and F-3, F-5, respectively (Figure 1a), before photolysis. Partial photolysis of the mixture in the NMR tube (Figure 1b) results in the development of two multiplets at δ −141.1 and −161.9. When triplet products were preferentially enhanced by photolyzing the mixture in the presence of a triplet sensitizer (acetophenone), two additional multiplets at δ −141.8 and −163.3 appear (Figure 1c) which can be assigned to the amine produced through hydrogen abstraction by triplet nitrene. An independently synthesized **12** shows ^{19}F NMR absorptions at δ 140.7 and 162.1 (Figure 1d) coinciding with the cyclohexane adduct produced photolytically. Thus, ^{19}F NMR can be an effective tool in differentiating insertion and noninsertion products. The relative integration of fluorine signals with respect to total fluorine integration reflects the relative efficiency of insertion *vs* noninsertion. The two multiplets of low intensity at δ 150.5 and 138.3 (Figure 1c) can be assigned to the azo compound formed by the recombination of triplet nitrenes.[15] Photolysis of **6** in methanol gives exclusively triplet products (azo and amine derivatives, not shown in Figure 1), the ^{19}F NMR of which is similar to that of triplet sensitized products. It is well established that methanol catalyzes the intersystem crossing leading to triplet products.[13c] The differentiation between insertion and noninsertion products, especially the amines, can also be achieved by ^{19}F−^{19}F homodecoupling procedures. Decoupling of F-2 and F-6 in the photolyzed mixture containing cyclohexane adduct and amine by irradiating at the center frequency, δ −141.1, changes the coupling pattern of the peak at δ −161.9 to a simple doublet corresponding to ^{1}H coupling (J = 3.5 Hz) in the C−H insertion adduct. For the amine, irradiating at the center frequency δ −141.8 resulted in the collapse of the multiplet at δ −163.7 to a triplet corresponding to ^{1}H coupling (J = 4.5 Hz). This decoupling procedure of

(24) Hibert, F. K.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296.

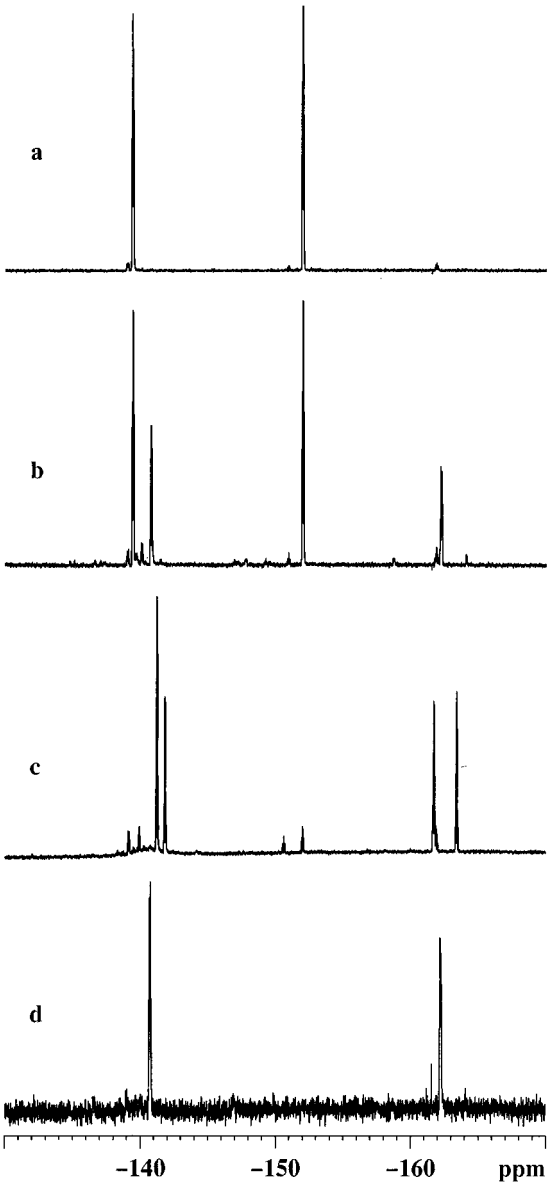


Figure 1. ^{19}F NMR spectrum of **6** in (a) cyclohexane (before photolysis); (b) photolysis in NMR tube for <5 min; (c) photolysis in the presence of triplet sensitizer (acetophenone); (d) ^{19}F NMR of independently synthesized cyclohexane adduct.

identifying insertion and noninsertion products is quite useful when fluorinated probes are attached to proteins where the photoproducts are difficult to isolate and characterize.

Conjugation of 6 with HSA. Equimolar solutions of HSA and **6** in aqueous buffer at pH 6.8 were incubated for 1 h before injecting into a SEC-HPLC column. Before photolysis, HSA elutes at 8.2 min and the photoprobe **6** at 10.8 min (Figure 2a). This separation demonstrates that the probe is not bound to HSA prior to photolysis. Photolysis (Figure 2b,c) results in disappearance of the probe accompanied by an increase in the absorbance in the protein peak. Denaturing of the photolyzed protein-photoprobe mixture with sodium dodecyl sulfate (SDS) has no effect other than dilution on the chromatogram features (Figure 2d). Although these absorbance changes do not constitute a proof of covalent attachment, they are consistent with the similar behavior seen in the photolabeling of HSA, IgG,[16b] and B72.3[17] where covalent bonding was confirmed with ^{14}C-labeling.

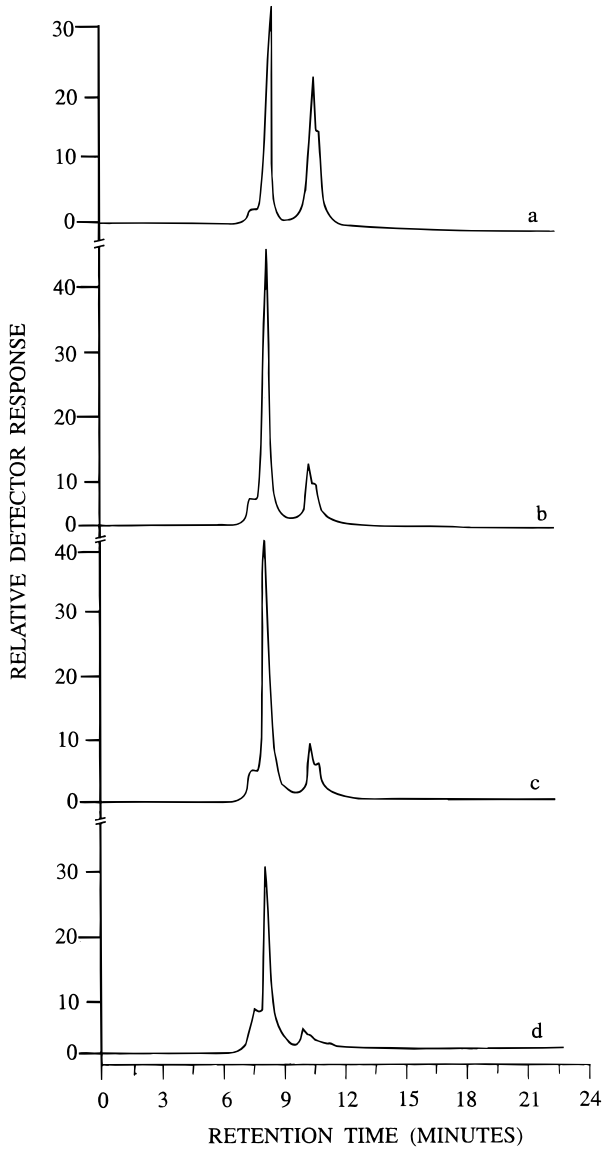


Figure 2. Size exclusion chromatogram (SEC-HPLC) of **1** with HSA (both 4×10^{-5} M) in pH 6.8 buffer: (a) before photolysis, (b) 15 s exposure, (c) 30 s exposure, (d) after treatment with SDS. The eluting solvent was 0.02 M monobasic sodium phosphate buffer containing 0.05 M sodium sulfate (pH 6.8) at a flow rate of 1 mL/min. Relative detector response refers to division of pen deflection on a strip chart recorder.

The distribution of products from photolysis of perfluoroaryl azides depends on the reactivity of the singlet nitrene and the environment in which it is generated. In a constrained medium (e.g., liquid crystalline or matrix isolated materials) even triplet nitrene can produce formal insertion products through abstraction of hydrogen from the substrate followed by radical combination. The noninsertion products are readily separated from native or photoconjugated proteins under SEC-HPLC conditions by virtue of the great disparity in molecular mass. For example, the triplet nitrene-derived amine derivative from photolysis of **6** in buffer elutes at 11.5 min under the HPLC conditions employed (not shown in Figure 2). The absence of this product, even after SDS denaturation of HSA, is consistent with a high efficiency for covalent attachment of the probe to the protein.[16b] Since covalent binding of nitrene-based photoprobes to proteins usually exceeds the C−H bond insertion efficiency on cyclohexane,[16b,17] a high insertion efficiency

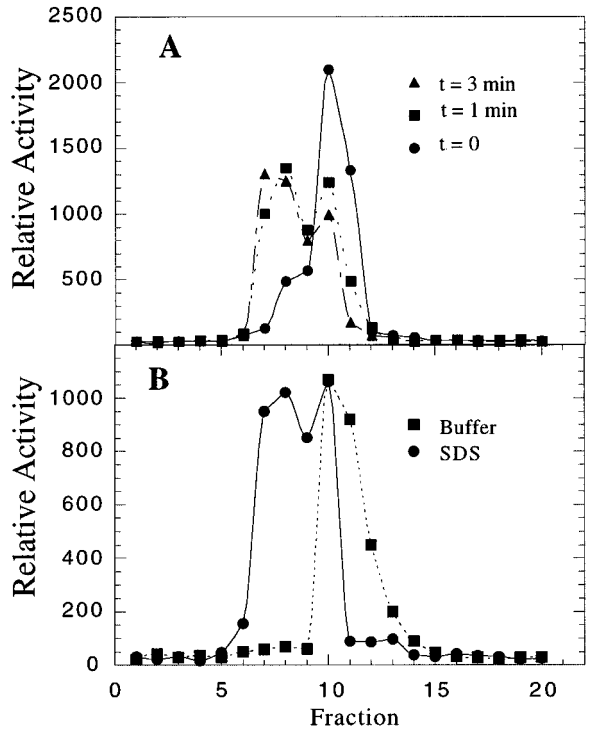


Figure 3. SEC-HPLC chromatograms of the ^{109}Pd complex **7**: (A) with HSA before photolysis (●), after 1 min photolysis (■), after 3 min photolysis (▲); (B) treatment of the 3 min photolysis mixture with SDS (●), and after photolysis of **7** in buffer without HSA (■).

of the singlet nitrene derived from **6** in HSA is consistent with the observed 82% efficiency for insertion into cyclohexane.

Complexation of ^{109}Pd with 6. The complexation of the activated $PdCl_4^{2-}$ sample containing ^{109}Pd with **6** was carried out in ethanolic solution. The complex, isolated by extraction into $CHCl_3$ and washing with saline, was characterized by paper chromatography as a neutral complex with a radiochemical purity of >98%.

Photoconjugation of the ^{109}Pd-Labeled 7 with HSA. The total concentration of the Pd complex stock solution ("cold" Pd and ^{109}Pd) is estimated to be 4.95×10^{-3}, assuming the 75% yield of complexation as found for unactivated Pd. Human serum albumin (HSA) (5 mL, 2×10^{-5} M) was mixed with of the ^{109}Pd complex (100 μL, $\sim 5 \times 10^{-3}$ M in total Pd complex) and incubated for 1 h. This solution with an approximate ratio of **7**/HSA of 5/1 was divided with part used for a control and part for the photoconjugation experiment. Samples (20 μL) of the mixture were injected onto the SEC-HPLC. Prior to photolysis, the photoprobe elutes between 10 and 11 min (Figure 3A) indicating the ^{109}Pd complex is separable from HSA which elutes at 7−9 min on this column. The small amount of activity appearing in the HSA region of the chromatogram is indicative of a minor prephotolysis binding of the ^{109}Pd complex to HSA, but the major fraction remains unattached to the protein before photolysis. The nature of the preassociation has not been elucidated. However, ^{31}P NMR of **7** in the presence of HSA indicates a single compound analogous to that observed for **7** in $CDCl_3$, suggesting that the Pd-chelating environment is not altered by the association. Sequential photolysis (1−3 min) of the mixture shifts a major fraction of the radioactivity to the HSA region of the chromatogram (Figure 3A). The nature of the association of ^{109}Pd activity with HSA was investigated by denaturing the protein with sodium dodecyl sulfate (SDS) which is expected to open the tertiary structure of the protein and release noncovalently bound molecules. Under these conditions (Figure 3B) there is no significant loss of activity in the HSA region, and we conclude the label is covalently attached to the protein. Previously,[16] it was found that the amine product from the triplet nitrene precursor is the only major side photoproduct in the absence of protein. Photolysis of **7** in the absence of HSA shows that this noninsertion product elutes at a similar retention time (t_R) as that of the unphotolyzed probe (Figure 3B). Although the resolution is inadequate to resolve **7** and the amine photoproduct, it does show that both are separable from the labeled HSA. Peak broadness, due to the eluent collection procedure, also precluded complete separation of the HSA and probe fractions of the chromatogram. However, if fractions 7−9 in Figure 3 are assumed to be exclusively protein and its labeled products, it can be estimated that 25% of the ^{109}Pd activity is bound to the protein after 1 h incubation before photolysis. Following a 3 min photolysis, 74% of the activity remains with the protein, and this value is still 71% after denaturation with SDS. Whether or not this preassociated activity becomes covalently bound to HSA during photolysis cannot be ascertained. Nevertheless, the 49% increase in labeling following photolysis sets a lower limit for the photoconjugation efficiency, with a possible efficiency as high as 74% if the preassociated ^{109}Pd complex is also covalently bound by photolysis.

^{19}F NMR Spectroscopy of the Photoconjugation of 6 with HSA. Application of ^{19}F NMR to distinguish between insertion and noninsertion reactions in proteins was attempted with **6** and HSA. The ^{19}F NMR spectrum of **6** (dissolved in a minimum amount of ethanol) and HSA in phosphate buffer shows two multiplets at δ −144.6 (F-2, F-6) and δ −151.9 (F-3, F-5, Figure 4a). A partial photolysis of this mixture produced two new multiplets at δ −146.4 and −161.2 (Figure 4b). The δ −161.2 multiplet matches the δ −162.1 feature observed for F-3 and F-5 for the independently synthesized cyclohexane adduct (Figure 1d). This observation may be rationalized as an insertion of the photoprobe into hydrophobic regions[25] of HSA. To confirm this hypothesis, the experiment was repeated in the absence of HSA where the only photoproduct is expected to be the triplet-derived amine. The spectrum of the prephotolysis mixture appears in Figure 4c. After photolysis (Figure 4d), a new multiplet appears at δ −163.4 which corresponds to the chemical shift of the triplet nitrene-derived amine. Although the difference in chemical shifts of F-3 and F-5 between the insertion and amine products is quite small (δ 1.3) it is adequate for differentiation by *in situ* NMR studies. However, the low signal to noise ratio and peak broadness precludes a quantitative evaluation of insertion efficiency by this method.

The chemical shifts of F-2 and F-6 are quite sensitive to the presence of HSA. In cyclohexane and in buffer without HSA, these resonances occur at δ −139.4 and −140.7, respectively, while in buffer with HSA present, the resonance occurs at δ −144.6. Although the origin of this shift is unclear, fluorine chemical shifts are known to be very sensitive to environment.[26] For example, Fenney et al.[27] have reported large changes of chemical

(25) Spector, A. A.; John, K.; Fletcher, J. J. *Lipid Res.* **1969**, *10*, 56.
(26) Lau, E. Y; Gerig, J. T. *J. Am. Chem. Soc.* **1996**, *118*, 1194.

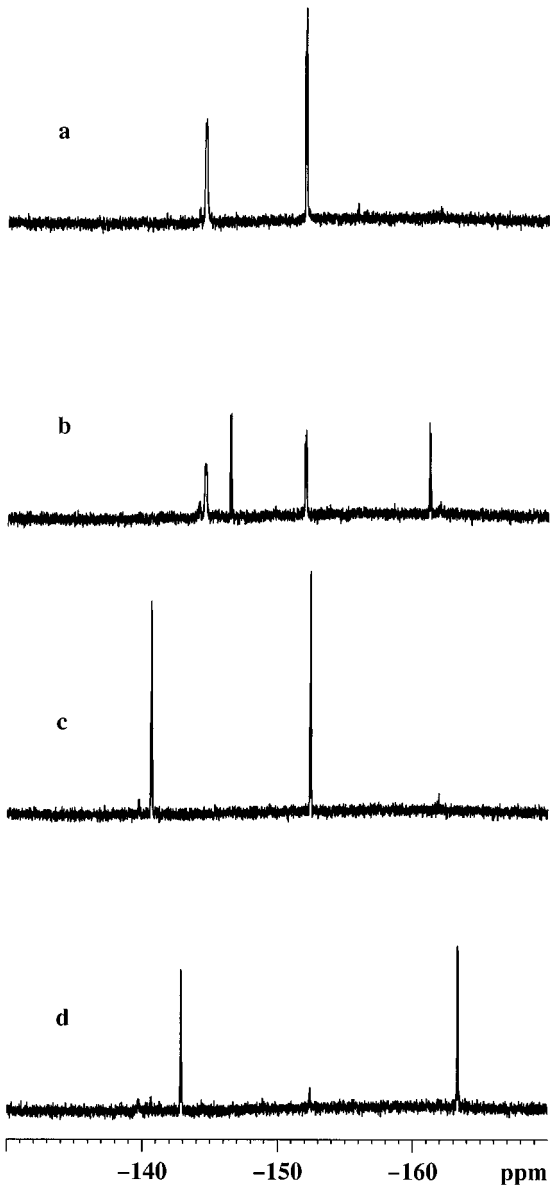


Figure 4. ^{19}F NMR of **6** with HSA (a) before photolysis control, (b) after partial photolysis, (c) **6** in buffer without HSA before photolysis, (d) after 3 min photolysis of c. All ^{19}F NMR chemical shifts are referenced to $CFCl_3$.

shifts in ^{19}F-labeled proteins which may be due to conformational as well as local field effects. The preferential small shift of F-2 and F-6 (δ 3.9) may be related to the prephotolysis orientation of the probe in the hydrophobic pocket of the protein. The much larger shifts of F-3 and F-5 after photolysis, however, are a clear indication of covalent modification of the protein with the photoprobe.

Conclusion

Incorporation of perfluoroaryl azides into phosphorus hydrazide ligating systems represent a new approach for the formulation of versatile bifunctional chelating agents carrying both photoactivable and chelating moieties. The amide-coupled phosphorus hydrazide derivatives appear to possess global reactivity by showing a high C−H insertion yield (78% and 82% for **5** and **6**, respectively) on cyclohexane. The high quantum yields for photoprobe binding is of considerable significance in photolabeling of proteins since long exposure may not be warranted for labeling biomolecules. *In situ* ^{19}F NMR provides a convenient method for differentiation between insertion and noninsertion products in proteins and model solvents.

Despite some prephotolysis binding between HSA and **7**, the ^{109}Pd-labeled **7** provides a convenient radiochemical probe to investigate the photoconjugation of proteins. The high efficiency for covalent binding to HSA and the small yields of the polar noninsertion photoproduct coupled with the facile separation of the covalently bound radiolabeled heterobifunctional chelating agent from the noncovalently bound photoproducts greatly enhances the potential applications of photolabeling in nuclear medicine. Although triplet nitrene products dominate when azides are photolyzed in hydroxylic media, these appear not to be significant in the presence of HSA. Our hypothesis is that the hydrophobic photoprobe is protected from solvent exposure by association with the hydrophobic regions of the protein. The extent to which this protection will continue with less hydrophobic proteins remains to be investigated.

Experimental Section

All synthetic procedures were conducted in a dry nitrogen atmosphere using standard Schlenk tube techniques and prepurified solvents. Reactions involving the synthesis of azides were carried out in subdued light by wrapping the flasks with aluminum foil. Phosphorus hydrazide **1** was prepared as reported earlier.[21b] NMR spectra were recorded in $CDCl_3$ on a 300 MHz spectrometer, and chemical shifts are reported in ppm downfield from $SiMe_4$ for ^1H NMR. The ^{31}P NMR chemical shifts are reported with respect to 85% H_3PO_4 as an external standard, and positive shifts lie downfield from the standard. ^{19}F NMR chemical shifts are reported with respect to $CFCl_3$ as an external standard. Infrared spectra were recorded in KBr pellets. Elemental analysis for the new compounds were done by Oneida Research Services, Inc., New York. UV/visible spectra were recorded either in cyclohexane or in acetonitrile.

Protein assays were performed by injecting 20 μL samples onto a size exclusion column (Biorad Biosil SEC 250). The eluent was a phosphate (PBS) buffer (0.02 M sodium phosphate, monobasic containing 0.05 M sodium sulfate, pH 6.8) pumped at the rate of 1 mL/min. Experiments performed with HSA and the bifunctional ligand before ^{109}Pd labeling were analyzed by 254 nm UV detection of the eluent. For mixtures of HSA and the radiolabeled complex, the eluent was collected in 1 mL fractions and subsequently counted with a NaI counter.

Photolysis was carried out with a 200 W high pressure Hg lamp. The focused beam passed through a water filter and a glass cut-off filter to limit excitation to wavelengths greater than 320 nm. Compounds **5**−**7** have similar absorption spectra with λ_{max} near 265 nm (Table 1), diminishing to <10% of the λ_{max} value at 320 nm. Thus, photolysis was carried out in the spectral region where proteins have minimal absorbance. A standard silica cuvet was utilized for labeling experiments which typically required 2 min irradiations for >90% completion. *In situ* experiments were performed by placing the NMR tube such that it intersected a portion of the light beam. The *in situ* studies typically required 10 min for >90% destruction of the photoprobe due to the small fraction of the beam area intercepted by the NMR tube. The photolytic decomposition of the photolabels was monitored by HPLC using a 30 mm × 4.7 mm C-18 reverse phase column. t_R values are reported in minutes using eluent CH_3CN-H_2O in ratio 2:1 at a flow rate of 1 mL/min. Solutions were purged with prepurified nitrogen for several minutes before photolysis.

(27) Feeney, J.; McCormick, J. E.; Bauer, C. J., Birdsall, B. Moody, C. M.; Starkman, B. A.; Young, D. W.; Francis, P.; Halvin, R. H.; Arnold, W. D.; Oldfield, E. *J. Am. Chem. Soc.* **1996**, *118*, 8700.

Synthesis of (C_6H_5)P(S)($NMeNH_2$)($NMeNHCH_2$-C_6H_4-3-NO_2) (3). Monofunctionalized phosphorous hydrazide **2** (2.8 g, 7.7 mmol) prepared earlier[22] and a trace of bromocresol green were dissolved in CH_3OH (50 mL), and $NaBH_3CN$ (2.9 g, 46.2 mmol) was added. The solution immediately turned deep blue. The pH of the solution was adjusted to ~4.0 by adding 2 N HCl−CH_3OH dropwise with stirring until the yellow color was restored. After 6−8 h, $NaBH_3CN$ (1.93 g, 30.8 mmol) in methanol was added and the solution stirred overnight. The excess $NaBH_3CN$ was destroyed with 5 N HCl. The product was extracted into CH_2Cl_2 and dried with $MgSO_4$, and the solvent was evaporated *in vacuo* to yield a pale yellow crystalline solid (2.39 g, 85%). Mp: 104−106 °C; IR: 3309, 3225, 3056, 2943, 1520, 1428, 1337, 1104, 999, 717 cm^{-1}; ^1H NMR: δ 2.76 (d, J = 12.37 Hz, 3H), 3.01 (d, J = 10.34 Hz, 3H), 3.40 (br s, 2H), 3.89 (d, J = 12.51 Hz, 1H), 4.04 (br, 1H), 4.07 (d, J = 12.21 Hz, 1H), 7.42−7.55 (m, 5H), 8.02−8.10 (m, 4H); ^{31}P NMR: δ 85.2. Anal. Calcd for $C_{15}H_{20}N_5O_2PS$: C, 49.31; H, 5.52; N, 19.17. Found: C, 49.21; H, 5.34; N, 19.27.

Synthesis of 4-Azido-2,3,5,6-tetrafluorobenzoyl Chloride (4). A mixture of 4-azidotetrafluorobenzoic acid (5 g, 21.18 mmol) and excess SO_2Cl_2 (10 mL) in dry hexane (250 mL) was refluxed until the acid was dissolved completely, indicating the completion of the reaction. The solvent was removed, and the residue was distilled (100 °C/0.5 mmHg) using a Kügelrohr apparatus to give a yellow liquid (79%). The physical constants of the compound coincided well with the earlier report.[14a]

Synthesis of (C_6H_5)P(S)($NMeNHCOC_6F_4N_3$)($NMeN{=}CHC_6H_4$-3-NO_2) (5). A solution of **4** (910 mg, 3.59 mmol) in CH_2Cl_2 was slowly added dropwise to the solution of **2** (1 g, 2.75 mmol) in CH_2Cl_2 at 0 °C in the presence of excess Et_3N (560 mg, 5.53 mmol) and stirred at 25 °C for 2 h. The mixture was washed with water (25 mL) and brine (25 mL) after which it was dried using sodium sulfate. The solvent was evaporated *in vacuo*, and the crude product was purified on a silica gel column by eluting with 30% ethyl acetate−hexane buffered with 1% Et_3N to give the amide **5** (1.28 g, 80%). Mp: 60−63 °C; IR: 3069, 2914, 2126, 1700, 1647, 1533, 1486, 1349, 985, 787, 734 cm^{-1}; ^1H NMR: δ 3.09 (d, J = 10.36 Hz, 3H), 3.46 (d, J = 8.87 Hz, 3H), 7.4−8.2 (m, 9H), 7.80(s,1H), 8.28 (m, 1H); ^{31}P NMR: δ 81.8; ^{19}F NMR: δ −140.8 (m, 2F), −150.4 (m, 2F). Anal. Calcd for $C_{22}H_{17}N_8F_4O_3PS$: C, 45.52; H, 2.95; N, 19.30. Found: C, 45.36; H, 3.21; N, 18.97.

Synthesis of (C_6H_5)P(S)($NMeNHCOC_6F_4N_3$)($NMeNHCH_2C_6H_4$-3-NO_2) (6). The procedure was similar to that used for the preparation of **5**. A CH_2Cl_2 solution of **4** (2.08 g, 8.1 mmol) and **3** (1.92 g, 5.25 mmol) reacted to give **6** (2.51 g, 82%) as a pale yellow solid. Mp: 95−96 °C; IR: 3267, 3055, 2858, 2126, 1689, 1639, 1520, 1337, 1252, 984, 731, 682, 622, 519 cm^{-1}; ^1H NMR: δ 2.83 (d, J = 11.12 Hz, 3H), 3.14 (d, J = 9.96 Hz, 3H), 3.76 (d, J = 11.87 Hz, 1H), 4.01(br, 1H), 4.04 (d, J = 12.33 Hz, 1H), 7.40−7.48 (m, 5H), 7.65 (s, 1H), 7.95 (s, 1H), 8.07−8.15 (m, 3H); ^{31}P NMR: δ 83.8; ^{19}F NMR: −140.6 (m, 2F), −150.2 (m, 2F). Anal. Calcd for $C_{22}H_{19}N_8F_4O_3PS$: C, 45.37; H, 3.29; N, 19.24. Found: C, 45.69; H, 3.29; N, 19.49.

Synthesis of Palladium Complex 7. A solution of [$PdCl_2$($PhCN)_2$] (0.380 g, 1 mmol), in CH_2Cl_2 (50 mL) was added slowly to the solution of **6** (0.582 g, 1 mmol) also dissolved in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature for 15−30 min, and the solvent was partially evaporated *in vacuo*. The concentrated solution was treated with dry *n*-hexane. The resulting precipitate was filtered, washed (3 × 30 mL) with *n*-hexane, dried, and redissolved in hot alcohol layered with *n*-hexane. Slow evaporation gave **7** as orange brown crystals (0.721 g), 75%. Mp: 175 °C dec; IR: 2128, 1532, 1685, 1462, 1277, 800, 792, 598 cm^{-1}; ^1H NMR: δ 3.22 (2 overlapping doublets, 6H), 4.12 (d, J = 13.4 Hz, 1H), 4.85 (d, J =13.5 Hz, 1H), 7.49−8.79 (m, 10H); ^{31}P NMR: δ 88.2; ^{19}F NMR δ −140.5 (m, 2F), −151.4 (m, 2F). Anal. Calcd for $C_{22}H_{19}N_8F_4O_3PSPdCl_2$ C, 34.83; H, 2.53; N, 14.78. Found: C, 34.49, H, 2.54, N, 14.45.

Synthesis of Methyl 4-(Cyclohexylamino)-2,3,5,6-tetrafluorobenzoate (8). A solution of methyl 4-azido-2,3,5,6-tetrafluorobenzoate (0.124 g, 0.5 mmol) in cyclohexane (100 mL) was photolyzed for 4 h. The crude photolysis products were separated by column chromatography using hexane, ethyl acetate, and methanol in the ratio of 20:1:1. The amine **8** (0.09 g, 57%) crystallized upon cooling. Mp: 85 °C; ^1H NMR: δ 1.22 (m 3H) 1.31 (m 2H); 1.58 (m, 1H), 1.76 (m, 2H), 2.03 (m, 2H), 3.61 (m, 1H), 3.91 (s, 3H), 4.02 (m, 1H); ^{19}F NMR: δ −144.2 (m, 2F), −161.2 (m, 2F); IR: 3368, 3007, 2930, 1714, 1638, 1541, 1311, 1082, 881, 742 cm^{-1}. Anal. Calcd for $C_{14}H_{15}F_4NO_2$ C, 55.08; H, 4.95; N, 4.59. Found: C, 55.21; H, 4.72; N, 4.49.

In order to prepare adequate quantities of **8** for syntheses of other cyclohexylamino derivatives an independent synthetic procedure was also employed.[16b]

Synthesis of 4-(Cyclohexylamino)-2,3,5,6-tetrafluorobenzoic Acid (9). The cyclohexane adduct **8** (5 g, 16.3 mmol) was dissolved in CH_3OH (100 mL), and the adduct was hydrolyzed using 30% aqueous NaOH (25 mL) by stirring overnight at 25 °C. The solution was acidified by 2 N HCl at 0 °C to pH < 1 and extracted into $CHCl_3$ layer (3 × 30 mL) and dried using $MgSO_4$ and evaporated to a white powder 9 (85%). Mp: 163−165 °C; IR: 3389, 2937, 1687, 1638, 1527, 1305, 1249, 985, 763, 714 cm^{-1}; ^1H NMR: δ 1.20 (m, 3H), 1.32 (m, 2H), 1.57 (m, 1H), 1.75 (m, 2H), 2.04 (m, 2H), 3.60 (m, 1H), 4.04 (m, 1H), 11.5 (s, 1H); ^{19}F NMR: δ −139.5 (m, 2F), −165.1 (m, 2F). Anal. Calcd for $C_{13}H_{13}F_4NO_2$ C, 53.59; H, 4.50; N, 4.81. Found: C, 53.12; H, 4.43; N, 4.76.

Synthesis of 4-(Cyclohexylamino)-2,3,5,6-tetrafluorobenzoyl Chloride (10). A mixture of 4-(cyclohexylamino)-2,3,5,6-tetrafluorobenzoic acid (**9**) (400 mg, 1.37 mmol) and SO_2Cl_2 (1.5 mL) in dry hexane (35 mL) was refluxed for 1 h. The solvent was removed, and the crude acid chloride **10** was used as such for preparation of the cyclohexane adduct **11**. IR: 3400, 2921, 2851, 1752, 1639, 1541, 1301, 984, 738, 710 cm^{-1}; ^1H NMR: δ 1.19 (m, 3H), 1.33 (m, 2H), 1.55 (m, 1H), 1.74 (m, 2H), 2.04 (m, 2H), 3.61 (m, 1H), 4.11 (m, 1H); ^{19}F NMR: δ −143.6 (m, 2F), −166.4 (m, 2F).

Synthesis of Cyclohexane Adduct 11. To the entire sample of **10** dissolved in CH_2Cl_2 (5 mL) was added a mixture of **2** (300 mg, 0.82 mmol) and Et_3N (170 mg, 1.68 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C under a N_2 atmosphere. The reaction mixture was allowed to come to room temperature, stirred for 2 h, diluted with CH_2Cl_2 (100 mL), washed with water (20 mL) and brine (20 mL), and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure yielded an oil which was purified on a silica gel column by eluting with 20% ethyl acetate−hexane buffered with 1% Et_3N to give **11** (392 mg) in 74% yield. Mp: 65−66 °C; IR: 3337, 3062, 2929, 2815, 1689, 1647, 1499, 1344, 1146, 956, 731, 674 cm^{-1}; ^1H NMR: δ 1.07−1.42 (m, 5H), 1.60−1.76 (m, 3H), 1.95−2.02 (m, 2H), 3.09 (d, J = 10.19 Hz, 3H), 3.43 (d, J = 8.89 Hz, 3H), 3.56 (m, 1H), 3.93 (m, 1H), 7.44−7.55 (m, 4H), 7.67 (s, 1H), 7.81−8.1 (m, 4H), 7.84(s, 1H), 8.26 (m, 1H); ^{31}P NMR: δ 80.7; ^{19}F NMR: δ −143.8 (m, 2F), −161.1 (m, 2F); ^{13}C NMR: 157.7, 148.4, 146.9(m), 143.0(m), 138.1(m), 137.0, 135.4(d) [J($^{31}P-^{13}C$), 11.9 Hz], 134.4(m), 132,2, 132.0 [J($^{31}P-^{13}C$), 3.7 Hz], 131.6, 129.6, 128.2, 128.0, 122.9, 121.7, 53.4 [CH, J($^{13}C-^{19}F$), 4.8 Hz], 37.5 [J($^{31}P-^{13}C$), 7.4 Hz], 34.2, 31.8 [J($^{31}P-^{13}C$), 10.2 Hz], 25.3, 24.5. Anal. Calcd for $C_{28}H_{29}N_6F_4O_3PS$: C, 52.83; H, 4.59; N, 13.20. Found: C, 52.62, H, 4.54, N, 13.29. HPLC t_R 4.7 min.

Synthesis of Cyclohexane Adduct 12. The procedure for **11** was followed using **3** (75 mg, 0.27 mmol), Et_3N (60 mg, 0.59 mmol), **10** (obtained from **9** (150 mg), and $SOCl_2$ (0.5 mL)) to give the crude product **12**. Purification on a silica gel column by eluting with 30% ethyl acetate and hexane yielded **12** (108 mg) in 78% yield. Mp: 55−56 °C; IR: 3330, 3246, 3055, 2921, 2851, 1682, 1647, 1520, 1344, 1104, 977, 724, 689 cm^{-1}; ^1H NMR: δ 1.10−1.50 (m, 5H), 1.55−1.80 (m, 3H), 1.99−2.05 (m, 2H), 2.83 (d, J = 10.83 Hz, 3H), 3.12 (d, J = 11.28 Hz, 3H), 3.60 (m, 1H), 3.78 (d, J = 12.26 Hz, 1H), 4.04 (d, J = 12.29 Hz, 1H), 4.07 (m, 1H), 4.15 (br, 1H), 7.33−7.55 (m, 5H), 7.63 (s, 1H), 7.91 (s, 1H), 7.95−8.16 (m, 3H); ^{31}P NMR: δ 83.2; ^{19}F NMR: δ −143.8 (m, 2F), −161.0 (m, 2F); ^{13}C NMR: δ 158.4, 147.7, 146.6 (m), 142.7 (m), 138.9, 138.1 (m), 134.9, 134.4 (m), 132.4 [J($^{31}P-^{13}C$), 10.7 Hz], 131.8 [J($^{31}P-^{13}C$), 2.5 Hz], 130.9, 128.8, 127.7, 127.5, 123.6, 121.9, 53.3 [CH, J($^{13}C-^{19}F$), 4.6 Hz], 51.7, 38.3 [J($^{31}P-^{13}C$), 6.2 Hz], 34.3 [J($^{31}P-^{13}C$), 12.2 Hz], 34.0,

25.1, 24.4. Anal. Calcd for $C_{28}H_{31}N_6F_4O_3PS$: C, 52.66; H, 4.89; N, 13.16. Found: C, 52.95; H, 4.75; N, 12.96. HPLC t_R 4.9 min.

Synthesis of Cyclohexane Adduct 13. A solution of $[PdCl_2(PhCN)_2]$ (0.380 g, 1 mmol) in CH_2Cl_2 (30 mL) was added slowly to the solution of **12** (0.638 g, 1 mmol) also dissolved in CH_2Cl_2 (30 mL). The mixture was stirred at room temperature for 15−30 min, and the solvent was partially evaporated *in vacuo*. The concentrated solution was treated with dry *n*-hexane, and the precipitate was filtered and washed (3 × 30 mL) with *n*-hexane, dried, dissolved in hot acetonitrile, and slowly evaporated to give orange brown crystals (0.711 g) in 87% yield. Mp: 225 °C dec; IR: 3395, 2921, 2867, 1690, 1521, 1361, 1321, 987, 797, 728, 596 cm^{-1}; ^1H NMR: δ 1.08−1.34 (m, 5H), 1.55−1.80 (m, 3H), 1.95 (m, 2H), 3.36 (d, J = 8.06 Hz, 3H), 3.54 (d, J = 9.73 Hz, 3H), 3.62 (m, 1H), 3.95 (br s, 1H), 4.21 (d, J = 12.83 Hz, 1H), 4.78 (br, 1H), 4.87 (d, J = 13.47 Hz, 1H), 7.4−8.1 (m, 10H); ^{31}P NMR: δ 91.2; ^{19}F NMR: δ −143.6 (m, 2F), −160.6 (m, 2F). Anal. Calcd for $C_{28}H_{31}N_6F_4O_3PSPdCl_2$: C, 41.22; H, 3.83; N, 10.30. Found: C, 41.38; H, 3.71; N, 10.42. HPLC t_R 1.9 min.

Synthesis of the ^{109}Pd Complex. ^{109}Pd was obtained from a Pd metal target irradiated at the Missouri University Research Reactor (MURR) for 15 min in a thermal flux of 8 × 10^{13} neutrons/cm^2 s^{-1}. The entire Pd sample containing ^{109}Pd (10 mCi/mg) was dissolved in *aqua regia* and evaporated to dryness in a boiling water bath. The resulting $PdCl_4^{2-}$ and $^{109}PdCl_4^{2-}$ was dissolved in 1 mL of saline and evaporated to dryness. The sample was diluted with saline (1 mL) and adjusted to pH 5 by NaOH.

The ^{109}Pd complex of **6** was prepared by slowly adding an ethanolic solution of **6** (15 mg in 1 mL, 25.8 μmol) to approximately 7 mCi of $^{109}PdCl_4^{2-}$ in 1 mL of saline. The mixture was incubated for 30 min before extraction into 1 ml of $CHCl_3$. This solution was dried under a stream of dry N_2, washed three times with 1 mL of saline to remove unreacted $^{109}PdCl_4^{2-}$, and redissolved in saline (1 mL). Radiochemical purity was determined by spotting 5 μL of the complex solution on paper chromatography strips (Gelman Pads 10 cm × 0.7 cm) and developing with acetone, ethyl acetate, and saline. After these solvents reached the top of the strip, the strips were removed and air dried. Each strip was cut into three equal sections and counted using a NaI(Tl) counter (Ortec system). The same solvent systems were used for the $^{109}PdCl_4^{2-}$ stock solutions as a control. Radiographic scans were performed on a Bioscan (system 200 imaging scanner) radioanalytic system. In the isolated complex, the ^{109}Pd activity migrated with acetone (R_f = 9.0, 98.1 ± 1.2%) and ethyl acetate (R_f = 9.0, 99 ± 0.5%), but remains at the origin with saline (R_f = 2.0, 99 ± 1.3%). In contrast the ^{109}Pd activity in $PdCl_4^{2-}$ showed exactly the opposite behavior, staying at the origin for acetone (R_f = 2.0, 91 ± 0.3%) and ethyl acetate (R_f = 2.0, 98 ± 1.3%), but moving with saline (R_f = 9.0, 95 ± 2.1%).

Acknowledgment. The work was supported by funds provided by DOE Grant DEFG0289ER 60875. NMR data were collected on instruments purchased with funds from NSF grants 8908304 and 9221835. We appreciate the help of Dr. Guo and Mr. Gao of the NMR facility for help with the decoupling experiments. The authors thank Dr. Silvia Jurisson for providing access to the NaI counter and the Bioscan analysis system.

JO961867B