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Fluorous Analogue of Chloramine-T: Preparation, X-ray Structure Determination, and Use as an Oxidant for Radioiodination and s-Tetrazine Synthesis

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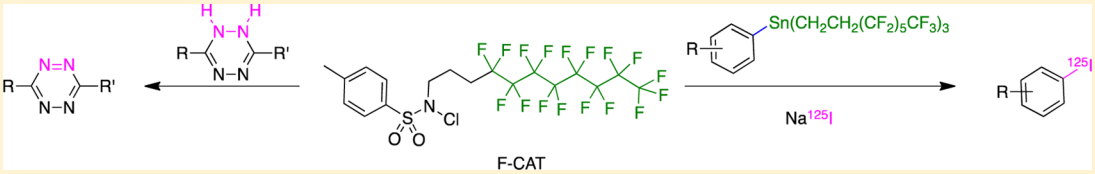
Supporting Information



ABSTRACT: A fluorous oxidant that can be used to introduce radioiodine into small molecules and proteins and generate iodinated tetrazines for bioorthogonal chemistry has been developed. The oxidant was prepared in 87% overall yield by combining a fluorous amine with tosyl chloride, followed by chlorination using aqueous sodium hypochlorite. A crystal structure of the oxidant, which is a fluorous analogue of chloramine-T, was obtained. The compound was shown to be stable for 7 days in EtOH and for longer than three months as a solid. The oxidant was effective at promoting the labeling of arylstannanes using [^{125}I]NaI, where products were isolated in high specific activity in yields ranging from 46% to 86%. Similarly, iodinated biologically active proteins (e.g., thrombin) were successfully produced, as well as a radioiodinated tetrazine, through a concomitant oxidation-halodemetalation reaction. Because of its fluorous nature, unreacted oxidant and associated reaction byproducts can be removed quantitatively from reaction mixtures by passing solutions through fluorous solid phase extraction cartridges. This feature enables rapid and facile purification, which is critical when working with radionuclides and is similarly beneficial for general synthetic applications.

INTRODUCTION

Iodine is one of the only elements that has commercially available isotopes that can be used for single photon emission computed tomography (SPECT, ^{123}I)[1,2] and positron emission tomography (PET, ^{124}I) imaging,[3] targeted radionuclide therapy (^{131}I),[4,5] and pharmacokinetic, pharmacodynamic,[6] and in vitro assay experiments (^{125}I). For drug development, particularly for biologics, radiotracer techniques based on isotopes like ^{125}I have a number of advantages over newer mass spectrometry and fluorescence-based methods, particularly with respect to avoiding matrix effects and the need for extensive method development.[7]

Synthetic strategies used to introduce radioactive isotopes of iodine must be rapid, high yielding, and require minimal purification and handling. For small molecules, the predominant synthetic method involves oxidative demetalation, whereas for proteins, oxidative labeling of tyrosine residues or the use of prosthetic groups, such as the Bolton−Hunter reagent[8,9] or *N*-succinimidyl-3-iodobenzoate,[10,11] for labeling lysine residues are the principal methods of choice. Although generally effective, these procedures typically require HPLC or a comparable biopurification technique to remove unreacted starting materials, including excess oxidant and impurities, which increases contamination and exposure risks and reduces overall yields.

Addressing these issues, new synthetic methods for rapid introduction of iodine and in situ purification have recently been reported. These include polymer-supported[12−16] and fluorous labeling[17−20] methods that allow for expedited "HPLC-free" preparation of iodine-based radiopharmaceuticals. Although a great deal of emphasis has been placed on creating new labeling constructs, the nature of the oxidants used for iodination reactions has remained unchanged for some time.[21] Peroxide and peracids are regarded as the ideal choice because they ultimately form benign reaction side products. However, they often give inconsistent yields and can degrade proteins.[22] Iodogen, chloramine-T (CAT), and iodobeads have been shown to be effective reagents for labeling proteins[9] and small molecules,[23] but difficulties associated with the removal of excess oxidant, formation of unwanted byproducts, and slow reaction rates due to the heterogeneous nature of certain reactions reduces their effectiveness and can obviate any

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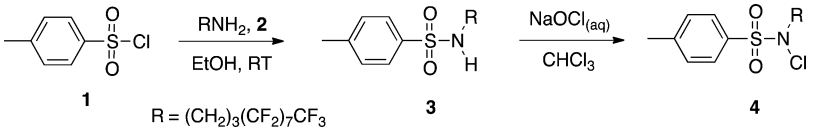
Scheme 1. Synthesis of *N*-chloro-*N*-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecyl)-4-methylbenzenesulfonamide (F-CAT) 4



advantages gained using polymer, fluorous, or other newer labeling methods.[12,24]

We sought to develop a new oxidant capable of introducing iodine into both small molecules and proteins that can be removed from reaction mixtures in a more convenient manner than is possible with current reagents. This led to the preparation and characterization of a new high fluorine content (fluorous) oxidant that can be selectively separated from labeled materials by passing the reaction solution through a fluorous solid-phase extraction cartridge. Although fluorous chemistry has not become a routine part of general synthetic chemistry and drug development, it is well-suited for radiochemistry given the small scale of the reactions, which typically take place at micromolar to nanomolar concentrations, and the need for rapid and facile purification methods. Not only can the fluorous oxidant reported here be used to label arylstannanes and proteins, it was also shown to be very effective for producing functionalized tetrazines, which are emerging as highly effective synthons for bioorthogonal labeling reactions.[25−28]

RESULTS AND DISCUSSION

Preparation and Characterization. A review of the literature revealed that a limited number of fluorous oxidants have been reported, and that these are not suitable for iodination reactions, which must be rapid, selective, and high yielding.[29−35] We therefore opted to prepare a fluorous analogue of chloramine-T (F-CAT) because this oxidant has been used effectively with a wide range of substrates. The first step involved treating *p*-toluenesulfonyl chloride with excess of commercially available fluorous amine **2** in ethanol (Scheme 1).[36] After 30 min, the desired product was isolated by silica gel chromatography in 89% yield. The NMR and HRMS data were consistent with the formation of sulfonamide **3**, which was obtained as a crystalline solid. *N*-chlorination was achieved by the treatment of a chloroform solution of **3** with aqueous sodium hypochlorite (6%, w/v). The *N*-chloro fluorous-sulfonamide **4** was isolated by simple extraction, and the desired product was obtained in nearly quantitative yield. ^{1}H NMR revealed that the signal corresponding to the NH group in **3** (4.51 ppm) was absent following chlorination, and the IR spectrum lacked the NH stretch (3263 cm^{-1}) observed in sulfonamide **3**, which is in agreement with what has been reported previously for *N*-alkyl sulfonamides.[37,38] In the ^{13}C NMR spectrum, the signal corresponding to the methylene carbon adjacent to the N−Cl group shifted downfield compared to that in **3**, which is consistent with NMR data for chlorination of nonfluorous sulfonamides.[39,40]

Single crystals of compounds **3** and **4** were obtained by slow evaporation of saturated chloroform solutions, and the associated crystal structures were determined. The complete structural data for **3** and **4**, as well as ORTEP drawings, can be found in the Supporting Information. The key bond lengths and angles for **4** are presented in Tables 1 and 2 in comparison to the corresponding data reported for CAT.[41] The previous published crystal structure of CAT revealed a salt that formed an aquo-bridged dimer,[41] whereas **4** is neutral and monomeric with S−O bond distances being similar (1.424(3) and 1.428(3) Å). The S−N bond length (1.677(3) Å) was longer than in CAT (1.590(2) Å), whereas the N−Cl bond distance in **4** (1.733(3) Å) was shorter. The differences are in part due to the ionic nature of CAT, which is also evident in the melting points (167−170 °C versus 77−79 °C) of the two compounds.

Table 1. Comparison of Select Bond Lengths for CAT[41] and 4 (F-CAT)

CAT		F-CAT (4)	
bond	length (Å)	bond	length (Å)
S−O (1)	1.455(2)	S−O (1)	1.428(3)
S−O (2)	1.439(2)	S−O (2)	1.424(3)
S−N	1.590(2)	S−N	1.677(3)
N−Cl	1.750(2)	N−Cl	1.733(3)

Table 2. Comparison of Select Bond Angles for CAT[41] and 4 (F-CAT)

CAT		F-CAT (4)	
bond	angle (deg)	bond	angle (deg)
S−N−Cl	110.9(1)	S−N−Cl	111.9 (3)
O(2)−S−O(1)	116.1(1)	O(2)−S−O(1)	120.23 (18)
O(2)−S−N	103.6(1)	O(2)−S−N	106.48 (17)
O(2)−S−C(1)	103.3(1)	O(2)−S−C(1)	109.36 (18)
O(1)−S−C(1)	105.8(1)	O(1)−S−C(1)	108.50 (18)
N−S−C(1)	109.6(1)	N−S−C(1)	107.94 (17)

With respect to stability, HPLC analysis of an ethanol solution of **4** after 24 h (Figure 1A) showed no significant decomposition. After 7 days in solution, a new peak at 8.4 min (Figure 1B) appeared, which corresponded to ∼10% of **3**. Nonetheless, F-CAT is stable for more than three months when stored as a solid at room temperature.

Radiochemistry. To evaluate the ability of the oxidant to promote labeling of small molecules, we used a series of fluorous arylstannanes. The advantage of using fluorous stannanes over simple trialkyl tin derivatives is that the excess starting stannane and the oxidant can be removed selectively by passing the reaction mixture through a fluorous solid-phase extraction cartridge. The specific approach employed minimizes sample handling and involves loading the fluorous tin derivative on fluorous silica prior to the addition of the oxidant and iodide. After a set period of time, the desired product was eluted using a fluorophobic solvent (EtOH-H_2O).

As an initial test of the reactivity of F-CAT, the radio-iodination of fluorous-tin benzoic acid **5a** loaded onto fluorous silica was performed (Scheme 2). Determination of the optimal reaction time involved running parallel reactions and washing the cartridges at different time points with 80:20 v/v EtOH/H_2O, which elutes all nonfluorous materials, and then performing HPLC analysis on the eluents. After 30 min, HPLC (Figure 2) showed complete consumption of iodide, a

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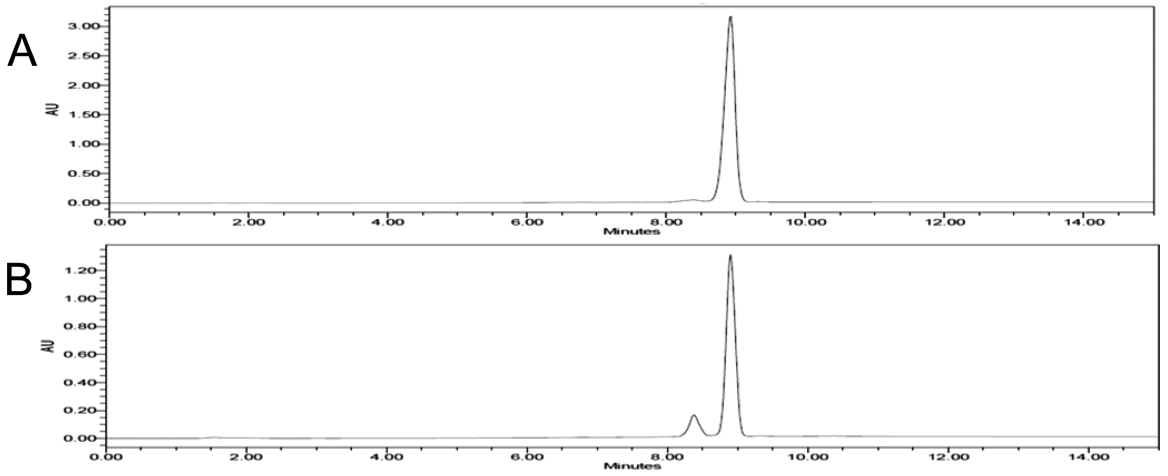
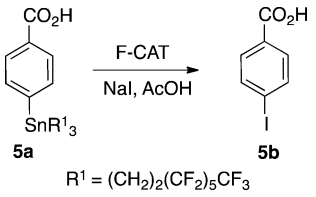


Figure 1. UV-HPLC chromatograms of a solution of compound **4** in ethanol after (A) 24 h and (B) 7 days. The peaks at t_R = 8.4 and 8.9 min correspond to compounds **3** and **4**, respectively.

Scheme 2. Model Reaction Used to Evaluate F-CAT as an Oxidant



single peak in the gamma chromatogram, and no detectable amount of fluorous oxidant in the corresponding UV-HPLC chromatogram, where the limit of detection was 2 μg/mL. When the amount of oxidant used was doubled (100 versus 200 μg of **4**), the isolated yield of **5b** increased from 69 ± 5% to 82 ± 4%. Further increases in the amount of F-CAT did not improve the isolated yield. In all experiments, **5b** was obtained in greater than 98% radiochemical purity and high specific activity (>240 Ci/mmol).

Fluorous oxidant **4** was used subsequently to label a range of different arylstannanes whose synthesis we reported previously,[20] including precursors of known radiopharmaceuticals, such as meta-iodobenzylguanidine. All experiments were performed in triplicate, and the products were analyzed by gamma and UV HPLC to determine radiochemical purity and to detect the presence of any residual oxidant. Isolated radiochemical yields ranged from 46 to 86% (Figure 3) with radiochemical purities greater than 97%. In no case was there evidence of any oxidant or precursor in the final product, and the radiolabeling yields were generally higher than those reported using CAT as the oxidant.[20] This is largely due to the fact that a larger quantity of F-CAT can be used compared to CAT without being concerned about breakthrough of the excess oxidant during SPE purification, which would lead to contamination of the product.

Protein Labeling. The use of CAT as an oxidant for iodination of biomolecules has been widely reported.[10,42,43] However, there is concern about direct exposure of proteins to the oxidant that is soluble in aqueous reaction media.[43,44] Consequently, the water-insoluble oxidant iodogen is often used[45] because it can be employed as a thin film coated onto the wall of the reaction vial, which minimizes direct interaction

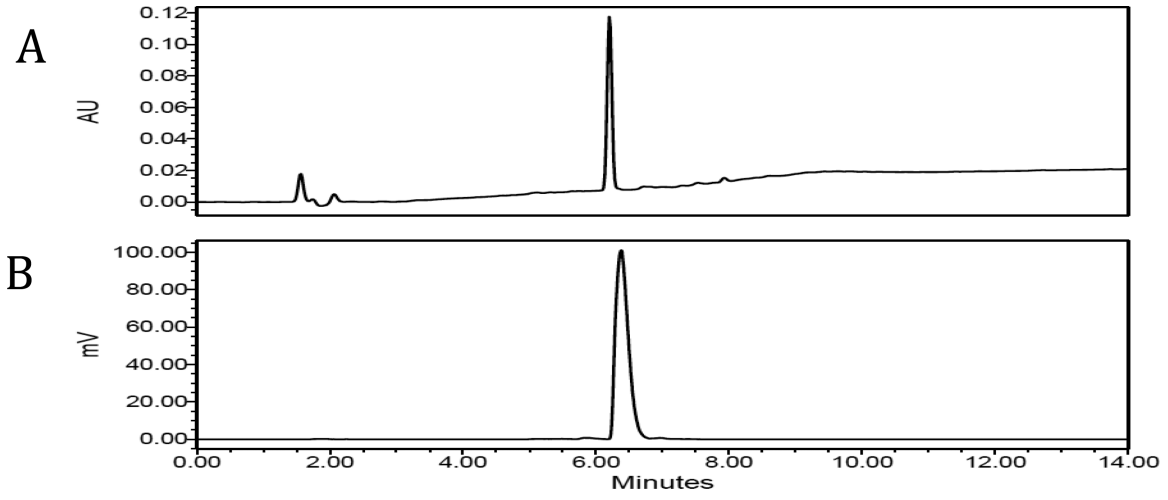


Figure 2. HPLC traces of ^{125}I-iodobenzoic acid **5b** coinjected with an authentic sample of 4-iodobenzoic acid (elution method B). (A) UV-HPLC chromatogram. (B) γ-HPLC chromatogram. Note that fluorous starting material **5a** elutes at 12.5 min and that the γ and UV detectors are connected in series.

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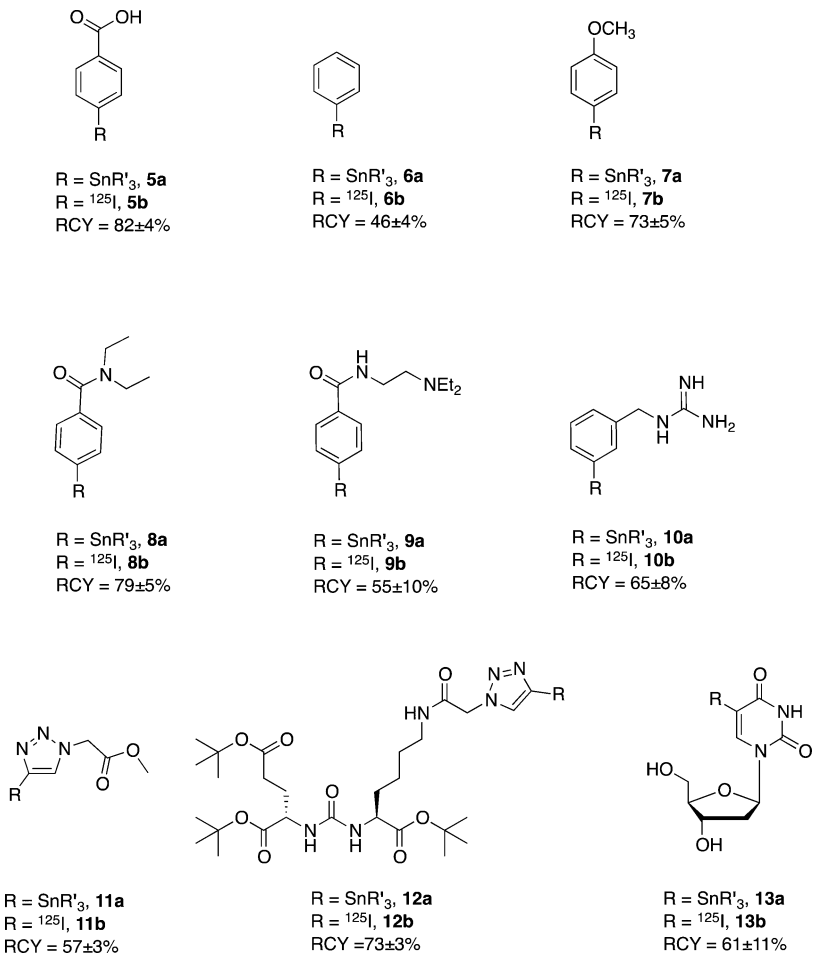

Figure 3. Structures of the fluorous precursors and corresponding radioiodinated compounds prepared using F-CAT **4**. RCY = isolated radiochemical yield (n = 3). R′ = $CH_2CH_2(CF_2)_5CF_3$.

with the biomolecule being labeled. Given the insoluble nature of fluorous compounds in media typically employed for biomolecule iodination reactions, the ability of **4** to effectively label proteins was investigated.

Two proteins were selected as model candidates: thrombin and a fusion protein of human serum albumin coupled to the C-terminus of the hirudin variant 3 protein (HSACHV3).[46,47] Thrombin is the final effector enzyme generated by the coagulation cascade, and it activates platelets and converts fibrinogen to active fibrin.[48] It is tightly regulated by protease inhibitors of the serpin protein family, which trap thrombin in covalent complexes and whose formation can be monitored via SDS-PAGE and autoradiography.[47] HSACHV3 is a fusion protein under investigation as a fully latent, plasmin-activatable, long-lasting hirudin of potential benefit in thrombotic disorders that have resistance to natural or pharmacological clot lysis.[46,49] Many fusion proteins based on albumin or on the Fc portion of immunoglobulin G are either in clinical development or have reached the clinic.[50−52]

Thrombin and HSACHV3 were labeled separately using equivalent amounts of **4** and iodogen under identical reaction conditions. The ^{125}I-labeled proteins were purified and concentrated using centrifugal filters, and the radioactivity in both the protein concentrates and supernatant solutions were measured using a calibrated NaI(Tl) well counter. SDS-page and in vitro functional assays were performed to compare the purity and function of the products produced using the two different oxidants. Thrombin activity following ^{125}I-labeling using either F-CAT or iodogen was quantified by assessing the rate of cleavage of chromogenic substrate S-2238 over a range of labeled thrombin concentrations (14, 7, and 3.5 nM). The rates were compared to unmodified thrombin as the positive control and buffer as the negative control. At all concentrations of labeled thrombin produced using either oxidant, there was no significant difference among the mean rates of substrate cleavage (Supporting Information, Figure S22). For analysis of labeled HSACHV3, the isolated products from F-CAT or iodogen labeling were first incubated with plasmin. The fusion protein was designed to be cleaved by plasmin, releasing the active hirudin (HV3) moleucule.[46] Following incubation, the resulting reaction mixture was analyzed by SDS-PAGE and autoradiography (Figure 4). The results showed that ^{125}I-HSACHV3 produced using F-CAT **4** was indistinguishable from that made using iodogen. What was also evident was a reduction in lower molecular weight impurities.

Tetrazine Labeling. As an alternative to direct protein labeling, pretargeting strategies that employ bioorthogonal coupling reactions are increasingly being used to develop molecular imaging probes and to study the distribution of new biological agents.[53−55] Of the many reactions being evaluated, the transcyclooctene (TCO) and tetrazine (Tz) coupling strategy has been shown to be particularly effective.[25,27,56−65] Here, a TCO-labeled targeting vector is administered and allowed to bind to its target in vivo and to clear from nontarget

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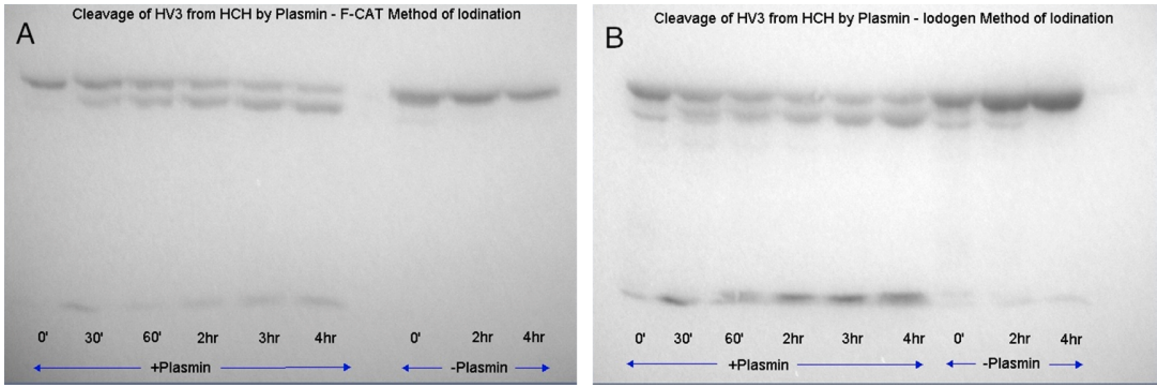

Figure 4. Autoradiography of SDS-PAGE gel showing the products produced as a function of time by cleavage (+plasmin) of HV3 from ^{125}I-HSACHV3 prepared using either (A) F-CAT or (B) iodogen. Controls run in the absence of plasmin (−Plasmin) are also shown. Largest MW band = HSACHV3, next band = HSA (human serum albumin) fragment, and lowest MW band = HV3 fragment. Additional small low molecular weight bands are evident with the iodogen method.

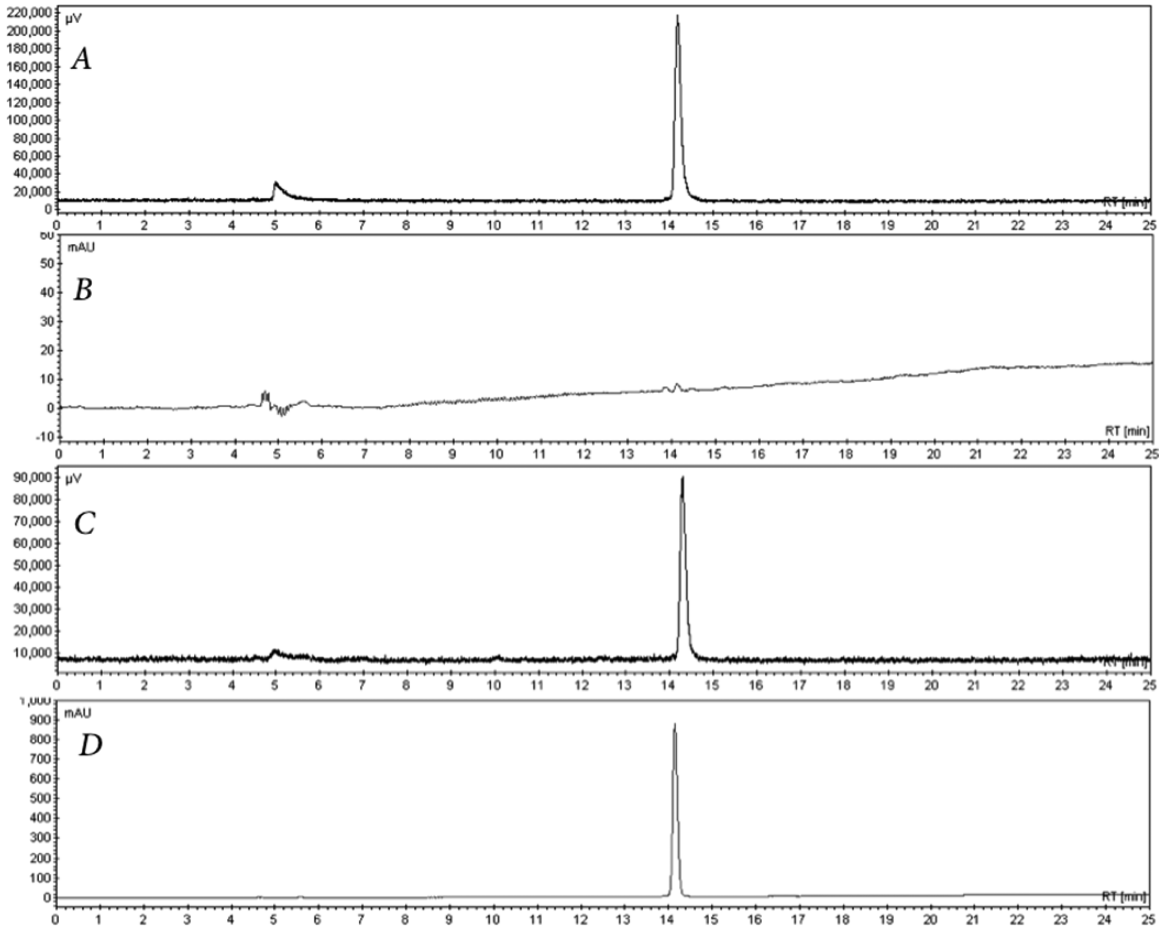

Figure 5. (A) γ-HPLC chromatogram of [^{125}I]iodotetrazine **14b** (R_t = 14.2 min). (B) UV-HPLC chromatogram of the same reaction mixture. (C) γ-HPLC and (D) UV-HPLC chromatograms of **14b** spiked with the nonradioactive reference standard.

tissues prior to administration of a radiolabeled tetrazine. The two components undergo a rapid inverse electron demand Diels−Alder reaction, thereby forming a covalent bond between the radiolabeled tetrazine and the TCO-modified targeting vector. This approach has a number of advantages over conventional targeting approaches for molecular imaging and has produced impressive results using a range of different isotopes.[59−66]

An iodinated tetrazine could be used as a convenient synthon to label biomolecules for in vitro assays and pharmacokinetic and pharmacodynamic studies. One critical advantage of the approach is that a nonradioactive TCO-labeled biomolecule could be prepared, purified, and stored until needed. Leveraging the rapid kinetics, high yields, and bioorthogonal nature of the TCO-tetrazine reaction, the labeled form can be generated in situ as needed by simply adding an iodinated tetrazine. To determine if iodination using F-CAT would

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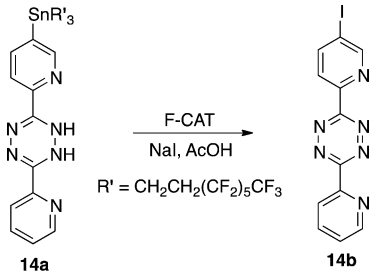
provide a convenient method to produce and purify an ^{125}I-labeled tetrazine, we synthesized 3-(pyridin-2-yl)-6-(5-(trisperfluorostannyl)pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazine **14a** (fluorous-dihydrotetrazine) and loaded it onto a fluorous silica support. F-CAT was then employed to simultaneously oxidize **14a** to the tetrazine and promote the halodemetalation reaction with [^{125}I]NaI (Scheme 3). This approach was used, as

Scheme 3. F-CAT-Mediated, Concomitant Oxidation and Labeling Reaction to Produce Iodinated Tetrazine 14b for Use in Bioorthogonal Coupling Reactions

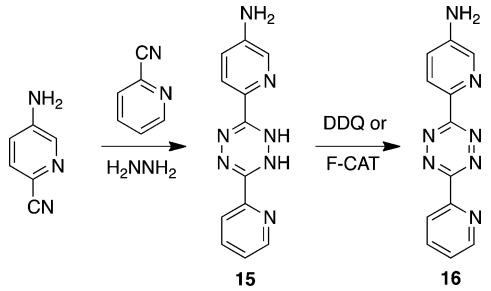


opposed to labeling a tin-tetrazine derivative directly, because the fluorous-dihydrotetrazine is stable for extended periods of time when stored at −10 °C. The reaction was allowed to proceed for 30 min, after which the desired product was eluted using an EtOH-water solution in a 69 ± 4% (n = 4) radiochemical yield and greater than 98% radiochemical purity without the need to employ purification by HPLC. The HPLC retention time of the product matched that of the authentic nonradioactive standard (Figure 5), and there was no evidence of any oxidant or precursor in the final product.

To verify that the iodinated tetrazine was suitably reactive, we treated **14b** with (*E*)-cyclooct-4-enol (TCO-OH) in MeOH/H_2O for 15−20 s. Analytical HPLC analysis of the reaction mixture revealed no evidence of residual **14b**, and the formation of multiple peaks associated with the different Diels−Alder adducts and subsequent oxidation products were seen, consistent with literature reports on comparable tetrazine reactions (Figure 6A).[25,65,67] The reaction was also repeated in the presence of a small amount of the nonradioactive analogue of **14b**, and the mixture was analyzed by HPLC (Figure 6B), where there was good concordance between the gamma and UV chromatograms.

The chemistry used to prepare **14b** suggested that F-CAT would also be useful for the synthesis of tetrazines themselves. Tetrazines are typically prepared by combining nitriles with hydrazine to form the corresponding 1,4-dihydro-*s*-tetrazine that is then oxidized. A range of oxidants can be used for the final step including sodium nitrite.[25,68,69] For widely used bifunctional tetrazine **16**, the preparation (Scheme 4) reported

Scheme 4. Preparation of 6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine 16 Using DDQ or F-CAT



by Fox and co-workers[25] involved the use of DDQ, because nitrite can oxidize the amino group. While successful, we and others found that the DDQ-based method produced a number of byproducts that complicated purification.[70] When **15** was treated with F-CAT, the reaction was free from any unexpected impurities, and the desired product, which was easily separated from the fluorous oxidant, and was isolated in 86% yield from the dihydrotetrazine.

CONCLUSION

A new fluorous oxidant that is an analogue of chloramine-T was synthesized and shown to be an effective reagent for labeling small molecules and proteins with ^{125}I. In the case of biomolecules, F-CAT can be used as a water insoluble analogue of chloramine-T and a substitute for iodogen. The fluorous chloramine-T derivative **4** is stable as a solid for months and in solution for up to 7 days and can be prepared in large quantities in two steps from commercially available starting materials. The

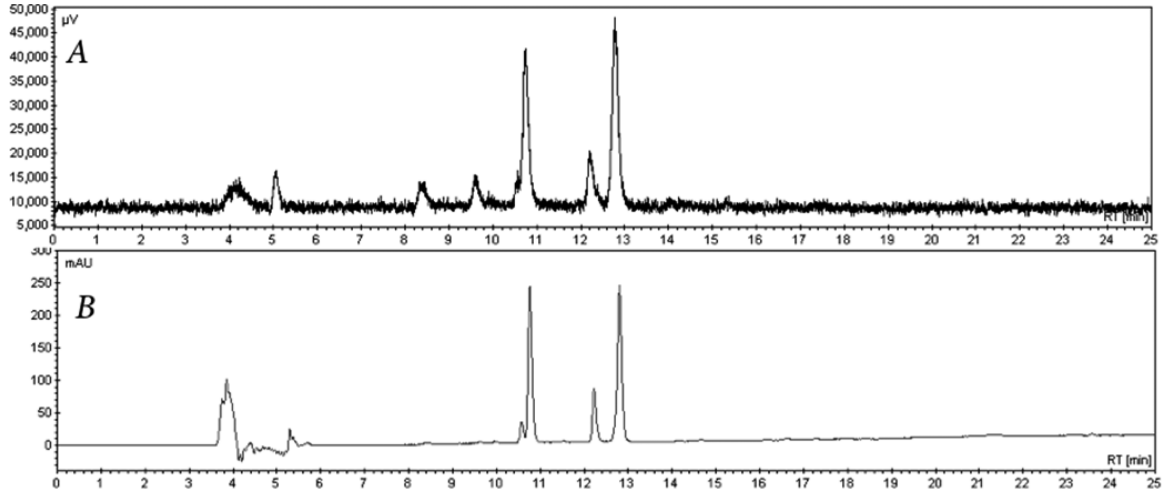


Figure 6. (A) γ-HPLC chromatogram of the reaction mixture containing **14b**, its nonradioactive analogue, and (*E*)-cyclooct-4-enol. (B) UV-HPLC chromatogram of the same reaction mixture.

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fluorous nature of the oxidant is such that high purity radioiodinated compounds, including labeled tetrazines for use in bioorthogonal chemistry, can be prepared without HPLC purification with high effective specific activity and free from any residual precursor and oxidant.

EXPERIMENTAL SECTION

Reagents. Unless otherwise stated, all chemical reagents were purchased from commercial sources and used as received without further purification. Compounds **5a** to **14a** and **15** were prepared as previously described.[20,25,67] FC-72 was purchased from 3M, whereas 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecan-1-amine **2** was purchased from Fluorous Technologies Inc. Sodium [^{125}I]iodide with a specific activity of ∼17 Ci/mg was purchased from the McMaster Nuclear Reactor (Hamilton, ON, Canada). ***Caution!*** *^{125}I is radioactive and should only be handled in an appropriately equipped and licensed facility.*

Instrumentation. NMR spectra were recorded with chemical shifts reported as δ values in ppm relative to the residual proton signal of the deuterated solvent or the carbon signal of the solvent. HRMS data were obtained using ESI time-of-flight (TOF) instrumentation. A dose calibrator was used for measuring the amount of radioactivity employed during the radiosynthesis protocols. Analytical HPLC (methods A and B) was performed using a binary HPLC system fitted with a PDA detector (254 nm), a γ-detector (dwell time of 5 s in a 10 μL loop), and a Phenomenex Gemini-C18 column (4.6 × 100 mm, 110 Å, 5 μm) at a flow rate of 1.0 mL/min. Analytical HPLC (method C) was performed using an instrument fitted with a γ-detector, an analog interface module, and a Phenomenex Gemini-C18 column (4.6 × 100 mm, 110 Å, 5 μm). The wavelength for UV detection was set at 254 nm, and the dwell time in the gamma detector was 5 s in a 10 μL loop. The mobile phase was composed of solvent A = H_2O (0.1% TFA) and solvent B = CH_3CN (0.1% TFA). Method A: 0−7 min, 20−100% B; 7−15 min, 100% B. Method B: 0−7 min, 20−100% B, 7−14 min, 100% B. Method C: 0−5 min, 20−100% B, 5−14 min, 100% B. For calibration curves, each calibration solution was evaluated in triplicate and the data analyzed by the least-squares method. The limit of quantitation and the limit of detection were calculated using the standard deviation method.[71]

Synthesis of Compounds 3, 4, 16. *N-(4,4,5,5,6,6,7,7,8,8,9,9,-10,10,11,11,11-Heptadecafluoroundecyl)-4-methylbenzenesulfonamide (**3**).* *p*-Toluenesulfonyl chloride **1** (0.095 g, 0.5 mmol) was weighed into a 10 mL round-bottom flask equipped with a stir bar. Ethanol (95%, 2 mL) was added, and the solution was stirred until **1** was completely dissolved. 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecan-1-amine **2** (0.477 g, 1 mmol) was added to the solution with stirring at room temperature. The initial colorless reaction mixture became heterogeneous after a few minutes. Stirring was continued until the reaction was complete (monitored by TLC in *n*-hexane:EtOAc, 3:1 v/v). The mixture was subsequently concentrated under reduced pressure, and the product was isolated by silica gel column chromatography using a gradient of 10−30% *n*-hexane:EtOAc. The fractions containing the product were combined, and the solvent was removed to afford **3** as a white crystalline solid. Yield (0.281 g, 89%); mp 108−111 °C; FTIR (KBr) 3263, 2957, 1959, 1600 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.75 (d, J = 8.3 Hz, 2H, Ar−H), 7.32 (d, J = 8.2 Hz, 2H, Ar−H), 4.51 (t, J = 6.4 Hz, 1H, NH), 3.05 (q, J = 6.7 Hz, 2H, NCH_2), 2.43 (s, 3H, Ar−CH_3), 2.15−2.06 (m, 2H, CH_2CF_2), 1.82−1.77 (m, 2H, CH_2); ^{13}C NMR (150 MHz, $CDCl_3$) δ 143.9, 136.9, 130.0, 127.2, 42.4, 28.2 (m), 21.6, 21.1; HRMS (ESI-TOF) m/z calcd for $C_{18}H_{15}NO_2SF_{17}$ $[M + H]^+$ 632.0552, found 632.0541.

*N-Chloro-N-(4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecyl)-4-methylbenzenesulfonamide (**4**).* Sulfonamide **3** (0.252 g, 0.4 mmol) was dissolved in $CHCl_3$ (10 mL) in a 50 mL round-bottom flask equipped with a stir bar. Aqueous NaOCl (6% w/v, 10 mL) was added, and the biphasic reaction mixture was stirred vigorously at room temperature. Stirring was continued until the reaction was complete (monitored by TLC using 3:1 v/v hexane:EtOAc). The reaction mixture was transferred into a separatory funnel; the $CHCl_3$ layer was separated, and the aqueous layer was extracted with $CHCl_3$ (3 × 5 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation to afford a white solid. Yield (0.260 g, 98%); mp 77−79 °C; FTIR (KBr) 2930, 1958, 1595 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, J = 8.2 Hz, 2H, Ar−H), 7.40 (d, J = 8.2 Hz, 2H, Ar−H), 3.33 (t, J = 6.3 Hz, 2H, NCH_2), 2.48 (s, 3H, Ar−CH_3), 2.27−2.18 (m, 2H, CH_2CF_2), 2.03−1.98 (m, 2H, CH_2); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.7, 129.8, 129.6, 55.6, 27.8 (m), 21.7, 18.3; HRMS (ESI-TOF) m/z calcd for $C_{18}H_{17}N_2O_2SF_{17}Cl$ $[M + NH_4]^+$ 683.0428, found 683.0439.

*3-(5-Aminopyridin-2-yl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**16**).* To a solution of 3-(5-aminopyridin-2-yl)-6-(pyridin-2-yl)-1,4-dihydro-*s*-tetrazine **15** (50 mg, 0.20 mmol) in acetonitrile (5 mL) was added F-CAT **4** (146 mg, 0.22 mmol), and the reaction mixture was allowed to stir at 40 °C. After 12 h, the solvent was evaporated under reduced pressure; the residue was dissolved in methanol (300 μL), and the product (43 mg, 86%) was isolated as a red colored solid using a FluoroFlash Cartridge and eluted with MeOH/H_2O (1:10, v/v). Characterization data matched that reported in the literature.[25]

General Procedures. *Preparation of Coated Fluorous Silica.* Fluorous silica (200 mg) was added to a solution of fluorous precursor in chloroform (1 mL, 10 mg/mL), and the mixture was agitated by hand until a slurry was formed. After sitting at room temperature overnight, the resulting powder (30 mg) was added to a polypropylene SPE tube that contained 500 mg of unmodified fluorous silica previously washed with DMF (1 mL), H_2O (5 mL), and 80:20 (v/v) EtOH/H_2O (5 mL).

Small Molecule Radiolabeling. To an SPE cartridge containing the coated fluorous silica connected to a Sep-Pak C18 Plus cartridge (820 mg sorbent/cartridge), 10% acetic acid in EtOH (50 μL) was added followed by F-CAT **4** (100 μL, 2 mg/mL in ethanol) and Na^{125}I (10 μL, 1.85 GBq/mL in 0.1 M NaOH). After 30 min at room temperature, $Na_2S_2O_{5(aq)}$ (50 μL, 0.2 M) was added, and the system was washed with water (5 mL) and a 0.09 M H_3PO_4 solution of EtOH/H_2O (200 mL of EtOH, 50 mL water, 1.5 mL of concd H_3PO_4), and 1 mL fractions were collected and counted. HPLC retention times were compared to those for nonradioactive authentic standards.

Protein Radiolabeling. Eppendorf tubes were treated with 10 μL of oxidant solution (2.0 mg/mL of iodogen in $CHCl_3$ or 7.7 mg/mL of F-CAT **4** in $CHCl_3$), and the solution was allowed to evaporate. A magnetic stir bar was added followed by protein (10 μL, 107 μg) in ice-cold phosphate buffered saline (PBS) (280 μL) and [^{125}I]NaI (10 μL, 37 MBq), and the mixture was stirred slowly for 10 min. The reaction was quenched by the addition of $Na_2S_2O_3$ (0.1 M, 100 μL) and diluted to a final volume of 1 mL with PBS. The content of the eppendorf tube was transferred to an Amicon Ultra-4 centrifugal filter unit and the eppendorf tube was rinsed twice with ice-cold PBS, which was added to the centrifugal filter unit. The total volume made up to 4 mL. The sample was centrifuged at 5000*g* for 10 min in an Eppendorf 5473 centrifuge. The filtrate was transferred to a scintillation vial, and the protein residue in the sample reservoir was diluted with ice-cold PBS (4 mL). The centrifugation process wasrepeated twice. After the final centrifugation, the residual protein was reconstituted in ice-cold PBS (1 mL) and transferred to an eppendorf tube for analysis.

Thrombin Assay. Chromogenic substrate S2238 (Chromogenix, Instrumentation Laboratory Company, Lexington, MA 02421−3125, USA) was diluted to 100 μM in PPNE kinetics buffer (20 mM sodium phosphate pH 7.4, 100 mM NaCl, 0.1 mM EDTA, and 0.1% w/vol polyethylene glycol). Samples (14, 7, and 3.5 nM) of thrombin, and labeled thrombin prepared using iodogen and F-CAT were prepared in PPNE kinetics buffer. Samples (30 μL) and buffer control (30 μL) were added to microtiter wells. Diluted substrate S2238 (270 μL) portions were added to each well, and the optical density at 405 nm was read immediately and at 15 s intervals for 5 min on the plate reader. Blank measurements of 300 μL of PPNE buffer alone were also taken.

HSACHV3 Cleavage and SDS-PAGE. Samples (10 μM) of HSACHV3, iodogen-labeled HSACHV3, and F-CAT-labeled

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HSACHV3 were prepared in PPNE buffer and then diluted to a final concentration of 376 nM with the total volume in each eppendorf vial being 200 μL. Plasmin (Enzyme Research Laboratories Inc., South Bend, IN 46628, USA) was diluted to 12 μM in PPNE buffer and added to each vial at a final concentration of 600 nM. Immediately upon adding plasmin at t_0, a 20 μL aliquot was removed from the reaction vial and add to a tube containing 20 μL PPNE and 13 μL of 4× SDS gel sample buffer, and the vial was placed in a 37 °C water bath. At 30 min and 1, 2, 3, and 4 h, the reaction vial was agitated before a 20 μL aliquot was removed and treated as the aliquot obtained at t_0. A previously prepared 8% acrylamide gel was loaded with the 20 μL samples. After staining, destaining, and drying, the gel was exposed to film overnight.

X-ray Structure Determination of 3 and 4. A crystal for each compound suitable for data collection was selected and mounted in paratone oil on a MiTeGen head and then placed in the cold stream of the diffractometer. Data were collected at 173 K using omega scans on a Bruker APEX2 platform with a SMART 6000 area detector and rotating anode generating Cu Kα radiation (λ = 1.54178 Å). Data were processed using SAINT,[72] corrected for absorption using redundant data (SADABS), and then solved using direct methods with the SHELXTL[73] program suite in the space group P2(1)/n. All nonhydrogen atoms were refined anisotropically; hydrogen atoms were placed in idealized positions riding on their constituent atoms and updated after each cycle of refinement. In the last stages of refinement, the largest residual peak (0.49 eÅ^{-3}) was associated with F15.

■ ASSOCIATED CONTENT

Supporting Information

HRMS, ^{1}H and ^{13}C NMR spectra, and HPLC chromatograms for various compounds, thrombin activity assay, and X-ray crystallographic data for **3** and **4.** The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00988.

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Notes

The authors declare no competing financial interest.

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