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One-Pot Triple Functionalization of Carbon Nanotubes

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Abstract: Carbon nanotubes (CNTs) are very promising as carriers for the delivery of bioactive molecules. The multifunctionalization of CNTs is necessary to impart multimodalities for the development of future CNT-based multipotent therapeutic constructs. In this context, we report the first example of covalent trifunctionalization of different types of CNTs. Our strategy is a simple and efficient methodology based on the simultaneous functionalization of the nanotube surface with three different active groups. The reaction is performed in one step by arylation with diazonium salts generated in situ. The CNTs are functionalized with benzylamine moieties blocked with three different protecting groups that can be selectively removed under specific conditions. The trifunctionalized CNTs were characterized by TEM, thermogravimetric analysis, and Raman and UV/Vis/NIR spectroscopy, while the amine loading was determined by using the Kaiser test. The sequential removal of the protecting groups of the amine functions allows the grafting of the molecules of interest on the nanotube surface to be controlled.

Keywords: carbon nanotubes · drug delivery · nanotechnology · one-pot reactions · protecting groups

Introduction

Due to their unique electrical,[1] mechanical,[2] and optical properties,[3] carbon nanotubes (CNTs) have played an important role in the rapidly developing field of nanotechnology. Applications in many fields, including biology[4] and nanomedicine,[5] for drug delivery[6] and imaging[7] have been thoroughly investigated. Due to their lack of solubility and strong tendency to aggregate, it is imperative to functionalize the surface of CNTs to fully exploit their properties. Both covalent and noncovalent approaches have been developed to increase dispersibility and to introduce chemical functionalities on their sidewalls and tips.[8] In particular, the multiple functionalization of CNTs is necessary to achieve multimodalities. For instance, it is of interest to use CNTs as multimodal drug delivery systems because CNTs have shown high potential as carriers of small drugs,[9] peptides,[10] proteins,[11] and nucleic acids.[12] The development of nanovectors able to carry one or more therapeutic agents with targeting and imaging capability is essential, for example, in the treatment of cancer or different types of infections.[13] In addition, the molecular targeting of CNT-based delivery systems to specific cell populations is of great importance to increase the therapeutic efficiency and reduce side effects by avoiding collateral consequences for healthy tissues.[14] Moreover, the attachment of a tracking probe, such as a fluorophore[15] or a radionuclide,[14a,16] may provide optical signals for imaging and localization of the CNT–drug conjugates. Several strategies have been developed for the double functionalization of CNTs.[9a,17] For example, we have recently covalently tethered methotrexate (MTX) to the sidewalls of oxidized multiwalled carbon nanotubes (MWCNTs), while a fluorophore was attached to the carboxylic functions that are mainly located at the tips.[18] We observed internalization of the MTX–CNT conjugates into human breast cancer cells with an enhanced antitumor activity.[17e] Recently, Heister et al. reported the functionalization of single-walled carbon nanotubes (SWCNTs) with three molecules of interest: 1) the anticancer drug doxorubicin, 2) a monoclonal antibody able to target the tumor marker carcinoembryonic antigen, and 3) fluorescein for imaging.[19] The antibody and fluorescein were covalently linked to protein bovine serum albumin, which was then bound to carboxylic groups of oxidized SWCNTs, whereas doxorubicin was subsequently adsorbed on the nanotube surface. To avoid a premature release of the therapeutic agent, to better control the amount of different functionalities on the nanotubes, and to increase the stability of the CNT-based conjugates, a reliable approach is to modify CNTs by covalent functionalization. For this purpose, we describe herein a simple methodology for the covalent introduction of three different functional groups on the sidewall of different types of CNTs, both SWCNTs and MWCNTs, that were pristine, purified, or oxidized. The functional groups contain amino functions blocked by three quasi-orthogonal protecting groups. We also report the selective de-

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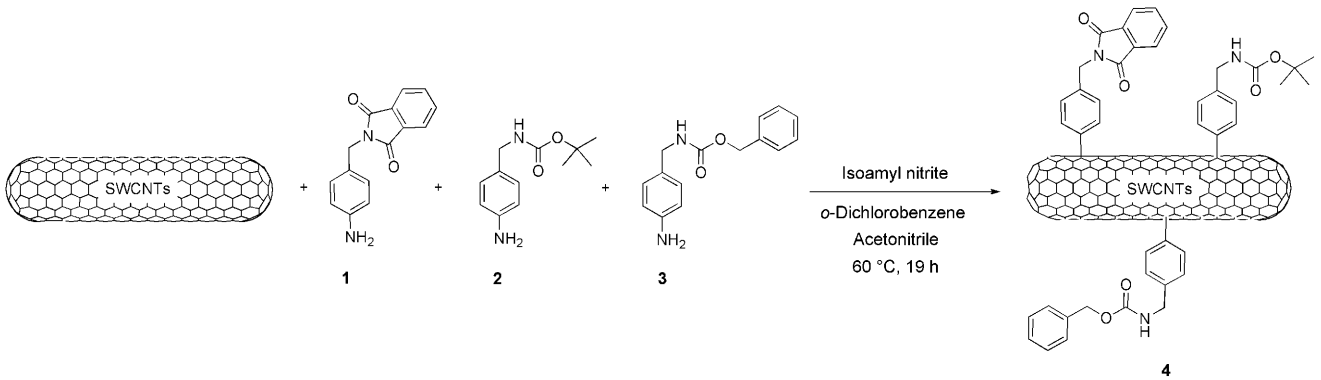
protection of each amine function under specific conditions. This allows the control of the attachment of three molecules of interest to CNTs in a sequential manner. Thus, the trifunctionalization approach would afford the possibility to covalently attach a drug, a targeting ligand, and a fluorophore. Tethering the drug to the nanotube backbone through a cleavable linker is also envisaged because it will facilitate the controlled release at the site of action.[17e,20] To the best of our knowledge, the triple covalent functionalization of CNTs has not yet been reported. We believe that this approach will expand the potential of carbon nanotubes in the biomedical and nanomedicine fields.

Results and Discussion

Trifunctionalization of SWCNTs: Our strategy for the triple functionalization of CNTs relies on the simultaneous reaction of an equimolar mixture of three aryldiazonium salts with the nanotube surface. The aryldiazonium salts are generated in situ and contain amine functions blocked with three different protecting groups. The arylation of CNTs with diazonium salts is an efficient method for the functionalization of CNTs, originally developed by the group of Tour.[21] The reaction can be performed under various conditions with the possibility of scaling up.[22] We have prepared 4-aminomethyl-anilines **1**–**3** protected with phthalimide (Pht), *tert*-butyloxycarbonyl (Boc), and benzyloxycarbonyl (Z) moieties, respectively (Scheme 1). These protecting groups were selected because conditions exist to remove them sequentially without affecting those remaining on the nanotube surface (see below).[17a,23] In a typical experiment, pristine SWCNTs (*p*-SWCNTs) were sonicated in *o*-dichlorobenzene (ODCB) for a short time by using a sonication tip (see the Experimental Section). A solution of an equimolar mixture of 4-aminomethyl-anilines **1**–**3** in acetonitrile was added to this suspension. The dispersion was sonicated again for 5 min with a tip and for 30 min in a water bath. After bubbling with argon, isoamyl nitrite was added and the reaction mixture was heated at 60 °C for 19 h. After cooling to room temperature, the suspension was diluted with methanol and filtered over a polytetrafluoroethylene (PTFE) membrane with 0.45 μm pore size. The solid recovered on the filter was dispersed in methanol, sonicated for 30 min in a water bath, and filtered again over a PTFE membrane. This sequence was repeated twice with DMF, methanol, dichloromethane, and diethyl ether. The resulting solid was dried under vacuum.

Characterization of trifunctionalized SWCNTs: The trifunctionalized SWCNTs (*f*-SWCNTs) **4** were characterized by different techniques, including TEM, thermogravimetric analysis (TGA), and Raman and UV/Vis/NIR spectroscopy. First of all, it is important to note that trifunctionalization imparts better dispersibility of the SWCNTs (Figure S1 in the Supporting Information). Indeed, the solubility of **4** is significantly improved in organic solvents, such as DMF, relative to *p*-SWCNTs. The suspension of *p*-SWCNTs is not stable because reaggregation takes place rapidly and SWCNTs settle down. On the contrary, the dispersion of **4** is highly stable for several hours. A TEM image of **4** shows that the nanotubes are present in small bundles (Figure 1 a). The morphology of the nanotubes after the functionalization process does not seem to have been altered by the chemical treatment. TGA results for **4** and *p*-SWCNTs performed under a nitrogen atmosphere are shown in Figure 1 b. As expected, the weight loss of *p*-SWCNTs is minor as CNTs are robust under nitrogen atmosphere, whereas **4** loses 25 % of the total weight at 700 °C. The weight loss can be attributed to the functional groups attached to the nanotube surface.

The Raman spectra of *p*-SWCNTs and **4** are given in Figure 1 c. The multiple peaks seen in the radial breathing mode (RBM) are due to the distribution of tube diameters in the sample; the frequency of the RBM is inversely proportional to the diameter (Figure S2 in the Supporting Information).[24] The weak band centered at 1292 cm^{-1} is attributed to disorder-induced mode (D band), mainly due to sp^3-hybridized carbon atoms in the hexagonal sp^2-hybridized carbon atom framework of the nanotube sidewall. The spectrum of **4** exhibits an increased intensity of the D band (I_D) relative to that of the G band (I_G) at 1593 cm^{-1}. It is an expected result of the introduction of functional groups covalently bound to the nanotube surface, wherein a significant number of sp^2-hybridized carbon atoms have been converted



Scheme 1. One-pot trifunctionalization of SWCNTs.

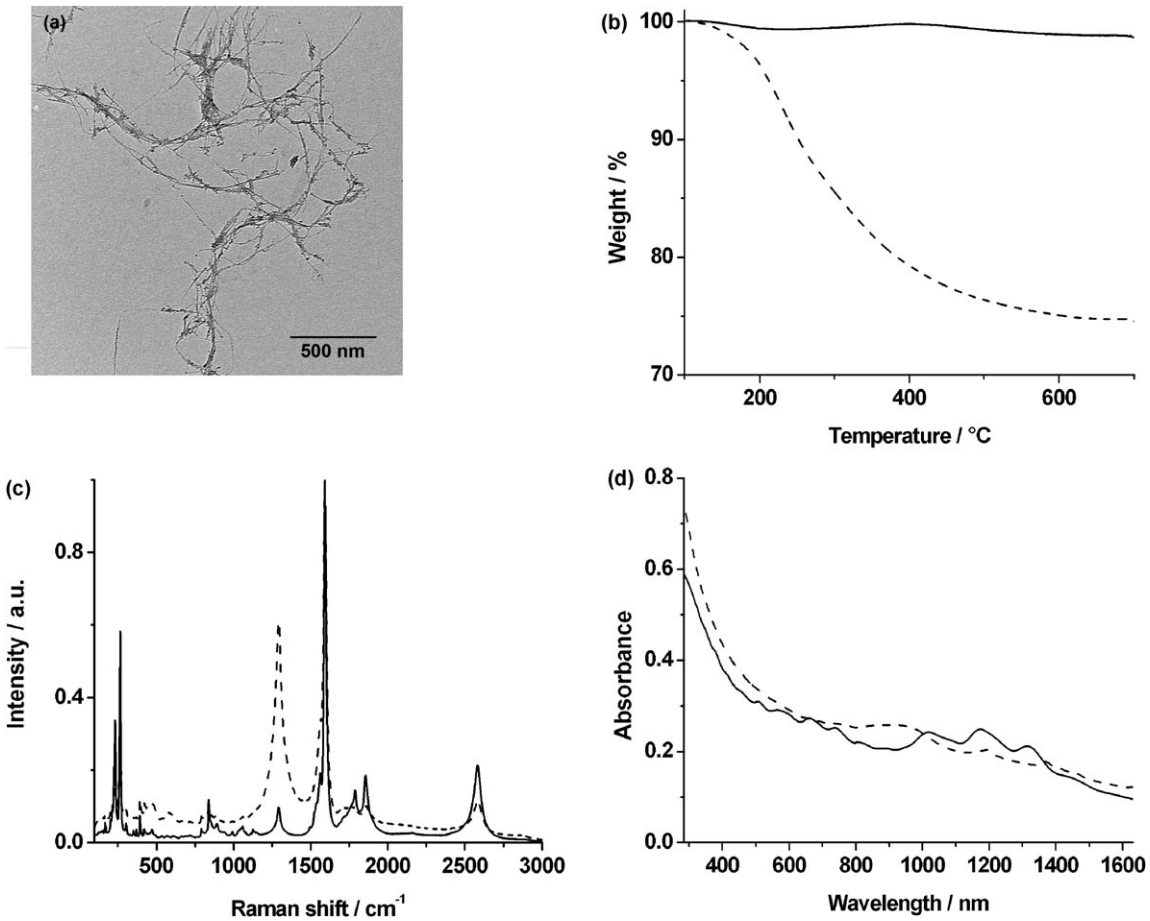
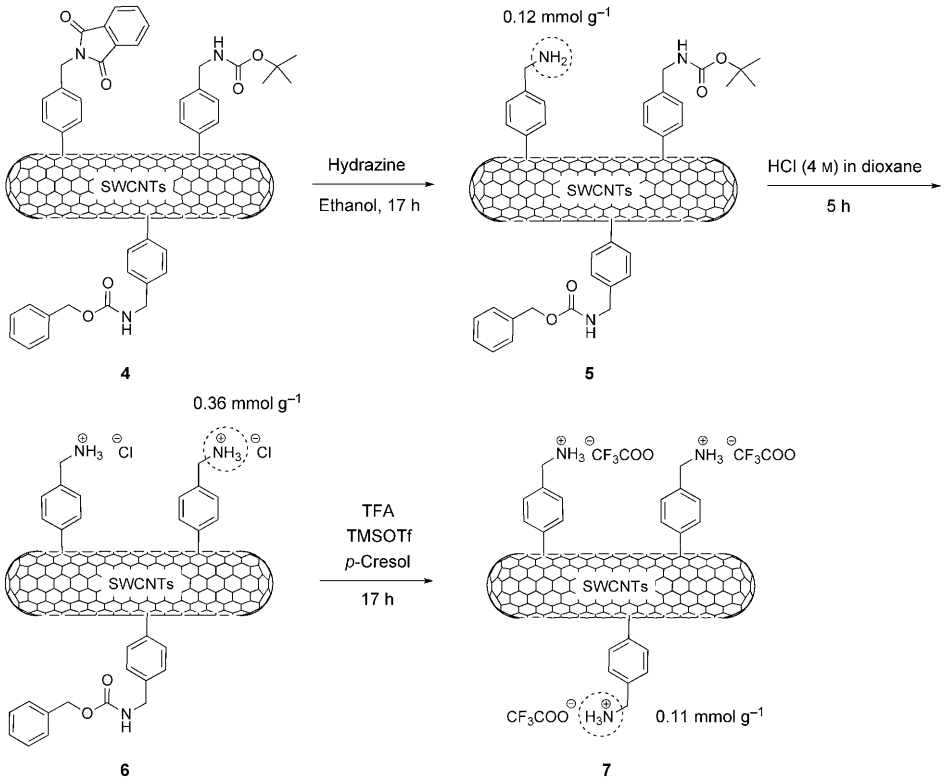


Figure 1. a) A TEM image of **4**. b) TGA of *p*-SWCNTs (——) and **4** (-----) under a nitrogen atmosphere (heating rate 10 °C min^{-1}). c) Raman spectra (785 nm, normalized on the G-band) of *p*-SWCNTs (——) and **4** (-----). d) UV/Vis/NIR absorption spectra of *p*-SWCNTs (——) and **4** (-----) in DMF.

to sp^3 hybridization.[25] The I_D/I_G ratio increased from 0.1 to 0.6 after functionalization of the SWCNTs. Moreover, the overall intensity of the RBM peaks decreased to give further confirmation of the covalent character of the functionalization of the nanotube surface (Figure S2 in the Supporting Information). UV/Vis/NIR analyses of *p*-SWCNTs and **4** are given in Figure 1d. The absorption spectrum of *p*-SWCNTs shows the characteristic van Hove singularities in the density of states (DOS), which are attributed to band-gap transitions.[26] These features are significantly reduced in the spectrum of **4**. This is indicative of significant electronic perturbation of the SWCNTs due to disruption of the extended π network and change in hybridization of a high number of carbon atoms. This effect is consistent with covalent functionalization of the nanotube sidewall, which is in agreement with observations from Raman spectroscopy analysis.[21]

Sequential removal of the three protecting groups: The three protecting groups were sequentially removed under specific conditions (Scheme 2). The Pht group was cleaved by treatment of **4** with a solution of hydrazine in ethanol to afford **5** (see the Experimental Section). Under these conditions, both Boc and Z moieties are stable. Then, the Boc group was removed by using a 4 M solution of HCl in dioxane to give **6**. This treatment is enough acidic to cleave the Boc group, but not strong enough to remove the Z moiety. Finally, a mixture of trifluoroacetic acid (TFA), trimethylsilyl trifluoromethanesulfonate (TMSOTf), and *p*-cresol was used to cleave the Z group, leading to **7**. At each step, the amine loading was determined by using the Kaiser test. This is a colorimetric test commonly used in peptide synthesis to assess the amount of primary amine functions (see Supporting Information).[27] The values obtained by the Kaiser test are reported in Scheme 2 and in Table 1, entry 1.

Even if an equimolar amount of the three anilines **1**–**3** was employed in the functionalization reaction with the *p*-SWCNTs, the degree of functionalization of the nanotube surface with each of the three benzylamines was not identical (Figure S3a in the Supporting Information). This could be explained by the fact that the reagents were used in excess and that side reactions between the corresponding diazonium salts, which are highly reactive, could occur during the functionalization reaction. However, the equimolarity of the mixture of the three anilines **1**–**3** can, in principle, be modified to tune the relative degree of functionalization and



Scheme 2. Sequential removal of the three protecting groups of **4** and the corresponding amine loadings determined by the Kaiser test.

Table 1. Loading capacity of **4**, **9**, **10**, and **12** prepared in organic or aqueous media.

Entry	Compound	Pht[a]	Boc[a]	Z[a]	Total NH_2
		NH_2 loading [mmol g^{-1}] ([%])			[mmol g^{-1}]
1	**4**	0.12 (21)	0.36 (61)	0.11 (18)	0.59
2	**9**	0.11 (39)	0.11 (39)	0.06 (22)	0.28
3	**10**	0.05 (14)	0.17 (43)	0.17 (43)	0.39
4	**12**[b]	0.13 (51)	0.03 (13)	0.09 (36)	0.25
5	**12**[c]	0.17 (24)	0.49 (73)	0.02 (3)	0.68

[a] Protecting group removed. [b] Reaction performed in organic media. [c] Reaction performed in aqueous media.

to vary the ratio between the three benzylamines linked to the surface of the nanotubes.

For comparison, we then performed one-pot trifunctionalization on oxidized SWCNTs (*ox*-SWCNTs) **8** (Figure S4 in the Supporting Information). Pristine SWCNTs were first treated with 3 M nitric acid at reflux for 70 h. This treatment reduces the length of the CNTs, which is particularly important for biomedical applications.[5] The one-pot trifunctionalization was applied to **8** and the three different protecting groups of the trifunctionalized oxidized SWCNTs (*f-ox*-SWCNTs) **9** were sequentially removed by using the same conditions described above. The amine loading values and the corresponding percentage of each of the three protected amines, determined by the Kaiser test, are reported in Table 1, entry 2, and displayed in Figure S3b in the Supporting Information. The proportion between the protected amines is remarkably different between **4** and **9** (cf. entries 1 and 2 in Table 1). This result can be explained by a difference in terms of reactivity of *p*- and *ox*-SWCNTs. However, the order of reactivity of **1**–**3** is similar in both cases: **2** (Boc) $\geq$ **1** (Pht) $>$ **3** (Z). We assume that the Pht- and Z-protected 4-aminomethylanilines **1** and **3** are likely to adsorb on the nanotube surface through π–π interactions, thereby lowering their reactivity, contrary to the bulky Boc-protected 4-aminomethylaniline **2**.

The triple functionalization of shortened SWCNTs has an additional advantage because the carboxylic functions introduced by oxidation, mainly at the tips,[18] can be further derivatized to prepare even more sophisticated multifunctionalized CNTs. The COOH groups may indeed be used as anchor point of a fourth molecule of interest or for the grafting of molecules to further enhance the dispersibility of the nanotubes.

Trifunctionalization of MWCNTs: Following this straightforward approach to modify carbon nanotubes with multiple functions, we have explored the possibility to extend our strategy to MWCNTs. The MWCNTs are indeed reactive towards diazonium salts.[28] Initially, we used purified MWCNTs and repeated the reactions described for SWCNTs. The *f*-MWCNTs **10** were characterized by TGA and TEM (Figures S5 and S6 in the Supporting Information, respectively). The amine loading values determined by the Kaiser test after sequential removal of the protecting groups are reported in Table 1, entry 3. Boc-protected **2** is still the most reactive aniline (Figure S3c in the Supporting Information). In a comparative study, one-pot trifunctionalization was also applied to *ox*-MWCNTs **11**. For this purpose, MWCNTs were first oxidized to shorten them to 315 nm on average.[29] TEM images show that the oxidative treatment dramatically reduces the nanotube length. The one-pot trifunctionalization reaction was performed in two different solvent systems, namely, in water and in a mixture of *o*-dichlorobenzene and acetonitrile (Figure S7 in the Supporting Information). Water is a green alternative to organic solvents and it is advantageous for potential scaleup. The *f-ox*-MWCNTs **12** prepared under both conditions were analyzed again by TEM and TGA (Figures S8 and S9 in the Supporting Information, respectively). The three protecting groups

were sequentially removed and the corresponding amine loading values determined by the Kaiser test are summarized in Table 1, entries 4 and 5, and represented in Figures S3d and S3e in the Supporting Information. The total amine loading is higher for **12** when the reaction is performed in water (0.68 mmol g^{-1} versus 0.25 mmol g^{-1}), which is in agreement with observations from TGA weight loss (Figure S9 in the Supporting Information). As a consequence, by changing the conditions, it is possible to modulate the level of functional groups on oxidized and shortened MWCNTs.

Conclusion

We have developed a methodology to covalently functionalize CNTs with three different active groups in one step by arylation with diazonium salts. The CNTs are functionalized with benzylamine moieties blocked with three different protecting groups that can be selectively removed under specific conditions. The trifunctionalized CNTs were characterized by TEM, TGA, and Raman and UV/Vis/NIR spectroscopy, and the level of functionalization was assessed by using the Kaiser test. The trifunctionalization approach is simple, rapid, and versatile because it can be applied to different types of CNTs, in terms of diameter, number of walls (SWCNTs, MWCNTs), and length (pristine and shortened CNTs). Further derivatization of the amine groups with a small drug, a fluorophore, and a targeting agent is currently being performed in our laboratory to use these multimodal CNTs for targeted drug delivery. We assume that the sequential removal of the protecting groups of the amine functions will allow the grafting of the molecules of interest onto the nanotube surface to be precisely controlled.

Experimental Section

Materials and methods: SWCNTs were purchased from Unidym (HiPco Single-Walled Carbon Nanotubes, Lot# R1912) and MWCNTs were purchased from Nanocyl (Thin MWCNT 95+% C purity, Nanocyl 3100, batch no. 071119, average diameter and length: 9.5 nm and 1.5 μm, respectively). All reagents and solvents were purchased from different commercial suppliers and used as received. TEM was performed on a Hitachi 600 microscope with an accelerating voltage of 75 kV. The CNTs were dispersed in ethanol by sonication, deposited on a carbon TEM grid, and dried. Thermogravimetric analyses were performed by using a TGA Q500 TA instrument with a ramp of 10 °C min^{-1} under N_2 using a flow rate of 60 mL min^{-1}. Raman spectroscopy analysis was performed on an inVia Raman microscope (Renishaw) by using 785 nm laser light. The UV/Vis/NIR spectra were recorded on a Varian Cary 5000 spectrophotometer. When stated, suspensions of CNTs were sonicated either in a water bath (Transsonics Digitals Elma, 20 W, 40 kHz) or by using tip sonication (Vibra-Cel Ultrasonic Processor).

Preparation of 4: Pristine SWCNTs (47 mg) were dispersed in ODCB (47 mL) and sonicated by using a tip (5 min cycle, 5 s ON, 5 s OFF, 30% amplitude). The suspension was further sonicated in a water bath (15 min). An equimolar mixture of the anilines **1** (148 mg, 0.59 mmol), **2** (130 mg, 0.59 mmol), and **3** (150 mg, 0.59 mmol) in acetonitrile (24 mL) was added to the suspension of *p*-SWCNTs. The resulting dispersion was sonicated by using a tip (5 min cycle, 5 s ON, 5 s OFF, 30% amplitude) and then by using a water bath for 30 min. Argon was bubbled in the suspension for 10 min. Isoamyl nitrite (0.37 mL, 2.8 mmol) was added and the reaction mixture was immediately heated at 60 °C for 19 h. After cooling to room temperature, the suspension was diluted with methanol (100 mL) and filtered over a PTFE (0.45 μm, Omnipore JHWP, Millipore) membrane. The solid recovered on the filter was dispersed in methanol (300 mL), sonicated for 30 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. This sequence was repeated twice with DMF, methanol, dichloromethane, and diethyl ether. The resulting solid was dried under vacuum.

Preparation of 5: Deprotection of the Pht group: Hydrazine hydrate (28 μL) was added to a suspension of **4** (7 mg) in ethanol (8 mL). The dispersion was sonicated in a water bath for 5 min and stirred for 17 h. Then, the suspension was diluted with methanol (75 mL), sonicated for 5 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. The solid recovered on the filter was dispersed in methanol (75 mL), sonicated for 10 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. This sequence was repeated twice with methanol, dichloromethane, and diethyl ether. The resulting solid was dried under vacuum.

Preparation of 6: Deprotection of Boc group: A suspension of **5** (5.9 mg) in HCl (3.9 mL, 4 M in dioxane (purchased from Sigma–Aldrich)) was sonicated in a water bath for 5 min and stirred for 5 h. Then, the mixture was diluted with diethyl ether (50 mL), sonicated for 5 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. The solid recovered on the filter was dispersed in methanol (50 mL), sonicated for 5 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. This sequence was repeated twice with methanol, dichloromethane, and diethyl ether. The resulting solid was dried under vacuum.

Preparation of 7: Deprotection of Z group: A mixture of **6** (3 mg) in TFA (195 μL), TMSOTf (52 μL), and *p*-cresol (26 mg) was stirred for 17 h. Then, the mixture was diluted with methanol (50 mL), sonicated for 5 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. The solid recovered on the filter was dispersed in methanol (50 mL), sonicated for 5 min in a water bath, and filtered over a PTFE (0.45 μm) membrane. This sequence was repeated twice with DMF, methanol, dichloromethane, and diethyl ether. The resulting solid was dried under vacuum.

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