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Antioxidant Single-Walled Carbon Nanotubes

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Abstract: Single-walled carbon nanotubes (SWCNTs) and ultrashort SWCNTs (US-SWCNTs) were functionalized with derivatives of the phenolic antioxidant, butylated hydroxytoluene (BHT). By using the oxygen radical absorbance capacity (ORAC) assay, the oxygen radical scavenging ability of the SWCNT antioxidants is nearly 40 times greater than that of the radioprotective dendritic fullerene, DF-1. In addition, ORAC results revealed two divergent trends in the antioxidant potential of SWCNTs, depending on the type of functionalization employed. When existing pendant sites on US-SWCNTs were further functionalized by either covalent or noncovalent interactions of the existing pendant sites with a BHT derivative, the amount of BHT-derivative loading proportionately increased the overall antioxidant activity. If, however, functionalization occurred via covalent functionalization of a BHT-derivative directly to the SWCNT sidewall, the amount of BHT-derivative loading was inversely proportional to the overall antioxidant activity. Therefore, increasing the number of pendant sites on the SWCNT sidewalls by covalent functionalization led to a concomitant reduction in ORAC activity, suggesting that the nanotube itself is a better radical scavenger than the BHT-derivatized SWCNT. Cytotoxicity assays showed that both nonfunctionalized and BHT-derivatized SWCNTs have little or no deleterious effect on cell viability. Therefore, SWCNTs may be attractive agents for antioxidant materials and medical therapeutics research.

Introduction

The development of water-soluble single-walled carbon nanotubes (SWCNTs)[1−9] has opened the door for the exploitation of the unique features of SWCNTs in biological settings. In particular, their notable aspect ratio renders them ideal scaffolds for the attachment of payloads and other entities to their modifiable, ample surface area sidewalls. Furthermore, SWCNTs have been shown to undergo rapid cellular uptake.[10−16] Another property of SWCNTs that has been scarcely exploited to date is the ability of the structures to behave as radical scavengers.[17] Radical scavenging may occur via radical addition to the curved, sp^2-hybridized carbon nanotube framework.[18,19]

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It is known that C_{60}-derivatives are efficient radical scavengers,[20] and several studies utilizing functionalized, water-soluble C_{60}-derivatives have accounted for this effect in vivo.[21−25] SWCNTs, having electron affinity similar to that of their fullerene counterparts, have been added to polymer matrices to retard oxidation.[26] Indeed, new generations of potent radical scavenging scaffolds could be important in materials technologies which often suffer from oxidative decomposition pathways. Additionally, antioxidant chemotherapy has been shown to be an essential component in the treatment of numerous disorders including stroke,[27] traumatic brain injury,[28] radiation exposure sickness,[29,30] Alzheimer's disease,[31] and coronary artery disease[32] to name a few; thus, generating a new series of potent radical scavenging scaffolds could prove to be important in medicine if the scaffolds could be shown to be nontoxic, deliverable to the site of interest, and then cleared so as not to disrupt the favorable biological radical cascades. Here, we show that SWCNTs act as antioxidants in biological settings both in their pristine forms and in phenolic-functionalized structures.

Because we and others have shown SWCNTs to be multifunctional,[33−35] it is possible to functionalize individual SWCNTs with solubility enhancers, targeting agents, fluorescent tags, and/or therapeutic payloads, for example, toward the goal of a SWCNT nanovector.[36] In this work, two types of SWCNTs have been functionalized with analogues of the phenolic antioxidant, butylated hydroxytoluene (BHT).[37] The sidewalls of long Pluronic-wrapped[38] pristine SWCNTs were directly studied along with their BHT-derivative functionalized forms. Pluronic (BASF Corporation) is a triblock copolymer of poly(ethylene glycol)/poly(propylene glycol)/polyethylene glycol (PEG/PPG/PEG). Another type of SWCNT studied was the ultrashort SWCNTs (US-SWCNTs)[39] that have carboxylic acids located along the sidewalls and ends of the structures; these carboxylic acid sites were used to append BHT-derivatives. By using the total oxygen-radical absorbance capacity (ORAC) assay,[40,41] we show here that both types of SWCNTs are extremely effective antioxidants. Two contrasting trends in the antioxidant potential of SWCNTs were noted, apparently as a consequence of the type of functionalization employed. When both covalent and noncovalent functionalizations with the BHT-derivatives to the carboxylic acids sites were performed, the antioxidant activity increased with increasing BHT-derivative moieties. However, when functionalization occurred directly on the sidewalls of the pristine SWCNTs through diazonium chemistry, the radical scavenging activity decreased with increasing BHT-derivative functionality. The latter case indicates that the radical scavenging activity of the SWCNT sidewalls was greater than the scavenging activity of the BHT-derivative appended system. Therefore, SWCNTs are themselves powerful antioxidants, as recently suggested by ab initio studies.[17b] Because the SWCNTs in the study were soluble in biologically relevant salts and it was also demonstrated that concentrations of SWCNTs up to 330 nM or 83 mg/L had little-to-no cytotoxicity on the cell line studied, SWCNTs are attractive agents for antioxidant therapy research.

Materials and Methods

Materials. THF was freshly distilled over sodium/benzophenone in a nitrogen atmosphere. Other solvents were distilled over calcium hydride under a nitrogen atmosphere. Anhydrous *N*,*N*-dimethylformamide and other chemicals and starting materials were purchased from Sigma-Aldrich and used without further purification unless otherwise stated. NANOPure water was obtained from a Barnstead water purification system and had a resistivity of 18 MΩ/cm or greater. SWCNTs were obtained from the HiPco laboratory at Rice University.[42,43] 2,6-Di-*tert*-butyl-4-(2-aminoethyl)phenol (amine-BHT) **1**[44] and US-SWCNTs **2**[39] were synthesized by using known procedures. The moist filter cake of US-SWCNTs was transferred to a scintillation vial and pulverized with a Teflon stir rod over light heat on a hot plate resulting in a fine, dry powder that was dried in a vacuum desiccator overnight. The Raman diamondoid-to-graphitic (D/G) ratio was 0.8 and the thermogravimetric analysis (TGA, 10 °C/min, Ar, up to 900 °C) mass loss was 43%. From the TGA, it was estimated that 1/5 of the SWCNT carbons[45] were functionalized with a carboxylic acid group, as described previously.[39] The average US-SWCNT length of 60 nm was determined by atomic force microscopy as described previously.[39,46] The X-ray photoelectron spectroscopy atomic concentrations were as follows: C 1s, 68%; O 1s, 31%; N 1s, 1%; and S 2p, 0.4%, as previously described for these heavily carboxylated nanotubes.[39]

Continuous Flow Setup for Aqueous Dialysis.[46] A deionized (DI) water supply was split into five separate lines by using

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polypropylene T-joints and Nalgene tubing (80 mm. i.d.). Each line was fed to the bottom of five separate 1 L beakers, and the beakers were placed in a plastic tray (102 cm × 61 cm × 7.6 cm) fitted with three drains at one end; the other end was slightly elevated to promote drainage. The water source was turned on, and the beakers were allowed to continuously overflow. The flow rate was adjusted to prevent overflowing the tray.

PEGylation of US-SWCNTs (PEG-US-SWCNTs) (3). An oven-dried 100 mL round-bottom flask equipped with a stir bar was charged with **2** (0.063 g, 5.2 mequiv C) and anhydrous DMF (50 mL). The mixture was vigorously stirred for 15 min under a nitrogen atmosphere. *N*,*N*′-Dicyclohexylcarbodiimide (DCC, 1.08 g, 5.2 mmol) was added, followed by methoxy polyethylene glycol amine (0.50 g, 0.1 mmol, MW 5000) and 4-dimethylaminopyridine (DMAP, 11 mg, 0.09 mmol). The mixture was stirred overnight and purified by dialysis (dialysis bag MWCO 50000) first in DMF for one day to remove water-insoluble organic byproducts, then for four days in flowing DI water for further purification and replacement of the organic solvent by water. Product **3** was repeatedly filtered through several layers of Kimwipes to remove remaining particulates. The concentration of the resultant material, determined by Beer's law analysis using an empirically derived extinction coefficient of 0.0104 L/mg at λ_{max} 763 nm, was 572 mg/L. The material was further diluted to 83 mg/L by using DI water for ORAC and cell-toxicity assays. The TGA mass loss was 95%. TGA analysis estimates 1/21 SWCNT carbons functionalized with PEG.

Ionically Bound Amine-BHT Derivatized PEG-US-SWCNTs (4). Compound **3** (0.6 mg, 0.05 mequiv C) was added to a 50 mL round-bottom flask equipped with a stir bar. Amine-BHT **1** (0.012 g, 0.05 mmol) was dissolved in DMF (1 mL) and added to the mixture. The mixture was stirred overnight. No film was visible on top of the reaction solution, indicating that the lipophilic amine-BHT had been solubilized through ionic association with the carboxylic acid groups on **3**. The material was purified by dialysis (dialysis bag MWCO 50000) in flowing DI water for five days to afford **4**. The concentration of the resultant material, determined by Beer's law analysis using an empirically derived extinction coefficient of 0.0104 L/mg at λ_{max} 763 nm, was 450 mg/L. The material was further diluted to 83 mg/L by using DI water for ORAC and cell-toxicity assays.

Covalently Bound Amine-BHT Derivatized PEG-US-SWCNTs (5). DCC (26 mg, 0.13 mmol) was quickly added to a stirring solution of 3 mg of PEG-US-SWCNT **3** (prepared as above, but further reacted in the same pot prior to purification for this specific process) under a nitrogen atmosphere in dry DMF (6 mL). After 10 min, **1** (16 mg, 0.064 mmol) was added, followed by DMAP (3 mg, 0.03 mmol). The mixture was left to stir overnight at room temperature. The mixture was purified in the same way as **4**. The concentration of the resultant material, determined by Beer's law analysis using an empirically derived extinction coefficient of 0.0104 L/mg at λ_{max} 763 nm, was 210 mg/L. The material was further diluted to 83 mg/L by using DI water for ORAC and cell-toxicity assays.

PEG Control (6). A total of 14.0 mg of 5000 MW methoxy polyethylene glycol was dissolved in 61.4 mL of DI water. The resulting solution was 0.228 mg/mL and represents the amount of PEG attached to an 83 mg/L concentration of US-SWCNTs.

(2,6-Di-*tert*-butyl-4-bromophenoxy)trimethylsilane (7).[47] An oven-dried 100 mL round-bottom flask equipped with a stir bar was charged with commercially available 2,6-di-*tert*-butyl-4-bromophenol (2.85 g, 10.0 mmol) and THF (50 mL) and then cooled to −78 °C. *n*-Butyllithium (6.00 mL, 15 mmol, 2.5 M in hexane) was slowly added, and the mixture was stirred for 1 h. After 1 h, chlorotrimethylsilane (1.84 g, 16.9 mmol) was added to the mixture. The reaction was allowed to stir for 1 h while warming to room temperature and was then poured into water. The product was extracted with hexanes twice, and the combined organic layers were washed with water. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed from the filtrate under reduced pressure. The product was purified by column chromatography (silica gel, hexanes as eluent) to provide 3.39 g of **7** (95%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.32 (s, 2H), 1.38 (s, 18H), 0.38 (s, 9H).

(2,6-Di-*tert*-butyl-4-iodophenoxy)trimethylsilane (8). An oven-dried 100 mL round-bottom flask equipped with a stir bar was charged with compound **7** (3.40 g, 9.5 mmol) and 50 mL of ether. The mixture was cooled to −78 °C, and *tert*-butyllithium (1.83 g, 28.5 mmol, 17.8 mL of a 1.6 M solution in pentane) was slowly added. The resulting solution was stirred for 1 h, and 1,2-diiodoethane (5.36 g, 19 mmol) was added. The mixture was stirred at −78 °C for 1 h and then allowed to warm to room temperature. The solution was poured into water and extracted with hexanes. The combined organic layers were washed with water and dried with $MgSO_4$. The product was filtered, and the solvent was removed from the filtrate under reduced pressure. The resulting material (1.47 g) was a mixture of **7** and the desired product **8** (51%), and it was used without purification. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.34 (s, 2H), 1.38 (s, 18H), 0.38 (s, 9H).

1-[(2,6-Di-*tert*-butyl-4-phenoxy)trimethylsilane]phenylethyne-(3,3-diethyl)triazene (9). An oven-dried 100 mL round-bottom flask equipped with a stir bar was charged with compound **8** (1.47 g of the above mixture), 1-acetylenephenyl(3,3-diethyl)-triazene[48] (0.40 g, 2.0 mmol), $PdCl_2(PPh_3)_2$ (0.042 g, 0.6 mmol), CuI (0.025 g, 1.3 mmol), triethylamine (2 mL), and well-degassed THF (30 mL). The contents were stirred at 60 °C until TLC analysis showed conversion of **8**. The mixture was filtered and poured into saturated NH_4Cl and extracted with dichloromethane. The combined organic layers were washed with water and dried with $MgSO_4$. The product was filtered, and the solvent was removed from the filtrate under reduced pressure. The product was purified by column chromatography (silica gel, 1:3 dichloromethane to hexanes as eluent) to yield 0.83 g (78%) of the desired product **9**. FTIR (cm^{-1}) 2955, 2872, 1495, 1467, 1421, 1397, 1326, 1269, 1255, 1236, 1200, 1165, 1123, 1108, 1079. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.41 (d, J = 8.4, 2H), 7.34 (s, 2H), 7.29 (d, J = 8.4, 2H), 3.78 (q, J = 14.3, 4H), 1.45 (s, 18H), 1.27 (t, J = 14.3, 6H), 0.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) 153.9, 150.9, 141.3, 132.4, 129.5, 120,5, 120.2, 115.5, 90.3, 88.3, 35.4, 31.4, 4.0. HRMS (EIS) $C_{29}H_{44}N_3OSi$ (+1 H): calcd, 478.3254; found, 478.3245.

1-(2,6-Di-*tert*-butyl-4-phenol)phenylethyne(3,3-diethyl)triazene (Triazene OPE-BHT) (10). Compound **9** dissolved in dichloromethane (30 mL) and tetra-*n*-butylammonium fluoride (3 mL, 3 mmol, 1.0 M in THF) were added to a 100 mL round-bottom flask equipped with a magnetic stir bar, and the mixture was stirred overnight at room temperature. The color changed from red to green. The product was isolated by filtering the solution through a silica gel plug and washing with 1:1 dichloromethane and hexane to give an orange solution. The solvent was removed under reduced pressure to provide a red solid, **10** (0.48 g, 90%). M.P. 61−63 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) 7.49 (d, J = 8.4, 2H), 7.38 (d, J = 8.4, 2H), 7.36 (s, 2H), 3.78 (q, J = 14.3, 4H), 1.45 (s, 18H), 1.27 (t, J = 14.3, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, ppm) 154.2, 150.6, 136.1, 132.1, 128.6, 120.3, 114.4, 90.2, 34.4, 30.2, 30.0. HRMS (EIS) $C_{26}H_{36}N_3O$ (+ 1H): calcd, 406.2858; found, 406.2856.

Pluronic-Wrapped SWCNTs (11). Raw HiPco SWCNTs (0.09 g) and Pluronic F 108NF Prill Poloxamer 338 (donated by the BASF Corporation, 2.25 g) in NANOPure water (225 mL) were homogenized for 1 h by using a homogenizer shaft driven by a Dremel Multipro model 395 motor and model 225 flexible shaft. The SWCNTs were then sonicated by using a cup-horn sonicator (Cole Parmer Ultrasonic Processor Model CP 750) for 10 min at 78%

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amplitude, and the mixture was then ultracentrifuged for 4 h at 29 000 rpm. The SWCNTs in solution were decanted, and the solid material was discarded. The concentration of the resultant material, determined by Beer's law analysis using an empirically derived extinction coefficient of 0.0305 L/mg at λ_{max} 763 nm, was 42 mg/L. The material was further diluted to 28 mg/L by using 1 wt% Pluronic in DI water for ORAC and cell-toxicity assays.

OPE-BHT Derivatized SWCNTs (12). The pH of 1 wt% (in NANOPure water) Pluronic-wrapped SWCNTs **11** (50 mL) was adjusted with enough concentrated HCl to lower the pH to 2. Compound **10** (25 mg, 0.62 mmol) was dissolved in acetonitrile (2 mL) and then added to the SWCNT solution.[33] The mixture was stirred for 20 min, and the pH was then adjusted to 10 by adding NaOH (40% aqueous) dropwise. The above procedure was repeated on the same SWCNT solution three additional times. The mixture was then dialyzed (dialysis bag MWCO 50000) in Pluronic (1 wt% in NANOPure water) for five days to purify the material, affording **12**, a black solution with a rust-colored tint. The concentration of the resultant material, determined by Beer's law analysis using an empirically derived extinction coefficient of 0.0305 L/mg at λ_{max} 763 nm, was 42 mg/L. The material was further diluted to 28 mg/L by using 1 wt% Pluronic in DI water for ORAC and cell-toxicity assays. Raman D/G ratio: 0.45; TGA mass loss: 35%. TGA analysis estimates 1/48 SWCNT carbons functionalized with OPE-BHT.

1 wt% Pluronic in Water Control (13). A total of 0.4 g of Pluronic was dissolved in 40 mL of NANOPure water.

ORAC Assay.[40] All solutions were prepared daily in 75 mM phosphate buffer at pH 7.4. Fluorescein sodium salt (FL) was prepared at 0.2 μM from a 4 mM aqueous stock (prepared fresh monthly and stored in the dark at 4 °C). α,α′-Azodiisobutyramidine dihydrochloride (AAPH) was prepared at 0.15 M in water and kept in an ice bath until added to the system. Racemic 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) was prepared at 400 μM in water.

The experiments were performed in a black-sided, clear-bottomed 96-well plate. In order to account for the background and any fluorescence loss during the overnight experiments, phosphate buffer was substituted for AAPH and FL in two wells. Therefore, each sample was analyzed in three wells as follows.

1. Assay = 120 μL FL + 20 μL sample + 60 μL AAPH
2. Control 1 (minus AAPH) = 120 μL FL + 20 μL sample + 60 μL phosphate buffer
3. Control 2 (minus FL) = 120 μL phosphate buffer + 20 μL sample + 60 μL AAPH

In the appropriate wells, the FL, sample, and phosphate buffer were added. Each experimental run included trolox and phosphate buffer as samples. The plate was then incubated at 37 °C for 15 min in a Safire2 plate reader (Tecan Systems Inc.). Then, the ice-cold AAPH was added to the appropriate wells. The fluorescent intensity at 530 nm, 485 nm excitation, was monitored every minute for 6 h.

The background (control 2) was subtracted from the assay and control 1 well results. The assay well results were divided by the control 1 well results. The area under the curve (AUC) for the resultant values was determined electronically. Trolox mass equivalents (TME) were calculated by using eq 1.[40]

$$\frac{\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{PBS}}}{\mathrm{AUC}_{\mathrm{trolox}} - \mathrm{AUC}_{\mathrm{PBS}}} \times \frac{\mathrm{trolox}_{\mathrm{mass}}}{\mathrm{sample}_{\mathrm{mass}}} = \mathrm{TME} \qquad (1)$$

All PEG-US-SWCNTs solutions were at 83 mg/L, and all Pluronic-wrapped SWCNT solutions were at 28 mg/L; solutions were diluted by 10 for the ORAC assay to give concentrations of 8.3 and 2.8 mg/L, respectively. The concentration of trolox used in the assay was 10 mg/L. Initial SWCNT concentrations were calculated by using empirically derived extinction coefficients. Each sample was run a total of nine times with the above treatment. Averages and standard deviations were calculated.

Cytotoxicity of SWCNT Formulations. Human renal epithelial (HRE) and HepG2 liver cells were utilized to assay acute cytotoxicity induced by all BHT-derivatized and nonderivatized SWCNTs. The cells were plated at 1×10^5 cells/well in a 12-well tissue-culture treated plate. The cells were allowed to attach overnight at 37 °C in 5% CO_2. The samples were added at a dose concentration of 109 nM (28 mg/L) for Pluronic-wrapped SWCNTs and 332 nM (83 mg/L) for PEG-US-SWCNTs. Triton-X at 1 wt% in water was utilized as the toxic control. After 24 h exposure to the SWCNT solutions, the cells were removed from the plate with trypsin. Cell viability was assayed by utilizing a Beckman Coulter Vi-Cell XR employing a trypan blue permeability assay. The viable-cell counts were normalized to the phosphate buffered saline (PBS) control.

Results and Discussion

Binding of Amine-BHT Ionically and Covalently to PEG-US-SWCNTs. In order to provide an appropriate scaffold for the ionic, acid/base attachment of amine-BHT **1** (Scheme 1), US-SWCNTs **2** were made by cutting purified SWCNTs in a mixture of oleum and nitric acid.[39] Carboxylic acid groups located at the functionalized sites provide a handle for further functionalization of the SWCNTs.[49,50] To render the already water-soluble US-SWCNTs **2** PBS-soluble, they were PEGylated to produce **3**. Following this process, the PEG-US-SWCNTs **3** were combined with amine-BHT **1** to yield **4** via an acid/base ionic interaction[51] as shown in Scheme 1.

For the covalent derivatization of PEG-US-SWCNTs (Scheme 2), DCC, methoxy polyethylene glycol amine, and DMAP were added in this order to a solution of US-SWCNTs in dry DMF under nitrogen with stirring. The reaction was left overnight at room temperature, and the following day, a portion of this solution was withdrawn and purified for analysis to verify the synthesis of the intermediate **3**. To the remaining solution, more DCC was added, followed by amine-BHT **1** and DMAP. After an overnight reaction, the product was purified in a fashion identical to that of **4** to yield **5**. Methoxy polyethylene glycol amine was added first to occupy enough acid groups needed for PBS solubility. By adding the amine-BHT last, the system could be treated with excess amine-BHT for maximal reaction with the remaining acid moieties.

The number of PEG groups per nanotube could easily be determined through TGA by heating the PEG-US-SWCNT at a rate of 10 °C/min under argon and recording the weight loss. Under these conditions, the nanotube itself will not lose any weight, and any weight loss is attributed to functionalities on the SWCNT. The weight loss from the US-SWCNT (done in the same manner as previously described) was subtracted from this value to determine the amount of PEG attached to the SWCNTs. Determination of the loading of amine-BHT on the PEG-US-SWCNTs was not feasible by a direct TGA determination. Because of the small size of the amine-BHT molecule relative to the 5000 MW PEG groups, TGA is not sensitive enough to discern the relatively small amount of weight loss due to amine-BHT when over 95% weight loss is already observed for the precursor **3**. Therefore, to estimate the upper limit of amine-BHT groups that can be bound to the PEG-US-SWCNTs, the number of open acid groups after attachment of PEG was estimated from the TGA thermogram. According to

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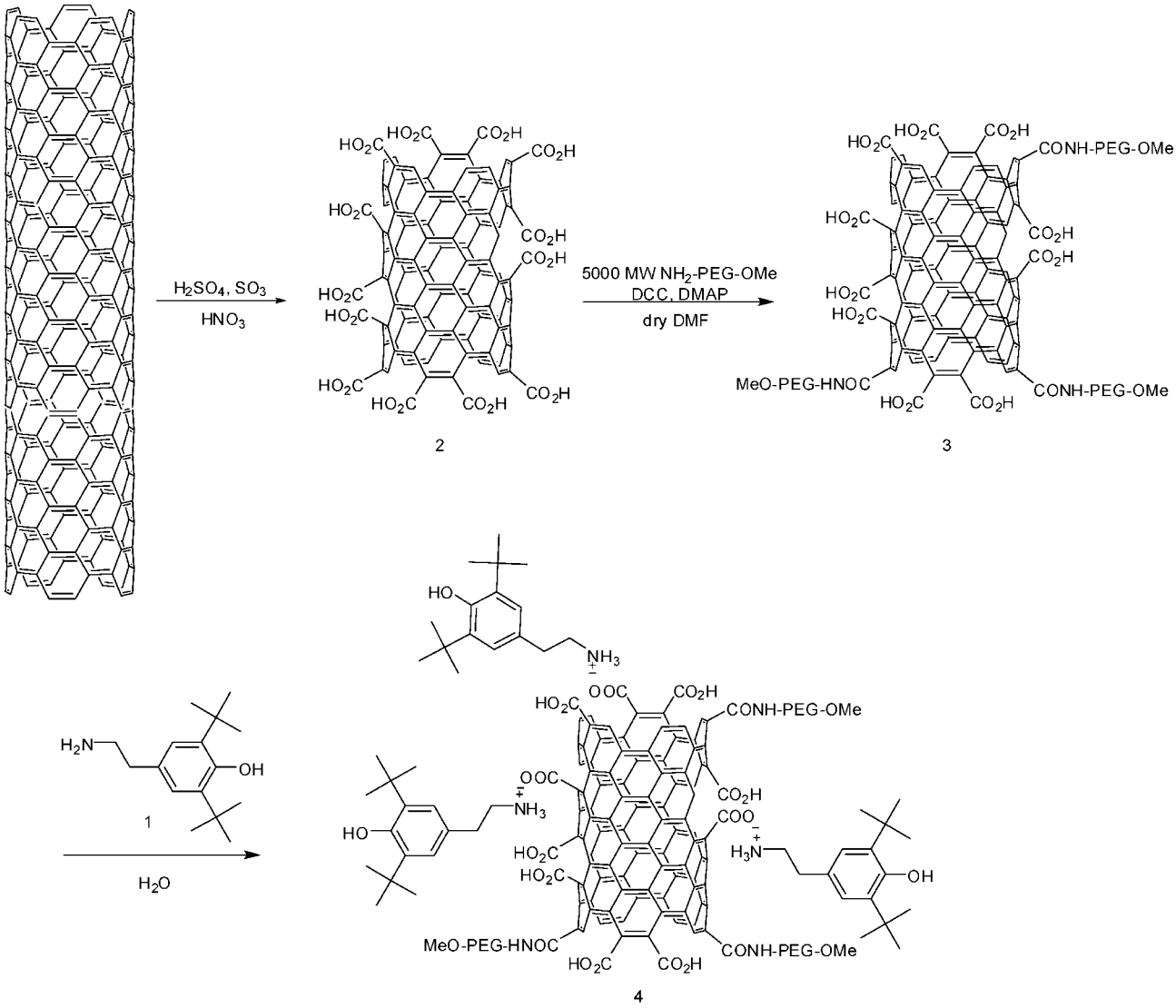
Scheme 1. Production of **4**[a]



[a] SWCNTs were cut by using oleum and nitric acid to give US-SWCNT **2**. The US-SWCNTs were PEGylated to give PEG-US-SWCNT **3**. Amine BHT **1** was acid−base bound to the PEG-US-SWCNT to give **4**.

the calculation (see Supporting Information), the upper limit for amine−BHT binding is 900 molecules per PEG-US-SWCNT.

OPE-BHT Derivatized SWCNTs. An alternative approach to functionalize SWCNTs with BHT-derivatives was to attach the BHT molecules directly to the SWCNT sidewalls. For this purpose, in situ diazonium formation and functionalization via the triazene moiety was utilized.[52] Briefly, triazene-OPE-BHT **10** (Scheme 3) was added to Pluronic-wrapped SWCNT solution **11** under stirring. The pH of the solution was adjusted to 2 to convert the triazene to the diazonium salt, and the pH was subsequently adjusted to 10 to complete the reaction (Scheme 4). Here, this step was repeated three more times to maximize the functionalization of SWCNTs with BHT moieties. OPE-derivatized SWCNTs **12** were subsequently dialyzed in 1 wt% Pluronic to remove impurities and excess reagent; but after dialysis, the mixture still remained rusty brown in color, an appearance that is atypical of the SWCNT alone. When OPE-BHT derivatized SWCNTs were washed with organic solvents to achieve a clean bucky paper for analysis, a clear orange filtrate consisting of organic byproducts was obtained. NMR analysis of the filtrate revealed that there was no excess OPE-BHT in solution. The bucky paper was washed repeatedly until the filtrate was colorless.

ORAC of SWCNT Formulations. The ORAC assay[40,41] monitors a fluorescent probe's loss of fluorescent intensity in the presence of oxygen radicals. When oxygen-radical scavengers are added to the system, the fluorescent intensity persists until the radical scavenger is consumed. The assay readout compares the radical scavenging ability of test compounds to a known radical scavenger, trolox, a water-soluble vitamin E derivative. For these experiments, FL and AAPH were used as the fluorescent probe and as thermally activated radical initiator, respectively. The TME value is a unit that expresses the tested antioxidant's radical scavenging ability relative to a given mass of trolox. Trolox is given a TME of 1. Through calculation of TMEs, one can compare results to other radical

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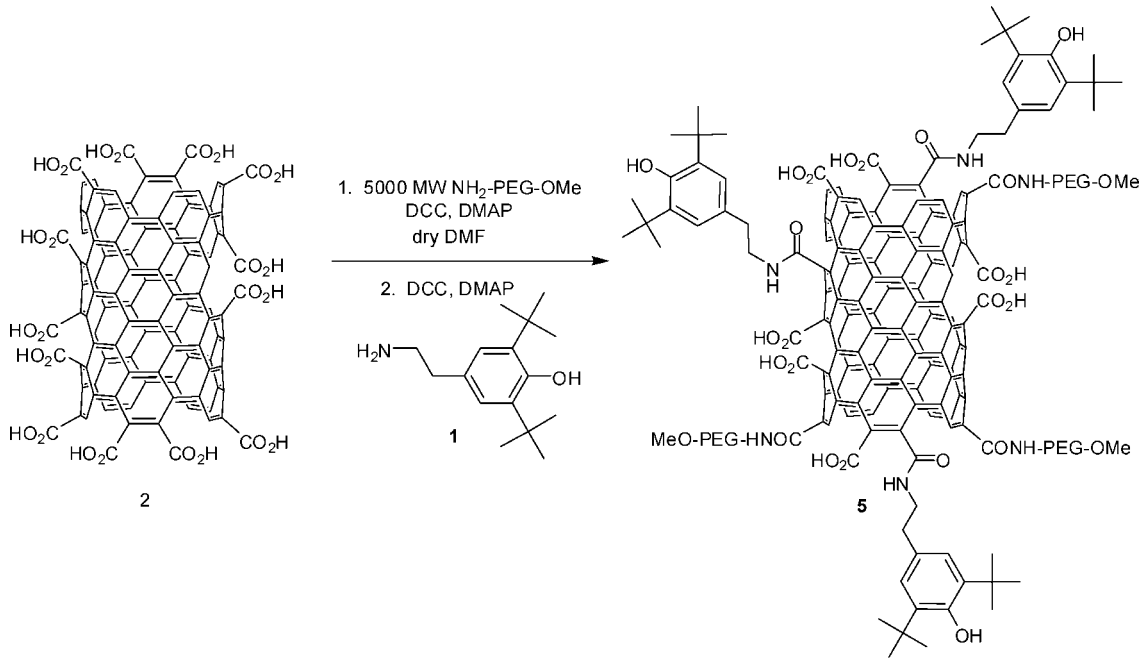
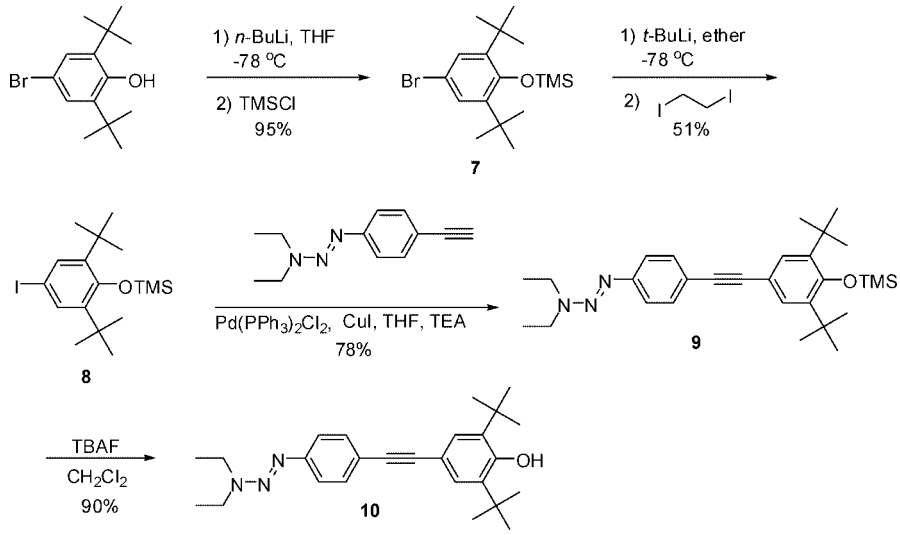
Scheme 2. Covalent Derivatization of PEG-US-SWCNTs[a]



[a] US-SWCNTs **2** were PEGylated, and amine-BHT **1** was also covalently attached to the carboxylic acids to give amine-BHT derivatized PEG-US-SWCNT **5**.

Scheme 3. Synthesis of Triazene OPE-BHT **10**



scavengers throughout the literature. Trolox equivalents (TE) are calculated by using molarity instead of mass. We chose to use TME instead of TE for a more accurate and fair comparison between SWCNTs and the free molecule trolox, because precise determination of SWCNT moles is not possible because of their polydispersity, and if based on moles, SWCNTs are grossly off-scale in their radical sequestration ability, as shown in Table 1.

Figure 1 depicts the ORAC results of all SWCNT samples assayed. Even the poorest SWCNT antioxidant, PEG-US-SWCNTs without BHT-derivative addends, is nearly 40 times more effective at scavenging radicals than dendrite-fullerene DF-1, which was shown to be radioprotective to zebrafish via an antioxidant mechanism.[21] In a related plot from which Figure 1 is determined, Figure 2 illustrates the effectiveness of the US-SWCNT series **3**, **4**, and **5** versus trolox and DF-1; the relative persistence of fluorescence intensity observed with the SWCNTs over the other samples is indicative of their powerful antioxidant capability.

Contrary to what we expected, increased loading of BHT-derivatives was not directly proportional to the antioxidant capacity of the SWCNTs in all cases. Our original goal was to load as many BHT-derivatives as possible onto the SWCNT or US-SWCNT scaffolds in order to produce large local concentrations of the BHT-derived antioxidants, by using the SWCNT primarily as a scaffold. It is important to note that BHT and its derivatives used here are water-insoluble. However, through the various associations of BHT-derivatives with SWCNTs and US-SWCNTs, the hydrophobic molecules were solubilized in water, permitting the application of the antioxidant derivatives to the future in vitro studies. For the US-SWCNT series, the results were as

Scheme 4. Triazene OPE-BHT **10** Covalently Attached to the Pluronic-Wrapped SWCNT **11** To Give OPE-BHT Derivatized SWCNTs **12**

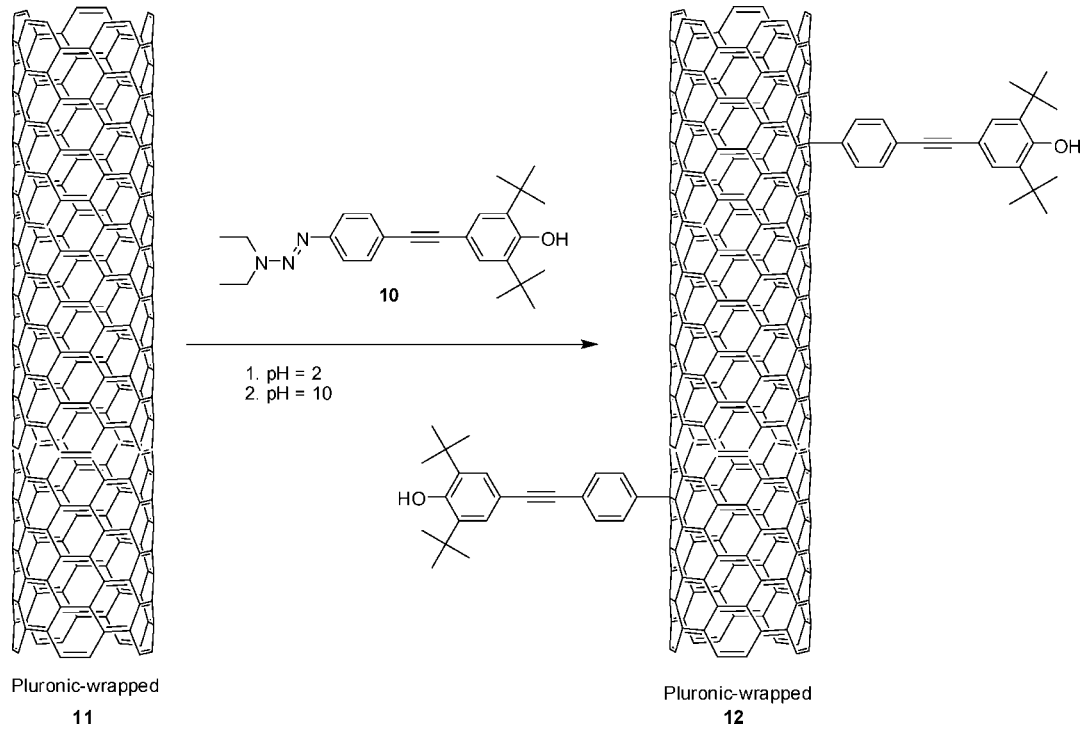


Table 1. TE for DF-1, PEG-US-SWCNTs **3**−**5**, Pluronic-Wrapped SWCNT **11**, and OPE-BHT Derivatized SWCNT **12**[a]

sample no.	trolox equiv (TE)
DF-1	2.32
3	221
4	1240
5	532
11	14046
12	9911

[a] TE values for US-SWCNT and SWCNT samples were adjusted to account for the contribution of the corresponding solubilizer controls, PEG, **6** and Pluronic, **13**, respectively, by subtracting the TE value found for each solubility control. Error range is 15%.

expected: the amount of BHT-derivative functionalization was proportional to the antioxidant activity as assessed by the TME values. Because ionic binding upon these scaffolds is more efficient than covalent binding,[51,53] it is not surprising that amine-BHT ionically bound to PEG-US-SWCNTs as in **4** is a more effective antioxidant than amine-BHT covalently bound to PEG-US-SWCNTs as in **5**. In contrast, it was found that the antioxidant activity of the Pluronic-wrapped SWCNTs was inversely proportional to the amount of BHT-derivative present. These findings are attributed to the mode of functionalization used for each series. In the case of the US-SWCNTs, the amine-BHT moieties were appended to carboxylic acids already present on the nanotube; the addition of amine-BHT did not alter the sidewall of the SWCNT itself. However, in the case of the Pluronic-wrapped SWCNTs, triazene-OPE-BHT was used to directly functionalize the sidewall of the Pluronic-wrapped SWCNTs. That is, with every addition of OPE-BHT, a new defect site was introduced on the sidewall. It is known that functionalization decreases the radical scavenging ability of fullerenes, because the amount of pristine conjugation available for radical addition is in turn decreased, and the bandgap of the structure is commensurately raised.[54,55] In spite of this, we hypothesized that the antioxidant potential afforded by each additional BHT-derivative would be the largest determinant in the overall radical scavenging activity of the SWCNT. However, according to the data, the BHT-derivatives were not potent-enough antioxidants to counter the antioxidant activity that was lost with each defect introduced to the sidewall of the SWCNTs through the covalent functionalization. This is evidenced by the TME values of the SWCNT samples **11** and **12** in Figure 1. Even though SWCNT **12** is functionalized with many OPE-BHT moieties, pristine SWCNT **11** has a higher total antioxidant capacity.

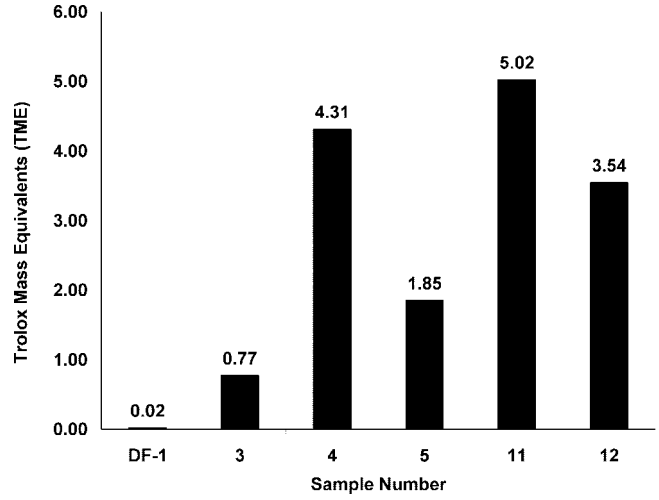


Figure 1. TME for DF-1, PEG-US-SWCNTs **3**−**5**, Pluronic-wrapped SWCNT **11**, and OPE-BHT derivatized **12**. TME values for US-SWCNT and SWCNT samples in this figure were adjusted to account for the contribution of the corresponding solubilizer controls, PEG, **6** and Pluronic, **13**, respectively, by subtracting the TME value found for each solubility control (Figure 2). In this way, the TME values listed on the plot are independent of the solubility enhancing addend. Error range is 15%.

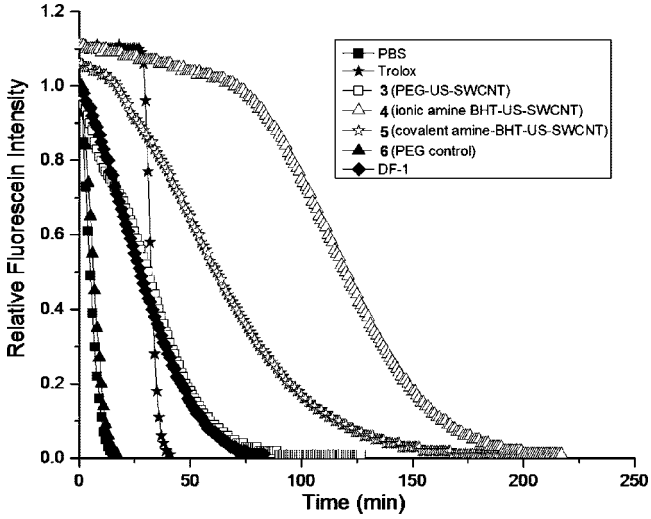


Figure 2. Relative fluorescein intensity versus time. Fluorescein-intensity persistence is proportional to the strength of the antioxidant.

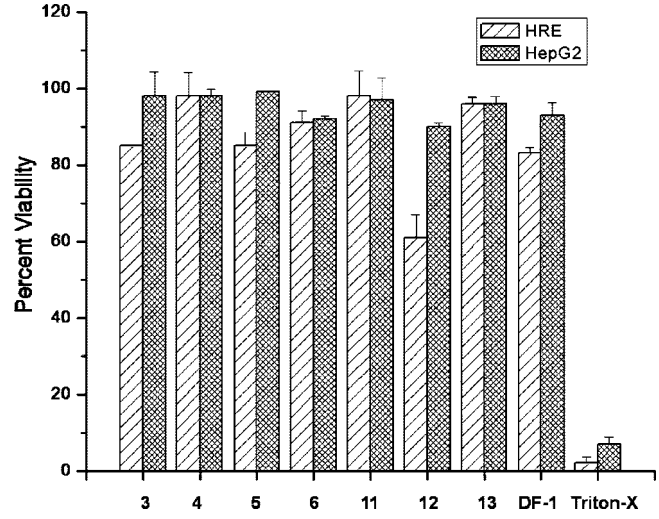


Figure 3. Cytotoxicity of SWCNT solutions **3**, **4**, **5**, **11**, and **12** as compared to that of DF-1. Triton-X, the toxicity control, is a standard surfactant known to be cytotoxic. PEG and Pluronic controls **6** and **13,** respectively, are also shown.

To glean the radical scavenging ability attributable to the US-SWCNT itself and not from the attached solubilizing PEG moieties, the antioxidant contribution of the PEG was calculated (see Supporting Information). Briefly, this was done by calculating the amount of PEG attached to the US-SWCNT, dissolving that amount of PEG in water (**6**), and testing it side-by-side in the ORAC assay. As can be seen in Figure 2, the area under the curve is enhanced for PEG-US-SWNTs **3** relative to that of the PEG control **6**. This means that all additional radical scavenging observed with PEG-US-SWCNTs **3** over PEG control **6** can be attributed to the US-SWCNT itself; therefore, even US-SWCNTs with heavily compromised sidewalls are still able to sequester oxygen radicals. The same treatment as the one outlined above was done to determine what contribution the Pluronic medium has toward the TME of Pluronic-wrapped SWCNTs by also testing a solution of 1 wt% Pluronic in water (**13**) in the ORAC assay. Although the TME was higher than that for PEG control **6**, the radical scavenging contribution was still small relative to that of the Pluronic-wrapped SWCNT. This observation indicates that the vast majority of the SWCNTs' antioxidant activity is independent of the solubilizer/surfactant.

Cytotoxicity of SWCNT Formulations. To assess an acute cytotoxic response of the various SWCNT solutions, HRE and HepG2 liver cells were exposed to the samples for 24 h. Renal and liver cells were chosen for the in vitro assays because the kidney and liver play an important role in the in vivo clearance of SWCNTs.[16,56−58] Figure 3 shows that no SWCNT solutions cause acute toxicity to HepG2 cells. No agglomeration of the SWCNTs was seen in any of the samples after being exposed to the cells for 24 h. OPE-BHT-SWCNT **12** shows some slight toxicity to HRE cells. This may be due to a minor organic[59] byproduct of the reaction of triazene-OPE-BHT **10** with Pluronic-wrapped SWCNTs **11** that yielded **12.** As noted earlier, the byproduct gave **12** a rust-colored tint and was not completely removable from the functionalized SWCNTs after dialysis in 1 wt% Pluronic solution.

Conclusion

Through use of the ORAC assay, SWCNTs were shown to be potent antioxidants. When functionalized with the radical scavenger BHT-derivatives through existing functionalities on the sidewall, the antioxidant activity of the system is increased. If, however, the BHT-derivative functionalization occurs through covalent addition to the sidewall, the antioxidant activity of the system is decreased. These observations confirm the radical scavenging activity of the pristine SWCNTs. The solubilized SWCNTs tested here induced little cytotoxic response; hence, application of SWCNTs toward antioxidants in materials and medical therapeutics warrants further studies.

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Supporting Information Available: Calculation of antioxidant contributions of PEG and the BHT-derivative loading on US-SWNTs along with IR, ^{1}H and ^{13}C NMR, and HRMS of **9** and IR, ^{1}H NMR, and HRMS of **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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