

Supporting information

for

A Novel SWCNT Platform bearing DOTA and β -Cyclodextrin Units. “One Shot” Multidecoration under Microwave Irradiation

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1. Materials and methods

All chemicals were purchased from Alfa-Aesar Italy and used without further purification. β -CD was kindly provided by Wacker Chemie (Germany).

DOTAMA-aldehyde (**4**) and DOTAMA-azido *tris*(*t*-butyl ester) derivatives were prepared following a previously reported procedure.^{S1,S2}

MW-assisted reactions were carried out in a SynthWAVE reactor (MLS GmbH, Milestone Srl).

Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or by heating after spraying with 5% H₂SO₄ in ethanol or phosphomolybdic acid. Product purification was performed by flash-chromatography (CombiFlash RfsTeledyne ISCO) on appropriate cartridges (silica or RP18).

NMR spectra were recorded on a Bruker Avance 300 (operating at a magnetic field strength of 7T) at 25°C; chemical shifts were calibrated to the residual proton and carbon resonance of deuterated solvents: CDCl₃ (¹H = 7.26 ppm, ¹³C = 77.16 ppm) or DMSO-*d*₆ (¹H = 2.54 ppm, ¹³C = 40.45 ppm).

Ultrapure water, prepared by Milli-Q Century System (Millipore, USA), was used throughout the entire study.

UV absorption measurements were performed in a UV-Vis dual-beam spectrophotometer (Agilent Technologies Cary 60, G6860AA) equipped with 1 cm path length quartz cuvette.

Non-complexed Php was separated from the functionalized SWCNT derivatives by filtration over a cellulose acetate membrane (0.2 μm) syringe filter (CPS Analytica, Italy).

Thermogravimetric analyses were performed in a thermogravimetric Analyzer TGA 4000 (Perkin Elmer) at 10 $^{\circ}\text{C}/\text{min}$ operating under air with alumina crucibles containing 10–20 mg of SWCNT derivatives. Total mass loss was attributed to functional groups which were covalently attached to the sidewalls. The number of functional groups was calculated considering a fixed thermogram temperature, the residual weight of functionalized SWCNTs (W_R), its mass loss (W_L) and the molecular weight of the organic fragment (M_F) on its sidewalls = $(\%W_R/12) / (\%W_L/M_F)$.

"CellTiter 96 Proliferation Assay" (Promega, Madison, WI, USA) kit for cell viability assays was used according to the manufacturer's instructions. Absorbances were measured at 490 nm using a Microplate Reader (Model 680, BIORAD).

2. Synthetic Procedures

2.1. *t*-Butyl 2-((*t*-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanoate (**1**).

Perchloric acid (1.8 mL, 30 mmol) was added to a suspension of *L*-tyrosine (3.63 g, 20.0 mmol) in *t*-butyl acetate (50 mL) at 0 $^{\circ}\text{C}$. The reaction mixture was stirred at room temperature for 24 h. After the addition of a saturated NaHCO_3 aqueous solution (50 mL), the mixture was extracted with ethyl acetate (3x50 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under vacuum to afford *L*-tyrosine *t*-butyl ester as a crude oily product which was used in the next step without further purification.

Di-*t*-butyl dicarbonate (5.2 g, 23.83 mmol) was added to a solution of *L*-tyrosine *t*-butyl ester crude product (4.75 g, 20 mmol) and triethylamine (6.34 mL, 4.63 g, 45.74 mmol) in CH_2Cl_2 (30 mL) at 0 $^{\circ}\text{C}$. The reaction mixture was stirred for 12 h at room temperature and then evaporated under reduced pressure. The residue was portioned between CH_2Cl_2 (3x100 mL) and water (100 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash-chromatography (CombiFlash, petroleum ether 40–60/EtOAc 8:2 v/v) to afford **1** (yield 90%) as a colourless oil. ^1H NMR (300 MHz, CDCl_3): δ 7.23 (d, $J = 8.5$,

2H aromatic), 6.99 (d, $J = 8.5$, 2H, aromatic), 5.04 (m, 1H, mobile proton), 4.49-4.31 (m, 1H, -CH₂CHNH-), 2.96 (d, $J = 5.32$ Hz, 2H, -CH₂CH), 1.42 (m, 18H, (CH₃)₃C-) ppm; ¹³C NMR (75 MHz, CDCl₃): δ 172.43, 156.4, 155.1, 130.6, 129.4, 114.8, 82.2, 80.0, 54.7, 37.7, 28.3, 28.0 ppm. MS(ESI): m/z calcd for C₁₈H₂₇NO₅ [M + H]⁺: 337.18, found: 337.16.

2.2. *t*-Butyl-2-((*t*-butoxycarbonyl)amino)-3-(4-(prop-2-yn-1-yloxy)phenyl) propanoate (2).

Propargyl bromide (1.45 g, 1.14 mL, 12.21 mmol) was added dropwise to a THF solution (15 mL) of *t*-butyl 2-((*tert*-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanoate **1** (520 mg, 1.54 mmol) and Cs₂CO₃ (337.5 mg, 2.44 mmol) at 0 °C. The resulting mixture was heated in a multimode MW reactor (SynthWAVE) at 60 °C (50 W) under N₂ atmosphere (2 bar) for 2 h, then concentrated under reduced pressure. The residue was portioned between CH₂Cl₂ (3x100 mL) and water (100 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by flash-chromatography (CombiFlash, petroleum ether 40-60/EtOAc 8:2 v/v) to afford the title compound as a colourless oil (yield 60%). ¹H NMR (300 MHz, CDCl₃): δ 7.19 (d, $J = 8.5$, 2H, aromatic), 6.90 (d, $J = 8.5$, 2H, aromatic), 4.99 (d, $J = 8.0$ Hz, 1H, mobile H), 4.68 (d, $J = 2.38$, 2H, -CH₂CCH), 4.47-4.35 (m, 1H, -CH₂CHNH-), 3.06 (d, $J = 5.91$, 2H, -CH₂CHNH), 2.51 (t, $J = 2.38$, 1H, -CH₂CCH), 1.43 (s, 9H, (CH₃)₃C-), 1.41 (s, 9H, (CH₃)₃C-) ppm. ¹³C NMR (75 MHz, CDCl₃): δ 172.4, 156.4, 155.1, 130.6, 129.4, 114.8, 82.0, 80.0, 78.6, 77.5, 55.8, 54.7, 37.6, 28.3, 28.0 ppm. MS(ESI): m/z calcd for C₂₁H₂₉NO₅ [M + H]⁺: 375.20, found: 375.18.

2.3. 2-Amino-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoic acid (2a).

A solution of *t*-butyl-2-((*t*-butoxycarbonyl)amino)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoate **2** (340 mg, 0.91 mmol) in trifluoroacetic acid (50-75% in CH₂Cl₂; 50 mL) was stirred at room temperature for 2 h. The reaction mixture was then evaporated under reduced pressure to afford **2a** as a colourless oil. ¹H NMR (300 MHz, DMSO-*d*₆): δ 7.19 (d, $J = 8.5$, 2H, aromatic), 6.90 (d, $J = 8.5$, 2H, protons), 4.68 (d, $J = 2.38$, 2H, -CH₂CCH), 4.47-4.35 (m, 1H, -CH₂CHNH₂-), 3.6 (s, 1H, -CH₂CCH), 3.06 (dd, $J = 12.5$, 1H, -CH₂^ACHNH₂), 2.76 (dd, $J = 12.3$ Hz, 1H, -CH₂^BCHNH), 2.12 (s, 2H, -NH₂) ppm. ¹³C-NMR (75 MHz, DMSO-*d*₆): δ 172.43, 155.1, 130.6, 129.4, 114.8, 78.6, 75.5, 55.8, 54.7, 37.6 ppm. MS(ESI): m/z calcd for C₁₂H₁₃NO₃ [M + H]⁺: 219.08, found: 219.06.

2.4. CuAAC between 6-monoazido-6-deoxy- β -CD and 2-amino-3-(4-(prop-2-yn-1-yloxy)phenyl) propanoic acid to afford conjugate (3).

2-amino-3-(4-(prop-2-yn-1-yloxy)phenyl)propanoic acid (400 mg, 1.8 mmol), 6-monoazido-6-deoxy- β -CD (382.8 mg, 0.33 mmol), CuSO₄ (18 mg, 0.08 mmol) and *L*-ascorbic acid (25.88 mg, 0.15 mmol), were suspended in DMF (10 mL), sonicated for 5 min in a US bath and heated in a MW reactor (SynthWAVE) at 70 °C (70 W, mean power) under N₂ atmosphere (2 bar) for 2 h. The reaction outcome was monitored by TLC using the following eluent: *i*PrOH/H₂O/EtOAc/NH₄OH, 5:3:1:1. After concentration under reduced pressure to half of the original volume, the addition of acetone (10 mL) afforded a suspension that, after filtration on a Hirsch funnel, afford a solid product. The crude product was purified by flash chromatography (CombiFlash, RP18 cartridge, eluent gradient profile: H₂O, H₂O/CH₃OH (98 : 2; 95 : 5; 9 : 1; 8 : 2); CH₃OH), affording 364.12 mg of pure **3** (yield 80%). ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.21 (s, 1H, H-5, *triazole*), 7.24 (dd, *J* = 8.43 Hz, 2H, *aromatic*), 7.09 (dd, *J* = 8.45 Hz, 2H, *aromatic*), 5.92-5.64 (m, 14H, O(2)H, O(3)H), 5.10 (d, *J* = 13.2 Hz, 1H, H-1'), 4.92-4.77 (m, 7H, H-1, H-6 ^A), 4.65 (s, 2H, -OCH₂C-*tyr*), 4.61-4.45 (m, 7H, O(6)H, H-6 ^B), 4.34 (m, 1H, O(6)H["]), 4.04 (m, 2H, H-5', -CH₂CHNH₂-*tyr*), 3.84-3.18 (m, overlaid with H₂O, H-6, H-5, H-4, H-3, H-2), 3.06 (m, 2H, -CH₂^ACHNH₂-*tyr*, H-6 ^A), 2.92 (m, 1H, H-6["]B), 2.76 (dd, *J* = 12.5 Hz, 1H, -CH₂^BCHNH₂ *tyr*), 2.12 (s, 2H, -NH₂) ppm. ¹³C NMR (75 MHz, DMSO-*d*₆): δ 172.4, 155.1, 149.1 (C-4, *triazole*), 130.6, 129.4, 122.35 (C-5, *triazole*), 114.8, 103.21 (C-1), 102.35 (C1'), 83.4, 82.3, 81.2, 80.6 (C-4), 72.88, 72.29, 71.97 (C-2, C-3, C-5), 71.51 (C-5'), 61.12, 60.05 (C-6), 55.8, 54.7, 50.07 (C-6'), 37.6 ppm. MS(ESI): *m/z* calcd for C₅₄H₈₂N₄O₃₇ [M+H]⁺: 1378.46, found: 1378.44.

2.5. Synthesis of functionalized SWNTs (5) and (6).

Pristine SWNTs (50 mg) were suspended in DMF (15 mL), in a quartz vessel (for the MW reactor), mixed with 1 mmol of the corresponding tyrosine derivative **3** (380 mg, 0.28 mmol), or native tyrosine itself (100 mg, 0.55 mmol) and the DOTAMA- aldehyde-*tris*(*t*-butyl ester) derivative **4** (468 mg, 0.7 mmol) or octanal (180 mg, 1.4). The reaction mixture was sonicated for 5 min and then heated in a multimode MW reactor (SynthWAVE) at 100 °C for 2 h (**5**) or at 130 °C for 1.5 h (**6**) under N₂ atmosphere (2 bar) (85-130 W mean power respectively). After cooling to room temperature, the crude product was suspended in CH₂Cl₂ (20 mL) and the mixture sonicated for 5 min, and separated by centrifugation. The collected black solid was washed thoroughly in THF (25 mL) and acetone (25 mL), and then finally dried under high vacuum affording **5** (48.7 mg) or **6** (26.9 mg) functionalized SWNTs.

2.6. Synthesis of functionalized SWNTs (7).

Propargyl bromide (480 mg, 0.38 mL, 4.04 mmol) was added dropwise to a THF suspension (15 mL) of functionalized SWCNTs **6** (25 mg) and Cs₂CO₃ (337.5 mg, 1 mmol) at 0°C. The reaction was heated in a multimode MW reactor (SynthWAVE) at 60 °C (50 W) under N₂ atmosphere (2 bar) for 2 h. The crude was centrifuged and washed by cycles of sonication and centrifugation with CH₂Cl₂ (15 mL) and methanol (15 mL), and finally dried under high vacuum affording 18 mg of **7**.

2.7. Synthesis of functionalized SWNTs (8).

The reaction between **7** (15 mg) and 6-monoazido-6-deoxy-β-CD (127.3 mg, 0.11 mmol) was carried out in a multimode MW reactor (SynthWAVE). The reaction mixture, which also contained CuSO₄ (6 mg, 0.03 mmol) and *L*-ascorbic acid (8.6 mg, 0.05 mmol), was suspended in DMF (5 mL), sonicated for 5 min in a US bath and heated at 60 °C (70 W) under N₂ atmosphere (2 bar) for 1 h. The crude product was centrifuged and washed by cycles of sonication and centrifugation with CH₂Cl₂ (15 mL) and acetone (15 mL). The collected solid was washed with a Na₄EDTA solution (50 mM, 2 mL, 3 times), then with water (2 mL, 2 times), and finally dried under high vacuum affording 13 mg of **8**.

2.8. Synthesis of functionalized SWNTs (9).

The reaction between **8** (12 mg) and the DOTAMA-azido tris(*t*-butyl ester) derivative (76.66 mg, 0.11 mmol) was carried out in a multimode MW reactor (SynthWAVE). The reaction mixture, which also contained CuSO₄ (6 mg, 0.03 mmol) and *L*-ascorbic acid (8.6 mg, 0.05 mmol), was suspended in DMF (7.5 mL), sonicated for 5 min in a US bath and heated in MW at 60 °C (70 W) under N₂ atmosphere (2 bar) for 1 h. The crude was centrifuged and washed by cycles of sonication and centrifugation with CH₂Cl₂ (15 mL) and acetone (15 mL). The collected solid was washed with a Na₄EDTA solution (50 mM, 2 mL, 3 times), then with water (2 mL, 2 times), and finally dried under high vacuum affording 10 mg of **9**.

3. Analytical β-CD and DOTA quantification.

3.1. Measurement of phenolphthalein complexation

A buffer solution was prepared with sodium carbonate (13.2 g) and sodium bicarbonate (2.1 g) dissolved in ultrapure water (250 mL, pH = 10.5). Phenolphthalein (Php) powder was dissolved in ethanol to obtain a 5 mM Php stock solution and β -CD powder was dissolved in ultrapure water to obtain a 0.88 mM β -CD stock solution. The Php stock solution was diluted with the buffer solution (pH = 10.5) to achieve a constant Php concentration of 0.008 mM and mixed together with the β -CD stock solution to achieve β -CD concentrations of 0, 7.9, 9.6, 11.3, 13, 14.7, 16.4, 18.1 mmol L⁻¹. The absorbance of the calibration solutions of CDs were measured at the wavelength of 553 nm at room temperature.

SWCNT-CD-DOTA *tris*(*t*-butyl ester) adducts **5** and **9** were dispersed in the buffered Php solution (0.008 mM, 5 mL). The mixture was stirred for 15 min at room temperature and filtered. Absorbance was recorded (553 nm).

3.2 Complexation procedure of (**5**) and (**9**) with GdCl₃.

8 mg of modified SWCNTs (adduct **5** or **9**) were suspended in CH₂Cl₂ and TFA (200 μ L) was added. The mixture was shaken over a swinging plate, at room temperature, for 4 hours. The solid was collected by centrifugation and washed with CH₂Cl₂ (2 mL, 3 times). After drying, the SWCNT adducts were mixed with 2 mL of a 25 mM GdCl₃ solution and the pH was adjusted to 6 (if needed). The mixture was sonicated for 5 min in an US cleaning bath and then shaken over a swinging plate for 12 hours. The mixture was then centrifuged and the solid washed with a Na₄EDTA solution (50 mM, 2 mL x 3) and then with water (2 mL x 2). The solid was finally dried in an oven (70 °C).

4. Cell viability assays

Samples of SWCNT adducts **5** and **9** were suspended by sonication in double distilled sterile water and used for cellular assays *in vitro*. Their effects on cell viability were evaluated on five human cell lines: 1) A549 human lung carcinoma cells; 2) Huh7: human hepatocellular carcinoma cells; 3) Hep2: epithelial cells of human laryngeal carcinoma; 4) Helf: human embryonic fibroblasts; 5) Hela: epithelial cells of human adenocarcinoma. Both Hep2 and A549 cell lines were cultured with Minimum Essential Medium (PAA, 4061 Pasching, Austria) with 10% Foetal Calf Serum (Gibco/BRL, Gaithersburg, MD) and 1% Zell Shield (Minerva Biolabs GmbH, Berlin, Germany). The Helf cell line was maintained in Minimum Essential Medium with 10% Foetal Calf Serum and supplemented with 1% Sodium Pyruvate (Gibco/BRL, Gaithersburg, MD) and 1% Zell Shield. The

Huh7 and Hela cell lines were cultured with Dulbecco's Modified Eagle's medium High Glucose (PAA, 4061 Pasching, Austria) supplemented with 10% Fetal Calf Serum and 1% Zell Shield. Cells were incubated in the presence of 5% CO₂ at 37 °C.

For cell viability assays, the cells were treated with different concentrations, ranging from 0.45 to 1000 µg/mL, of adducts **5** and **9** and the rate of cell viability was monitored after 24 hours, using the "CellTiter 96 Proliferation Assay" (Promega, Madison, WI, USA) kit according to manufacturer's instructions. Absorbances were measured at 490 nm using a Microplate Reader (Model 680, BIORAD). The effect of the formulation on cell viability at different concentrations was expressed as a percentage, by comparing treated cells with cells incubated with the culture medium alone.

References

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