Aqueous Compatible Fullerene-Doxorubicin Conjugates

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Abstract

Covalent conjugates of fullerene C_{60} and the highly effective anticancer drug doxorubicin (DOX) were prepared and studied. The conjugation was through the amide linkage to preserve the intrinsic properties of DOX and fullerene cage. As designed, the conjugates with hydrophilic ethylene glycol spacers exhibited much improved aqueous compatibility, with significant solubility in water-DMSO mixtures. The anti-neoplastic activities of DOX were apparently unaffected in the conjugates according to evaluations *in vitro* with a human breast cancer cell line.

Introduction

Fullerenes are extensively investigated and widely acknowledged for their unique properties as excellent electron acceptors, potent radical scavagers, and others.[1–4] There has been much interest in exploiting these properties for potential biomedical applications of fullerenes.[1,3,5] For example, fullerenes have been studied for their therapeutic effects on neurodegenerative diseases,[6] as agents for DNA cleavage,[7] and as nitric oxide synthase inhibitors.[8] Among intersting recent investigations on potentially using fullerenes in medicine was the work by Wilson and coworkers, in which C_{60} was covalently conjugated with the widely used anticancer drug paclitaxel for *in vitro* pharmacological evaluations.[9] Similar conjugates of fullerenes with other popular drugs, including the highly effective anticanecer drug doxorubicin (DOX, Scheme 1), have been explored or proposed for various medicinal purposes.[10,11] In particular, there were many reports on using polyhydroxylated C_{60}, often called fullerenols,[12] to mitigate DOX-induced toxic side-effects.[13] Beyond fullerenes, carbon nanotubes have been conjugated with DOX for the delivery of the drug to take advantage of the enhanced cellular uptake, selectivity to cancer cells, and pH regulated release.[14,15]

A major issue in the effort on using fullerene-DOX conjugates for improved drug formulation is on their aqueous solubility or compatibility. DOX in its natural form is marginally soluble in water,[16] and the widely used commercial formulation is a salt with hydrochloric acid (under the trade name Adriamycin, Scheme 1).[17] The fullerene cage is even less hydrophilic, hardly helpful to DOX in terms of compatibility with physiological media. Therefore, a special consideration is required in the conjugation of C_{60} with DOX to impart sufficient hydrophilicity into the conjugates for their desired bioavailability. Here we report the covalent conjugation of DOX with methano-C_{60} derivatives (Scheme 2), for which the molecular structures were determined unambiguously by using NMR and other techniques. As designed, the conjugates with hydrophilic linkages exhibited significantly improved aqueous compatibility, amenable to their targeted bioapplications. The anti-neoplastic activities of DOX were apparently

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preserved in the conjugates according to evaluations *in vitro* with human breast cancer cells. The results set the stage for further development of fullerene conjugates in the delievery of DOX for benefits similar to those found in the conjugation of fullerene with paclitaxel[12] and those demonstrated with the use of fullerenols.[13]

Results and Discussion

The conjugate **I** was synthesized to test the amidation of the carboxylic acid moiety in the methano-C_{60} derivative **1** by DOX (Scheme 3). The amide linkage was selected with the consideration of literature reports that the amidation of the primary amine in DOX had relatively minor effect on the anti-neoplastic activities of the drug.18 The synthesis was relatively straightforward, but the conjugate was found to be insoluble in water or aqueous solvent mixtures. This was hardly surprising since neat DOX (without the HCl salt in the commercial formulation) was known to have little solubility in water,[16] even less for the fullerene. In order to increase the desired aqueous compatibility without inducing any major changes to the chemical structures (thus intrinsic properties) of the fullerene cage and/or DOX, hydrophilic moieties in the form of oligomeric ethylene glycol spacers were added to the conjugates (Scheme 2).

For the conjugate **II**, the methano-C_{60} adduct (with the unsymmetrical malonic ester **3**) was obtained in the classical Bingel-Hirsch reaction (Scheme 4),[19,20] with the observed yield (40%) comparable to those in other similar additions. Upon the selective hydrolysis of the terminal *t*-butyl ester, the resulting carboxylic acid was again activated by using the agent DCC (*N,N'*-dicyclohexylcarbodiimide) for amidation with DOX′-NH_2 (Scheme 4).

The molecular structure of **II** was confirmed by results from ^{1}H- and ^{13}C-NMR measurements and mass spectroscopy analyses. The ^{1}H-NMR spectrum of the conjugate (Figure 1) was close to a superposition from those of the underlying fullerene and DOX. In the ^{13}C-NMR spectrum of **II**, the 26 sp^2 signals with a characteristic intensity distribution pattern for the fullerene cage suggested that the conjugate maintained the C_s symmetry (the same as that of **5** in Scheme 4).[21] The signal for the two methano-fullerene bridgehead carbons (sp3) was at 71.5 ppm, and the other carbon of the cyclopropyl ring at 52.0 ppm. The matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) MS pattern of **II** was compared with the prediction on the basis of isotopic populations (IsoPro 3.0). Shown in Figure 2 is an excellent match, with the expected parent peak mass of 1632.3 dalton (**II**+Na). This was further confirmed in the electrospray ionization (ESI) MS characterization, with both **II**+Na (1,632.32 vs 1,632.29 calculated) and **II** (1,610.21 vs 1,610.30 calculated) peaks observed.

The observed UV/vis absorption spectrum of the conjugate **II** was also close to the superposition from those of the methano-C_{60} and DOX′-NH_3Cl (Figure 3). Thus, the molar absorptivity at the spectral maximum of **II** could be approximated as the sum of those for the methano-C_{60} and DOX′-NH_3Cl at the same wavelength, which allowed the use of observed absorbance to estimate the concentration of **II** in a solution (or the solubility of **II** in a saturated solution). The presence of the hydrophilic spacer in **II** made the conjugate more dispersible in water, but still practically insoluble. However, the conjugate was found to be soluble in some aqueous mixtures with a polar organic solvent, such as those with dimethyl sulfoxide (DMSO). The saturated solutions of **II** in water-DMSO mixtures of different compositions were prepared by pushing an excess amount of **II** into a given mixture, followed by vigorous high-speed centrifuging to keep the supernatant for absorption spectral measurements. The solubility results thus obtained are shown in Figure 4. In the 1:1 (v/v) water-DMSO mixture, for example, the solubility of **II** is ~1.5 mg/mL. The conjugate is obviously more soluble in mixtures with more DMSO (Figure 4).

The conjugate **III** with two hydrophilic tethers and two DOX units sandwiching a C_{60} cage was designed for further improved aqueous compatibility, as well as the doubling in DOX loading per conjugate. As illustrated in Scheme 4, the malonic ester **4** was similarly added to C_{60} in the classical Bingel-Hirsch reaction, followed by the selective hydrolysis and then amidation reaction with DOX. The relatively more difficult coupling of the methano-C_{60} with two DOX molecules was reflected in the lower product yield, 42% in comparison with 65% found in the coupling for **II**. Again, the conjugate **III** was confirmed unambiguously in the characterization with ^{1}H- and ^{13}C-NMR techniques (Figure 5) and MS measurements (MALDI-TOF: 2,364.5; and ESI-MS/MS: 2,364.538, vs the expected 2,364.536 for **III**+Na).

Despite being only marginally soluble in water (less than 0.1 mg DOX-equivalent per mL), the conjugate **III** exhibited significantly improved aqueous compatibility. Again in the 1:1 (v/v) water-DMSO mixture, for example, the DOX-equivalent solubility was doubled (Figure 4). The improved aqueous compatibility of **III** over **II** was significant with respect to their bioevaluations. Specifically, both **II** and **III** were tested in experiments for their cytotoxicity evaluations, but those for **II** were very difficult and inconclusive (due a large part to significant aggregation of the conjugate in cell culture media). On the other hand, the same experiments for **III** were successful, enabling a comparison of anti-neoplastic activities between the conjugate and free DOX (the commercial formulation DOX′-NH_3Cl).

The viability of human breast cancer MCF-7 cells in the presence of **III** or free DOX was evaluated in the MTT assay (mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by succinic dehydrogenase[22]). A solution of **III** or free DOX in DMSO was diluted with the cell culture medium to the targeted concentration just prior to the cell exposure. The MCF-7 cells cultured in the free medium were taken as the control. The results of the MTT assay were obtained in terms of the relative cell viability (% of the control). As shown in Figure 6, the viability of MCF-7 cells exposed to the conjugate **III** decreased in a dose-dependent fashion, with about half of the cell viability inhibited at the DOX-equivalent concentration of 25 μg/mL. Between the conjugate **III** and free DOX (the commercial formulation DOX′-NH_3Cl), the observed anti-neoplastic activities toward the breast cancer cell line were rather similar (Figure 6). This is interesting because a commonly observed phenomenon in the delivery of DOX by nanoscale carriers such as polymeric nanoparticles,[23] carbon nanotubes,[14] or nanodiamond,[24] has been a decrease in the anti-neoplastic activities of the drug. Mechanistically, the anti-neoplastic function of DOX molecules has been attributed to their intercalation into DNA to inhibit the macromolecular biosynthesis, the inhibition of enzyme helicase to interfere with DNA unwinding, and/or the inhibition of topoisomerase II to induce DNA damage.[25] The conjugate **III** is structurally flexible due to the ethylene glycol spacers, making the attached DOX units readily available for their biological functions. On the other hand, the extremely hydrophobic nature of the fullerene cage probably makes the wrapping of the cage by the hydrophilic spacers a favorable configuration, which thus keeps the conjugate compact in size, not to sterically hinder the activities of the DOX units. These structural characteristics might have contributed to the observed comparable *in vitro* performance of the conjugate to that of free DOX in the aqueous soluble formulation.

In summary, DOX could be covalently conjugated with methano-C_{60} through the amide linkage to preserve the intrinsic properties of DOX and fullerene cage. The improvement in aqueous compatibility could be accomplished by introducing hydrophilic ethylene glycol spacers into the conjugate structure, which imparted significant solubility of the conjugate in water-DMSO mixtures. The conjugate with two hydrophilic spacers was sufficiently aqueous compatible to enable bio-evaluations *in vitro*, from which the results suggested comparable anti-neoplastic activities of the conjugate with those of free DOX against the human breast cancer cells. The fullerene-spacer-DOX design apparently serves as a versatile platform for

conjugates of desired properties, including availability of the drug, structural flexibility with hydrophilic and hydrophilic moieties for different cellular domains, etc. The platform may be further developed for conjugates of improved performances in both delivery and activities (including those for the mitigation of induced toxicities) of the drug. In particular, these conjugates are structurally uniquely defined, potentially offering pharmacological benefits that are not available in more complex and/or mixture-based systems such as those with fullerenols. [13]

Experimental Section

Materials

Fullerene C_{60} sample (>98%) from Bucky USA was purified on a silica gel column with toluene as eluent. *N*-hydroxysuccinimide (NHS), trifluoroacetic acid (TFA), triethylamine (TEA), and *N,N′*-dicyclohexylcarbodiimide (DCC) were purchased from Acros, carbon tetrabromide from Alfa Aesar, ethyl-malonyl chloride from Aldrich, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) from Avocado Research Chemicals, and malonic dichloride from TCI America. Toluene, THF, and DMF were dried over molecular sieves, and then toluene and THF were freshly distilled over sodium and DMF distilled under reduced pressure before use. Other solvents were either spectrophotometry/HPLC grade or purified via simple distillation. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories.

Measurement

NMR spectra were measured on Bruker Avance 300 MHz and 500 MHz NMR spectrometers. MALDI-TOF MS analyses were performed on a Bruker AutoFlex system, and 2,5-dihydroxybenzoic acid was used as the matrix. Electrospray ionization MS results were obtained on a QSTAR XL hybrid LC/MS/MS system. UV/Vis absorption spectra were recorded on Shimadzu UV-2501 and UV-3100 spectrophotometers.

I

A solution of the methano-C_{60} **1** (100 mg, 0.128 mmol)[26] in CH_2Cl_2/dioxane (1:1 v/v, 100 mL) was prepared, and *N*-hydroxysuccinimide (30 mg, 0.26 mmol) was added. To the mixture was added dropwise (within ~10 min) a solution of DCC (60 mg, 0.29 mmol) in CH_2Cl_2/dioxane (1:1 v/v, 5 mL). After stirring for 12 h at room temperature, the reaction mixture was concentrated in vacuum and then separated by flash chromatography on silica gel with toluene as eluent to yield **2** as a brown solid (60 mg, 53% yield). Separately, a solution of DOX′-NH_3Cl (14.5 mg, 0.025 mmol) in dry DMF (2 mL) was prepared, and to the solution was added TEA (4.14 μL, 0.03 mmol). Upon the mixture being stirred at room temperature for 2 min under argon, a solution of **2** (22 mg, 0.025 mmol) in dry DMF (2 mL) was added dropwise (within ~1 min). The resulting mixture was stirred in the dark at room temperature for 48 h. The subsequent separation of the reaction mixture by flash chromatography on silica gel with a chloroformmethanol mixture (95:5, v/v) as eluent yielded **I** as a reddish brown solid (16 mg, 49% yield). ^{1}H NMR (CDCl_3, 500 MHz, CDCl_3): δ14.10 (s, 1H), 13.28 (s, 1H), 8.07 (d, *J* = 8 Hz, 1H), 7.81 (t, *J* = 8 Hz, 1H), 7.41 (d, *J* = 8 Hz, 1H), 7.29 (d, 1H), 5.63 (d, *J* = 3.5 Hz, 1H), 5.36 (s, 1H), 4.80 (t, *J* = 4.5 Hz, 2H), 4.64 (s, 1H), 4.50 (bs, 2H), 4.33 (q, *J* = 6.5 Hz, 1H), 4.10 (s, 3H), 3.91 (bs, 1H), 3.34 (d, *J* = 17.5 Hz, 1H), 3.09 (d, *J* = 19 Hz, 1H), 3.02 (t, 1H), 2.38 (d, 1H), 2.25 (m, 3H), 2.01 (m, 1H), 1.40 (d, J = 6.5 Hz, 3H) ppm; ^{13}C NMR (75.46 MHz, CDCl_3): δ 214.0, 188.0, 161.12, 156.23, 155.75, 148.44, 146.12, 145.62, 145.25, 145.15, 145.05, 144.68, 144.59, 144.53, 144.28, 143.94, 143.70, 143.32, 143.12, 142.96, 142.80, 142.42, 142.25, 142.09, 141.13, 140.96, 140.88, 140.20, 136.18, 135.62, 133.57, 133.41, 119.94, 118.50, 111.79, 100.86, 70.22, 69.59, 67.08, 65.55, 56.71, 46.19, 41.33, 35.81, 34.05, 29.68, 16.87 ppm. MALDI-TOF MS (M+Na)[+]: 1327.1 (1327.16 calculated).

II

A solution of C_{60} (600 mg, 0.83 mmol) in toluene (600 mL) was prepared, and to the solution was added the malonic ester **3** (300 mg, 0.71 mmol), CBr_4 (240 mg, 0.72 mmol), and DBU (0.12 mL, 0.80 mmol). The resulting mixture was stirred at room temperature for 20 h, followed by the removal of solvent on a rotary evaporator. The crude reaction mixture was separated on a silica gel column with first toluene as eluent to remove unreacted C_{60} and then ethyl acetate to obtain the methano-C_{60} adduct. The sample was dissolved in CH_2Cl_2 (50 mL) to mix with TFA (50 mL). The mixture was stirred for 30 min, followed by solvent removal to yield the selectively hydrolyzed adduct **5** (270 mg, 36% yield).

A solution of **5** (100 mg, 0.092 mmol) in CH_2Cl_2 (40 mL) was prepared, and to the solution was added *N*-hydroxysuccinimide (11 mg, 0.097 mmol) and then dropwise a solution of DCC in CH_2Cl_2 (5 mL, 4 mg/mL). The mixture was stirred at room temperature for 12 h, filtered, concentrated, and then re-dissolved in dry DMF (2 mL). Separately, a solution of DOX′-NH_3Cl (50 mg, 0.086 mmol) in dried DMF (20 mL) was prepared, and to the solution was added TEA (12.5 μL, 0.088 mmol). After brief stirring (about 2 min) at room temperature under argon, the DMF solution of the activated **5** above was added dropwise. After stirring in the dark at room temperature for 48 h, the reaction mixture was concentrated and separated on a silica gel column with chloroform-methanol (95:5, v/v) as eluent to obtain **II** as a reddish brown solid (96 mg, 65% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 14.0 (s, 1H), 13.28 (s, 1H), 8.05 (d, *J* = 8 Hz, 1H), 7.80 (t, *J* = 7.5 Hz, 1H), 7.41 (d, *J* = 9Hz, 1H), 7.24 (d, *J* = 9 Hz, 1H), 5.54 (d, *J* = 4 Hz, 1H), 5.32 (s, 1H), 4.79 (d, *J* = 2 Hz, 2H), 4.70 – 4.68 (m, 2H), 4.59 (q, *J* = 14.5 Hz, 2H), 4.22 – 4.12 (m, 2H), 4.10 (s, 3H), 3.95 (d, *J* = 4.5 Hz, 2H), 3.91 (t, *J* = 4.5 Hz, 2 H), 3.75 – 3.67 (m, 14 Hz), 3.29 (d, *J* = 19 Hz, 1H), 3.04 (d, *J* = 18.5 Hz, 2H), 2.38 (d, *J* = 15 Hz, 2H), 2.18 (dd, *J* = 4 Hz, 14.5 Hz, 1H), 1.97 (dt, *J* = 4 Hz, 13.5 Hz, 1H), 1.85 (dd, *J* = 4 Hz, 13.25 Hz, 1H), 1.51 (t, *J* = 7 Hz, 3H), 1.31 (d, *J* = 6.5 Hz, 3H) ppm; ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 213.9, 187.1, 186.7, 169.2, 163.6, 163.4, 161.07, 156.3, 155.7, 145.3, 145.27, 145.24, 145.18, 145.13, 144.88, 144.69, 144.66, 144.61, 144.57, 144.55, 143.88, 143.84, 143.09, 143.06, 143.02, 142.99, 142.93, 142.20, 142.16, 141.89, 141.82, 140.95, 140.89, 139.15, 138.92, 135.77, 135.57, 133.72, 119.89, 118.46, 111.64, 111.48, 100.9, 71.52, 70.92, 70.75, 70.63, 70.55, 70.50, 70.33, 70.20, 69.69, 69.17, 68.82, 67.46, 66.13, 65.60, 63.5, 56.7, 52.0, 44.9, 35.7, 34.0, 29.6, 17.0, 14.2 ppm.

III

C_{60} (230 mg, 0.32 mmol) was dissolved in toluene (500 mL), and to the solution was added the malonic ester **4** (200 mg, 0.29 mmol), CBr_4 (97 mg, 0.29 mmol), and DBU (60 μL, 0.40 mmol). The mixture was stirred at room temperature for 30 min, followed by the solvent removal. The crude reaction mixture was separated on a silica gel column with first toluene as eluent and then ethyl acetate to obtain the methano-C_{60} adduct (190 mg, 46% yield). A portion of the sample (100 mg, 0.071 mmol) was dissolved in CH_2Cl_2 (50 mL) to mix with TFA (50 mL). The mixture was stirred for 30 min to yield the hydrolyzed adduct **6** quantitatively.

Similarly, **6** (100 mg, 0.77 mmol) was activated by *N*-hydroxysuccinimide (18 mg, 0.156 mmol) with DCC (33mg, 0.16 mmol), followed by the coupling with DOX′-NH_3Cl (78 mg, 0.134 mmol) in dry DMF to obtain **III** as a reddish brown solid (75 mg, 42% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 13.9 (s, 2H), 13.28 (s, 2H), 8.01 (d, *J* = 7.5 Hz, 2H), 7.80 (t, *J* = 7.5 Hz, 2H), 7.41 (d, *J* = 9 Hz, 2H), 7.24 (d, *J* = 9 Hz, 2H), 5.52 (d, *J* = 4 Hz, 2H), 5.27 (s, 2H), 4.77 (s, 4H), 4.71–4.64 (m, 4H), 4.62 (bs, 2H), 4.20–4.13 (m, 4H), 4.09 (s, 6H), 3.97–3.91 (m, 8H), 3.76–3.66 (m, 28H), 3.41 (bs, 2H), 3.19 (d, *J* = 19 Hz, 2H), 2.88 (d, *J* = 18.5 Hz, 2H), 2.36 (d, *J* = 15 Hz, 2H), 2.19 (dd, *J* = 4 Hz, 13.5 Hz, 2H), 1.97–1.92 (m, 2H), 1.79 (dd, *J* = 4 Hz, 13.25 Hz, 2H), 1.34 (d, *J* = 6.5 Hz, 6H) ppm; ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ

213.9, 186.9, 186.5, 169.3, 163.4, 161.0, 156.2, 155.6, 145.3, 145.2, 145.1, 144.9, 144.6, 144.5, 143.8, 143.1, 143.0, 142.9, 142.2, 141.8, 140.9, 139.0, 135.7, 135.4, 133.8, 133.7, 119.8, 118.5, 111.5, 111.3, 100.9, 71.4, 70.9, 70.7, 70.6, 70.5, 70.3, 70.2, 69.7, 69.1, 68.8, 67.5, 66.2, 65.6, 56.7, 52.1, 44.9, 35.6, 33.9, 29.6, 17.0 ppm.

Bioassay

The human breast cancer MCF-7 cells (kindly provided by Dr. G. Huang in the Department of Biological Sciences at Clemson University) were cultured in Eagle's Minimum Essential medium supplemented with 10% (v/v) fetal bovine serum and 1% of penicillin-streptomycin. The cells were cultivated in 75 cm^2 flasks at 37 °C in a humidified atmosphere (5% CO_2 and 95% air).

In the MTT assay on cell viability, DMSO solutions of the conjugate and free DOX were prepared with the same DOX-equivalent concentration of 1.1 mg/mL. The solutions, filtered with sterile syringe filters (Millipore, USA), were diluted with the cell culture medium to the targeted concentrations just prior to the cell exposure. MCF-7 cells were plated in 96-well plates (2×10^4 cells per well) and incubated for 24 h. The conjugate and free DOX were introduced to the cells at DOX-equivalent concentrations of 1, 5, 10, and 25 μg/mL. Cells cultured in the free medium were taken as the control. After 24 h, the supernatants were discarded, and MTT (0.5 mg/mL in culture medium, 100 μL) was added to each well, followed by incubation for another 4 h. The formed formazan was dissolved by adding sodium dodecyl sulfate (SDS) solution (10%, also containing 5% isobutanol and 10 mM HCl, 100 μL). The optical density (OD) of formazan at 570 nm was recorded on a microplate reader (μQuant, Bio-Tek, USA). The relative cell viability was obtained as a percentage of $OD^{Test}/OD^{Control}$, where OD^{Test} and $OD^{Control}$ are ODs of the exposed sample and the control, respectively.

Acknowledgments

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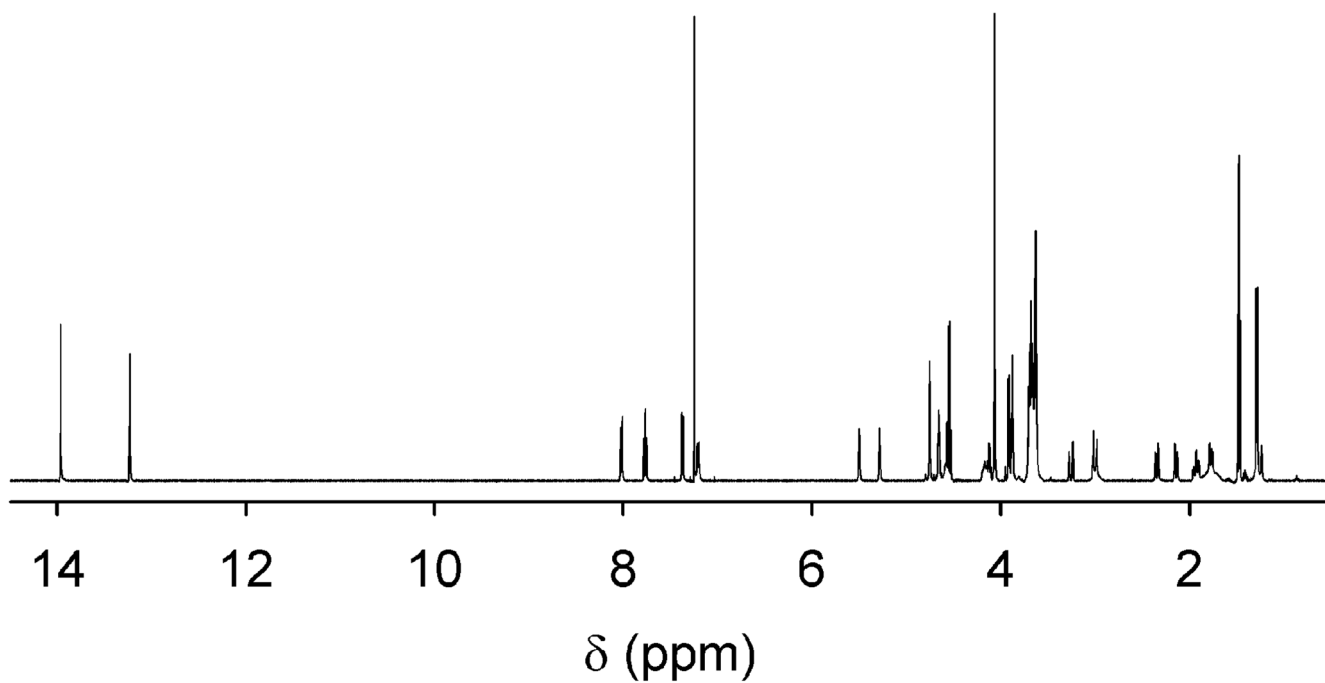
26. Isaacs L, Diederich F. Helv Chim Acta 1993;76:2454.

Figure 1.
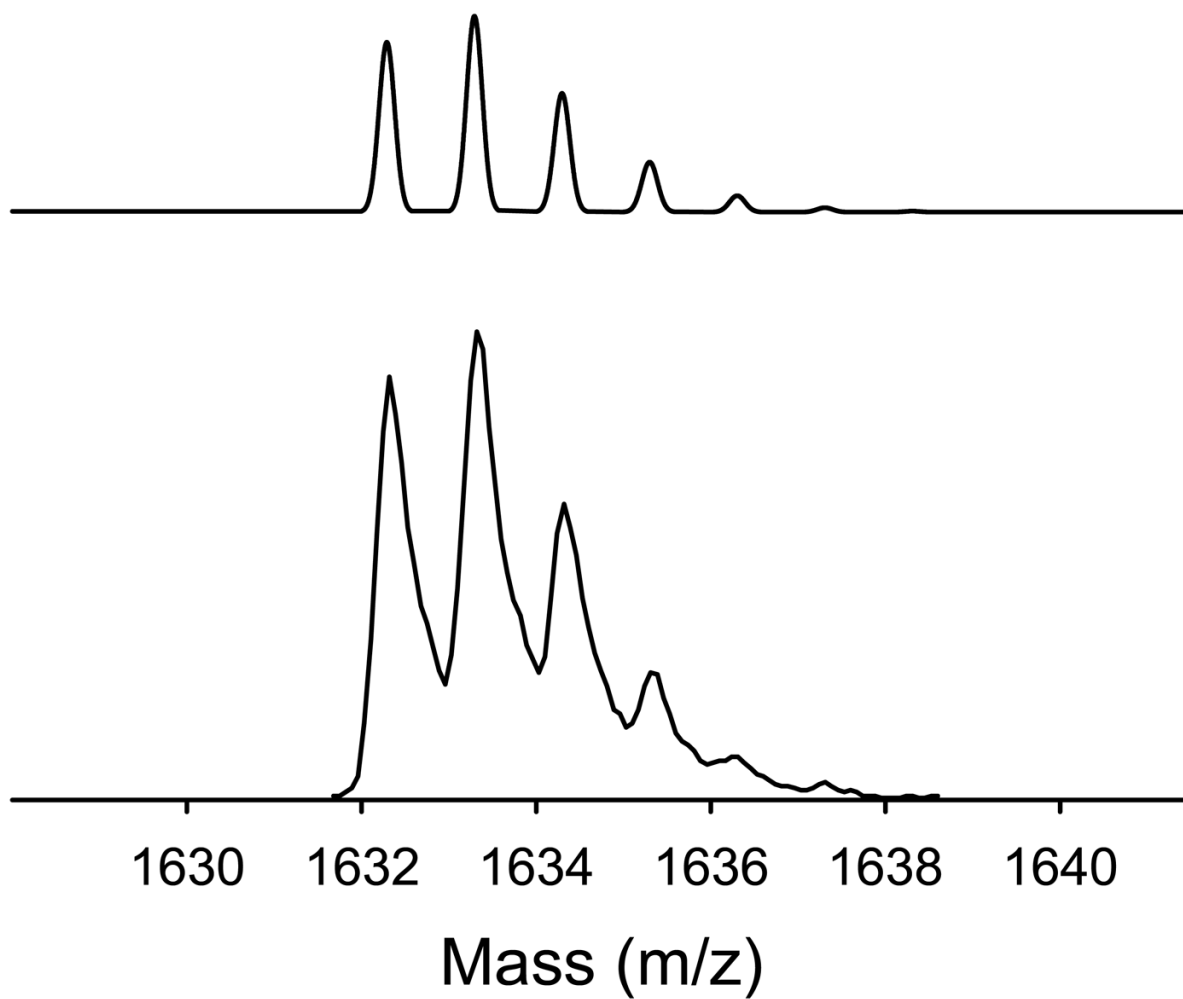The ^{1}H NMR spectrum of the conjugate **II** in deuterated chloroform.

Figure 2.
The observed MALDI-TOF MS trace of the conjugate **II** (in 2,5-dihydroxybenzoic acid matrix, bottom) is compared with that from calculation (IsoPro 3.0, top).
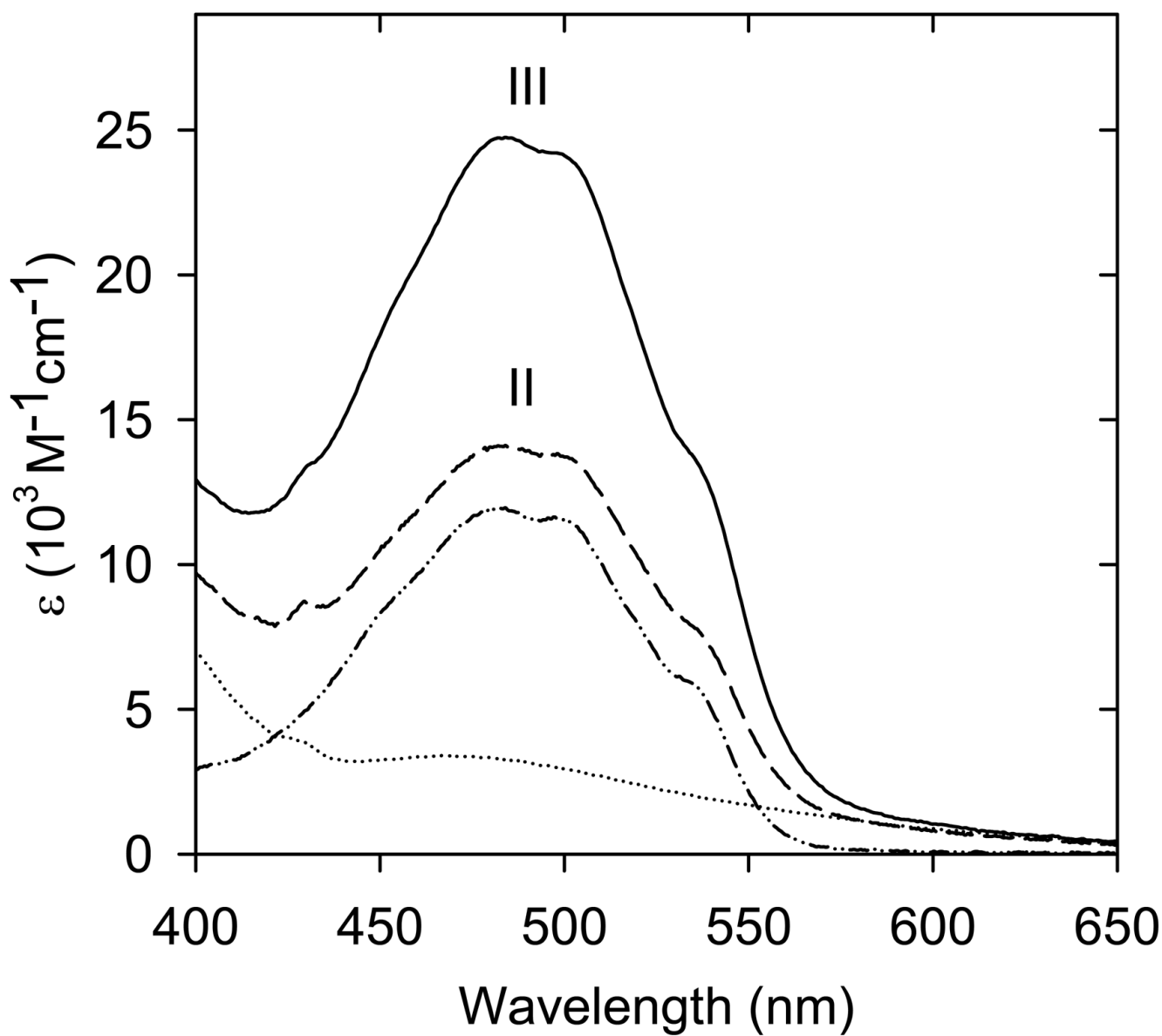
Figure 3.
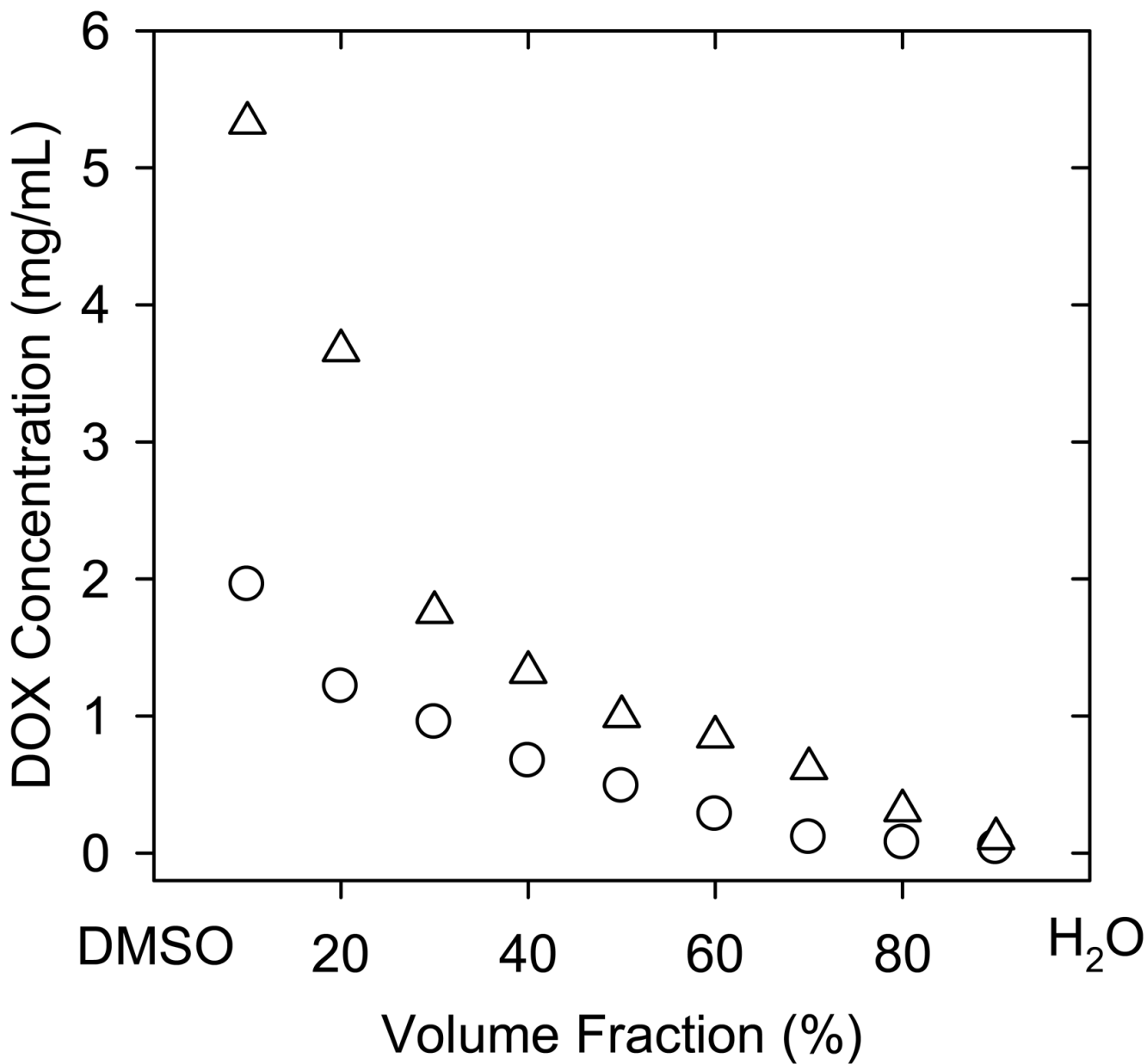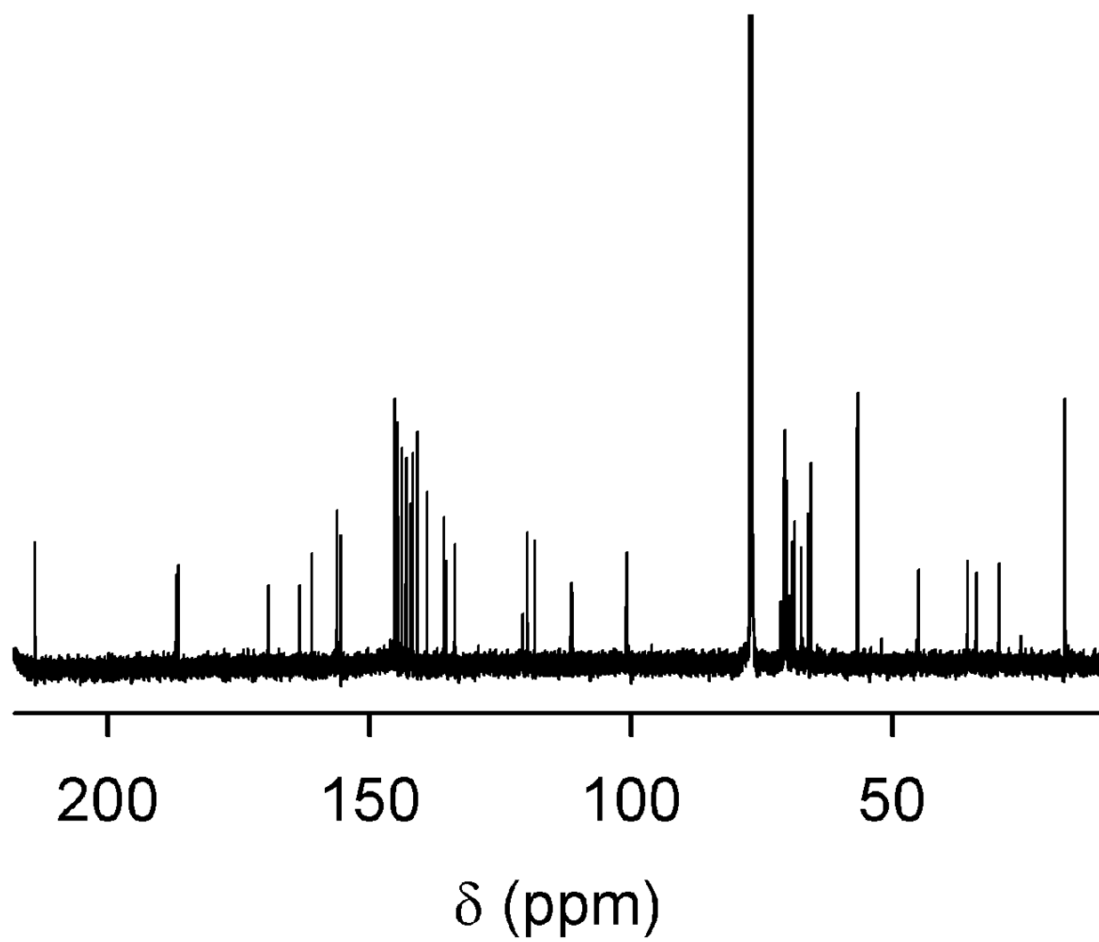UV/vis spectra of **III** (solid line), **II** (dash line), DOX-NH$_3$Cl (dash-dot line), and **5** (dot line) in DMSO solutions.

Figure 4.
The DOX-equivalent solubility of **II** and **III** as a function of the volume fraction in water-DMSO mixtures.

Figure 5.
The ^{13}C NMR spectrum of the conjugate **III** in deuterated chloroform.
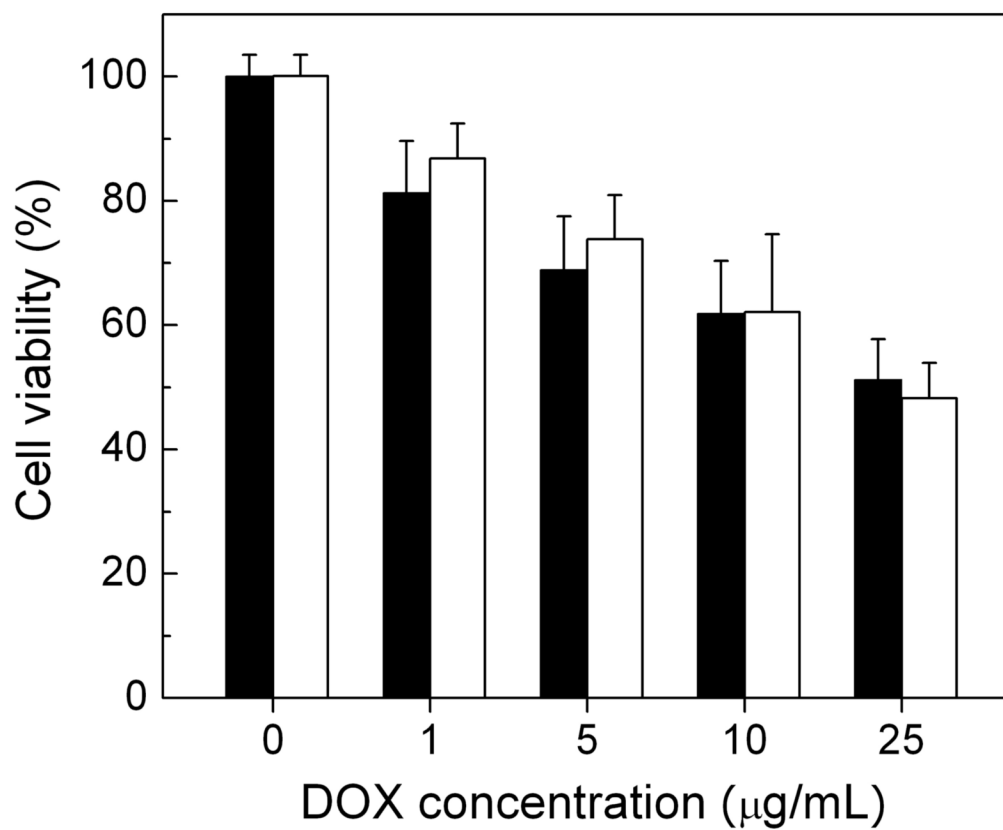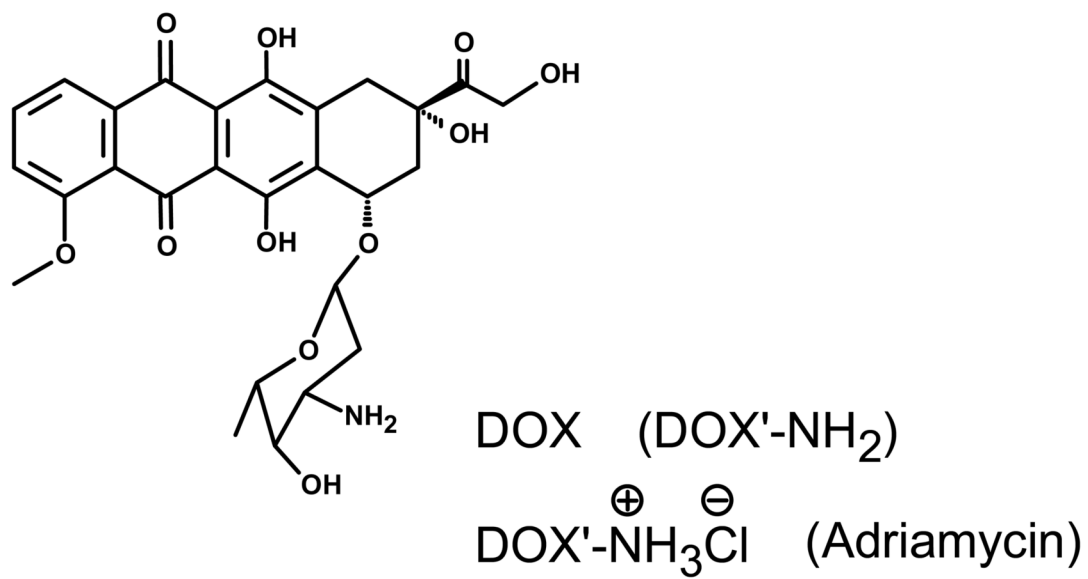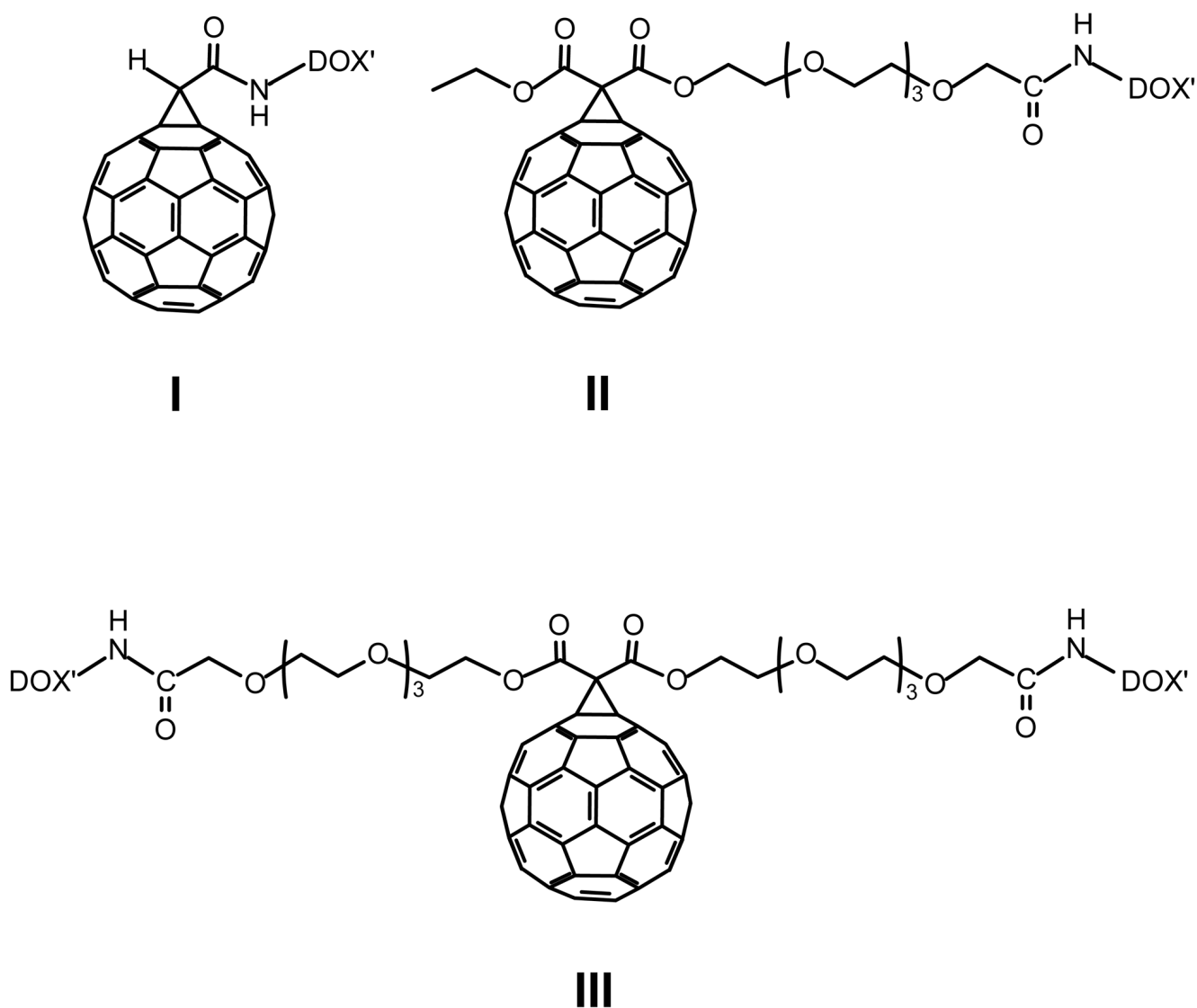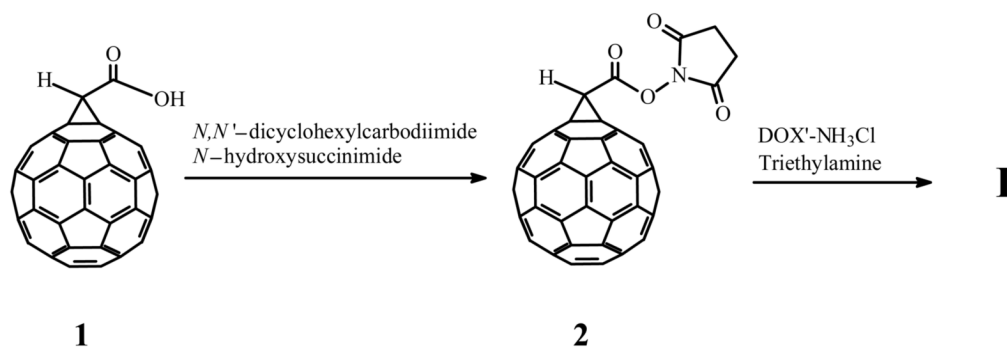
Figure 6.
The cell viability of MCF-7 cells after exposure to DOX′-NH$_3$Cl (black) and the conjugate
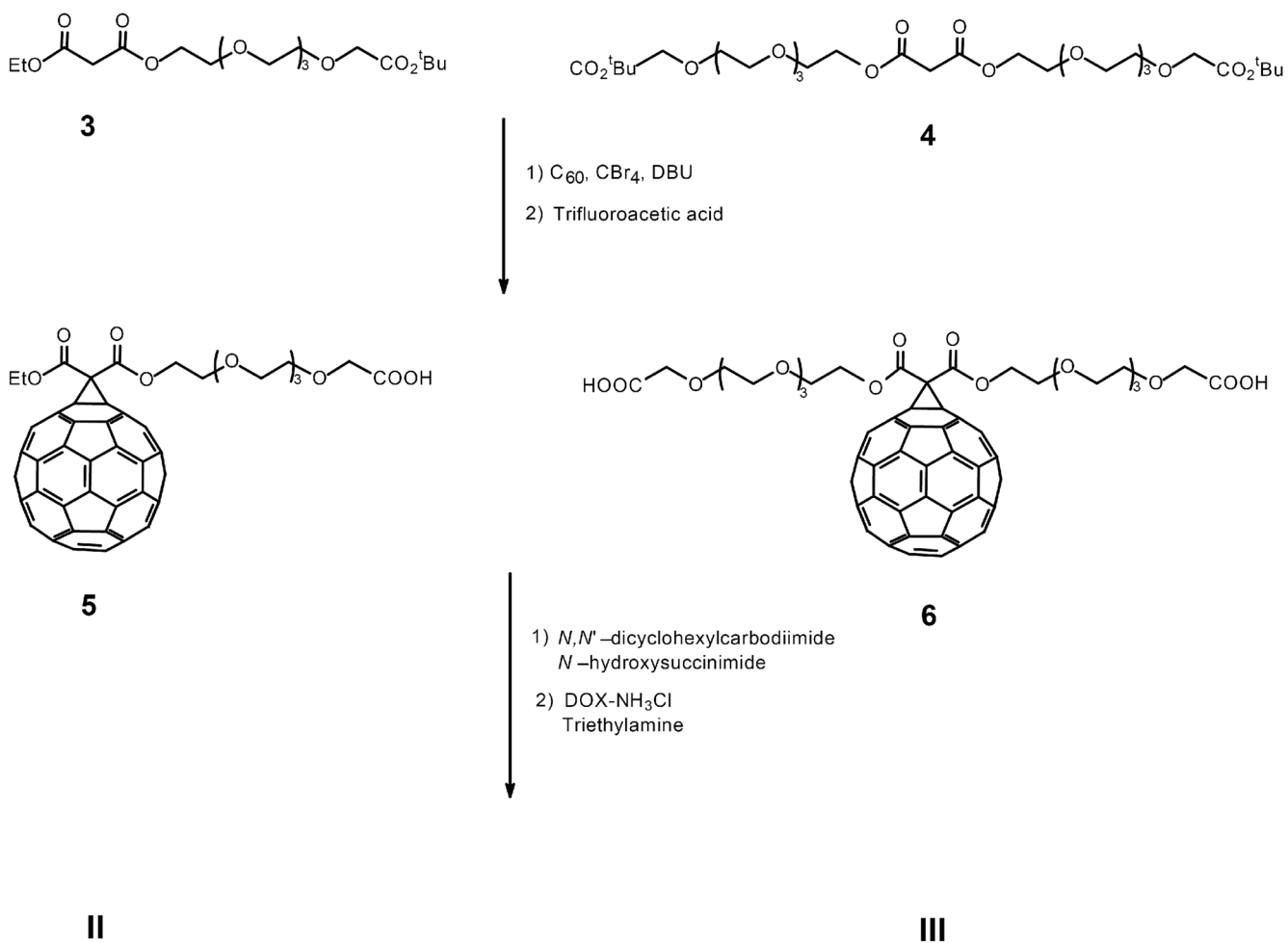**III** (white) at various DOX-equivalent concentrations. Data presented as mean ± SD (n=4).

DOX   (DOX'-NH$_2$)

DOX'-NH$_3^{\oplus}$Cl$^{\ominus}$ (Adriamycin)

Scheme 1.

Scheme 2.

Scheme 3.

3

4

1) C_{60}, CBr_4, DBU

2) Trifluoroacetic acid

5

6

1) N,N'–dicyclohexylcarbodiimide
N–hydroxysuccinimide

2) DOX-NH_3Cl
Triethylamine

II

III

Scheme 4.