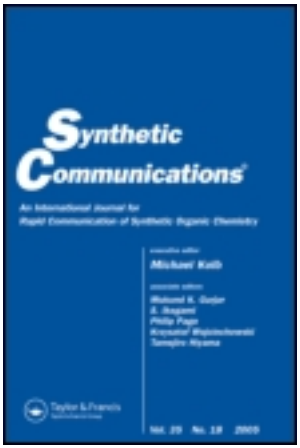

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Efficient Synthesis of Doxorubicin Melanotransferrin p97 Conjugates Through SMCC Linker

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ABSTRACT

Doxorubicin–succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) **3**, prepared by treating doxorubicin (**1**) with SMCC **2**, is treated with 2-mercaptoacetic acid to give doxorubicin–SMCC–sulfo-acetic acid **5**. Treating with benzotriazol-1-yl-*N*,*N*,*N*′,*N*-tetra-methyluronium tetrafluoroborate (BTTU), the carboxy group of **5** is activated, and reacts efficiently with the amino group of melanotransferrin p97 to afford the expected doxorubicin-p97 conjugate **6**, which is a potential agent capable to cross the blood–brain barrier (BBB) to treat brain tumors.

Key Words: Doxorubicin; Blood–brain barrier; Melanotransferrin; Vector-mediated approach; SMCC linker; Bioconjugate.

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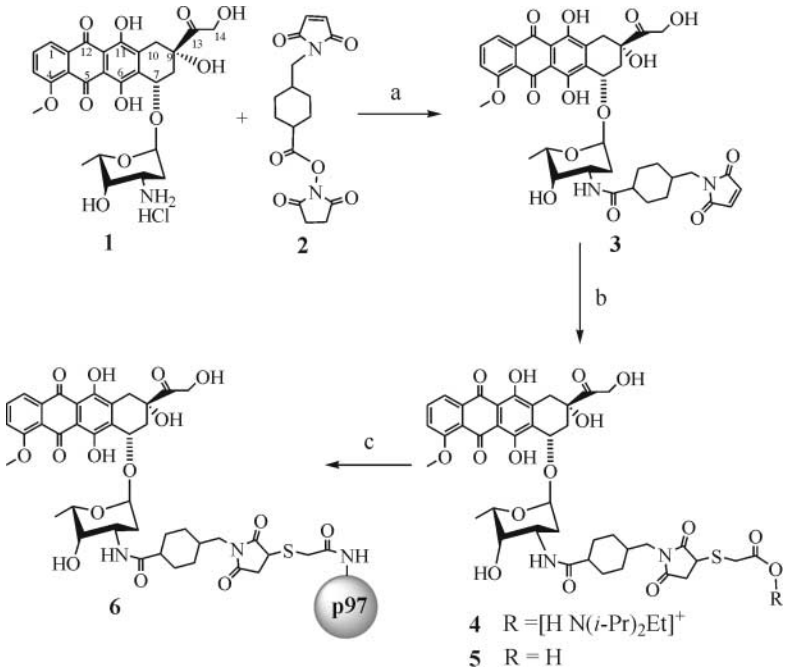
INTRODUCTION

Doxorubicin (**1**, dox) is a widely used anti-cancer drug for the treatment of human cancers such as leukemia, breast carcinoma, etc.[1] However, a serious problem in using this drug in cancer chemotherapy is its toxicity to normal tissues, especially its toxic dose-related side effects, such as cumulative cardiotoxicity, myelosuppression, nephrotoxicity, and extravasation.[1b,1c] Moreover, dox is known to be excluded from the brain by the blood–brain barrier (BBB), and therefore could not be directly used to treat brain cancers. Melanotransferrin (p97) has been found to localize in capillary endothelial cells of the human brain and to play an important role in the transport of iron across the BBB.[2] This provides a base for a vector-mediated approach in delivery of therapeutics to the brain. We previously synthesized dox-p97 conjugates through its 3′-amino, 13-ketone, and 14-hydrxoxy group, and used them for brain drug delivery study.[3] In this paper, we reported the novel synthesis of doxorubicin-p97 conjugate through a succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker.

RESULTS AND DISCUSSIONS

SMCC, a heterobifunctional cross-linker, is widely used for the synthesis of bioconjuates.[4] In a typical procedure, the *N*-succinimidyl ester of SMCC **2** is first coupled with the amino group of a drug to afford the corresponding product with a maleimide group. The SMCC-modified drugs is then treated with the sulfuryl-modified proteins through Michael-type conjugation addition reaction to give the expected bioconjugates. Consequently, proteins used for such purpose have to be pre-modified by sulfuryl agents such as *N*-succinimidyl *S*-acetylthioacetate (SATA) or 2-iminothiolane (2-IT). To avoid the protein pre-modified step, a process which usually causes about 10% loss of the protein, we are interested in developing so-called "one-step" synthesis, i.e., the expected bioconjugates are synthesized by coupling the modified drug directly with p97 so as to reduce the loss of protein during the reaction and purification. Thus, our novel procedure, as shown in Sch. 1, consists of the preparation of dox–SMCC, the reaction with 2-mercaptoacetic acid, and the direct coupling reaction with p97.

Dox (**1**) is well-known for its sparse solubility in a variety of organic solvents. We discovered that, after stirring with Hünig's base (diisopropylethylamine) in DMF at ambient temperature for about 15–30 min, the original suspension of dox (**1**) in DMF became a clear solution, which was coupled with the SMCC **2** in DMF at room temperature. After stirring in



Scheme 1. Reagents and conditions: (a) diisopropylethylamine/DMF, r.t. 12 hr, 96%; (b) diisopropylethylamine/DMF, 2-mercaptoacetic acid, r.t. 2 hr, 96%; (c) H_2O, ion-exchange resin, 71%; (d) diisopropylethylamine/DMF, benzotriazol-1-yl-*N*,*N*,*N*′,*N*-tetramethyluronium tetrafluoroborate (BTTU), r.t. 2 hr, then p97 in PBS, pH = 7.4, r.t. 12 hr. MSR = 8.2, protein recovery 91%.

the dark overnight, TLC indicated that the reaction was completed (spot-to-spot conversion). The solvent was then removed at reduced pressure (30–40 mmHg) under 35–40°C. The residue was taken up in dichloromethane and purified by column chromatography to afford the expected dox–SMCC **3** in 96%.

Upon treatment with 2-mercaptoacetic acid in a basic solution (2 N NaOH) at room temperature for 30 min, dox–SMCC **3** was converted to product **5** in about 30% yield. Seeking for a more efficient method for the synthesis of **5**, we interested a variety of bases, and found that the best yield of **4** (96%) was obtained with diisopropylethylamine. Thus, compound **3** was dissolved in methylene chloride and treated with mercaptoacetic acid and diisopropylethylamine. The solution was stirred at ambient temperature in the dark for 2 hr and monitored by TLC (dichloromethane/methanol/acetic

acid, 7/2/1, v/v/v). The reaction mixture was concentrated under vacuum and the residue was taken up in methanol. Precipitation occurred when the resulting methanol solution was concentrated. The solid was collected by filtration and identified as the corresponding ammonium salt **4**. The free acid was subsequently regenerated by passing a solution of the ammonium salt in a small amount of methanol through a short pad of weakly acidic ion-exchange resin eluting with water. The free acid **5** was obtained in 71% yield. Upon treatment with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (BTTU) in DMF, the carboxy group of **5** was activated and could be directly coupled with the amino groups of p97 in phosphate buffer solution (PBS), pH 7.4 room temperature 12 hr. The dox-p97 conjugate **6** is purified by dialysis against PBS, pH = 7.4. Molecule substituted ratio (MSR) is to be 8.2 as measured by UV-VIS method as described previously.[3] The protein recovery is 91%. The conjugate is characterized by FPLC, SDS–PAGE, and Western-blotting, which demonstrated that the conformation of p97 is preserved. In vitro and in vivo analyses are currently under study.

EXPERIMENTAL SECTION

Reagents were purchased or obtained from commercial sources and used as received. Proton and carbon nuclear magnetic resource spectra were obtained on a Bruker AC 300 spectrometer at 300 MHz, using tetramethylsilane as the internal standard. Infrared spectra were run on a Perkin Elmer Spectrum 1000 spectrometer. UV-VIS spectra were recorded on a Beckman DU640 photo diode array spectrophotometer (instrumental precision ± 2 nm) in the solvents indicated. IR spectra were recorded on a Shimadzu FTIR 8400. FPLC was run in a AKTA Purifier™ FPLC (using UNICORN™ version 3.10 as operation system, obtained from Amersham Pharmacia Biotech) using Mono Q® HR 10/10 ion-exchange column (from Pharmacia Biotech Inc.) with PBS buffer (0.01 M, pH = 7.4) and 1 M NaCl–0.001 M PBS buffers as the mobile phases or using BIOSEP™ 300 size exclusion column (from Phenomenex, Inc) and 0.01 M PBS buffer (pH = 6.80). Elemental analyses, high and low resolution mass spectra were obtained from Department of Chemistry, the University of British Columbia. Thin-layer chromatographic (TLC) analysis was performed on 1 × 3 in. Whatman Silica Gel GE plates (250 μ thick). The TLC plate was visualized by UV light (254 nm) and/or staining with 20% phosphomolybdic acid reagent. Doxorubicin HCl salt was purchased from Albany Molecule Research Inc, New York. Dialysis Cassettes Slide-A-Lyzer (10K, 10,000 MWCO), Dialysis Tubing SnakSkin™ (10,000 MWCO), and SMCC were purchased from Biolynx Inc., Brockville, Ontario.

Dox–SMCC (3). In a 250-mL round-bottomed flask containing a magnetic stirrer bar, anhydrous DMF (150 mL), doxorubicin HCl salt (**1**, 795 mg, 1.371 mmol), SMCC (**2**, 550 mg, 1.645 mmol), and diisopropylethylamine (0.36 mL, 2.057 mmol) were added. The reaction flask was wrapped with aluminum foil and the mixture was stirred at room temperature overnight under nitrogen. The solvent was removed under vacuum at 35–40°C. The residue was taken up by dichloromethane (200 mL) and washed with brine (50 mL), followed by drying over anhydrous magnesium sulfate. The solids were filtered, the filter cake was washed with dichloromethane (3 × 50 mL), and the filtrate and washings were combined and concentrated under vacuum until viscous oil was obtained. The residue was purified by silica gel chromatography eluting with 0–5% methanol/methylene chloride to yield a red solid (998 mg, 96%): m.p. 162–172°C; $[\alpha]_D^{25} + 189.20^\circ$ (*c* 0.12, CH_2CI_2); ESI MS *m*/*z* 761 $[C_{39}H_{42}N_2O_{14} - H]^-$; R_f 0.31 (94/6 CH_2CI_2/MeOH); UV (CH_2CI_2) λ_{max} 202, 233, 252, 288, 478, 495 nm. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 0.83$ (m, 2H), 1.19 (m, 4H), 1.63 (m, 4H), 1.84 (m, 1H), 2.18 (m, 3H), 2.92 (d, $J = 12$ Hz, 2H), 3.19 (d, $J = 6.8$ Hz, 2H), 3.92 (m, 1H), 3.97 (s, 3H), 4.14 (q, $J = 6.6$ Hz, 1H), 4.57 (s, 2H), 4.77 (d, $J = 5.6$ Hz, 1H), 4.91 (m, 1H), 5.21 (s, 1H), 5.44 (s, 1H), 5.77 (s, 1H), 6.99 (s, 2H), 7.40 (d, $J = 8.0$ Hz, 1H), 7.62 (dd, $J = 3.3$, 3.4 Hz, 1H), 7.90 (m, 2H), 13.26 (s, 1H), 13.99 (s, 1H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6) δ 16.9, 28.3, 28.5, 29.3, 29.6, 31.9, 36.0, 36.3, 43.0, 43.4, 44.6, 54.8, 56.5, 63.6, 66.7, 68.0, 69.9, 74.9, 100.4, 110.5, 110.7, 118.9, 119.6, 119.9, 133.9, 134.2, 134.6, 135.4, 136.1, 154.4, 156.0, 160.7, 171.1, 174.2, 186.4, 213.6 ppm. Anal. Calcd for $C_{39}H_{42}N_2O_{14} \cdot 0.5CH_2C1_2$: C, 58.92; H, 5.38; N, 3.48. Found: C, 59.14; H, 5.49; N, 3.26.

Dox–SMCC–*S*-acetic acid ammonium salt 4. To a 500-mL round-bottom flask containing a magnetic stirrer bar was added CH_2Cl_2 (300 mL), dox–SMCC (**3**, 980 mg, 1.285 mmol), Hünig's base (0.34 mL, 1.927 mmol), and 2-mercaptoacetic acid (0.14 mL, 1.927 mmol). The reaction flask was wrapped with aluminum foil and the mixture was stirred at room temperature for 2 hr under nitrogen, then concentrated under vacuum. The residue was taken up in methanol (30 mL) and the resulting methanol solution was re-concentrated. Precipitation occurred during the concentration. However, the slurry was further concentrated until about 3 mL of total volume remained. The solid was collected by vacuum filtration and washed with *i*-PrOH/EtOAc (3 × 3 mL) to yield 1.178 g (96%) of the ammonium salt as a red solid: m.p. 141–150°C; $[\alpha]_D^{25} + 2l8.9^\circ$ (*c*, 0.25, MeOH/EtOAc, 2/1 v/v); ESI MS *m*/*z* = 853 $[C_{49}H_{65}N_3O_{16}S–C_8H_{19}N - H]^-$; R_f 0.43 (88/10/2 CH_2CI_2/MeOH/AcOH); UV (CH_2Cl_2) λ_{max} 234, 251, 288, 400, 477 nm; ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 0.87$ (m, 2H), 1.11–1.23 (m, 19H), 1.37–1.65 (m, 6H), 1.84 (m, 1H), 2.05–2.25 (m, 3H), 2.60 (d, $J = 3.5$ Hz, 1H),

2.84–3.05 (m, 3H), 3.10–3.20 (m, 3H), 3.35–3.55 (m, 4H), 3.95 (m, 4H), 4.05 (m, 1H), 4.16 (q, $J = 6.6$ Hz, 1H), 4.57 (s, 2H), 4.85 (m, 1H), 5.21 (s, 1H), 5.45 (br, 1H), 7.40 (d, $J = 8.0$ Hz, 1H), 7.62 (dd, $J = 3.3$, 3.4 Hz, 1H), 7.85 (m, 2H), 13.25 (br, 1H), 13.99 (s, 1H) ppm. ^{13}CNMR (75 MHz, DMSO-d_6) δ 17.4, 18.6, 28.8, 28.9, 29.7, 33.8, 35.7, 43.8, 44.4, 45.1, 48.9, 56.9, 64.0, 67.1, 68.6, 70.3, 75.3, 100.8. 100.9, 111.1, 119.3, 120.1, 120.3, 134.9, 135.8, 136.5, 154.8, 156.4, 161.1, 171.0, 174.6, 175.7, 177.1, 186.7, 186.8, 214.1 ppm. Anal. Calcd for $C_{49}H_{65}N_3O_6S \cdot 3H_2O$: C, 56.69; H, 6.89; N, 4.05. Found: C, 56.62; H, 6.86; N, 4.17.

Dox–SMCC–*S*-acetic acid 5. A sample of the dox–SMCC–*S*-acetic acid ammonium salt (**4**, 242 mg) was dissolved in MeOH (2 mL). The solution was loaded onto Dowex weakly acidic ion-exchange resin (8.34 g), prepacked in a column ($1.6 \times 32\,cm^2$) and eluted with water. The red aqueous solution was extracted with CH_2C1_2 (3×60 mL). The extracts were combined and dried over Na_2SO_4. The solids were vacuum filtered, and the filter cake was washed with CH_2Cl_2 (3×10 mL). The filtrate and washings were combined and the solvent was completely removed under vacuum to afford 150 mg (71%) of the free acid as a red solid: m.p. l43–150°C; $[\alpha]_D^{25} + 202.50°$ (*c*, 0.12, MeOH/CH_2C1_2, 1/1, v/v); ESI MS $m/z = 877$ [$C_{41}H_{46}N_2O_6S + Na$]; R_f 0.29 (88/10/2 CH_2Cl_2/MeOH/AcOH); UV (MeOH/CH_2CI_2, 1/1, v/v) λ_{max} 203, 234, 252, 286, 478, 495, 529 nm; ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 0.86$ (m, 2H), 1.18 (m, 5H), 1.35–1.65 (m, 1H), 1.61–1.67 (m, 3H), 1.85 (m, 1H), 2.00–2.25 (m, 3H), 2.50 (m, 2H), 2.90 (q, $J = 12.5$ Hz, 2H), 3.17–3.20 (m, 3H), 3.38–3.46 (m, 3H), 3.62 (d, $J = 15$ Hz, 1H), 3.96 (s, 4H), 4.05 (s, 1H), 4.14 (s, 1H), 4.58 (s, 2H), 4.62 (s, 1H), 4.75 (s, 1H), 4.91 (s, 1H), 5.21 (s, 1H), 5.35 (s, 1H), 7.28 (d, $J = 10$ Hz, 1H), 7.58 (d, $J = 5$ Hz, 3.4 Hz, 1H), 7.85 (m, 2H), 12.69 (br, 1H), 13.19 (s, 1H), 13.90 (s, 1H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 16.9$, 28.4 28.5, 29.2, 32.6, 35.2, 35.3, 43.4, 44.0, 44.7, 56.5, 63.6, 66.7, 68.1, 69.8, 74.9, 100.3, 110.5, 110.6, 119.6, 119.9, 133.9, 134.5, 135.4, 154.4, 155.9, 160.7, 170.5, 174.2, 175.0, 176.4, 186.2, 186.3, 213.4 ppm; Anal. Calcd for $C_{41}H_{46}N_2O_{16}S$: C, 57.60; H, 5.42; N, 3.28. Found: C, 57.87; H. 5.59; N, 2.97.

Synthesis of Dox-p97 Conjugate 6

A mixture of dox–SMCC–*S*-acetic acid **5** (6.9 mg, 7.826 μmol, 50 equiv. of p97), BTTU (6.3 mg, 0.01956 mmol, 2.50 equiv. of **5**), triethylamine (0.04 mL), DMF (1 mL) was stirred at room temperature for 60 min. TLC confirmed the reaction completed (MeOH/CH_2Cl_2, v/v, 5/95). The solution is saved for the following reaction.

p97 ($c = 1.23$ mg/mL, 12 mL, 1.56×10^{-4} mM) was added to a 50 mL flask. To this solution, BTTU-activated doxorubicin compounds **5** prepared from the above (1 mL, 7.8 μmol, 50 equiv. of p97) mixed with 3.7 mL DMF (~28% total concentration) was added over a period of 2 min under vigorous stirring. The mixture was then stirred at room temperature and monitored by FPLC (Mono Q column, wavelength: 280 and 480 nm). It was found that after 20 hr at room temperature all starting p97 was converted to the conjugate. During the first few hours, FPLC showed several peaks that were assigned to the starting p97, and conjugates with different ratio of doxorubicin conjugated to p97. The conjugate peaks finally became to one major peak after 20 hr at room temperature. The product was then purified by dialysis using SnakeSkin™ tubing (MWCO = 10 K) against PBS (10 mM, pH = 7.4) to give the expected conjugate **6** with MSR 8.2, protein recovery 91%.

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