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Conjugation of Doxorubicin to Monoclonal Anti-carcinoembryonic Antigen Antibody via Novel Thiol-directed Cross-linking Reagents

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Abstract—In order to improve the available methods to produce an immunoconjugate the use of longer heterobifunctional cross-linking reagents were investigated. Two new maleimidobenzoyl spacers have been synthesized in a one step process from 4-maleimidobenzoic acid. The new heterobifunctional cross-linking reagents were fully characterized by their IR, ^{1}H NMR, ^{13}C NMR and mass spectra. These spacers are selectively attached to NH_2-3' of the daunosamine moiety of doxorubicin. The spacer-doxorubicin derivatives were also characterized by ^{1}H NMR spectrometry before coupling to thiol groups of thiolated anti-carcinoembryonic antigen (CEA) monoclonal antibody (11-285-14). These conjugates contain 1.51–3.44 molecules of drug for each molecule of monoclonal antibody (MAb).

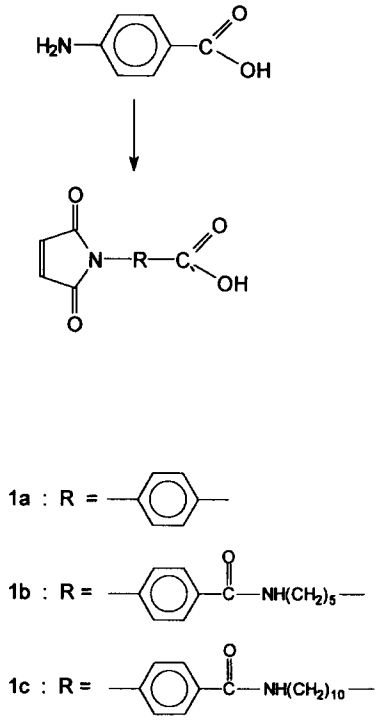
Introduction

Doxorubicin (Dox) is widely used as an anti-cancer drug because of its great therapeutic value in treating a number of human cancers.[1] However, a serious impediment in using Dox in cancer chemotherapy is its toxicity to normal tissues, especially its dose-dependent cardiotoxicity.[2] A possible way to overcome the toxic limitations of Dox and to increasing its selectivity is to use monoclonal antibodies (MAbs) as carriers. The efficacy of drug–MAb immunoconjugates *in vitro* and *in vivo* and the methods of drug conjugation to MAbs have been reviewed.[3–6] The development of MAb–anthracycline conjugates has been extensively discussed by Hermentin *et al.*[7] Heterobifunctional cross-linking reagents are increasingly used for their regiospecificity in coupling a drug to MAb for immunotargeted chemotherapy. The maleimidobenzoyl spacers have proven to be useful in the preparation of anthracycline–MAb conjugates.[8] Therefore, we designed and synthesized two new maleimidobenzoyl cross-linking agents which enable the conjugation of the amino group of Dox and thiolated MAb, resulting in stable conjugates with amide and thioether bonds. The present work was undertaken to assess the influence of the length of the cross-linking reagents on the conjugation process as well as to establish a clear and precise procedure for conjugation with this type of linker. It is well known that much of the work in this field lacks important experimental details and thorough characterization of the various intermediates used in the conjugation procedure.

Results and Discussion

The maleimido derivatives, 4-maleimidobenzoic acid (**1a**, MBA), *N*-(4-maleimidobenzoyl)-6-aminocaproic acid (**1b**, MBCA) and *N*-(4-maleimidobenzoyl)-11-aminoundecanoic acid (**1c**, MBUA) were designed to cross-link the amine group of Dox to the thiol groups of the antibody (Schemes 1 and 2). Before using 1,1,1,3,3,3-hexamethyldisilazine, several synthetic approaches were unsuccessful in preparing MBCA and MBUA. 1,1,1,3,3,3-Hexamethyldisilazane is a suitable reagent for direct silylation of amino acids which are insoluble in ordinary organic solvents. Thus, reactions can be carried out in a homogeneous organic phase. These heterobifunctional spacers consist of two reactive functional groups, maleimide and carboxylic acid, separated by a phenyl, *N*-benzoylaminopentyl or *N*-benzoylaminodecyl spacer. The maleimide and carboxylic acid functional groups were chosen because they exhibit the desired reactivities with amines and thiols, respectively. Moreover, these groups are chemically compatible, yielding stable cross-linking reagents. The *N*-benzoylaminopentyl or *N*-benzoylaminodecyl spacer arms in MBCA and MBUA were designed to potentially limit the possibility of steric hindrance between Dox and MAb and thereby improve the conjugation process. All maleimido derivative spacers, **1a–c**, were easy to prepare (Scheme 1) and stable during handling. They are very useful for coupling drugs containing amino groups to proteins containing thiols, and the resulting amide and thioether bonds provide stable linkage of the conjugate. The spacers, activated

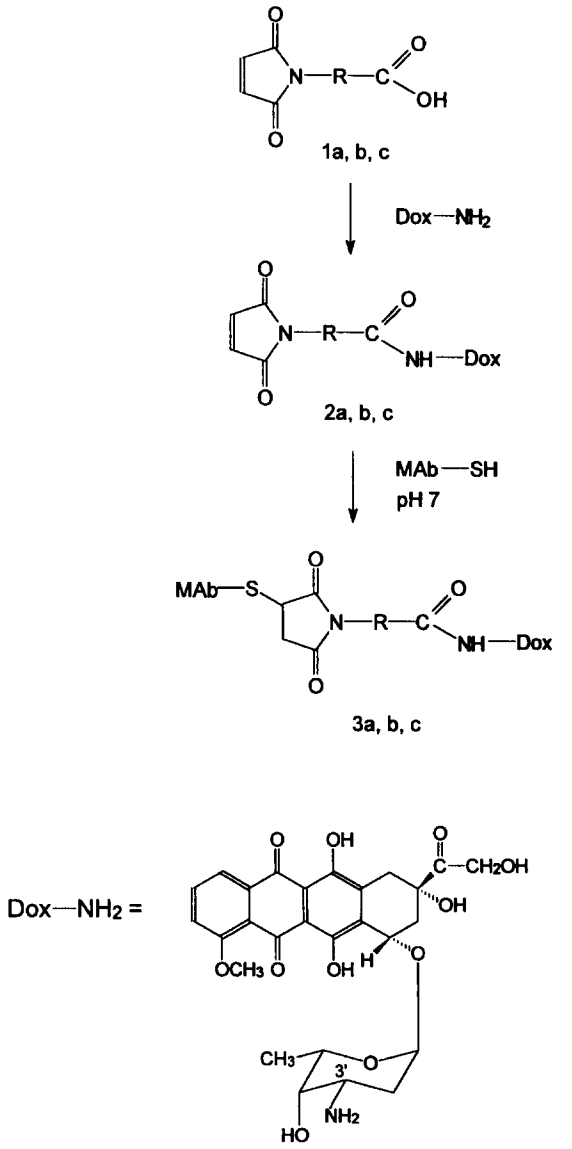
by isobutyl chloroformate, were regioselectively introduced to Dox-NH_2 in a straightforward manner and in reasonable yield (Scheme 2).



Scheme 1. Synthesis of the spacers.

The pH dependence of the coupling reactions between maleimide and thiol was investigated by addition of **1a**, **b** or **c** to cysteine thiol at two different pHs, 7.0 and 6.5. The deprotonated thiol is a strong nucleophilic species for the Michael addition to the double bond of maleimide.[9] As expected, the rate of coupling (cysteine-to-linker condensation) was slow at pH 6.5 and rapid at pH 7.0. At pH 7.0, more deprotonated thiols exist to achieve the desired Michael addition (Fig. 1A–C). The addition products absorb near 266 nm at which wavelength maleimides **1a–c** do not. Therefore, the progress of the coupling reaction was followed by the increase with time of the absorbance near 266 nm. However, maleimide undergoes base-catalyzed hydrolysis to maleamic acid.[10] The rate of hydrolysis was rapid at pH 8.0 and slow at pH 7.0 and nonexistent at pH 6.5 (Fig. 1D–F). Maleamic acid absorbs near 286 nm at which wavelength the maleimides **1a–c** do not. The based-catalyzed hydrolysis of the maleimides was followed by the increase over time of the absorbance near 286 nm. Thus, based on these observations, pH 7.0 was chosen to be the optimal condition for the maleimide–thiol coupling reaction.

The Dox–maleimide linkers, **2a–c** were reacted with thiolated MAb of anti-CEA (11-285-14) to give a conjugate containing 1.51–3.44 molecules of drug per molecule of MAb. Previous studies of direct drug linking to MAb 11-285-14 has shown that the maximum drug to antibody molar ratios that could be obtained were 1.2.[11] The present results show that it is possible to



Scheme 2. Introduction of the spacers to doxorubicin and monoclonal antibodies.

improve the drug per antibody ratio using these heterobifunctional cross-linking reagents. We initially hypothesized that the increase in length of the linker would prevent strong steric interaction between the drug and the antibody allowing a greater drug:antibody ratio. However, the length of the new linkers did not influence the conjugation process very much. Steric hindrance does not seem to play an important role in the conjugation process.

While we appreciate that the amine group on the sugar portion of doxorubicin is not the most suitable position to link doxorubicin to an antibody molecule, we chose this route to demonstrate that it is possible to characterize the drug–linker intermediate correctly and to show that such an approach would work with a complex drug such as doxorubicin.[8] We feel that the importance of this work lies in the ability to link the heterobifunctional linker to a drug in a stepwise manner, thereby facilitating purification and characterization of

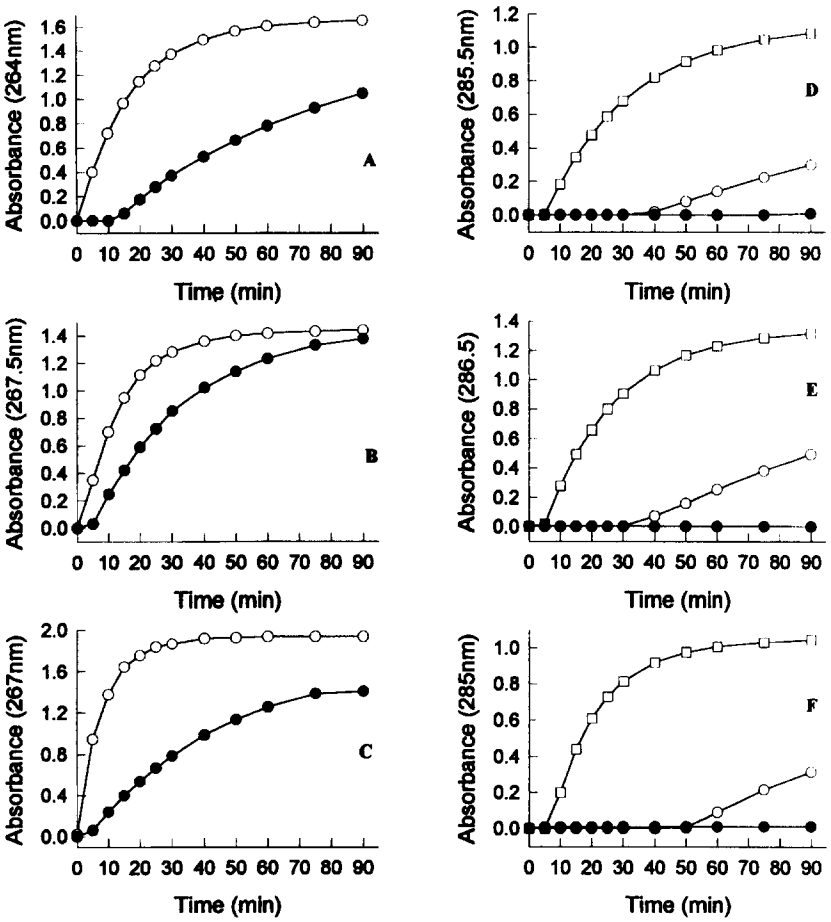


Figure 1. Optimal condition of maleimide reaction with thiol. Panels A–C show the reaction of MBA, MBCA and MBUA, respectively, with cysteine. Maleimide (100 μM) was reacted with cysteine (450 μM) in 0.1 M phosphate buffer at pH 7.0 (○) and 6.5 (●). Panels D–F show the base-catalyzed hydrolysis of MBA, MBCA and MBUA, respectively. Maleimide (100 μM) was incubated in 0.1 M phosphate buffer at pH 8.0 (□), 7.0 (○) and 6.5 (●).

the drug–linker moiety. The ability of doxorubicin to be released from the linker under physiological conditions is under investigation and the efficacy and selectivity of the resulting immunoconjugates *in vitro* is described in the following paper.

The use of these novel maleimidobenzoyl spacers for conjugation is in its initial stage of development and one of our aims in undertaking this study was to develop versatile linkers which may also be suitable for attaching toxic agents which may not need to be released to exert their toxic effects, e.g. radioisotopes.

Experimental

Materials

Anhydrous reactions were performed under an inert atmosphere of nitrogen. Unless otherwise noted, starting material, reactant and solvents were obtained commercially from Aldrich and were used as such or purified and/or dried by standard means.[12] Organic solvents were dried over magnesium sulphate ($MgSO_4$), evaporated on a rotatory evaporator and under reduced pressure. All reactions were monitored by thin-layer chromatography (TLC). The plates were visualized by UV fluorescence. Commercial TLC plates were Sigma T6145 (polyester silica gel 60 Å 0.25 mm). Flash chromatography was performed according to the method of Still *et al.* on Merck grade 60 silica gel, 230–400 mesh.[13] Mp were recorded on an Electrothermal 9100 apparatus and are uncorrected. The IR spectra were taken on a Nicolet model 205 FT-IR spectrophotometer. MS assays (m/z) were obtained using a VG Micromass 7070 HS instrument with an ionization energy of 70 eV. Elemental analysis was conducted by Microanalysis Laboratories Limited, Markham, Ontario, Canada. NMR spectra were obtained in deuterated dimethyl sulfoxide, methanol and chloroform on a General Electric GE 300-NB (300 MHz) instrument: chemical shifts were measured relative to internal standards: tetrameth-ylsilane (TMS, δ 0.0 ppm) for ^1H and ^{13}C NMR spectra.

Synthesis

*4-Maleimidobenzoic acid (MBA) (**1a**; Scheme 1).* 4-Maleimidobenzoic acid was prepared by the procedure of Hermentin *et al.*[8] 4-Aminobenzoic acid (20 g, 146 mmol) suspended in anhydrous acetone (120 mL) was solubilized by the addition of distilled methanol (20

mL). Then, maleic anhydride (16.49 g, 168 mmol) in anhydrous acetone (40 mL) was added dropwise and stirred at room temperature for 1 h. The yellow precipitate was filtered, washed with acetone, and vacuum-dried (yield, 32.7 g, 95%). A portion of the precipitate (20 g, 84 mmol) was treated with acetic anhydride (40 mL) and anhydrous sodium acetate (3.4 g, 41 mmol) at 50 °C for 2 h. The solution was evaporated to dryness and stirred with H_2O (500 mL) at 70 °C for 2 h. The precipitate was filtered off and vacuum-dried to yield 14.45 g (78%) of **1a**: mp 238–240 °C; IR (KBr) 3350–2600 (CO_2H), 1799 and 1715 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6): δ 13.1 (*s*, 1H, CO_2H), 8.05 (*d*, 2H, *J* = 8.5 Hz, Ar, H-2, H-6), 7.51 (*d*, 2H, *J* = 8.5 Hz, Ar, H-3, H-5), 7.23 (*s*, 2H, maleimido-H); ^{13}C NMR (DMSO-d_6): δ 169.52 (C=O, maleimide), 166.65 (CO_2H), 135.50 (Ar, C-4), 134.86 (C=C, maleimide), 129.89 (Ar, C-2, C-6), 129.50 (Ar, C-1), 126.11 (Ar, C-3, C-5); MS, m/z: 217 (M^+) Anal. Calcd for $C_{11}H_7NO_4$: C, 60.83; H, 3.25; N, 6.45. Found: C, 60.97; H, 3.36; N, 6.31.

N-*(4-Maleimidobenzoyl)-6-aminocaproic acid (MBCA) (**1b**; Scheme 1).* Compound **1b** was prepared directly from **1a** by coupling to 6-aminocaproic acid. A suspension of 6-aminocaproic acid (1.00 g, 7.62 mmol) in 1,1,1,3,3,3-hexamethyldisilazane (1.77 mL, 8.39 mmol), containing 1 drop of concentrated sulfuric acid, was heated at reflux (120–130 °C) under nitrogen until complete dissolution. Afterwards, the reaction mixture was boiled for a further 30 min.[14] After cooling, benzene (10 mL), triethylamine (0.36 mL, 2.58 mmol), and chlorotrimethylsilane (0.32 mL, 2.53 mmol) were added and the mixture was stirred overnight at room temperature to yield the trimethylsilyl ester of *N*-trimethylsilyl-6-aminocaproic acid. MBA (0.83 g, 3.82 mmol) was suspended in 20 mL CH_2Cl_2 and cooled to 0 °C before the addition of triethylamine (0.97 mL, 6.96 mmol) and isobutyl chloroformate (0.91 mL, 7.01 mmol). The mixture was stirred for 1 h at 0 °C and then added to a solution of silylated intermediate in 25 mL CH_2Cl_2. Stirring was continued for 4 h at room temperature. The reaction mixture was diluted with EtOAc (150 mL), washed with 0.1 N HCl (2 × 100 mL), saturated NaCl (2 × 100 mL), then dried ($MgSO_4$) and evaporated to give crude maleimide. The product was purified by flash chromatography, using two mixtures of solvent (initially 1:3 Me_2CO:hexane and then 0.2:2:3 HOAc:Me_2CO:hexane), to yield 0.69 g (55%) of **1b**: mp 156–157 °C; IR (KBr) 3500–2500 (CO_2H), 3466 and 3318 (NH), 3086 (Ar), 2931 and 2868 (alkane) and 1701 (C=O) cm^{-1}; ^1H NMR (Me_2CO-d_6): δ 7.99 (*d*, 2H, *J* = 8.6 Hz, Ar, H-2, H-6), 7.48 (*d*, 2H, *J* = 8.6 Hz, Ar, H-3, H-5), 7.07 (*s*, 2H, maleimido-H), 3.42 (*t*, 2H, *J* = 7.1 Hz, CH_2N), 2.31 (*t*, 2H, *J* = 7.4 Hz, CH_2CO), 1.65 (*m*, 4H, CH_2 × 2), 1.44 (*m*, 2H, CH_2); ^{13}C NMR (Me_2CO-d_6): δ 174.64 (C=O, CO_2H), 170.24 (C=O, maleimide), 166.47 (C=O, CONH), 135.40 (C=C, maleimide), 135.20 (Ar, C-4), 134.89 (Ar, C-1), 128.43 (Ar, C-2, C-6), 126.66 (Ar, C-3, C-5), 40.09, 34.08, 29.98, 27.11, 25.30 (alkane); MS, m/z: 330 (M^+). Anal. Calcd for $C_{17}H_{18}N_2O_5$: C, 61.81; H, 5.49; N, 8.48. Found: C, 61.69; H, 5.39; N, 8.33.

N-*(4-Maleimidobenzoyl)-11-aminoundecanoic acid (MBUA) (**1c**; Scheme 1).* The procedure described in the preparation of **1b** was repeated with 11-aminoundecanoic acid (1.54 g, 7.65 mmol), 1,1,1,3,3,3-hexamethyldisilazane (2.58 mL, 12.2 mmol), triethylamine (0.36 mL, 2.58 mmol), and chlorotrimethylsilane (0.32 mL, 2.53 mmol). The product was purified by flash chromatography, using two mixtures of solvent (initially 1:3 and then 2:3 Me_2CO:hexane), to yield 0.71 g (46%) of **1c**: mp 162–164 °C; IR (KBr) 3500–2500 (CO_2H), 3473 and 3318 (NH), 3079 (Ar), 2945 and 2868 (alkane), 1715 (C=O) cm^{-1}; ^1H NMR (7:3 Me_2CO-d_6:HOAc-d_4): δ 7.99 (*d*, 2H, *J* = 8.6 Hz, Ar, H-2, H-6), 7.50 (*d*, 2H, *J* = 8.6 Hz, Ar, H-3, H-5), 7.04 (*s*, 2H, maleimido-H), 3.40 (*t*, 2H, *J* = 7.1 Hz, CH_2N), 2.32 (*t*, 2H, *J* = 7.4 Hz, CH_2CO), 1.62 (*m*, 4H, CH_2 × 2), 1.36–1.29 (*bs*, 12H, CH_2 × 6); ^{13}C NMR (7:3 Me_2CO-d_6:AcOH-d_4): δ 170.47 (C=O, CO_2H), 169.50 (C=O, maleimide), 168.14 (C=O, CONH), carbon of C=C (maleimide) hidden by HOAc, 134.43 (Ar, C-4), 128.87 (Ar, C-1), 126.95 (Ar, C-2, C-6), 126.79 (Ar, C-3, C-5), 40.89, 34.38, 27.79, 25.68 (C, alkane, 6 carbons hidden by acetone and HOAc); MS, m/z: 400 (M^+). Anal. Calcd for $C_{22}H_{28}N_2O_5$: C, 65.98; H, 7.05; N, 7.00. Found: C, 65.90; H, 7.01; N, 6.92.

*Introduction of linker **1a**, **2a**, and **3a** into doxorubicin (Scheme 2).*

The spacers **1a–c** were each activated by isobutyl chloroformate and reacted with the amine group of Dox to form the amide bonding. Briefly, doxorubicin free base (from 5 mL of 2 mg mL^{-1} of Dox·HCl salt in 0.9% saline, 0.017 nmol) was extracted with $CHCl_3$ from 1 mL 1% $NaHCO_3$ until the aqueous layer was free of the strong orange color. The extracts were dried over Na_2SO_4, filtered, and evaporated. MBA (**1a**, 0.2 g, 0.92 mmol) was suspended in 2 mL CH_2Cl_2. Triethylamine (144 μL, 1.03 mmol) and isobutyl chloroformate (132 μL, 1.02 mmol) were added and stirred at 0 °C for 1 h. The activated MBA (150 μL, 26.18 μmol) was added to the Dox-NH_2 in 5 mL CH_2Cl_2:MeOH (95:5) and stirred at room temperature for 0.5 h. The desired product was purified by TLC, using 9:1 $CHCl_3$:MeOH to yield 6.28 mg (49%) of **2a**: ^1H NMR ($CDCl_3$): δ 14.00 (*s*, 1H, OH-11), 13.27 (*s*, 1H, OH-6), 8.05 (*d*, 1H, *J* = 7.1 Hz, H-1), 7.83 and 7.45 (two *d*, 4H, *J* = 8.6 Hz, *para*-substituted phenyl group), 7.79 (*t*, 1H, *J* = 8.0 Hz, H-2), 7.39 (*d*, 1H, *J* = 8.3 Hz, H-3), 6.87 (*s*, 2H, maleimido-H), 6.51 (*d*, 1H, *J* = 8.4 Hz, NH-3'), 5.58 (*d*, 1H, *J* = 3.5 Hz, H-1'), 5.35 (*m*, 1H, H-7), 4.78 (*d*, 2H, *J* = 3.6 Hz, CH_2-14), 4.58 (*s*, 1H, OH-4'), 4.35 (*m*, 1H, H-3'), 4.23 (*q*, 1H, *J* = 6.7 Hz, H-5'), 4.07 (*s*, 3H, OCH_3), 3.28 (*d*, 1H, *J* = 18.9 Hz, H-10β), 3.03 (*d*, 1H, *J* = 18.9 Hz, H-10a), 1.32 (*d*, 3H, *J* = 6.6 Hz, CH_3-6').

*Compound **2b**.* 7.6 mg yield (51%); ^1H NMR ($CDCl_3$): δ 13.97 (*s*, 1H, OH-11), 13.26 (*s*, 1H, OH-6), 8.04 (*d*, 1H, *J* = 6.8 Hz, H-1), 7.85 and 7.46 (two *d*, 4H, *J* = 8.6 Hz, *para*-substituted phenyl group), 7.79 (*t*, 1H, *J* = 8.1 Hz, H-2), 7.38 (*d*, 1H, *J* = 8.2 Hz, H-3), 6.88 (*s*, 2H, maleimido-H), 6.44 (*t*, 1H, *J* = 5.6 Hz, NH), 5.90 (*d*,

1H, J = 8.7 Hz, NH-3'), 5.49 (*s*, 1H, H-1'), 5.29 (*m*, 1H, H-7), 4.75 (*d*, 2H, J = 2.7 Hz, CH_2-14), 4.60 (*s*, 1H, OH-4'), 4.13 (*m*, 2H, H-3', H-5'), 4.07 (*s*, 3H, OCH_3), 3.58 (*bs*, 2H, CH_2N), 3.25 (*d*, 1H, J = 18.9 Hz, H-10β), 3.02 (*d*, 1H, J = 18.9 Hz, H-10a), 1.24 (*d*, 3H, J = 6.7 Hz, CH_3-6').

Compound 2c. 7.13 mg yield (45%); ^{1}H NMR ($CDCl_3$): δ 13.97 (*s*, 1H, OH-11), 13.27 (*s*, 1H, OH-6), 8.06 (*d*, 1H, J = 7.0 Hz, H-1), 7.86 and 7.46 (two *d*, 4H, J = 8.6 Hz, *para*-substituted phenyl group), 7.79 (*t*, 1H, J = 7.8 Hz, H-2), 7.39 (*d*, 1H, J = 8.4 Hz, H-3), 6.88 (*s*, 2H, maleimido-H), 6.42 (*t*, 1H, J = 5.2 Hz, NH), 5.89 (*d*, 1H, J = 8.7 Hz, NH-3'), 5.49 (*d*, 1H, J = 2.7 Hz, H-1'), 5.29 (*m*, 1H, H-7), 4.75 (*d*, 2H, J = 3.5 Hz, CH_2-14), 4.60 (*s*, 1H, OH-4'), 4.13 (*m*, 2H, H-3', H-5'), 4.08 (*s*, 3H, OCH_3), 3.58 (*bs*, 2H, CH_2N), 3.46 (*m*, 2H, CH_2), 3.25 (*d*, 1H, J = 18.9 Hz, H-10β), 3.03 (*d*, 1H, J = 18.9 Hz, H-10a), 1.24 (*d*, 3H, J = 7.8 Hz, CH_3-6').

pH Optimization of maleimide reaction (1a–c)

1 mM maleimide **1a–c** were prepared from a 10 mM stock solution in DMF. 300 µL of 1 mM maleimide (**1a, b** or **c**) was incubated with 2.7 mL of 0.5 mM DL-cysteine in 0.1 M phosphate buffer, pH 6.5 and 7.0, containing 1 mM EDTA. The reaction of thiol with maleimide was followed between 240 and 300 nm within 1.5 h. Hydrolyses of maleimide **1a–c** were measured by incubating 300 µL of 1 mM maleimide in 2.7 mL of 0.1 M phosphate buffer, containing 1 mM EDTA, pH 6.5, 7.0 and 8.0. Base-catalyzed conversion of the maleimide to maleamic acid was followed from 240 to 300 nm within 1.5 h.

Thiolation of MAbs

MAbs were thiolated by 2-iminothiolane (2-IT).[15] MAbs (1.49 mg mL^{-1} in 0.1 M phosphate buffer, 1 mM EDTA, pH 8.0) were mixed with 2-IT (0.1 M) in molar ratio 1:100. The reaction was incubated for 2 h at room temperature. The thiolated MAbs were freed of excess 2-IT by passage through a Sephadex G-25 column (25 × 0.9 cm) equilibrated with 0.1 M phosphate buffer, 1 mM EDTA, pH 7.0 and then concentrated by Amicon membrane filtration filters (Millipore, Bedford, MA). The number of thiol groups was determined as 6.84/MAb by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).[16]

Conjugation of thiolated MAb with maleimide (3a–c)

In a typical experiment, 10–20 equivalents of Dox–maleimide linker in DMF were added to MAb (1.23 mg mL^{-1}). The conjugation reaction was allowed to proceed for 1.5 h at room temperature. Conjugated drug was separated from unreacted Dox–maleimide spacers by passage through a Sephadex G-25 column (25 × 0.9 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0). Conjugate fractions were concentrated using Amicon membrane filters (Millipore, Bedford, MA).

Determination of drug/antibody molar ratios

The molar extinction coefficients of Dox–maleimide linkers **2a–c** were determined in DMF and summarized in Table 1. Differences in extinction coefficient from the conversion of maleimido group to substituted succinimidyl group was neglected. The Dox/MAb molar ratios were determined by measuring the absorbance at 495 and 280 nm, respectively, and are summarized in Table 2.

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Table 1. Molar extinction coefficient (ε) of Dox derivatives **2a–c**

Compound	λ_{max} (nm)	ε	ε_{280}	ε_{495}	Solvent
2a	267	16853	12508	12380	DMF
2b	267	16248	11413	13236	DMF
2c	267	16017	11754	13061	DMF
IgG	280	214600			PBS

Dox 3'-*N*-amine derivatives (**2a–c**).

Table 2. Spectrophotometric evaluation of the Dox–11-285-14 conjugates

C	C A 280nm	C A 495nm	Dox A 280nm	MAb A 280nm	Dox (µM)	MAb (µM)	Dox/ MAb
3a	0.73	0.12	0.12	0.61	9.70	2.82	3.44
3b	1.51	0.13	0.11	1.40	9.82	6.52	1.51
3c	0.96	0.14	0.12	0.84	10.72	3.91	2.74

C = Conjugate, A = absorbance.

References

1. Arcamone, F. *Doxorubicin Anticancer Antibiotics;* Academic Press: New York, 1981; Chapter 2.

2. Wiernik, P. H. In *Anthracyclines. Current Status and New Developments;* Crooke S. T.; Reich S. D., Eds; Academic Press: New York, 1980; pp. 273–294.

3. Ford, C. H. J.; Casson, A. G. *Cancer Chemother. Pharmacol.* **1986**, *17*, 197.

4. Ghose, T. I.; Blair, A. H.; Kulkarni, P. N. *Methods Enzymol.* **1983**, *93*, 280.

5. Ghose, T. I.; Blair, A. H. *Crit. Rev. Ther. Drug Carrier Syst.* **1987**, *3*, 263.

6. Pramod, K. G. *J. Pharm. Sci.* **1990**, *79*, 949.

7. Hermentin, P.; Seiler, F. R. *Behring Inst. Mitt.* **1988**, *82*, 197.

8. Hermentin, P.; Doenges, R.; Gronski, P.; Bosslet, K.; Kraemer, H. P.; Hoffmann, D.; Zilg, H.; Steinstraesser, A.; Schwarz, A.; Kuhlmann, L.; Luben, G.; Seiler, F. R. *Bioconjugate Chem.* **1990**, *1*, 100.

9. Liu, T. Y. In *The Role of Sulfur in Proteins; The Proteins*, 3rd ed.; Nerrath, H.; Hill, R. L., Eds; Academic Press of San Francisco: USA,1977; Vol. III, pp. 267–268.

10. Knight, P. *Biochem. J.* **1979**, *179*, 191.

11. Richardson, V. J.; Ford, C. H. J.; Tsaltas, G.; Gallant, M. E. *Eur. J. Cancer Clin. Oncol.* **1989**, *25*, 633.

12. Perrin, D. D.; Armarego, L. F. *Purification of Laboratory Chemicals;* 3rd ed.; Pergamon Press: Oxford, 1988.

13. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

14. Birkofer, L.; Ritter, A. *Angew. Chem. Int. Ed.* **1965**, *4*, 417.

15. King, T. P.; Li, Y.; Kochoumian, L. *Biochemistry* **1978**, *17*, 1499.

16. Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49.

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