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Facile synthesis of maleimide bifunctional linkers

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Abstract—The synthesis of maleimide containing derivatives capable of serving as linkers for the conjugation of proteins is described. These compounds differ from previously reported linkers by having multiple sites available for drug attachment. Compounds **3a**, **3b**, and **3d** were synthesized from the corresponding amino alcohols by the addition of two equivalents of *t*-butyl bromoacetate to a series of amino alcohols followed by the introduction of the maleimide under Mitsunobu reaction conditions and ester hydrolysis.

Maleimide linkers[1] such as the readily available **1** and **2** have become very useful in the area of bioconjugate chemistry. These linkers allow the conjugation of sulfhydryl containing molecules such as peptides having cysteine residues, that react with the maleimide functionality, to a second molecule via an amide or ester bond (Fig. 1). This allows the construction of various moieties including peptide-conjugate haptens, immobilized antibodies or enzymes, immuno-conjugates or -toxins, and immunodiagnostic agents.[2]

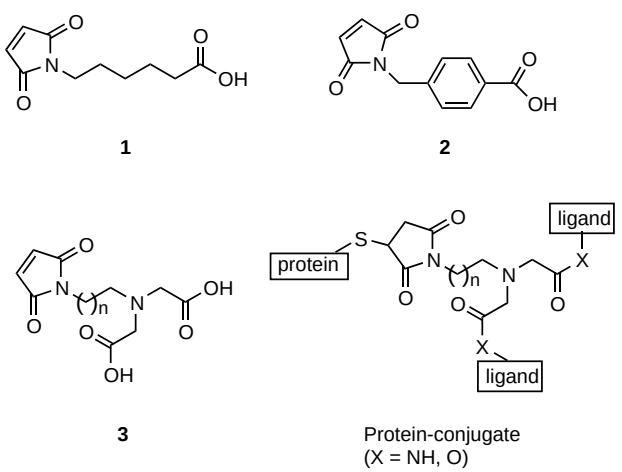
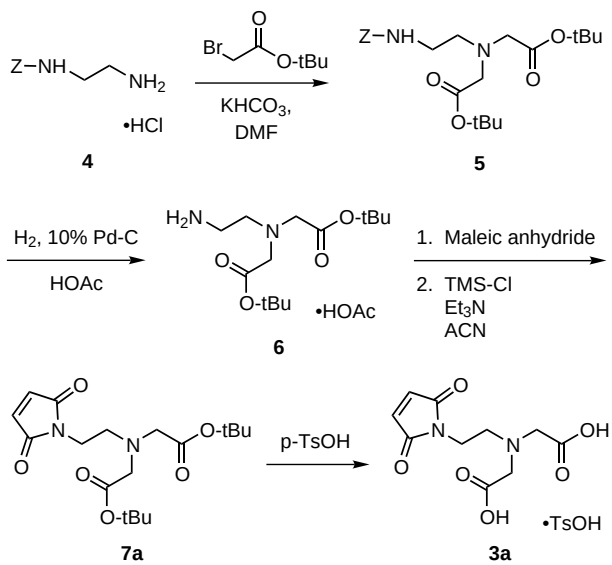


Figure 1. Maleimide linkers and protein conjugate.

Maleimide is a very good linker since it reacts exclusively with cysteine SH groups over other nucleophilic amino acids.[3] Because cysteine is usually present in only discrete numbers, this results in nearly homogenous conjugates where the stoichiometry and site of attachment are predictable. The activity and physical properties of the protein are thus preserved by reducing deleterious conjugation and by maintaining the native pI.

In an effort to synthesize immunoconjugates with improved activity[4] we have designed the maleimide bifunctional linker **3**. Our primary interest was develop-



Scheme 1. Standard synthesis of **3a** (Z = benzyloxycarbonyl).

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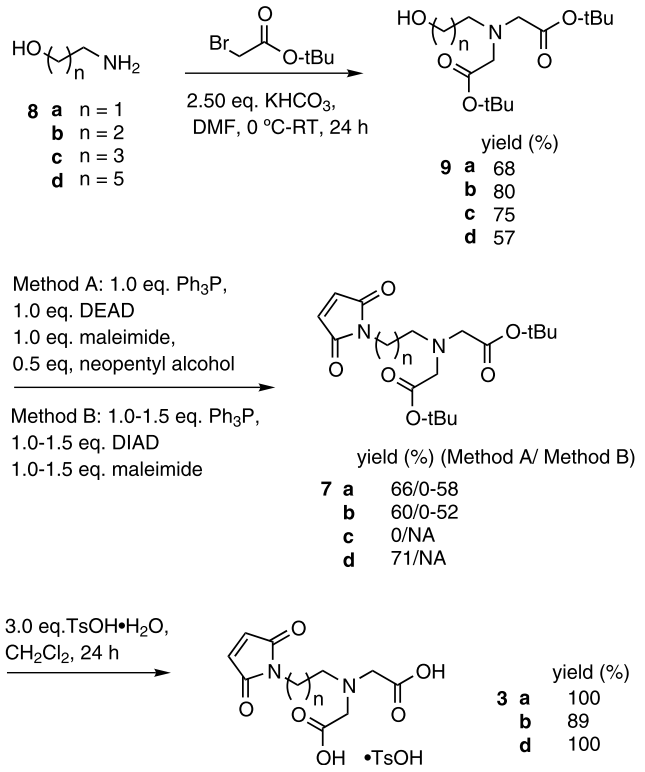


Scheme 2. Modified synthesis of series **3**.

ing a linker which would increase drug loading onto a MAb in the presence of a limited number of available cysteine conjugation sites without causing aggregation. Linker **3** offers the following advantages over **1** and **2** by: (i) the presence of a water solubilizing amine functionality; (ii) the ability to couple two components at a time to the MAb (cf. protein-conjugate, Fig. 1) and (iii) a straight-forward synthesis that can be modified, if necessary, to optimize the linker length. A MAb such as BR96, which generates eight thiol groups on mild DTT reduction would yield a conjugate with a possible mole ratio of 16 drugs per MAb when used with linker **3**.[5]

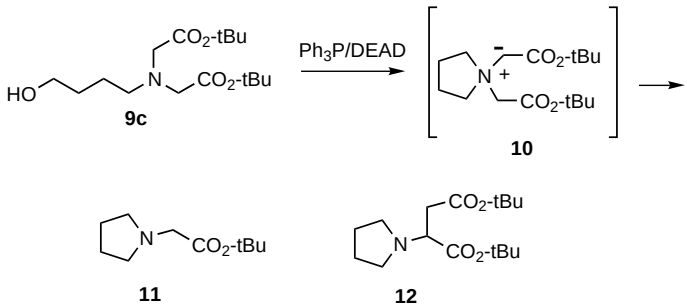
The synthesis of **3a** using standard literature methods requires manipulation of protecting groups on the diaminoalkane, alkylation, and installation of the maleimide via condensation of maleic acid.[6] Thus, **4** was dialkylated with *tert*-butyl bromoacetate to give **5**, which was then deprotected to **6**. Reaction with maleic anhydride followed by dehydration with TMS-Cl/triethylamine yielded the maleimide **7a**. Removal of the *t*-butyl esters was accomplished with *p*-toluenesulphonic acid (Scheme 1).[7] Alternatively, we employed our method for synthesizing *N*-alkyl maleimides, namely the Mitsunobu reaction.[8] Thus, selective protection is not required since the maleimide is introduced via displacement of a hydroxyl group onto an amino alcohol (Scheme 2). This allows the synthesis to be accomplished in just three steps starting from the appropriate amino alcohol.

Amino alcohols **8a–d** were readily alkylated with two equivalents of *tert*-butyl bromoacetate to provide the corresponding bis-esters **9a–d** in good yield. Our modified Mitsunobu conditions (method A), wherein 0.5 equiv. of neopentyl alcohol is used as a non-reacting ligand for phosphorous, gave favorable yields for **7a–b** and **7d**.[9] When unmodified conditions (method B) were used the yields were lower and occasionally, depending on the addition order and reagent stoichiometry, no product was obtained.

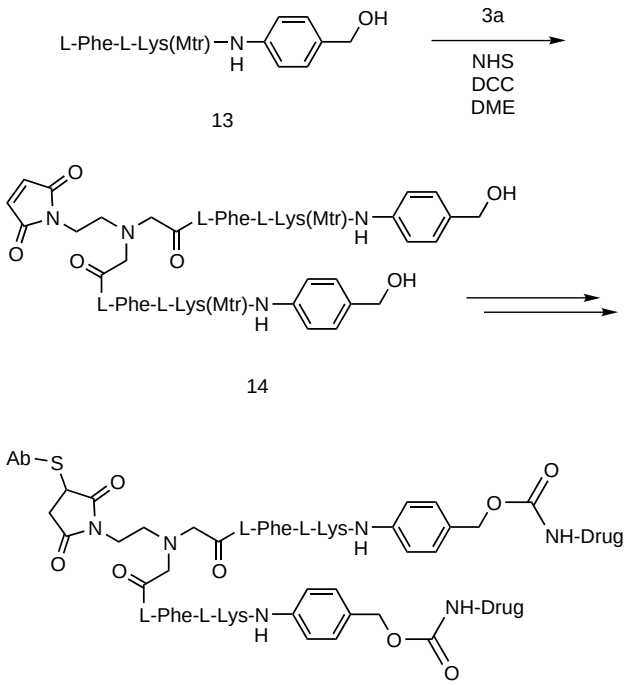
Unexpectedly, the Mitsunobu reaction was not successful for **9c**,[10] rather, a side reaction appeared to be interfering. Instead of the expected product (not shown), **11** and **12** were isolated from the crude reaction mixture in modest yield (~30% combined yield). The by-products, **11** and **12**, can arise from a Stevens rearrangement of the putative zwitterion **10** shown in Scheme 3. In the absence of maleimide and neopentyl alcohol, the reaction of **9c** with Ph_3P and DEAD[11] gives **12** exclusively.[12]

The synthesis of **3a–b,d** was completed by acid mediated removal of the *tert*-butyl ester groups with *p*-toluenesulphonic acid.[13] The resulting linkers can be coupled directly to amines using DCC. As an example, **3a** was coupled to the protected dipeptide amide **13**[14] to give **14** in good yield (Scheme 4). Compound **14** may be further elaborated to couple an NH_2- or OH-containing cytotoxic drug **15**.[14] A full description of this chemistry will be the subject of a future publication.[7]

In conclusion, we have demonstrated a facile three-step synthesis of a unique set of maleimide linkers **3a–b,d**[15]



Scheme 3.



Scheme 4.

which, utilizing standard disconnections, require five–six steps to accomplish. Our synthesis utilizes modified Mitsunobu reaction conditions to install a maleimide moiety in one step onto appropriately substituted amino alcohols. Linkers **3a**–**b**,**d** may be coupled to cytotoxic drugs and used to construct MAb conjugates.

References

1. Wong, S. S. *Chemistry of Protein Conjugation and Cross-linking*; CRC Press: Boca Raton, 1991 and references cited therein.
2. For reviews and recent examples, see: (a) Dubowchik, G. M.; Walker, M. A. *Pharmacol. Ther.* **1999**, *83*, 67–123; (b) Pai, L. H.; Pastan, I. *J. Am. Med. Assoc.* **1993**, *269*, 78; (c) Rusiecki, V. K.; Warne, S. A. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 707; (d) Janda, K. D.; Ashley, J. A.; Jones, T. M.; McLeod, D. A.; Schloeder, D. M.; Weinhouse, M. I. *J. Am. Chem. Soc.* **1990**, *112*, 8886; (e) Pietersz, G. A. *Bioconjugate Chem.* **1990**, *1*, 89; (f) Maggio, E. T. *Enzyme-Immunoassays*; CRC Press: Boca Raton, 1980.
3. Maleimide reacts approximately 1000-times faster with thiols than with amines at neutral pH and below. At this pH the amino groups of lysine and arginine are mostly protonated.
4. Trail, P. A.; Willner, D.; Hellström, K. E. *Drug Develop. Res.* **1995**, *34*, 196.
5. (a) Willner, D.; Trail, P. A.; Hofstead, S. J.; King, H. D.; Lasch, S. J.; Braslawsky, G. R.; Greenfield, R. S.; Kaneko, T.; Firestone, R. A. *Bioconjugate Chem.* **1993**, *4*, 521; (b) King, H. D.; Yurgaitis, D.; Willner, D.; Firestone, R. A.; Yang, M. B.; Lasch, S. J.; Hellstrom, K. E.; Trail, P. A. *Bioconjugate Chem.* **1999**, *10*, 279.
6. For a recent example, see: Reddy, P. Y.; Kondo, S.; Toru, T.; Ueno, Y. *J. Org. Chem.* **1997**, *62*, 2652 and references cited therein.
7. Experimental details for this synthesis are reported in Dubowchik et al., submitted for publication.
8. (a) Walker, M. A. *Tetrahedron Lett.* **1994**, *35*, 665; (b) Walker, M. A. *J. Org. Chem.* **1995**, *60*, 5352.
9. Representative synthetic procedure for **7a**: In a 1000 mL 3-neck round bottomed flask, equipped with two dropping funnels, Ph_3P (27.0 g, 0.103 mol) was dissolved in 700 mL of THF and cooled to −78°C. DEAD (16.22 mL, 0.103 mol) was added dropwise via a dropping funnel and the resulting light yellow solution stirred 5 min. Compound **9a** (29.7 g, 0.103 mol) was dissolved in 100 mL of THF and added dropwise over 15 min using the second dropping funnel. Neopentyl alcohol (4.54 g, 0.052 mol) was added in one portion followed by maleimide (10 g, 0.103 mol). The flask was removed from the cooling bath and allowed to stir overnight. The reaction mixture was concentrated to 1/4 the original volume by rotary evaporation and purified by flash column chromatography with 6:1 hexanes/EtOAc on SiO_2. Pure **7a** (25 g, 66% yield) was isolated as a solid mp 64–66°C. ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9), 2.85 (t, 2, $J=6.4$), 3.36 (s, 4), 3.54 (t, 2, $J=6.5$), 6.62 (s, 2). ^{13}C NMR ($CDCl_3$) δ 28.08, 35.68, 51.42, 55.34, 80.92, 134.03, 170.35, 170.82. **7b**: Solid mp 51–52°C. ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9), 1.62 (p, 2, $J=7.1$), 2.61 (t, 2, $J=7.0$), 3.29 (s, 3), 3.48 (t, 2, $J=7.1$), 6.57 (s, 2). ^{13}C NMR ($CDCl_3$) δ 27.74, 35.82, 51.28, 55.50, 80.78, 134.01, 134.92, 170.41, 170.69. **7d**: Oil. ^{1}H NMR ($CDCl_3$): δ 1.04 (m, 6), 1.22 (s, 18), 1.30 (m, 2), 2.42 (dd, 2, $J=7.27$), 3.17 (s, 4), 3.26 (t, 2, $J=7.2$), 6.45 (s, 2). ^{13}C NMR ($CDCl_3$): δ 26.49, 26.55, 27.69, 27.99, 28.33, 37.64, 53.83, 55.72, 80.61, 133.86, 170.54, 170.66.
10. We have found that the Mitsunobu procedure is difficult for amino alcohols which have an amine group which is optimally positioned for cyclization and/or has a higher p*K*a.
11. Abbreviations: DEAD (diethylazodicarboxylate), DIAD (diisopropylazodicarboxylate), Mtr (methoxytrityl), DCC (dicyclohexylcarbodiimide).
12. For a more detailed discussion of this side reaction see: Walker, M. A. *Tetrahedron Lett.* **1996**, *37*, 8133.
13. *p*-Toluenesulphonic acid (TsOH) was found to be superior to trifluoroacetic acid in this procedure and also provides the products as solid *p*-tosylate salts, making them convenient to handle and purify.
14. (a) Dubowchik, G. M.; Radia, S. *Tetrahedron Lett.* **1997**, *38*, 5257; (b) Dubowchik, G. M.; Firestone, R. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3341; (c) Dubowchik, G. M.; Mosure, K.; Knipe, J. O.; Firestone, R. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3347; (d) De Groot, F. M. H.; de Bart, A. C. W.; Verheijen, J. H.; Scheeren, H. W. *J. Med. Chem.* **1999**, *42*, 5277.
15. Analytical data **3a**: ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3), 3.51 (m, 2), 3.80 (m, 2), 4.21 (s, 4), 7.01 (s, 2), 7.10 (d, 2, $J=8.0$), 7.50 (d, 2, $J=8.0$), 11.09 (br s, 2). ^{13}C NMR (DMSO-d_6): δ 20.86, 32.28, 52.54, 53.66, 125.58, 128.39, 134.96, 138.47, 144.65, 167.44, 170.84. MS (HR ESI) calcd for $C_{10}H_{12}N_2O_6{\cdot}H^+$ (MH^+): 257.0774. Found: 257.0779. **3b**: ^{1}H NMR (DMSO-d_6): δ 1.88 (m, 2), 2.28 (s, 3), 3.20 (m, 2), 3.42 (m, 2), 4.12 (s, 4), 7.04 (s, 2), 7.11 (d, 2, $J=8.0$), 7.48 (d, 2, $J=8.0$). ^{13}C NMR (DMSO-d_6): δ 20.75, 23.55, 34.23, 53.68, 54.21, 125.46, 128.07, 134.58,

137.71, 145.49, 167.69, 171.01. Anal. calcd for $C_{11}H_{14}N_2O_6 \cdot C_7H_8SO_3$: C, 48.86; H, 5.01; N, 6.33. Found: C, 48.66; H, 5.07; N, 6.21. **3d**: ^{1}H NMR (DMSO-d_6): δ 1.08 (m, 4), 1.45 (m, 2), 1.59 (m, 2), 2.28 (s, 3), 3.18 (m, 2), 3.37 (t, 2, $J=6.9$), 4.14 (s, 4), 7.01 (s, 2), 7.11 (d, 2, $J=8.0$), 7.48 (d, 2, $J=8.0$). ^{13}C NMR (DMSO-d_6): δ 20.75, 22.98, 25.29, 25.58, 27.72, 36.89, 54.11, 55.94, 125.46, 128.06, 134.45, 137.72, 145.48, 167.63, 171.08. MS (HR ESI) calcd for $C_{14}H_{20}N_2O_9 \cdot H^+$ (MH^+): 313.1400. Found: 313.1408.