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Synthesis of a homotrifunctional conjugation reagent based on maleimide chemistry

Omar K. Farha,[†] Richard L. Julius[†] and M. Frederick Hawthorne[*]

Department of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, CA 90095-1569, USA

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Abstract—A novel homotrifunctional conjugation reagent, 1,3,5-tris-(*N*-maleimidomethyl)benzene has been synthesized in high yield with minimum purification. The reactivity of this compound was examined by using L-cysteine as a nucleophile.

1. Introduction

Maleimide-containing compounds are a widely utilized class of substrates employed in chemical and biological applications due to their reactivity in Michael-addition reactions and their dienophilic nature.[1] This reactivity is exemplified by the fact that the maleimido group is the reagent of choice for functionalizing thiols, including the cysteine residues of a protein.[2] In addition of being an excellent Michael-acceptor, maleimides are also highly chemoselective, reacting 10^3 times faster with a thiol group than an amine group at neutral pH and below.[3] Consequently, maleimides are model reagents for use in a biological setting. In the preponderance of proteins or peptides, free thiols are uncommon, providing ideal targets for modification at a defined site. Modern techniques of peptide synthesis or protein engineering allow facile incorporation of a cysteine residue at a defined location in a peptide or protein.[4] Such cysteines may be located on the protein surface allowing for straightforward site-specific attachment with predictable stoichiometry without significantly altering biological activity.

The exploitation of purposely introduced as well as wild-type thiol functionality through multifunctional conjugation reagents involving both intramolecular and intermolecular cross-linking has been an invaluable tool for the study of proteins. Homobifunctional conjugation reagents have been used as molecular rulers[5] to allow the determination of the distance between two cross-linked amino acid residues.[6] This information can be pertinent to the determination of the tertiary and quaternary structure of proteins,[7] and is especially valuable for proteins that are difficult to crystallize[8] or to refine low-resolution crystal structures.[9] These intramolecular cross-linking distances are also useful to probe the conformational states of an allosteric enzyme[10] in addition to characterizing transmembrane transport channels[11] and hormone receptors.[12] Intermolecular protein cross-linking has shown similar efficacy in elucidating protein–protein interactions,[13] improving both the pharmacodynamics[14] and potency[15] of protein therapeutics as well as facilitating the development of enzyme–antibody conjugates for enzyme immunoassay.[16]

Similarly, homotrifunctional conjugation reagents have been used to characterize the drug binding domains of ATP-binding cassette transporters[17] and to define the catalytically active form of membrane enzymes.[18] However, the usefulness of current tris-maleimido compounds is hindered by inefficient synthetic routes and the utilization of a nucleophilic nitrogen as the central core atom.[19] In an effort to expand upon these successes, a versatile, stable trishomofunctional conjugation reagent based upon maleimide chemistry was developed and is described here.

Several literature precedents exist for N-alkylation of a variety of imides under Mitsunobu conditions.[20] The

Keywords: Maleimide; Cysteine; Diels–Alder; Mitsunobu; Thiol; Homotrifunctional conjugation reagent; 1,3,5-Tris(hydroxymethyl)-benzene; 1,3,5-Tris-(*N*-maleimidomethyl)benzene.
* Corresponding author. Tel.: +1 310 825 7378; fax: +1 310 825 5490; e-mail: mfh@chem.ucla.edu
† Both authors contributed equally to this work.

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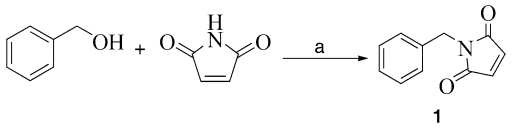
Mitsunobu method has several advantages including in situ generation of the reactive species characterized by a very labile leaving group under mild reaction conditions. However, the scope of N-alkylation of imides was limited to substrates that have no electrophilic reaction centers, such as phthalimide and succinimide, due to the requirement of nucleophilic triphenyl phosphine as a Mitsunobu reactant. Walker overcame this obstacle by changing the order of reagent addition in a modified Mitsunobu procedure to produce high yields of N-alkylated maleimides using alcohols as convenient starting materials.[1,21] However, this method was only applied to simple alkyl systems.[1,21] In this letter, we expand this simple synthetic approach utilizing Mitsunobu reaction conditions by combining the reversible Diels–Alder addition of furan to maleimide[22] to serve as a protective group for the maleimide double bond against nucleophiles. This allows the synthesis of the desired homotrifunctional maleimide conjugation reagent (**6**) derived from 1,3,5-tris(hydroxymethyl)benzene.

2. Results and discussion

The majority of literature methods describing the synthesis of N-substituted maleimides involve reaction of a primary amine with maleic anhydride followed by subsequent dehydration of the intermediate maleamic acid.[1] This reaction is limited by the required harsh reaction conditions as well as the necessity of employing primary amines as starting materials.[1,23] Even under suitable circumstances, yields can be compromised by the low solubility of the maleamic acid and the weak nucleophilicity of the amines.[1,23] Previous reports of the synthesis of tris-maleimido amines, tris(2-malemidoethyl)amine,[19a] and tris(4-maleimidophenyl)amine,[19b] suffer from low yields, 29% and 12%, respectively. This is presumably due to difficulties resulting from the ring closure step as well as the reaction of the nucleophilic amine core with the maleimide double bond.

Three alternative methods for direct N-alkylation of maleimides have been proposed. The first, by Lerner and Schwartz,[24] involves generation of the silver or mercury salt of maleimide followed by reaction with an alkyl bromide. However, this reaction was hindered by the stability of the salts, which generally led to low yields and only with simple alkyl chains.[1,24] A recent report by Turnbull describing the direct use of benzyl halides in the presence of K_2CO_3 has its own limitation due to the reactivity of the benzyl halides.[25] In an effort to avoid these problems the method described by Walker using alcohols as starting materials for direct N-alkylation of maleimide under mild Mitsunobu conditions was adopted.[1,21]

Using benzyl alcohol in a model system, direct N-alkylation of maleimide was attempted, as shown in Scheme 1. As previously reported,[1] the order of addition of reactants was found to be important to reaction yield. When to PPh_3, initially present, was added DEAD (diethyl azodicarboxylate), alcohol, and finally maleimide (in the order given), **1** was isolated at 86% yield.



1

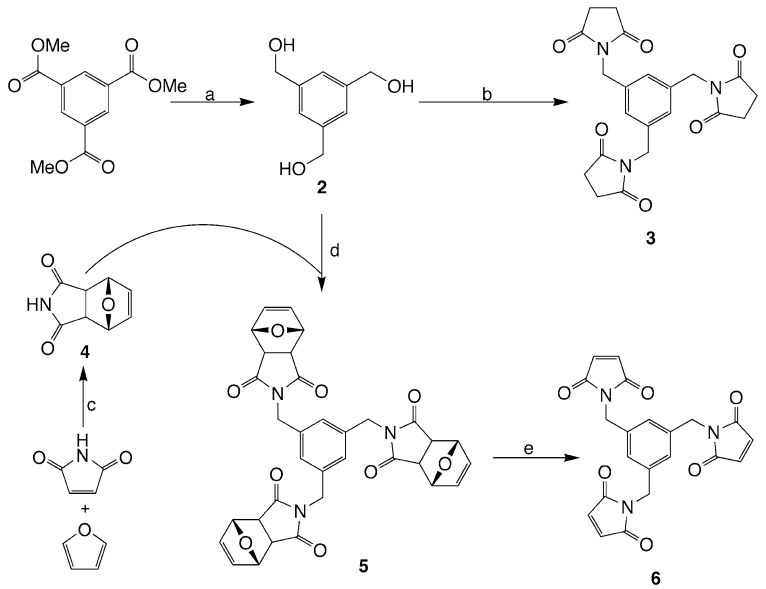
Scheme 1. Reagents: (a) THF, PPh_3, and DEAD.

Synthesis of the tris-maleimido derivative, **6**, began with the reduction of trimethylbenzene-1,3,5-tricarboxylate by lithium aluminum hydride, as shown in Scheme 2, to produce **2**, in 90% yield.[26] Subsequent reaction of **2** under Mitsunobu conditions with maleimide resulted in the production of a red-brown polymeric material insoluble in all common laboratory solvents. Consequently, a model Mitsunobu reaction was investigated in which succinimide was substituted for maleimide (Scheme 2). Succinimide exhibits structural and chemical features similar to those of maleimide, but without the ability to undergo Michael addition. The reaction of succinimide with **2** under Mitsunobu conditions produced **3**[27] in 77% yield, validating the sensitivity of the unprotected maleimide double bond to any free nucleophiles. The fortuitous precipitation of **3** (and later, **5**[27]) from the reaction mixture eliminated the need for a laborious chromatographic isolation of product from spent and excess reagents associated with the Mitsunobu reaction.[28]

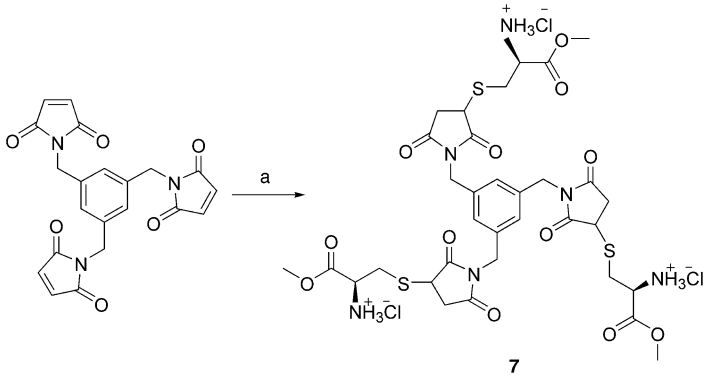
Protection of the maleimide double bond was accomplished through a Diels–Alder reaction using furan as a diene and maleimide as a dienophile. Both the *endo* and *exo* isomers of **4** were isolated, however, both were useful for this application. Production of **4** allowed the reaction of **2** and **4** without the possibility of nucleophile-initiated polymerization. The reaction of **2** with **4** under the conditions used to produce **3** similarly produced **5** in 70% yield while no polymerization products were observed. Heating **5** in anisole for 1 h at the reflux temperature accomplished the retro-Diels–Alder reaction and led to the isolation of the final product, **6**,[29] in 82% yield after column chromatography.

The reactivity and efficacy of the final product **6** in thiol conjugation was investigated using its reaction with L-cysteine methyl ester hydrochloride under rigorously oxygen-free conditions illustrated in Scheme 3. L-Cysteine methyl ester hydrochloride was chosen as a simple representative thiol-containing molecule with peripheral functional groups suitable for further functionalization while at the same time providing an idealized demonstration of peptide conjugation. Overnight reaction of **6** with L-cysteine methyl ester hydrochloride in 1:1 water/acetonitrile at room temperature afforded the tris-conjugated **7**,[30] confirmed by APCI-MS, in greater than 90% yield as determined by ^1H NMR integration.

In summary, production of molecules containing multiple maleimide functionalities using common techniques is encumbered by a variety of factors, including the stability and reactivity of the starting materials as well as by solubility issues. Described here is the facile synthesis of a novel homotrifunctional conjugation reagent, **6**, based on maleimide chemistry, which necessitates a min-



Scheme 2. Reagents and conditions: (a) THF, $LiAlH_4$, and H_2O; (b) THF, PPh_3, DEAD, succinimide; (c) H_2O; (d) THF, PPh_3, DEAD; (e) anisole, Δ.



Scheme 3. Reagents: (a) H_2O, MeCN, L-cysteine methyl ester hydrochloride.

imum of purification. The application of a Diels–Alder reaction with furan to protect the maleimide double bond from nucleophilic attack combined with a modified Mitsunobu reaction to provide N-alkylation of the maleimide–furan adduct produced **5**. Subsequent thermal cleavage of the furan protecting group afforded the desired product **6** in good yield. The reactivity of the maleimide moieties present in **6** was investigated through its tris-conjugation with L-cysteine methyl ester hydrochloride, a model for peptide conjugation, which also provides a suitable reagent for further derivation. APCI-MS confirmed tris-addition to the maleimide functions, validating the utility of **6**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.02.032.

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27. General procedure for the preparation of compounds **3** and **5**: A 250 mL round-bottom flask was charged with PPh_3 (1.22 g, 4.58 mmol) to which was added 10 mL of THF. The resulting clear solution was cooled to −78 °C and DEAD (0.70 mL, 4.58 mmol) was added slowly. The yellow solution was stirred for 5 min and then **2** (0.25 g, 1.52 mmol) was added over 2 min and stirred for 10 min. Succinimide (453 mg, 4.58 mmol) or **4** (1.00 g, 6.02 mmol) was dissolved in 20 mL THF then added to the reaction mixture. The resulting solution was allowed to remain at −78 °C for an additional 10 min. The reaction was stirred at room temperature for 12 h. The product, **3** or **5**, separated from solution as a white solid, which was removed by filtration and dried under vacuum to yield 0.50 g (77%) of **3** or 0.65 g (70%) of **5**. Compound **3**: white solid. Mp 165.0–169.5 °C. ^{1}H NMR (400 MHz, CD_2Cl_2): 2.69 (12H), 4.57 (6H), 7.18 (3H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): 28.2, 41.7, 127.66, 136.8, 176.9; HRMS (EI^+) *m*/*z* calcd for $C_{21}H_{21}N_3O_6$: 411.1430, found: 411.1430. Compound **5**: white solid. Decomposition point 120 °C. ^{1}H NMR (400 MHz, CD_2Cl_2): 3.51 (6H), 4.37 (6H), 5.27 (6H), 6.27 (6H), 6.97 (3H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): 41.7, 46.0, 79.5, 127.7, 134.5, 136.3, 174.5; HRMS (APCI) *m*/*z* calcd for $C_{21}H_{15}O_6N_3Na^+$ (M+Na−3 furan): 428.0853, found: 428.0841.
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29. A typical procedure is as follows. A suspension of 0.25 g (0.41 mmol) of **5** in 15 mL of anisole was heated at 145 °C for 1 h. The reaction was cooled to room temperature and then chromatographed on silica employing hexanes to remove the anisole followed by EtOAc to elute **6**. The solvents were removed via rotary evaporation and the product was dried under vacuum to yield 0.13 g (82%) of **6** as a white solid. Mp 173.0–175.0 °C. ^{1}H NMR (400 MHz, CD_2Cl_2): 4.60 (6H), 6.70 (6H), 7.16 (3H); ^{13}C NMR (100.6 MHz, CD_2Cl_2): 40.8, 127.3, 134.1, 137.1, 170.1; HRMS (MALDI) *m*/*z* calcd for $C_{21}H_{15}O_6N_3Na^+$: 428.0853, found: 428.0846.
30. The test reaction of **6** with L-cysteine methyl ester hydrochloride was performed as follows. Solvents were deoxygenated by using the following method: The sample container, provided with a magnetic stirrer, was subject to a partial vacuum that was broken with argon. This process was repeated four times. A deoxygenated water solution (1 mL) of L-cysteine methyl ester hydrochloride (40 mg, 0.23 mmol) was added to a deoxygenated solution of **6** (30 mg, 0.074 mmol) in acetonitrile (1 mL) and was stirred under argon for 12 h. The solution was concentrated via rotary evaporation and the resulting residue was lyophilized to give a white solid. APCI-MS calcd for $C_{33}H_{45}N_3O_{12}S_3Cl_3H^+$: 879.3, found: 879.5.