VIP Bioconjugation Very Important Paper

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Exocyclic Olefinic Maleimides: Synthesis and Application for Stable and Thiol-Selective Bioconjugation

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Abstract: *Michael addition reactions between biological thiols and endocyclic olefinic maleimides are extensively used for site-specific bioconjugation. The resulting thio-succinimidyl linkages, however, lack stability because of their susceptibility to thiol exchange. Reported herein is that in contrast to their endocyclic counterparts, exocyclic olefinic maleimides form highly stable thio-Michael adducts which resist thiol exchange at physiological conditions. A high-yielding approach for synthesizing a variety of exocyclic olefinic maleimides, by 4-nitrophenol-catalyzed solvent-free Wittig reactions, is reported. Mechanistic studies reveal that the catalyst facilitates the formation of the Wittig ylide intermediate through sequential proton donation and abstraction. Overall, this report details an improved thiol bioconjugation approach, a facile method for synthesizing exocyclic olefinic maleimides, and demonstrates that phenolic compounds can catalyze ylide formation.*

Modern bioconjugation approaches owe their tremendous success to chemoselective organic reactions which proceed in high yields under physiological conditions.[1] One such reaction, Michael addition of thiols to endocyclic C=C bonds of maleimides (**A**; Figure 1 a), is the mainstay of site-specific protein bioconjugation. Indeed, both of the antibody-drug conjugates that are currently on the market employ this chemistry.[2] Unfortunately, however, recent work has demonstrated a major drawback of this methodology—the resulting thio-succinimidyl adducts (**B**) are prone to breakdown as a result of thiol exchange reactions[3] which involve an initial reverse Michael reaction to form the starting maleimide (**C**) and subsequent Michael addition with thiols present in vivo, including cysteine residues of proteins such as serum albumin,[3a] to generate side products (**D**).

The observation that thiol exchange on the ring-opened carboxylates **E** and **F** (Figure 1 a) is much slower than on the intact conjugate **B**,[3b–d,4] has led to the development of maleimide derivatives which form thio-succinimidyl rings and then hydrolyze rapidly to form relatively stable ring-opened thiol adducts.[3b,d] Although extremely successful on antibody-drug conjugates,[3b,d] this approach is not ideal for applications, such as studies on the cellular internalization of cell-penetrating peptides,[5] which depend on the net charge of the bioconjugate, as it results in the introduction of an additional negative charge through the installation of a carboxylate group into the ring-opened products (**E** and **F**). Another detrimental effect of ring opening is that it alters the spectral properties of fluorophores attached to biomolecules through thio-succinimidyl linkages, as previously observed for *N*-1-pyrene maleimides.[6] These drawbacks have motivated researchers to explore alternative scaffolds for thiol bioconjugation.[7] Although these new approaches are promising, it remains to be seen whether these linkages will demonstrate suitable pharmacokinetic and metabolic profiles for in vivo applications,[8] as compared to maleimide conjugates which are routinely utilized for such applications. Consequently, the development of stable thiol-adduct-forming maleimide derivatives which contain only subtle modifications on the tried and tested maleimide scaffold, and do not rely on thio-succinimidyl ring opening to impart stability, is highly desirable.

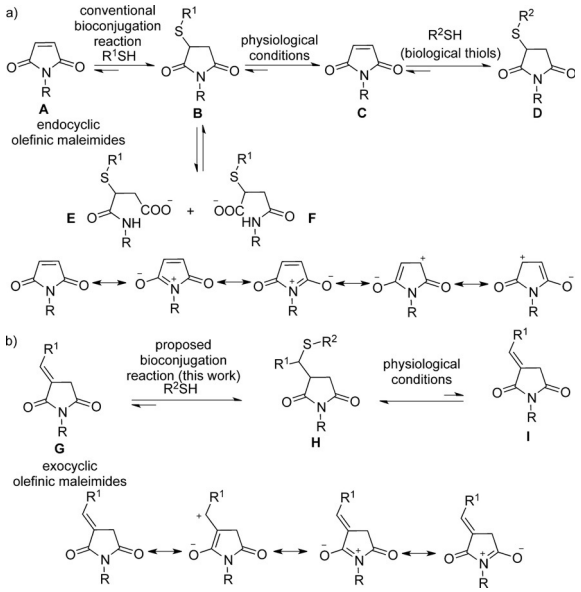

Figure 1. Thiol bioconjugation with maleimides. a) Conventional methodology using endocyclic olefinic maleimides. b) Our proposed approach using exocyclic olefinic maleimides. Resonance structures of each scaffold are provided in the bottom panels.

We sought to achieve this goal by designing maleimide derivatives that would form thiol adducts resistant reverse Michael reactions, thereby preventing thiol exchange. We reasoned that exocyclic olefinic maleimides (**G** in Figure 1 b) could possess this attribute as their reverse Michael products (**I**) are not as well resonance-stabilized (bottom panel of both Figure 1 a and b).

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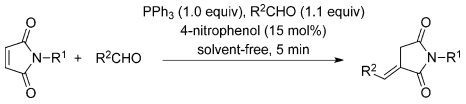
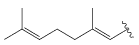
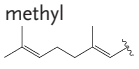
To test this hypothesis, we first needed to synthesize exocyclic olefinic maleimides. Literature searches indicated that most state-of-art approaches are low yielding.[9] Additionally, the most commonly used method, involving 1,4-addition of PPh_3 to endocyclic olefinic maleimides followed by Wittig reaction with aldehydes, requires high reaction temperatures, which are incompatible with low-boiling aldehydes.[10] To overcome these drawbacks, we sought to utilize efficient catalysts for this reaction. Motivated by previous reports that another reaction, which proceeds by 1,4-addition of PPh_3 to Michael acceptors (the PPh_3-catalyzed Morita–Baylis–Hillman reaction), is catalyzed by phenolic compounds,[11] we treated benzaldehyde, 2,6-dichlorobenzaldehyde, and 4-bromobenzaldehyde with endocyclic *N*-ethyl and *N*-benzyl maleimides in the presence of PPh_3 and 15 mol% of either phenol or 4-nitrophenol as potential catalysts (see Table S1 in the Supporting Information). In the presence of 4-nitrophenol, all the reactions were complete within 5 minutes, exclusively yielding *E* isomers of the desired exocyclic olefinic maleimides. Notably, the reactions proceeded under solvent-free conditions, thus rendering this approach environmentally friendly. In the absence of catalysts, only 10–23% yield was obtained (see Table S1). We successfully employed this methodology for the synthesis of 23 structurally diverse exocyclic olefinic maleimides in moderate to high yields (Table 1), thus establishing its compatibility with aromatic aldehydes containing both electron-withdrawing (**1**–**4**, **14**, **20**, and **21**) and electron-donating (**15** and **16**) groups, conjugated aldehydes (**13**, **17, 18**, and **23**), sterically-hindered aldehydes (**7**, **9**, **12**, **13**, and **19**), and low-boiling aldehydes (**8** and **22**).

Table 1: Our approach for the synthesis of exocyclic olefinic maleimides.



Product	R^1	R^2	Yield [%]
1	Bn	4-nitrophenyl	79
2	Bn	3-nitrophenyl	87
3	Bn	4-bromophenyl	87
4	Bn	2,6-dichlorophenyl	92
5	Bn	phenyl	86
6	Bn	propyl	93
7	Bn		82
8	Bn	methyl	79
9	Bn	*tert*-butyl	56
10	Bn	2-pyridyl	52
11	Bn	benzyl	65
12	Bn	isopropyl	78
13	Bn		74
14	Bn	4-fluorophenyl	78
15	Bn	4-methylphenyl	77
16	Bn	3-methoxyphenyl	80
17	Bn		71
18	Bn		82
19	Et		74
20	Et	4-nitrophenyl	88
21	Et	2,6-dichlorophenyl	86
22	Et	methyl	70
23	Et		82

This remarkable catalytic effect of 4-nitrophenol motivated us to investigate the mechanistic basis of its action. ^{31}P NMR studies on the uncatalyzed reaction revealed that upon adding *N*-benzyl maleimide to PPh_3 (molar ratio 1:1), the peak at $\delta = -4.8$ ppm for PPh_3 slowly disappeared over 32 hours and was replaced by peaks at $\delta = +12.2$ and $+13.3$ ppm (see Figure S1a–e in the Supporting Information). These chemical shift values are consistent with those of positively charged phosphines[12] and were attributed to the phosphonium enolate **J** and the phosphonium ylide **K** (Figure 2a). In contrast, in the presence of 4-nitrophenol, a single peak at $\delta = +13.9$ ppm was observed within 1 minute (see Figure S1f). ^1H NMR analysis of this sample (see Figure S2) revealed that this species was **K**, thus demonstrating that 4-nitrophenol profoundly facilitates ylide formation, which upon addition of the aldehyde (butyraldehyde in this case) undergoes a Wittig reaction to generate Ph_3PO (peak at $\delta = +32.8$ ppm in Figure S1g). When deuterium-enriched 4-nitrophenol (50 mol% deuteration determined by NMR spectroscopy; see Figure S3) was used in equimolar catalyst/substrate ratio in a reaction between 4-bromobenzaldehyde and *N*-benzyl maleimide, 50% deuteration of the product **24** was observed (see Figure 2c and Figure S4), thus strongly suggesting that the catalyst acts by donating its phenolic proton to **J** to form **L** (Figure 2b). The conjugate base of the catalyst thus formed can then abstract a proton from **L** to form **K**.

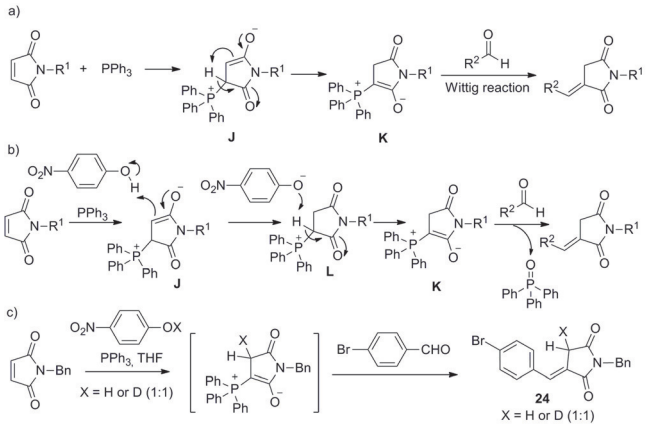


Figure 2. Mechanism of conversion of endocyclic olefinic maleimides into exocyclic olefinic maleimides. a) Uncatalyzed reaction. b) 4-Nitrophenol-catalyzed reaction. c) Deuterium-labeling experiment.

To compare the reverse Michael addition rates of thiol conjugates of exocyclic and endocyclic olefinic maleimides, we designed a continuous spectrophotometric assay involving chromogenic adducts of these scaffolds (compounds **25** and **26** in Figure 3; see Figure S5). Reverse Michael reactions on both these compounds would yield the 4-nitrothiophenolate anion (A_{max} at 410 nm), thus allowing monitoring of the reaction progress. Incubation of **25** at pH 7 at 20 °C resulted in

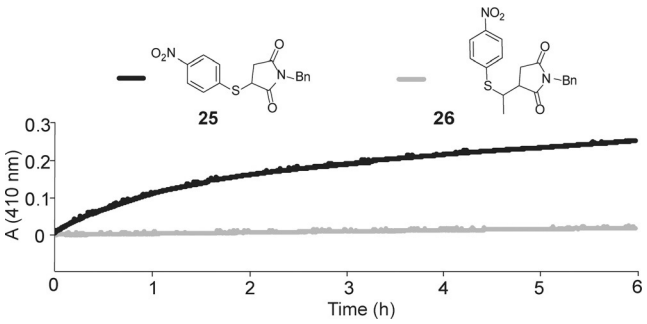


Figure 3. Kinetic assays for reverse Michael reactions on chromogenic thiol adducts of endocyclic (**25**) and exocyclic (**26**) olefinic maleimides.

a steady increase in $A_{410\,nm}$ over 6 hours, whereas $A_{410\,nm}$ for **26** remained unchanged, thus demonstrating the higher stability of the exocyclic olefinic maleimide adduct.

We next evaluated the thiol reactivity and selectivity of exocyclic olefinic maleimides in aqueous solutions by treating **8** with *N*-acetyl cysteine and *N*-acetyl lysine. These studies (see Figure S6) revealed that exocyclic olefinic maleimides react rapidly and selectively with *N*-acetyl cysteine under physiological conditions. Encouraged by these results, we explored the potential of these scaffolds for protein bioconjugation by synthesizing a fluorescent exocyclic olefinic maleimide derivative (**27** in Figure 4a; see Scheme S1) and

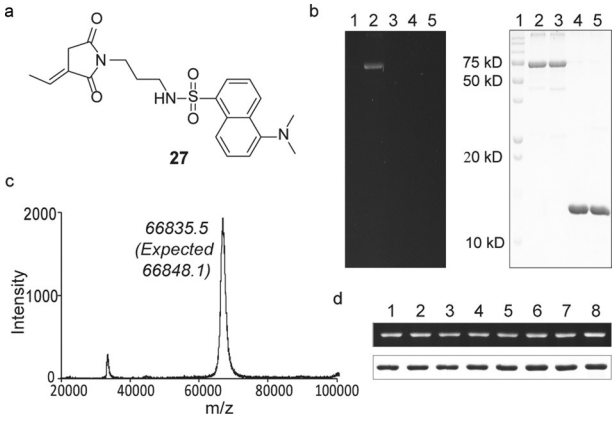


Figure 4. Protein labeling with exocyclic olefinic maleimides. a) The exocyclic dansyl fluorophore used for labeling. b) SDS-PAGE analysis of labeling: fluorescence (left) and Coomassie staining (right). Lane 1: Molecular weight marker, 2: BSA with **27**; 3: BSA without **27**; 4: Lysozyme with **27**; 5: Lysozyme without **27**. c) MALDI spectrum of BSA-**27** conjugate. d) Evaluation of thiol exchange on BSA-**27** conjugate: SDS-PAGE analysis (top: fluorescence and bottom: Coomassie staining) of aliquots taken at different time points from a mixture of BSA-**27** conjugate (0.25 mM) and *N*-acetyl cysteine (10 mM), in sodium phosphate buffer (pH 7, 125 mM). Lane 1: 0 d (before adding *N*-acetyl cysteine), 2: 1 d, 3: 2 d, 4: 3 d, 5: 4 d, 6: 5 d, 7: 6 d, 8: 7 d.

treating it with bovine serum albumin (BSA), a protein which contains 1 free-cysteine residue, at pH 7 (for a detailed procedure see the Supporting Information). SDS-PAGE analysis of this mixture revealed that BSA was rendered fluorescent (lane 2, Figure 4b). In contrast, when lysozyme, a protein that does not contain any free-cysteines (but contains other nucleophilic residues including 6 lysines, 11 arginines, and 1 histidine) was treated with **27**, no labeling was observed (lane 4, Figure 4b). MALDI mass spectrometry on the BSA-**27** conjugate yielded an *m*/*z* value consistent with the attachment of a single dansyl moiety to the protein (Figure 4c). Taken together, these studies convincingly demonstrate that exocyclic olefinic maleimides react selectively with cysteine residues of proteins.

To evaluate its propensity for thiol exchange, we incubated the BSA-**27** conjugate with 40-fold molar excess of a thiol trap (*N*-acetyl cysteine) to drive thiol exchange. SDS-PAGE analysis revealed no time-dependent reduction in the fluorescence intensity of the conjugate over 7 days at 37 °C (Figure 4d), thus suggesting negligible thiol exchange with the thiol trap. This result is in sharp contrast to that obtained in a similar experiment with the endocyclic olefinic maleimide protein conjugate, Cimzia, which showed significant thiol exchange.[3b] The suitability of these scaffolds for bioconjugation was further emphasized by our experiments with glutathione and *N*-acetyl cysteine as model thiols, which demonstrated favorable conjugation kinetics at physiological pH and high conjugate stability under reducing conditions, in presence of excess thiol, and under acidic and basic conditions (see Figure S7).

In summary, we have developed a high-yielding, straight forward, and generally applicable synthetic route for exocyclic olefinic maleimides by developing a modified solvent-free Wittig reaction catalyzed by phenolic compounds. Mechanistic studies demonstrate that the catalyst acts by facilitating the formation of an ylide intermediate by acid-base chemistry. We report that exocyclic olefinic maleimides are attractive scaffolds for site-specific bioconjugation as they react specifically with thiols at physiological conditions to form stable linkages which resist thiol-exchange-mediated breakdown. In addition to bioconjugation applications, the advances in the synthesis of exocyclic olefinic maleimides reported herein will facilitate realization of the tremendous potential of these scaffolds for the preparation of heterocyclic compounds as underscored by their previous use for the synthesis of substituted maleic anhydrides,[10a] endocyclic olefinic maleimides,[10b,13] succinimides,[14] and pyrrolidines,[14] and encourage exploration of their considerable therapeutic potential.[15]

Acknowledgments

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Keywords: bioconjugation · Michael addition · olefins · sulfur · Wittig reactions

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