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Development of novel linkers to conjugate pharmacophores to a carrier antibody

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ABSTRACT

We have developed modified maleimide novel linkers with improved chemical stability that could potentially be used in conjugating various pharmacophores such as oligo nucleotides, peptides, and proteins to antibodies to afford novel biologics with well-defined therapeutic benefits and improved pharmacokinetic properties. These linkers expand the array of tools available for bioconjugation of pharmacophores to antibodies.

Protein based drugs have enabled targeted modulation of biologically relevant targets not easily druggable by small molecules. There is a growing interest in developing chemical, biochemical, and genetic tools for doing 'medicinal chemistry' with proteins in order to optimize their pharmaceutical properties. One of the areas of focus is developing new bioconjugation chemistries that enable modulation of specific residues on a protein with improved pharmacokinetic and pharmacodynamic properties. Many of these proteins have been modified chemically for various purposes such as; proteins attached to polyethylene glycol as novel therapeutics; proteins attached to chemical probes for studying cellular function; proteins with photoreactive group to study the protein binding site; and proteins with artificial cofactors to impart novel activity.[1] There is an ongoing need for additional techniques to selectively modify proteins to further develop conjugates as therapeutics, probes or sensors.

If a protein has solvent accessible cysteine thiol, one can take advantage of the reactivity of thiol to selectively modify the functional group. The thiol group is a more reactive nucleophile than amine towards certain electrophiles, such as maleimide under physiological conditions. Maleimide offers several advantages including high selectivity towards thiol under neutral and acidic conditions in the presence of an amine,[2] ease of synthesis and reasonable aqueous solubility. Thiols in proteins and peptides react with maleimides in a Michael reaction with very high efficiency to form succinimide rings. In the absence of an accessible surface thiol functional group, a widely used protein modification is the replacement of a suitable surface accessible amino acid with a cysteine. The cysteine can be introduced relatively easily in most of the proteins and conjugated to maleimide with relative ease. However, after the maleimide conjugation with a thiol, the resulting succinimide ring is susceptible to ring-opening hydrolysis in aqueous buffers, or a retro-Michael reaction followed by reaction with other thiols (such as albumin or glutathione) in vivo. These liabilities could influence drug stability as well as PK/PD profile of the molecules. While the implications of succinamide ring-opening in vivo are not well understood, it can present a problem in drug product stability. In this publication we disclose modified maleimide analogs with improved chemical stability, while maintaining the efficiency of maleimide reactions towards thiol nucleophiles in Michael reaction. We also disclose the synthesis of new linkers with the modified maleimide ring that would be suitable for conjugating proteins to an antibody.[3]

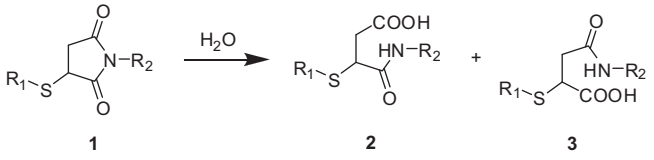

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Figure 1. Hydrolysis of an amide bond.

The succinimide ring amide bond undergoes hydrolysis to form acid products at either of the two carbonyl groups even under neutral conditions (Fig. 1).[4–6]

It is known that the amide bond's stability towards hydrolysis is affected by the succinimide ring's substituents.[7] The stability of

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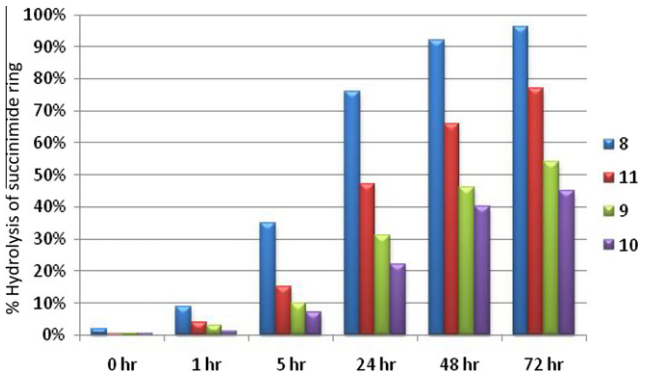


Figure 2. Stability studies of succinimide groups at pH 7.5 (100 mM Histidine, 200 mM Glycine, and 200 mM Sucrose buffer), 40 °C. The incubation mixture was analyzed using LC–MS.

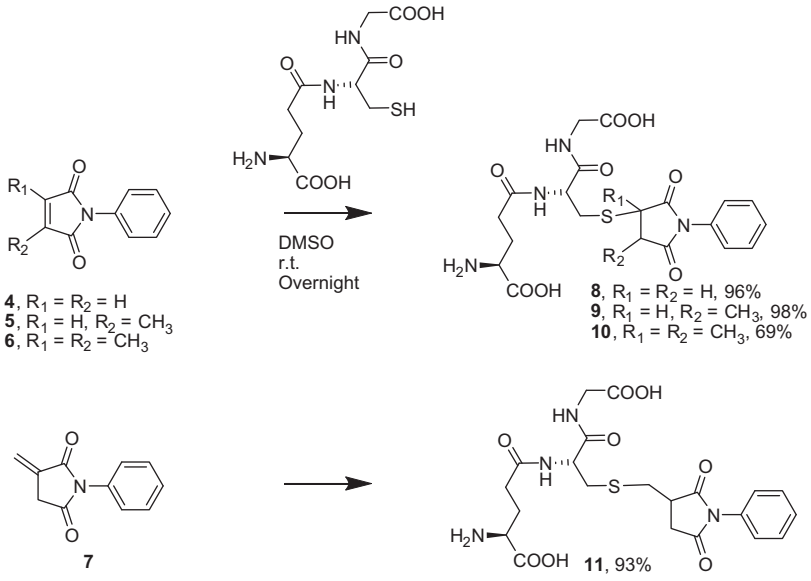
methyl and dimethyl succinimide rings towards hydrolysis was studied under basic conditions, although there was no example of thiol substituted succinimide rings and their stability.[8,9] Subsequently, it was recognized that thiol-substituted succinimide rings undergo ring opening hydrolysis to form mixture of acids under neutral conditions.[10] The substitution of a methyl group on the succinimide ring improves the amide bond stability towards hydrolysis. We undertook the first systematic study to examine the stability of thio-maleimide ring in neutral buffer, with the aim of developing more stable succinimide linkers suitable for bioconjugations. These new linkers offer a potential advantage for conjugating various small molecules, oligonucleotides, peptides and proteins to antibodies with improved stability in buffered solutions.

The preliminary hydrolysis studies were carried out on glutathione conjugated *N*-phenyl succinimide compounds as shown in Figure 2. The syntheses of these compounds were outlined in Scheme 1. Appropriately substituted *N*-phenyl succinimides (**4**–**7**)[11] were treated with reduced glutathione to give compounds **8**–**11**. Compound **9** has one methyl group on a carbon adjacent to a carbonyl group, and **10** has methyl groups on each carbon. Compound **11** has a methylene bridge between glutathione and succinimide ring. While compounds **8**–**10** can potentially undergo retro-Michael reaction, the extra methylene group in **11** would slow down this molecule from undergoing a retro-Michael reaction. These modifications were selected to provide improved stability to the succinimide ring without affecting the efficiency of the Michael reaction with a thiol.

The stability of these glutathione adducts (**8–11**) was studied at pH 7.5 at 40 °C in a formulation buffer (100 mM Histidine, 200 mM Glycine, and 200 mM Sucrose buffer). The materials were analyzed for the formation of succinimide ring-opened acids as well as retro-Michael products. The incubation mixture did not show any fragments as a result of retro-Michael reaction or peptide hydrolysis. The maleimide analog **8** underwent complete hydrolysis of the succinimide ring in 72 h, and was the least stable among the four followed by the itaconimide analog **11**. While dimethyl maleimide analog was the most stable, the formation of the product **10** was the least efficient (69% after 16 h at room temperature). It appears that by simply substituting a methyl group on the succinimide ring, the stability can be improved without sacrificing the conjugation efficiency.

Next we examined the effect of groups substituted on the nitrogen of the succinimide ring and their role in facilitating a water molecule to attack either carbonyl groups of the succinimide ring. We evaluated four different connectors as shown below using the model compounds **12**–**15** (Fig. 3). We used glutathione as a model peptide in all four compounds. Methyl maleimide ring was chosen due to its improved stability and comparable reactivity as maleimide towards thiol for further development. Three different linkers were connected to the nitrogen of the methyl maleimide ring. These nitrogen atom substituents were selected because these modifications were of interest for incorporation in the linkers (**16**–**19**) to be used for bioconjugation applications. These linkers (**16**–**19**) have a maleimide ring on one end and a propanoyl azetidin-2-one group on the other end.[12]

In recent publications we have demonstrated successfully that one could combine a carrier monoclonal antibody such as aldolase antibody with favorable pharmacokinetic properties and a small molecule or peptide with therapeutic benefit to generate CovX bodies that have desirable pharmacokinetic properties of the antibody and therapeutic effect of peptides.[13–15] The aldolase antibody has a lysine at position 93 of the heavy chain and located deep in the hydrophobic binding pocket on each of the two Fab arms.[16] We have developed phenylpropanoylazetidin-2-one to



Scheme 1. Reagents and Conditions: (a) reduced glutathione (1.2 equiv), DMSO, rt, overnight.

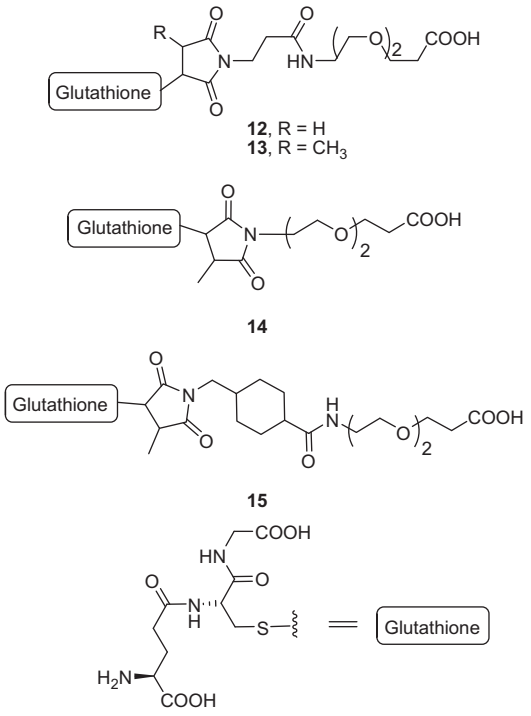
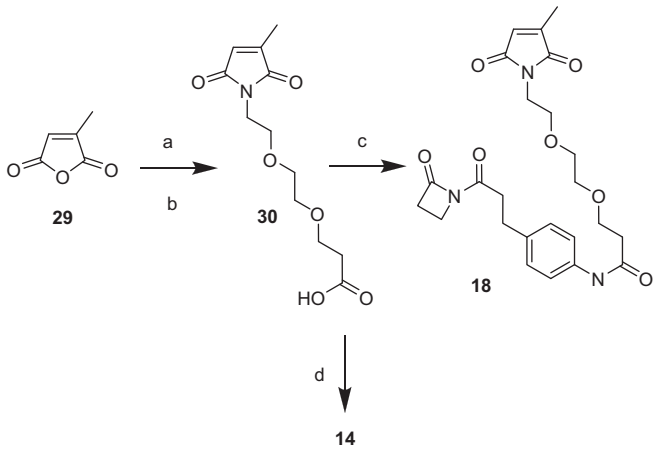


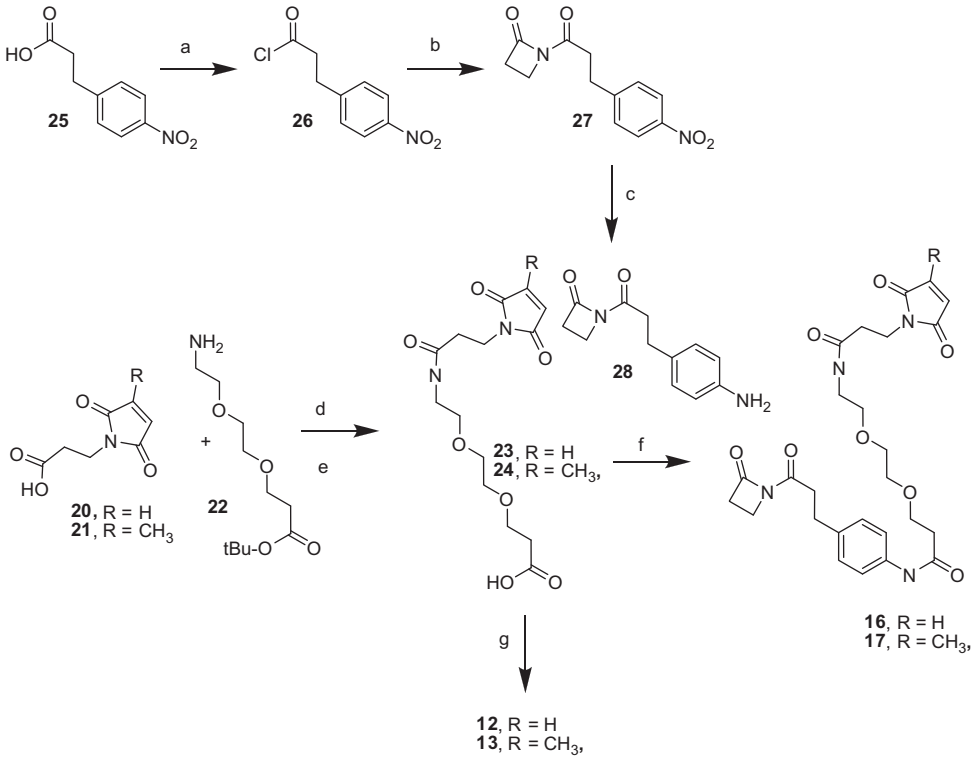
Figure 3. Structures of glutathione-maleimide linker.

react selectively with Lys-93 located deep in the hydrophobic pocket and not with any other lysine on the surface of the antibody or protein.[17] We propose linkers **16**–**19** which would be useful for conjugating either peptides or proteins to the aldolase antibody.



Scheme 3. Reagents and conditions: (a) **22**, DIC, HOBT, 80 °C; (b) HCl, CH_3CN, rt; (c) **28**, HBTU, DIEA, DMF, rt; (d) glutathione, DMSO, rt.

The synthesis of compounds **12** and **13** and linkers **16** and **17** are shown in Scheme 2. Nitrophenyl acid chloride **26** was prepared from **25** as described.[18] An amide proton in azetidin-2-one was deprotanated with *n*-butyl lithium in tetrahydrofuran and reacted with the acid chloride **26** to give **27**. The nitro group in **27** was reduced to an amine using palladium on carbon in methanol at 35 °C to give **28**. Maleimide acids (**20** and **21**) were activated with *N*-hydroxy succinimide and reacted with PEG amine **22**.[19] The tertiary butyl group was cleaved using 15% trifluroacetic acid in dichloromethane to give acids **23** and **24**, respectively. The acids (**23** and **24**) were treated with glutathione to give glutathione adducts **12** and **13**. The acids **23** and **24** were activated with HBTU and treated with amine **28** to give linkers **16** and **17**, respectively.



Scheme 2. Reagents and conditions: (a) $SOCl_2$, toluene, rt; (b) azetidin-2-one, n-BuLi, THF, −78 °C to rt; (c) H_2, 1 N HCl, Pd/C, methanol, 35 °C; (d) *N*-hydroxysuccinimide, diisopropylcarbodiimide, THF, rt; (e) 15% TFA in CH_2Cl_2, rt; (f) **28**, HBTU, DIEA, DMF, rt; (g) glutathione, DMSO, rt.

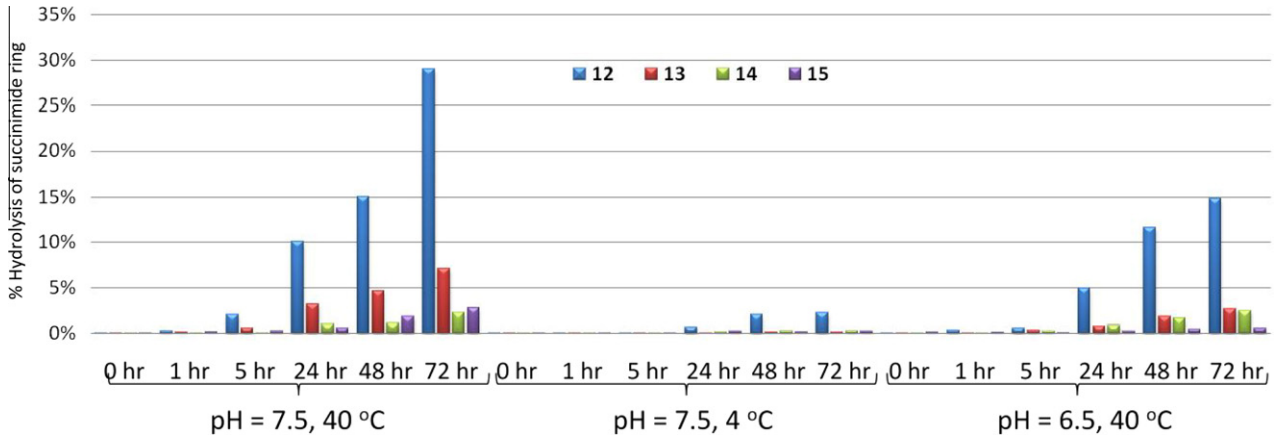
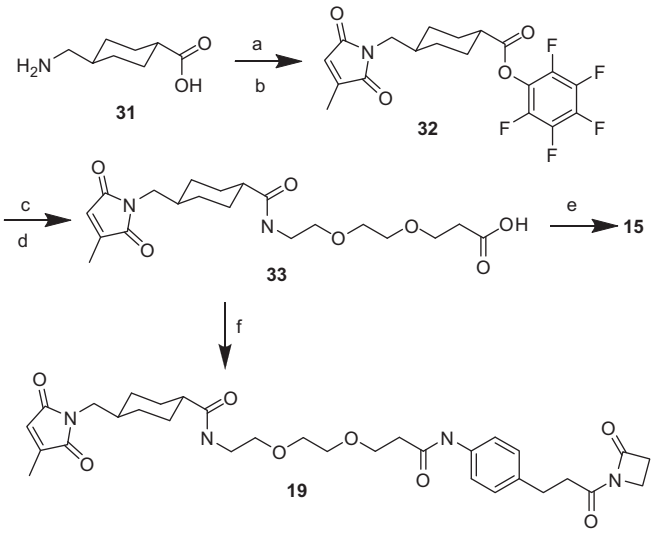


Figure 4. Hydrolytic stability studies of succinamide ring in a formulation buffer (100 mM Histidine, 200 mM Glycine, and 200 mM Sucrose) at pH 7.5, 40 °C; 7.5, 4 °C; and 6.5, 40 °C . The formation of the succinimide ring-opened acid products was measured using LC–MS.



Scheme 4. Reagents and conditions: (a) **29**, DMF, rt; (b) DIPEA, DMF, rt; (c) **22**, NMM, THF, rt; (d) 50% TFA, CH_2Cl_2, rt; (e) glutathione, DMSO, rt; (f) **28**, HBTU, HOBT, NMM, DMF, rt.

The synthesis of glutathione analog of linker **14** and the synthesis of linker **18** were started from citraconic anhydride (**29**) as shown in Scheme 3. A solution of citraconic anhydride, **29**, PEG amine **22** in DMF was stirred at room temperature for 2 h. Diisopropylcarbodiimide and HOBT were added and the reaction was heated at 80 °C for 2 h that resulted in higher yield of an ester intermediate compared to acetic acid approach.[20] The ester intermediate was treated with 50% 6 N HCl in acetonitrile to afford **30**. The resulting acid **30** was treated with glutathione in DMSO to give glutathione adduct **14**. Acid **30** was coupled to amine **28** using HBTU and DIEA to give **18**.

The synthesis of **15** and **19** are shown in Scheme 4. A solution of citraconic anhydride (**29**) and trans-4-aminomethyl cyclohexane carboxylic acid (**31**) in dimethylformamide was stirred at room temperature for 6 h. The reaction solution was cooled to 0 °C, DIPEA followed by pentafluorophenyl trifluoroacetate in DMF were added. The reaction mixture was warmed to room temperature and stirred for another 16 h under N_2 to give **32**. Compound **32** was treated with PEG amine **22** in the presence of *N*-methyl morpholine to give a crude intermediate which was treated with 50% trifluoroacetic acid in dichloromethane to afford the acid intermediate **33** as white solid after HPLC purification. The acid **33** was treated with glutathione in DMSO to afford **15** in quantitative yield. The acid **33** was coupled to **28** using HBTU in the presence of HOBT in DMF to afford linker **19**.

The ring stability of glutathione adducts **12**–**15** was studied under the same conditions as described above. Figure 3 shows the result of succinimide ring stability studies at neutral pH. When the phenyl ring in **8** and **9** was replaced with an aliphatic chain as shown in **12** and **13** we saw improved stability of the succinimide ring. While the stability improvement from compound **8** to **12** was modest, the stability improvement from **9** to **13** was much more pronounced. These improvements in stability were anticipated since electron withdrawing groups such as phenyl would facilitate the hydrolysis of an amide bond in succinimide. We noticed further improvement in stability when propionyl group was replaced with more hydrophobic alkyl chain (**14**) and cyclohexyl chain (**15**).

We also examined the stability of compounds **12**-**15** at lower temperature, 4 °C at pH 7.5 and at lower pH 6.5 at 40 °C (Fig. 4). By lowering the temperature, from 40 to 4 °C, the stability improved approximately 15-fold. Similarly, by lowering the pH from 7.5 to 6.5, the stability of the ring increased approximately twofold.

The studies showing the improved stability in the succinimide ring would help to develop appropriate buffer systems for administering the compound as well as storage conditions. We also examined the stability of the succinimide ring in different buffers at the same pH and at the same temperature and observed the type of buffer has effect on the stability of the succinimide ring.

In summary, we have developed four different maleimide linkers (**16**–**19**). The methyl maleimide group in linkers **17**–**19** offer improved stability towards hydrolysis without compromising the reaction efficiency towards thiols (data not shown). These linkers have the required aqueous solubility to dissolve in buffered solutions for conjugation. These linkers have an azetidin-2-one group that selectively reacts with lysine-93 in aldolase antibody and modified maleimide groups to react selectively with a thiol group in a pharmacophore. These new linkers expand the list of available linkers in the bioconjugation field that could be used to conjugate small molecules/oligonucleotides/proteins/peptides to an antibody. We have successfully used these linkers to conjugate both peptides and proteins to the carrier antibody and evaluated their activity both in vitro and in vivo. The application of these linkers in conjugating a protein therapeutic to an antibody for generating novel therapeutics will be disclosed in future.

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