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SYNTHESIS OF REAGENTS FOR THE ONE STEP INCORPORATION OF HYDRAZIDE FUNCTIONALITY ONTO THE LYSINE RESIDUES OF PROTEINS, AND THEIR USE AS LINKERS FOR CARBONYL CONTAINING MOLECULES

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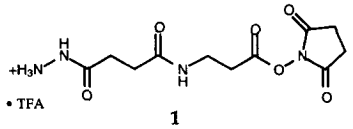
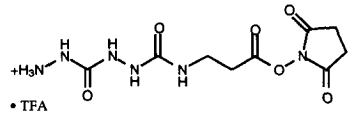
Abstract: Two new reagents, **1** and **2**, were prepared for the one step incorporation of hydrazide functionality onto the lysine residues of proteins. Their utility as linking reagents was demonstrated by their use in the coupling of two model aldehydes, and the anticancer agent doxorubicin, to a monoclonal antibody.



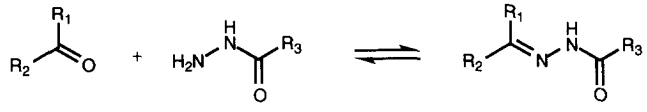
1



2

Introduction

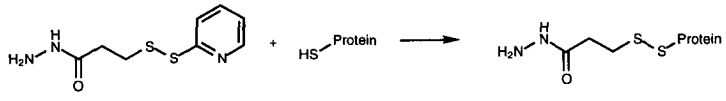
Hydrazone linkages are a useful way of attaching, sometimes reversibly, small molecules to proteins.[1-3] The hydrazones are formed between hydrazide (or hydrazide-like) functional groups, and aldehydes or ketones.



R_1, R_2, R_3 = H, alkyl or aryl

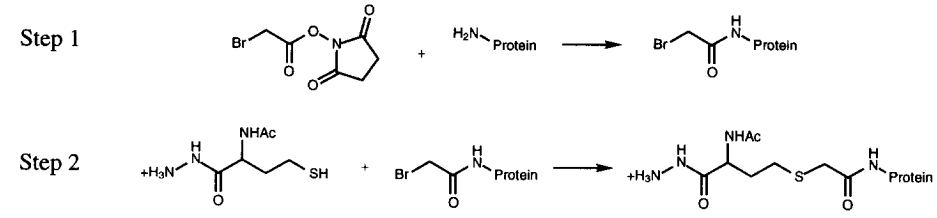
When the carbonyl component is on the small molecule, the hydrazide or hydrazide-like group to be linked is often separately incorporated into the protein.[1-4] If free sulfhydryl groups are available a one-step modification is possible.[4] An example is shown in Scheme 1.

Scheme 1



If, as is commonly the case, no sulfhydryl groups are available, or lysine amine substitution is specifically sought, current methodology requires a two step process such as that shown in Scheme 2.[2]

Scheme 2

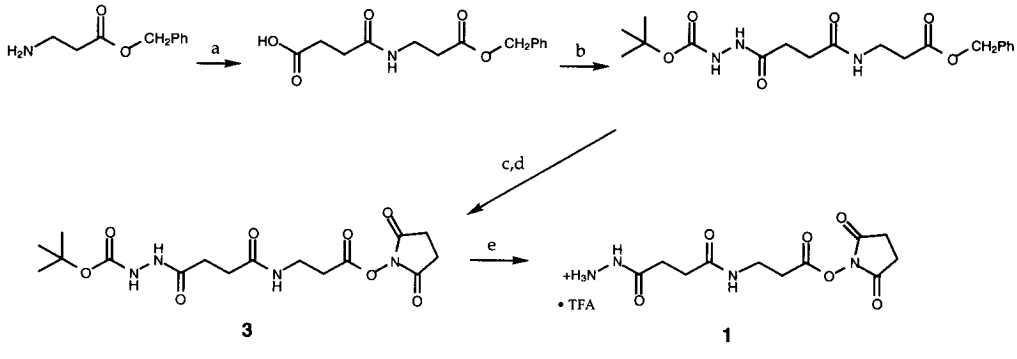


In this paper we describe the preparation and utilization of two new reagents, **1** and **2**, which permit a simple ***one-step*** incorporation of hydrazide functionality onto the lysine residues of proteins, and subsequent conjugation of these hydrazides to carbonyl containing molecules. Because of differences in the hydrolytic stability of the hydrazones formed with **1** and **2**[1] each of these should be useful conjugation reagents.

Preparation of reagents

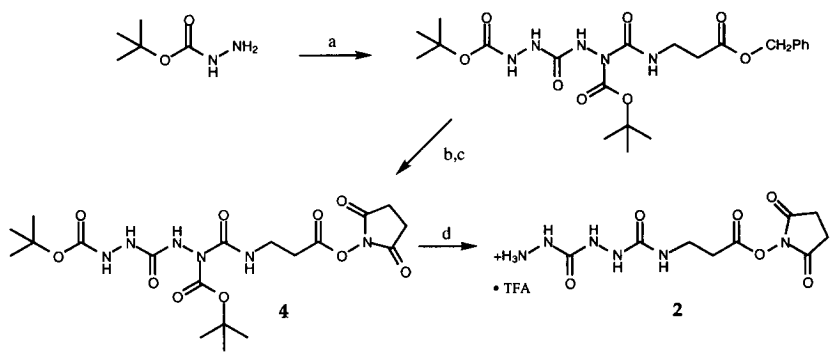
Our approach to a one-step substitution of lysine residues with hydrazide or hydrazide-like functional groups was based on the premise that in its protonated form a hydrazide would be relatively unreactive towards active esters, and the two functional groups could temporarily coexist in the same molecule (i.e. **1** or **2**). When added to a solution of protein at basic pH the active ester component would selectively acylate lysine primary amines (which are in large excess) in preference to the now unprotonated, but less reactive, hydrazide. The compounds were synthesized as outlined in Schemes 3 and 4.

Scheme 3



Reagents and conditions: (a) succinic anhydride (1 equiv), pyridine, 20 h, rt (86%); (b) N,N' disuccinimidyl oxalate (1 equiv), pyridine (1 equiv), acetonitrile, 2 h, rt, then t-butyl carbazate (1 equiv), 20 h, rt (73%); (c) 1,4-cyclohexadiene (2 equiv), 5% Pd/C, EtOH, 40 min, 60° (100%); (d) N-hydroxysuccinimide (1 equiv), dicyclohexylcarbodiimide (1 equiv), dichloromethane/dioxane, 4 h, rt (72%); (e) 1:1 trifluoroacetic acid:dichloromethane, 0.5 h.

Scheme 4



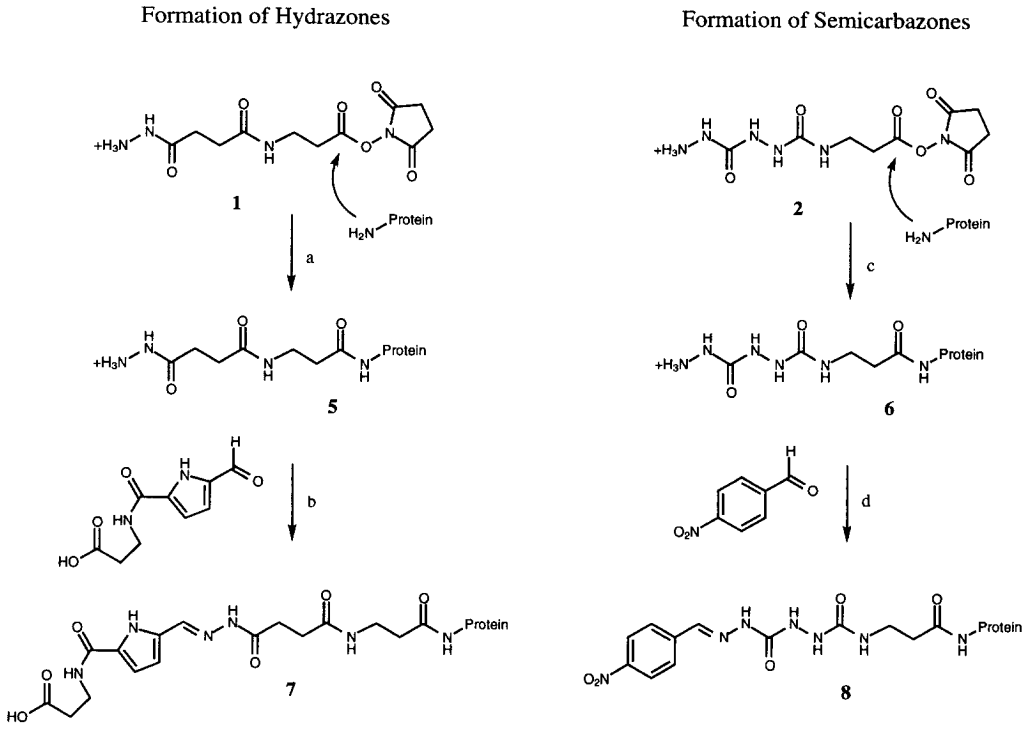
Reagents and conditions: (a) triethylamine (2 equiv), triphosgene (0.33 equiv), chloroform, 0.5 h, rt, then beta-alanine benzyl ester·pTsOH (1 equiv), triethylamine (1 equiv), chloroform, 24 h, rt (53%); (b) 1,4 cyclohexadiene (5 equiv), 5% Pd/C, EtOH, 70°, 1.5 h (41%); (c) N-hydroxysuccinimide (1 equiv), dicyclohexylcarbodiimide (1 equiv), dioxane, 2 h, rt (70%); (d) 1:1 trifluoroacetic acid:dichloromethane, 0.5 h.

Compound **3**[5], the protected precursor to **1**, was made by conventional chemistry. Synthesis of **4**[6], the analagous precursor to **2**, was based on chemistry described by Kaneko, et al.[1] Both linkers had a beta alanine spacer incorporated to prevent self-destruction of active ester intermediates by intramolecular cyclization of the nitrogen in the hydrazide unit. After chromatographic purification of these intermediates a final trifluoroacetic acid deprotection afforded the trifluoroacetate salt of **1** and **2**. These products were used without any further purification in the protein functionalization step.

Use of 1 and 2

To demonstrate their use in the one step incorporation of hydrazide-like functionality into a protein, **1** and **2** were each reacted with the monoclonal antibody CC49[7] to afford **5** and **6**. Simple small molecule aldehydes were then coupled to the functionalized protein to simultaneously show the utility of **1** and **2** as linker agents and to quantitate the degree of hydrazide incorporation into the protein. Compound **5** was quantitated with beta alanine pyrrole-2-carboxaldehyde and compound **6** with p-nitrobenzaldehyde to give the hydrazone and semicarbazone adducts **7** and **8**, respectively. The steps are outlined in Scheme 5.

Scheme 5



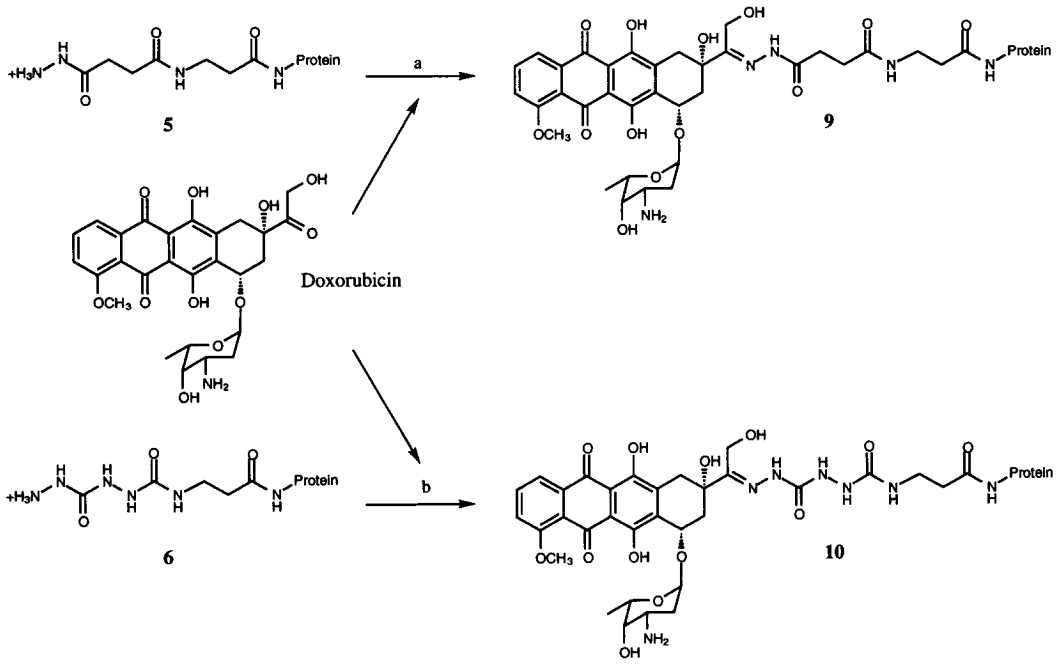
Reagents and conditions: (a) CC49 (1 equiv, 130 μM in 0.1 M borate buffer, pH 8.6), **1** (10 equiv), 0.5 h, rt (96%); (b) **5** (1 equiv, 6.6 μM in 0.1 M sodium acetate, pH 4.6), beta alanine pyrrole 2-carboxaldehyde (300 equiv), 20 h, rt (100%); (c) CC49 (1 equiv, 130 μM in 0.1 M borate buffer, pH 8.6), **2** (10 equiv), 1.5 h, rt (70%); (d) **6** (1 equiv, 6.6 μM in 0.1 M sodium acetate, pH 4.6), p-nitrobenzaldehyde (75 equiv), 20 h, rt (90%).

Compounds **5** through **8** were purified from small molecular weight reagents and by-products by size exclusion chromatography on Sephadex G25 columns. UV analysis of **7** and **8** (using molar absorptivities for the unmodified protein and respective hydrazones) was employed to quantitate the degree of hydrazide incorporation in **5** and **6**. For compound **1** a tenfold molar excess of **1** to protein yielded **5** with an average of 6.4 hydrazide groups per antibody molecule. Compound **2**, also at a tenfold molar excess, yielded **6** with an average of 5.1 substitutions per antibody molecule.

Conjugation of doxorubicin to 5 and 6

The functionalized proteins **5** and **6** were solvent exchanged into 0.1 M sodium acetate, pH 4.6, in preparation for conjugation to doxorubicin as shown in Scheme 6.

Scheme 6



Reagents and conditions: (a) **5** (1 equiv, 133 μM in 0.1 M sodium acetate, pH 4.6), doxorubicin hydrochloride (300 equiv), 20 h at 30° (32%); (b) **6** (1 equiv, 87 μM in 0.1 M sodium acetate, pH 4.6), doxorubicin hydrochloride (230 equiv), 20 h at 32° (31%).

Compounds **5** and **6** reacted at mildly acidic pH with a large excess of doxorubicin to give **9** and **10**, respectively. Conjugates were purified from excess doxorubicin and protein aggregate by chromatography over Sephadex G25 (15% acetonitrile, 85% phoshphate buffered saline, pH 7.4) followed by Superose 12 (phosphate buffered saline, pH 7.4). UV analysis showed that **5** gave **9** with an average incorporation of 3 doxorubicin molecules for each molecule of antibody CC49. Compound **6** afforded **10** with an average of 3.1 doxorubicin molecules per molecule of CC49.

Summary

In this communication we describe the preparation and use of reagents that permit a simple one-step incorporation of hydrazide and hydrazide-like groups onto lysine amines in proteins. Both reagents are readily prepared. Their utility as linkers between small molecules and proteins was demonstrated by using them to link simple aldehydes and an antitumor agent, doxorubicin, to a monoclonal antibody.

Acknowledgement

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References and Notes

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2. King, T. P.; Zhao, S. W.; Lam, T. *Biochemistry* **1986**, *25*, 5774.
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4. Zara, J.; Pomato, N.; McCabe, R. P.; Bredehorst, R.; Vogel, C-W. *Bioconjugate Chem.* **1995**, *6*, 367.
5. **3**: ^{1}H-NMR (360 MHz; $CDCl_3$, δ ppm): δ = 8.17 (s, 1H, NH), 6.80 (br s, 1H, NH), 6.73 (br s, 1H, NH), 3.73 (q, 2H), 2.96 (br s, 6H), 2.67 (s, 4H), 1.57 (s, 9H).
6. **4**: ^{1}H-NMR (360 MHz; $CDCl_3$, δ ppm): δ = 9.38 (br s, 1H, NH), 7.78 (br s, 1H, NH), 6.82 (br s, 1H, NH), 6.57 (br s, 1H, NH), 3.79 (m, 2H), 3.04 (m, 2H), 2.96 (s, 4H), 1.60 (s, 9H), 1.58 (s, 9H).
7. CC49 reacts with the high molecular weight mucin designated TAG72. The antibody was originally described by Muraro, R.; Kurokil, M.; Wunderlich, D.; Poole, D. J.; Colcher, D.; Thor, A.; Greiner, J. W.; Simpson, J. F.; Molinolo, A.; Noguchi, P.; Schlom. J. *Cancer Res.* **1988**, *48*, 4588.

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