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Synthesis of new spin labels for Cu-free click conjugation

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ABSTRACT

New pyrroline nitroxides attached to a terminal acetylenic sulfone, a dibenzocyclooctyne or a cyclooctyne carboxylic acid were synthesized and tested in Cu-free click reactions to conjugate these new spin labels with 4-azido-TEMPO, azidophenylalanine and an azidophenylalanine-containing protein.

The incorporation of nitroxides into various biological and non-biological structures followed by EPR analysis has emerged as an important technology. The spin labeling method, introduced by McConnell and co-worker,[1] is an effective tool to explore structure, dynamics and interactions of complex biomolecules such as proteins, nucleic acids, and polysaccharides. Spin labels can be site-specifically introduced into proteins by covalent chemical bond formation with sulfhydryl-specific reagents, such as the reaction of methanethiosulfonates with cysteine,[2] or by genetic incorporation.[3] However, this particular labeling approach is not useful when native, reactive thiols are present in the protein of interest. To circumvent this limitation, it has recently become possible to investigate biomolecules in vivo or in vitro by using bioorthogonal chemical reactions (i.e., reactions based on functional groups not found in biology). Such reactions must have fast rates under physiological conditions and be inert to the myriad of functionalities present in biomolecules.[4] To that end, an orthogonal spin labeling strategy based on the genetically encoded amino acid *p*-acetyl-L-phenylalanine was recently introduced, in which a ketoxime-linked spin label was generated by selective modification of the ketone group with a hydroxylamine nitroxide.[5]

In addition to *p*-acetyl-L-phenylalanine, it is now possible to genetically encode over seventy unnatural amino acids,[6] including many with bioorthogonal functional groups (e.g., *p*-azido-L-phenylalanine). These unnatural amino acids allow several other bioorthogonal reactions for attaching spin labels beyond the aforementioned oxo-hydroxylamine conjugation, such as Staudinger ligations,[7] Diels–Alder reactions with alkenes, and azide–alkyne reactions, which are 1,3-dipolar cycloadditions of organic azides with alkynes.[8,9] In the presence of Cu(I), azide–alkyne coupling has been established as one of the most versatile means for the covalent assembly of complex molecules[10] and tagging fluorophores to biomolecules.[11]

Although bioorthogonal techniques are quite widespread in fluorescence labeling,[12] their applications in spin labeling are currently limited. Our laboratory reported a series of paramagnetic monofunctional and bifunctional azides, and monofunctional acetylenes for modifying biomolecules in Cu(I) catalyzed reactions.[13] However, it would be most useful to develop nitroxide reagents that can react with azides or acetylenes under physiological conditions in the absence of cytotoxic Cu(I) ions., that is, Cu-free click conjugation. In this report, we consider spin labels with an acetylene function that undergo facile reaction with azides in the absence of Cu(I). Because the chemical properties of stable free nitroxide radicals limit the range of reactions and procedures applicable, it was obvious that the 'clickable' (acetylene) part could not be formed on the nitroxide ring, but that the nitroxide should be attached to an appropriate acetylene moiety. We have explored two different strategies to promote the [3+2] cycloaddition under mild conditions: (1) use of an alkyne activated with an electron-withdrawing group,[14,15] and (2) use of a strained alkyne.[16] As far as we know, this is the first report of the synthesis and model reactions for azide specific, biocompatible spin label reagents.

Treatment of compound **1**[17] with chlorosulfonic acid at 0 °C in an aromatic electrophilic substitution reaction[18] gave sulfonyl

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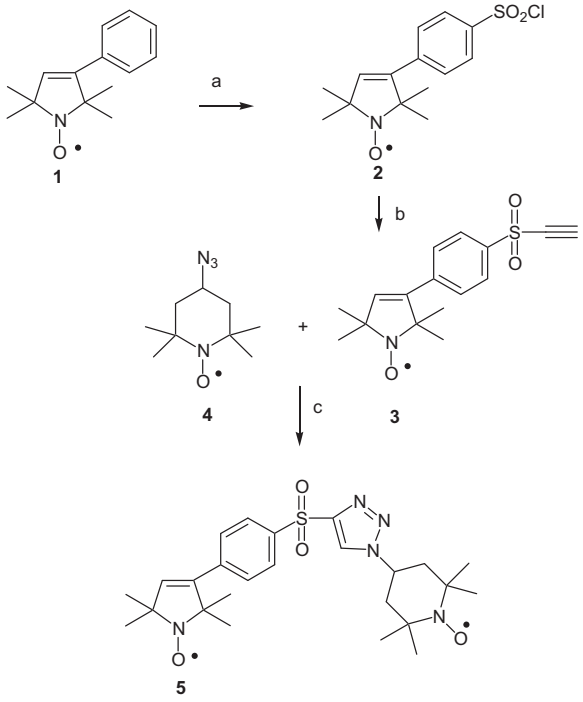
chloride **2** in 73% yield. Reaction of compound **2** with HCl gas in ethyl acetate containing an equivalent amount of ethanol gave the hydroxylamine salt, which was treated with 3 equiv of aluminum chloride and 3 equiv of bis(trimethylsilyl)acetylene in dichloromethane[19] to give compound **3** (HO-4429) in 15% yield. Reaction of compound **3** with 4-azido-TEMPO (**4**)[20] in CH_2Cl_2 at room temperature yielded biradical **5** as a mixture of the 1,4-disubstituted triazole as the main isomer and a non-separable minor (1,2-disubstituted) isomer,[14] suggesting that compound **3** is a candidate for azido-specific spin labeling (Scheme 1).

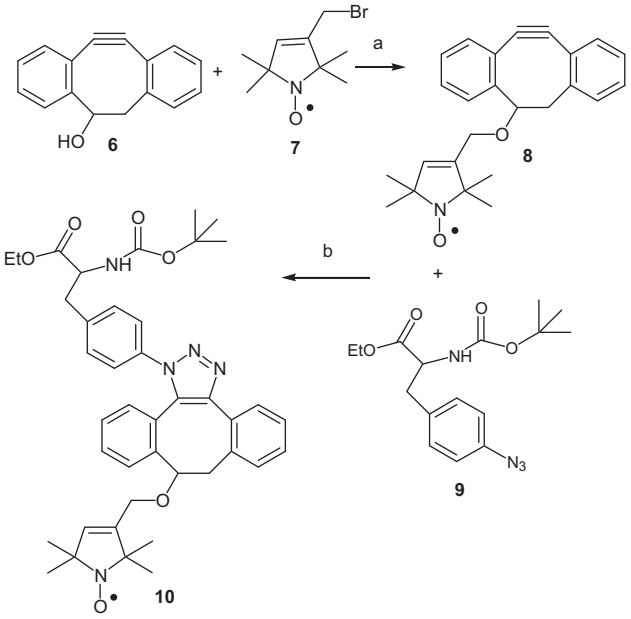
Because ethynyl sulfones are powerful Michael acceptors[19] that can react with many nucleophiles, we explored using strained acetylenes, namely cyclooctynes, for generating azide specific nitroxide reagents in order to improve selectivity. Currently, several cyclooctyne rings have been reported for copper-free click chemistry.[21] For modification with a nitroxide, we chose the dibenzocyclooctyne, 11,12-didehydro-5,6-dihydrodibenzo[*a,e*]cyclooctene-5-ol (**6**),[22] which was alkylated with the allylic bromide[23] **7** in the presence of NaH in a THF/DMF (10:7) mixture. The resulting ether **8** (HO-4389) showed limited water solubility, but underwent a [3+2] cycloaddition reaction in CH_2Cl_2 at ambient temperature with N-protected D,L-4-azidophenylalanine ester (**9**)[24] to give an inseparable mixture of regioisomers of compound **10** (Scheme 2).

Another possibility was the application of 1-fluorocyclooct-2-ynecarboxylic acid (**11**),[25] an easily available cyclooctyne moiety, to react with a paramagnetic amine. Treatment of **11** in THF with 1,1′-carbonyldiimidazole (CDI) gave an imidazolide that was not isolated, but used directly for acylating **12**[26] to give amide **13** (HO-4451) in 39% yield. Reaction of compound **13** in CH_2Cl_2 with the protected azidophenylalanine **9** gave a 1:1 mixture of triazole regioisomers of compound **14**, suggesting its applicability for modification of azides (Scheme 3).

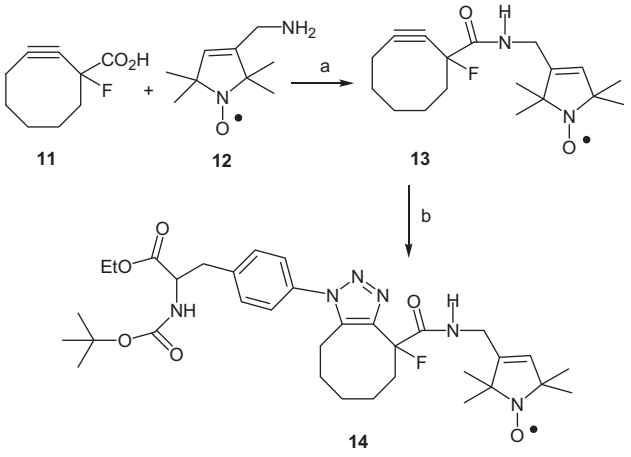
The utility of compound **13** for modifying azide-containing proteins was demonstrated by reacting it with a T4 lysozyme mutant



Scheme 1. Reagents and conditions: (a) HSO_3Cl, excess, 0 °C→rt, 2 h, then work-up, PbO_2 (0.2 equiv), O_2, 10 min, 73%; (b) HCl gas, excess, EtOH (1.0 equiv), EtOAc, then evaporation, $AlCl_3$ (3 equiv), $Me_3SiC{\equiv}CSiMe_3$ (3 equiv), CH_2Cl_2, 0 °C→ rt, 24 h, then work-up with H_2O, $NaNO_2$ (1 equiv), 15%; (c) **4** (1 equiv), CH_2Cl_2, rt, 12 h, 23% (isolated).



Scheme 2. Reagents and conditions: (a) THF, NaH (1.0 equiv) 30 min, 0 °C, then **7** (1.0 equiv), DMF, 40 °C, 1 h, rt 12 h, 43%; (b) **9** (1.0 equiv), CH_2Cl_2, rt, 1 h, 45% (isolated).



Scheme 3. Reagents and conditions: (a) CDI (1.1 equiv), THF, reflux 10 min, then **12** (1.0 equiv), reflux, 30 min, 39%; (b) **13** (1.0 equiv), **9** (1.0 equiv), CH_2Cl_2, rt, 1 h, 38% (isolated).

containing *p*-azido-L-phenylalanine at site 109 (T4L 109*p*-AzF). The resulting X-band EPR spectrum of the triazole-linked nitroxide side chain (Fig. 1) shows restricted nitroxide motion, as evidenced by the reduced intensities of the low- and high-field resonance lines relative to the central line, indicating that compound **13** is linked to the protein.

In conclusion, we have synthesized new, azido-specific, biocompatible nitroxide reagents suitable for generating spin labels via Cu-free click chemistry. All of the new labels were tested in model reactions, and their triazole adducts were isolated for mass spectrometric identification. The utility of compound **13** was demonstrated by modifying a *p*-azido-L-phenylalanine-containing protein for the assembly of spin label-protein conjugates. This orthogonal azide and cyclooctyne click reaction spin labeling methodology proved superior to modification of ketones with a nitroxide hydroxylamine[5] in that the reaction is faster and takes place at neutral pH. It is anticipated that highly specific spin label reagents can be

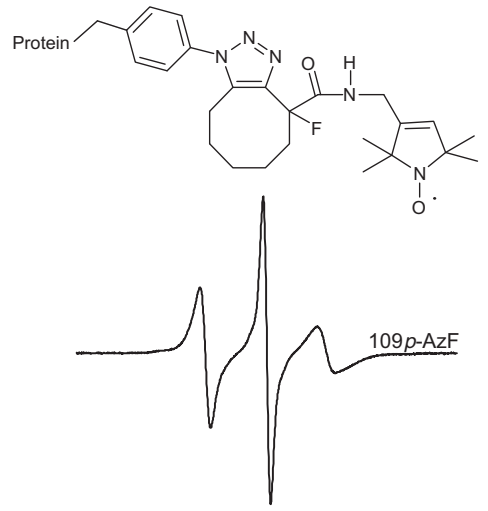


Figure 1. Room temperature EPR spectrum of a T4L 109*p*-AzF modified with compound **13** in a 30% sucrose solution (sucrose was added to the protein solution only to increase the solution viscosity, which aids in spectral analysis). Expression of the T4L 109*p*-AzF mutant was performed as described by Fleissner et al.[5] using the pEVOL-*p*AzF plasmid[27] and 2 mM *p*-azido-L-phenylalanine. Sweep width is 100 Gauss.

prepared using this methodology, and the synthesis and biophysical studies of other azido-specific spin label reagents[28] for click chemistry is currently an ongoing area of research in our laboratories.

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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.2011.03.077.

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