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Synthesis of Unnatural Amino Acids Functionalized with Sterically Shielded Pyrroline Nitroxides

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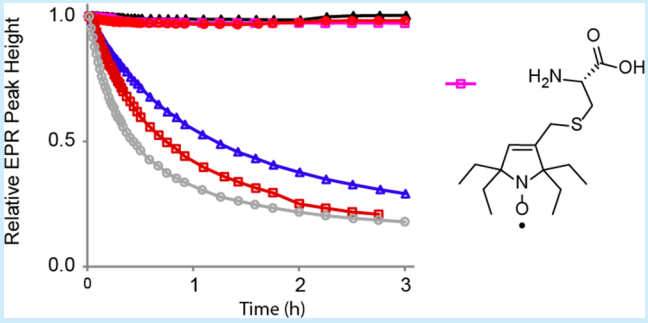
Supporting Information

ABSTRACT: A series of unnatural amino acids functionalized with sterically shielded pyrroline nitroxides were synthesized. Their reduction by ascorbate/glutathione indicates that L-cysteine functionalized with *gem*-diethylpyrroline nitroxide is reduced at the slowest rate and is comparable to that measured for the most resistant to reduction pyrroline and pyrrolidine nitroxides.



The spin labeling of proteins by incorporating unnatural amino acids functionalized with nitroxides has many potential advantages over the established, site-directed spin labeling (SDSL) methods.[1,2] Unnatural amino acid spin labels offer access to novel approaches to spin labeling of proteins via nonsense codon suppression of an introduced stop codon: (1) via a chemically aminoacylated tRNA[3,4] or flexizyme-prepared aminoacylated tRNA[5,6] and (2) via genetic encoding of unnatural, spin-labeled amino acids in live cells.[7] However, despite the tremendous promise for genetic encoding strategies, a major challenge remains in the survival of such probes in the reducing conditions during the ribosome-mediated protein synthesis, resulting in partially irreversible chemical reduction of the nitroxide to the corresponding diamagnetic hydroxylamine.[8] Thus, chemical modifications of the spin labels are required to render them resistant to such reduction before they can be applied for the structure–function study of expressed proteins in biological environments.

To this end, we[9,10] and others[11,12] recently prepared sterically shielded pyrrolidine nitroxides that are by far the most resistant to ascorbate and ascorbate/glutathione mediated reduction[9] and, therefore, would be valuable as modified unnatural amino acid spin labels. Note that functionalization of L-amino acids with racemic pyrrolidine nitroxides would give two possible stereoisomeric products, but in the case of pyrroline nitroxides, without stereocenters, only one enantiomer for the product is expected.

Here, we report the synthesis of amino acids **1**–**4**, functionalized with sterically shielded pyrroline nitroxides (Figure 1), and their reduction kinetics by ascorbate/glutathione. For reference, we study reduction kinetics of pyrroline nitroxides **5** and **6** (Figure 1).

Synthesis of amino acid **1** is outlined in Scheme 1. Dibromination of **7**,[13,14] according to the previously published procedure, provided **8**.[11] Subsequent Favorskii rearrangement

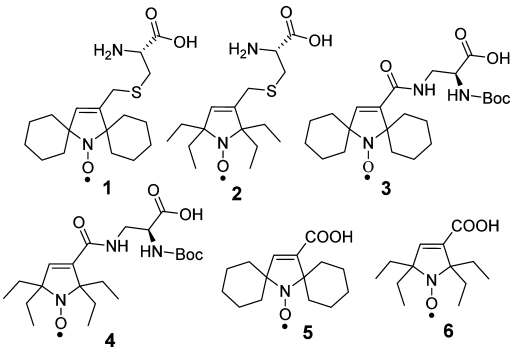


Figure 1. Amino acids **1**–**4** and pyrroline nitroxides **5** and **6**.

of **8** gave 5-membered ring amine **9**. Selective reduction of **9** using Red-Al provided allylic alcohol amine **10**, which was then selectively oxidized with *m*-CPBA (1.6 equiv) to yield pyrroline nitroxide **11**.[15] Note that the conversion of **8** to **10** was accomplished by modifications of reaction conditions for the analogous *gem*-dimethylamines.[17,18] Reaction of the allylic alcohol nitroxide **11** with MsCl gave mesylate nitroxide **12**.[15] Nucleophilic substitution of **12** with the sulfhydryl group of L-cysteine provided the target amino acid **1**.

The synthesis of amino acid **2** is outlined in Scheme 2. In the first step, alcohol amine **13**[9,15,16] is selectively oxidized to ketone amine **14**[15] using Dess–Martin reagent (DMP).[19,20] Subsequent steps leading to mesylate nitroxide **18** are implemented in an analogous way to that for spirocyclohexyl nitroxide **12**. Given the increased steric hindrance of *gem*-diethyl groups, we were surprised to attain selective oxidation of amine **16** with *m*-CPBA (1.8 equiv) to provide pyrroline

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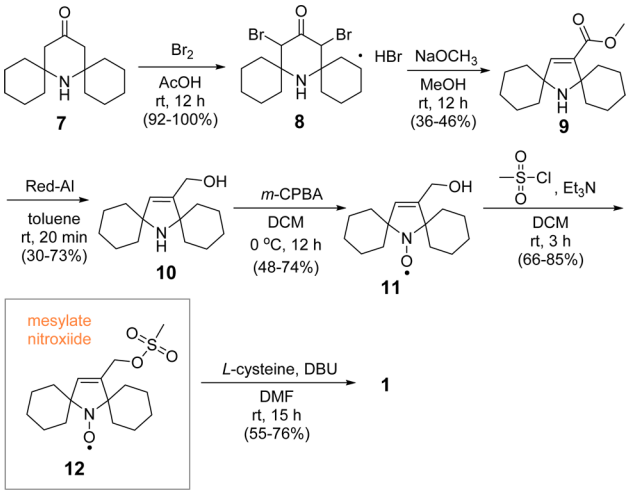
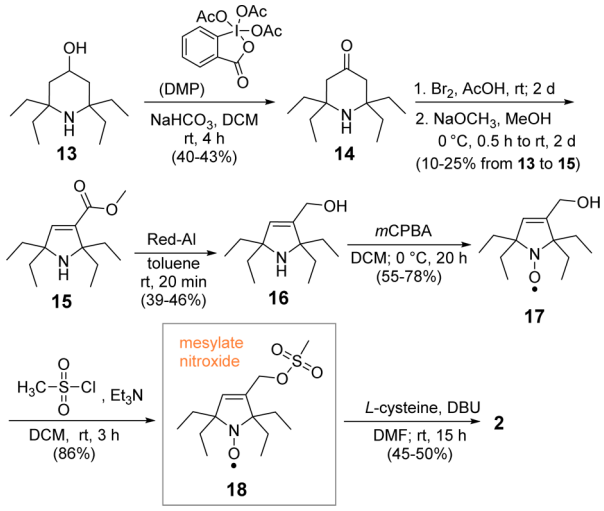
Scheme 1. Synthesis of Amino Acid 1



Scheme 2. Synthesis of Amino Acid 2



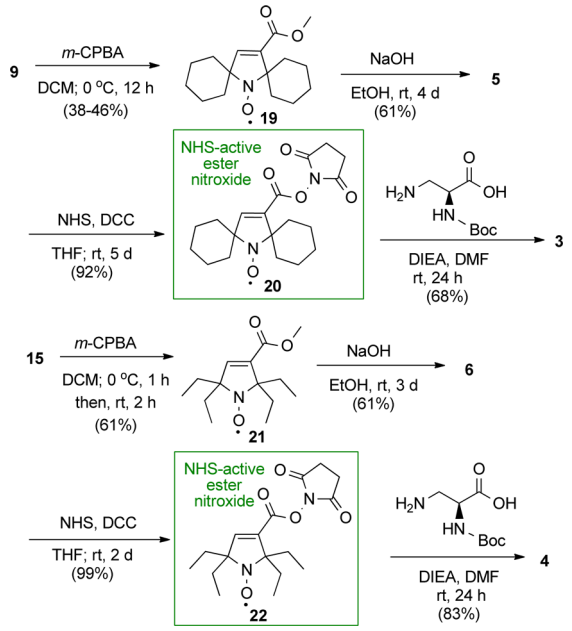
nitroxide **17** in 78% yield (100% spin purity) and negligible amounts of alkene epoxidation byproduct. Finally, reaction of **18** with L-cysteine gave the target amino acid **2**.

The synthesis of *N*-Boc-protected amino acids **3** and **4** as well as pyrroline nitroxides **5**[15] and **6** is outlined in Scheme 3. In the initial steps, the Favorskii rearrangement products **9** and **15** (Schemes 1 and 2) are subjected to oxidation with *m*-CPBA to provide corresponding nitroxides **19** and **21**. Ester hydrolysis of **19** and **21** gives the pyrroline nitroxides **5** and **6**, respectively. The carboxylic acids in nitroxides **5** and **6** are converted to *N*-hydroxysuccinimidyl (NHS) esters by standard methods, using NHS and DCC, to give the corresponding NHS-active esters **20**[15] and **22**, which are then coupled with *N*-Boc-dab to yield target *N*-Boc-protected amino acids **3** and **4**, respectively.

The high purity of the bulk samples of amino acid nitroxides **1**–**4**, as well as pyrroline nitroxides **5** and **6**, used for kinetic studies, is determined by paramagnetic ^{1}H NMR spectroscopy (Table S2, Supporting Information) and EPR spin concentrations. Pyrroline nitroxide **3** and **4** are reduced, using a large excess of ascorbate and reduced glutathione (GSH), to the corresponding diamagnetic hydroxylamines, and their structures are confirmed by ^{1}H NMR spectroscopy.[21]

EPR spectra of amino acid nitroxides **1**–**4** in chloroform show triplet patterns due to ^{14}N hyperfine splitting, $a_N \approx 14$–

Scheme 3. Synthesis of *N*-Boc-Protected Amino Acids 3 and 4



16 G, and *g* values of about 2.006, similar to those for the reference pyrroline nitroxides **5** and **6**.

Rates of reduction for pyrroline nitroxides **1**–**6** are studied under pseudo-first-order conditions using a 20-fold excess ascorbate and 25-fold exccess of GSH in pH 7.4 PBS buffer. Addition of GSH leads to higher conversion of nitroxide to hydroxylamine and provides only slightly increased initial rates of the reduction of nitroxides, compared to that in the absence of GSH (ascorbate only).[9,11] Second-order rate constants, *k*, are obtained by monitoring the decay of the low-field EPR peak height of nitroxides at 295 K (Figure 2 and Table 1). Single

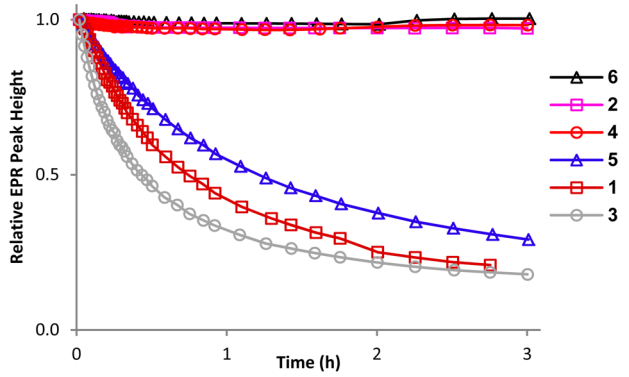


Figure 2. Reduction profiles of 0.2 mM nitroxides with 4 mM ascorbate and 5 mM GSH in 25 mM PBS pH 7.4 at 295 K.

integrated peak heights are examined and found to produce similar values of *k* for most nitroxides (Table S1, Supporting Information).[9] For comparison, the rate of reduction of the *gem*-dimethylpyrrolidine nitroxide (3-carboxy-PROXYL),[9,11,22] is measured under identical conditions (Table 1).

gem-Diethylpyrroline nitroxides **2** ($k \approx 0.001$ M^{-1} s^{-1}), **4** ($k \approx 0.003$ M^{-1} s^{-1}), and **6** ($k \approx 0.001$ M^{-1} s^{-1}) are reduced at significantly slower rates than the corresponding spirocyclohexyl nitroxides **1** ($k \approx 0.08$ M^{-1} s^{-1}), **3** ($k \approx 0.14$ M^{-1} s^{-1}),

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Table 1. Second-Order Rate Constants, k (M^{-1} s^{-1}), for Initial Rates of Reduction of Pyrroline Nitroxides (0.2 or 1 mM) with 20-fold Excess Ascorbate and 25-fold Excess of GSH in PBS (25 or 125 mM) pH 7.4 at 295 K[a,b]

0.2 mM spirocylohexyl nitroxides		1.0 mM *gem*-diethyl nitroxides	
	k		k
1	0.0845 ± 0.0018	**2**	0.0014 ± 0.0005
3	0.1370 ± 0.0023	**4**	0.0025 ± 0.0003
5[c]	0.0509 ± 0.0039	**6**	0.0012 ± 0.0000

[a]Mean ± two standard deviations from three measurements; see Table S1 in the Supporting Information. [b]k = 0.0603 ± 0.0025 M^{-1} s^{-1} was measured for 3-carboxy-PROXYL under identical conditions. [c]k = 0.07 M^{-1} s^{-1} was reported for **5**, based on imprecise temperature control, which could vary 4−5° (ref 11).

and **5** ($k \approx 0.05$ M^{-1} s^{-1}). More importantly, all three *gem*-diethylpyrroline nitroxides, which require increased concentration (from 0.2 to 1 mM) to observe measurable initial decay, show a very small decrease in concentration, leaving 97+ % of 0.2 mM nitroxide intact after 3 h (Figure 2). These results imply that amino acids functionalized with *gem*-diethylpyrroline nitroxides have great potential for spin labeling via the ribosome-mediated protein synthesis.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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