Synthesis of Unnatural Amino Acids Functionalized with Sterically Shielded Pyrroline Nitroxides

Ying Wang  
*University of Nebraska-Lincoln*

Joseph T. Paletta  
*University of Nebraska-Lincoln*

Kathleen Berg  
*University of Nebraska-Lincoln*

Erin Reinhart  
*University of Nebraska-Lincoln*

Suchada Rajca  
*University of Nebraska-Lincoln*, srajca1@unl.edu

See next page for additional authors

Follow this and additional works at: [http://digitalcommons.unl.edu/chemistryrajca](http://digitalcommons.unl.edu/chemistryrajca)
Authors
Ying Wang, Joseph T. Paletta, Kathleen Berg, Erin Reinhart, Suchada Rajca, and Andrzej Rajca
Synthesis of Unnatural Amino Acids Functionalized with Sterically Shielded Pyrroline Nitroxides

Ying Wang, Joseph T. Paletta, Kathleen Berg, Erin Reinhart, Suchada Rajca, and Andrzej Rajca*

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304, United States

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 tRNA3,4 or flexizyme-prepared aminoacylated tRNA5,6 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, we9,10 and others11,12 recently prepared sterically shielded pyrrolidine nitroxides that are by far the most resistant to ascorbate and ascorbate/glutathione mediated reduction9 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 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 139,15,16 is selectively oxidized to ketone amine 1415 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 nitroxide 17.

Figure 1. Amino acids 1−4 and pyrroline nitroxides 5 and 6.
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 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, 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 1H 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 1H NMR spectroscopy.21

EPR spectra of amino acid nitroxides 1−4 in chloroform show triplet patterns due to 14N hyperfine splitting, $a_{14N} \approx 14 - 16$ 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 excess 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 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-dimethylpyrroline nitroxide (3-carboxy-PROXYL) is measured under identical conditions (Table 1).

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.

\[ k \approx 0.08 \text{ M}^{-1} \text{s}^{-1}, \] \[ k \approx 0.14 \text{ M}^{-1} \text{s}^{-1}, \]
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 Kᵃᵇ

|            | 0.2 mM spirocyclohexyl nitroxides | 1.0 mM gem-diethyl nitroxides |
|------------|-----------------------------------|-------------------------------|
|            | k                                  | k                             |
| 1          | 0.0845 ± 0.0018                     | 2                             |
| 3          | 0.1370 ± 0.0023                     | 0.0025 ± 0.0003               |
| 5          | 0.0509 ± 0.0039                     | 0.0012 ± 0.0000               |

*“Mean ± two standard deviations from three measurements; see Table S1 in the Supporting Information. k² = 0.0603 ± 0.0025 M⁻¹ s⁻¹ was measured for 3-carboxy-PROXYL under identical conditions. 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 ≈ 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.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: arajca1@unl.edu.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was primarily supported by a National Institutes of Health consortium grant (US4GM087519). Additional support was provided by the National Science Foundation (Grant Nos. CHE-1012578 and CHE-1362454), in particular for the research of undergraduate students (K.B. and E.R.). E.R. was supported in part by the Research Experiences for Undergraduates (REU) Program of the National Science Foundation (CHE-1156560). We thank Robert Nickel, an undergraduate at UNL, who was supported by the UCARE program, for his contribution to the early stages in the synthesis of amino acids. We thank Yao Liu, an undergraduate at UNL, who was supported by the UCARE program, for her contribution to the synthesis of 7 and 15. We thank Christopher Richardson (Hamilton College), who was supported by the above referenced REU Program, for his synthesis of nitroxide 19.

**REFERENCES**

(1) Hubbell, W. L.; Lopez, C. J.; Altenbach, C.; Yang, Z. Curr. Opin. Struct. Biol. 2013, 23, 725–733.

(2) Munnihan, E. C.; Yokoyama, K.; JoAnne Stubbe, J. A. F1000 Biol. Rep. 2009, 1, 88.

(3) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. Proc. Natl. Acad. Sci. U.S.A. 2019, 91, 2910–2914.

(4) Shafer, A. M.; Kalai, T.; Liu, S. Q. B.; Hideg, K.; Voss, J. C. Biochemistry 2004, 43, 8470–8482.

(5) Goto, Y.; Katoh, T.; Suga, H. Nat. Protoc. 2011, 6, 779–790.

(6) Goto, Y.; Suga, H. Methods Mol. Biol. 2012, 848, 465–478.

(7) Schmidt, M. J.; Borbas, J.; Drescher, M.; Summerer, D. J. Am. Chem. Soc. 2014, 136, 1238–1241.

(8) Belkin, S.; Mehlhorn, R. J.; Hideg, K.; Hankovsky, O.; Packer, L. Arch. Biochem. Biophys. 1987, 256, 232–243.

(9) Paletta, J. T.; Pink, M.; Foley, B.; Rajca, S.; Rajca, A. Org. Lett. 2012, 14, 5322–5325.

(10) (a) Rajca, A.; Wang, Y.; Boska, M.; Paletta, J. T.; Olankitwanit, A.; Swanson, M. A.; Mitchell, D. G.; Eaton, S. J.; Eaton, G. R.; Rajca, S. J. Am. Chem. Soc. 2012, 134, 15724–15727. (b) Correction: Rajca, A.; Wang, Y.; Boska, M.; Paletta, J. T.; Olankitwanit, A.; Swanson, M. A.; Mitchell, D. G.; Eaton, S. J.; Eaton, G. R.; Rajca, S. J. Am. Chem. Soc. 2014, 136, 3318–3318.

(11) Kiriluyk, I. A.; Polienko, Y. F.; Krumkacheva, O. A.; Strizhakov, R. K.; Gatilov, Y. V.; Grigor’ev, I. A.; Bagryanskaia, E. G. J. Org. Chem. 2012, 77, 8016–8027.

(12) Morozov, D. A.; Kiriluyk, I. A.; Komarov, D. A.; Goti, A.; Bagryanskaia, I. Y.; Kuratieva, N. V.; Grigor’ev, I. A. J. Org. Chem. 2012, 77, 10688–10698.

(13) Sakai, K.; Yamada, K.; Yamasaki, T.; Kinoshita, Y.; Mito, F.; Utsumi, H. Tetrahedron 2010, 66, 2311–2315.

(14) Rajca, A.; Kathirvelu, V.; Roy, S. K.; Pink, M.; Rajca, S.; Sarkar, S.; Eaton, S. S.; Eaton, G. R. Chem.—Eur. J. 2010, 16, 5778–5782.

(15) Spirocyclohexyl nitroxides 5, 11, 12, and 20 and gem-diethylamines 13 and 14 were prepared using different synthetic routes in refs 11 and 16, respectively.

(16) Wetter, C.; Gierlich, J.; Knoop, C. A.; Müller, C.; Schulte, T.; Studer, A. Chem.—Eur. J. 2004, 10, 1156–1166.

(17) Hatano, B.; Araya, H.; Yoshimura, Y.; Sato, H.; Ito, T.; Ogata, T.; Kújma, T. Heterocycles 2010, 81, 349–356.

(18) Krishna, M. C.; DeGraff, W.; Hanksowski, O. H.; Sär, C. P.; Kálai, T.; Jekö, J.; Russo, A.; Mitchell, J. B.; Hideg, K. J. Med. Chem. 1998, 41, 3477–3492.

(19) Meyer, S. D.; Schreiber, S. L. J. Org. Chem. 1994, 59, 7549–7552.

(20) Boeckman, R. K., Jr.; Shao, P.; Mullins, J. J. Org. Synth. 2000, 77, 141–152.

(21) Compound 4 (~5 mM) could be only partially reduced to hydroxylamine with ~70% of its gem-diethyl nitroxide remaining. For ~3 mM 3 reduced under similar conditions, only ~1% of its spirocyclohexyl nitroxide remained (Supporting Information).

(22) Vianello, F.; Momo, F.; Scarpa, M.; Rigo, A. Magn. Reson. Imaging 1995, 13, 219–226.
Supporting Information

Synthesis of Unnatural Amino Acids Functionalized with Sterically Shielded Pyrroline Nitroxides

Ying Wang, Joseph T. Paletta, Kathleen Berg, Erin Reinhart, Suchada Rajca, Andrzej Rajca*

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304.

E-mail address: arajca1@unl.edu

Table of Contents

1. Experimental Section: General Procedures and EPR spectroscopy (pp. S2–S5).
   1.a General procedures and materials.
   1.b EPR spectroscopy and kinetic studies (Table S1).
   1.c Analysis of paramagnetic $^1$H NMR spectra (Table S2).

2. Experimental Section: Synthesis of Pyrroline Nitroxides and Amino Acids 1 – 4 (pp. S6–S29).

3. Spectra of Nitroxides (EPR, Paramagnetic $^1$H NMR, and IR) and Spectra of Diamagnetic Synthetic Intermediates (pp. S30–S58).

4. Supporting References (p. S58–S59).
1. Experimental Section.

1.a General procedures and materials.

Throughout the following paragraphs labels “JTP-4-08f1G” and alike correspond to sample or experiment codes directly traceable to the laboratory notebooks or raw data.

Chemicals and per-deuterated solvents for NMR spectroscopy were obtained from commercial sources and used as received unless otherwise indicated. Amino acids, such as L-cysteine Boc-dap-OH, were obtained from a commercial source. Purification and titration of m-CPBA were followed as previously described. Dess-Martin periodinane (DMP) was prepared as previously reported. Column chromatography was carried out on flash grade silica gel, using 0 – 20 psig pressure. Preparative TLC (PTLC) was carried out using tapered silica plates with a preadsorbent zone. Reverse-phase column chromatography (0–20 psig pressure) was carried out on regular column with reverse-phase silica gel (octadecyl, C\textsubscript{18}, bonded silica gel, 14% carbon content, 35-75 micron particle size, 150 Å pore size) with the mixture of methanol/water/ammonia (28–30% aq.) as the eluent, monitoring by analytical reverse-phase HP-TLC plates (octadecyl, C18, bonded silica gel, tapered with a preadsorbent zone).

NMR spectra were obtained using commercial spectrometers (\textsuperscript{1}H, 400, 500 and 600 MHz) using methanol-\textsuperscript{d}\textsubscript{4} (CD\textsubscript{3}OD) and chloroform-\textsuperscript{d} (CDCl\textsubscript{3}) as solvent. The 500 MHz instrument was equipped with a cryoprobe. The chemical shift references were as follows: \textsuperscript{1}H methanol-\textsuperscript{d}3, 3.31 ppm, \textsuperscript{1}H chloroform, 7.26 ppm. Typical 1D FID was subjected to exponential multiplication with an exponent of 0.3 Hz (For selected spectra smaller values of LB were used to resolve closely spaced resonances, as indicated in the spectral data summaries). IR spectra were obtained using a commercial instrument, equipped with an ATR sampling accessory. MS analyses were carried out at the local mass spectrometry facility.

1.b EPR spectroscopy and kinetic studies.

EPR spectroscopy. CW X-band EPR spectra for nitroxides in solution were acquired on an X-band EPR instrument, equipped with a frequency counter and nitrogen flow temperature control (130–300 K). The spectra were obtained using a dual mode cavity; all spectra were recorded using an oscillating magnetic field perpendicular (TE\textsubscript{102}) to the swept magnetic field. DPPH powder (\textit{g} = 2.0037) was used as a \textit{g}-value reference.
**Kinetic studies.** The results of kinetic measurements are summarized in Table S1 (this Section). The ascorbate solution was made with ascorbic acid, diethylenetriaminepentaacetic acid (DTPA, 0.1 mM), sodium hydroxide, and sodium phosphates (<30 ppm transition metals) at pH 7.4, measured with pH/ion analyzer. Phosphate buffer was made with sodium phosphates and DTPA (0.1 mM) at pH 7.4 and used to make nitroxide solutions. Typically, kinetic runs were carried out with a 20-fold molar excess of ascorbate and 25-fold molar excess of GSH. Prior to a typical kinetic run, solutions of nitroxide and ascorbate/GSH were filtered through a 0.45 µm nylon syringe filter, combined in equal portions, vortexed for 6 seconds, and then the resultant mixture was drawn into an EPR-quality quartz capillary tube (0.6-mm I.D.). The capillary was stoppered with parafilm and placed in a 5-mm O.D. EPR sample tube in the cavity of X-band spectrometer. The peak height (PH, Table S1) and the integrated peak height (IPH, Table S1) of the low-field line of the triplet were measured as a function of time. Microwave power was kept under 6.5 mW and temperature was controlled at 295 K with nitrogen flow system. Typical EPR parameters for kinetics of *gem*-diethyl nitroxides: power 15 dB, modulation amplitude 4 G, sweep width 20 G, 2 scans, receiver gain 4.48E+04, conversion time 20.48 ms, time constant 20.48 ms, and sweep time 10.49 s. Typical EPR parameters for kinetics of spirocyclohexyl nitroxides: power 15 dB, modulation amplitude 4 G, sweep width 30 G, 2 scans, receiver gain 1.00E+05, conversion time 20.48 ms, time constant 20.48 ms, and sweep time 10.49 s.
Table S1. Kinetics of reduction of 0.2 or 1 mM nitroxides with 20-fold molar excess of ascorbate and 25-fold molar excess of GSH.

| Compd Label | Sample Run No. | Data Label | Initial Kinetics (<1 h) | Range of fit | 
|-------------|----------------|------------|-------------------------|-------------|
|             |                |            | k × 10^4 (M^-1 s^-1)    | Remaining radical (%) |
|             |                |            | k × 10^4 (M^-1 s^-1)    | Time (s)    |

**Notes:**
- *k* = 0.07 M^-1 s^-1 was reported for 5 at rt, however, the temperature was not well controlled in this measurement.53

**Table Data:**

| Compd Label | Sample Run No. | Data Label | Initial Kinetics (<1 h) | Range of fit | 
|-------------|----------------|------------|-------------------------|-------------|
|             |                |            | k × 10^4 (M^-1 s^-1)    | Remaining radical (%) |
|             |                |            | k × 10^4 (M^-1 s^-1)    | Time (s)    |
1.c Analysis of paramagnetic $^1$H NMR spectra.

Table S2. Analyses of paramagnetic $^1$H NMR spectra for pyrroline nitroxides and amino acids 1 – 4.

| Compd. | Sample label | Paramagnetic integrals | Diamagnetic integrals | Int $(\text{H}_2\text{O})$ | Concentration of nitroxide (M) | Minimum apparent purity (%)$^a$ |
|--------|--------------|------------------------|-----------------------|---------------------------|-----------------------------|--------------------------------|
| 11     | YW11-72col   | 1.000, 4.420, 6.748, 0.758, 2.408, 10.868, 3.707, 0.595 | 30.504 | 0.054, 0.108, 0.272 | 0.434 | 0.73 | 99 |
| 12     | YW11-77col1  | 1.309, 1.948, 3.000, 0.349, 0.907, 2.054, 1.134 | 10.842 | 0.065, 0.047, 0.121 | 0.233 | 0.70 | 98 |
| 1     | YW11-88colA2 | 0.748, 1.040, 1.390, 1.439, 1.163, 1.759, 0.423 | 7.962 | 0.205, 0.047, 0.031, 0.088, 0.052, 0.636, 0.320 | 1.379 | 0.095 | 85$^b$ |
| 17    | YW12-71col   | 1.000, 1.859, 0.948, 0.190 | 3.997 | 0.001, 0.018, 0.096 | 0.115 | 0.30 | 97 |
| 18    | YW12-78col   | 2.919, 3.935, 1.840, 3.000, 0.972, 1.035 | 13.701 | 0.003, 0.048, 0.261, 0.062 | 0.371 | 0.54 | 97 |
| 2     | YW13-08crp2  | 3.902, 1.627, 2.000, 0.615, 0.578, 0.831, 0.607 | 10.16 | 0.038, 0.152, 0.079 | 0.269 | 0.14 | 97 |
| 19    | CRR-1-55-f2  | 1.000, 2.086, 3.047, 4.668, 2.813, 1.590, 2.074, 0.692, 1.111, 0.574 | 19.655 | 0.069, 0.760 | 0.829 | 1.8 | 96 |
| 5     | JTP-10-8-fl  | 1.000, 0.142, 6.541, 3.498, 4.395 | 15.576 | 0.010, 0.179, 0.130 | 0.319 | 1.4 | 98 |
| 20    | JTP-10-14-fl | 0.710, 4.000, 1.718, 0.855, 0.932 | 8.215 | 0.060, 0.275 | 0.335 | 0.109 | 0.5 | 96 |
| 3     | YW13-11col   | 0.264, 1.335, 2.530, 0.741, 0.853, 12.000, 2.222, 0.491 | 20.436 | 0.008, 0.483, 0.121 | 0.612 | 0.10 | 97 |
| 21    | JTP-10-20-fl | 5.654, 5.614, 3.000, 1.771, 0.751, 0.487, 1.043 | 18.320 | 0.035, 0.253, 0.309, 0.008 | 0.605 | 2.5 | 97 |
| 6     | JTP-10-21-fl | 10.000, 14.717, 3.408, 3.313 | 31.438 | 0.310, 0.268, 0.024, 0.106 | 0.708 | 0.045 | 1.3 | 98 |
| 22    | JTP-10-23-fl | 1.554, 3.062, 4.554, 4.000, 0.024, 1.670, 0.311, 2.054 | 17.229 | 0.079, 0.163, 0.079, 0.153, 0.321, 0.063 | 0.858 | 0.178 | 0.8 | 95 |
| 4     | YW13-17col   | 1.998, 0.380, 1.309, 2.338, 1.120, 1.115, 12.000, 1.080 | 21.34 | 0.027, 0.029, 0.085 | 0.141 | 0.17 | 99 |

$^a$Minimum apparent purity (%) is computed under the assumption that some of the paramagnetic peaks are missed (or under-integrated) because they are too broadened to be detected at relatively low concentrations of nitroxides.  

$^b$Very low concentration (<0.095 M, saturated solution) of nitroxide amino acid 1 was used because of its low solubility in common organic solvents (see: footnote “a”).
2. Experimental Section: Synthesis of Pyrroline Nitroxides and Amino Acids 1 – 4.

Scheme S1. Synthesis of amino acid 1.

YW10_80: Starting material, 7-aza-3,11-dithiadispiro[5.1.5.3]hexadecane-15-one (7) was prepared according to the previously published procedure. This reference was followed in preparation of 8. To the stirred solution of 7-Azadispiro[5.1.5.3]hexadecan-15-one (7) (label: BF2-71; 0.96 g, 4.1 mmol, 1.0 equiv) in acetic acid (2.0 mL), a solution of Br₂ (1.05 mL, 20.4 mmol, 5.0 equiv) in acetic acid (1.5 mL) was added drop by drop. A yellow precipitate was formed at the beginning and finally a red-orange mixture was obtained. The mixture was stirred at ambient temperature for overnight. The precipitate was filtered off, which was washed with acetic acid.
(totally 25 mL), then diethyl ether (3 mL × 2) and evacuated under high vacuum at ambient
temperature to yield the product 8 (1.99 g, yield: 100%; label: YW1080PPT) as a yellow powder
which was directly used for next step without further purification.

Summary for preparation of compound 9:

| Run | ID       | SM (g/mmol) | Na (mL/ equiv) | MeOH (mL) | Yield (g/%) | Label          |
|-----|----------|-------------|---------------|-----------|-------------|----------------|
| 1   | YW1040   | 1.68/3.5    | 0.41/5.0      | 14        | 0.48/43     | YW10-40crp1    |
| 2   | YW1085   | 1.99/5.1    | 0.84/7.2      | 18        | 0.51/46     | YW10-85-fr2&3  |
| 3   | YW1157   | 8.36/21.5   | 4.08/17.0     | 120       | 1.69/36     | YW11-57crp2&3  |

YW10_85: We followed this reference55 for preparation of gem-dimethyl pyrroline methyl ester.
In argon bag, metal sodium (0.84 g, 36.3 mmol, 7.16 equiv) was dissolved into anhydrous methanol
(12 mL) in a round bottom flask to give a sodium methoxide solution in methanol. The flask was
sealed, then an argon balloon was attached. To this solution in ice-water bath, the mixture of
starting material 8 (1.99 g, 5.1 mmol, 1.0 equiv; label: YW10-80cr) in 6.0 mL anhydrous methanol
was added dropwise at 0 °C in five portion during 50 min. The ice-water bath was removed, and the
reaction mixture was stirred at ambient temperature for 12 h. The mixture was concentrated under
reduced pressure until most the methanol was removed. The residue was re-dissolved in 10%
aqueous K₂CO₃, then extracted with ethyl ether. The ether layer was washed with brine (× 3), dried
over Na₂SO₄, concentrated under reduced pressure. Purification on silica gel flash column
chromatography (ether/hexanes, 1/12) to give the product 9, 0.51 g (label: YW10-85fr2&3; Yield:
46%), as a light yellow pasty. Rf = 0.24 (ether/hexanes, 1/12). ¹H NMR (CDCl₃, 400 MHz,
YW11-57crp2), δ = 6.85 (s, 1H), 3.74 (s, 1H), 2.13-2.05 (m, 2H), 1.66-1.28 (m, 18H). ¹³C-NMR
(CDCl₃, 100MHz, YW11-57crp2): δ = 165.0, 147.9, 139.4, 67.8, 66.4, 51.3, 40.0, 37.6, 25.6, 25.4,
23.4, 22.6. IR (ZnSe, cm⁻¹, YW11-57crp2): 2922, 2952, 1716, 1449, 1434, 1327, 1226, 1139,
1031, 1001, 915, 906, 765. LRMS-ESI (0.1% HCOOH in MeOH, YW11-57crp2), m/z (ion type,
% RA for m/z, 150–2000) at [M+H]⁺: 264.4 (100%), 265.3 (17%). TOF-HRMS-EI (1%
CH$_3$COONa in 3:1 (v/v) MeOH/H$_2$O; ion type, %RA for m/z, deviation from the formula; sample label: YW11-57crp2): Calcd. for $^{12}$C$_{16}$H$_{26}$NO$_2$ at [M+H]$^+$: 264.1964; found: 264.1969 (2.1 ppm).

Summary for preparation of compound 10:

| Run | ID     | SM (g/mmol) | Red-Al (mL/ equiv) | toluene (mL) | Yield (g/%) | Label         |
|-----|--------|-------------|--------------------|-------------|------------|---------------|
| 1   | YW1051 | 0.170/0.66  | 0.80/3.9           | 1.5         | 0.045/30   | YW1051fr3     |
| 2   | YW1096 | 0.250/0.95  | 0.80/2.7           | 2.0         | 0.12/52    | YW1096col     |
| 3   | YW1124 | 0.250/0.95  | 0.59/2.0           | 2.0         | 0.14/63    | YW1124col     |
| 4   | YW1162 | 1.987/5.52  | 4.8/2.1            | 12          | 0.81/73    | YW1162fr2,3&5 |

YW11_62: The procedure was slightly modified from the one which was published before for preparation of 3-(hydroxymethyl)-2,2,5,5-tetramethyl-3-pyrroline.$^{56}$ To the solution of ester 9 (label: YW11-57-crp1-3; 1.98 g, 7.52 mmol, 1.0 equiv) in anhydrous toluene, sodium bis(2-methoxyethoxy)aluminumhydride (Red-Al, 65% solution in toluene, 3.215 M; 4.8 mL, 15.4 mmol, 2.05 equiv) was added dropwise in argon atmosphere at $-40$ °C. The mixture was warmed up to ambient temperature and stirred for 20 min. The mixture was cooled back to 0 °C, quenched with aqueous KOH solution (20%, 10 mL), then extracted with ethyl ether. The organic layer was washed with brine ($\times$ 3), dried over Na$_2$SO$_4$, then concentrated under reduced pressure. Purification on silica gel flash column chromatography (ether/hexanes, 20/80 to 30/70) to give the product 10, 0.81 g (label: YW11-62fr2,3&5; Yield: 73%), as almost colorless pasty. $R_f = 0.23$ (ether/hexanes, 40/60). $^1$H NMR (CDCl$_3$, 400 MHz, YW11-62crp2), $\delta = 5.78$ (s, 1H), 4.20 (d, 2H, $J = 1.6$ Hz), 1.66-1.41 (m, 20H). $^{13}$C-NMR (CDCl$_3$, 100MHz, YW11-62crp2): $\delta = 147.9$, 129.4, 67.5, 66.2, 59.3, 41.0, 38.4, 25.8, 25.6, 23.9, 25.6. IR (ZnSe, cm$^{-1}$, YW11-62crp2): 3301, 2091, 2850, 1448, 1414, 1066, 1014, 908, 673. LRMS-ESI (0.1% HCOOH in MeOH, YW11-62crp2), m/z (ion type, % RA for m/z, 150–2000) at [M+H]$^+$: 236.3 (100%), 237.3 (17%). TOF-HRMS-EI (1% CH$_3$COONa in 3:1 (v/v) MeOH/H$_2$O; ion type, %RA for m/z, deviation from the formula; sample label: YW11-62crp2): Calcd. for $^{12}$C$_{15}$H$_{26}$NO at [M+H]$^+$: 236.2014; found: 236.2004 (-4.4 ppm).
Summary for preparation of compound 11:

| Run | ID   | SM (g/mmol) | mCPBA (g/equiv) | Product | Yield (mg%) | Spin Conc (%) | Label          | Comment                  |
|-----|------|-------------|-----------------|---------|-------------|---------------|----------------|--------------------------|
| 1   | YW1114 | 0.010/0.043 | 0.0089/1.20     | 0.0051/48 | 104         | YW11-14r2     |                |                          |
| 2   | YW1123 | 0.055/0.23  | 0.066/1.63      | 0.0344/58 | 96          | YW11-23col    |                |                          |
| 3   | YW1153 | 0.12/0.52   | 0.17/1.91       | 0.047/36 | /           | YW12-53crp1   |                |                          |
| 4   | YW1170 | 0.19/0.80   | 0.23/1.64       | 0.63/74  | 99          | YW11-72col&col3 |                | Restricted 28 mg pure for characterization |
| 5   | YW1172 | 0.61/2.6    | 0.72/1.61       |          |             |                |                |                          |

YW11_23: To compound 10 (label: YW10-96col; 55.1 mg, 0.23 mmol, 1.0 equiv) in anhydrous DCM (2.2 mL) at argon atmosphere, *meta*-Chloroperoxybenzoic acid (mCPBA: commercial compound which washed with PBS buffer, YW1165; 66.0 mg, 0.38 mmol, 1.6 equiv) in anhydrous DCM (2.2 mL) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 24 h to give a yellow suspension mixture. The mixture was concentrated under reduced pressure. The residue was redissolved into ether, washed with saturated aqueous NaHCO₃ (× 3) then brine (× 2), dried over Na₂SO₄, then concentrated under reduced pressure. Purification on silica gel flash column chromatography (benzene/acetone, 100/3) to give the product 11, 34.4 mg (label: YW11-23col; Yield: 58%, spin concentration 96%) as yellow pasty which solidify after kept at freezer (–20 °C) for 3 days. \(R_f = 0.26\) (ether/hexanes, 40/60) and 0.48 (acetone/benzene, 10/90). M.p. 92–97 °C (under argon, YW11-72col); lit.: S3 yellow crystals, mp 106–108 °C. Paramagnetic \(^1\)H NMR spectrum for 11, see: Fig. S9. EPR (sample label: YW11-72col; 1.11 mM in CHCl₃): g-value = 2.006; \(a_N = 14.72\) G; spin concentration 99% (data label: YW1281r5&6, YW1324r5). IR (ZnSe, \(\text{cm}^{-1}\), YW11-72col): 3406, 2924, 2855, 1448, 1413, 1063, 1049, 997, 906, 829, 689; lit.: S3 3362, 3059, 2930, 2858, 1454, 1443, 1408, 1356, 1207, 1194, 1173, 1130, 1047, 1018, 1007, 905, 841, 673, 638, 608, 554. LRMS-ESI (0.1% TFA in DCM, YW11-72col), m/z (ion type, % RA for m/z, 120–800) at \([\text{M}]^+\): 250.4 (61%, \(N\)-oxomethanaminium); at \([\text{M}+2\text{H}]^+\): 252.3 (100%, protonated hydroxylamine). TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for m/z, deviation from the formula; sample label: YW11-72col): Calcd. for \(^{12}\text{C}_{15}\text{H}_{24}\text{N}_{23}\text{Na}_2\text{O}_2\) at \([\text{M}+\text{Na}]^+\): 273.1705; found: 273.1708 (1.2 ppm).
Summary for preparation of 12:

| Run | ID    | SM (g/mmol) | Sulfonyl Chloride (mL/equiv) | Et₃N (mL/equiv) | Product               |
|-----|-------|-------------|------------------------------|----------------|-----------------------|
| 1   | YW1130| 0.032/0.127 | 0.012/1.22                   | 0.022/1.25     | YW11-30col            |
| 2   | YW1177| 0.60/2.40   | 0.23/1.20                    | 0.44/1.31      | YW11-77col1&3        |

YW11_77: The starting material 11 (label: YW11-72col; 0.60 g, 2.4 mmol, 1.0 equiv) was placed in a Schlenk vessel, and then evacuated under high vacuum for 12 h. The vessel was charged with argon. To the vessel, anhydrous DCM (8 mL) was added, following the addition of triethylamine (redistilled, 0.44 mL, 3.2 mmol, 1.3 equiv). The resultant colorless solution was cooled down to –15 °C with ice/acetone bath, then methanesulfonyl chloride (225 μL, 2.9 mmol, 1.2 equiv) was added drop by drop, providing a yellow suspended mixture. The mixture was warmed up to ambient temperature and stirred for 3 h. The mixture was diluted with DCM, washed with 5% aqueous NaHCO₃ (× 3) then brine (× 2). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. Purification on silica gel flash column chromatography (benzene/acetone, 100/1) to give the product 12, 0.54 g (label: YW11-77col&col3; Yield: 68%, spin concentration 100%, data label: YW1186r5) as yellow solid. Rf = 0.48 (benzene/acetone, 95/5). M.p. 122–125 °C (under argon, YW11-77col1); lit.:S₃ yellow crystals, mp 125–127 °C. Paramagnetic ¹H NMR spectrum for 12, see: Fig. S12. EPR (YW11-77col1, 0.52 mM in CHCl₃): g-value = 2.006; αN = 14.69 G; spin concentration 100% (data label: YW1186r5&6, YW1324r8). IR (ZnSe, cm⁻¹, YW11-77col1): 2930, 2857, 1454, 1355, 1174, 966, 924, 907, 859, 833; lit.:⁵ S3 3024, 3003, 2945, 2926, 2856, 1445, 1414, 1355, 1173, 972, 961, 908, 841, 808, 737, 673, 528, 492. LRMS-ESI (0.1% TFA in DCM, YW11-77col1), m/z (ion type, %RA for m/z, 120–800) at [M]+: 328.3 (96%, N-oxomethanaminium); at [M+2H]+: 330.3 (100%, protonated hydroxylamine). TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, m/z, sample label: YW11-77col1): Calcd. for ¹²C₁₆H₂₆NO₄²³NaS at [M+Na]+: 351.1480; found: 351.1470 (-2.9 ppm).
Summary for preparation of amino acid 1:

| Run | ID      | SM (g/mmol) | Cysteine (g/mmol) | DBU (mL/equiv) | Product | Comment | Yield (mg/%) | Spin Conc (%) | Label       |
|-----|---------|-------------|-------------------|----------------|---------|---------|-------------|---------------|-------------|
| 1   | YW1114  | 0.034/0.10 | 0.020/1.60        | 0.028/1.82     | 37.2/102| 72      | YW11-78PPT2 |
| 2   | YW1183  | 0.105/0.32 | 0.062/1.61        | 0.087/1.82     | 86/76   | 89      | YW11-83col  |
| 3   | YW1188  | 0.165/0.50 | 0.97/1.60         | 0.135/1.81     | 97/55   | 100     | YW11-88colA2 |
| 4   | YW1231  | 0.166/0.51 | 0.97/1.58         | 0.140/1.85     | 93.6/52 | 94      | YW12-31colA |
|     |         |             |                   |                |         | 87      | YW11-88colB3 |

YW11_88: To L-cysteine (96.5 mg, 0.80 mmol, 1.60 equiv) crystals in a Schlenk vessel prefilled with argon, anhydrous DMF (2.6 mL) was added, following the addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, redistilled; 135 μL, 0.90 mmol, 1.81 equiv). The mixture was stirred at ambient temperature for 1 h, providing a homogeneous colorless solution with tiny undissolved L-cysteine crystal suspended. The mixture was cooled to 0 °C with ice-water bath. Then nitroxide 12 (sample label: YW11-77col&col3; 163.5 mg, 0.50 mmol, 1.0 equiv) in anhydrous DMF (1.2 mL) was added. The mixture was stirred at ambient temperature for 12 h. The resultant pale yellow milk mixture was concentrated under high vacuum, re-dissolved into methanol (3 mL), and centrifuged to remove the white precipitate. The obtained yellow homogenous solution was concentrated under reduced pressure. The residue was purified on silica gel (reverse phase, C18, 40% surface coverage) flash column chromatography (MeOH/H2O/(28%, aq.)NH4OH, 10/80/10) twice to give the product 1, 59.2 mg (label: YW11-88colA2) with spin concentration of 100% and 37.8 mg (label: YW11-88colB3) with spin concentration of 94% as yellow solid. Rf = 0.52 (reverse phase silica gel, C18, 14% C; MeOH/H2O/(28%)NH4OH, 60/30/10). M.p. 150–155 °C (under argon, YW11-88colA2). Paramagnetic 1H NMR spectrum for 1, see: Fig. S15. EPR (YW11-88colA2, 1.00 mM in MeOH): αN = 14.95 G; spin concentration 100% (EPR label: YW1189r3). IR (ZnSe, cm\(^{-1}\), YW11-88colA2): 3067, 2926, 2855, 1644, 1620, 1535, 1452, 1412, 1390, 1343, 906. Optical rotation (sample label: YW11-88colA2; 2.39 mg in 5.0 mL methanol): \([\alpha]_{520}^{20} = −10.5^\circ\). LRMS-ESI (0.1% HCOOH in MeOH, YW11-88colA2), m/z (ion type, % RA for m/z, 150–2000) at
[M+H]+: 354.3 (100%); at [2M+H]+: 707.3 (35%). TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for m/z, deviation from the formula; sample label: YW11-88colA2): Calcd. for ¹²C₁₈H₂₉N₂O₃²³Na at [M+Na]+: 376.1797; found: 376.1800 (0.9 ppm).

Scheme S2. Synthesis of amino acid 2

Summary for preparation of 14:

| Run | ID       | SM (g/mmol) | DMP (g/equiv) | NaHCO₃ (g) | Yield (g%) | Label       | Comments                                      |
|-----|----------|-------------|---------------|------------|------------|-------------|------------------------------------------------|
| 1   | YW1055   | 0.020/0.093 | 0.055/1.4     | 0.118      | 0.0079/40  | YW11-55crp1 |                                 |
| 2   | YW1128A  | 0.50/2.35   | 1.50/1.5      | 3.04/15.4  | 0.42/43    | YW11-28col  |                                 |
| 3   | YW1128B  | 0.51/2.37   | 1.51/1.5      | 3.13/15.7  | 1.59/99    | YW11-32cr   |                                 |
| 4   | YW1132A  | 0.79/3.72   | 2.37/1.5      | 4.69/15.0  | 1.77/78    | YW12-39cr   |                                 |
| 5   | YW1132B  | 0.78/2.69   | 2.36/1.5      | 4.66/15.0  | 1.09/98    | YW12-95cr   |                                 |
| 6   | YW1239A  | 1.16/5.47   | 3.50/1.5      | 6.92/15.1  | 1.30/1.6   | YW11-88col  |                                 |
| 7   | YW1239B  | 1.12/5.26   | 3.38/1.5      | 6.66/15.1  | 2.43/15.3  | YW12-95cr   |                                 |
| 8   | YW1295A  | 0.58/2.71   | 1.74/1.5      | 3.57/15.7  | 2.43/15.3  | YW12-95cr   |                                 |
| 9   | YW1295B  | 0.40/1.89   | 1.30/1.6      | 2.43/15.3  | 1.30/1.6   | YW12-95cr   |                                 |

* Purified by silica gel flash column chromatography at the first step. ** Passed through a very short gel flash column chromatography.  
† Did not purified. Crude NMR showed it was almost pure.
For reactions on very small scale, typically only tiny amount of water is needed to accelerate the reaction. In these cases, small amount of degassed water (for example, 10 μL) was added into comparatively large amount of anhydrous DCM (for example, 2.0 mL) in a vial under argon atmosphere, sonicated to give a milk solution, then part of the mixture was transferred to the reaction. Then, sonication and transfers in portion were repeated until enough water was added.

YW12_95A: To starting material 2,2,6,6-tetraethylpiperidin-4-ol (13) (0.579 g, 2.71 mmol, 1.0 equiv; sample label: EFR-1-18cr) in 50 mL round bottle flask, NaHCO₃ (Aldrich, 3.56 g, 42.5 mmol, 15.7 equiv) was added. The mixture was briefly evacuated under high vacuum for about 3 min, then charged with argon, following the addition of anhydrous DCM (from purification system, 13 mL). After the suspension mixture was cooled down to 0 °C, to this mixture, Dess–Martin periodinane (1.74 g, 4.1 mmol, 1.52 equiv) in anhydrous DCM (10 mL) was added drop by drop. The mixture was warmed up to ambient temperature, then to this mixture, degassed pure water (57.4 μL, 3.19 mmol, 1.18 equiv) was added dropwise within 45 min (9 drops, 1 drop per 5 min). After stirred at ambient temperature for totally 4 hours, the brown-yellow suspension was concentrated under reduced pressure. By the same approach, another reaction (experiment label: YW1295B) with 0.505 g starting material was also taken parallel. The residues were combined. To the residue, a mixture of 10% Na₂S₂O₄/(sat.) NaHCO₃ (1:1) was added until the pH was adjusted to 8–9, then taken up with ethyl ether (15 mL×4). The combined organic layers was washed with brine (×3) and water, dried over Na₂SO₄ and concentrated under reduced pressure again to provide the brown crude mixture 14, 1.09 g (crude yield: 98%), with crude ¹H NMR which showed it was almost pure.
Summary for preparation of **15**:

| Run | ID       | SM (g/mmol b) | SM Label | Br2 (1st step) (mL/ equiv) | Na (2nd Step) (g/equiv) | Yield (g%) | Label      |
|-----|----------|---------------|----------|----------------------------|-------------------------|------------|------------|
| 1   | YW1084   | 0.095/0.45    | YL1-71col| 0.19/8.3                   | /                       | 0.0276/26  | YW11-01crp2&3 |
|     | YW1101   | -/-0.45       | YW10-84cr | /                          | 0.45/39.5               |            |            |
| 2   | YW1141A  | 0.42/1.97     | YW11-28col| 0.84/8.3                   | 1.92                    | d          | /          |
| 3   | YW1141B  | 0.54/2.39     | YW11-32cr | 1.0/8.1                    | 2.7/40.0                | 0.184/10  | YW11-54crp1 |
| 4   | YW1244   | 1.77/8.38     | YW12-39cr | 3.5/8.1                    | /                       |            | /          |
|     | YW1246   | -/-4.19       | YW12-44cr | /                          | 3.92/40.7               | 0.50/25   | YW12-46col  |
|     | YW1251   | -/-4.19       | YW12-44cr | /                          | 9.95/41.0               |            | YW12-51col  |
| 5   | YW1297   | 1.08/-4.60    | YW12-95cr | 1.9/8.0                    | /                       | 4.33/0.9  | 0.163/15  |
|     | YW1307   | -/-4.60       | YW12-97cr | /                          | 3.92/40.7               | /          | YW13-07crp1&2 |

* Purified by silica gel flash column chromatography at the first step.  
* If the starting material was a crude compound, the molar amount of starting material was calculated according to the amount of its precursor.  
* Crude starting material was used.  
* Products decomposed after the crude seat in fridge for three weeks.  
* Yield for three steps including the reaction of oxidation by DMP.

**YW12_44, YW12_46 & YW12_51:**

Bromination: To compound **14** (crude material obtained from the previous step, 1.77 g, 8.38 mmol, 1.0 equiv; sample label: YW12-39cr) in 25 mL round bottle flask, acetic acid (7.0 mL) was added. The brown mixture was cooled down to 0 °C. To the flask, bromine (Br2, 3.5 mL, 68.0 mmol, 8.1 equiv) was added at 0 °C drop by drop. The obtained dark brown mixture was warmed up to ambient temperature and stirred under the light-free condition for 48 h. The dark solution was evaporated with the flowing of nitrogen, and the exhaust was absorbed with 1:1 (v/v) saturated Na2S2O3(aq.)/NaOH (aq.) solution. Then DCM (10 mL) was added and evaporated with nitrogen again. By repeating this for 2~3 times, most of the residual bromine was removed. The obtained brown pasty was evacuated under high vacuum line with two additional liquid nitrogen traps, providing brown foam. To remove the possible trace amounts of acetic acid, the foam was further evacuated under high vacuum for 24 h.

Favorskii rearrangement: In a nitrogen-filled glove bag, the foam from previous step as re-dissolved into 4 mL anhydrous DCM, and half of the brown solution (4.19 mmol, 1.0 equiv) was transferred to a Schlenk vessel, evaporated with the flowing of nitrogen and evacuated under high vacuum for 12 h then re-dissolved into 10 mL anhydrous methanol. In an argon-filled glove bag,
metal sodium (3.92 g, 170.4 mmol, 40.7 equiv) was dissolved into anhydrous methanol (60 mL) in a 250 mL round bottom flask to give a sodium methoxide solution in methanol. The flask was sealed and an argon balloon was attached. The solution was cooled down to 0 °C. Then to this solution, the brown solution of di-bromo compound was added dropwise at 0 °C, providing a light brown suspension. The reaction mixture was stirred at 0 °C for 30 min, then warmed up to ambient temperature and stirred for 48 h. The mixture was concentrated under reduced pressure until most the methanol was removed. The residue was re-dissolved into cool (0 °C) 100 mL 10% aqueous K₂CO₃, then extracted with ethyl ether (× 5). The combined ether layer was washed with brine (× 3), dried over Na₂SO₄, concentrated under reduced pressure. Using this procedure, the other half of dibromo crude compound was also converted to 15. Purification of the crude mixtures on silica gel flash column chromatography (ether/hexanes, 10/90) gave the product 15 in the total amount of 0.50 g as a light pale pasty (label: YW12-46col and YW12-50col; yield for conversion from 13 to 15: 25%). \( R_f = 0.53 \) (ether/hexanes, 20/80). \(^1\)H NMR (CDCl₃, 400 MHz, YW12-46col2), \( \delta = 6.72 \text{ (s, 1H)}, 3.72 \text{ (s, 3H)}, 1.73-1.64 \text{ (m, 4H)}, 1.54-1.51 \text{ (m, 4H)}, 0.88 \text{ (t, 6H, } J = 7.2 \text{ Hz)}, 0.83 \text{ (t, 6H, } J = 7.2 \text{ Hz)} \). \(^{13}\)C-NMR (CDCl₃, 100 MHz, YW12-46col2): \( \delta = 164.9, 148.8, 137.0, 72.2, 66.2, 51.4, 32.2, 31.5, 9.1, 8.9 \). IR (ZnSe, cm\(^{-1}\), YW12-46col): 2962, 2937, 2869, 1717, 1458, 1436, 1319, 1269, 1236, 1160, 1071, 996, 928, 763. TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for m/z, deviation from the formula; sample label: YW12-46col): Calcd. for \(^{12}\)C₁₄H₂₆NO₂ at \([\text{M+H}]^+\): 240.1964; found: 240.1968 (1.9 ppm).
Summary for preparation of 16:

| Run | ID      | SM (g/mmol) | Red-Al* (mL/equiv) | toluene (mL) | Yield (g/%) | Label      |
|-----|---------|-------------|--------------------|--------------|-------------|------------|
| 1   | YW1237  | 0.059/0.25  | 0.30/2.5           | 0.5          | 0.020/39    | YW12-37col |
| 2   | YW1259  | 0.48/2.0    | 2.9/3.0            | 3.5          | 0.20/46     | YW12-59fr1 |

* 65% (w/w, 3.215 M) solution in toluene.

YW12_59: To the solution of ester 15 (label: YW11-01crp2, YW12-46col, YW12-51col; 0.48 g, 2.0 mmol, 1.0 equiv) in anhydrous toluene, sodium bis(2-methoxyethoxy)aluminumhydride (Red-Al, 65% solution in toluene, 3.215 M; 2.9 mL, 6.1 mmol, 3.0 equiv) was added dropwise at –40 °C under the flowing of argon. The mixture was warmed up to ambient temperature and stirred for 20 min. Then the mixture was cooled back to 0 °C, quenched with aqueous KOH solution (20%, 5 mL), and extracted with ethyl ether. The organic layer was washed with brine (× 3), dried over Na₂SO₄ and concentrated under reduced pressure. Purification on silica gel flash column chromatography (acetone/DCM, 10/90 to 25/75) to give the product 16, 0.20 g (label: YW12-59fr1; Yield: 46%), light-yellow pasty. \( R_f = 0.35 \) (ether/hexanes, 40/60). \(^1\)H NMR (CDCl₃, 400 MHz, YW12-37col), δ = 5.63 (s, 1H), 4.07 (t, 2H, \( J = 1.6 \) Hz), 1.52–1.41 (m, 8H), 0.84 (t, 12H, \( J = 7.6 \) Hz). \(^1^3\)C-NMR (CDCl₃, 100MHz, YW12-37col): δ = 145.2, 130.1, 77.8, 69.1, 59.6, 32.3, 32.1, 9.1, 8.9. IR (ZnSe, cm⁻¹, YW12-37col): 3321, 2961, 2921, 2876, 1458, 1413, 1378, 1100, 1040, 992, 921, 845. LRMS-ESI (0.1% HCOOH in MeOH, YW12-37col), m/z (ion type, % RA for m/z, 150–1000) at [M+H]^+: 212.4 (100%), 213.4 (12%). TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for m/z, deviation from the formula; sample label: YW12-37col): Calcd. for \(^{12}\)C₁₃H₂₆NO at [M+H]^+: 212.2014; found: 212.2018 (1.7 ppm).
Summary for preparation of 17:

| Run | ID    | SM (g/mmol) | mCPBA (g/equiv) | Yield (mg/%) | Spin Conc. (%) | Label      |
|-----|-------|-------------|-----------------|--------------|----------------|------------|
| 1   | YW1243| 0.0056/0.026| 0.0074/1.61     | 0.0031/55    | 97             | YW12-43col |
| 2   | YW1265| 0.0175/0.083| 0.0258/1.80     | 0.0104/56    | 90             | YW12-65col |
| 3   | YW1271| 0.177/0.84   | 0.265/1.83      | 0.148/78     | 103            | YW12-71col |

YW12_71: To compound 16 (label: YW12-59fr1; 176.6 mg, 0.84 mmol, 1.0 equiv) in anhydrous DCM (9.0 mL) at argon atmosphere, meta-chloroperoxybenzoic acid (m-CPBA: commercial compound washed with PBS buffer, S1 YW1165; 264.8 mg, 1.53 mmol, 1.8 equiv) in anhydrous DCM (6.0 mL) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 20 h to give a yellow suspension mixture. The mixture was concentrated under reduced pressure. The residue was re-dissolved into ether, washed with saturated aqueous NaHCO₃ (× 3) then brine (× 2), dried over Na₂SO₄, and then concentrated under reduced pressure. Purification on silica gel flash column chromatography (benzene/acetone, 9/1) to give the product 17, 148.2 mg (label: YW12-71col; Yield: 78%, spin concentration 103% (original one from calculation)) as yellow pasty. 

\[ R_f = 0.25 \] (ether/hexanes, 40/60) and 0.23 (acetone/benzene, 10/90). Paramagnetic \(^1\)H NMR spectrum for 19, see: Fig. S33. EPR (sample label: YW12-71col; 2.3 mM in CHCl₃): \( g \)-value = 2.006; \( a_N \) = 14.31 G; spin concentration 103% (data label: YW1274r5&6). IR (ZnSe, cm\(^{-1}\), YW12-71col): 3408, 2967, 2937, 2879, 1458, 1415, 1377, 1111, 1051, 1025, 934, 906, 828, 679. TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for \( m/z \), deviation from the formula; sample label: YW12-71col): Calcd. for \(^{12}\)C\(^{13}\)H\(^{24}\)N\(^{23}\)NaO\(_2\) at \([M+Na]^+\): 249.1705; found: 249.1709 (1.7 ppm).
Summary for preparation of 18:

YW12_78: To starting material 17 (label: YW12-65col & YW12-71col; 147.5 mg, 0.65 mmol, 1.0 equiv) in a schlenk vessel pre-filled with argon, anhydrous DCM (8 mL) was added, following the addition of triethylamine (redistilled, 120 μL, 0.86 mmol, 1.3 equiv). The resultant colorless solution was cooled down to –15 °C with ice/acetone bath, then methanesulfonyl chloride (Aldrich, 61 μL, 0.79 mmol, 1.2 equiv) was added drop by drop, providing a yellow suspended mixture. The mixture was warmed up to ambient temperature and stirred for 3 h, then diluted with DCM, washed with 5% aqueous NaHCO₃ (× 3) then brine (× 2). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. Purification on silica gel flash column chromatography (benzene/acetone, 100/2) to give the product 18, 172 mg (label: YW12-78col; Yield: 87%, spin concentration 103% (original datum from calculation), data label: YW1281r2-3) as yellow solid. Rf = 0.36 (benzene/acetone, 95/5). M.p. 58–61 °C (under argon, YW12-78col). Paramagnetic ¹H NMR spectrum for 18, see: Fig. S27. EPR (YW12-78col, 2.67 mM in CHCl₃): g-value = 2.006; αN = 14.25 G; spin concentration 103% (original one from calculation, data label: YW1281r2-3). IR (ZnSe, cm⁻¹, YW12-78col): 2969, 2939, 2880, 1459, 1417, 1354, 1173, 967, 923, 859, 827. TOF-HRMS-EI (1% CH₃COONa in 3:1 (v/v) MeOH/H₂O; ion type, %RA for m/z, deviation from the formula; sample label: YW12-78col): Calcd. for ¹³C₁₄H₂₆NO₄²³NaS at [M+Na]⁺: 327.1480; found: 327.1477 (-1.0 ppm).
Summary for preparation of spin-labeled amino acid 2:

| Run | ID    | SM (mg/μmol) | L-cysteine (mg/equiv) | DBU (μL/equiv) | Product | Yield (mg%) | Spin Conc (%) | Label        | Comment                  |
|-----|-------|--------------|-----------------------|----------------|----------|-------------|---------------|--------------|--------------------------|
| 1   | YW1282| 10.2/33.5    | 6.5/1.6               | 9/1.8          | 5.8/50   | 62          | YW12-82col    |              |                          |
| 2   | YW1292| 6.2/20.4     | 3.9/1.6               | 6/2.0          | 1.94/28  | 63          | YW12-92col-t3 |              |                          |
| 3   | YW1308| 25.2/82.8    | 16.0/1.6              | 25/2.0         | 3.95/14  | 99          | YW13-08crp1   |              |                          |

YW13_08: To L-cysteine (Aldrich, 16.0 mg, 132 μmol, 1.6 equiv) crystal in a schlenk vessel prefilled with argon anhydrous DMF (180 μL) was added, following the addition of 1,8-Diazabicyclo[5.4.0] undec-7-ene (DBU, Aldrich, redistilled; 25 μL, 167 μmol, 2.0 equiv). The mixture was stirred at ambient temperature for 1 h, providing a homogeneous colorless solution. The mixture was cooled to 0 °C with ice-water bath. Then nitroxide 18 (sample label: YW12-78col; 25.2 mg, 82.8 μmol, 1.0 equiv) in anhydrous DMF (150 μL) was added. The mixture was stirred at ambient temperature for 15 h. The resultant pale yellow milk mixture was centrifuged. The supernatant homogenous yellow solution was concentrated with the flowing of nitrogen, then evacuated under high vacuum. The residue which could dissolved into 0.15 mL methanol to form a homogenous solution was purified on silica gel (reversed phase, C18, 40% surface coverage) flash column chromatography (MeOH/H2O/(28%)NH4OH, 10/80/10) to give the product 2 of 3.95 mg (label: YW13-08crp1) with spin concentration of 99% and 8.92 mg (label: YW13-08crp2) with spin concentration of 102% (original datum from calculation) as yellow solid. \( R_f = 0.80 \) (reversed phase silica gel, C18, 14% C; MeOH/H2O/(28%)NH4OH, 60/30/10). M.p. 163–165 °C (crystalline-like particles after evaporated from methanol and smashed; under argon, YW13-08crp2). Paramagnetic \(^1\)H NMR spectrum for 2, see: Fig. S30. EPR (YW13-08crp1, 0.97 mM in MeOH): \( g\)-value = 2.006; \( \alpha_N = 14.67 \) G; spin concentration 99% (data label: YW1313r5). IR (ZnSe, cm\(^{-1}\), YW13-08crp2): 3124, 2968, 2936, 2869, 1628, 1507, 1460, 1394, 1341, 1095, 935, 832, 668. TOF-HRMS-EI (in 3:1 (v/v) MeOH/H2O; ion type, %RA for m/z, deviation from the formula; sample label: YW13-08crp2): Calcd. for \(^{12}\)C\(_{16}\)H\(_{29}\)N\(_2\)O\(_3\)\(_{23}\)NaS at [M+Na]\(^{+}\): 352.1797;
found: 352.1802 (1.5 ppm). Optical rotation (sample label: YW13-08crp1&2; 10.36 mg in 2.3 mL methanol): $[\alpha]_{20}^D = -7.7^\circ$.

**Scheme S3.** Synthesis of N-Boc-protected amino acids 3 and 4

| Reaction label | SM (mg/mmol) | $m$-CPBA (g/mmol) | DCM (mL) | Time (h) | Yield (mg/%) | Product label | Spin conc. (%) |
|---------------|--------------|-------------------|----------|----------|-------------|---------------|---------------|
| CRR-1-45      | 59/0.225     | 0.158/0.915       | 3        | 35       | 18.9/30     | CRR-1-45-f10  | 97            |
| CRR-1-55      | 205/0.778    | 0.530/3.071       | 11       | 45       | 99.0/46     | CRR-1-55-f2   | 102*          |
| JTP-7-78      | 487/1.849    | 1.317/7.63        | 18.2     | 74       | 195.9/38    | JTP-7-78      |               |
| JTP-9-3       | 1595/6.058   | 4.314/24.99       | 60       | 60       | 938/56      | JTP-9-3-f1    | 106*          |
| JTP-9-97      | 497/1.893    | 1.275/7.39        | 20       | 46       | 205/39      | JTP-9-97-f1   |               |

CRR-1-55: A 25 mL three neck round bottom flask was charged with ester-amine 9 (204.8 mg, 0.778 mmol), evacuated, charged with nitrogen and dissolved in DCM (4 mL), the resulting solution was cooled in an ice water bath and a solution of $m$-CPBA in DCM (0.530 g, 3.071 mmol...
in 7 mL) was added dropwise over 5 minutes. The resulting green solution was stirred at rt for 45 h, over which time the solution became yellow, and finally was evaporated. The resulting solid was dissolved in diethyl ether (25 mL) and washed at 0 °C with saturated aqueous sodium bicarbonate solution (3 × 20 mL), then a 1.5 M aqueous solution of sodium bisulfate (2 × 10 mL), then saturated aqueous sodium bicarbonate solution again (2 × 10 mL). After drying over magnesium sulfate, evaporation in rotary evaporator gave a yellow crude oil which was purified by column chromatography (silica gel, chloroform/ethyl ether/hexanes, 1:1:18) to give nitroxide 19 as yellow crystals (99 mg, 46 %). mp 92–93 °C. EPR; spin concentration ~100%. LR-ESI MS (0.1 % TFA in DCM): m/z ion type (%RA for m/z = 150–800): 262.4 [M-O]⁺ (100%). Paramagnetic ¹H NMR spectrum for 19, see: Fig. S33. EPR (sample label: CRR-1-55-f2; 1.0 mM in CHCl₃): g-value = 2.006; aN = 14.59 G; spin concentration 102% (data label: JP819r7&13). IR (diamond, cm⁻¹, JTP-9-3-f1): 2925, 2856, 1920, 1626, 1436, 1357, 1310, 1237, 1192, 1163, 1118, 1063, 1040, 1024, 257, 908, 840, 775, 760, 731. TOF-HRMS- EI (methanol/sodium acetate, label: JTP-9-3-f1): m/z ion type (%RA for m/z = 95-820): 301.1641 [M+Na]⁺ (75%, 4.3 ppm for C₁₆H₂₄NO₃Na).

![nitroxide 19](image)

| Reaction label | SM           | SM (mg/mmol) | NaOH (mg/mmol) | EtOH (mL) | Yield (mg/%) | Product label |
|---------------|--------------|--------------|----------------|-----------|--------------|---------------|
| JTP-10-2      | JTP-9-97-f1  | 8/0.028      | 7/0.17         | 0.3       | Yes by HRMS  |               |
| JTP-10-8      | JTP-9-97-f1  | 130/0.468    | 138/3.425      | 5.8       | 76/61        | JTP-10-8-f1   |

JTP-10-8: 19 (130 mg, 0.468 mmol), sodium hydroxide (138 mg, 3.43 mmol), and ethanol (5.8 mL) were added to a vial, and then stirred at rt for 4 days. Subsequently, the yellow solution was concentrated. The resultant oil was diluted with water (2 mL) and washed with diethyl ether (2 × 2 mL), acidified with aqueous sodium bisulfate (1.5 M, 3 mL), and extracted chloroform (3 × 2 mL). The organic phase was dried over sodium sulfate and evaporated, yielding a crude yellow solid which was purified by column chromatography (silica gel, diethyl ether/chloroform, 1:24). The yellow band corresponding to the product (the only intensely colored band) was collected, to give nitroxide 5 as a yellow solid (76 mg, 61%). M.p. 203 °C (decomp.); lit.: S3 light-yellow

S21
crystals, mp 196–198 °C (ethyl acetate). Paramagnetic ¹H NMR spectrum for 5, see: Fig. S35.
EPR (sample label: JTP-10-8-f1; 0.4 mM in PBS): g-value = 2.005; $\alpha_N = 16.03$ G; spin concentration 102% (data label: JP864r4&9).
IR (diamond, cm⁻¹, JTP-10-8-f1): ~3000 (broad), 2956, 2929, 2863, 2849, 1719, 1626, 1446, 1421, 1293, 1229, 1167, 1116, 995, 932, 907, 859, 780, 759, 684, 668; lit.: S3 2955, 2932, 2862, 1722, 1626, 1449, 1421, 1294, 1231, 1171, 1117, 995, 933, 908, 860, 845, 779, 760, 685.
TOF-HRMS-EI (methanol/sodium acetate, label: JTP-10-8-f1): $m/z$ ion type (%RA for $m/z = 50-300$): 264.1605 [M]$^+$ (11%, 2.0 ppm for C₁₅H₂₂NO₃).

| Reaction label | SM (mg/mmol) | NHS (mg/mmol) | DCC (mg/mmol) | THF (mL) | Yield (mg/%) | Product label | Spin conc. (%) |
|----------------|--------------|---------------|---------------|---------|--------------|---------------|----------------|
| JTP-10-14      | 66/0.319     | 34/0.295      | 31/0.319      | 3.5     | 73/92        | JTP-10-14-f1  | 100            |
| JTP-10-12      | 8/0.031      | 7/0.062       | 11/0.055      | 0.4     | Yes TLC      | JTP-10-8-f1   |                |

JTP-10-14: Nitroxide 5 (58 mg, 0.219 mmol), N-Hydroxysuccinimide (34 mg, 0.295 mmol), and $N,N'$-Dicyclohexylcarbodiimide (66 mg, 0.319 mmol) were placed in a 10 mL conical flask, and then briefly evacuated and filled with nitrogen gas. THF (3.5 mL, freshly distilled from sodium and benzophenone) was added and the resulting yellow solution was stirred at rt, protected from light, for 5 days. The reaction mixture was evaporated in rotary evaporator and filtered through cotton with dichloromethane, evaporation of which yielded a crude yellow oil. The crude was dissolved in chloroform and purified by column chromatography (silica gel, chloroform/acetone, 97:3). The product was the only colored (yellow) band. Evaporation of solvents yielded active ester 20 as a yellow solid (73 mg, 92%, label JTP-10-14-f1). A portion (32 mg) was purified by column chromatography (silica gel, chloroform/acetone, 97:3), yielding a yellow solid (31 mg, label JTP-10-14-f1). M.p. 198–201 °C; lit.: S3 yellow crystals, mp 203–204 °C (methanol).

Paramagnetic ¹H NMR spectrum for 20, see: Fig. S37. EPR (sample label: JTP-10-14-f1; 1.0 mM in CHCl₃): g-value = 2.006; $\alpha_N = 14.46$ G; spin concentration 100%. IR (diamond, cm⁻¹, JTP-10-14-f1): 2930, 2858, 1768, 1738, 1447, 1368, 1305, 1202, 1080, 1066, 1047, 993, 964, 908, 892, 741; lit.: S3 2934, 2856, 1767, 1738, 1620, 1450, 1427, 1371, 1306, 1225, 1203, 1084, 1069,
993, 964, 895, 766, 743, 648, 594. TOF-HRMS-ESI (methanol/sodium acetate, label: JTP-10-14-f1): m/z ion type (%RA for m/z = 90-900): 416.1921 [M+CH$_3$OH+Na]$^+$ (98%, 0.6 ppm for C$_{20}$H$_{29}$N$_2$O$_6$Na), 438.1829 [M+CH$_3$O+Na$_2$]$^+$ (87%, 20 ppm for C$_{20}$H$_{28}$N$_2$O$_6$Na$_2$).

Summary for preparation of N-Boc-protected amino acid 3:

| Run | ID     | SM (mg/μmol/equiv) | Boc-dap-OH (mg/equiv) | Hüning’s base (DIEA) (μL/equiv) | Yield (mg%) | Spin Conc. (%) | Label         |
|-----|--------|-------------------|-----------------------|---------------------------------|-------------|----------------|---------------|
| 1   | YW1310 | 9.3/25.7/1.1      | 4.8/1.0               | 14/3.4                          | 20.3/48     | 95             | YW13-11col    |
| 2   | YW1311 | 28.6/79.1/1.1     | 14/6.1/1.0            | 43/3.4                          | 11.6/27     | 97             | YW13-11col2   |

YW13_10 and YW13_11: To aminoacid Boc-dap-OH (Bachem; 4.8 mg, 23.5 μmol, 1.0 equiv) in a schlenk vessel under argon atmosphere, N,N-Diisopropylethylamine (DIEA; 14 μL, 80.2 μmol, 3.4 equiv) was added. The mixture was cooled to 0 °C. Then to the vessel, nitroxide active ester 20 (9.3 mg, 25.7 μmol, 1.1 equiv) in 0.18 mL anhydrous DMF was added dropwise. The suspension mixture was stirred at ambient temperature with light-free for 24 h. The obtained yellow homogenous solution was concentrated by the flowing of nitrogen, then evacuated under high vacuum for 12 h to give a yellow pasty. With the same procedure, another reaction with 28.6 mg of 20 was also prepared. The two crude pasty were combined and purified on silica gel flash column chromatography (DCM/MeOH/AcOH, 96/4/1) to give the product 3 (20.3 mg, label: YW13-11col) with spin concentration of 95% and 11.6 mg (label: YW13-11col2) with spin concentration of 97% as yellow solid (Yield: 68%). $R_f = 0.38$ (DCM/MeOH/AcOH, 95/5/1). M.p. 186–188 °C (under argon, YW13-11col2). Paramagnetic $^1$H NMR spectrum for 3, see: Fig. S39. EPR (YW13-11col2, 0.99 mM in MeOH): g-value = 2.006; $\alpha_N = 14.89$ G; spin concentration 97% (data label: YW1312r5). IR (ZnSe, cm$^{-1}$, YW13-11col): 3426, 3348, 2935, 2858, 1748, 1669, 1618, 1538, 1502, 1430, 1366, 1314, 1230, 1149, 848, 782, 666. LRMS-ESI (0.1% HCOOH in MeOH, YW13-11col2), m/z (ion type, % RA for m/z, 150–2000) at [M+H]$^+$: 451.5 (22%); [M+Na]$^+$: 473.7 (17%); at [2M+Na]$^+$: 923.9 (100%). TOF-HRMS-EI (0.1% TFA in 3:1 (v/v) MeOH/H$_2$O; ion type, %RA for m/z, deviation from the formula; sample label: YW13-11col): Calcd. for
(sample label: YW13-11col.CB; 23.93 mg in 2.0 mL methanol): \([\alpha]_D^{20} = -2.5^\circ\).  

\[ ^{12}\text{C}_{23}\text{H}_{35}\text{N}_3^{23}\text{Na}_2\text{O}_6 \text{ at } [\text{M–H+2Na}]^+ : 495.2321; \text{ found: } 495.2335 (2.8 \text{ ppm}) . \]

JTP-10-20: A small vial was charged with ester-amine 15 (163 mg, 0.681 mmol) under nitrogen, and dissolved in DCM (1.9 mL), the resulting solution was cooled in an ice water bath and a solution of \(m\)-CPBA in DCM (307 mg, 1.779 mmol in 3.4 mL) was added dropwise. The resulting green solution was stirred for 1 h, then the ice bath was removed and stirred for 2 h at rt, over which time the solution became yellow, and finally was evaporated. The resulting solid was dissolved in diethyl ether (5 mL) and washed with an aqueous solution of sodium bisulfate (1.5 M, 2 \times 3 mL), then saturated aqueous sodium bicarbonate solution (3 \times 5 mL). After drying over sodium sulfate, evaporation in rotary evaporator gave a yellow crude oil which was purified by column chromatography (silica gel, diethyl ether/pentane, 1:9) to give nitroxide 21 as a yellow oil (128 mg, 74 \%). Paramagnetic \(^1\text{H} \text{ NMR} \) spectrum for 21, see: Fig. S42. EPR (sample label: JTP-10-20-f1; 0.5 mM in CHCl\(_3\)): \(g\)-value = 2.006; \(a_N = 14.22 \text{ G} \) IR (diamond, cm\(^{-1}\), JTP-10-20-f1): 2966, 2938, 2879, 1719, 1427, 1375, 1276, 1247, 1207, 1165, 1084, 962, 940, 783, 755.

TOF-HRMS-ESI (methanol/sodium acetate, label: JTP-10-20-f1): \(m/z\) ion type (%RA for \(m/z = 80-920\)): 277.1664 [M+Na]\(^+\) (75\%, 3.6 ppm for C\(_{14}\)H\(_{24}\)NO\(_3\)Na).

\[ \text{JTP-10-21: Charge 21, sodium hydroxide (147 mg, 3.675 mmol), and ethanol (4.3 mL) to a vial and stir at rt for 3 days before concentrating the yellow solution. The resulting oil was diluted} \]
with water (5 mL) and washed with chloroform (3 × 5 mL), acidified with aqueous sodium bisulfate (1.5 M, 3 mL), and extracted chloroform (3 × 5 mL). The organic phase was dried over sodium sulfate and evaporated, yielding a crude yellow solid which was purified by column chromatography (silica gel, diethyl ether/chloroform, 1:24). The yellow band corresponding to the product (the only intensely colored band) was collected, to give nitroxide 6 as a yellow solid (63 mg, 61%). M.p. 126–129 °C. Paramagnetic ^1H NMR spectrum for 6, see: Fig. S44. EPR (sample label: JTP-10-21-f1; 0.4 mM in PBS): g-value = 2.006; a_N = 15.76 G; spin concentration 93%. IR (diamond, cm^{-1}, JTP-10-21-f1): ~3000 (broad), 2979, 2944, 2878, 1685, 1624, 1445, 1427, 1288, 1255, 1174, 1089, 943, 749, 680. TOF-HRMS- EI (methanol/sodium acetate, label: JTP-10-21-f1): m/z ion type (%RA for m/z = 50-300): 240.1600 [M]^+ (15%, 0.1 ppm for C_{13}H_{22}NO_{3}).

![Chemical structure of nitroxide 6 and active ester 22](image)

| Reaction label | SM (mg/mmol) | SM (mg/mmol) | NHS (mg/mmol) | DCC (mg/mmol) | THF (mL) | Yield (mg%) | Product label |
|----------------|--------------|--------------|----------------|----------------|----------|------------|---------------|
| JTP-10-23      | JTP-10-23    | 41/0.362     | 42/0.362       | 56/0.270       | 3        | 57/99      | JTP-10-23-f1  |

JTP-10-23: Nitroxide 6 (41 mg, 0.171 mmol), N-hydroxysuccinimide (42 mg, 0.362 mmol), and N,N'-dicyclohexylcarbodiimide (56 mg, 0.270 mmol) were charged to a 10 mL conical flask, briefly evacuated and filled with nitrogen. THF (3 mL, freshly distilled from sodium and benzophenone) was added and the resulting yellow solution was stirred at rt, protected from light, for 2 days. The reaction mixture was evaporated in rotary evaporator and filtered through cotton with dichloromethane, evaporation of which yielded a crude yellow oil. The crude was dissolved in chloroform and purified by column chromatography (silica gel, chloroform/acetone, 97:3). The product was the only colored (yellow) band. Evaporation of solvents yielded active ester 22 as a yellow solid (57 mg, 99%). M.p. 90.5–92.0 °C. Paramagnetic ^1H NMR spectrum for 22, see: Fig. S46. EPR (sample label: JTP-10-23-f1; 0.8 mM in CHCl_3): g-value = 2.006; a_N = 14.13 G; spin concentration 96% (data label: JP875r2&4). IR (Diamond, cm^{-1}, JTP-10-23-f1): 2972, 2939, 2881, 1769, 1736, 1622, 1458, 1421, 1369, 1235, 1197, 1136, 1065, 992, 978, 942, 910, 881, 841,
Summary for preparation of N-Boc-protected amino acid 4:

| Run | ID    | SM (mg/μmol/ equiv) | Boc-dap-OH (mg/ equiv) | Hüning’s base (DIEA) (μL/ equiv) | Yield (mg/%) | Spin Conc. (%) | Label       |
|-----|-------|---------------------|------------------------|----------------------------------|--------------|----------------|-------------|
| 1   | YW1317| 28.2/83.6/1.1       | 15.5/1.0               | 45/3.4                           | 26.8/83      | 100            | YW13-17col  |

YW13_17: To amino acid Boc-dap-OH (15.5 mg, 75.9 μmol, 1.0 equiv) in a Schlenk vessel under argon atmosphere, N,N-Diisopropylethylamine (DIEA; 45 μL, 0.26 mmol, 3.4 equiv) was added. The mixture was cooled to 0 °C. Then to the vessel, nitroxide active ester 22 (28.2 mg, 83.6 μmol, 1.1 equiv) in 0.20 mL anhydrous DMF was added dropwise. The suspension mixture was stirred at ambient temperature with light-free for 24 h. The obtained yellow homogenous solution was concentrated by the flowing of nitrogen, then evacuated under high vacuum for 12 h to give a yellow pasty. It was purified on silica gel flash column chromatography (DCM/MeOH/AcOH, 96/3.5/1). The resultant solution was concentrated under reduced pressure. Azeotropic distillation of the concentrated solution (with some residual TFA) with methanol (5 mL × 5) and DCM (5 mL × 3) rapidly, then re-dissolved in 0.2 mL DCM, evaporated with the flowing of nitrogen, and evacuated under high vacuum to give the product 4 of 26.8 mg as yellow foam (label: YW13-17col; Yield: 83%) with spin concentration of 100%. $R_f = 0.38$ (DCM/MeOH/AcOH, 95/5/1). M.p. 58–67 °C (under argon, YW13-17col). Paramagnetic $^1$H NMR spectrum for 4, see: Fig. S48. EPR (YW13-17col, 0.97 mM in MeOH): $g$-value = 2.006; $\alpha_N = 14.59$ G; spin concentration 97% (data label: YW1318r5). IR (ZnSe, cm$^{-1}$, YW13-11col): 3336, 2970, 2935, 2877, 1697, 1655, 1611, 1520, 1367, 1300, 1250, 1160, 1048, 1021, 936, 851, 774, 747. LRMS-ESI (0.1% HCOOH in MeOH, YW13-17col), m/z (ion type, % RA for m/z, 150–2000) at [M+H]$^+$: 427.5 (29%); [M+Na]$^+$: 449.7 (11%); at [2M+Na]$^+$: 975.9 (100%). TOF-HRMS-EI (0.1% TFA in 3:1 (v/v) MeOH/H$_2$O; ion type, %RA for m/z, deviation from the formula; sample label: YW13-11col): Calcd. for
$^{12}$C$_{21}$H$_{35}$N$_{3}$O$_{6}$ at $[\text{M}-\text{H}\text{+2Na}]^+$: 471.2321; found: 471.2340 (4.0 ppm). Optical rotation (sample label: YW13-11col.CB; 23.93 mg in 2.0 mL methanol): $[\alpha]_{D}^20 = -3.1^\circ$.

**Scheme S4**. Reduction of N-Boc-protected amino acids 3 and 4

YW13_22: N-Boc-protected amino acid 3 (label: YW13-11col.CB; 2.16 mg, 4.79 µmol, 1.0 equiv) was dissolved into 1.5 mL phosphate buffered saline (PBS; 125 mM) to give a homogenous solution with initial spin concentration of 3.27 mM (data label: YW1322r3-4). To this solution, L-glutathione reduced (36.86 mg, 120 µmol, 25.0 equiv) was added, following the addition of L-ascorbic acid (16.82 mg, 95.5 µmol, 19.9 equiv). The pH value of this solution was adjusted rapidly by NaOH powder to 7.3. The mixture was stirred at ambient temperature with the protection from light for 3 h.

In parallel, to amino acid 4 (label: YW13-17col; 2.25 mg, 5.28 µmol, 1.0 equiv) in 1.0 mL PBS (125 mM) with initial spin concentration of 5.20 mM (data label: YW1322r5-6), L-glutathione reduced (40.62 mg, 132 µmol, 25.0 equiv) and L-ascorbic acid (18.36 mg, 104 µmol, 19.7 equiv) was added in sequence. After its pH value was adjusted to 7.3 rapidly, it was stirred at ambient temperature with the protection from light for 3 h.

EPR spin concentration measurement showed that, after 3 hours, the spin concentration of the solution of 3 decreased to 0.0198 mM (conversion: 99.4%), and that of 4 to 3.462 mM (conversion:
Then respectively, the solutions were acidified with 0.5 M NaHSO₄ to pH 4, extracted with ethyl acetate (2 mL× 6). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure then evacuated under high vacuum to give the residue from the reaction of 3 of 1.23 mg (label: YW13-11col.CBR), and from 4 of 1.91 mg (label: YW13-17colR). The residue were dissolved into CD₃OD and characterized by ¹H NMR (Fig S1 and S2).

YW13-11col.CBR (1.23 mg in 0.14 mL 99.98D% MeOD)  
NAME     YW13-11col.CBR  
EXPNO                 1  
PROCNO                1  
Date_          20140814  
Time               2.34  
INSTRUM           spect  
FIDRES         0.122266 Hz  
AQ            4.0894966 sec  
RG               210.59  
DW               62.400 usec  
DE                10.00 usec  
TE                298.2 K  
D1           1.00000000 sec  
-------- CHANNEL f1 --------  
SFO1        400.1324710 MHz  
SI                65536  
WDW                  EM  
SSB                   0  
LB                 0.30 Hz  
CB                    0  
PC                 1.00  

Figure S1. ¹H NMR spectrum (400 MHz, CD₃OD) of the extracted residue from the reduced solution of amino acid 3 (label: YW13-11col.CBR).
YW13-17col.R (1.91 mg in 0.13 mL 99.98D% MeOD)

Figure S2. $^1$H NMR spectrum (400 MHz, CD$_3$OD) of the extracted residue from the reduced solution of amino acid 4 (label: YW13-17colR).
3. Spectra of Nitroxides (EPR, Paramagnetic $^1$H NMR, and IR) and Spectra of Diamagnetic Synthetic Intermediates.

Figure S3. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of compound 9 (label: YW11-57crp2).

Figure S4. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of compound 9 (label: YW11-57crp2).
Figure S5. IR spectrum (ATR, ZnSe) of compound 9 (label: YW11-57crp2).

Figure S6. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 10 (label: YW11-62crp2).
Figure S7. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 10 (label: YW11-62crp2).

Figure S8. IR spectrum (ATR, ZnSe) of compound 10 (label: YW11-62crp2)
Figure S9. $^1$H NMR spectrum (500 MHz, 0.73 M in CDCl$_3$) of 11 (label: YW11-72col).

Figure S10. IR spectrum (ATR, ZnSe) of compound 11 (label: YW11-72col)
Figure S11. LRMS-ESI spectrum (solvent: 0.1% TFA in DCM) of compound 11 (label: YW11-72col)

Figure S12. $^1$H NMR spectrum (500 MHz, 0.70 M in CDCl$_3$) of 12 (label: YW11-77col1).
Figure S13. IR spectrum (ATR, ZnSe) of compound 12 (label: YW11-77col1)

Figure S14. LRMS-ESI spectrum (solvent: 0.1% TFA in DCM) of 12 (label: YW11-77col1)
Figure S15. $^1$H NMR spectrum (400 MHz, 0.095 M) of saturated 1 (label: YW11-88colA2) in CD$_3$OD.

Figure S16. IR spectrum (ATR, ZnSe) of compound 1 (label: YW11-88colA2)
Figure S17. LRMS-ESI spectrum (solvent: 0.1% HCOOH in MeOH) of compound 1 (label: YW11-88colA2)

Figure S18. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 15 (label: YW12-46col2).
Figure S19. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 15 (label: YW12-46col2).

Figure S20. IR spectrum (ATR, ZnSe) of compound 15 (label: YW12-46col)
**Figure S21.** $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 16 (label: YW12-37col).

**Figure S22.** $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 16 (label: YW12-37col).
Figure S23. IR spectrum (ATR, ZnSe) of compound 16 (label: YW12-37col)

Figure S24. $^1$H NMR spectrum (400 MHz, 0.30 M in CDCl$_3$) of 17 (label: YW12-71col).
Figure S25. IR spectrum (ATR, ZnSe) of compound 17 (label: YW12-71col).

Figure S26. LRMS-ESI spectrum (solvent: 1% TFA in DCM) of 17 (label: YW12-71col)
Figure S27. $^1$H NMR spectrum (400 MHz, 0.54 M in CDCl$_3$) of 18 (label: YW12-78col).

Figure S28. IR spectrum (ATR, ZnSe) of compound 18 (label: YW12-78col).
Figure S29. LRMS-ESI spectrum (solvent: 1% TFA in DCM) of 18 (label: YW12-78col).

Figure S30. $^1$H NMR spectrum (400 MHz, 0.14 M in CD$_3$OD) of 2 (label: YW13-08crp2).
Figure S31. IR spectrum (ATR, ZnSe) of compound 2 (label: YW13-08crp2).

Figure S32. LRMS-ESI spectrum (solvent: 0.1% HCOOH in MeOD) of 2 (label: YW13-08crp1)
Figure S33. $^1$H NMR spectrum (500 MHz, 1.8 M in CDCl$_3$) of 19 (label: CRR-1-55-f2).

Figure S34. IR spectrum (ATR, diamond) of compound 19 (label: JTP-9-3-f1).
Figure S35. $^1$H NMR spectrum (500 MHz, 1.4 M in CDCl$_3$) of 5 (label: JTP-10-8-f1).

Figure S36. IR spectrum (ATR, diamond) of compound 5 (label: JTP-10-8-f1).
Figure S37. $^1$H NMR spectrum (500 MHz, 0.5 M in CDCl$_3$) of 20 (label: JTP-10-14-f1).

Figure S38. IR spectrum (ATR, diamond) of compound 20 (label: JTP-10-14-f1).
Figure S39. $^1$H NMR spectrum (400 MHz, 0.10 M in CD$_3$OD) of 3 (label: YW13-11col).

Figure S40. IR spectrum (ATR, ZnSe) of compound 3 (label: YW13-11col)
Figure S41. LRMS-ESI spectrum (solvent: 0.1% HCOOH in MeOH) of 3 (label: YW13-11col2).

Figure S42. $^1$H NMR spectrum (500 MHz, 2.5 M in CDCl$_3$) of 21 (label: JTP-10-20-f1).
Figure S43. IR spectrum (ATR, diamond) of compound 21 (label: JTP-10-20-f1).

Figure S44. $^1$H NMR spectrum (500 MHz, 1.3 M in CDCl$_3$) of 6 (label: JTP-10-21-f1).
Figure S45. IR spectrum (ATR, diamond) of compound 6 (label: JTP-10-21-f1).

Figure S46. $^1$H NMR spectrum (500 MHz, 0.8 M in CDCl$_3$) of 22 (label: JTP-10-23-f1).
Figure S47. IR spectrum (ATR, diamond) of compound 22 (label: JTP-10-23-f1).

Figure S48. $^1$H NMR spectrum (400 MHz, 0.17 M in CD$_3$OD) of 4 (label: YW13-17col).
Figure S49. IR spectrum (ATR, ZnSe) of compound 4 (label: YW13-17col)

Figure S50. LRMS-ESI spectrum (solvent: 0.1% HCOOH in MeOH) of 4 (label: YW13-17col)
Figure S51. EPR (X-band) spectrum of **11** (sample label: YW11-72col; 1.11 mM in CHCl$_3$; EPR label: YW1324r5, parameters: power, 30 dB, 12.59 µW; modulation amplitude 0.8 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 5.02 × 10$^4$).

Figure S52. EPR (X-band) spectrum of **12** (sample label: YW11-77col1; 0.99 mM in CHCl$_3$; EPR label: YW1324r8, parameters: power, 30 dB, 12.59 µW; modulation amplitude 0.8 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 5.02 × 10$^4$).

Figure S53. EPR (X-band) spectrum of **1** (sample label: YW11-88colA2; 1.0 mM in MeOH), used for spin concentration determination (EPR label: YW1189r3, parameters: power, 20 dB, 2.046 mW; modulation amplitude 1.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 7.96 × 10$^4$).
Figure S54. EPR (X-band) spectrum of 19 (sample label: CRR-1-11-f1; 1.0 mM in CHCl₃; EPR label: JP819r7, parameters: power, 30 dB, 12.59 µW; modulation amplitude 2.0 G; conversion time 40.96 ms; time constant 1.28 ms; resolution in X, 1024 points; receiver gain, 2.00 × 10⁴).

Figure S55. EPR (X-band) spectrum of 5 (sample label: JTP-10-8-f1; 1.2 mM in CHCl₃; EPR label: JP864r4, parameters: power, 30 dB, 12.59 µW; modulation amplitude 2.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 1.12 × 10⁴).

Figure S56. EPR (X-band) spectrum of 20 (sample label: JTP-1 0-14-f1; 1.1 mM in CHCl₃; EPR data label: JP864r5, parameters: power, 30 dB, 12.59 µW; modulation amplitude 2.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 1.42 × 10⁴).
Figure S57. EPR (X-band) spectrum of 21 (sample label: JTP-10-20-f1; 0.5 mM in CHCl$_3$; EPR label: JP1102r4, parameters: power, 20 dB, 2.046 mW; modulation amplitude 0.5 G; conversion time 40.96 ms; time constant 1.28 ms; resolution in X, 1024 points; receiver gain, $5.64 \times 10^3$).

Figure S58. EPR (X-band) spectrum of 6 (sample label: JTP-10-21-f1; 0.9 mM in PBS; EPR label: JP869r2, parameters: power, 15 dB, 6.469 mW; modulation amplitude 2.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, $5.64 \times 10^3$).

Figure S59. EPR (X-band) spectrum of 22 (sample label: JTP-10-23-f1; 0.8 mM in CHCl$_3$; EPR label: JP875r2, parameters: power, 30 dB, 12.59 µW; modulation amplitude 2.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, $8.93 \times 10^3$).
Figure S60. EPR (X-band) spectrum of 3 (sample label: YW13-11col2; 0.99 mM in CH₃OH; EPR label: YW1312r5, parameters: power, 20 dB, 2.046 mW; modulation amplitude 1.0 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 7.96 × 10⁴).

Figure S61. EPR (X-band) spectrum of 19 (sample label: YW12-71col; 2.25 mM in CHCl₃; EPR label: YW1274r10, parameters: power, 30 dB, 12.59 µW; modulation amplitude 0.5 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 2.24 × 10⁴).

Figure S62. EPR (X-band) spectrum of 20 (sample label: YW12-78col; 2.66 mM in CHCl₃; EPR label: YW1281r3, parameters: power, 30 dB, 12.59 µW; modulation amplitude 0.5 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 1.59 × 10⁴).
Figure S63. EPR (X-band) spectrum of 2 (sample label: YW13-08crp2; 0.97 mM in CH$_3$OH; EPR label: YW1313r5, parameters: power, 20 dB, 2.046 mW; modulation amplitude 0.5 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 7.96 × 10$^4$).

Figure S64. EPR (X-band) spectrum of 4 (sample label: YW13-17col; 0.97 mM in CH$_3$OH; EPR0 label: YW1318r5, parameters: power, 20 dB, 2.064 mW; modulation amplitude 0.5 G; conversion time 40.96 ms; time constant 10.24 ms; resolution in X, 1024 points; receiver gain, 7.96 × 10$^4$).

4. Supporting References.

S1. Rajca, A.; Shiraishi, K.; Rajca, S. Chem. Commun. 2009, 4372–4374.

S2. Boeckman, R. K.; Shao, P.; Mullins, J. M. Org. Synth. 2004, Coll. Vol. 10, 696–701.

S3. Kirilyuk, I. A.; Polienko, Y. F.; Krumkacheva, O. A.; Strizhakov, R. K.; Yurii V. Gatilov, Y. V.; Grigor’ev, I. A.; and Elena G. Bagryanskaya, E. G. J. Org. Chem. 2012, 77, 8016–8027.

S4. 7-Aza-3,11-dithiadispiro[5.1.5.3]hexadecane-15-one: Sakai, K.; Yamada, K.; Yamasaki, T.; Kinoshita, Y.; Mito, F.; Utsumi, H. Tetrahedron 2010, 66, 2311–2315.
S5. Hatano, B.; Araya, H.; Yoshimura, Y.; Sato H.; Ito, T.; Ogata, T.; Kijima, T. *Heterocycles* **2010, 81**, 349–356.

S6. Krishna, M. C.; DeGraff, W.; Hankovszky, O. H.; Sár, C. P.; Kálai, T.; Jekő, J.; Russo, A.; Mitchell, J. B.; Hideg, K. *J. Med. Chem.* **1998, 41**, 3477–3492.

S7. Meyer, S. D.; Schreiber, S. L. *J. Org. Chem.* **1994, 59**, 7549–7552.

S8. Boeckman, R. K. Jr.; Shao, P.; and Mullins, J. J. *Organic Syntheses*, **2000, 77**, 141–152.