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Synthesis of new conformationally rigid paramagnetic α-amino acids

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Abstract—A pyrroline nitroxide based cyclic tetrasubstituted α-amino acid and paramagnetic homoproline and their derivatives are described. Introduction of a new paramagnetic protecting group to follow the incorporation of an amino acid into peptides by EPR is also suggested.

Electron spin resonance (ESR) is one of the most important tools for studying structure and function of proteins and biomolecules.[1] Nowadays there are two fundamental methods to attach a paramagnetic label in a site-specific fashion to a protein. One possibility is making cysteine mutants followed by labeling with a methanethiosulfonate spin label.[2] This method is referred to as site directed spin labeling (SDSL). Another approach is solid phase protein synthesis, where natural and unnatural amino acids[3] are incorporated in a step by step process.[4] A new trend with increasing significance is the nonsense suppression technology to build in unnatural amino acids.[5] For ESR studies of proteins, a variety of paramagnetic α-amino acids (TOAC,[6] I,[7,8] II[9]), β-amino-acids (POAC,[6] β-TOAC[10]) and γ-amino acids (III)[11] have been synthesized (Fig. 1). In several cases naturally occurring amino acids were modified by alkylation or acylation with functionalized nitroxides to obtain a paramagnetic protein building block.[12] TOAC (4-amino-1-oxyl-2,2,6,6-tetramethyl-piperidine-4-carboxylic acid) by far the most popular among the above mentioned amino acids, is easily available from 1-oxyl-4-oxo-2,2,6,6-tetramethyl-piperidine. This paramagnetic amino acid is considered to be a β-turn and 3_{10}-helix inducer and serves as a good reporter of backbone flexibility.[13] TOAC was incorporated into α-melanocyte stimulating hormone without loss of biological activity.[14] TOAC was used in the synthesis of paramagnetic fullerenes[15] as well as of nitroxyl peptides to catalyze enantioselective oxidation.[16] However, the six-membered ring of TOAC is sensitive to reduction/oxidation and acidic media, the latter required for Merrifield solid phase synthesis.[3] It is known that pyrroline nitroxides are less sensitive toward these experimental conditions.[17] In our

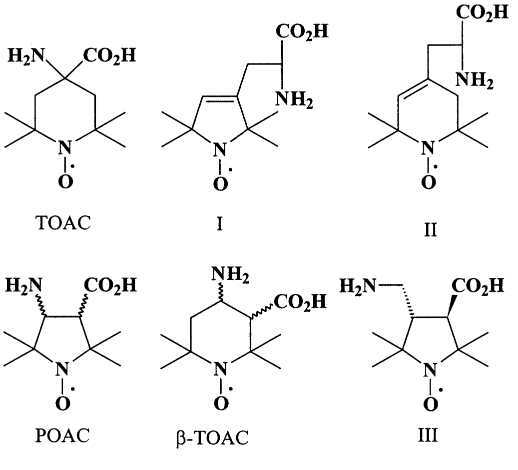

Figure 1. Chemical structure of paramagnetic amino acids.

Keywords: amino acids; nitroxides; protecting group; TOAC.

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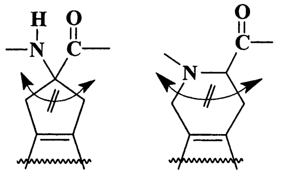


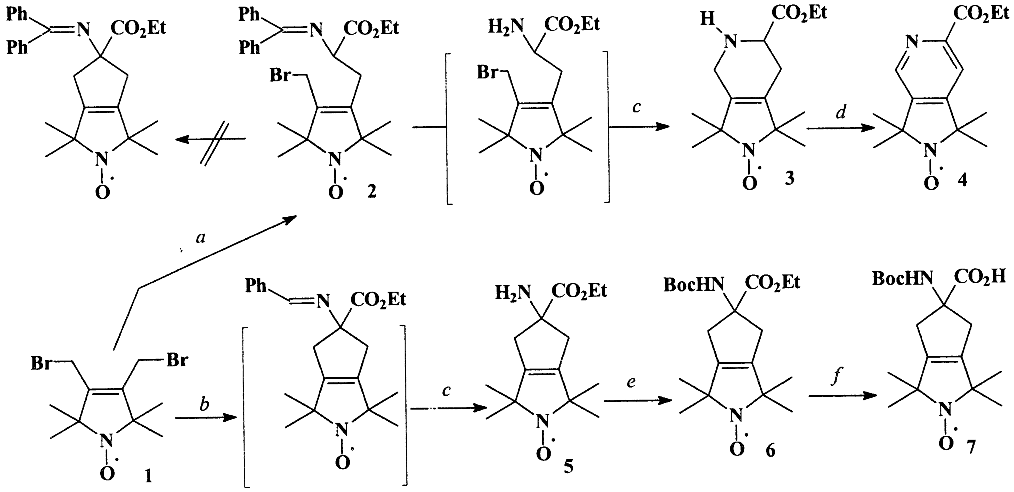
Figure 2. Conformationally rigid amino acids.

laboratory several carbocycle and heterocycle anellated pyrroline nitroxides have been synthesized,[18] so it was obvious that we should aim to make a new conformationally rigid paramagnetic amino acid which combines the advantages of TOAC and pyrroline nitroxides (Fig. 2).

In this paper we report the synthesis of a pyrroline TOAC abbreviated as 'PTOAC' by us. Twofold alkylation of ethyl *N*-diphenylmethylene glycine with paramagnetic bis(allylic bromide) **1**[18a] under phase transfer conditions[19] gave the monoalkylated product **2**, which could be readily hydrolyzed under acidic conditions to the corresponding amine, alkaline treatment of which yielded the cyclic six-membered α-aminoester **3**.[20] Oxidation of compound **3** with activated MnO_2 gave the pyrroline nitroxide anellated pyridine 2-carboxylic acid ethyl ester **4**, synthesized earlier in our laboratory.[18b] The benzylidene derivative of glycine ethyl ester was treated with 2.2 equivalent of NaHDMS in THF at −78°C followed by twofold alkylation with compound **1** and acidic hydrolysis[21] giving α,α-disubstituted α-amino acid ester **5** in moderate yield. It was found in preliminary experiments with compound **7** that the *N*-Boc group can be removed without reduction of nitroxide to diamagnetic hydroxylamine. Treatment of compound **5** with di-*tert*-butyldicarbonate gave the acid sensitive *N*-Boc derivative **6** which could be hydrolyzed with NaOH in EtOH to the *N*-Boc protected amino acid **7** (Scheme 1).

Compound **3** can be regarded as a paramagnetic derivative of DL-pipecolic acid, a homolog of proline which is a widespread non-proteinogenic amino acid in plants, moreover its incorporation into a biologically active peptide in place of L-proline was successful.[22] Considering this achievement, we decided to explore the chemistry of this paramagnetic proline-like building block, therefore its *N*-Boc derivatives **8** and **9** were synthesized in processes analogous to these leading to compounds **6** and **7**.

The carboxycyclohexyloxy amine-masking group was introduced earlier,[23] but has not found wide application. We believe that its paramagnetic derivative 2,2,6,6-tetramethyl-piperidine could be useful to monitor the solid phase amino acid synthesis by EPR. The paramagnetic protecting group TmpcCl can be synthesized from the easily available 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) and triphosgene.[24] The acid chloride was converted to acid azide $TmpcN_3$,[25] which is considered to be a milder reagent, with NaN_3 under phase transfer conditions. Acylation of amino acid ester **3** either with TmpcCl or $TmpcN_3$ gave biradical **10**, which could be hydrolyzed by aqueous NaOH to acid **11** without hydrolysis of the carbamate. The principle behind the use of Tmpc is that this group can be observed by its EPR spectrum when it is attached to an amino acid introduced at the *N*-terminal of a peptide. Upon hydrolytic cleavage of the Tmpc protecting group its increased mobility is revealed by the changes in triplet line shape (or, in the case of a paramagnetic amino acid, the quintet disappears) indicating the successful deprotection and binding of the amino acid in question, which may be the critical issue in the case of unnatural amino acids. The TmpcCl itself can be considered as a cheap acylating spin label reagent.[26] To fulfil the requirements of Nvoc and Fmoc chemistry, we also synthesized the photo-cleavable 3,4-dimethoxy-6-



Scheme 1. *Reagents and conditions:* (a) $Ph_2C=NCH_2CO_2Et$ (1.0 equiv.), 10% aq. NaOH (2.0 equiv.), CH_2Cl_2, Bu_4NHSO_4 (0.1 equiv.), rt, 2 h, 78%; (b) $PhCH=NCH_2CO_2Et$, THF, −78°C, NaHMDS (2.2 equiv., 1.0 M in THF) 45 min, then **1**, −78°C→rt, 2 h; (c) 5% aq. H_2SO_4 (1.1 equiv.), EtOH, 30 min rt, then NaOH to pH 8, 15–84%; (d) MnO_2, $CHCl_3$, reflux, 1 h, 53%; (e) Boc_2O (1.0 equiv.), THF, 40°C, 30 min, 36%; (f) 10% aq. NaOH (1.1 equiv.), EtOH, 78°C, 30 min, then EtOH evaporated, aq. H_2SO_4 to pH 3, 48%.

nitrobenzyl (Nvoc) carbamate and base sensitive *N*-protected 9-fluorenylmethoxycarbonyl (Fmoc) derivative. Treatment of compound **3** with $Ba(OH)_2$ followed by saturation with CO_2 yielded compound DL-**12**. Acylation of DL-**12** with Fmoc-succinate in the presence of $KHCO_3$ gave amino acid **13** with a base (e.g. piperidine) sensitive *N*-protecting group and acylation of **12** with 6-nitroveratryl-chloroformate in aqueous dioxane in the presence of K_2CO_3 yielded compound **14** with a photo-cleavable protecting group (Scheme 2).[27]

In conclusion, we have synthesized two new conformationally rigid amino acids for use in the synthesis of paramagnetic peptides: a new TOAC-like pyrroline nitroxide based achiral paramagnetic amino acid, which can be directly used in peptide labeling, and a paramagnetic racemic homoproline and their *N*-protected derivatives.[28] These last ones should be resolved before their use in biological systems. We suggest the introduction of a paramagnetic protecting group, Tmpc, to follow the amino acid incorporation process by EPR. The applications of the spin labels described above are in progress and will be reported separately.

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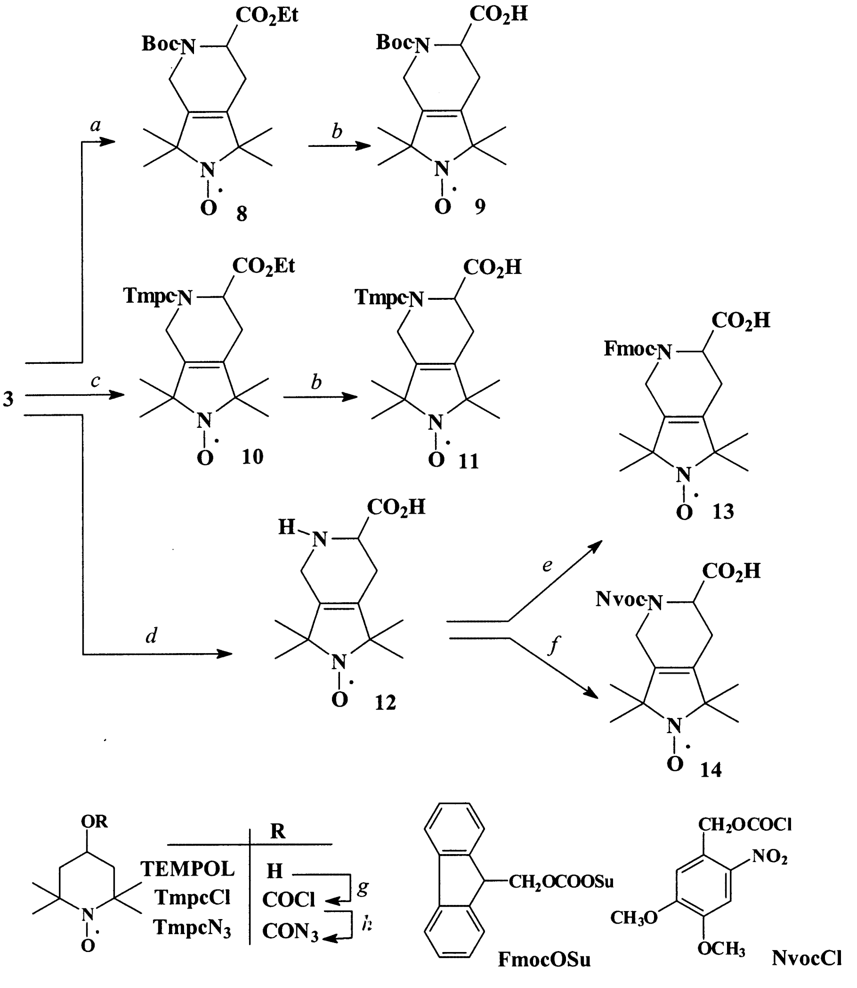

Scheme 2. *Reagents and conditions*: (a) Boc_2O (1.0 equiv.), THF, 40°C, 30 min, 68%; (b) 10% aq. NaOH (1.1 equiv.), EtOH, 78°C, 30 min, then EtOH evaporated, aq. H_2SO_4 to pH 3, 61–68%; (c) TmpcCl (1.0 equiv.), Et_3N (1.0 equiv.), THF, 65°C, 3 h, 55%; (d) $Ba(OH)_2\cdot 8\ H_2O$ (1.1 equiv.), $MeOH/H_2O$ (1:1), 65°C, 30 min, then excess dry ice, filtration, evaporation, 48%; (e) Fmoc-*O*-Su (1.0 equiv.), dioxane/H_2O (2:1), $KHCO_3$ (2.0 equiv.), rt, 4 h, then 5% aq. H_2SO_4 to pH 3, 75%; (f) 6-nitroveratryl chloroformate (1.1 equiv.), K_2CO_3 (2.0 equiv.), dioxane, 18-crown-6 (0.05 equiv.), rt, 6 h, then evaporated, 5% aq. H_2SO_4 to pH 3, 55%; (g) triphosgene (0.5 equiv.), pyridine (1.0 equiv.), CH_2Cl_2, −20°C, 15 min, then rt, 30 min, 59%; (h) NaN_3, 18-crown-6, dioxane, rt, 3 h, 47%.

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26. **Synthesis of TmpcCl**: To a stirred solution of TEMPOL (860 mg, 5.0 mmol) and dry pyridine (400 mg, 5.0 mmol) in dry CH_2Cl_2 (10 mL) triphosgene (742 mg, 2.5 mmol) dissolved in CH_2Cl_2 (5 mL) was added dropwise at −20°C. The mixture was stirred at this temperature for 15 min, then at ambient temperature for 30 min. The precipitated pyridine hydrochloride was filtered off, the solvent evaporated and the residue was crystallized from hexane/Et_2O to give an orange-pink solid 797 mg (68%), mp 49–51°C, calcd for $C_{10}H_{17}ClNO_3$: C, 51.18; H, 7.30; N, 5.97, found: C, 51.20; H, 7.29; N, 6.03. IR (nujol) ν: 1770 cm^{-1}. MS (EI) m/z: 234 (M^+, 7), 220 (9), 109 (77), 41 (100). This compound was stable for several weeks stored in refrigerator at 0°C.
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28. Compounds were characterized by MS, NMR, IR and elemental analysis. Spectra were consistent in each case with the assigned structures. To obtain high resolution NMR spectra of the radicals they were reduced by co-dissolved PhNHNHPh additive. Physical and spectroscopic data of selected compounds: **2**: mp 99–101°C; calcd for $C_{27}H_{32}BrN_2O_3$: C, 63.28; H, 6.29; N, 5.47, found: 63.18; H, 6.19; N, 5.50; MS (EI) m/z: 512/514 (M^+, 2/2), 266 (27), 193 (43), 167 (100). **3**: oil; calcd for $C_{14}H_{23}N_2O_3$: C, 62.90; H, 8.67; N, 10.48, found: 63.02; H, 8.70; N, 10.55; IR (neat) ν: 3300, 1730 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 4.21 (2H, q, $J=7.1$ Hz, O-CH_2), 3.57 (1H, dd, $J_1=8.4$ Hz, $J_2=5.0$ Hz, CH), 3.42–3.30 (2H, m, NCH_2), 2.30–2.14 (2H, m, CH_2), 1.28 (t, 3H, $J=7.1$ Hz, Et-CH_3), 1.23 (s, 3H, C-CH_3), 1.21 (s, 3×3H, C-CH_3); MS (EI) m/z: 267 (M^+, 28), 253 (27), 237 (28), 194 (100). **5**: mp 57–59°C; calcd for $C_{14}H_{23}N_2O_3$: C, 62.90; H, 8.67; N, 10.48, found: C, 63.10; H, 8.75; N, 10.39; IR (neat) ν: 3350, 3300, 1720 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 4.24 (2H, q, $J=7.2$ Hz, O-CH_2), 2.96 (2H, br d, C*H*H 'eq'), 2.37 (2H, br d, CH*H* 'ax'), 1.54 (6H, s, C-CH_3), 1.50 (6H, s, C-CH_3), 1.31 (3H, t, $J=7.2$ Hz, Et-CH_3); ^{13}C NMR ($CDCl_3$) δ: 175.8 (carboxy), 141.0 (br, bridgehead), 74.4 (br, *C*CH_3), 62.0 (OCH_2), 41.7 (br, CH_2), 23.1 (br, C*C*H_3), 22.8 (br, C*C*H_3), 14.2 (Et-CH_3); MS (EI) m/z: 267 (M^+, 15), 253 (30), 237 (35), 164 (100). **7**: mp 169–171°C, calcd for $C_{17}H_{27}N_2O_5$: C, 60.16; H, 8.02; N, 8.25, found: C, 60.14; H, 8.06; N, 8.18; MS (EI) m/z: 339 (M^+, 1), 251 (7), 205 (17), 57 (100). **9**: mp 136–138°C; calcd for $C_{17}H_{27}N_2O_5$: C, 60.16; H, 8.02; N, 8.25, found: C, 60.22; H, 8.10; N, 8.20; IR (nujol) ν: 1720, 1600 cm^{-1}; MS (EI) m/z: 339 (M^+, 1), 325 (2), 269 (5), 57 (100). **10**: mp 103–105°C; calcd for $C_{24}H_{39}N_3O_6$: C, 61.69; H, 8.44; N, 9.03, found: C, 61.75; H, 8.40; N, 9.10; IR (nujol) ν: 1730, 1600 cm^{-1}; MS (EI) m/z: 465 (M^+, 16), 435 (6), 311 (27), 124 (100). **11**: mp 145–146°C; calcd for $C_{22}H_{35}N_3O_6$: C, 60.39; H,

8.06; N, 9.60, found: C, 60.35; H, 8.03; N, 9.51; IR (nujol) *ν*: 1720, 1605 cm^{-1}; MS (EI) *m*/*z*: 437 (M$^+$, 1), 235 (21), 221 (100), 205 (60). **12**: mp 209–211°C, decomp.; calcd for $C_{12}H_{19}N_2O_3$: C, 59.48; H, 9.15; N, 11.56, found: C, 59.51; H, 8.10; N, 8.07; IR (nujol) *ν*: 3300, 1600 cm^{-1}; MS (EI) *m*/*z*: 239 (M$^+$, 14), 225 (30), 209 (18), 177 (100). **13**: mp 110–112°C; calcd for $C_{27}H_{29}N_2O_5$: C, 70.26; H, 6.33; N, 6.07, found: C, 70.15; H, 6.29; N, 5.95; IR (nujol) *ν*: 1720, 1600 cm^{-1}; MS (EI) *m*/*z*: 461 (M$^+$, 1), 447 (1), 431 (2), 178 (100). **14**: mp 124–126°C; calcd for $C_{22}H_{28}N_3O_9$: C, 55.23; H, 5.90; N, 8.78, found: C, 55.25; H, 6.01; N, 8.80; IR (nujol) *ν*: 1705, 1570, 1505 cm^{-1}; MS (EI) *m*/*z*: 478 (M$^+$, 4), 448 (3), 219 (44), 136 (100).