# Click Reactions with Nitroxides

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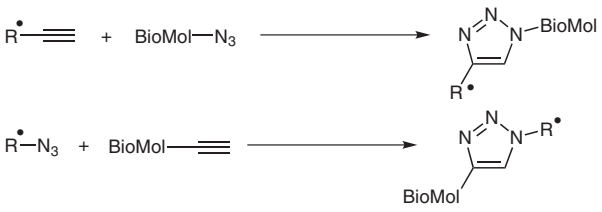
**Abstract:** Copper-catalyzed azide-alkyne 1,3-dipolar cycloadditions (CuAAC) with paramagnetic azide and alkyne building blocks are described. This method provides a route to the synthesis of complex spin-labeled molecules such as amino acids, carbohydrates, drug molecules and biradicals.

**Key words:** acetylenes, amino acids, azides, click chemistry, free radicals

The incorporation of nitroxides into various biological and non-biological structures, followed by EPR analysis, has emerged as an important technology during the last two decades. The spin-labeling method, introduced by McConnell and co-workers,[1] is an effective tool with which to explore the structure, dynamics and interactions of complex biomolecules such as proteins, nucleic acids, enzymes, and polysaccharides.[2–4]

Spin-labels can be introduced into selected parts of macromolecules by covalent chemical bond formation with site-directed specific reagents, such as methanethiosulfonates,[5] or by genetic site-directed incorporation.[6]

The concept of 'click chemistry' was introduced by Kolb, Finn and Sharpless[7] as a 'near perfect' (very selective, modular, high-yield and wide in scope) carbon–heteroatom bond forming reaction. The 1,3-dipolar cycloadditions of organic azides with alkynes in the presence of Cu(I) or Ru-catalysts fulfill the click criteria,[8,9] albeit that the basic azide-alkyne 1,3-dipolar cycloaddition has been well known since the works of Huisgen.[10] This reaction is an established procedure for triazole synthesis in heterocyclic chemistry[11] and its copper-catalyzed variation has been established as one of the most wide-spread means for the covalent assembly of complex molecules.[12]




**Scheme 1** Possible modification of biomolecules (Biomol) with stable free nitroxide radical (R) spin-labels

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In the technique of site-directed spin-labeling, nitroxide spin-labels are selectively introduced into proteins by replacing a native amino acid with cysteine, followed by selective modification of the sulfhydryl group with a thiol-specific reagent, most often a methanethiosulfonate.[13] Proteins that contain native and reactive cysteine residues pose a problem that is generally solved by replacing the native cysteine residues with inert amino acids. Since this is often not practical, a method for site-selectively introducing a nitroxide side chain based on chemistry foreign to natural proteins would be extremely valuable. Click chemistry is a possible candidate. In this study we evaluate the applicability of both paramagnetic azides and acetylenes in copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reactions (Scheme 1) that can potentially be used to introduce nitroxide side chains into proteins in a manner similar to that used earlier with fluorophores.[14] This chemistry was used by Jawalekar and coworkers for the modification of adenosine using the paramagnetic azide **1**.[15,16] In this paper we report an extension of the click reaction to other biomolecules (BioMol) and nitroxide-containing click reaction partners.

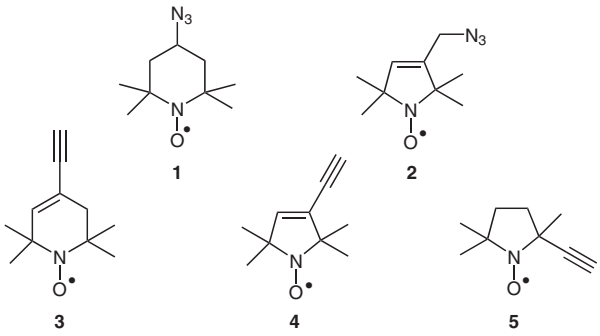
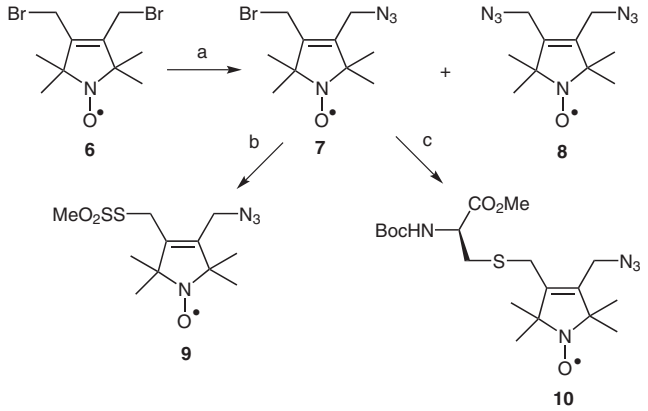



**Figure 1** Paramagnetic azides and acetylenes used for click reactions

Paramagnetic azides **1**[15] and **2**,[17] and acetylenes **3**,[18] **4**[19] and **5**,[20] have been synthesized previously (Figure 1). Azides are available by nucleophilic substitution from mesylates. Acetylenes can be prepared by reaction of 4-oxo-2,2,6,6-tetramethylpiperidin-1-yloxil with the lithium salt of triisopropylsilylacetylene, followed by elimination and deprotection.[18] Alternatively, an elimination reaction of a 1,2-dibromo derivative[17] or Grignard reaction of ethynylmagnesium bromide with 2,2,5-trimethyl-3,4-dihydo-2*H*-pyrrol-1-oxide can be employed.[20]

In addition to these monofunctional click reaction partners, cross-linking building blocks can also be accessed from the dibromo compound **6**.[19] Thus, reaction of compound **6** with one equivalent of sodium azide in aqueous acetone, yielded the mixture of monoazido[21] and diazido derivatives **7** and **8**, respectively (Scheme 2).




**Scheme 2** *Reagents and conditions*: (a) $NaN_3$ (1.2 equiv), aq acetone, 40 °C, 30 min.; **7** (16%), **8** (40%); (b) $NaSSO_2Me$ (2.0 equiv), aq acetone, 50 °C, 30 min (59%); (c) *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (1.0 equiv), $K_2CO_3$ (1.0 equiv), $CHCl_3$, reflux, 1 h (48%).

The former can be functionalized further with nucleophilic substitution by heating with sodium methanethiosulfonate ($NaSSO_2CH_3$) in aqueous acetone, to yield compound **9** as a cross-linking spin-label with both thiol-specific and acetylene-specific functional groups. S-Alkylation of *N*-Boc-L-cysteine methyl ester with compound **7** in chloroform, in the presence of potassium carbonate, afforded amino acid **10** as an unnatural, paramagnetic L-amino acid with an azido side chain capable of further conjugation in a click reaction (Scheme 2).

Reaction of equimolar amounts of compounds **1** and **3**, in the presence of copper(I) iodide (0.4 equiv) in dimethyl sulfoxide at 40 °C, gave the biradical compound **11** (Scheme 3). This reaction was applied to introduce a paramagnetic group into biomolecules with an acetylene function, such as 17α-ethynylestradiol (**12**) or mestranol (**14**), to give spin-labeled steroid derivatives **13** and **15**, respectively. Reaction of compound **8** with two equivalents of 17α-ethynylestradiol (**12**) and copper(I) iodide (0.8 equiv), gave the cross-linked bisteroid compound **16**. Alternative conditions for this reaction were found to be treatment of a 2:1 mixture of the acetylene and azide in the presence of ascorbate (1.5 equiv) and aqueous copper(II) sulfate solution in *N*,*N*-dimethylformamide (Method B). An excess of ascorbate was required because of consumption of the reducing agent by nitroxide reduction to *N*-hydroxylamine. This reaction can be applied to the synthesis of paramagnetically modified amino acids containing an acetylene moiety. For example, treatment of the protected *S*-propargyl cysteine[22] (**17**) with azide **1**, in the presence of copper(I) iodide in dimethyl sulfoxide, yielded the spin-labeled L-cysteine derivative **18**. In the above examples, biomolecules with an acetylene moiety were modified with spin-labeled azides. This idea can be inverted; for example, azido-containing biomolecules can be modified by paramagnetic acetylenes such as compound **4**. Thus, reaction of **4** with β-azido-pentaacetyl-D-glucopyranozide (**19**)[23] or with protected DL-4-azidophenylalanine **21**,[24] gave the spin-labeled carbohydrate derivative **20** and the spin-labeled DL-paramagnetic amino acid **22**, respectively (Scheme 3).

In conclusion, we have demonstrated with several examples that both paramagnetic azides and both paramagnetic acetylenes are reasonable click reaction partners. All react with the corresponding biomolecules under mild conditions and the reaction can be accomplished using relatively simple procedures. The application of this [3+2] cycloaddition reaction for spin-labeling of a native protein at properly modified side chains is underway.

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on a Fisons EA 1110 CHNS elemental analyzer. The IR (Specord 85) spectra were in each case consistent with the assigned structure. Mass spectra were recorded on a Thermoquest Automass Multi and VG TRIO-2 instruments in the EI mode. The ESI MS were recorded on Perkin–Elmer Sciex API III instrument (0.1% TFA in $H_2O$–MeCN, 1:1; flow rate: 0.2 mL/min). ESR spectra were taken on Miniscope MS 200 in $10^{-4}$ M $CHCl_3$ solution and all mono-radicals gave a triplet line $a_N$ = 14.7–16.5 G. Optical rotations were recorded at 25 °C, with a Perkin–Elmer 343 polarimeter. Flash column chromatography was performed using Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel $GF_{254}$. Compounds **12**, **14**, *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester and all other reagents were purchased from Fluka or Aldrich. Compounds **1**,[15] **2**,[17] **3**,[18] **4**,[19] **5**,[20] **6**,[18] **17**,[22] **19**,[23] and **21**[24] were prepared according to published procedures.

**3-Azidomethyl-4-bromomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (7) and Bis-3,4-diazidomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (8)**

To a solution of compound **6** (3.26 g, 10.0 mmol) in acetone (20 mL), $NaN_3$ (780 mg, 12.0 mmol) in $H_2O$ (5 mL) was added and the mixture was heated at 40 °C for 30 min. The mixture was diluted with $H_2O$ (20 mL), acetone was evaporated off in vacuo, the aqueous phase was extracted with EtOAc (2 × 20 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography (hexane–$Et_2O$, 2:1). The first band contained the dibromo compound **6**, the second band consisted of compound **7** [$R_f$ = 0.31 (hexane–$Et_2O$, 2:1)], then compound **8** [$R_f$ = 0.25 (hexane–$Et_2O$, 2:1)].

**7**

Yield: 460 mg (16%); yellow solid; mp 58–60 °C.

IR (Nujol): 2100 ($N_3$), 1650 (C=C) $cm^{-1}$.

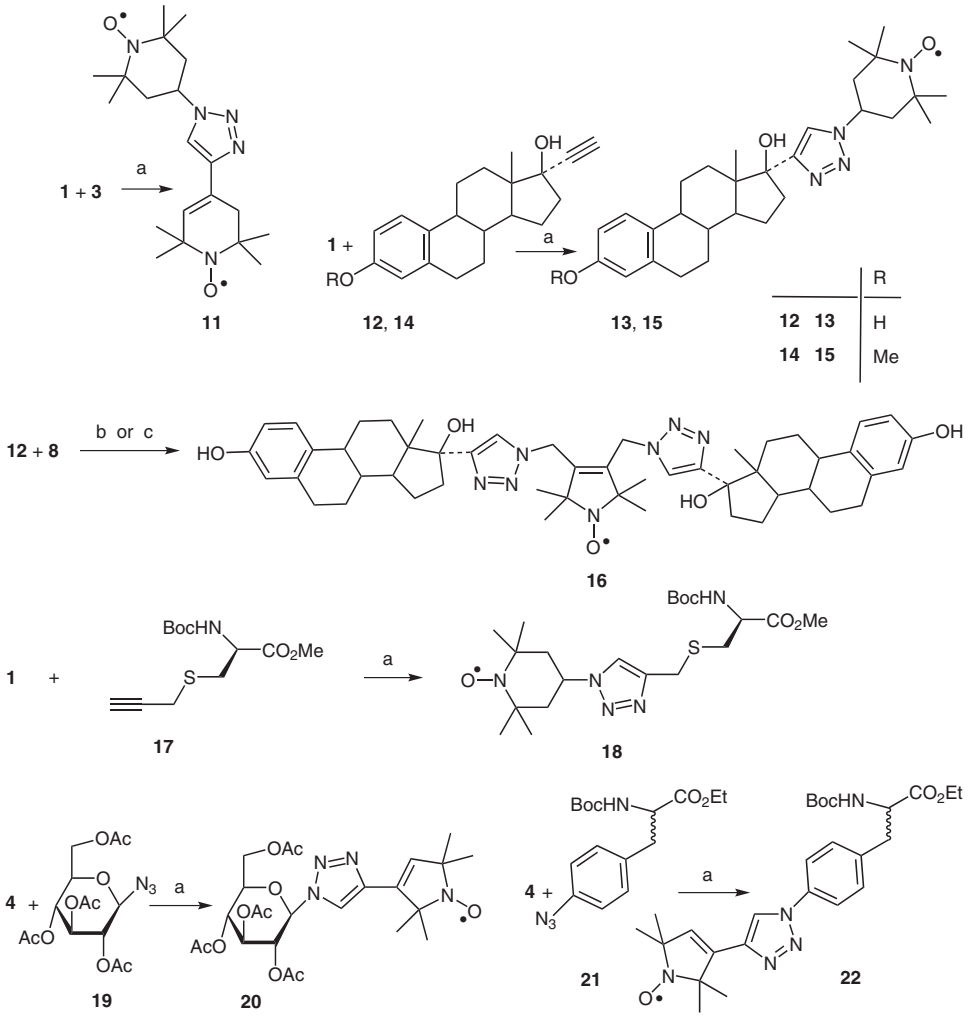
MS (EI): *m*/*z* (%) = 287/289 (9/9) [$M^+$], 228/230 (18/18), 193 (40), 152 (68), 41 (100).

Anal. Calcd for $C_{10}H_{16}BrN_4O$: C, 41.68; H, 5.60; N, 19.44. Found: C, 41.55; H, 5.71; N, 19.25.

**8**

Yield: 1.12 g (45%); yellow solid; mp 95–97 °C.

IR (Nujol): 2100 ($N_3$), 1660 (C=C) $cm^{-1}$.




**Scheme 3** *Reagents and conditions*: (a) CuI (0.4 equiv), DMSO, 40 °C, 30–60 min (32–79%); (b) CuI (0.8 equiv), **12** (2.0 equiv), DMSO, 40 °C, 1 h (48%); (c) aq 10% $CuSO_4$ (0.2 equiv), sodium ascorbate (2.2 equiv), DMF, r.t., 2 h, under $N_2$, then $MnO_2$ (1.0 equiv), $O_2$, 15 min (73%).

MS (EI): *m*/*z* (%) = 250 (64) [M+], 235 (11), 193 (73), 77 (100).

Anal. Calcd for $C_{10}H_{16}N_7O$: C, 47.99; H, 6.44; N, 39.17. Found: C, 47.89; H, 6.35; N, 39.21.

### 3-Azidomethyl-4-methanethiosulfonylmethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (9)

To a solution of compound **7** (286 mg, 1.0 mmol) in acetone (10 mL) and $H_2O$ (3 mL), $NaSSO_2CH_3$ (270 mg, 2.0 mmol) was added and the solution was heated at 50 °C until complete consumption of starting material was observed (~30 min). After cooling, the acetone was evaporated off, $H_2O$ (7 mL) was added and the aqueous phase was extracted with $CHCl_3$ (2 × 10 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated, then the residue was purified by flash column chromatography ($CHCl_3$–$Et_2O$, 2:1) to give the title compound.

Yield: 188 mg (59%); orange solid; $R_f$ = 0.27 ($CHCl_3$–$Et_2O$, 2:1); mp 32–34 °C.

IR (Nujol): 2100 ($N_3$), 1650 (C=C) $cm^{-1}$.

MS (EI): *m*/*z* (%) = 319 (5) [M+], 305 (4), 166 (12), 79 (100).

Anal. Calcd for $C_{11}H_{19}N_4O_3S_2$: C, 41.36; H, 6.00; N, 17.54. Found: C, 41.18; H, 6.02; N, 17.50.

### Methyl 3-(1-Oxyl-4-azidomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylsulfanyl)-2-*tert*-butoxycarbonylaminopropionate Radical (10)

A mixture of compound **7** (286 mg, 1.0 mmol) and *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (235 mg, 1.0 mmol) and $K_2CO_3$ (138 mg, 1.0 mmol) in $CHCl_3$ (10 mL) was stirred under reflux for 1 h. After cooling, the inorganic salts were filtered off and washed with $CHCl_3$ (5 mL). The filtrate was washed with brine (10 mL), and the organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography (hexane–EtOAc, 2:1) to yield compound **10**.

Yield: 212 mg (48%); yellow oil; $R_f$ = 0.30 (hexane–EtOAc, 2:1); $[\alpha]_D^{25}$ –17.6 (*c* 0.49, MeOH).

IR (neat): 3330 (NH), 2105 ($N_3$), 1730, 1700 (C=O) $cm^{-1}$.

MS (EI): *m*/*z* (%) = 442 (48) [M+], 412 (1), 299 (4), 182 (37), 150 (90), 57 (100).

Anal. Calcd for $C_{19}H_{32}N_5O_5S$: C, 51.57; H, 7.29; N, 15.82. Found: C, 51.55; H, 7.38; N, 15.66.

### Cu-Catalyzed 1,3-Dipolar Addition; General Procedure

A solution of azide **1**, **8**, **19** or **21** (2.0 mmol) and acetylene **3**, **4**, **12**, **14** or **17** (2.0 mmol or 4. 0 mmol for reaction with **8**) and CuI (152 mg, 0.8 mmol or 304 mg, 1.6 mmol for compound **8**) was heated to

40 °C in DMSO (8 mL) and kept at this temperature until complete consumption of starting materials was observed (indicated by TLC; 30–60 min). The solution was diluted with $H_2O$ (30 mL) and the precipitate was filtered and purified further by flash column chromatography. When no precipitation occurred, the mixture was extracted with EtOAc (2 × 10 mL) and the organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography ($CHCl_3$–$Et_2O$, 2:1 or $CHCl_3$–MeOH, 9:1) to yield compounds **13**, **15**, **16**, **18**, **20** or **22** as solids in 32–79% yields.

**4-{1-(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1*H*-[1,2,3]triazol-4-yl}-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-1-yloxyl Biradical (11)**

Compounds **1** (2.0 mmol, 394 mg) and **3** (356 mg, 2.0 mmol) were used to obtain compound **11**, which was purified by column chromatography ($CHCl_3$–$Et_2O$, 2:1).

Yield: 420 mg (56%); pink solid; mp 129–132 °C; $R_f$ = 0.23 ($CHCl_3$–$Et_2O$, 2:1).

IR (Nujol): 1675 (C=N), 1650 (C=C) $cm^{-1}$.

ESR ($10^{-4}$ M solution in $H_2O$ containing 1% MeOH) consisted of 11 lines of 5:12:13:1:5:12:5:1:13:12:5 intensity ratio.

MS (EI): *m*/*z* (%) = 375 (8) [$M^+$], 346 (22), 330 (20), 140 (80), 74 (100).

Anal. Calcd for $C_{20}H_{33}N_5O_2$: C, 63.97; H, 8.86; N, 18.65. Found: C, 64.05; H, 8.75; N, 18.53.

**17-{4-(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1*H*-[1,2,3]triazol-4-yl}-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[a]phenanthren-3,17-diol Radical (13)**

Compounds **1** (2.0 mmol, 394 mg) and **12** (592 mg, 2.0 mmol) were used to obtain compound **13**, which was purified by column chromatography ($CHCl_3$–MeOH, 9:1).

Yield: 424 mg (43%); orange solid; mp 162–164 °C; $R_f$ = 0.33 ($CHCl_3$–MeOH, 9:1); $[\alpha]_D^{25}$ +31 (*c* 0.16, MeOH).

IR (Nujol): 3300 (OH), 1610, 1580 (C=C) $cm^{-1}$.

MS (EI): *m*/*z* (%) = 493 (2) [$M^+$], 476 (7), 270 (34), 140 (80), 41 (100).

Anal. Calcd for $C_{29}H_{41}N_4O_3$: C, 70.56; H, 8.37; N, 11.35. Found: C, 70.48; H, 8.44; N, 11.33.

**4-{4-(17-Hydroxy-3-methoxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[a]phenanthren-17-yl)-1*H*-[1,2,3]triazol-1-yl}-2,2,6,6-tetramethylpiperidin-1-yloxyl Radical (15)**

Compounds **1** (2.0 mmol, 394 mg) and **14** (620 mg, 2.0 mmol) were used to obtain compound **15**.

Yield: 608 mg (60%); orange solid; mp 106–108 °C; $R_f$ = 0.41 ($CHCl_3$–MeOH, 9:1); $[\alpha]_D^{25}$ +29 (*c* 0.16, MeOH).

IR (Nujol): 3300 (OH), 1610, 1590 (C=C) $cm^{-1}$.

MS (EI): *m*/*z* (%) = 507 (1) [$M^+$], 492 (5), 284 (5), 227 (100), 124 (55), 41 (59).

Anal. Calcd for $C_{30}H_{43}N_4O_3$: C, 70.97; H, 8.54; N, 11.04. Found: C, 70.88; H, 8.62; N, 10.95.

**3,4-Bis-{4-(3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[a]phenanthren-17-yl)-1*H*-[1,2,3]triazol-1-ylmethyl}-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl Radical (16)**

*Method A:* Compounds **8** (2.0 mmol, 500 mg) and **12** (1.18 g, 4.0 mmol) were used to obtain compound **16**.

Yield: 538 mg (32%); beige solid; mp 220–222 °C; $R_f$ = 0.50 ($CHCl_3$–MeOH, 4:1); $[\alpha]_D^{25}$ +63 (*c* 0.17, MeOH).

IR (Nujol): 3350 (OH), 1655 (C=C) $cm^{-1}$.

MS (ESI): *m*/*z* = 843 $[M + H]^+$.

Anal. Calcd for $C_{50}H_{64}N_7O_5$: C, 71.23; H, 7.65; N, 11.63. Found: C, 71.05; H, 7.54; N, 11.68.

*Method B:* A mixture of diazide **8** (125 mg, 0.5 mmol), α-ethynylestradiol **12** (296 mg, 1.0 mmol), sodium L-ascorbate (217 mg, 1.1 mmol) and 10% aq $CuSO_4$ (0.16 mL, 0.1 mmol) in DMF (7 mL), was stirred under $N_2$ at r.t. for 2 h. The mixture was poured onto a $H_2O$-ice mixture (30 mL) and allowed to reach r.t. The mixture was filtered and the collected solid was washed with $H_2O$ (10 mL). The solid was dried at r.t. then dissolved in a mixture of $CHCl_3$–MeOH (2:1, 30 mL), $MnO_2$ (43 mg, 0.5 mmol) was added and $O_2$ was bubbled through for 15 min. The $MnO_2$ was filtered off and the solvent was removed under vacuum to afford compound **16** with the same spectroscopic and physical data as listed above for Method A.

Yield: 307 mg (73%); beige solid.

**Methyl (*S*)-2-*tert*-Butoxycarbonylamino-3-[{1-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)-1*H*-[1,2,3]triazol-4-yl}methylthio]propionate Radical (18)**

Compounds **1** (2.0 mmol, 394 mg) and **17** (546 mg, 2.0 mmol) were used to obtain compound **18**, which was purified by column chromatography ($CHCl_3$–MeOH, 9:1).

Yield: 742 mg (79%); pink solid; mp 101–103 °C; $R_f$ = 0.28 ($CHCl_3$–MeOH, 9:1); $[\alpha]_D^{25}$ –7 (*c* 0.17, MeOH).

IR (Nujol): 3400 (NH), 1740, 1700 (C=O) $cm^{-1}$.

MS (ESI): *m*/*z* = 471 $[M + H]^+$.

Anal. Calcd for $C_{21}H_{36}N_5O_5S$: C, 53.60; H, 7.71; N, 14.88. Found: C, 53.53; H, 7.64; N, 14.75.

**1-β-D-{4-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-1*H*-[1,2,3]triazol-1-yl}-2,3,4,6-tetra-*O*-acetylglucopyranose Radical (20)**

Compounds **4** (328 mg, 2.0 mmol) and **19** (746 mg, 2.0 mmol) were used to obtain compound **20**.

Yield: 730 mg (68%); pale-yellow solid; mp 88–89°C; $R_f$ = 0.34 ($CHCl_3$–MeOH, 9:1); $[\alpha]_D^{25}$ –50 (*c* 0.18, MeOH).

IR (Nujol): 1760, 1750 (C=O), 1630 (C=C) $cm^{-1}$.

MS (ESI): *m*/*z* = 538 $[M + H]^+$.

Anal. Calcd for $C_{24}H_{33}N_4O_{10}$: C, 53.63; H, 6.19; N, 10.24. Found: C, 53.80; H, 6.11; N, 10.41.

**Ethyl 2-*tert*-Butoxycarbonylamino-3-{4-[4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-1*H*-[1,2,3]triazol-1-yl]phenyl}propionate Radical (22)**

Reaction of D/L-amino acid **21** (468 mg, 2.0 mmol) and compound **4** (328 mg, 2.0 mmol) afforded a brown residue after work-up. Chromatographic purification ($CHCl_3$–$Et_2O$, 2:1) gave compound **22**.

Yield: 637 mg (64%); pale-yellow solid; mp 125–126 °C; $R_f$ = 0.35 ($CHCl_3$–$Et_2O$, 2:1).

IR (Nujol): 3280 (NH), 1700, 1695 (C=O), 1620 (C=C) $cm^{-1}$.

MS (ESI): *m*/*z* = 499 $[M + H]^+$.

Anal. Calcd for $C_{26}H_{36}N_5O_5$: C, 62.63; H, 7.28; N, 14.05. Found: C, 62.58; H, 7.14; N, 14.10.

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