

A novel target spin trap for sulfhydryl-containing polypeptides

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Synthesis and characterizations.

All reactions with moisture- and air-sensitive compounds were carried out under dry nitrogen atmosphere (ultra-high purity) using standard Schlenk techniques. All Chemicals are commercially available and were used upon receipt unless otherwise stated.

(a) 4-(chloroacetamino) benzaldehyde **1**.

A solution of 4-amino-benzaldehyde (4.5 g, 37.1 mmol) and pyridine (3.5 g, 44.2 mmol) in 140 ml of dry ether was slowly added to chloro acetyl chloride (3.6 ml, 45.2 mmol) in 100 ml of dry ether under nitrogen atmosphere at 0 °C (ice bath). After stirring for 4h, the reaction mixture was washed with water (3×100 ml) and dried over anhydrous Na₂SO₄ overnight. The solvent was removed under reduced pressure. The crude product was crystallized from ether to give **2** as a pink solid (3.77g, 19.1 mmol, 51.5%). ¹H NMR (d₆-acetone): δ 4.316 (2H, s), 7.908 (4H,m), 9.954 (H, s), 10.253 (H, s). ¹³C NMR (d₆-acetone): δ 120.16, 131.56, 133.38, 144.87, 166.16, 191.89. MS (EI, *m/z*): 199 (M⁺, 32%), 197 (100), 148 (23), 121 (64), 120 (75).

(b) *N*-[4-(chloroacetamino) benzylidene]-*N*-tert-butylamine *N*-oxide **2**.

N-tert-butylhydroxylamine was extracted with ether after the addition of a saturated NaHCO₃ aqueous solution to *N*-tert-butylhydroxylamine hydrochloride until pH 8.0. The organic layer was dried with anhydrous Na₂SO₄ and the solvent was evaporated. To a solution of **2** (2.7 g, 13.7 mmol) in 150 ml of benzene was added *N*-tert-butylhydroxylamine (1.8 g, 20.6 mmol). The reaction mixture was refluxed for 2 days, then filtered and concentrated. The resultant was purified by silica gel column chromatography, eluting with a solution of dichloromethane and ethanol (95% and 5%, respectively). The compound **3** was obtained as a white powder (3.0 g, 11.2 mmol, 81.7%). ¹H NMR (CDCl₃): δ 1.595 (9H, s), 4.176 (2H, s), 7.523 (H, s), 7.624 (2H, d, J = 8.4 Hz), 8.282 (2H, d, J = 8.5 Hz), 8.583 (H, s). ¹³C NMR (CDCl₃): δ 28.29, 42.96, 70.71, 119.37, 127.64, 129.68, 129.95, 138.30, 163.98. MS (EI, *m/z*): 270 (M⁺, 11.5%), 268 (34), 239 (20), 237 (68), 214 (27), 212 (100).

(c) *N*-[4-(iodoacetamino) benzylidene]-*N*-tert-butylamine *N*-oxide **3**.

The mixture of the compound **3** (3.0 g, 11.2 mmol) and sodium iodine (8.4 g, 56 mmol) was dissolved in 200 ml of acetone and stirred during 80 h under refluxing in darkness. The brown red color of the reaction mixture was filtered, concentrated *in vacuo* and then 200 ml of ethyl acetate was added. The resulting solution was washed with 3×100 ml of water and dried on anhydrous Na₂SO₄ overnight. After removal of the solid by

filtration, the solvent was evaporated *in vacuo*. The colorless or pale yellow products were obtained (2.7 g, 7.5 mmol, 67%). Decomposition temperature: 151 °C. ¹H NMR (CD₃OD): δ 1.61 (9H, s), 3.89 (2H, s), 7.69 (2H, d, J = 8.7 Hz), 7.89 (H, s), 8.33 (2H, d, J = 8.7 Hz), 9.75 (H, s). ¹³C NMR (CD₃OD): δ 29.45, 44.12, 71.88, 121.21, 128.87, 132.95, 135.65, 143.28, 170.69. MS (EI, *m/z*): 360 (M⁺, 34%), 329 (60), 304 (100).

Coupling of the nitrone 3 to sulfhydryl-containing polypeptides.

Coupling with glutathione.

The nitrone 3 (0.1472 g, 0.41 mmol) in 6 ml of ethanol was added to a solution of glutathione (0.1002 g, 0.32 mmol) and 10 ml of 0.1 M Na-phosphate buffer (pH 7.4). The reaction mixture was stirred overnight at 25 °C in darkness. Then 10 ml of chloroform was added to the reaction mixture, the latter was shaken and the lower phase was removed. The procedure was repeated about 6 times until, assessed by thin layer chromatography, the nitrone 3 in the chloroform disappeared. The aqueous phase was concentrated *in vacuo* and purified by Sephadex LH-20, eluting with water. The white solid was obtained (0.1210 g, 70.1%). ¹H NMR (D₂O), δ 1.54 (9H, s), 2.1 (2H, m), 2.48 (2H, m), 2.98 (1H, m), 3.16 (1H, m), 3.48 (2H, s), 3.70 (2H, m), 3.75 (1H, s), 4.60 (2H, s), 7.61 (2H, d), 7.92 (1H, s), 8.22 (2H, d). ¹³C NMR (D₂O), δ 24.8, 27.3, 28.2, 32.6, 44.5, 54.1, 55.3, 65.4, 71.8, 121.8, 127.3, 132.7, 137.7, 141.1, 172.2, 172.9, 175.1, 176.0, 177.4. MS (ESI, *m/z*): 562.3 ([M + Na]⁺, 100%), 540.3 ([M + H]⁺, 71.6).

Coupling with BSA.

The nitrone 3 (40.4 mg) was added to 750 µl of 1 mM BSA dissolved in 0.02 M sodium-phosphate buffer pH 7.4. Then, the solution was stirred for 3h and allowed to stand overnight at 30 °C in darkness. Finally excess of the nitrone 3 was removed by sephadex G-25.

UV-vis measurements.

Aqueous solutions of BSA (6.5 µM), the nitrone 3 (12.0 µM) and BSA-PBN (protein concentration is 8.2 µM) were respectively added to a quartz cell with 1 cm of path length. Then, each spectrum was recorded from 200 nm to 400 nm, using a Hitachi U-3310 UV-visible spectrophotometer.

Spin trapping studies.

ESR Measurements. ESR spectra were recorded at room temperature on a Bruker ESP 300 spectrometer equipped with an X-band resonator (9.4GHz). The spectrometer settings were normally at the modulation amplitude of 0.1 mT, modulation frequency of 100 kHz, microwave power of 12.9 mW, and microwave frequency of 9.5 GHz. The UV photolysis was performed by a 200 W high-pressure mercury lamp.

Spin trapping with GS-PBN.

(a) spin trapping methyl and α-hydroxyethyl radicals. In aqueous media, methyl and α-hydroxyethyl radicals were generated in the presence of GS-PBN (25 mM) by a

standard Fenton system [0.2% H₂O₂, 2 mM EDTA, 5 mM FeSO₄] in the presence of DMSO (v/v, 10%) and ethanol (v/v, 17%) respectively, to give the corresponding spin adducts.

(b) spin trapping alkoxy radicals. *tert*-Butoxyl radical was generated by UV photolysis of a solution of di-*tert*-butyl peroxide (1M) in the presence of GS-PBN (25 mM) in DMSO. The spin adducts of ethoxyl radical were produced by adding a small amount of Pb(OAc)₄ to a solution of GS-PBN (25 mM) and ethanol in DMSO.

(c) spin trapping para-chlorophenyl radicals. In aqueous media, p-ClPh• was generated by UV photolysis of para-chlorophenyl diazonium tetrafluoroborate in the presence of GS-PBN (25 mM).

(d) spin trapping sulfite radicals. The sulfite radicals were generated and spin trapped by adding sodium sulfite (20 mM) and sodium dichromate (10 mM) into 25 mM of GS-PPN.

Spin trapping with BSA-PBN.

In the sodium-phosphate buffer pH 6.5, p-ClPh• was generated by UV photolysis of a small amount of para-chlorophenyl diazonium tetrafluoroborate in the presence of BSA-PBN (0.5 mM). In the proteolytic experiment, pronase (5 mg) was added to the solution of 200 µl of the spin adduct. The solution was allowed to stay for 24 h at room temperature.