Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information for:

Synthesis of C_2-Chiral Bifunctionalised Spin Labels and Their Application to Troponin C

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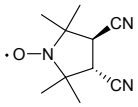
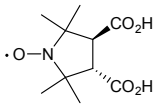
Isomerisation of *cis*-dinitrile *cis*-**2** to *trans*-dinitrile (±)-**2**.



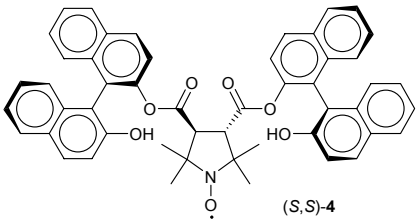
To a mixture of *cis*-**2** (4.0 g, 21 mmol), Bu^{t}OH (100 mL), and THF (100 mL), Bu^{t}ONa (200 mg, 2 mmol) was added. After stirring at room temperature for 1 h, water (150 mL) was added to the mixture. The mixture was extracted with EtOAc, and the organic layer was washed with saturated NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by SiO_2 flash chromatography (EtOAc-hexane, 1:4) to give *trans*-(±)-**2** as a yellow solid (1.86 g, 46%) and *cis*-**2** as an orange solid (0.99 g, 25%).

trans-Dicarboxylic acid (±)-**3**.

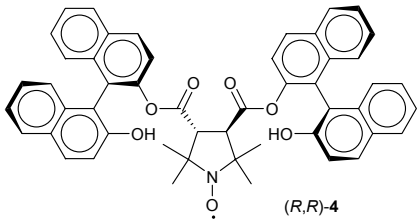


A mixture of (±)-**2** (1.0 g, 5.2 mmol) and 2M NaOH (60 mL) was heated at 90˚C overnight. After being acidified to pH 2 with 5% HCl, the mixture was extracted with ether (30 mL × 2). The extracts were dried over Na_2SO_4 and evaporated to give (±)-**3** as a yellow powder (0.73 g, 61%): Elemental analysis calcd. for $C_{10}H_{16}NO_5$: C, 52.17; H, 7.00; N, 6.08. Found: C, 51.92; H, 6.98; N, 5.82.

Binaphthyl esters (*S*,*S*)-**4** and (*R*,*R*)-**4**.



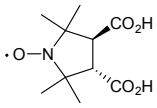
(*S*,*S*)-**4**



(*R*,*R*)-**4**

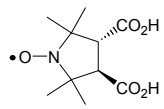
To a mixture of (±)-**3** (1.18 g, 5.13 mmol), CH_3CN (20 mL), and CH_2Cl_2 (20 mL), (*R*)-1,1'-bi-2-naphthol (3.23 g, 11.29 mmol), EDC·HCl (2.06 g, 10.77 mmol), and DMAP (126 mg, 1.03 mmol) were successively added at 0˚C. The mixture was stirred at 0˚C for 3 h and at room temperature for 2 days. The reaction mixture was evaporated, and the residue was dissolved in $CHCl_3$ (50 mL). The solution was washed with saturated $NaHCO_3$, and then dried over Na_2SO_4. After evaporation, the residue (3.39 g, 86%) was dissolved in $CHCl_3$ (20 mL). Diethyl ether was added until crystallisation began. After standing at room temperature overnight, yellow crystals of (*S*,*S*)-**4** were collected by suction (1.35 g, 40%). The filtrate was evaporated and the residue containing (*R*,*R*)-**4** and (*S*,*S*)-**4** was purified by SiO_2 flash chromatography ($CHCl_3$-EtOH, 99:1) to give (*R*,*R*)-**4** (1.24 g, 51%) and (*S*,*S*)-**4** (0.24 g, 7%, total yield: 1.59 g, 47%). (*S*,*S*)-**4**: de >99%; $[\alpha]_D^{23}$ +23 (*c* 0.2, $CHCl_3$); δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 0.19 (s, 6H, CH_3), 0.79 (s, 6H, CH_3), 2.72 (s, 2H, CHCO); HR-FAB-MS: *m*/*z* calcd for $C_{50}H_{40}NO_7$ (M^+) 766.2805, found 766.2811; Elemental analysis calcd for $C_{50}H_{40}NO_7 \cdot 2(C_2H_5)_2O$: H, 6.61; C, 76.13; N, 1.53. Found: H, 6.44; C, 76.21; N, 1.56. (*R*,*R*)-**4**: de 97%; $[\alpha]_D^{23}$ +129 (*c* 0.2, $CHCl_3$); δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 0.04 (s, 6H, CH_3), 0.50 (s, 6H, CH_3), 2.99 (s, 2H, CHCO); HR-FAB-MS: *m*/*z* calcd for $C_{50}H_{40}NO_7$ (M^+) 766.2805, found 766.2775.

Dicarboxylic acid (*S*,*S*)-**3**: Typical procedure for hydrolysis of **4**.



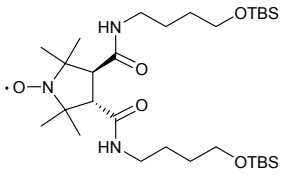
A mixture of (*S*,*S*)-**4** (400 mg, 0.52 mmol) and 2M NaOH (13 mL) was stirred at 90˚C overnight. After neutralized with 5% HCl, the mixture was washed with ether (10 mL × 3) to remove binaphthol. The aqueous layer was acidified to pH 2, and then extracted with ether (10 mL × 3). The extracts were washed with saturated HCl, dried over Na_2SO_4, and evaporated to give (*S*,*S*)-**3** as an orange powder (98 mg, 82%): $[\alpha]_D^{23}$ -67 (*c* 0.98, MeOH).

Dicarboxylic acid (*R*,*R*)-**3**.



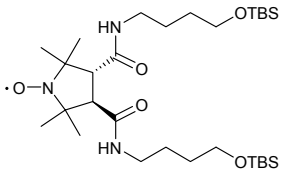
An orange powder (67%): $[\alpha]_D^{23}$ +64 (*c* 0.70, MeOH).

Silyl ether (*S*,*S*)-**5**: Typical procedure for amide formation from **3**.



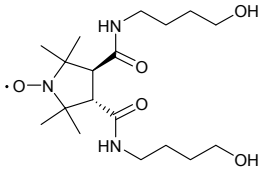
To a mixture of $TBSO(CH_2)_4NH_2$ (407 mg, 2.0 mmol), (*S*,*S*)-**3** (230 mg, 1.0 mmol), and CH_2Cl_2 (10 mL), Et_3N (293 μL, 2.1 mmol), HOBt (284 mg, 2.1 mmol), and DCC (433 mg, 2.1 mmol) were successively added at 0˚C. After stirring at room temperature overnight, the mixture was evaporated, and then EtOAc (20 mL) was added to the residue. The mixture was filtrated and the filtrate was successively washed with 4% citric acid, 4% $NaHCO_3$, and saturated NaCl. The organic layer was dried over Na_2SO_4 and evaporated to give (*S*,*S*)-**5** as a yellow oil (530 mg, 88%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 0.04 (s, 12H, $Si(CH_3)_2$), 0.88 (s, 18H, $SiC(CH_3)_3$), 1.05 (s, 6H, CH_3), 1.28 (s, 6H, CH_3), 1.48-1.53 (m, 8H, CH_2), 2.96 (s, 2H, CHCO), 3.08 (m, 2H, C*H*$_2$NH), 3.34 (m, 2H, C*H*$_2$NH), 3.60 (br, 4H, CH_2O); δ_C (270 MHz, $CDCl_3$) 18.42, 21.48, 21.69, 25.71, 26.03, 26.22, 27.35, 30.14, 39.49, 53.18, 62.65, 63.84, 170.18.

Silyl ether (*R*,*R*)-**5**.



An yellow oil (90%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 0.04 (s, 12H, $Si(CH_3)_2$), 0.88 (s, 18H, $SiC(CH_3)_3$), 1.05 (s, 6H, CH_3), 1.28 (s, 6H, CH_3), 1.48-1.53 (m, 8H, CH_2), 2.96 (s, 2H, CHCO), 3.08 (m, 2H, C*H*$_2$NH), 3.34 (m, 2H, C*H*$_2$NH), 3.60 (br, 4H, CH_2O); δ_C (270 MHz, $CDCl_3$) 18.42, 21.48, 21.69, 25.71, 26.03, 26.22, 27.35, 30.14, 39.49, 53.18, 62.65, 63.84, 170.18.

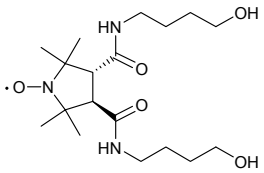
Diol (*S*,*S*)-**6**: Typical procedure for desilylation of **5**.



A THF solution of TBAF (1M, 0.53 ml, 0.53 mmol) was added to a solution of (*S*,*S*)-**5** (267 mg, 0.44 mmol) in THF (10 mL) at room temperature. The mixture was stirred for 2 h and then evaporated. The residue was purified by SiO_2 flash chromatography (EtOAc-MeOH, 10:1) to give (*S*,*S*)-**6** as a yellow powder (94 mg, 0.25 mmol, 57%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 1.01 (s, 6H, CH_3), 1.29 (s, 6H, CH_3), 1.54 (m, 8H, CH_2), 3.04 (s, 2H, CHCO), 3.12 (m, 2H, C*H*$_2$NH), 3.24 (m, 2H, C*H*$_2$NH), 3.55 (m, 4H, CH_2O); δ_C (270 MHz, $CDCl_3$) 22.33, 26.98,

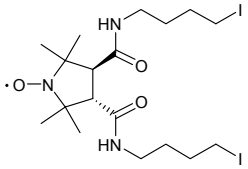
27.67, 30.94, 40.42, 53.98, 62.47, 64.62, 172.88; Elemental analysis calculated for $C_{18}H_{34}N_3O_5$: C, 58.04; H, 9.20; N, 11.28. Found: C, 57.30; H, 9.27; N, 11.03.

Diol (*R*,*R*)-**6**.



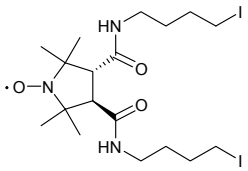
An yellow powder (51%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 1.01 (s, 6H, CH_3), 1.29 (s, 6H, CH_3), 1.54 (m, 8H, CH_2), 3.04 (s, 2H, CHCO), 3.12 (m, 2H, C*H*$_2$NH), 3.24 (m, 2H, C*H*$_2$NH), 3.55 (m, 4H, CH_2O); δ_C (270 MHz, $CDCl_3$) 22.33, 26.98, 27.67, 30.94, 40.42, 53.98, 62.47, 64.62, 172.88.

Diiodide (*S*,*S*)-**7**: Typical procedure for iodination of **6**.



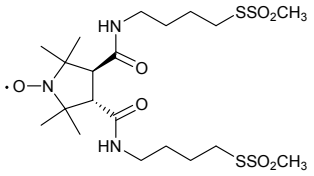
To a solution of (*S*,*S*)-**6** (94 mg, 0.25 mmol) in pyridine (5 mL), MsCl (78 μL, 1.00 mmol) was added at 0˚C. The mixture was stirred at room temperature for 4 h. After adding water (10 mL), the mixture was extracted with EtOAc (10 mL × 2). The combined extracts were washed with saturated NaCl (10 mL), dried over Na_2SO_4, and evaporated. A mixture of the residue, NaI (150 mg, 1.0 mmol), and acetone (10 mL) was refluxed for 10 h. After evaporation, the residue was dissolved in EtOAc (10 mL), and the solution was washed with 10% $Na_2S_2O_3$ and saturated NaCl. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by SiO_2 flash chromatography ($CHCl_3$-MeOH, 10:1) to give (*S*,*S*)-**7** as a yellow powder (85 mg, 0.14 mmol, 57%): HR-FAB-MS: *m*/*z* calcd for $C_{18}H_{33}I_2N_3O_3$ $(M+H)^+$ 593.0611, found 593.0604; Elemental analysis calculated for $C_{18}H_{32}I_2N_3O_3$: C, 36.50; H, 5.45; N, 7.09; I, 42.85. Found: C, 37.46; H, 5.30; N, 7.29; I, 40.21; $[\alpha]_D^{23}$ -47 (*c* 0.66, $CHCl_3$).

Diiodide (*R*,*R*)-**7**.



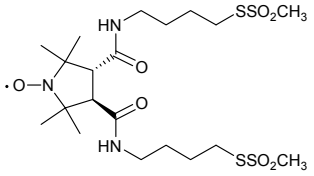
An yellow powder (32%): $[\alpha]_D^{23}$ +48 (*c* 0.32, $CHCl_3$)

Dimethanethiosulphonate (*S*,*S*)-**1**: Typical procedure for methanethiosulphonation of **7**.



To a solution of (*S*,*S*)-**7** (66 mg, 111 μmol) in DMSO (4 mL), $NaSSO_2CH_3$ (45 mg, 333 μmol) was added. The mixture was stirred at room temperature overnight. The mixture was diluted with $CHCl_3$ (20 mL), and then the solution was washed with water, dried over $MgSO_4$, and evaporated. The residue was purified by SiO_2 flash chromatography (EtOAc-methanol, 10:1) to give (*S*,*S*)-**1** as a yellow oil (48 mg, 86 μmol, 73%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 1.05 (s, 6H, CH_3), 1.31 (s, 6H, CH_3), 1.62 (m, 8H, CH_2), 2.97 (s, 2H, CHCO), 3.17 (t, 4H, C*H*$_2$NH), 3.27 (m, 4H, CH_2S), 3.33 (s, 6H, SO_2CH_3).

Dimethanethiosulphonate (*R*,*R*)-**1**.



An yellow oil (78%): δ_H (270 MHz, $CDCl_3$, with 1.5 mol equiv of 1,2-diphenylhydrazine) 1.05 (s, 6H, CH_3), 1.31 (s, 6H, CH_3), 1.62 (m, 8H, CH_2), 2.97 (s, 2H, CHCO), 3.17 (t, 4H, C*H*$_2$NH), 3.27 (m, 4H, CH_2S), 3.33 (s, 6H, SO_2CH_3).

X-ray crystallographic data for (*S*,*S*)-**4**·$(C_2H_5)_2O$

The crystal structure has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number, CCDC 256206.

empirical formula	$C_{58}H_{60}O_2N$
formula weight	915.11
crystal system	orthorhombic
space group	$C222_1$ (#20)
a, Å	11.098(4)
b, Å	14.847(7)
c, Å	29.42(1)
V, Å^3	4847(3)
Z	4

F(000)	1948.00
D_{calc}, g/cm^3	1.254
T, °C	–100
crystal size, mm	0.30 × 0.30 × 0.10
μ (MoKα), cm^{-1}	0.84
diffractometer	Rigaku RAXIS-RAPID
radiation	MoKα (0.71075 Å)
$2\theta_{max}$, deg	55.0
no. of reflns measd	21835
no. of reflns obsd	8672 [$I > 3.0\sigma(I)$]
no. of variables	339
R [a]	0.051
R_w [b]	0.065
GOF[c]	0.999
Flack parameter	0(1)

[a] $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $R_w = \{\Sigma_w(|F_o| - |F_c|)^2/\Sigma_w F_o^2\}^{1/2}$. [c] Goodness of fit on F^2.

Expression and Purification of TnC

For site-directed spin labelling 94 serine residue was mutated to cysteine residue by the method of Mutan-Super Express Km kit (TaKaRa shuzo). And the chicken TnC gene fragment was ligated into *NcoI/BamHI*-digested pET15b vector, and used to transform competent *Escherichia coli* cell, BL21 (DE3). The cells were grown at 37˚C in LB medium and were induced with 1.0 mM isopropyl D-thiogalactopyranoside (IPTG) and incubated for 8 h. The harvested cells were homogenized into 50 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), and then sonicated using a sonicator (Heatsystems Inc.). Ammonium sulphate (60% w/v) was added to *E. coli* cell extract and the solution was centrifuged at 15,000 × g for 10 min. The TnC remained in the supernatant was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, 2 mM EDTA. It was loaded onto a Q- Sepharose Fast Flow column (Amersham Biosciences, 2.5 cm × 10 cm, flow rate: 2 mL/min) and eluted with a linear 0 - 0.8 M NaCl gradient. The fractions containing TnC were identified by absorption at 214 nm and SDS-polyacrylamide gel electrophoresis. Purified TnC fractions were dialyzed against 10 mM MOPS buffer, pH 7.0 containing 100mM KCl. The protein concentrations were measured with the method of BCA protein assay.

Spin labelling of TnC

The TnC mutant was covalently labelled with (*S*,*S*)- and (*R*,*R*)-**1** at two cysteine residues (94Cys and 101Cys) on TnC mutant (S94C). The TnC mutants were treated in 5 mM DTT to reduce disulphide bonds or oxidized SH residues. Then, DTT was removed by applying Sephadex G-25 (Amersham Biosciences, 1.5 cm × 20 cm, flow rate: 1mL/min). The final concentration of the spin labels added in the TnC solution was 100 μM. The labeling reaction was allowed to proceed for 48 h at 4˚C and then it was stopped and unreacted labels were removed by applying Sephadex G-25 desalting column (Amersham Biosciences, 1.5 cm × 20 cm, flow rate: 1mL/min), and TnC which was unspecifically labeled by only one disulphide linkage with the spin label was removed by applying Activated Thiol Sepharose 4B (Amersham Biosciences, 1.5 cm × 10 cm, flow rate: 0.5 mL/min). For EPR measurements, the samples were concentrated to 50μM.

EPR measurement

EPR spectra from spin-labeled TnCs were collected using an ELEXSYS E500 CW-EPR spectrometer (Bruker Biospin). X band, first-derivative absorption EPR spectra were obtained with following instrument settings; microwave power, 5 mW; modulation amplitude, 2 G; sweep time, 40 s; microwave attenuation, 16 dB; time constant, 20 ms; conversion time, 40 ms; receiver gain, 60 dB and averaged scans, 10–30. TnC solution (10 μL) was placed in the capillary cell (1 mm × 10 cm) mounted in a cylindrical dielectric resonator (ER4118X-MD5-W1) at 25˚C. EPR spectra in the presence or absence of $CaCl_2$ (2 mM) were analyzed as described by Freed and collaborators using stochastic Liouville approach.