Synthesis of C_2-chiral bifunctionalised spin labels and their application to troponin C†

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An enantiomeric pair of C_2-chiral bifunctionalised spin labels having a pyrrolidine nitroxide moiety, whose configurations were determined by X-ray crystal diffraction analysis, was prepared and applied to troponin C whose binding mode of double disulfide linkage was proved by EPR spectroscopy.

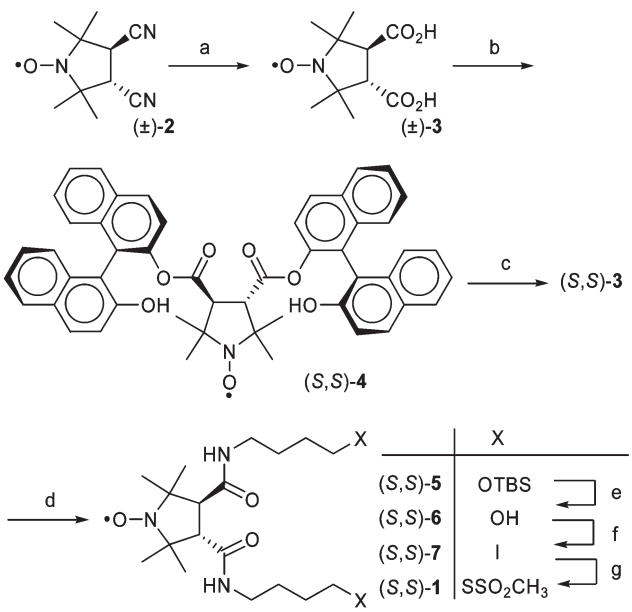
To investigate the physiological motion of protein, spin labels can be used as an orientation indicator.[1] For this purpose, the angle between the principal axis of an unpaired electron orbital of a spin label and the long axis of protein should be unambiguously determined.[2] Monofunctional spin labels cannot be employed because their free mobility is too large, but bifunctionalised spin labels (BSL) are suitable for the EPR experiment. In the course of our investigation of troponin C (TnC) dynamics, which is a key protein regulating muscle contraction and relaxation, BSL that can be bound between two SH groups on TnC are desirable. A spin label of C_2-chiral structure is ideal because it binds to protein in single mode and diastereomeric isomerism cannot occur. Moreover, it is advantageous to obtain both enantiomers because either isomer may give desirable binding orientation for the measurement.

Some types of BSL have been synthesized and utilized for the EPR study of proteins,[3] and some optically active pyrrolidine-based nitroxides bearing 3,4-substituents have been also synthesized.[4,5] However, the BSL reported so far are not suitable for protein dynamics experiments by EPR because two different structural isomers could be formed depending on the direction of the spin label to the protein, and we designed nitroxide labels (*S*,*S*)- and (*R*,*R*)-**1**. They consist of C_2-chiral pyrrolidine nitroxide and two methanethiosulfonate groups linked with an amide bond and four methylene groups. The fitting between the spin labels and the SH groups on TnC was preliminarily suggested by molecular modelling.

We chose known *trans*-dinitrile (±)-**2** as the starting material for the spin labels.[6] In the synthesis of (±)-**2**, a concomitant *cis*-isomer was converted to (±)-**2** by treatment with NaOBu^t. Basic hydrolysis of (±)-**2** provided dicarboxylic acid (±)-**3** in 61% yield (Scheme 1), from which a diastereomeric mixture of ester **4** was synthesized with (*R*)-1,1′-bi-2-naphthol in 62% yield.[4] Isomer (*S*,*S*)-**4** crystallised when a solution of the diastereomeric mixture in $CHCl_3$ was carefully treated with diethyl ether in 40% yield. The de of (*S*,*S*)-**4** was proved to be >99% by ^{1}H NMR monitoring of the signal of the pyrrolidine ring protons. The signal of (*S*,*S*)-**4** (δ 2.72) was clearly distinguished from that of (*R*,*R*)-**4** (δ 2.99). From the filtrate, (*R*,*R*)-**4** was separated by SiO_2 chromatography in 32% yield. The de of (*R*,*R*)-**4** was found to be 97%, also by ^{1}H NMR.‡ The configuration of (*S*,*S*)-**4** was unambiguously established by X-ray crystal diffraction analysis based on the *R* configuration of the binaphthyl moiety. Crystallographic data showed that a crystal of (*S*,*S*)-**4** contains 2 equiv of diethyl ether which are omitted in Fig. 1.§

Alkaline hydrolysis of (*S*,*S*)-**4** afforded dicarboxylic acid (*S*,*S*)-**3** in 60% yield, which was converted to diamide (*S*,*S*)-**5** with $TBSO(CH_2)_4NH_2$ in 88% yield. Successive desilylation, mesylation, and iodination gave iodide (*S*,*S*)-**7** in an overall yield of 32%. Finally, bifunctionalised spin label (*S*,*S*)-**1** was obtained in 58% yield from (*S*,*S*)-**7** with $NaSSO_2CH_3$. The *R*,*R*-counterpart was also prepared through the same reaction sequence.

A chicken TnC mutant expressed in *E. coli* cells, S94C, having two cysteine residues (94Cys and 101Cys) was employed for the label experiment with (*S*,*S*)-**1** and (*R*,*R*)-**1** (Fig. 2). The two cysteine residues reside on the central E-helix domain of TnC and



Scheme 1 *Reagents and conditions*: (a) 2N NaOH, 90 °C, 61%; (b) (*R*)-1,1′-bi-2-naphthol, EDC·HCl, DMAP, CH_2Cl_2, CH_3CN, 0 °C, 62%; (c) 2N NaOH, 90 °C, 60%; (d) $TBSO(CH_2)_4NH_2$, DCC, HOBt, Et_3N, CH_2Cl_2, 0 °C, 88%; (e) TBAF, THF, rt, 76%; (f) MsCl, pyridine, 0 °C, then NaI, acetone, reflux, 45%; (g) $NaSSO_2CH_3$, DMSO, rt, 58%.

† Electronic supplementary information (ESI) available: experimental details, spectral data, and crystallographic data. See http://www.rsc.org/suppdata/cc/b4/b418016j/

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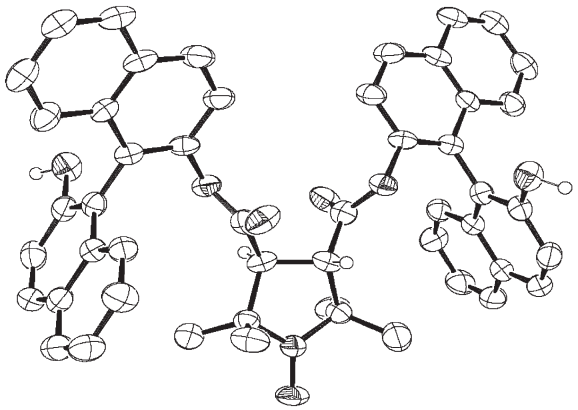

Fig. 1 ORTEP drawing of (*S*,*S*)-**4**.

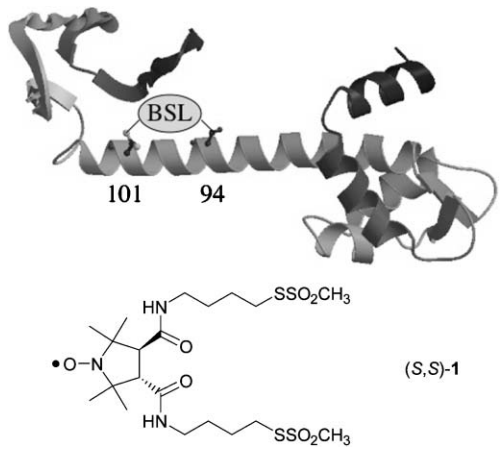

Fig. 2 Labelling positions of BSL on chicken skeletal TnC and C_2-chiral spin label (*S*,*S*)-**1**. The label site is on the central helix (E-helix) of TnC and the distance between 94 and 101 cysteine residues is 11 Å.

the distance between their S atoms is 11 Å. The mutant was treated in 5 mM DTT to reduce disulfide bonds or oxidized SH residues. Then DTT was removed by applying a Sephadex G-25 desalting column (1.5 cm × 20 cm, Amersham Biosciences), and the mutant was labelled with the present spin labels. The final concentration of the spin labels added in the TnC solution was 100 μM. The labelling reaction was allowed to proceed at 4 °C for 48 h and then stopped. The unreacted labels were removed by applying a Sephadex G-25 column. TnC molecules which were unspecifically labelled by only one disulfide linkage with the spin label and still had an active methanethiosulfonate group were removed by applying Activated Thiol Sepharose 4B (1.5 cm × 10 cm, Amersham Biosciences). For EPR measurements, the samples were concentrated to 50 μM.

EPR spectra showed that (*S*,*S*)-**1** and (*R*,*R*)-**1** were largely immobilized when they labelled the TnC protein (Fig. 3B). Small sharp peaks were completely removed by passing through the thiol resin column. Final spectra from (*S*,*S*)-**1** and (*R*,*R*)-**1** (Figs. 3C and D) showed a high immobilization ($2A'_{zz}$ = 53 and 49 gauss, respectively) as compared to a monofunctional label ($2A'_{zz}$ = 34 gauss, data not shown). According to Stoke's hydrodynamic theory, the rotational correlation time τ for TnC (molecular weight

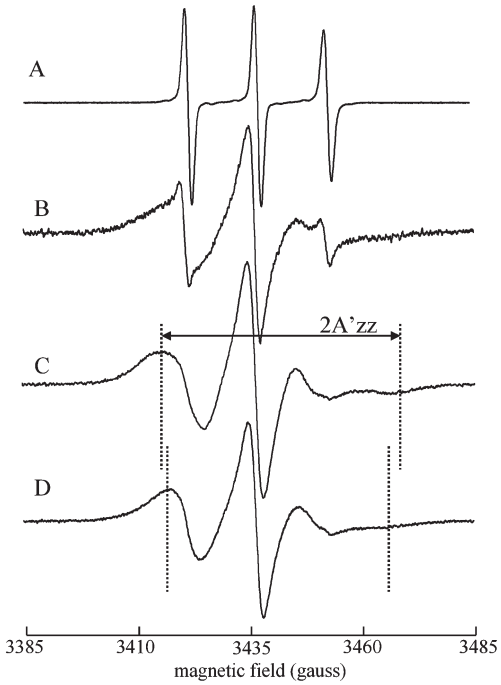

Fig. 3 EPR spectra of TnC labelled with (*S*,*S*)-**1** and (*R*,*R*)-**1**: (A) (*S*,*S*)-**1** itself; (B) (*S*,*S*)-**1** labelled TnC which was purified with a gel-filtration column; (C) (*S*,*S*)-**1** labelled TnC which was further purified with a thiol column; (D) (*R*,*R*)-**1** labelled TnC which was further purified with a thiol column ($2A'_{zz}$, the distances between two dashed lines, show the mobility of spin labels).

of 18 000) at 25 °C in water is 7×10^{-9} s by assuming an equivalent sphere.[7] Using the equation[2] of $5.4 \times 10^{-10} (1 - A'_{zz}/A_{zz})^{-1.36}$ and assuming $2A_{zz}$ = 72 gauss (A'_{zz} in frozen solution at −25 °C), the τ of 5×10^{-9} s was calculated for (*S*,*S*)-**1** and (*R*,*R*)-**1**. The observed spectra were similar to one simulated with a single motional component according to the stochastic Liouville approach by Freed and colleagues for Brownian diffusion.[8] A good agreement in τ suggests that spin label motion reflects the motion of the whole protein molecule. However, a small spectral difference in (*S*,*S*)-**1** and (*R*,*R*)-**1** arises from a diastereomeric interaction between chiral spin labels and protein: (1) the anisotropic motion is characterised by different orientation of spin labels relative to the main rotational axis of the protein molecule, (2) the molecular shapes of TnC are slightly different by modification with (*S*,*S*)-**1** and (*R*,*R*)-**1**, and (3) slight segmental motion of a spin label relative to the whole protein molecule is different in (*S*,*S*)-**1** and (*R*,*R*)-**1** attached sites.

Here, we demonstrated the synthesis of new bifunctionalised spin labels and their application to spin labelling of TnC. Bifunctionalised compounds (*S*,*S*)-**1** and (*R*,*R*)-**1** are well suited for angular analysis of an oriented sample and slow motional studies of a whole protein molecule or whole labelled domains by EPR spectroscopy.

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Notes and references

‡ Synthesis of (*S*,*S*)-**4** and (*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.

§ Crystal data for (*S*,*S*)-**4**·$2(C_2H_5)_2O$: $C_{58}H_{60}O_9N$, M = 915.11, orthorhombic, a = 11.098(4), b = 14.847(7), c = 29.42(1) Å, V = 4847(3) Å^3, T = 173 K, space group $C222_1$ (#20), Z = 4, D_{calc} = 1.254 g cm^{-3}, $F(000)$ = 1948.00, μ(MoKα) = 0.84 cm^{-1}, Flack parameter = 0(1), 21835 reflections measured, 21681 unique (R_{int} = 0.068). The final agreement factors are R = 0.051 and Rw = 0.065 for 8672 observed reflections with $I > 3.00\sigma(I)$. CCDC 256206. See http://www.rsc.org/suppdata/cc/b4/b418016j/ for crystallographic data in .cif or other electronic format.

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