Spin labels are small molecules that attach to larger molecules of interest and that stabilize a free radical. The spin labels are most likely to be used to report on their microenvironment, accessibility, motional dynamics or to enable nanometre-scale distance measurements by electron paramagnetic resonance (EPR) spectroscopy. Alternatively, they may be used as paramagnetic relaxation enhancing reagents in nuclear magnetic resonance (NMR) spectroscopy. It is therefore often desirable to have a spin label that demonstrates little conformational freedom while having enough flexibility to avoid disruption of the protein fold.

Commonly these labels stabilize a nitroxyl radical as part of a 5 or 6 membered carbon framework and are hence called nitroxide spin labels. These are functionalized to react at the site of interest. For proteins this site is often the thiol of cysteine. The labels can be made to covalently attach reversibly or irreversibly with short or long linkers. Attachment chemistries

**Scheme 1** Proposed method for labeling proteins containing multiple free cysteines but with only one vicinal pair using a next generation maleimide. The maleimide and succinimide linkages are shown. In this work R is the nitroxide-containing framework (pyrrolinoxyl or pyrrolidinoxyl) and X a Br or OPh leaving group. The NGM labels made are shown as 1, 2, 3 and 4.
Here we report on the synthesis of spin labels based on next generation maleimides. Following literature precedence, these should bind to pairs of cysteines which are close in space ("vicinal") via a succinimide bridge but bind to single cysteines with a sulfur-maleimide bond which can be reversed, see Scheme 1. The labels proposed here are different to the existing next generation maleimide spin labels, TPMP and TPM-P proposed by Baker and co-workers as spinostic reagents. In that work they did not use succinimide bridging but instead had two leaving groups to result in a maleimide bridged product. Here we wish to create spin labels that will give a different chemical reactivity, depending on whether single or pairs of cysteines are bound, to enable selective labeling of only pairs of cysteines (Scheme 1).

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**Scheme 2** Reagents and conditions: (a) Ethyl chloroformate, Et$_2$N, toluene, quantitative; (b) NaBH$_4$, EtOH, 83%; (c) TiCl$_4$, Et$_2$N, DCM, 67%; (d) NH$_2$, MeOH, 80%; (e) Bromomaleic anhydride (1 equiv.), Ac$_2$O, 47%; (f) phenol (15 equiv), t-BuOK (1.2 equiv.), dioxane, 92%.

Proteins which are excreted from the biological cell rarely contain cysteines suitable for labeling. Therefore cysteines can be engineered into sites of interest in proteins and labeled using site directed spin labeling (SDSL). However many excreted proteins contain disulfide bonds, where two cysteines are close in space rather than necessarily sequence, and intracellular proteins contain cysteines much more frequently. These situations are not ideal for designing SDSL experiments. One method to overcome this problem is to incorporate unnatural amino acids into the protein sequence. These have bioorthogonal reactivities and can therefore undergo selective labeling, alternatively there is a published example of a nitroxyl radical being incorporated directly. This field is progressing but at the moment the technology cannot be readily applied to any protein, and the spin labels are on long flexible linkers which is a disadvantage for many applications.

**Scheme 3** Reagents and conditions: (a) Bromomaleic anhydride (1 equiv.), ether, 87%; (b) NaOAc (1 equiv.), Ac$_2$O, 64%; (c) phenol (15 equiv), t-BuOK (1.2 equiv.), dioxane, 92%.

Next generation maleimides have a leaving group and this can be altered to tune for desired properties. It has previously been shown that bromide may leave so rapidly that adjacent pairs of cysteines will each bind a maleimide marker whereas an O-phenyl leaves less efficiently, thus favoring the intramolecular reaction and formation of a succinimide bridged product. We have therefore made each label type with Br (1 and 3) and OPh (2 and 4) leaving groups on the maleimide. The two families consist of either a pyrrolinoyl (1 and 2) or pyrrolidinoxy (3 and 4) carbon framework stabilizing the radical. The
pyrrolidinoxyl has an extra stereocentre but one less carbon in the linker between the nitroxide ring and the nitrogen of the maleimide. An advantage of the pyrrolinoxyl ring is that it is expected to have greater stabilizing properties for the nitroxyl radical.

Carboxylic acid was Scheme 2) was converted into alcohol 7 via the intermediate mixed anhydride 6. This route was favoured over lithium aluminium hydride reduction which can lead to low yield and a lack of reproducibility. The two-step procedure has previously been used by Kirlyuk et al. for the synthesis of 2,5-bis(spirocyclohexane)-substituted nitroxides and in our hands gave 7, reproducibly, at 83% yield. Alcohol 7 was then converted to tosylate 8, and the tosyl group was substituted with ammonia, yielding amine 9. Efficient amination (80%) was achieved using a 7 M solution of ammonia in methanol. Amine 9 was transformed using a one-step procedure into bromomaleimide 1 in the presence of bromomaleimic anhydride using acetic acid as the solvent. This reaction gave 1 with 47% yield and the nitroxyl group was shown to be unaffected by the reaction conditions, i.e. no reduction was observed by continuous wave (CW) EPR spectroscopy.

When this one-step bromomaleimide formation reaction was attempted on the commercial aminopyrrolidinoxyl radical the formation of a complex mixture was observed. This mixture did not give any signal in CW EPR spectroscopy, suggesting that reduction of the nitroxyl moiety had occurred. Thus, 3 was synthesized from amine 10, using a two-step reaction pathway (Scheme 3). This procedure consists of the synthesis of a mixture of compounds 11 and 11’, using bromomaleic anhydride, followed by an intramolecular cyclisation giving compound 3 with an overall yield of 56%.

In both families of NGM labels, the OPh derivative (2 and 4) was synthesized, with an excellent yield, from the bromo precursor (6 and 10 respectively) using phenol in the presence of t-BuOK.

Continuous wave (CW) EPR spectroscopy of the compounds did not demonstrate any decrease in radical content over the course of the reactions. The structure of the compounds was confirmed through NMR spectroscopy, mass spectrometry and X-ray crystallography. The crystallographic structures are presented in Figure 1. The crystals were racemic and diffracted well to give single independent results for 1, 2 and 4. Crystals of 3 gave less clear results. However, HPLC, TLC and NMR results for 3 did not indicate that it was less pure than the other end products.

To demonstrate that the labels bind to proteins through loss of either their bromo or O-Phenyl groups, and to see if there were any clear differences in mobility between the families, were added to Sperm Whale Myoglobin S3C. This was an available protein containing one cysteine residue for labeling at position 3, which is a serine in the wild type. A 10 times excess of spin label was incubated with the protein for 1 hour at room temperature or at 4°C overnight. Size exclusion chromatography was then employed to remove unreacted label. LC-MS confirmed that all four labels bound to myoglobin and gave the expected mass increase, with no free protein observed (Figure 2). The CW EPR spectra for these samples show that the labels have a reduced mobility compared to the free labels which gave a characteristic sharp three-line spectrum. The broader spectra
for the shorter linked spin labels, 3 and 4, demonstrate that they have a further reduced conformational freedom compared to the longer linkers of 1 and 2, see figure 3.3.18

In conclusion, four new nitroxide-containing spin labels have been synthesized with next generation maleimide functionalities. This may lead to the ability to selectively bind the spin label tovicinal cysteines and trials are ongoing.

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Supporting Information
Yes

Primary Data
The magnetic resonance data supporting this publication can be accessed at http://dx.doi.org/10.17630/19ac1929-adee-323baeddff0e5. CCDC 1468786 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures. The primary data for the LC-MS analysis have been deposited to the PrometXchange Consortium via the PRIDE partner repository with the dataset identifier PXD003824. 19

References and Notes
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(3)-[[Ethoxycarbonyl]oxy[carbonyl]-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-oxyl] (Ethylchloroformate (2.9 g; 26.7 mmol) was added dropwise to a stirred cold (-10 °C) solution of 5 (3.6 g; 19.5 mmol) in a mixture of dry toluene (155.0 mL) and triethylamine (3.5 mL; 35.5 mmol). After stirring for 50 minutes, the solvent was evaporated and the residue was triturated with ether. The precipitate was filtered off, washed with ether. The organic layer was concentrated in vacuo and the residue was recrystallized from hexane yielding 6 (50 g; quantitative) as a yellow solid. HRMS ESI (m/z) calcd for C14H17NO2, 256.1179 found 256.1176.
3-Hydroxymethyl-2,2,5,5-tetramethylpyrrole-2-n-oxyl (A solution of NaBH4 (1.5 g; 39.0 mmol) in ethanol was cooled down with an ice bath and 6 (5.0 g; 19.5 mmol) was added portion wise upon stirring. After stirring for 2 h, the solvent was evaporated under reduced pressure and the residue was diluted with water and extracted with ether. The extract was dried and concentrated to give 7 (28 g; 85%) as a yellow powder. 'H NMR (400 MHz, acetone d6/D2O + 1.5 eq. Na2SO3) 6 1.14 (6, H), 6 6.16 (6, H), 6 4.02 (d, J = 1.7 Hz, 2H), 5.50 (6, 1H), 12.11 PMRS ESI (m/z) calcd for C14H23NO3, 193.1073 found 193.1070.
2,5-Dihydro-2,2,5,5-tetramethyl-3-[[methylphenyl]sulfonyl]oxy[ethyl]1-pyrrol-1-oxyl] (A) solution of 7 (2.5 g; 14.5 mmol) and trimethylamine (1.9 mL; 19.3 mmol) in dry dichloromethane (40.0 mL) was cooled at -10°C, and then tosyl chloride (2.8 g; 14.5 mmol) was added portion wise upon vigorous stirring. The solution was stirred for 3 h at rt, washed with water and with a sat. solution of NaHCO3, dried and concentrated under vacuo. The crude was purified by column chromatography (cyclohexane/EtOAc) yielding 8 (3.5 g; 66%) as a yellow solid. HRMS ESI (m/z) calcd for C14H23NO3S, 347.1162 found 347.1154.
3-(Aminomethyl)-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-oxyl] A solution of 8 (1.5 g; 4.6 mmol) in anhydrous methanol was added dropwise to ammonia solution (75.0 mL; 7 N in MeOH). The mixture was stirred for 2 h at rt, then left to stand overnight. The solvent was evaporated under reduced pressure. The residue was treated with a buffer solution (60.0 mL; mixture of citric acid and NaHPO4) at pH 5 and extracted with ether. The aqueous layer was saturated with sodium hydroxide and extracted with ether. The extract was dried and concentrated yielding 9 (706.0 mg; 90 %) as an orange oil. 'H NMR (400 MHz, CDCl3 + phenylhydrazine to reduce the nitroxide radical to a diamagnetic N-hydroxylamine,22 since we found that our product was degraded by Na2S(O2)6 1.24 (6, 6H), 1.25 (6, 6H).
3.31 (d, J = 1.8 Hz, 2H), 5.42 (s, 1H); HRMS ESI (m/z) calcd for C_{38}H_{40}O_{12}N_{3}S: 701.2408.

3-[(3-bromo-2,5-dihydro-1H-pyrrrole-2,5-dione-1-yl)methyl]-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yloxy (I)

Bromomalonic anhydride (388.2 μL; 4.2 mmol) was dissolved in acetic acid (7.0 mL). Nitrosoyl 9 (707.9 mg; 4.2 mmol) in acetic acid (7.0 mL) was added, and the reaction was heated at 80°C for 3 h. The solvent was removed under vaccuo and the mixture was purified by column chromatography (cyclohexane/EtOAc) to give the bromomalonides 1 (644.7 mg; 47%) as an oil. 1H NMR (400 MHz, CDCl_3): δ 1.38 (s, 6H), 1.45 (s, 6H), 4.17 (d, J = 1.4 Hz, 2H), 5.40 (s, 1H), 6.94 (s, 1H); HRMS NSI (m/z) calcd for C_{39}H_{39}O_{13}BrNa: 738.0417 found 738.0417; mp = 146-147°C.

3-[(3-bromo-2,5-dihydro-1H-pyrrrole-2,5-dione-1-yl)-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yl oxy] (3)

To a stirred solution of amine 10 (489.0 mg; 3.1 mmol) in dry ether (24.7 mL) bromomalonic anhydride (286.7 μL; 3.1 mmol) was added. The reaction was left stirring at rt for 3 h. The precipitate was filtered and washed with ether yielding a mixture of 11 and 11′ (910.0 mg; 87%) as a yellow solid.

The mixture of 11 and 11′ (910 mg; 2.7 mmol) and sodium acetate (222.2 mg; 2.7 mmol) were dissolved in acetic anhydride (13.5 mL) and heated at 60-70°C for 3 h. The reaction mixture was then concentrated, dissolved in DCM and filtrated. The filtrate was concentrated and purified by column chromatography (cyclohexane/EtOAc) to give 3 (853 mg; 64%) as an orange solid.

1H NMR (400 MHz, CDCl_3): δ 1.08 (s, 3H), 1.25 (s, 3H), 1.26 (s, 3H), 1.36 (s, 3H), 1.82 (dd, J = 12.5, 8.8 Hz, 1H), 2.93 (dd, J = 12.5, 11.0 Hz, 1H), 4.47 (dd, J = 11.0, 8.8 Hz, 1H), 6.89 (s, 1H); HRMS ESI (m/z) calcd for C_{39}H_{38}O_{14}N_{2}BrNa+: 532.1394 found 532.1371; mp = 101-102°C.

Phenoxymaleimide 2 and 4: General procedure

To molten phenol (13.2 mmol), potassium tert-butoxide (1.1 mmol) in dry dioxane (0.8 mL) was added dropwise and the solution was left stirring for 10 min at 40 °C. Then a solution of bromomalonide 1 or 3 (0.8 mmol) in dry dioxane (0.8 mL) was added dropwise and the resulting mixture was stirred at 40 °C for 30 minutes. After this time, the solvent was evaporated under reduced pressure. The mixture was purified by column chromatography (cyclohexane/EtOAc) to give the corresponding phenoxymaleimide.

3-[(3-phenoxycarbonyl-2,5-dihydro-1H-pyrrrole-2,5-dione-1-yl)methyl]-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yl oxy (2) yield: 279 mg (92%); Aspect: light yellow powder; 1H NMR (400 MHz, CDCl_3): δ 1.32 (s, 6H), 1.40 (s, 6H), 4.14 (d, J = 1.4 Hz, 2H), 5.34 (s, 1H), 5.48 (s, 1H), 7.36-7.29 (m, 2H), 7.51-7.43 (m, 3H); HRMS NSI (m/z) calcd for C_{38}H_{39}O_{13}N_{3}: 722.2474 found 722.2473; mp = 92-94°C.

3-[(3-phenoxycarbonyl-2,5-dihydro-1H-pyrrrole-2,5-dione-1-yl)-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yl oxy] (4) yield: 345 mg (92%); Aspect: yellow solid; 1H NMR (400 MHz, CDCl_3): δ 1.13 (s, 3H), 1.24 (s, 3H), 1.27 (s, 3H), 1.36 (s, 3H), 1.80 (dd, J = 12.4, 8.7 Hz, 1H), 2.98 (dd, J = 11.2, 12.4 Hz, 1H), 4.46 (dd, J = 11.2, 8.7 Hz, 1H), 5.29 (s, 1H), 7.37-7.30 (m, 2H), 7.54-7.43 (m, 3H); HRMS ESI (m/z) calcd for C_{39}H_{39}O_{13}N_{3}: 532.1394 found 532.1371; mp = 120-122°C.