Photoswitch inhibitors of α-chymotrypsin—increased substitution and peptidic character in peptidomimetic boronate esters†

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A series of peptidomimetic boronate esters containing the photoisomerisable azobenzene group has been synthesised and assayed against the serine protease α-chymotrypsin. Compounds with borophenylalanine inhibition groups were found to be inhibitors with micromolar activity, while those with aryl boronate inhibition groups were inactive. Selected compounds were isomerised by UV or visible light to obtain enriched states of the (*Z*) or (*E*) isomers, respectively, and assayed. A change in activity on photoisomerisation was observed, however some decomposition of the boronate group on irradiation was also observed, limiting reversibility.

Introduction

The physical and chemical properties of materials can be controlled at the molecular level using molecular switches that operate under a variety of conditions and in a variety of situations. Numerous switching systems have been developed that control the movement or reactivity of molecules based on pH,[1] light[2] and electrochemical[3] switches, for example. Our research aims to control optically biological systems using photoswitchable enzyme inhibitors. We and others have reported examples of inhibitors of serine proteases that combine an azobenzene photoswitch with a group that binds to the enzyme of interest: for example, boronic acid **1** was found to be 3 times less active after UV irradiation (inhibition constant, K_i, of 41 μM *vs* 11 μM) to give a predominance of the *cis* isomer,[4] while the α-keto ester **2** was found to be 2 times more active (K_i of 130 *vs* 240 nM) after irradiation to give the *cis* isomer.[5] In this paper, we extend this concept with the preparation and testing of peptidomimetic-based photoswitchable inhibitors of proteases that are amenable to incorporation into a peptide sequence as a means to potentially increase potency and specificity for one protease over another. These systems are also amenable to attachment to other materials, such as metal surfaces, polymers or nanoparticles. Photoswitch molecules attached to such materials might form the basis for molecular computing or reversible biosensor technologies. We also hoped to gain some insight into those structural factors that influence the potency of *cis vs trans* azobenzenes given the opposing results obtained for **1** and **2**.

Initially, two groups of mono-substituted azobenzenes (Fig. 1) were synthesised: those containing a simple aryl boronate (**3**–**5**) based on literature photoswitch inhibitor **1**, and a novel series containing the boronate ester analogue of phenylalanine (**6**–**8**).[6] The second series was prepared in an attempt to better target α-chymotrypsin, where this enzyme is known to prefer aromatic residues (*e.g.*, the benzyl group of Phe) at the P_1 position of substrates and inhibitors[7] (see notation of Schechter and Berger[8]). These compounds were assayed against α-chymotrypsin and found to be active micromolar inhibitors. Based on these results the disubstituted azobenzenes **9** and **10** (Fig. 2) were designed, synthesised and tested as compounds suitable for incorporation into a peptide sequence and for surface attachment.

Results and discussion

Further to the design of the photoswitchable inhibitors we anticipated that potency, specificity and photoswitchability might be further controlled by incorporating an amino acid with a hydrophobic side chain (*e.g.* leucine) into the structure at P_2, this provides potential to bind to the S_2 protease subsite[9] (see **4**, **5**, **7**, and **8**). The design of **4** and **5** assumes that the aryl boronic acid group occupies the S_1 subsite. Compounds **3**–**5** were synthesised by standard peptide coupling methods (Schemes 1–3) and assayed against chymotrypsin using a spectrophotometric assay. Surprisingly, these compounds were found to be inactive against chymotrypsin up to their solubility limits (~0.5 mM), despite their structural similarity to reported inhibitor **1**. This suggests that the fit of **1** into the enzyme active site is significantly enhanced by the shape and/or hydrogen bonding attributable to the sulfonamide group. However, replacing the amide of **4** with a sulfonamide to give **5** did not replicate this effect (see Table 1). It is possible that the incorporation of a boronate ester into **3**–**5** might account for the reduction in potency relative to **1**. However, the observed >50 fold decrease in activity is not consistent with literature, where boronate esters and their corresponding free acids are reported to possess very similar activities.[6,10] Our results suggest that **1**, although an effective photoswitch, cannot be simply extended as a peptide analogue.

Better results were obtained using compounds **6**–**8**, which contain a boronate ester analogue of phenylalanine. Compound **6** was synthesised by coupling azobenzene **11** to the pinacol ester of borophenylalanine **13** (Scheme 1). Difficulties were initially encountered on attempting to perform amide coupling reactions with **13** using normal peptide coupling reagents such as 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI)

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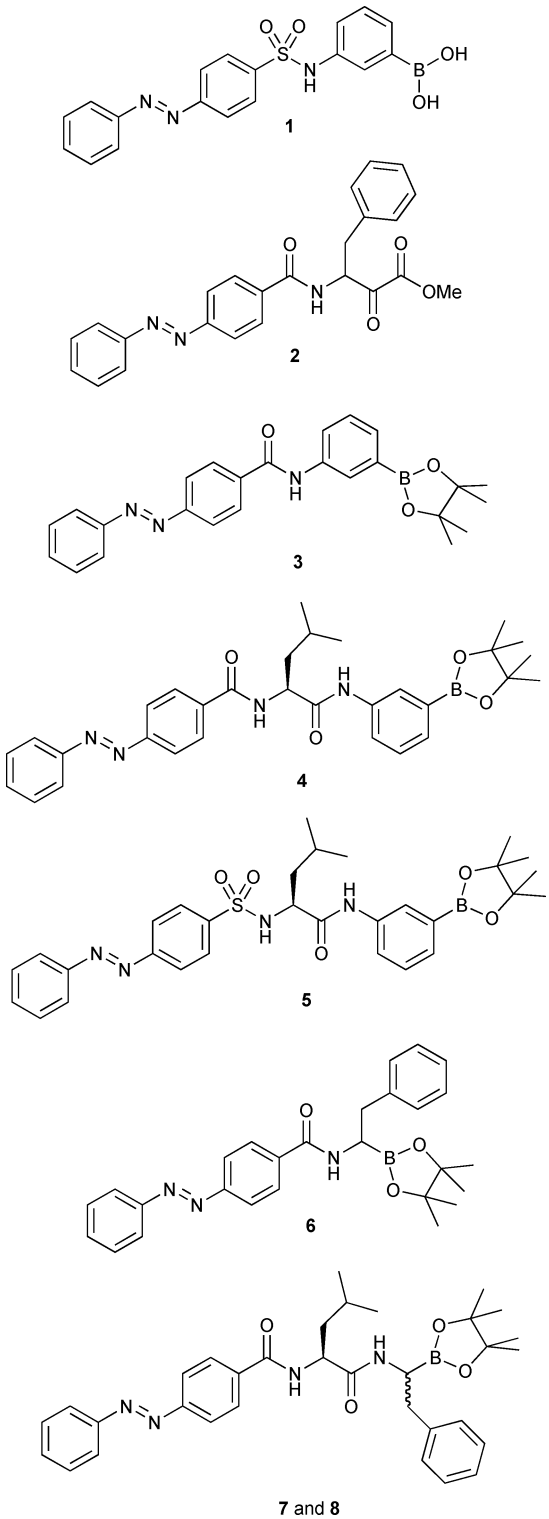


Fig. 1 Monosubstituted azobenzenes.

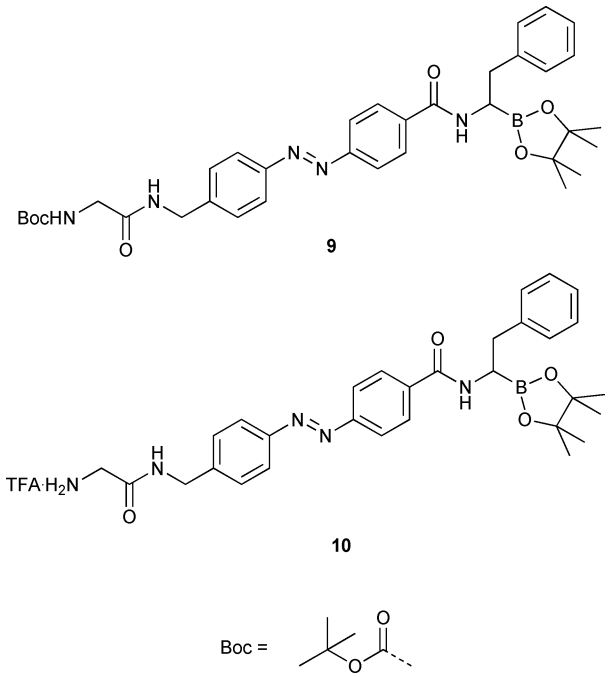
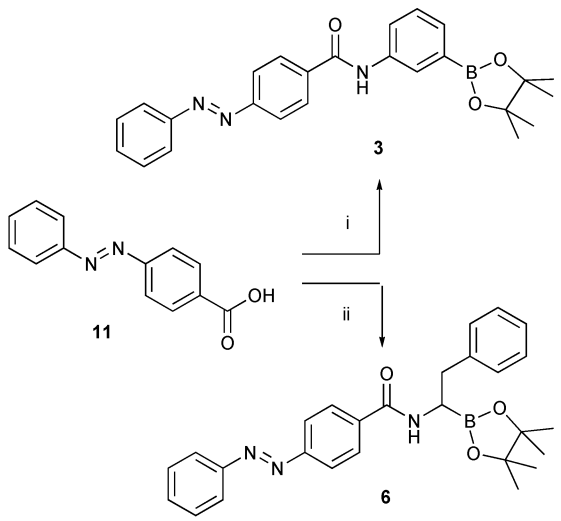
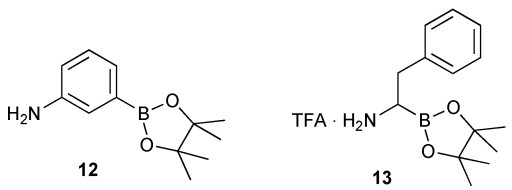


Fig. 2 Disubstituted azobenzenes.



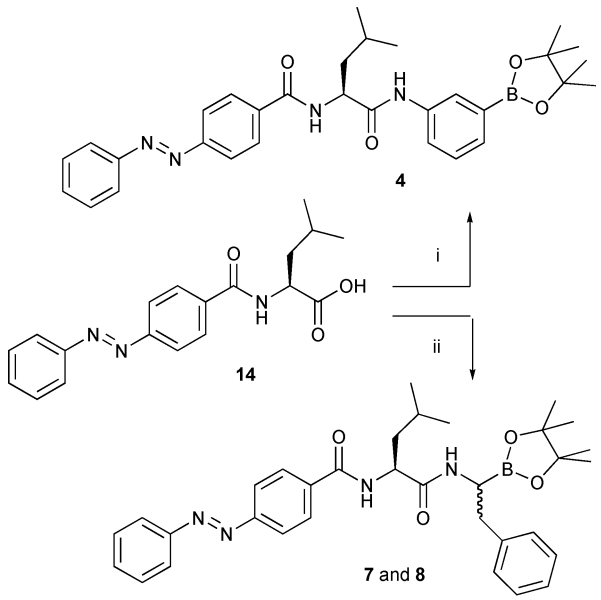
i) HATU, DIEA, **12**, DMF, 53%; ii) i-BuOCOCl, NMM, **13**, THF, 51%



Scheme 1

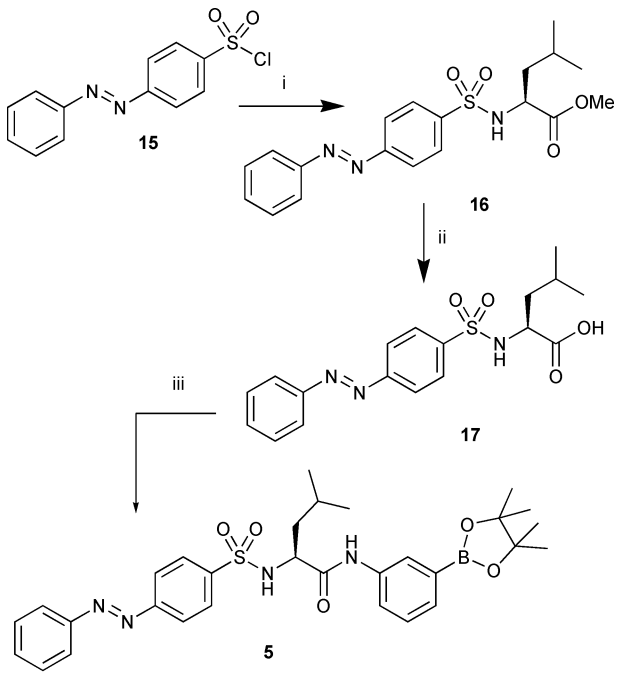
and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), although the mixed anhydride coupling method with isobutyl chloroformate gave acceptable yields. Diastereomers **7** and **8** were synthesised similarly from **14** and **12** in good yield (Scheme 2). Separation of the diastereomers was problematic since they decomposed on silica. However, careful chromatography on deactivated silica allowed separation of **7** and **8** in up to 86% de (see Table 1), although with poor recovery yields (<10%) due to decomposition.

The ^{1}H NMR of synthetic samples of **6**, **7**, and **8** revealed them to be present almost exclusively as the *E* isomers (>95%). Assay of these samples against chymotrypsin gave IC_{50} values of 3, 1.3 and 18 μM, respectively (see Table 1 entries vi, viii, and ix).



i) EDCI, HOBt, **12**, DCM, 43%; ii) i-BuOCOCl, NMM, **13**, THF, 76%

Scheme 2



i) Leu-OMe, DIEA, DCM, 91%; ii) LiOH, THF, H_2O, 63%;
iii) HATU, DIEA, **12**, DMF, 28%.

Scheme 3

Encouragingly, the potency of compounds **6** and **7** is similar to the activity of acetylborophenylalanine ityself ($K_i = 2.1$ μM), a well-studied inhibitor of chymotrypsin.[11] This result is perhaps better than might have been expected, and it implies that activity is not significantly impaired by the presence of the azobenzene group or the protection of the boronic acid as the pinacol ester. It is possible that the azobenzene group may even enhance activity by providing either a good fit into the shallow hydrophobic S_2 subsite, or an extended mimic of a β-strand, the preferred conformation of peptide substrates of proteases.[12] This could then offset any possible reduction in activity due to the boronic acid protection. The configuration of compounds **7** and **8** could not be assigned from NMR data. However, the more active isomer **7** is assigned the (*R*) configuration, as this corresponds to (i) the configuration of natural phenylalanine and (ii) that commonly found in the more active isomers of related inhibitors.[13] Interestingly, the most active diastereomer **7** was found to have approximately twice the potency of racemic **6**. This suggests that incorporation of leucine into the inhibitor structure does not significantly enhance binding through interaction of leucine with the S_2 subsite. It is, however, possible that any increase in binding conferred by leucine is off-set by the affinity of the hydrophobic azobenzene group for the S_2 subsite (as in **6**) rather than the S_3 subsite (as in **7**).

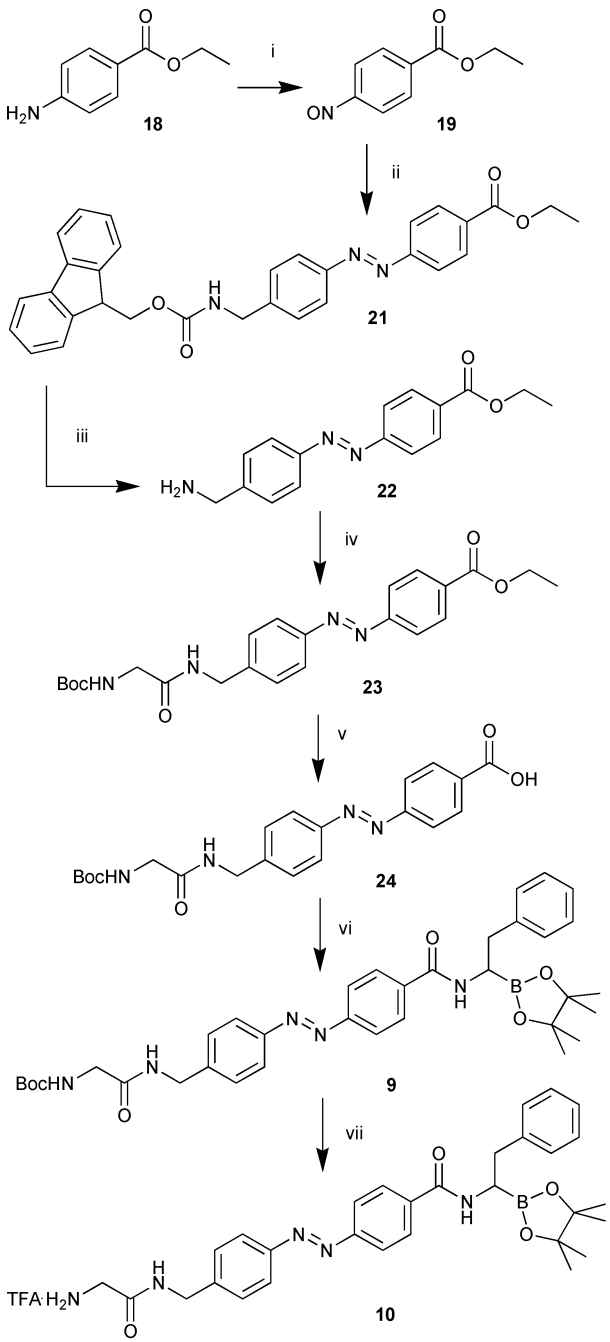
Table 1 Activities of inhibitors[a]

Entry	Compound	Inhibition constant/μM[b]
i	**1**	2.1[c]
ii	**2** (26%, *Z* isomer)	0.24[c]
iii	**3**	>500
iv	**4**	>500
v	**5**	>400
vi	**6**	3.0
vii	**6** (*Z* isomer)	4.7
viii	**7** and **8**, 92 : 8 ratio	1.3
ix	**7** and **8**, 7 : 93 ratio	18
x	**9**	10
xi	**10**	10

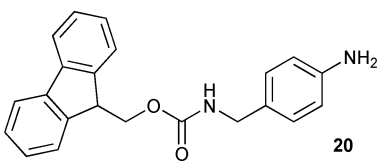
[a] >95% (*E*)-isomer, unless specified; [b] IC_{50}, unless specified; [c] K_i

A sample of (*Z*) **6**, obtained by chromatography from a mixture of (*E*) and (*Z*) isomers formed from pure (*E*) on exposure to ambient light conditions, was also assayed to assess the influence of the configuration of the azobenzene on activity. This isomer was less active (Table 1 entry vii), a result consistent with earlier reports on the photoswitching of the related boronic acid **1**, where the (*E*) isomer was the more active.

The disubstituted azobenzenes **9** and **10** were synthesised based on the successful inhibitor **6** (Scheme 4). These structures consist of the borophenylalanine enzyme binding group, the azobenzene photoswitch and an amino acid (glycine) at the N-terminus. The added amino acid is intended to enhance the peptidic character of the inhibitors in order to better mimic a natural substrate, as well as adding extra bulk to the photoswitch with the aim of improving photoswitching. The corresponding free amine is also potentially useful for attachment of further substituents and for surface attachment work.[14] In addition, the Boc protecting group of **9** allows assessment of the effect of substitution on binding at a site well removed from the main binding and photoswitching region of the inhibitor. Inhibitor **9** was synthesised in six steps from commercially available **18** and previously reported **20**.[15] Amine **18** was oxidised by catalyst $Mo(O_2)_2O{\cdot}H_2O{\cdot}HMPA$[16] to the nitroso derivative **19**, which was then heated with **20** in HOAc to form azobenzene **21**. The Fmoc protecting group was found to cause solubility and purification difficulties if carried into further steps. Therefore, it was removed at this stage to give **22**, and the more



i) $Mo(O_2)_2O(H_2O)$HMPA, DCM, H_2O_2 ii) HOAc, **20**, 22% over 2 steps; iii) DMF, piperidine iv) Boc-glycine, DMF, HATU, DIEA, 88% over 2 steps; v) LiOH, THF, H_2O, qu; vi) **13**, iBuOCOCl, NMM, DMF, 60%; vii) DCM, TFA, 49%



Scheme 4

convenient Boc group was introduced by coupling to Boc-glycine, giving **23**. Deprotection of the ester was unsuccessful under normal conditions (LiOH, THF–H_2O, 0 °C, 2 h), presumably due to the stabilising effect of the aromatic group, however, heating and longer reaction times afforded acid **24** in good yield. Boronate ester **9** was synthesised by mixed anhydride coupling of **24** with **13**, and **10** was formed by Boc deprotection. Assay of **9** and **10** gave identical inhibition constants (10 μM, Table 1 entries x and xi), several times weaker than the monosubstituted parent **6**, but significantly more potent than **3**–**5**. This suggests that the extra substitution of the azobenzene group only slightly impairs the 'fit' of the inhibitor into the active site. The extra bulk of the Boc group in **9** is tolerated, suggesting that the specificity of the enzyme binding site does not extend further than the glycine in these inhibitors.

The inhibitors **6** and **9** were photoisomerised and the two resultant photostationary states assayed against chymotrypsin in order to assess their efficacy as photoswitches. Good photoisomerisation of both compounds was obtained on UV irradiation with a 500 W mercury arc lamp through a UV filter with a narrow wavelength band centred at 340 nm, giving rise to up to 74% (*Z*) isomer for **6**, and 68% (*Z*) for **9**. Assays were performed during a series of irradiations in order to assess the impact of photoswitching and potential decomposition. A sample of inhibitor was dissolved in acetonitrile, irradiated with UV light, visible light (>360 nm), then UV light, and aliquots were removed and assayed initially and after each irradiation. For inhibitor **6** the decomposition outweighed any photoswitching effect so that activity decreased after each irradiation cycle. Irradiation of a sample of **6** with UV for several hours gave a mixture that was partially purified by flash chromatography. NMR analysis of this revealed that the pinacol group had been lost. As this decomposition product showed no (or very weak) activity it is assumed that the entire boronate group was lost, or significantly altered by irradiation. Better results were obtained for **9**, and photoswitching was observed with limited decomposition at each cycle. As shown in Fig. 3 the activity increased from 10 μM (for sample containing <5% (*Z*) isomer) to 7 μM after UV isomerisation to give 60% (*Z*) isomer. The activity then decreased to 13 μM on isomerisation back to 85% (*E*) isomer, and finally increased to 10 μM following a second UV irradiation.

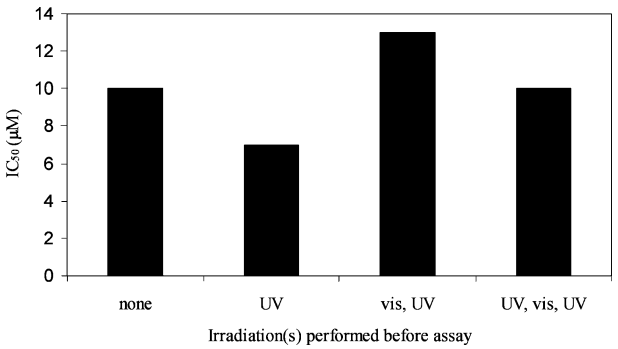


Fig. 3 Semi-reversible photoswitching of inhibitor **9**.

Conclusions

A new series of boronate ester inhibitors of chymotrypsin has been developed and tested. Compounds with a disubstituted

azobenzene core that exhibit photoswitching of enzyme activity are presented (see compounds **9** and **10**). While the magnitude of photoswitching is not improved by increasing the substitution and peptidic character of inhibitors, it is possible to retain photoswitching in such extended chain inhibitors. This opens up possibilities for such compounds to be used in useful molecular switching applications. Some decomposition of the boronate group was observed on irradiation, however the peptidomimetic design of these compounds is modular and so easily modified, to allow simple alteration of the warhead group in order to improve stability. The amino acids used can also be easily altered, which should allow specific photoswitch inhibitors for a variety of proteases to be developed using these basic methods. The last point to note is that the exact fit of the inhibitors into the active site appears to be particularly sensitive to molecular shape or conformation. This is evidenced by the fact that in some cases (**1** and **6**) the (*E*) azobenzene is the more active isomer, while for others (**2** and **9**) the (*Z*) isomer is more active. A direct correlation does not seem to exist between the nature of the warhead or linker, and the relative activities of the (*Z*) and (*E*) isomers. Rather, it is dependent upon the gross structure of the inhibitor and hence the nature of its overall 'fit' in the active site.

Experimental

General

NMR spectra were obtained on a Varian Inova spectrometer, operating at 500 MHz for ^{1}H NMR and at 126 MHz for ^{13}C NMR, or on a Varian Unity 300 spectrometer, operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Two-dimensional NMR experiments including COSY and HSQC were used to assign spectra, and were obtained on the Varian Inova spectrometer operating at 500 MHz. Electrospray ionisation mass spectra were detected on a Micromass LCT TOF mass spectrometer operating in electrospray mode with 50% acetonitrile–H_2O as solvent. Dry DMF was purchased from Acros. Dry THF was distilled from sodium or potassium, dry DCM was distilled from CaH. EtOAc, NMM, HOAc and EtOH were distilled before use. HPLC grade acetonitrile was purchased from BDH. All other commercial reagents were used as received.

Photoisomerisation

Compounds were photoisomerised by irradiation with UV or visible light from a 500 W mercury arc lamp. The light beam was filtered through water to reduce heat, and through either an Edmund optics 340 nm interference filter for UV, or a Corning 0–51 visible filter for visible light. Samples were dissolved in d3-acetonitrile, and irradiated for 70 min with UV light, or for 30 min with visible light. The ratio of (*E*) : (*Z*) isomers was determined before and after irradiations by ^{1}H NMR analysis using the integrals of well separated peaks. The major isomer was assigned the thermodynamically more stable (*E*) configuration based on literature precedence.[4,5] The minor (*Z*) isomer characteristically gave rise to upfield signals for the aryl protons which were enhanced on irradiation. 2D NMR was used to confirm the presence of minor isomers in some representative cases. All experiments involving photoisomerisation were carried out in dim lighting conditions, *i.e.* glassware wrapped in foil and with the lights turned off.

Enzyme assays

Buffer solution (Tris): Tris(hydroxymethyl)aminomethane (1.21 g), $CaCl_2 \cdot 6H_2O$ (0.44 g) and Triton X-100 (0.05 g) were dissolved in Milli-Q deionised water (75 mL), adjusted to pH 7.8 with 1 M NaOH solution and made up to 100 mL with Milli-Q water.

Substrate solution: *N*-succinyl–$(Ala)_2$–Pro–Phe–4-nitroanilide (21 mg) was dissolved in Tris buffer solution (10 mL) by ultrasonication. The solution was stored at −18 °C for up to two weeks. The concentration of the solution was determined at the start of each day from its UV spectrum ($\varepsilon_{315} = 14\,000$ L mol^{-1} cm^{-1})

Enzyme solution: a stock solution was prepared from α-chymotrypsin (15 mg) in HCl solution (10 mL, pH 3, made up by dilution of conc. HCl with Milli-Q water). The stock solution was stored at −18 °C for up to 1 month. Each day an enzyme solution was prepared: stock solution (200 μL) and Triton X-100 (25 mg) were made up to 50 mL with Milli-Q water.

Inhibition of α-chymotrypsin was determined with Suc–Ala–Ala–Pro–Phe–4-nitroanilide as the substrate by an assay procedure developed from the technique described by Geiger,[17] except that the order of addition of enzyme and substrate was inverted, and only one substrate concentration was used, in order to obtain inhibition constants as IC_{50} rather than K_i. Briefly, inhibitors were dissolved in acetonitrile at a series of dilutions ranging from 0.5–500 μM as appropriate for each compound. For each rate measurement, inhibitor solution (or acetonitrile blank, 50 μL), substrate solution (60 μL) and buffer solution (910 μL) were mixed in a cuvette, and incubated for 5 min at 25 °C. Enzyme solution (30 μL) was added, and the absorbance at 405 nm was monitored for 5 min. An absorbance *vs* time plot was obtained, and the slope used to find the initial rate. From the difference between the initial rate and the initial rate for the acetonitrile blank, the percent inhibition was calculated. This experiment was repeated over as many inhibitor concentrations as required to obtain a good straight line plot of percent inhibition *vs* log [inhibitor concentration], from which the IC_{50} was interpolated. In the case of the assay of photoisomerised azobenzene (*Z*)-isomers, assays were performed as above except with d3-acetonitrile instead of acetonitrile.

Synthesis

4-(Phenylazo)-*N*-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzamide (3). To a mixture of 4-(phenylazo)benzoic acid **11** (200 mg, 0.88 mmol), 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzenamine **12** (194 mg, 1 eq.) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 370 mg, 1.1 eq.) was added DMF (5 mL) then DIEA (340 μL, 2.2 eq.). The solution was stirred for 16 h then diluted with ethyl acetate (50 mL), washed with water (50 mL × 2), dried over $mgSO_4$ and concentrated. The crude product was purified by flash chromatography, eluting with DCM followed by 1 : 9 EtOAc–DCM then 1 : 5 EtOAc–DCM, giving **3** (200 mg, 53%, >95% *E* by ^{1}H NMR) as an orange solid: mp 162–164 °C. Found: C, 70.19; H, 6.04; N, 9.93, calcd for $C_{25}H_{26}BN_3O_3$: C 70.27, H 6.13, N 9.83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (12H, s,

(CMe_2)$_2$), 7.37 (1H, t, $J = 7.8$), 7.50 (3H, m), 7.60 (1H, d, $J = 7.2$), 7.87–8.04 (8H, m), 8.23 (1H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.9, 84.0, 123.09, 123.10, 123.3, 126.2, 128.0, 128.7, 129.2, 131.0, 131.7, 136.5, 137.3, 152.5, 154.3, 164; *m/z* (ES) 428.2162, calcd for $C_{25}H_{27}{}^{11}BN_3O_3$ (MH^+) 428.2145.

(*S*)-*N*-(4-Methyl-1-oxo-1-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan - 2 - yl)phenylamino)pentan-2-yl)-4-(phenyldiazenyl)benzamide (4). A solution of (*S*,*E*)-4-methyl-2-(4-(phenylazo)benzamido)pentanoic acid **14**[18] (165 mg, 0.49 mmol), **12** (108 mg, 1 eq.), EDCI (130 mg, 1 eq.) and HOBt (92 mg, 1 eq.) in DCM (5 mL) was stirred for 16 h then washed with water (50 mL), brine (50 mL), and filtered to give **4** (113 mg, 43%, >95% *E* by ^{1}H NMR) as an orange solid: mp 225–227 °C, ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 1.05 (6H, t, $J = 6.8$, CHMe_2), 1.38 (12H, s, (CMe_2)$_2$) 1.72 (1H, m), 1.80–2.00 (2H, m), 4.77 (1H, m, COC*H*), 7.42 (2H, m), 7.71 (3H, m), 7.92 (1H, d, $J = 6.6$), 8.05 (5H, m), 8.25 (2H, d, $J = 8.4$ Hz), 8.92 (1H, d, $J = 7.2$), 10.30 (1H, s); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 21.6 (CHMe_2), 23.2 (CHMe_2), 24.8(2C, (CMe_2)$_2$ and *C*HMe_2), 41 (obscured by solvent peak, *C*H$_2$), 53.2 (CO*C*H), 83.8 (*C*Me$_2$)$_2$), 122.4, 122.9, 125.6, 128.5, 129.1, 129.4, 129.7, 132,2, 136.3, 138.8, 152.0, 153.5, 165.9, 171.5; *m/z* (ES) 541.2958, calcd for $C_{31}H_{38}{}^{11}BN_4O_4$ (MH^+) 541.2986.

(*S*)-4-Methyl-2-(4-(phenylazo)phenylsulfonamido)-*N*-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)pentanamide (5). A mixture of **12** (50 mg, 0.23 mmol), **17** (100 mg, 1 eq.) and HATU (95 mg, 1.1 eq.) was dissolved in DMF (5 mL) and DIEA (87 μL, 2.2 eq.) was added. The reaction mixture was stirred overnight then diluted with EtOAc (50 mL), washed with water (50 mL), brine (50 mL), dried over mgSO_4 and concentrated. The crude material was purified by flash chromatography, eluting with 1 : 19 EtOAc–DCM to give **5** (43 mg, 28%, >95% *E* by ^{1}H NMR) as an orange solid: mp 75–80 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.75 (3H, d, $J = 5$, CHMe_2), 0.87 (3H, d, $J = 5.5$, CHMe_2), 1.31 (12H, m, (CMe_2)$_2$), 1.54 (1H, t, $J = 9$), 1.65 (2H, m), 3.88 (1H, m, COC*H*), 5.45 (1H, m, N*H*), 7.24 (1H, t, $J = 7.8$), 7.49–7.54 (4H, m), 7.60 (1H, s), 7.64 (1H, d, $J = 7$), 7.86 (1H, s), 7.90–7.96 (4H, m), 8.03 (2H, d, $J = 7.5$) ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4 (CHMe_2), 22.9 (CHMe_2), 24.4 (*C*HMe$_2$), 24.8 ((CMe_2)$_2$), 42.2 (*C*H$_2$), 56.4 (CO*C*H), 83.9 ((CMe_2)$_2$), 123.1, 123.3, 123.4, 125.9, 128.4, 128.5, 129.2, 131.1, 132.1, 136.4, 140.5, 152.3, 154.8, 169.2; *m/z* (ES) 577.2665, calcd for $C_{30}H_{38}{}^{11}BN_4O_5S$ (MH^+) 577.2656.

***N*-(2-Phenyl-1-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-ethyl)-4-(phenylazo)benzamide (6).** To a solution of **11** (190 mg, 0.84 mmol) in THF (5 mL) was added isobutylchloroformate (108 μL, 1 eq.) then NMM (183 μL, 2 eq.). The reaction mixture was stirred for 10 min, then a solution of 4,4,5,5-tetramethyl-α-(phenylmethyl)-1,3,2-dioxaborolane-2-methanamine, trifluoroacetic acid salt **13**[6] (300 mg, 1 eq.) in THF (5 mL) was added. The resulting mixture was stirred for 16 h, then diluted with EtOAc (50 mL), washed with water (50 mL) then brine (50 mL) and concentrated. The crude material was purified by flash chromatography, eluting with 3 : 7 EtOAc–DCM to give **6** (192 mg, 51%, >95% *E* by ^{1}H NMR) as an orange solid: mp 205–206 °C. Found: C, 71.13; H, 6.55; N, 9.31, calcd for $C_{27}H_{30}BN_3O_3$: C 71.22, H 6.64, N 9.23%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (6H, s, (CMe_2)$_2$), 1.32 (6H, s, (CMe_2)$_2$), 2.83 (1H, dd, $J = 11.8$ and 14.3, CHCH_2), 3.08 (1H, dd, $J = 4.3$ and 14.3, CHCH_2), 3.15 (1H, m, C*H*CH$_2$), 6.96 (1H, s, N*H*), 7.27 (3H), 7.36 (2H, t, $J = 7.8$), 7.53 (3H), 7.87 (2H, d, $J = 8.5$), 7.94 (4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 25.0, 25.3, 37.3 (CHCH_2), 46.3 (*C*HCH$_2$), 81.0, 122.9, 123.2, 126.3, 128.7, 128.9, 129.0, 129.2, 131.9, 140.8, 152.4, 155.2, 170.4; *m/z* (ES) 456.2451, calcd for $C_{27}H_{31}{}^{11}BN_3O_3$ (MH^+) 456.2458.

(*Z*)-*N*-(2-Phenyl-1-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ethyl)-4-(phenylazo)benzamide ((*Z*)-6). A solution of (*E*)-**6** (10 mg, 0.02 mmol) in 3 : 7 EtOAc–DCM (2 mL) was exposed to ambient indoor lighting and daylight for 2 d, giving a mixture of (*E*) and (*Z*) **6**. Separation by flash chromatography, eluting with 3 : 7 EtOAc–DCM, gave (*Z*)-**6** (1 mg, 7%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27 (6H, s, (CMe_2)$_2$), 1.28 (6H, s, (CMe_2)$_2$), 2.76 (1H, dd, $J = 12,14$), 3.03 (1H, dd, $J = 14$, 4.3), 3.09 (1H, m), 6.80 (3H, m), 6.85 (2H, d, $J = 8.5$), 7.18 (1H, t, $J = 9.3$), 7.23 (5H, m), 7.33 (2H, t, $J = 7.5$), 7.62 (2H, d, $J = 8.5$).

(*S*)-*N*-(4-Methyl-1-oxo-1-(2-phenyl-1-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ethylamino)pentan-2-yl)-4-(phenylazo)benzamide (7 and 8). A solution of **14**[18] (100 mg, 0.29 mmol) in THF (4 mL) was cooled to 0 °C, then isobutylchloroformate (40 μL, 1 eq.) and NMM (65 μL, 2 eq.) were added. The reaction mixture was stirred for 10 min then a solution of **13**[6] (106 mg, 1 eq.) in THF (4 mL) was added. The mixture was stirred for 16 h, diluted with EtOAc (50 mL), washed with water (50 mL) then brine (50 mL), dried over $MgSO_4$ and concentrated. The crude material was purified by silica column chromatography on deactivated silica (containing 35% water w/w), eluting with 3 : 7 EtOAc–DCM to give a mixture of **7** and **8** (125 mg, 76%) as an orange solid. The mixture of diastereomers was further purified three times by column chromatography to give **7** (8 mg, 5%, 86% de, >95% *E* by ^{1}H NMR)) as an orange solid: mp 55–60 °C, ^{1}H NMR (500 MHz, CD_3OD) δ 0.98 (3H, d, $J = 6.5$, CHMe_2), 1.00 (3H, d, $J = 5$, CHMe_2), 1.14 (12H, m, (CMe_2)$_2$), 1.68–1.87 (3H, m), 2.60 (1H, dd, $J = 9.3$, 12.8), 2.80–2.88 (2H, m), 4.89 (1H, m, COC*H*), 7.12–7.23 (5H, m), 7.55 (3H, m), 7.94–8.03 (6H, m), *m/z* (ES) 569.3307, calcd for $C_{33}H_{42}{}^{11}BN_4O_4$ (MH^+) 569.3299; and **8** (5 mg, 3%, 86% de, >95% *E* by ^{1}H NMR)) as an orange solid: mp 60–64 °C, ^{1}H NMR (500 MHz, CD_3OD) δ 0.96 (3H, d, $J = 6$, CHMe_2), 0.99 (3H, d, $J = 6.5$, CHMe_2), 1.10 (6H, s, (CMe_2)$_2$), 1.15 (6H, s, (CMe_2)$_2$), 1.68 (1H, m), 1.78 (1H, m), 1.87 (1H, m), 2.62 (1H, dd, $J = 8.5$, 12), 2.84 (2H, m), 4.92 (1H, dd, $J = 5$, 10.5), 7.17 (1H, m), 7.26 (4H, m), 7.55 (4H, m), 7.94–8.04 (6H, m); *m/z* (ES) 569.3279, calcd for $C_{33}H_{42}{}^{11}BN_4O_4$ (MH^+) 569.3299.

***tert*-Butyl-2-oxo-2-(4-((4-(2-phenyl-1-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ethylcarbamoyl)phenyl)diazenyl)benzylamino)-ethylcarbamate (9).** A solution of **24** (30 mg, 0.07 mmol) in DMF (3 mL) was cooled in an ice bath and isobutylchloroformate (10 μL, 1 eq.) and NMM (16 μL, 2 eq.) were added. The reaction mixture was stirred for 10 min then a solution of **13** (26 mg, 1 eq.) in DMF (2 mL) was added. The resulting mixture was stirred for 16 h, warming from 0 °C to r.t, then diluted with EtOAc (50 mL), washed with water (50 mL × 2), brine (50 mL), dried over $MgSO_4$, filtered and concentrated. The crude material was purified by silica column chromatography on deactivated silica (containing 35% water w/w), eluting with EtOAc to give **9** (28 mg, 60%, >95% *E* by ^{1}H NMR) as an orange solid: mp 188–191 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (12H, m, (CMe_2)$_2$), 1.45

(9H, s, CMe_3), 2.83 (1H, dd, J = 12,14.1, CHCH_2), 3.08 (1H, dd, J = 14.1, 3.80, CHCH_2), 3.16 (1H, m, CHCH$_2$), 3.86 (2H, d, J = 6), 4.55 (2H, d, J = 5.5), 5.18 (1H, s, NH), 6.65 (1H, s, NH), 7.13 (1H, s, NH), 7.28 (3H, m), 7.37 (2H, t, J = 7.8), 7.41 (2H, d, J = 8), 7.88 (6H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.0 (CMe_2)$_2$), 25.1 (CMe_2)$_2$), 28.2 (CMe_3), 37.4 (CHCH$_2$), 42.7, 44.2, 46.8 (CHCH$_2$), 80.1 (CMe$_2$), 80.6 (CMe$_2$)$_2$), 122.7, 123.3, 126.0, 127.8, 128.4, 129.0, 129.2, 140.7, 142.1, 151.4, 154.8, 156.2, 169.9, 170.5, 207.0; m/z (ES) 642.3488, calcd for $C_{35}H_{45}{}^{11}BN_5O_6$ (MH$^+$) 642.3463.

4-((4-((2-Aminoacetamido)methyl)phenyl)diazenyl)-*N*-(2-phenyl-1-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-ethyl)benzamide (10). To a solution of **9** (17 mg, 0.03 mmol) in DCM (4 mL) was added TFA (1 mL). The reaction mixture was stirred for 30 min then concentrated and the crude material purified by flash chromatography, eluting with 1 : 3 EtOH–DCM to give **10** (14 mg, 82%, >95% E by ^{1}H NMR) as an orange solid: mp 157–160 °C, ^{1}H NMR (500 MHz, CD_3OD) δ 1.15 (12H, m, (CMe_2)$_2$), 2.73 (1H, dd, J = 9.1,13.8), 2.96 (1H, dd, J = 6.4, 13.8), 2.99 (1H, dd, J = 6.4, 9.1), 3.72 (2H, s), 4.49 (2H, s), 7.16 (1H, m), 7.27 (4H, m), 7.47 (2H, d, J = 8.1), 7.89 (2H, d, J = 8.1), 7.96 (2H, d, J = 8.1), 8.09 (2H, d, J = 8.1), m/z (ES) 542.2965, calcd for $C_{30}H_{37}{}^{11}BN_5O_4$ (MH$^+$) 542.2938.

(*S*)-Methyl-4-methyl-2-(4-(phenylazo)phenylsulfonamido)pentanoate (16). A mixture of 4-(phenylazo)benzenesulfonyl chloride **15**[19] (773 mg, 2.75 mmol) and (*S*)-leucine methyl ester (500 mg, 1 eq.) was dissolved in DCM (15 mL) then DIEA (1.44 mL, 3 eq.) was added. The reaction mixture was refluxed for 2 h, then cooled, diluted with EtOAc (100 mL), washed with water (100 mL) then brine (100 mL), dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography, eluting with DCM then 1 : 9 EtOAc–DCM to obtain **16** (979 mg, 91%, >95% E by ^{1}H NMR) as an orange solid: mp 131–134 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.91 (6H, m, CHMe_2), 1.53 (2H, t, J = 7.1, CH_2), 1.81 (1H, m, CHMe$_2$), 3.45 (3H, s, OMe), 4.03 (1H, m, COCH), 5.39 (1H, d, J = 9.6, NH), 7.54 (3H, m), 7.93–8.01 (6H, m); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.3, 22.7, 24.3, 42.2, 52.3, 54.4, 123.1, 123.2, 128.3, 129.2, 132.1, 141.1, 152.3, 154.6, 172.5; m/z (ES) 390.1485, calcd for $C_{19}H_{24}N_3O_4S$ (MH$^+$) 390.1488.

(*S*)-4-Methyl-2-(4-(phenylazo)phenylsulfonamido)pentanoic acid (17). To a solution of **16** (500 mg, 1.28 mmol) in THF (20 mL) was added a solution of LiOH (8 mL; 0.25 M in 2 : 1 THF–water). The reaction mixture was stirred for 16 h then diluted with water (100 mL), washed with DCM (100 mL), then acidified to pH 3 with 1 M HCl and extracted with DCM (100 mL). The organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by silica column chromatography, eluting with 1 : 1 EtOAc–DCM to give **17** (302 mg, 63%, >95% E by ^{1}H NMR) as an orange solid: mp 128–130 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (6H, m, CHMe_2), 1.48 (2H, m, CH_2), 1.74 (1H, m, CHMe$_2$), 3.97 (1H, m, COCH), 5.18 (1H, d, J = 9.9, NH), 5.35–5.90 (1H, br s, OH), 7.54 (3H, m), 7.96 (6H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.1, 22.7, 24.3, 41.9, 54.1, 123.2, 123.3, 128.3, 129.3, 132.2, 141.0, 152.3, 154.8, 176.6; m/z (ES) 376.1345, calcd for $C_{18}H_{22}N_3O_4S$ (MH$^+$) 376.1331. Unreacted **16** (114 mg, 23%) was also recovered from the column.

Ethyl 4-((4-((((9*H*-fluoren-9-yl)methoxy)carbonylamino)methyl)-phenyl)diazenyl)benzoate (21). A mixture of ethyl 4-aminobenzoate **18** (2 g, 12 mmol) and $MoO_5{\cdot}H_2O{\cdot}$HMPA (0.45 g, 0.1 eq.) was dissolved in DCM (20 mL) and a solution of H_2O_2 (6 mL; 30% in water) was added. The reaction mixture was stirred for 16 h then quenched by addition of excess $MgSO_4$, filtered and concentrated. The crude material was purified by flash chromatography, eluting with 1 : 9 EtOAc–DCM to obtain ethyl 4-nitrosobenzoate **19** as a yellow solid. To this compound was added **20**[15] (1.2 g, 3.49 mmol) then HOAc (25 mL). The resulting mixture was heated to 100 °C for 1 h, then cooled and filtered to collect an orange precipitate. The filtrate was diluted with DCM (100 mL), washed with sat. $NaHCO_3$ (100 mL × 3) then dried over $MgSO_4$ and concentrated. This crude material was combined with the precipitate and purified by silica column chromatography, eluting with 1 : 19 EtOAc–DCM to give **21** (1.345 g, 22% over 2 steps, >95% E by ^{1}H NMR) as an orange solid: mp 191–194 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (3H, t, J = 7, Me), 4.24 (1H, t, J = 6.3, CH$_2$CH), 4.40–4.53 (6H, m), 5.17 (1H, NH), 7.26–7.42 (6H, m), 7.61 (2H, d, J = 6.9), 7.77 (2H, d, J = 7.2), 7.93 (4H, m), 8.20 (2H, d, J = 8.7); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3 (Me), 44.7, 47.3 (CH$_2$$C$H), 61.3, 66.7, 120.0, 122.6, 123.5, 125.0, 127.1, 128.1, 130.6, 132.2, 141.4, 142.3, 143.8, 151.9, 155.0, 156.5, 166.1; m/z (ES) 506.2120, calcd for $C_{31}H_{28}N_3O_4$ (MH$^+$) 506.2080.

Ethyl 4-((4-((2-(*tert*-butoxycarbonylamino)acetamido)methyl)-phenyl)diazenyl)benzoate (23). To a solution of **21** (900 mg, 1.78 mmol) in DMF (12 mL) was added piperidine (3 mL). The reaction mixture was stirred for 15 min then diluted with EtOAc (100 mL), washed with water (100 mL) then brine (100 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give **22** as an orange solid which was not purified further. To this compound was added Boc-glycine (312 mg, 1 eq.), HATU (677 mg, 1 eq.) then DMF (10 mL) and DIEA (620 μL, 2 eq.). The reaction mixture was stirred for 16 h, diluted with EtOAc (100 mL) and washed with water (100 mL) brine (100 mL), dried over $MgSO_4$, filtered and concentrated. The crude material was purified by silica column chromatography, eluting with 1 : 1 EtOAc–DCM to give **23** (691 mg, 88% over 2 steps, >95% E by ^{1}H NMR) as an orange solid: mp 136–139 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41–1.47 (12H, CMe_3 and CH$_2$$Me$), 3.87 (2H, d, J = 5.5), 4.42 (2H, q, J = 5.4), 4.57 (2H, d, J = 6.0), 5.13 (1H, br, NH), 6.56 (1H, br, NH), 7.44 (2H, d, J = 8.0), 7.92 (2H, d, J = 8.0), 7.94 (2H, d, J = 8.5), 8.19 (2H, d, J = 8.5); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 28.2, 42.9, 44.5, 61.2, 80.4, 122.6, 123.4, 128.2, 130.5, 132.1, 141.8, 151.8, 154.9, 156.2, 166.0, 169.6; m/z (ES) 441.2108, calcd for $C_{23}H_{29}N_4O_5$ (MH$^+$) 441.2138.

4-((4-((2-(*tert*-Butoxycarbonylamino)acetamido)methyl)phenyl)-diazenyl)benzoic acid (24). To a solution of **23** (68 mg, 0.15 mmol) in THF (5 mL) was added a solution of LiOH (1 mL; 0.25 M in 2 : 1 THF–water). The reaction mixture was heated to 50 °C and stirred for 16 h then concentrated. The crude material was purified by flash chromatography, eluting with 1 : 99 HOAc–EtOAc to give **24** (55 mg, 87%, >95% E by ^{1}H NMR) as an orange solid: mp > 360 °C, ^{1}H NMR (500 MHz, CD_3OD) δ 1.45 (9H, s, CMe_3), 3.76 (2H, s), 4.49 (2H, s), 7.47 (2H, d, J = 8), 7.87 (4H, m), 8.08 (2H, d, J = 8); ^{13}C NMR (75 MHz, CD_3OD) δ 29.0 (CMe_3), 43.9, 45.1, 81.1 (CMe$_3$), 123.4, 124.3, 129.5, 131.5,

141.8, 144.0, 153.5, 155.3, 158.8, 173.1, 174.7; *m/z* (ES) 413.1805, calcd for $C_{21}H_{25}N_4O_5$ (MH^+) 413.1825.

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