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Azobenzene-Containing, Peptidyl α-Ketoesters as Photobiological Switches of α-Chymotrypsin

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Abstract—Three photoswitchable, peptidomimetic inhibitors of α-chymotrypsin have been synthesised. The compounds comprise an azobenzene, an α-ketoester and L-phenylalanine. The compounds were photoisomerised to give enriched states of the (*E*) and (*Z*) isomers and these states were assayed against α-chymotrypsin. The inhibitors were shown to be moderately active with switching ability of between two- and three-fold between the two isomer-enriched states. The behaviour of the inhibitors in solution was examined; specifically, their hydration and configurational stability.

Introduction

A photobiological switch is a biologically active compound with a built-in photochromic group that allows light-triggered switching of its biological target.[1] A variety of enzymes have been targeted with photobiological switches either by making the environment photoresponsive, by covalently modifying the enzyme with a photochromic group, or by employing a photoisomerisable effector molecule. For example, the activity of α-chymotrypsin has been photoregulated by its immobilisation on an azobenzene-containing polymer.[2] In another example, the activity of papain was modified by the covalent attachment of 4-(phenylazo)benzoic acid to the enzyme.[2b] An inhibitor comprising an azobenzene tethered to a simple aryl boronic acid has been shown to afford reversible inhibition of α-chymotrypsin.[3]

The photoisomerisable inhibitors described in this paper, compounds **1**, **2** and **3**, incorporate an azobenzene as the photochromic group and an α-ketoester as the inhibitory moiety (Fig. 1). A third and novel component of these switches is a peptidyl group designed to enhance binding to α-chymotrypsin. Photoswitchable affinity ligands of this type, once appropriately immobilised, would provide the basis of a novel approach to enzyme purification. α-Chymotrypsin was chosen as the initial target enzyme because (i) much is known about its inhibition and (ii) the general ideas developed here should be applicable to related proteases.

Results and Discussion

Peptidyl α-ketoesters are known potent inhibitors of serine proteases.[4] It has been postulated that the active site serine of the protease reacts with the ketone carbonyl to form a stable, but reversible, tetrahedral intermediate.[5] It has also been suggested that the peptidyl α-ketoesters bind to the active site as a hydrate which mimics the transition state associated with the normal catalytic mechanism of the enzyme.[6] The design of compounds **1**, **2** and **3** was based on previously reported examples of peptidyl-based-α-ketoester inhibitors of serine proteases.[4a] A benzyl group was

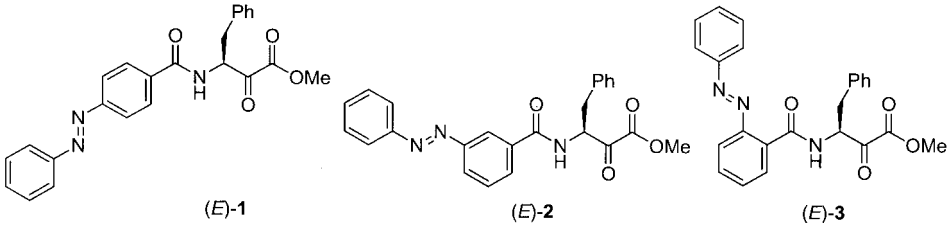

Figure 1.

Keywords: photobiological switch; α-ketoester; inhibition; α-chymotrypsin; photoisomerisation; azobenzene; peptidomimetic.
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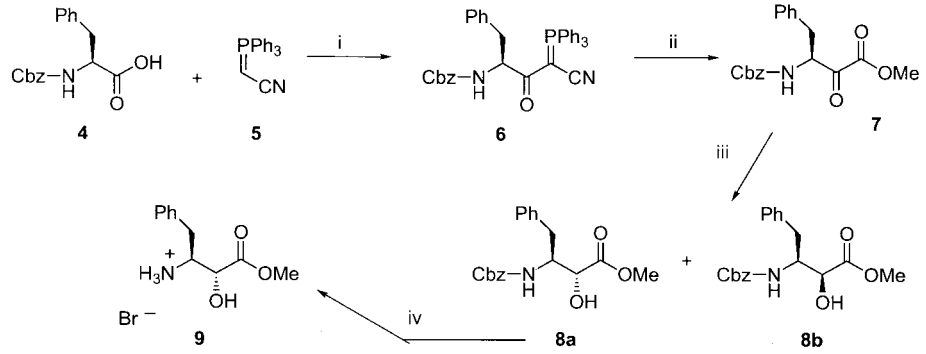
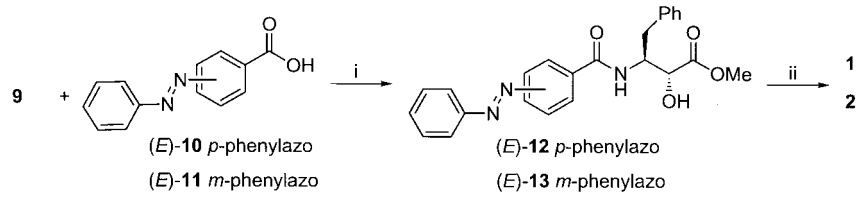


Scheme 1. (i) EDCI, DMAP, CH_2Cl_2, 20 h, 78%; (ii) O_3, MeOH, CH_2Cl_2, −78°C, 10 min, 77%; (iii) $Zn(BH_4)_2$, ether, −78°C, 2 h, **8a** 44%, **8b** 44%; (iv) HBr, AcOH, 20 min, 86%.



Scheme 2. (i) EDCI, HOBT, DIEA, DMF, CH_2Cl_2, 16 h; **12** 89%; **13** 39%; (ii) TEMPO, KBr, NaOCl, $NaHCO_3$, water, CH_2Cl_2; **1** quant; **2** 95%.

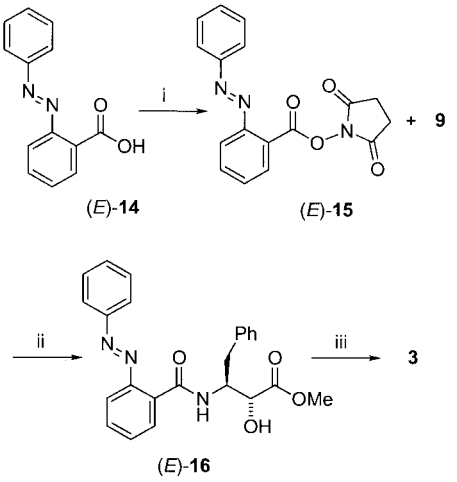
incorporated into these peptidomimetics to bind to the primary binding pocket (S_1 by the notation of Schechter and Berger)[7] of α-chymotrypsin, with the aim to correctly orient the electrophilic carbonyl in the active site.[8]

The irradiation of an azobenzene at a specific wavelength is known to give a photostationary state mixture of (*E*) and (*Z*) isomers, the composition of which is dependent on the ratio of extinction coefficients at that wavelength.[9] The basis of the current study was the supposition that differences in the molecular geometry and the dipole moment of the (*E*) and (*Z*) isomers[10] would result in differential binding to α-chymotrypsin and hence photoregulation of its activity. Compounds **1**, **2** and **3** were also designed to determine which regioisomer of the azobenzene provides the optimal geometry for binding to α-chymotrypsin.

Synthesis

The key step in the synthesis of compounds **1**, **2** and **3** required coupling the hydrochloride salt **9**, derived from **8a** (Scheme 1), with a (phenylazo)benzoic acid (Schemes 2 and 3). We initially sought an improved route to the key intermediate **8a**, and its epimer **8b**, due to the complexity and low reproducibility of existing methods[11–14] for their preparation. We have found that compounds **8** are best prepared from Cbz-L-phenylalanine over three steps utilising cyanoketophosphorane methodology developed by Wasserman et al.[15–17] As shown in Scheme 1, the cyanophosphorane **5** was acylated with Cbz-L-phenylalanine to give the cyanoketophosphorane **6** in 78% yield. Ozonolysis of **6**, in the presence of methanol, gave the methyl α-keto-ester **7** in 77% yield. This was followed by zinc borohydride reduction of **7** to give the key intermediates **8a** and **8b**, which were isolated separately after flash chromatography in yields of 44% and 44% respectively. The absolute configurations of the epimers were assigned on the basis of literature comparison of the ^{1}H NMR spectra and optical rotation data.[18,19]

A single epimer **8a** was used in subsequent steps to avoid complications that would arise from working with mixtures of isomers. The Cbz group of **8a** was removed by treatment with HBr in acetic acid to give the amine salt **9** in 86% yield (Scheme 1). This was then coupled with the (phenylazo)benzoic acids **10** or **11**,[20,21] in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI), to give **12** (89%) and **13** (39%) respectively (Scheme 2). The *ortho* isomer **16** proved more difficult to synthesise, however, it was ultimately prepared in 36%



Scheme 3. (i) DCC, *N*-hydroxysuccinimide, THF, 0°C, 16 h; (ii) **9**, DIEA, THF, 4 h, 36% over two steps; (iii) TEMPO, KBr, NaOCl, $NaHCO_3$, water, CH_2Cl_2, 88%.

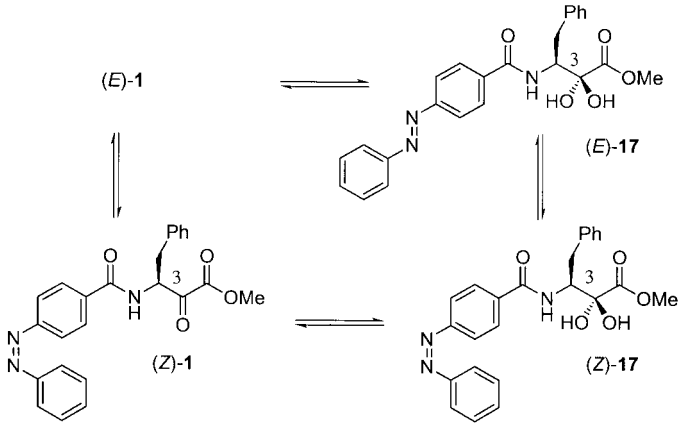


Figure 2. Hydration of compound **1**.

yield on coupling of the *N*-hydroxysuccinimide ester **15** with **9** (Scheme 3). The final step in the synthetic sequence involved oxidation of the α-hydroxyl group of **12**, **13** and **16** with 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO)[22,23] in aqueous sodium hypochlorite to give **1**, **2** and **3** in excellent yields (quant, 95%, 88% respectively).

^{1}H NMR analysis of the azo compounds **1**, **2** and **3** revealed a predominance of the (*E*) isomer (typically >90%) with traces of the (*Z*) isomer. It was observed that solutions of the azobenzenes reached a photostationary state[24] comprising both (*E*) and (*Z*) isomers on exposure to sunlight or fluorescent light. For example, a solution of **1** in d_3-acetonitrile afforded a mixture (3:1 by ^{1}H NMR spectroscopy) of the (*E*) and (*Z*) isomers after exposure to fluorescent lighting for 24 h. The formation of trace amounts of the (*Z*) isomers was not a problem in the subsequent enzyme assay studies since they were performed on photostationary states comprising (*E*) and (*Z*) isomers (refer to Isomerisation section), which were prepared in a standardised manner.

Hydration studies

The hydration properties of the target compounds were briefly explored since this is thought to play a role in the mechanism by which this class of compound inhibits serine proteases. For example, it has been suggested that the 'slow-tight binding' kinetics, which are often observed with α-ketoesters, results from a pre-binding equilibrium step to the ketone from the hdrate.[5] It has been widely reported that α-ketoesters[6] and α-ketoamides[22,25] undergo hydration in organic solutions containing water. In our case, it was found that a sample of **1** in d_3-acetonitrile[†] comprised (*E*)-**1** and the *gem*-diol (*E*)-**17** in a ratio of >19:1 (by ^{1}H NMR). After 26 h on the bench, the solution comprised (*E*)-**1**/(*Z*)-**1**/(*E*)-**17**/(*Z*)-**17** (Fig. 2) in the ratio 42:13:34:11, indicating both *gem*-diol formation and some photoisomerism. The solution was then diluted, dried over magnesium sulfate, evaporated and resuspended in dry deuterochloroform. ^{1}H NMR analysis of this sample revealed a shift in the equilibrium with (*E*)-**1**/(*Z*)-**1**/(*E*)-**17** being present in the ratio 81:13:6. ^{1}H NMR analysis of a solution of **1** in d_3-acetonitrile/D_2O (2:1) showed that it existed as hydrate (75%) and ketone (25%) after 10 min at room temperature, a ratio that did not change over a period of 450 min. These simple experiments suggest that the *gem*-diol would be the predominant, if not the exclusive, species under the conditions of the assay (see below).

Stability studies

There are conflicting reports on the epimerisation and/or racemisation of peptidyl-α-ketoamides and peptidyl-α-ketoesters. For instance, Harbeson et al. report that peptidyl-α-ketoamides epimerised on silica gel, whereas Wasserman and co-workers purified these compounds by flash chromatography on silica gel to afford single diastereomers.[15,16,22] In order to clarify this issue, studies sought to confirm the stability of **1** against racemisation under various conditions. The stability of this compound on silica was investigated. A sample of **1** with an ORD measurement of $-52\pm5°$ (*c* 0.3, acetonitrile) was passed down a silica column to give a sample with a rotation of $-57\pm5°$ (*c* 0.2, acetonitrile). The pH-dependence of the stability of **1** was also investigated by monitoring solutions of **1** in d_3-acetonitrile/D_2O acidified to pH 3 (2:1) and d_3-acetonitrile/D_2O buffered to pH 7.8 with HEPES buffer[‡] (2:1). The resonance due to H3 of all four species (*E*)-**1**, (*Z*)-**1**, (*E*)-**17** and (*Z*)-**17** was observed to decrease, due to deuterium exchange, over 30 h in the case of the pH 7.8 buffered solution but not for the acidified solution. This result is consistent with the finding of Harbeson et al., that α-ketoamides epimerise in general base conditions.[22] Over the time course of a typical enzyme assay (10 min) the extent of racemisation of **1** would, however, be expected to be negligible.

Isomerisation studies

Three photostationary states (PSS) were prepared for each of the compounds **1**, **2** and **3**; one resulting from ambient lighting, one from visible light and one from UV light. The

[†] The d_3-acetonitrile used in these experiments was not dried and contained traces of water.

[‡] The components of the buffered D_2O were at the same concentration as the enzyme assay buffer solution, i.e. HEPES 0.1 M, calcium chloride 0.02 M, Triton X-100 0.05% w/v.

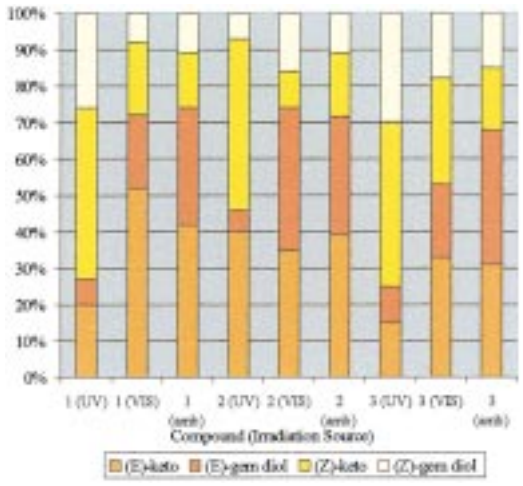


Figure 3. Photoisomerisation of inhibitors, **1**, **2** and **3**.

compositions of these PSS were determined by ^{1}H NMR spectroscopy in order to determine the compositions of the mixtures that were subsequently used to inhibit α-chymotrypsin. A solution of the compound (5 mg) in d_3-acetonitrile (150 μL) was irradiated for one hour with light from a high pressure mercury arc lamp that was filtered to allow passage of wavelengths between 330 and 370 nm for (*Z*) isomer enrichment and the components of the mixture were measured by integration of the methoxy resonances. The visible light PSS was then obtained by irradiation of the sample with wavelengths over 400 nm for (*E*) enrichment and the mixture composition was determined. The solution was allowed to photoequilibrate under fluorescent lighting for 24 h and the resulting mixture composition was measured. The results for the isomerisation of the compounds **1**, **2** and **3** are presented in Fig. 3. As evidenced by the histogram, each of the compounds underwent hydrate formation during isomerisation. For example, the UV PSS for **3** comprised 40% hydrated species, the visible PSS of **3** comprised 38% hydrated species and the ambient PSS of **3** had 52% of hydrated species present. The significance of hydrate formation in these experiments is secondary to the ratio of (*E*) and (*Z*) isomers, because these compounds are expected to exist as the hydrate in the aqueous conditions of the enzyme assay (see earlier for a discussion). The photostationary states arising from ambient light and visible light for **1** [amb 74% (*E*), vis 72% (*E*)] and **2** [amb 71% (*E*), vis 74% (*E*)] have comparable compositions, and the ambient PSS was used as an approximations to the visible light PSS in the enzyme assays for these compounds (Fig. 3). The ambient PSS for **3** is noticeably different from the visible PSS [amb 68% (*E*), vis 53% (*E*)] and as a result does not provide a good approximation for the visible PSS.

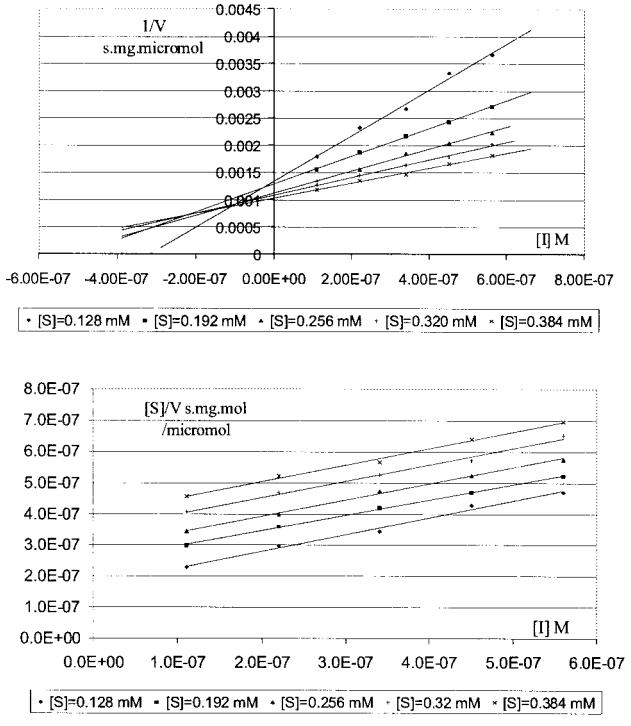


Figure 4. The Dixon and modified Dixon plots for the inhibition of α-chymotrypsin by the ambient light PSS of **2**.

Table 1. The inhibition constants for the inhibition of α-chymotrypsin by the UV and ambient light PSS of **1**, **2** and **3**.

	PSS (*E*):(*Z*)[a]	Percent (*Z*)	K_i (nM)	ΔK_i
1 ambient	3:1	26±5	240	110 μM
1 UV	1:3	73±5	130	2-fold
2 ambient	3:1	28±5	80	40 μM
2 UV	4:5	54±5	40	2-fold
3 ambient[b]	7:3	32±5	1200	840 μM
3 UV	1:3	75±5	360	3-fold

[a] (*E*)/(*Z*) compositions determined by ^{1}H NMR spectroscopy by integration of the methoxy resonances.
[b] The ambient light PSS of **3** does not provide a good approximation for the visible light PSS of **3**.

Inhibition studies

The ambient and UV photostationary states for each of the inhibitors were tested against α-chymotrypsin and the type of inhibition and the inhibition constants were determined by Dixon, modified Dixon and double-reciprocal analyses.[26,27] The Dixon (plot of 1/V vs [*I*]) and modified Dixon (plot of [*S*]/V vs [*I*]) plot for the inhibition of α-chymotrypsin by the ambient PSS of **2** are shown in Fig. 4 as a representative example. Dixon and modified Dixon plots together give both the inhibition constant and also the type of inhibition.[§] It is clear from these graphs that the ambient PSS of **2** inhibits the enzyme by a competitive mechanism, where the Dixon plot gives a good approximation to the inhibition constant (80 nM) with a tight scatter of line intersections in the fourth quadrant and the modified Dixon plot is composed of a set of parallel lines.

[§] **Competitive Inhibition.** Dixon plot, K_i is given as the [*I*]-axis value for the median of the line intersections in the fourth quadrant; Modified Dixon plot gives a series of parallel lines. **Uncompetitive Inhibition.** Dixon plot gives a series of parallel lines; Modified Dixon plot, K_i' is given as the [*I*]-axis value for the median of the line intersections in the fourth quadrant. **Mixed Inhibition.** Dixon plot, Ki is given as the [*I*]-axis value for the median of the line intersections in the fourth quadrant; Modified Dixon plot, Ki' is given as the [*I*]-axis value for the median of the line intersections in the third quadrant.

For each of the inhibitors, the UV PSS gave a lower value for the inhibition constant than the ambient PSS (see Table 1). For example the UV PSS of **1** gave K_i=130 nM and the ambient PSS gave K_i=240 nM. These results imply that the (*Z*) isomer was more active against α-chymotrypsin than the (*E*) isomer in each case.[‖] As might be expected for α-ketoester-based inhibitors of serine proteases,[4b] the type of inhibition for all of the compounds fitted most closely to a competitive model of inhibition. The results in Table 1 give an indication of the structure-activity relationship for azobenzene substitution. The *meta* isomer **2** was the most active ($K_i^{amb\ PSS}$ = 80 nM), followed by the *para* isomer **1** ($K_i^{amb\ PSS}$=240 nM) with the *ortho* isomer **3** being the least active ($K_i^{amb\ PSS}$=1200 nM). The isomer that showed the best switching ability with respect to α-chymotrypsin was **3**, for which there was a more than 3-fold difference in inhibition constant between the ambient and visible light PSS ($K_i^{amb\ PSS}$=1200 nM, $K_i^{UV\ PSS}$=360 nM).

Conclusions

We have prepared novel photobiological switches of α-chymotrypsin. The inhibitors **1**, **2** and **3** were designed to target α-chymotrypsin by the incorporation of (*L*)-phenylalanine to bind in the S_1 subsite of the enzyme. It is anticipated that other proteases could be targeted by simply altering the amino acid(s) in this P_1 position. The α-ketoesters **1**, **2** and **3** proved to be competitive inhibitors of α-chymotrypsin with a magnitude of enzyme switching between two- and three- fold by inhibition constant measurement. The most efficacious switching of α-chymotrypsin was achieved by **3**, for which the ambient PSS was a considerably less active than the UV PSS. This result implies that *ortho* substituted azobenzene provides an effective α-chymotrypsin switch in compounds of this type. The *meta* isomer **2** was the most active inhibitor in either of the photostationary states and this result suggests that *meta* substitution of either (*E*) or (*Z*) azobenzene provides the best binding with α-chymotrypsin for this type of compound. Further work in our laboratories is being directed at the development of photobiological switches that; (i) are more stable in the assay medium, (ii) show greater differential activity between the two PSS and (iii) will allow isolation and testing of pure (*E*) and (*Z*) isomers.

Experimental

The following reagents were obtained commercially: Cbz-(L)-phenylalanine (Sigma); cyanomethyltriphenylphosphonium chloride (Lancaster); EDCI (Aldrich); TEMPO (Aldrich); Succ-Ala-Ala-Pro-Phe-4-nitroanilide and α-nitroanilide and α-chymotrypsin (Sigma). Proton detected NMR spectra were obtained on either a Varian Unity 3000 spectrometer or a Varian XL300 spectrometer, both operating at 300 MHz. Carbon detected NMR were obtained on the XL300 spectrometer operating at 75 MHz. IR spectra were obtained using a Shimadzu 8201PC series FTIR. Mass spectrometry was performed on either a Kratos MS80 Mass Spectrometer or a Micromass LCT operating in Electrospray (ES) mode with 50:50 acetonitrile water as solvent. Optical rotation measurements were performed either on a JASCO J-20C recording spectropolarimeter with a 10 mm path length or a Perkin Elmer polarimeter Model 341 with a 100 mm path length. $[\alpha]_D^{20}$ values are given in units of g/100 ml. Absorbance measurements were made on a Hewlett–Packard 8452A diode array spectrophotometer, which was controlled by Hewlett–Packard 89532A UV-VIS operating software. The plastic cuvettes (1.5 ml) were held during the assays in a custom-made thermostatted cell block that was set at 25.0±0.2°C. Buffer, enzyme and substrate solutions were stored during use in a water bath thermoregulated at 25°C by a Techne Tempette TE-8A water bath thermostat. All buffer solutions were pH tested at 25°C with a custom-made digital pH meter that had been referenced with borax solution (0.01 M) to pH 9.18. All stock inhibitor solutions were prepared in acetonitrile (BDH HiperSolv™ 'Far UV' grade).

Oxidation of α-hydroxyesters with TEMPO/NaOCl. General procedure[22]

To TEMPO (ca. 0.1 equiv.), KBr (ca. 0.2 equiv) and water (ca. 0.01 equiv) was added a solution of the alcohol (1.0 equiv) in dichloromethane (ca. 0.1 M). The reaction mixture was stirred at 0°C. A buffered bleach solution was prepared by the addition of $NaHCO_3$ (300 mg, 3.6 mmol) to a 5.25% aqueous sodium hypochlorite solution (9.7 ml commercial bleach, 15.3 ml distilled water) and the resulting mixture was stirred until all of the solid had dissolved. The buffered bleach solution (one part) was added dropwise to the above reaction mixture (two parts) at 0°C. After rapid stirring for 30 min the reaction mixture was diluted with ethyl acetate. The organic phase was separated, washed with 0.5N aqueous HCl, saturated aqueous $NaHCO_3$, brine, dried over $MgSO_4$ and concentrated in vacuo. Where required the products were recrystallised from ethyl acetate and petroleum ether.

(3*S*)-2-Oxo-4-phenyl-3-[[[4-(phenylazo)benzene]carbonyl]-amino]butanoic acid methyl ester (1). The alcohol **12** (43 mg, 0.10 mmol) was oxidised with TEMPO/NaOCl as described in the General procedure to give **1** (43 mg, quant) as an orange solid: $[\alpha]_D^{20}=-52\pm5°$ (*c* 0.29 acetonitrile); mp 138–139°C; IR ($CHCl_3$) 1733, 1664 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.98 (4H, m, ArH), 7.84 (2H, m, ArH), 7.55 (3H, m, ArH), 7.17–7.30 (5H, m, ArH), 6.67 (1H, d, *J*=4.9 Hz, NH), 5.65 (1H, m, CH), 3.91 (3H, s, CH_3), 3.41 (1H, dd, *J*=5.9, 13.7 Hz, CH_A), 3.25 (1H, dd, *J*=6.6, 13.7 Hz, CH_B); ^{13}C NMR ($CDCl_3$) δ 191.5, 166.3, 160.7, 154.6, 152.5, 134.9, 134.9, 131.6, 129.4, 129.2, 128.9, 128.0, 127.5, 123.1, 123.0, 57.1, 53.3, 36.9; HRMS (EI) calcd for $C_{24}H_{21}O_4N_3$ (M^+) 415.1532, found 415.1539.

A sample of **1** (4 mg) was subjected to flash chromatography eluting with dichloromethane–ethylacetate (9:1) to give **1** (3 mg): $[\alpha]_D^{20}=-57\pm5°$ (*c* 0.20 acetonitrile).

(3*S*)-2-Oxo-4-phenyl-3-[[[3-(phenylazo)benzene]carbonyl]amino]butanoic acid methyl ester (2). The alcohol **13** (27 mg, 65 μmol) was oxidised with TEMPO/NaOCl as described in the General procedure to give **2** (26 mg, 95%)

[‖] Work is in progress to assay the pure (*E*) and (*Z*) isomers of an example of this class of compound.

as an orange solid: $[\alpha]_D^{20}=-53\pm2°$ (*c* 0.67 acetonitrile); IR ($CHCl_3$) 1734, 1666 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.21 (1H, s, ArH), 8.07 (1H, d, *J*=7.8 Hz, ArH), 7.94 (2H, d, *J*=6.5 Hz, ArH), 7.84 (1H, d, *J*=7.5 Hz, ArH), 7.56 (4H, m, ArH), 7.17–7.33 (5H, m, ArH), 6.76 (1H, d, *J*=6.7 Hz, NH), 5.65 (1H, m, CH), 3.90 (3H, s, CH_3) 3.41 (1H, dd, *J*=6.0, 14.0 Hz, CH_A), 3.25 (1H, dd, *J*=6.5, 14.0 Hz, CH_B); ^{13}C NMR ($CDCl_3$) δ 191.5, 166.4, 160.7, 152.5, 152.3, 134.9, 134.3, 131.5, 129.6, 129.4, 129.2, 128.8, 127.5, 126.4, 123.0, 121.0, 57.1, 53.3, 36.8; HRMS (EI) calcd for $C_{24}H_{21}O_4N_3$ (M^+) 415.1532, found 415.1531.

(3*S*)-2-Oxo-4-phenyl-3-[[[2-(phenylazo)benzene]carbonyl]-amino]butanoic acid methyl ester (3). The alcohol **16** (24 mg, 57 μmol) was oxidised with TEMPO/NaOCl as described in the General procedure to give **3** (21 mg, 88%) as an orange solid: $[\alpha]_D^{20}=+16\pm1°$ (*c* 0.94 acetonitrile); IR ($CHCl_3$) 1739, 1635 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.36 (1H, d, *J*=5.5 Hz, NH), 8.41 (1H, m, ArH), 7.78 (1H, m, ArH), 7.44–7.66 (7H, m, ArH), 6.95–7.09 (5H, m, ArH), 5.77 (1H, m, CH), 3.87 (3H, s, CH_3), 3.37 (1H, dd, *J*=5.9, 14.2 Hz, CH_A), 3.23 (1H, dd, *J*=6.4, 14.2 Hz, CH_B); ^{13}C NMR ($CDCl_3$) δ 191.4, 165.2, 160.8, 152.2, 149.8, 135.1, 132.2, 132.1, 131.6, 131.3, 130.6, 129.3, 129.2, 128.3, 127.0, 123.3, 115.9, 57.6, 53.1, 36.8; HRMS (EI) calcd for $C_{24}H_{21}O_4N_3$ (M^+) 415.1532, found 415.1524.

(4*S*)-4-[[(Benzyloxy)carbonyl]amino]-3-oxo-5-phenyl-2-triphenylphosphoranylidene-pentanenitrile (6). To a mixture of Cbz-L-phenylalanine, **4** (0.400 g, 1.3 mmol) in dichloromethane (14 ml) at 0°C were added EDCI (0.270 g, 1.4 mmol) and DMAP (0.016 g, 0.13 mmol), followed by the dropwise addition of a solution of cyanophosphorane **5** (0.81 g, 2.7 mmol) in dichloromethane (6 ml). The reaction mixture was allowed to warm to rt and was stirred under nitrogen for 20 h. The mixture was then poured over 1:1 dichloromethane/water (15 ml) and the phases were separated. The aqueous phase was extracted with dichloromethane (2×5 ml) and the combined organic extracts were washed with brine (30 ml), dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by flash chromatography, eluting with dichloromethane-ethyl acetate (4:1), to give **6** (0.61 g, 78%) as a white solid: mp 101–103°C; IR (film) 1716 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.61–7.66 (3H, m, PPh_3), 7.45–7.60 (12H, m, PPh_3), 7.30 (5H, m, ArH), 7.21 (5H, m, ArH), 5.53 (1H, d, *J*=7.8 Hz, NH), 5.19 (1H, m, CH), 5.06 (2H, s, $CbzCH_2$), 3.35 (1H, dd, *J*=4.8, 13.5 Hz, $CHCH_APh$), 3.08 (1H, dd, *J*=6.8, 13.5 Hz, $CHCH_BPh$); ^{13}C NMR ($CDCl_3$) δ 192.8, 155.4, 136.8, 133.6, 133.4, 133.2, 129.6, 129.2, 129.0, 128.3, 128.0, 127.7, 126.4, 122.4 (d, *J*=92.8 Hz, CN), 120.9 (d, *J*=16 Hz, P-C_q), 66.2, 57.2 (d, *J*=9 Hz, CH), 47.8 (d, *J*=125 Hz, P=C), 38.7; HRMS (FAB) calcd for $C_{37}H_{32}O_3N_2P$ $(M+1)^+$ 583.2150, found 583.2145.

(3*S*)-3-[[(Benzyloxy)carbonyl]amino]-2-oxo-4-phenylbutanoic acid methyl ester (7). A solution of **6** (538 mg, 0.95 mmol) in (7:3) dichloromethane/methanol (9 ml) at −78°C, was treated with ozone at a rate of 1–2 bubbles per second for 15 min. The solution was flushed with nitrogen for 10 min and then allowed to warm to rt. The solution was evaporated to dryness and the resulting residue was purified by flash chromatography, eluting with petroleum ether–ethyl acetate (3:2), to give **7** (249 mg, 77%) as a white solid: mp 68–73°C; IR (film) 1736, 1720 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.20–7.38 (10H, m, ArH), 7.09 (1H, d, *J*=7.5 Hz, NH), 5.27 (1H, m, CH), 5.07 (2H, s, $CbzCH_2$), 3.85 (3H, s, OCH_3), 3.23 (1H, m, $CHCH_APh$), 3.05 (1H, m, $CHCH_BPh$); ^{13}C NMR ($CDCl_3$) δ 191.8, 160.7, 155.5, 136.0, 134.7, 129.3, 128.7, 128.6, 128.5, 128.2, 127.3, 64.1, 58.1, 53.2, 37.1; HRMS (FAB) calcd for $C_{19}H_{20}O_5N$ $(M+1)^+$ 342.1340, found 342.1330.

(2*R*,3*S*) -and (2*S*,3*S*)-3-[[(Benzyloxy)carbonyl]amino]-2-hydroxy-4-phenylbutanoic acid methyl esters (8a, 8b). To a solution of **7** (30 mg, 0.10 mmol) in THF (2 ml) at −78°C was added a freshly prepared solution (0.15 M) of zinc borohydride (1.33 ml, 0.20 mmol) in ether. The reaction mixture was stirred at −78°C for 2 h before quenching with water (10 ml), followed by ethyl acetate (10 ml). The phases were separated and the aqueous phase was extracted with ethyl acetate (3×15 ml). The combined organics were washed with brine (20 ml), dried over $MgSO_4$ and concentrated in vacuo to afford a cream gum. Flash chromatography eluting with petroleum ether–ethyl acetate (5:1) afforded **8a** (15 mg, 44%) as a white solid: $[\alpha]_D^{20}=-60\pm5°$ (*c* 1.1 dichloromethane), lit. $[\alpha]_D^{20}=-82°$ (*c* 0.83 methanol);[18a] ^1H NMR ($CDCl_3$) δ 7.20–7.40 (10H, m, Ar), 5.10 (1H, d, *J*=9.8 Hz, NH), 5.04 (2H, s, $CbzCH_2$), 4.33 (1H, q, *J*=9.7 Hz, $CHCHOH$), 4.08 (1H, br s, $CHCHOH$), 3.70 (3H, s, CH_3), 3.20 (1H, d, *J*=3.0 Hz, OH), 2.92 (2H, m, $CHCH_2Ph$).

Further elution with petroleum ether–ethyl acetate (5:3) afforded **8b** (15 mg, 44%) as a white solid: $[\alpha]_D^{20}=-13\pm1°$ (*c* 0.69 methanol), lit. $[\alpha]_D^{20}=-6°$ (*c* 0.85 methanol);[18b] ^1H NMR ($CDCl_3$) δ 7.20–7.40 (10H, m, ArH), 5.22 (1H, d, *J*=9.3 Hz, NH), 5.04 (2H, s, $CbzCH_2$), 4.38 (1H, m, $CHCHOH$), 4.35 (1H, s, $CHCHOH$), 3.56 (3H, s, CH_3), 2.80 (2H, m, $CHCH_2Ph$).

(2*R*,3*S*)-2-Hydroxy-4-phenyl-3-[[[4-(phenylazo)benzene]-carbonyl]amino]butanoic acid methyl ester (12). Compound **8a** (795 mg, 2.31 mmol) was dissolved in a solution of HBr in acetic acid (33%, 1.0 ml) and the resulting mixture was stirred at rt for 20 minutes. The addition of ether (2 ml) caused the amine salt to precipitate. The mixture was kept at 0°C for 30 min and then filtered and washed with ether (3×2 ml) to give **9** (574 mg, 86%) as a white solid that was subsequently used without further purification: mp 160–161°C; ^1H NMR (D_2O) δ 7.22–7.35 (5H, m, ArH), 4.28 (1H, d, *J*=3.4 Hz, $CHCHOH$), 3.87 (1H, m, $CHCHOH$), 3.64 (3H, s, CH_3), 2.99 (2H, m, CH_2Ph); ^{13}C NMR (D_2O) δ 172.9, 134.8, 129.4, 129.2, 127.7, 68.5, 54.2, 53.1, 35.1. To a solution of **9** (100 mg, 0.34 mmol) and **10**[20] (71 mg, 0.31 mmol) in 1:1 dimethylformamide/dichloromethane (2 ml) at rt under nitrogen were added EDCI (78 mg, 0.41 mmol) and HOBT (63 mg, 0.47 mmol). The reaction mixture was stirred for 5 min and DIEA (60 μL, 0.34 mmol) was added. The reaction mixture was stirred 16 h and the solution was diluted with dichloromethane (5 ml), washed with 1N HCl (2×5 ml), saturated aqueous $NaHCO_3$ (5 ml), brine (5 ml), dried over $MgSO_4$ and concentrated in vacuo to afford **12** (127 mg, 89%) as an orange solid: mp 176°C; IR ($CHCl_3$) 1734, 1666 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.95 (4H, m, ArH), 7.82 (2H, d, *J*=8.3 Hz,

ArH), 7.54 (3H, m, ArH), 7.24–7.36 (5H, m, ArH), 6.44 (1H, d, J=9.1 Hz, NH), 4.82 (1H, m, CH), 4.22 (1H, d, J=2 Hz, C*H*OH), 3.77 (3H, s, CH_3), 3.31 (1H, d, J=4 Hz, OH), 3.05 (2H, m, CH_2); ^{13}C NMR ($CDCl_3$) δ 174.3, 166.5, 154.4, 152.5, 137.2, 135.9, 131.6, 129.4, 129.2, 128.7, 127.9, 126.9, 123.1, 122.9, 70.1, 53.4, 53.1, 38.0; Anal. calcd for $C_{24}H_{23}O_4N_3$, C 69.05, H 5.55, N 10.06; found C 68.80, H 5.66, N 10.24.

(2*R*,3*S*)-2-Hydroxy-3-4-phenyl-[[[3-(phenylazo)benzene]-carbonyl]amino]butanoic acid methyl ester (13). To a solution of **9**, prepared as described in the previous example (52 mg, 0.18 mmol) and **11**[20] (37 mg, 0.16 mmol) in 1:1 dimethylformamide/dichloromethane (1.5 ml) at rt under nitrogen were added EDCI (41 mg, 0.21 mmol) and HOBT (24 mg, 0.18 mmol) and DIEA (43 μL, 0.24 mmol) as described for **12**. The resulting residue was purified by flash chromatography, eluting with dichloromethane to afford **13** (26 mg, 39%) as an orange solid: IR ($CHCl_3$) 3435, 3031, 2956, 1734, 1668 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.18 (1H, s, ArH), 8.02 (1H, d, J=7.8 Hz, ArH), 7.92 (2H, d, J=8.1 Hz, ArH), 7.82 (1H, d, J=7.5 Hz, ArH), 7.53 (4H, m, ArH), 7.24–7.35 (5H, m, ArH), 6.68 (1H, m, NH), 4.86 (1H, m, C*H*CHOH), 4.24 (1H, s, C*H*OH), 3.75 (3H, s, CH_3), 3.09 (2H, m, CH_2); ^{13}C NMR ($CDCl_3$) δ 174.2, 166.5, 152.5, 152.4, 137.2, 135.2, 131.4, 129.4, 129.1, 128.7, 126.8, 125.6, 123.0, 121.2, 70.1, 53.4, 53.0, 37.9; HRMS (EI) calcd for $C_{24}H_{23}O_4N_3$ (M^+) 417.1689, found 417.1691.

(2*R*,3*S*)-2-Hydroxy-4-phenyl-3-[[[2-(phenylazo)benzene]-carbonyl]amino]butanoic acid methyl ester (16). A solution of DCC (77 mg, 0.40 mmol) in THF (1.5 ml) at 0°C was added to an ice-cold solution of acid **14**[21] (90 mg, 0.40 mmol) and *N*-hydroxysuccinimide (46 mg, 0.40 mmol) in THF (3 ml). The reaction mixture was stirred 2 h at 0°C then stored at 4°C overnight. The reaction mixture was filtered, washing with cold THF (2×1 ml). To the solution was added **9** (166 mg, 0.77 mmol) and DIEA (76 μL, 0.44 mmol) and the reaction mixture was stirred 1 h at 0°C and 4 h at rt. The reaction mixture was diluted with dichloromethane (20 ml), washed with saturated aqueous NH_4Cl (20 ml), brine (20 ml), dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by flash chromatography, eluting with dichloromethane, to afford an orange solid tentatively assigned as **15** (30 mg, 23%): ^{1}H NMR ($CDCl_3$) δ 8.06 (3H, m, ArH), 7.75 (2H, m, ArH), 7.48–7.59 (4H, m, ArH), 2.86 (4H, s, 2x CH_2); HRMS (ES) calcd for $C_{17}H_{13}O_4N_3K$ $(M+K)^+$ 362.054, found 362.055. Further elution with dichloromethane–ethyl acetate (19:1) gave **16** (62 mg, 37%) as an orange solid: mp 127–130°C; IR ($CHCl_3$) 3622, 3533, 3310, 1743, 1656 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.02 (1H, d, J=9.0 Hz, NH), 8.43 (1H, m, ArH), 7.97 (2H, d, J=8.1 Hz, ArH), 7.76 (1H, m, ArH), 7.54 (5H, m, ArH), 7.17–7.34 (5H, m, ArH), 4.90 (1H, m, C*H*CHOH), 4.22 (1H, s, C*H*OH), 3.68 (3H, s, CH_3), 3.35 (1H, br s, OH), 3.04 (2H, m, CH_2); ^{13}C NMR ($CDCl_3$) δ 174.2, 165.3, 152.3, 149.6, 137.5, 132.2, 131.8, 131.5, 131.3, 130.2, 129.3, 129.3, 128.5, 126.6, 123.4, 115.8, 70.0, 53.9, 52.8, 38.1; HRMS (EI) calcd for $C_{24}H_{23}O_4N_3$ (M^+) 417.1689, found 417.1692.

Hydration studies

A solution of **1** (5 mg) in d_3-acetonitrile (150 μL) was analysed by ^{1}H NMR at t=5 min to reveal (*E*)-**1** and (*E*)-**17** in a ratio of >19:1. After 26 h, (*E*)-**1**/(*Z*)-**1**/(*E*)-**17**/(*Z*)-**17** were observed in a ratio of 42:13:34:11. Water was then removed from the sample by diluting it with dichloromethane and drying over $MgSO_4$. The solvent was concentrated in vacuo and the sample was redissolved in dry $CDCl_3$. The solution was examined by ^{1}H NMR to reveal (*E*)-**1**/(*Z*)-**1**/(*E*)-**17** in the ratio 81:13:6. In an analogous experiment the composition of a solution of **1** (5 mg) in CD_3CN/D_2O (2:1, 150 μL) was monitored by ^{1}H NMR spectroscopy after 10 min to reveal a mixture of **1** and **17** in a ratio of 1:3 which was unaltered after a further 450 min.

Selected data for (*E*)-**1** from the mixture: ^{1}H NMR (CD_3CN) δ 7.92–8.08 (6H, m, ArH), 7.63 (3H, m, ArH), 7.27–7.39 (5H, m, ArH), 5.32 (1H, dd, J=5.4, 9.3 Hz, CH), 3.85 (3H, s, CH_3), 3.39 (1H, dd, J=5.4, 14.2 Hz, CH_A), 3.13 (1H, dd, J=9.3, 14.2, CH_B). Selected data for (*Z*)-**1** from the mixture: ^{1}H NMR (CD_3CN) δ 7.92–8.08 (2H, m, ArH), 7.63 (3H, m, ArH), 7.27–7.39 (5H, m, ArH), 6.89 (4H, m, H2, ArH), 5.24 (1H, m, CH), 3.81 (3H, s, CH_3). Selected data for (*E*)-**17** from the mixture: ^{1}H NMR (CD_3CN) δ 7.92–8.08 (6H, m, ArH), 7.84 (2H, d, J=8.8 Hz, NH), 7.63 (3H, m, ArH), 7.27–7.39 (5H, m, ArH), 4.79 (1H, dd, J=3.4, 11.7 Hz, CH), 3.78 (3H, s, CH_3), 3.35 (1H, m, CH_A), 3.10 (1H, m, CH_B). Selected data for (*Z*)-**17** from the mixture: ^{1}H NMR (CD_3CN) δ 7.92–8.08 (2H, m, ArH), 7.63 (3H, m, ArH), 7.27–7.39 (5H, m, ArH), 6.89 (4H, m, ArH), 4.63 (1H, m, CH), 3.74 (3H, s, CH_3).

Stability studies

Solutions of **1** (5 mg) were prepared in (2:1) d_3-acetonitrile/acidified D_2O-pH 3 by Universal indicator paper (150 μL) and (2:1) d_3-acetonitrile/buffered D_2O (150 μL). The buffered D_2O solution at pH 7.8 comprised HEPES buffer (0.1 M), $CaCl_2$ (0.02 M) and Triton X-100 (0.05% w/v). The two solutions were monitored over a period of 30 h by ^{1}H NMR spectroscopy. The combined multiplets due to H3 of (*E*)-**1**, (*Z*)-**1**, (*E*)-**17** and (*Z*)-**17** decreased by 40% over a period of 30 h in the case of pH 7.8 solution. No change was evident with the pH 3 sample.

Isomerisation studies

For the purposes of PSS composition measurement, solutions of the inhibitors in d_3-acetonitrile (typically 30 mM) were irradiated in quartz NMR tubes for 60 min. Isomer-enriched photostationary states were obtained by irradiation of the solution with filtered light from a 200 W high pressure mercury arc lamp. The light was filtered with an Oriel 59810 filter to allow passage of UV light ($330<\lambda<370$ nm) for (*Z*) isomer enrichment. For (*E*) enrichment the light was filtered with an Oriel 59494 filter ($\lambda>400$ nm) to allow the transmittance of visible light. At 500 mm from the light source the glass filter was held in a cylinder, at 540 mm from the light source a water filter of 10 mm thickness was held and at 580 mm from the light source the sample was held. The PSS compositions were measured by ^{1}H NMR spectroscopy by integration of the

methoxy resonances (as recorded earlier) immediately after irradiation of the solution.

Enzyme assay and kinetics

Buffer (HEPES) solution. HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid) (11.9 g, 0.05 mol), $CaCl_2 \cdot 6H_2O$ (2.2 g, 0.01 mol) and Triton X-100 (0.25 g) were dissolved in Milli-Q ionised water (400 ml) in a 500 ml volumetric flask. Sufficient 1 M NaOH solution was added (approx. 40 ml) to reach pH 7.8 and the solution was made up to 500 ml with more Milli-Q water.

Substrate solution. HEPES (11.9 g, 0.05 mol) and $CaCl_2 \cdot 6H_2O$ (2.2 g, 0.01 mol) were dissolved in Milli-Q ionised water (400 ml) in a 500 ml volumetric flask. Sufficient 1 M NaOH solution was added (approx. 40 ml) to reach pH 7.8 and the solution was made up to 500 ml with more Milli-Q water. *N*-Succinyl-$(Ala)_2$-Pro-Phe-4-nitroanilide (21 mg, 34 μmol) was dissolved in a sample of this solution (10 ml) by sonication for 10 min in cold water. The solution was stored for up to two weeks below 0°C. The concentration of the solution was determined at the start of each day from its UV spectrum (ϵ_{315}= 14 000 $Lmol^{-1}\,cm^{-1}$).

Enzyme solution. A stock solution of α-chymotrypsin was prepared from α-chymotrypsin (3.03 mg, 120 nmol) and Milli-Q water (1 ml). To a 100 ml volumetric flask was added stock solution (200 μL, 24 nmol), Triton X-100 (50 mg, 0.05% w/v) and conc. HCl (analytical grade, 10 μL, 1.2 mM) and Milli-Q water (about 50 ml). The flask was shaken at length and the solution was made to 100 ml with Milli-Q water. The enzyme solutions were prepared fresh each day.

Inhibition of α-chymotrypsin was determined with Succ-Ala-Ala-Pro-Phe-4-nitroanilinde as the substrate by an assay procedure developed from the technique described by Geiger,[28] except that HEPES buffer was used instead of TRIS buffer and that the order of addition of enzyme and substrate was inverted. Each measurement was made in duplicate. For the purposes of enzyme inhibition, solutions of the inhibitors in d_3-acetonitrile (15–90 μM) were irradiated in quartz cuvettes for 60 min as described in the Isomerisation under the Experimental section. Large data sets (5×[*S*], 5×[*I*]) were obtained in duplicate for the PSS composition resulting from ambient light. These data sets were fitted to the Michaelis–Menten model with double-reciprocal, direct linear, Dixon and modified Dixon plots. From these analyses the type of inhibition was determined and the kinetic parameters K_M^{app}, V_{max}^{app} and K_i were calculated. Smaller data sets (4×[*S*], 3×[*I*]) were obtained for the UV light photostationary states, enabling analysis by double-reciprocal and direct linear plots for the parameters K_M^{app}, V_{max}^{app} and K_i. The assumption that the visible and ambient light photostationary states had the same effect on the enzyme was tested by measuring the extent of inhibition at two inhibitor concentrations for the ambient light PSS with the results for the visible light PSS and it was found that the initial rates were the same within experimental error.

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