DOI: 10.1002/chem.200903369

Structural Optimization of Photoswitch Ligands for Surface Attachment of α-Chymotrypsin and Regulation of Its Surface Binding

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Abstract: A modified gold surface that allows photoregulated binding of α-chymotrypsin has previously been reported. Here the development of this surface is reported, through the synthesis of a series of trifluoromethyl ketones and α-keto esters containing the azobenzene group and a surface attachment group as photoswitch inhibitors of α-chymotrypsin. All of the compounds are inhibitors of the enzyme, with activity that can be modulated by photoisomerization. The best photoswitch shows a reversible change in IC_{50} inhibition constant of >5.3 times on photoisomerization. The trifluoromethyl ketone **1** exhibited excellent photoswitching and was attached to a gold surface in a two-step procedure involving an azide–alkyne cycloaddition. The resulting modified surface bound α-chymotrypsin to a degree that could be modulated by UV/Vis irradiation, showing "slow-tight" enzyme binding as observed for inhibitors in solution.

Keywords: azo compounds · enzyme inhibitors · photoswitching · surface chemistry · surface plasmon resonance

Introduction

Photoisomerization of the azobenzene group provides a platform for substantial control of biological activity, to give a variety of potential applications.[1,2] The incorporation of this functionality into a (photo)switchable surface, for example, shows much promise for the development of "intelligent" nanoscale biosystems.[3] Photoresponsive surfaces that are able to modulate the binding and release of molecules (an enzyme, for example) in reversible fashion offer potential for the development of new biosensors, advanced medical implants, biomolecular computers, and advanced protein purification procedures based on photoaffinity chromatography. However, most such systems reported to date[4–6] have lacked the versatility to merit development beyond initial feasibility studies. In order to make advances in this area we require general approaches for photoswitchable binding of biomolecules to surfaces in predictable and controllable ways. With this in mind we recently published a communication reporting the photoreversible binding of a protease (a class of enzyme that accepts a range of related peptide-based substrates) to a surface-bound inhibitor.[7] Reversible surface photoregulation was demonstrated for α-chymotrypsin with compound **1** (Figure 1), which contains a protease inhibition group (a phenylalanine-based trifluoromethyl ketone for specific targeting of α-chymotrypsin), an azobenzene switch (to allow reversible control of shape), an oligoethylene glycol (OEG) tether (to extend the inhibitor into solution), and a terminal alkyne for attachment to a surface-bound azide through a Huisgen cycloaddition reaction.[8,9] Photochemical irradiation with UV (or visible) light reversibly controls the geometry of the azobenzene constituent and hence protease binding to the surface, as shown schematically in Figure 1.[7] This modular design is amenable to surface attachment of a range of peptide-based structures to allow targeted binding of a range of proteases and other biological receptors: that is, it provides a general approach to a photoswitchable surface. α-Chymotrypsin was chosen for study because it is particularly well characterized and thus represents a prototypical protease.[10]

Here we report the preparation and testing of a number of trifluoromethyl ketone and α-keto ester photoswitch inhibitors (**1**–**3**, **5**–**8**, Figures 2 and 3) of α-chymotrypsin. These studies have begun to define, for the first time, the optimum inhibitory group and C-terminal acetylene-based

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200903369.

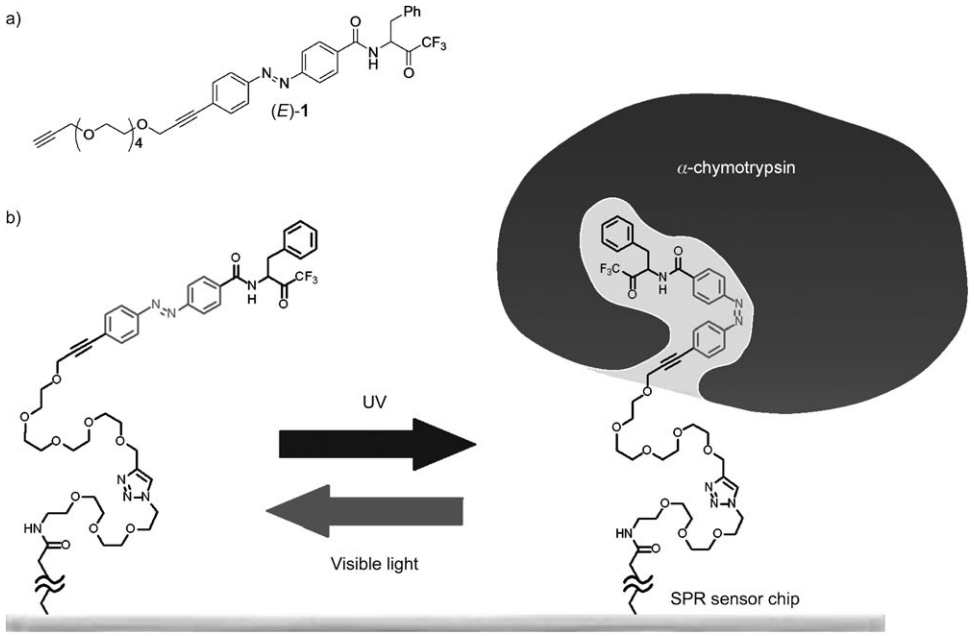


Figure 1. α-Chymotrypsin photoswitch. a) Inhibitor (*E*)-**1**. b) Schematic of surface photoswitching showing surface-attached (*E*)-**1** and α-chymotrypsin-bound surface-attached (*Z*)-**1**.

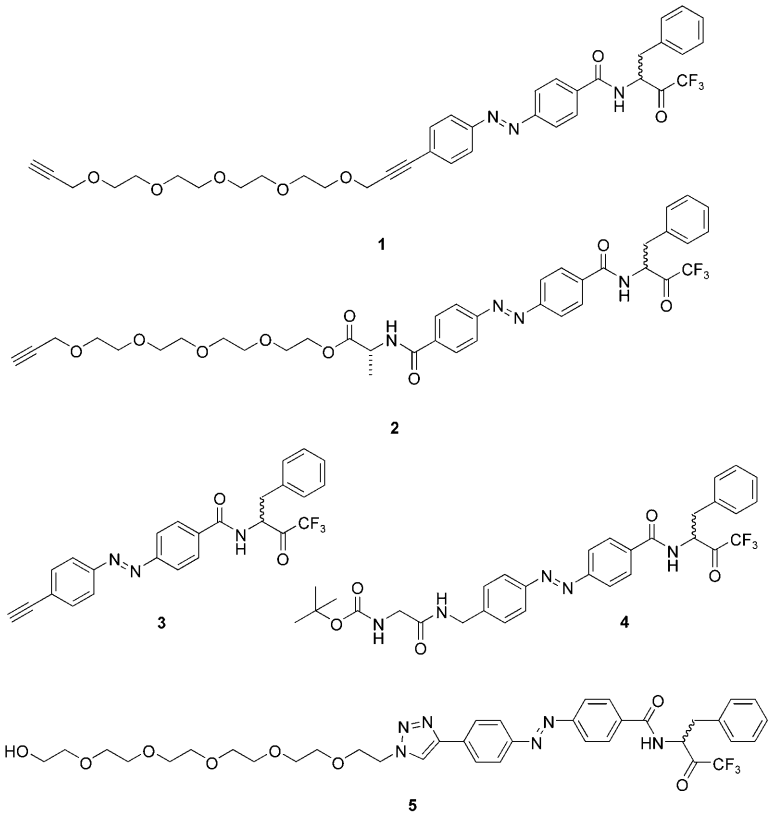


Figure 2. Trifluoromethyl ketone photoswitch inhibitors.

linker for simultaneous optimization of enzyme binding, photoswitching, and surface attachment. This is an important step in the generation of more versatile photoswitchable surfaces based on these structures. The general design of these compounds is based on the affinity of α-chymotrypsin for inhibitors/substrates containing aromatic amino acids in their P_1 positions and large hydrophobic groups in their P_2 positions.[11] As such, the phenylalanine-mimicking trifluoromethyl ketone/α-keto ester and the azobenzene group are expected to bind to the S_1 and S_2 subsites of the enzyme, respectively, whereas the OEG group should extend from the peptide recognition site for surface attachment without affecting enzyme binding. The azobenzene units employed are similar to those previously used in a range of photoswitch peptides.[12–15] Previous studies[11] have found that substitution of an azobenzene (as required for attachment of the OEG group) can significantly affect photoswitching, but little is known with regard to suitable substrates for surface studies. A range of substituents were therefore investigated here.

The trifluoromethyl ketones **1** and **2** each contain an extended OEG linker that attaches the terminal alkyne and the azobenzene, through a second acetylene and an amide, respectively. In comparison, analogue **3** has the terminal alkyne directly attached to the azobenzene core. The alkyne and amide groups were investigated because they present significantly different structures and because of their initial ease of synthesis as linkers. The α-keto esters **6** and **7**, each with an alternative inhibitory group to the otherwise identical trifluoromethyl ketones **1** and **3**, respectively, allow comparative studies to begin to identify optimum groups for surface attachment and photoswitchable binding. The trifluoromethyl ketone **5** provides a solution-based model for surface-bound **3**, whereas the previously reported trifluoromethyl ketone

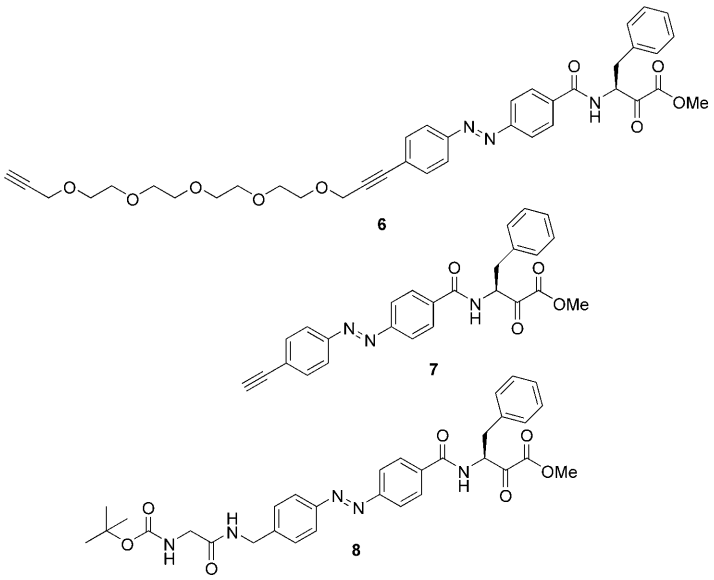


Figure 3. α-Keto ester photoswitch inhibitors.

4[16] and the α-keto ester analogue **8** provide further derivatives for comparative solution-based studies (see below).

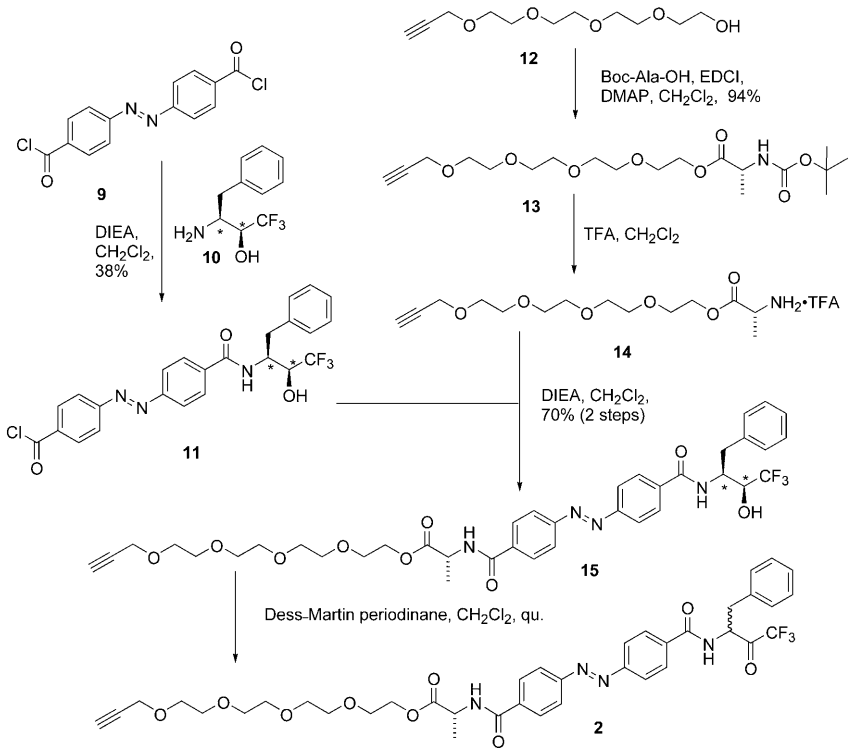
The extended C-terminal OEG linkers of **1**, **2**, and **6** were not expected to affect enzyme binding (or photoswitching) significantly because of the low specificity of α-chymotrypsin beyond the S_3 subsite.[11,17] However, the binding of α-chymotrypsin to compounds **3** and **7** after surface attachment might be expected to be significantly different, due to the likely participation of the less extended terminal alkyne/triazole group in enzyme binding. This paper thus begins to define the optimum chemical make-up of a photoswitchable peptide for surface attachment, which is critical if one is to begin to develop the generality of the approach. Additional work on extending our preliminary surface findings with further analysis and discussion on the binding of α-chymotrypsin to surface-attached **1** is also reported.

Results and Discussion

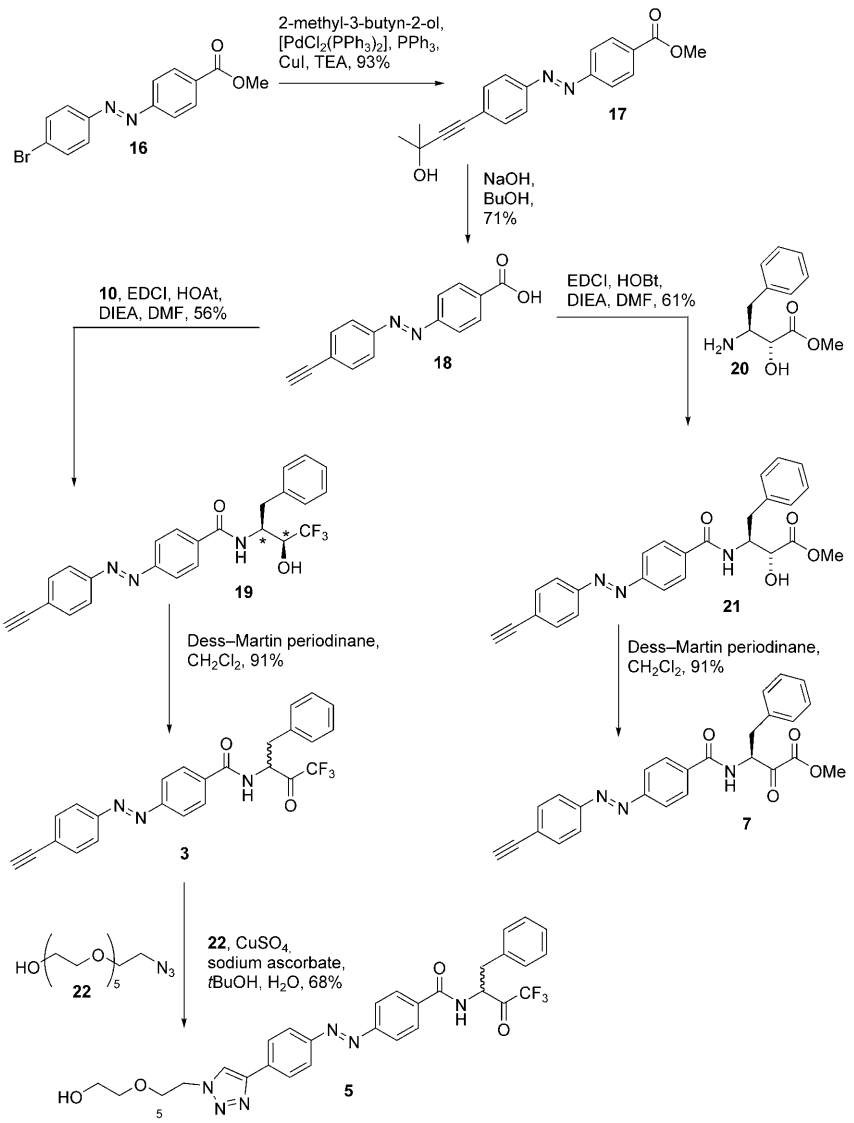
The trifluoromethyl ketone **2** was prepared from the symmetrical azobenzenedicarboxylic acid chloride **9**[18,19] and compound **12**[20] as shown in Scheme 1. Treatment of the acid chloride **9** with the amine **10**[21] in the presence of base (DIEA) gave the amide **11** in 38% yield. The alcohol **12** was treated with Boc-Ala-OH in the presence of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDCI) to give the amide **13** in 94% yield. This was then treated with TFA to give the primary amine **14**, which was treated with the acid chloride **11** in the presence of base (DIEA) to give the amide **15** in 70% yield over two steps. The alcohol **15** was finally oxidized to the ketone **2** with Dess–Martin periodinane[22] in quantitative yield.

The trifluoromethyl ketone **3** and the α-keto ester **7** were each prepared by means of a Sonogashira coupling reaction as the key step (Scheme 2). The bromoazobenzene **16**[23] was coupled to 2-methylbut-3-yn-2-ol in the presence of [Pd(PPh_3)$_2$$Cl_2$] to give the alkyne **17** in 93% yield. Both protecting groups were removed on hydrolysis with aqueous NaOH to give **18**, which was separately treated with **10** and with the α-hydroxy ester **20**[24] in the presence of EDCI as in similar syntheses[25] to give the amides **19** and **21**, respectively. Oxidation with Dess–Martin periodinane gave the ketones **3** and **7**, both in 91% yield. The triazole **5** was obtained by treatment of the azide **22** with the alkyne **3** in the presence of Cu^I catalyst formed in situ from $CuSO_4$ and sodium ascorbate.[26]

The extended derivatives **1** and **6** were prepared in a similar manner (Schemes 3 and 4, below). The dialkyne **23**[27] was treated with TMSCl to give the monoprotected alkyne **24** in 26% yield. Treatment of **24** with the bromoazobenzene **16** in the presence of [Pd(PPh_3)$_2$$Cl_2$] and CuI was attempted, but the desired product **26** was not obtained, possibly due to homocoupling of the alkyne **24**.[28] However,



Scheme 1. Preparation of the trifluoromethyl ketone **2**.



Scheme 2. Preparation of the trifluoromethyl ketones **3** and **5** and the α-keto ester **7**.

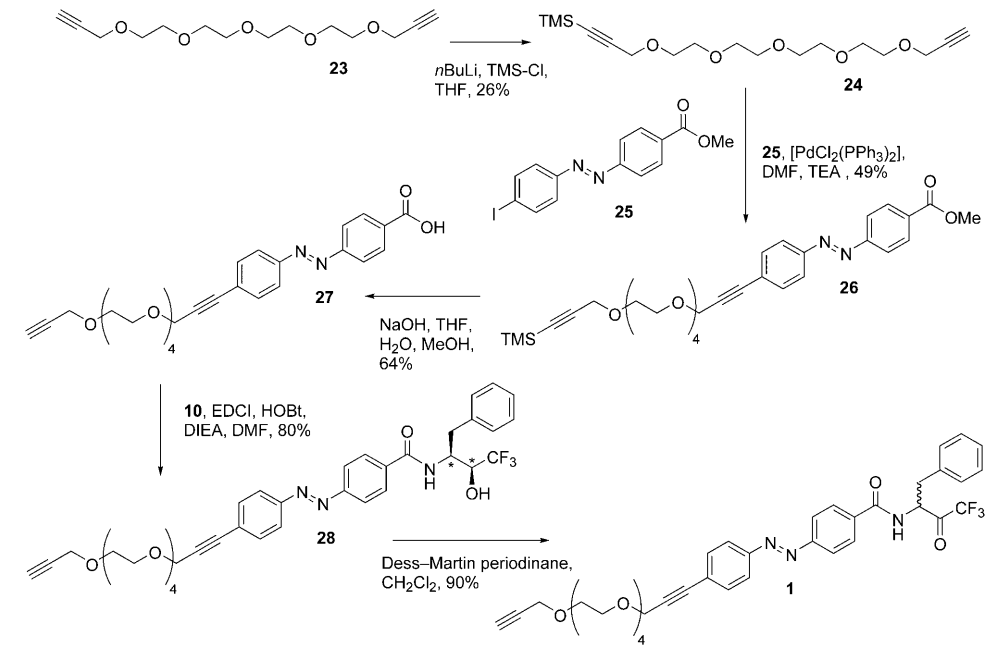
treatment of **24** with the corresponding iodide **25**[29] in the presence of $[Pd(PPh_3)_2Cl_2]$ gave the alkyne **26** in 49% yield. CuI was not added because copper has been reported to promote alkyne homocoupling.[30] NaOH hydrolysis of **26** gave the carboxylic acid **27** in 64% yield, and this was treated with the amine **10** in the presence of EDCI to give the amide **28** in 80% yield. Oxidation of **28** with Dess–Martin periodinane gave the desired trifluoromethyl ketone **1** in 90% yield.

The carboxylic acid **27** was also treated with the amine **20** (Scheme 4) to give the amide **29** in 58% yield. This was oxidized with Dess–Martin periodinane to give the α-keto ester **6** in 51% yield.

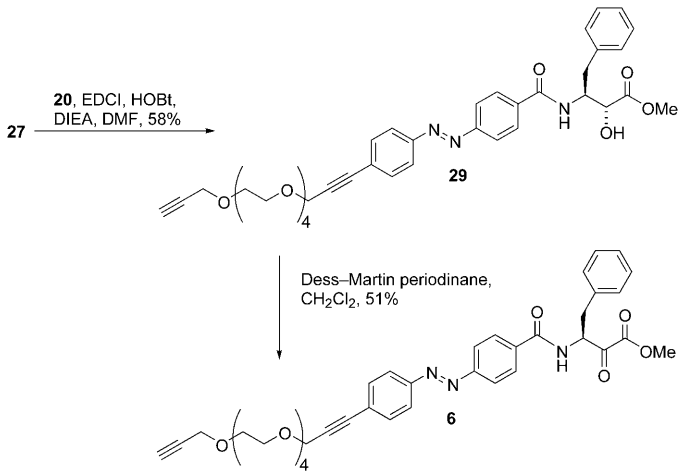
Coupling of **30**[11,16] with the amine **20** in the presence of EDCI, followed by oxidation of the resulting alcohol **31** with Dess–Martin periodinane, gave the α-keto ester **8** (Scheme 5).

Photoisomerization and inhibition assays were initially carried out in solution to assess the abilities of the designed compounds to photoswitch and to inhibit the enzyme. The compounds **1**–**8** were irradiated with UV light (320–380 nm) to obtain *Z*-enriched photostationary states. The *Z*-enriched states were then irradiated with visible light (>360 nm) to return the *E*-enriched photostationary states. ^{1}H NMR of these samples showed good photoisomerization (see Table 1) from an initial state of <10% *Z* isomer to 66–93% *Z* after UV irradiation and back to 8–21% *Z* after visible irradiation, with no observed decomposition (see Table 1).

The nature of the azobenzene substituent strongly influences the makeup of the photostationary states; compare, for example, compounds **2** and **4**, which have 93% and 66% of the Z isomer, respectively, after UV irradiation. These compounds are identical except in the nature of the attached azobenzene substituent. In contrast, the nature of the electrophilic "warhead" (enzyme-binding group) has little effect in this regard (compare compound pairs **1**/**6**, **3**/**7**, **4**/**8** in Table 1, with common azobenzene substituents, but different protease binding groups at their C termini). This observation is as would be expected, because photoisomerization is dependent on the electronic transitions of the azobenzene group, which are affected by the electron-withdrawing/donating effects of substituents.[31,32] Photoswitching is influenced both by the overlap of the UV/Vis absorption bands with the UV/Vis filters used (selected on the basis of good wavelength overlap with the appropriate bands; data not shown) and by the relative stabilities of the *E* and *Z* isomers. Each of the compounds studied here contains an electron-withdrawing amide substituent on one aromatic ring and a variable substituent on the other (alkyne, amide, CH_2-amide, or triazole). Whereas electron-donating substituents are known to red-shift absorption bands, often leading to better irradiation overlap, the electron-withdrawing or neutral substituents used here cannot be assumed to have a major effect on photoswitching without analysis of UV/Vis spectra. The best



Scheme 3. Preparation of the trifluoromethyl ketone **1**.



Scheme 4. Preparation of the α-keto ester **6**.

photoswitching (as measured by the decrease in the amount of *Z* isomer on visible irradiation) was exhibited by the alkynes **1** and **7**. Compounds with alkyne groups in the 4′-position (**1**, **3**, **6**, and **7**) generally gave better photoswitching than compounds with methylene (**4** and **8**) or triazole (**5**) groups in the 4′-position. Alkyne substituents have been shown to have no significant effect on UV/Vis absorption.[33] However, the conjugating effect of the triple bond may alter the influence of the electron-withdrawing amide substituent.

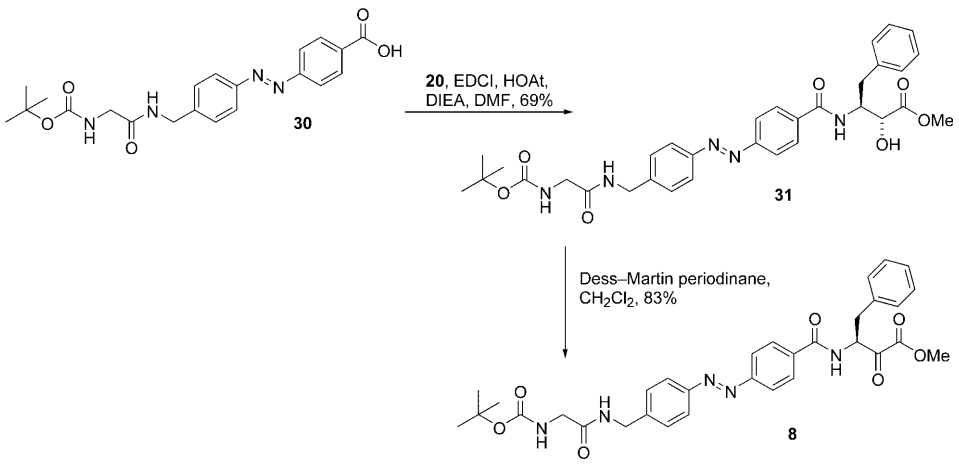
Comparisons with literature data are difficult, due to the wide variation in approaches to data reporting, but it can be noted that the CH_2-amide compounds **4** and **8** gave similarly good $E \rightarrow Z$ photoswitching to that previously reported for a compound with similar substituents,[14] but poor $Z \rightarrow E$ photoswitching.

The potential photoswitch inhibitors **1**–**8** were assayed against α-chymotrypsin as their initial *E*-enriched states, as their *Z*-enriched PSSs resulting from UV irradiation, and as their *E*-enriched PSSs after visible irradiation. The corresponding IC_{50} values were then calculated.

All the compounds were found to inhibit α-chymotrypsin, with IC_{50} values as shown in Table 2. Whereas the α-keto esters (**6**–**8**) exhibited normal inhibition characteristics, the inhibition by the trifluoromethyl ketones (**1**–**5**) appeared to increase over time. This slow increase in binding strength is consistent with the "slow-tight" binding mode.[34–36] In this case, it is likely that the slow binding is due to the active trifluoromethyl ketone species existing in equilibrium with their hydrates.[34] These hydrates were observed in the NMR spectra of selected trifluoromethyl ketones in (wet) acetone. To record steady-state hydrolysis rates, trifluoromethyl ketones were allowed to incubate with enzyme and substrate for 8 min before rate data were collected. Consequently, the IC_{50} values given for the trifluoromethyl ketones reflect the final "tight" binding. In all cases, the initial binding of trifluoromethyl ketones was too weak for accurate IC_{50} values to be obtained.

A range of structure/activity/photoswitching comparisons can be drawn from the data in Table 2. The α-keto esters (**6**–**8**) are more potent inhibitors than the trifluoromethyl ketones (**1**–**5**), with inhibition constants ranging from 0.23 to 2.2 μM for the α-keto esters and 12 to >85 μM for the trifluoromethyl ketones. This suggests that the α-keto ester group binds more rapidly and/or strongly to the Ser195 residue[17] of the active site. Both α-keto esters and trifluoromethyl ketones are more active as the *Z*-enriched PSSs obtained from UV irradiation, as previously reported for these classes of photoswitch inhibitors[16,37] and for a related boronate ester.[11]

It is clear that the structural requirements for potent binding of the trifluoromethyl ketones are very different from those of the α-keto esters, despite the fact that both inhibit serine proteases through their constituent electrophilic carbonyl groups. In particular, the *Z*-enriched forms of the trifluoromethyl ketones exhibit a narrow range of inhibition constants (12–17 μM) whereas the *E*-enriched forms vary considerably (19–85 μM), Therefore, the 4′-substituents have little impact on the potencies of the *Z*-trifluoromethyl ketones, but a major effect on those of the *E*-trifluoromethyl



Scheme 5. Preparation of the α-keto ester **8**.

Table 1. *E*/*Z* compositions obtained by UV/Vis photoisomerization of azobenzenes **1**–**8**.

Compound	Inhibitor type	Amount of *Z* isomer before irradiation [%]	Amount of *Z* isomer after UV irradiation [%]	Amount of *Z* isomer after visible irradiation [%]	Decrease in *Z* isomer after visible irradiation
1	trifluoromethyl ketone	6	85	8	11-fold
2	trifluoromethyl ketone	5	66	9	7.3-fold
3	trifluoromethyl ketone	5	87	12	7.3-fold
4[16]	trifluoromethyl ketone	<5	93	16	5.8-fold
5	trifluoromethyl ketone	7	76	18	4.2-fold
6	α-keto ester	6	88	14	6.3-fold
7	α-keto ester	<5	85	7	12-fold
8	α-keto ester	5	94	14	6.7-fold

Table 2. Inhibition constants (IC_{50}) for assay against α-chymotrypsin.

Compound	Inhibitor type	IC_{50} before irradiation [μM]	IC_{50} after UV irradiation [μM]	IC_{50} after visible irradiation [μM]	Decrease in activity after visible irradiation
1	trifluoromethyl ketone	>51	14	>51	>3.6
2	trifluoromethyl ketone	20	17	21	1.2
3	trifluoromethyl ketone	>85	16	>85	>5.3
4[16]	trifluoromethyl ketone	32	13	29	2.2
5	trifluoromethyl ketone	19	12	21	1.8
6	α-keto ester	2.0	0.73	2.2	3.0
7	α-keto ester	1.0	0.47	0.85	1.8
8	α-keto ester	0.71	0.23	0.59	2.6

ketones. In comparison, the inhibition constants of both the *E*- and the *Z*-enriched states of the α-keto esters vary significantly between compounds, showing that the natures of the 4′-substituents affect the potencies of both the *E*- and the *Z*-α-keto esters. As a result, the differences in activity between the *E* and *Z* isomers of the inhibitors (i.e., the extent of enzyme photoswitching) vary more between individual trifluoromethyl ketones than between individual α-keto esters. For example, the best and the worst photoswitching results are observed for trifluoromethyl ketones (**3**/**1** and **2**, respectively), whereas all of the α-keto esters exhibit intermediate photoswitching of 1.8- to 3.0-fold changes in activity between isomers.

A comparison of the 4′-azobenzene substituents in the trifluoromethyl ketones shows that the strongest binding is exhibited by the triazole **5** and the weakest by the alkynes **1** and **3**. Similarly, the alkynes **6** and **7** exhibit the weakest binding of the α-keto esters. However, the best enzyme photoswitching is exhibited by alkynes **1** and **3**, which give >3.6- and >5.3-fold changes in activity on photoisomerization, respectively. Because the *Z*-trifluoromethyl ketones all exhibit similar potencies, the excellent photoswitching of **1** and **3** is due to particularly weak binding of the *E* isomers of these compounds, relative to other *E*-trifluoromethyl ketones.

The trifluoromethyl ketones **1** and **3** exhibited the best enzyme photoswitching results. However, surface attachment of alkyne **3** would yield a triazole of identical local structure to **5** in the region expected to bind to the enzyme. Triazole **5** is a poor photoswitch, so it is likely that **3** would give poor photoswitching results after surface attachment, unless the poor photoswitching of **5** is due to an interaction with the terminal OH group. Consequently, the trifluoromethyl ketone **1** was chosen for use in surface enzyme photoswitching. The alkyne **1** has the additional advantage of containing an extended OEG group, which should facilitate enzyme binding by spacing the inhibitor from the surface (see discussion earlier).

The trifluoromethyl ketone **1** was attached to commercially available surface plasmon resonance (SPR) surfaces (detailed in ref. [7]). The binding of α-chymotrypsin to the surface with attached **1** was then monitored by SPR (also briefly discussed in Ref. [7]). Solutions of enzyme (0, 2.0, 6.0, and 18 μM) were separately injected over the surface for periods of 300 s to monitor enzyme binding to the surface, and after each experiment, buffer was run over the surface for 300 s to monitor detachment of enzyme (Figure 4, enzyme injection at $t=70$ s).

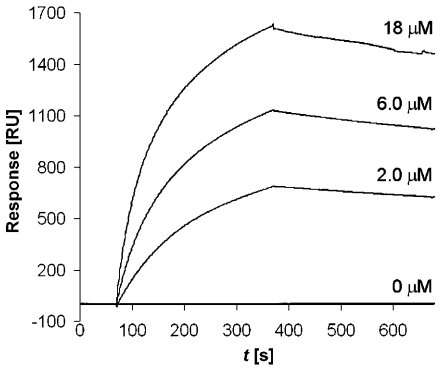


Figure 4. SPR sensorgrams for binding of α-chymotrypsin (0, 2.0, 6.0, 18 μM) to surfaces modified with **1**. SPR data simultaneously recorded for a control surface were subtracted from these plots.

Remaining enzyme was washed from the surface by injections of guanidine (6 M, 5 μL), followed by acetic acid (1 M, 10 μL), to regenerate the surface rapidly after each measurement. For each nonzero enzyme concentration, the SPR response increased over the period during which the enzyme was injected relative to the surface control lacking attached **1**. Furthermore, the response was enhanced for each increase in enzyme concentration (0→18 μM in Figure 4), which is consistent with attachment of enzyme to the surface. This represents binding of enzyme to approximately 10% of the theoretical maximum number of surface-attached inhibitor molecules,[7] at the highest enzyme concentration.

Calculation of the K_D affinity constant for enzyme binding to the surface-bound inhibitor from this data was attempted by use of a curve-fitting model (with the aid of Biacore Biaevaluation software). However, no good fit could be obtained by simultaneous modeling of all three concentration curves. Individual modeling of the association and dissociation regions for each curve gave calculated k_A, k_D, and K_D values as shown in Table 3.

Table 3. Apparent k_A, k_D, and K_D constants for binding of α-chymotrypsin to a surface containing attached inhibitor **1**, calculated by individual curve fitting to SPR data.

Enzyme concentration [μM]	k_A [$M^{-1}s^{-1}$]	k_D [s^{-1}]	K_D [μM]
2.0	3260±70	$3.24\times10^{-4}\pm4\times10^{-6}$	0.099±0.003
6.0	1400±20	$3.25\times10^{-4}\pm2\times10^{-6}$	0.233±0.005
18	590±4	$3.19\times10^{-4}\pm2\times10^{-6}$	0.541±0.007

The apparent affinity constant (K_D) increases with increasing enzyme concentration, corresponding to apparently weaker binding at higher enzyme concentrations. This is surprising, because a constant K_D value would be expected. The apparent kinetic dissociation rate constant (k_D) is approximately constant at all enzyme concentrations, but the apparent kinetic association rate constant (k_A) decreases with increasing enzyme concentration. This shows that the rate of association of α-chymotrypsin with surface-bound **1** is decreased at high enzyme concentrations. This trend of a decrease in the apparent association constant with increasing enzyme concentration has also been observed in solution-phase studies of the binding of trifluoromethyl ketones to α-chymotrypsin.[34] The decrease in k_A was attributed to an equilibrium between the active ketone inhibitor and an inactive hydrated form, as shown in the equation:

$$I_h \underset{H_2O}{\rightleftharpoons} I_k \overset{E}{\rightleftarrows} E\text{-}I \qquad (1)$$

where I_h represents hydrated inhibitor, I_k represents ketone inhibitor, E represents α-chymotrypsin, and E–I represents the enzyme–inhibitor complex. In aqueous solutions, the hydrated form I_h is known to be present in an approximately 4500-fold excess.[34] At a high concentration of enzyme, the concentration of ketone will be significantly decreased by reaction with the enzyme and it has previously been proposed that a relatively slow rate of hydrate to ketone dehydration should lead to a decrease in the actual concentration of ketone, dependent on the enzyme concentration. This would lead to a decrease in the apparent k_A value as the enzyme concentration increases, and thus an increase in the apparent K_D value. We propose that the surface-attached trifluoromethyl ketone in this study is exhibiting the same behavior. Alternative explanations for slow binding kinetics, such as those proposed for α-keto acid inhibitors of a hepatitis C protease,[35] do not explain the observed decrease in association rate at high enzyme concentrations.

Next, the enzyme-free surface was removed from the SPR instrument and irradiated with UV light (carried out as above for solution-phase irradiations) to photoisomerize surface attached (*E*)-**1** to (*Z*)-**1**. The surface was returned to the instrument, and injection of enzyme (2.0, 6.0, 18 μM) was repeated to assess the extent of enzyme binding after this photoisomerization (see Figure 5, curve 2 for 2.0 μM injection, Table 4 for maximum SPR responses, and Table 5 for apparent binding constants).

A significantly higher response (1200 RU for the 2.0 μM injection, Figure 5, curve 2) was observed than before irradiation (690 RU for the 2.0 μM injection, Figure 5, curve 1). Therefore, significantly more enzyme binds to the surface after UV irradiation. The apparent k_A and k_D constants after UV irradiation are greater than those observed before irradiation (compare Table 3 and Table 5), representing more rapid attachment and detachment of α-chymotrypsin from the surface. This is as would be expected for a larger proportion of the more active *Z* isomer at the surface. However, the increase in enzyme binding is only approximately twofold, much less than the 11-fold change in the proportion of *Z* isomer observed in solution (see Table 1). This is at least partially attributable to enzyme binding by the weaker *E* isomer that is expected to be predominantly present at the surface before UV irradiation, and possibly also to decreased *E*/*Z* photoswitching at the surface. These factors could not be assessed in detail for the surface-bound inhibi-

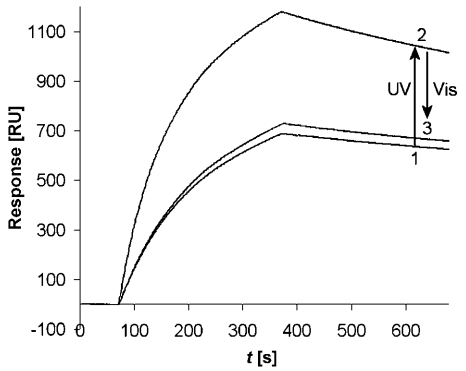


Figure 5. SPR sensorgrams for photoswitching of α-chymotrypsin (2.0 μM) binding to a surface modified with **1**. SPR data simultaneously recorded for a control surface were subtracted from these plots. 1) surface before irradiation, 2) surface after UV irradiation, and 3) surface after UV and then visible irradiation.

Table 4. Maximum SPR response (RU) for binding of α-chymotrypsin binding to surface attached inhibitor **1**, before and after UV/Vis irradiations.

Enzyme concentration [μM]	Maximum SPR response before irradiation (RU)	Maximum SPR response after UV irradiation (RU)	Maximum SPR response after visible irradiation (RU)
2.0	690	1200	720
6.0	1100	1700	1200
18	1600	2100	1700

Table 5. Apparent k_A, k_D, and K_D constants for binding of α-chymotrypsin to a surface containing attached inhibitor **1** after UV irradiation, calculated by individual curve fitting to SPR data.

Enzyme concentration [μM]	k_A [$M^{-1}s^{-1}$]	k_D [s^{-1}]	K_D [μM]
2.0	4220 ± 70	$5.02\times10^{-4}\pm1\times10^{-6}$	0.119 ± 0.002
6.0	1890 ± 20	$4.82\times10^{-4}\pm1\times10^{-6}$	0.255 ± 0.003
18	748 ± 6	$4.43\times10^{-4}\pm1\times10^{-6}$	0.593 ± 0.006

tor. After removal of attached enzyme as above, the surface was irradiated with visible light to "switch back" to the less active *E*-enriched state [(*E*)-**1**], and injection of enzyme was repeated. The response (720 RU for the 2.0 μM injection, Figure 5, curve 3, Table 6) was close to the initial response before UV irradiation, corresponding to a similar amount of enzyme binding.

Table 6. Apparent k_A, k_D, and K_D constants for binding of α-chymotrypsin to a surface containing attached inhibitor **1** after visible irradiation, calculated by individual curve fitting to SPR data.

Enzyme concentration [μM]	k_A [$M^{-1}s^{-1}$]	k_D [s^{-1}]	K_D [μM]
2.0	3060 ± 50	$3.31\times10^{-4}\pm1\times10^{-6}$	0.108 ± 0.002
6.0	1330 ± 10	$3.22\times10^{-4}\pm1\times10^{-6}$	0.242 ± 0.003
18	533 ± 3	$2.98\times10^{-4}\pm1\times10^{-6}$	0.559 ± 0.004

These results show that the surface can be reversibly photoswitched between two states, one of which binds a significantly larger amount of enzyme. Photochemical switching can therefore modulate binding of α-chymotrypsin to a surface.

Conclusion

A new series of azobenzene-containing electrophilic ketones has been synthesized and assayed against α-chymotrypsin as both their *E*- and *Z*-enriched photostationary states. All compounds (**1**–**8**) inhibited the enzyme with activity that could be modulated by irradiation. The α-keto esters exhibited 1.8- to 3.0-fold changes in activity on photoisomerization, whereas the trifluoromethyl ketones exhibited 1.2- to >5.3-fold changes in activity. The best enzyme photoswitching was exhibited by trifluoromethyl ketones with alkyne groups as 4′-substituents on their azobenzene groups, due to the relatively weak inhibition exhibited by the *E* isomers of these compounds. These results show that the photoswitching of azobenzene-based photoswitch inhibitors can potentially be optimized through the use of a relatively bulky 4′-azobenzene substituent (e.g., alkyne) that interferes with inhibitor binding by the *E* (or *Z*) form of the photoswitch. The best candidate, the trifluoromethyl ketone **1**, was attached to an SPR chip in a two-step procedure involving an amide coupling followed by an azide–alkyne cycloaddition. Enzyme binding to the modified surface was detected, but no K_D affinity constant could be calculated, due to "slow-tight" binding of enzyme to the surface-bound inhibitor. Irradiation of the surface with UV/Vis light modulated the amount of enzyme that attached to the surface. As in the cases of previously reported solution studies, a decrease in the apparent association constant is observed for the surface binding of trifluoromethyl ketones to α-chymotrypsin. This is attributable to an equilibrium between the active ketone inhibitor and an inactive hydrated form.

Experimental Section

General: NMR spectra were obtained with a Varian INOVA spectrometer, operating at 500 MHz for ^{1}H NMR and at 126 MHz for ^{13}C NMR, or with 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 with the Varian INOVA spectrometer operating at 500 MHz. Electrospray ionization mass spectra were detected with a Micromass LCT TOF mass spectrometer operating in electrospray mode with acetonitrile/H_2O (50%) as solvent. Dry THF was distilled from sodium or potassium, and dry DCM was distilled from CaH_2. EtOAc, NMM, AcOH, and EtOH were distilled before use.

Photoisomerization: Compounds were photoisomerized by irradiation with UV or visible light from a Mercury arc lamp (500 W). 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 CD_3CN (≈1.5 mg mL^{-1}), and irradiated for 70 min with UV light or for 30 min with visible light. The ratios of *E*/*Z* isomers before and after irradiation

were determined by ^{1}H NMR analysis by use of the integrals of well-separated peaks. The major isomer was assigned the thermodynamically more stable *E* configuration on the basis of literature precedence.[37,38] 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 photoisomerization were carried out in dim lighting conditions (that is, 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 deionized water (75 mL), adjusted to pH 7.8 with HCl solution (1 M), 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) with the aid of 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 ($\varepsilon_{315}=14000\ L\,mol^{-1}\,cm^{-1}$).

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,[39] 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} values rather than K_i values. Briefly, inhibitors were dissolved in CD_3CN 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 for α-keto esters or 10 min for trifluoromethyl ketones. Absorbance vs. time plots were obtained, and the slopes were used to find the initial rates for α-keto esters or the final rates for trifluoromethyl ketones. From the differences between these rates and the rates for the acetonitrile blanks, the percent inhibitions were calculated. Each 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} value was interpolated.

Surface plasmon resonance experiments: SPR was performed with a Biacore 2000 instrument. All solutions were filtered through a 0.22 μmfilter and centrifuged (1300 rpm, 10 s) before use.

Buffers used: HEPES [0.01 M, pH 7.4 containing NaCl (0.15 M) and EDTA (3 mM)], Tris [0.1 M, pH 7.8, containing $CaCl_2$ (0.02 M) and P20 surfactant (12.5 μL)].

Compound **1** was immobilized to an SPR chip as follows. A Biacore CM5 chip was docked in the SPR instrument and prepared by priming and running a sensorgram in HEPES buffer at 100 $\mu L\,min^{-1}$ flow rate, with injection of NaOH (50 mM, 20 μL × 2), HCl (10 mM, 20 μL × 2), and SDS (0.1%, 20 μL × 2). Surface modification was then carried out at 5 $\mu L\,min^{-1}$ flow rate by injection of EDCI/*N*-hydroxysuccinimide (35 μL), followed by 11-azido-3,6,9-trioxaundecan-1-amine (**32**, 11 $mg\,mL^{-1}$ in HEPES buffer, 3 × 5 μL) and then ethanolamine (1 M, pH 8.5, 35 μL). During attachment of **32**, the SPR resonance increased by 147 RU. The chip was then undocked and removed from the instrument and rinsed with water. A solution of **1** (0.3 mg, 0.44 μmol), CuBr (0.063 mg, 0.44 μmol), 2,6-lutidine (0.094 mg, 0.88 μmol), 2,2′-bipyridine (0.14 mg, 0.88 μmol), and sodium ascorbate (0.17 mg, 0.88 μmol) in DMF/H_2O (1:1, 150 μL) was pipetted onto the surface and left for 1 h at room temperature. The surface was then rinsed with DMF/H_2O (1:1), H_2O, EDTA (0.1 M), DMF/H_2O (1:1), and H_2O and was then redocked in the SPR instrument. A sensorgram was run in Tris buffer for several hours in order to obtain a stable baseline. A reference cell was prepared by the same method except with omission of the injection of **32**.

Enzyme binding to this modified surface was monitored by running a sensorgram in Tris buffer at 30 $\mu L\,min^{-1}$. A range of α-chymotrypsin solution concentrations were injected over the surface and monitored as follows:

An α-chymotrypsin solution (0, 2.0, 6.0, or 18 μM in Tris buffer, 150 μL) was injected over a period of 5 min, flow rate 30 $\mu L\,min^{-1}$. After the injection was complete, buffer was run for 5 min to monitor dissociation of enzyme from the surface, and then the surface was regenerated. Regeneration was carried out at 5 $\mu L\,min^{-1}$ in Tris buffer by the injection of guanidine hydrochloride (6 M, 5 μL) followed by acetic acid (1 M, 10 μL), and buffer was then run for 5 min.

For photoswitching experiments, the above enzyme binding assay was carried out, and the chip was then regenerated, removed from the instrument, rinsed with water, and irradiated with UV light (as detailed above for solution-phase photoisomerization) for 10 min. The chip was then replaced in the SPR instrument, and the enzyme binding assay was repeated. The photoisomerization process was repeated with visible light irradiation and this assay was repeated.

Details of synthesis and NMR spectra are given in the Supporting Information.

Acknowledgements

The authors thank Andrew Muscroft-Taylor for synthesis of compound **26**, Nathan Alexander for synthesis of compound **10**, and Fiona Clow for help with SPR. Financial support from the Royal Society of NZ, the Marsden Fund and the ARC (DP0771901) is also gratefully acknowledged. D.P. was funded by a TEC Bright Future Top Achiever Doctoral Scholarship.

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Received: December 9, 2009
Published online: May 7, 2010

