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# α-Ketoester-Based Photobiological Switches: Synthesis, Peptide Chain Extension and Assay against α-Chymotrypsin

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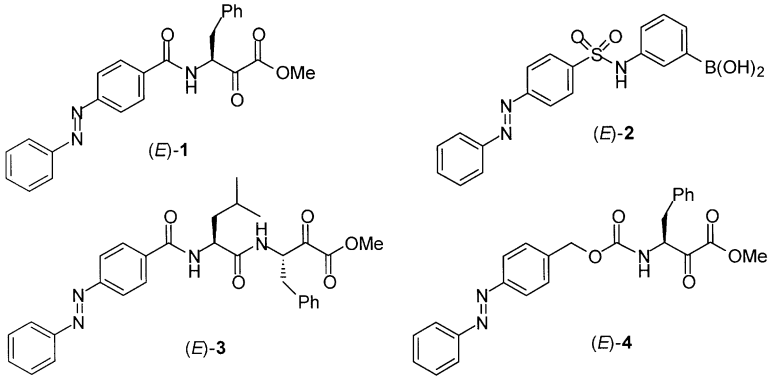
**Abstract**—The design, synthesis, photoisomerism and biological testing of two peptide-based photoswitchable inhibitors of α-chymotrypsin are presented. The use of a dipeptide recognition sequence gave a 'slow-tight binding' inhibitor, while the introduction of a carbamate linker to the azobenzene gave a modest enhancement in photoswitching of enzyme activity for the photostationary state enriched in the (*Z*)-isomer over the (*E*)-isomer. 

The activity of a biological system, such as an enzyme, can be modulated by incorporating a photobiological switch into its structure.[1] Examples of such photobiological switches include photoisomerisable enzyme inhibitors and co-factors,[2] covalently modified enzymes,[3] and enzymes immobilised on photoisomerisable polymers.[4] Photoisomerisable inhibitors of α-chymotrypsin have been reported in which an azobenzene group is tethered to an inhibitory group (e.g., an α-keto ester as in compound **1**[5] or a boronic acid as in **2**).[6] These compounds, while lacking extended peptide binding domains, are reported to exhibit modest photoswitching of enzyme activity for photostationary states enriched in either the (*Z*)-isomer or the (*E*)-isomer.

In this paper, we begin to address the effect of increasing the peptidic character (the enzyme binding domain) and also the nature of the azobenzene-based substituent (the photoswitch) on the potency[7] and switchability[8] of inhibitors of the type **1**. The literature inhibitor **1**[5] served as a template for the design of compounds **3** and **4**. Unlike **1**, compound **3** contains residues at both the $P_1$ and $P_2$ positions[9] [i.e., (L)-phenylalanine and (L)-leucine, respectively]. Compound **4** contains a carbamate

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linker to the azobenzene photoswitch, rather than an amide, as in **1**. This linker represents an analogue of the benzyloxycarbonyl (CBz) group, which has been used at the N-terminus of other α-ketoester-based serine protease inhibitors. It should also be noted that the nature of the azobenzene substituent has been reported to influence the *E*/*Z* composition of photostationary states (PSS) of compounds containing this photoswitch.[8] We now report synthesis, photoisomerisation and inhibition studies on these new photoswitchable inhibitors of α-chymotrypsin.

Compound **3** was prepared from 4-(phenylazo)benzoic acid **5** (85% yield over four steps) as detailed in Scheme 1. The sequenece involved coupling **5** with (L)-leucine methyl ester, followed by ester hydrolysis and subsequent coupling with the amine **10**[5] to give **8**. The hydroxyl group of **8** was then oxidized with TEMPO/NaOCl[5] to afford the desired α-ketoester **3** as a single diastereomer by $^1$H and $^{13}$C NMR spectroscopy. It is noteworthy that this reaction proceeded without evidence of epimerisation at the β-carbon (α to the ketone of **3**).[10] Compound **4** was synthesised via the carbamate **11**, which was itself prepared in one-pot by reaction of the alcohol **9** with triphosgene, followed by the addition of the amine **10**. The resulting alcohol **11** was oxidised to the α-ketoester **4** using the TEMPO/NaOCl procedure.

Separate solutions of **3** and **4**, in acetonitrile-$d_3$, were then photoisomerised, under three sets of conditions (see Table 1), giving isomer-enriched photostationary states, the compositions of which were quantified by $^1$H NMR spectroscopy.[11] Each of these acetonitrile-$d_3$ solutions was observed, by $^1$H NMR spectroscopy, to undergo hydration of the ketone functional group to give the corresponding *gem*-diol. This resulted in four species being present in each of the irradiated solutions (see Table 1). A *gem*-diol derivative is known to be the predominant species in an aqueous solution of an α-ketoester or α-ketoamide[12] (i.e., the conditions of an enzyme assay). Therefore, the ratio of ketone to hydrate in these solutions is not as important in determining the effect of the mixture on the enzyme as is the ratio of (*E*)- to (*Z*)-isomers.

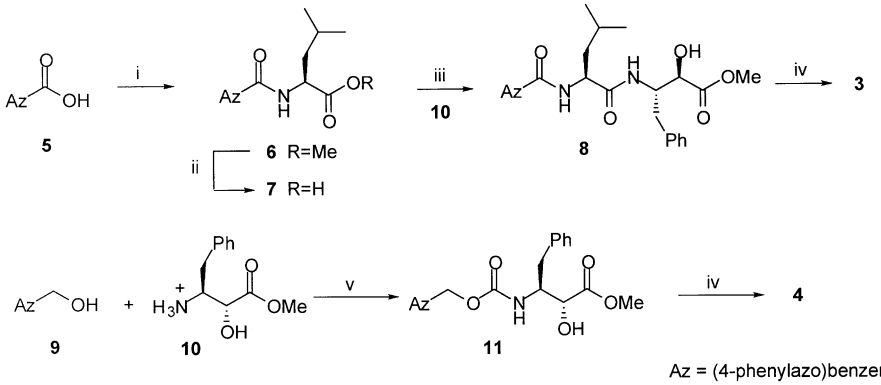
From the results in Table 1, it is apparent that the PSS arising from ambient light serves as a reasonably good approximation for the visible light PSS ($\lambda > 400$ nm) in all cases studied, a trend also noted by Vollmer et al.[13] It can also be seen that **1** and **3**, which have the same azobenzene substituent, gave similar *E*/*Z* ratios under each of the three sets of conditions. However, **4** exhibited comparatively more (*E*)-isomer in the ambient and visible PSS and less (*E*)-isomer in the UV PSS compared to **1** and **3**. Consequently, **4** possesses the best marginal switching between isomer-enriched states at the specified wavelengths of the three inhibitors.

Next, the inhibitors **3** and **4** were assayed[5] against α-chymotrypsin using both isomer-enriched photostationary states and the results obtained were compared to the known inhibitor **1** (see Table 2 for results on **1**[5] and **4**). Compounds **1** and **4** gave initial-rate kinetics that were consistent with competitive inhibition,[14] as might be anticipated for compounds comprising an electrophilic carbonyl moiety.[15] For both these inhibitors, the UV light PSS gave the lower inhibition constant, indicating that the (*Z*)-isomer was the more active

**Table 1.** Photostationary state (PSS) compositions of the inhibitors in acetonitrile-$d_3$

| Inhibitor | PSS composition at specified wavelength[a] (*E*-keto):(*E*-hydrate):(*Z*-keto):(*Z*-hydrate) | | |
|---|---|---|---|
| | $330 > \lambda > 370$ nm | $\lambda > 400$ nm | Ambient |
| **1**[5] | 20:7:47:26 (27:73) | 52:20:20:8 (72:28) | 42:32:15:11 (74:26) |
| **3** | 18:6:56:20 (24:76) | 44:30:18:8 (74:26) | 27:50:16:7 (77:23) |
| **4** | 16:6:56:22 (22:78) | 75:2:23:n.o. (77:23) | 80:2:18:n.o. (82:18) |

[a]Combined *E*/*Z* in parentheses.
n.o., not observed.




**Scheme 1.** Reagents and conditions: (i) (L)-leucine methyl ester hydrochloride, EDCI, HOBT, DIEA, DMF, $CH_2Cl_2$, 16 h, 95%; (ii) LiOH, MeOH, water, 30 min, qu; (iii) EDCI, HOBT, DIEA, DMF, $CH_2Cl_2$, 16 h, 90%; (iv) TEMPO, KBr, NaOCl, $NaHCO_3$, water, $CH_2Cl_2$, qu; (v) **9**, $(CCl_3O)_2CO$, DIEA, $CH_2Cl_2$, 0 °C to rt, then **10**, DIEA, $CH_2Cl_2$, rt, 30 min, 41%.

**Table 2.** The inhibition constants for the inhibition of α-chymotrypsin by the UV and ambient light PSS of inhibitors **1** and **4**

| | PSS *E*/Z | $K_i$ (μM) | $\Delta K_i$[a] |
|---|---|---|---|
| **1** ambient[5] | 74:26 | 0.24 | 0.11 μM (~2-fold) |
| **1** UV[5] | 27:73 | 0.13 | |
| **4** ambient | 82:18 | 0.77 | 0.48 μM (~3-fold) |
| **4** UV | 22:78 | 0.29 | |

[a]Difference between ambient and UV $K_i$ values.

isomer in each case. Although the UV light PSS of **4** proved to be approximately 2-fold less active than the UV light PSS of **1**, it did exhibit more effective switching of α-chymotrypsin than compound **1** (i.e., ~3-fold). This result is consistent with the relative *E*/*Z* compositions of **1** and **4** under ambient and UV conditions (see Table 2).

Inhibitor **3** was identified as a 'slow-tight binder' of α-chymotrypsin.[16] Inhibitors of this nature are identified by the slow onset of inhibition in assays with no pre-incubation of inhibitor with enzyme, but an increase in rate over time for pre-incubated assays.[17] Pre-incubation of **3** with enzyme followed by the addition of substrate gave a slow initial rate, $V_B = 90$ μmol$^{-1}$ mg$^{-1}$, which stabilised to a constant rate, $V_S = 170$ μmol$^{-1}$ mg$^{-1}$, after about 5 min. Addition of the enzyme to a mixture of inhibitor and substrate gave a high initial rate, $V_A = 490$ μmol$^{-1}$ mg$^{-1}$, which tended downward over time reaching a steady rate $V_S = 170$ μmol$^{-1}$ mg$^{-1}$ after about 5 min. The rate in the absence of inhibitor was measured at 950.[16] 'Slow-tight binding' has been observed for a number of α-ketoester, trifluoromethylketone and aldehyde inhibitors of serine proteases.[15,17,18] Evidence from the trifluoromethylketone and aldehyde inhibitors suggests that a pre-binding equilibrium from hydrate to the putative active species, ketone, leads to the slow onset of inhibition.[19]

In summary, the reported photobiological switch **1** was used as a template for the design and synthesis of the inhibitors **3** and **4**. Methodology is presented to incorporate an azobenzene switch into extended α-ketoester peptidomimetics such as in **3**. Inhibitor **4** displayed improved switching action of α-chymotrypsin compared with the template compound. This result is due, at least in part, to the change in azobenzene substitution between the two compounds, which led to more effective isomer enrichment of **4** by irradiation compared with compound **1**. Elaboration of **1** to give the dipeptide mimic **3** led to the synthesis of an inhibitor of α-chymotrypsin that displayed 'slow-tight binding' inhibition. These results provide an insight into the constraints of shape and polarity in the binding site of azobenzene-containing inhibitors of α-chymotrypsin.

## Acknowledgements

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11. A solution of the compound under study (5 mg) in acetonitrile-$d_3$ (150 μL) was irradiated for 1 h with light from a high pressure mercury arc lamp filtered to allow passage of wavelengths between 330 and 370 nm. The $^1$H NMR spectrum was recorded and the components of the mixture were identified by integration of the OMe resonances. The visible light PSS was then obtained by irradiation of the sample with wavelengths over 400 nm and the mixture composition was again determined. Finally, the solution was allowed to photoequilibrate under fluorescent lighting for 24 h and the resulting mixture composition was measured. The OMe resonance for the (*E*)-isomers is downfield by approx. 0.04–0.06 ppm relative to the (*Z*)-isomer. The OMe resonance for the keto isomers is downfield by approx. 0.06–0.08 ppm relative to the corresponding hydrates. See ref 5 for details.
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