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Photoregulation of α-Chymotrypsin Activity by Spiropyran-Based Inhibitors in Solution and Attached to an Optical Fiber

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Abstract: Here the synthesis and characterization of a new class of spiropyran-based protease inhibitor is reported that can be reversibly photoswitched between an active spiropyran (SP) isomer and a less active merocyanine (MC) isomer upon irradiation with UV and visible light, respectively, both in solution and on a surface of a microstructured optical fiber (MOF). The most potent inhibitor in the series (**SP-3 b**) has a C-terminal phenylalanyl-based α-ketoester group and inhibits α-chymotrypsin with a K_i of 115 nM. An analogue containing a C-terminal Weinreb amide (**SP-2 d**) demonstrated excellent stability and photoswitching in solution and was attached to the surface of a MOF. The SP isomer of Weinreb amide **2 d** is a competitive reversible inhibitor in solution and also on fiber, while the corresponding MC isomer was significantly less active in both media. The ability of this new class of spiropyran-based protease inhibitor to modulate enzyme activity on a MOF paves the way for sensing applications.

Introduction

An ability to detect the on/off binding of a bioligand to a complementary surface-bound receptor provides a basis of real-time sensors of wide applicability.[1] The associated "on" and "off" states are typically modulated photochemically, by means of a component azobenzene, or other photochromic substructure.[2] One problem with switchable sensors of this type is that they generally lack an ability to be tailored to a range of ligands, with each specific application requiring a different base system.[1b,e,2,3] For example, an azobenzene-based molecular glue has been reported to hybridize DNA strands with mismatch mutations on a gold surface.[3a] While this permits reversible control of DNA hybridization with an external light stimulus, the molecule only binds to a specific nucleotide sequence rather than a range of such structures.

More general and modular photoswitchable systems capable of targeting families of biomolecules, in a controlled and predictable manner, are needed to advance this area. With this in mind we recently reported a switchable surface containing an azobenzene core to which was attached a peptidomimetic trifluoromethyl ketone, the backbone structure of which can be tailored to inhibit a particular class or example of protease.[4] This system was immobilized onto gold surface by Huisgen 1,3-dipolar cycloaddition to provide an on/off switch and basis of a biosensor. Irradiation with UV (or visible light) reversibly controls the geometry of the azobenzene, and the associated inhibitory activity.[4,5]

The use of azobenzenes to regulate enzyme activity in this way is, however, somewhat limited since the two photostationary states often exhibit only a small difference in activity.[6] In addition, the fluorescence intensities of both switchable states are low,[7] such that detecting and measuring the extent of isomerization can be problematic. Finally, the *cis*-azobenzene usually undergoes spontaneous thermal isomerization back to the low-energy *trans*-isomer.[8] Given these limitations, there is a need to develop new photoswitchable systems.

We now present a new class of serine protease inhibitor based on a photoswitchable spiropyran core that mimics a peptide backbone as found in protease inhibitors. This structure is amenable to functionalization to target a particular protease, and also for attachment to a microstructured optical fiber (MOF) core surface for biosensor development (Figure 1). The fiber has a duel function; it facilitates irradiation of the spiropyran with light of a specific wavelength (532 nm) to bring about photoswitching and also the detection of changes in fluorescence produced on binding to the protease. There are reports on the nonspecific covalent attachment of a spiropyran to α-chymotrypsin in order to modulate its activity.[9] However, our system is unique in that we designed this structural unit to mimic the extended backbone of a competitive inhibitor. Proteases are known to almost universally recognize the backbone of substrates and inhibitors in a β-strand.[10] Binding in this geometry is dictated by the juxtaposition of active site binding pockets that accommodate the side chains of the peptide-based ligands.[10a]

Irradiation of the spiropyran core of these new inhibitors results in reversible ring opening (Figure 1), with an associated change in polarity and geometry[11] to affect inhibitor binding and hence potency. The change in dipole moment would be

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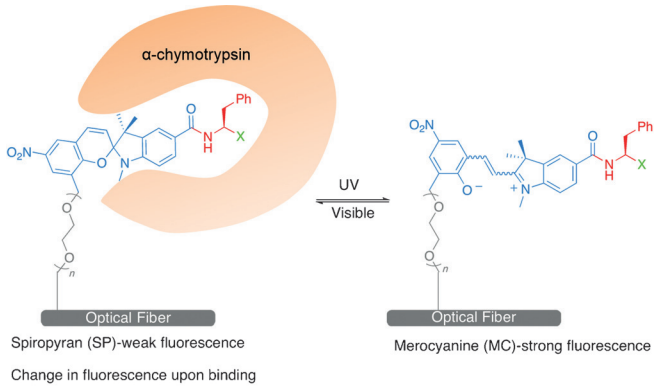


Figure 1. Schematic representation of spiropyran-based α-chymotrypsin inhibitors. The photoswitch is highlighted in blue, the sensing platform and linker grey, amino acid residue red and the C-terminal electrophile green.

expected to be greater than that observed with an azobenzene.[1b,12] A spiropyran has the added advantage that it is resistant to reductive environments and thermal relaxations in aqueous environment, while providing excellent fluorescence intensity to allow easy detection of inhibitor binding.[13] In addition, isomerization of a spiropyran is more efficient and the products are easier to detect compared to other photoswitchable systems such as an azobenzene.[13]

Results and Discussion

The system reported here and depicted in Figure 1, consists of four components: a photoswitchable spiropyran backbone core (blue), an amino acid-based group (red) capable of binding to the active site of a given protease for example, α-chymotrypsin (orange), an attached C-terminal electrophile (X, green) to bring about inhibition once bound, and a microstructured optical fiber (MOF)-based sensing platform to guide light and detect changes in fluorescence (grey). The key photoswitchable spiropyran core was designed to reversibly switch the inhibitor geometry and polarity to modulate active site binding. This group is known to provide an excellent fluorescence emission profile for sensing purposes,[13] where the ring-closed spiropyran (SP; Figure 1, left) generally exhibits weak fluorescence and the switched merocyanine isomer (MC; produced on UV irradiation, Figure 1, right) has an enhanced fluorescence emission[14] that can be detected through the MOF. The isomerization from MC to SP is promoted by visible light typically provided by a halogen lamp. We can then detect changes in fluorescence through the fiber upon selective active site binding of one isomer, anticipated to be the SP isomer given that α-chymotrypsin prefers nonpolar substrates.

The design allows for incorporation of suitable amino acid and sensing components to target a particular protease and/or sensing application. Here we use a phenylalanine-based amino acid mimic since such a group is known to bind in the primary S_1 pocket[15] (and also other neighboring pockets) of our target protease, α-chymotrypsin. Both an aldehyde and a α-ketoester were investigated as C-terminal electrophilic groups capable of interacting with the protease. α-Chymotrypsin was chosen for study as a model protease, since much is known about its structure and inhibition.[16] The overall system also contains a spacer to link the photoswitchable inhibitor to the sensing platform (in grey, Figure 1). A relatively short spacer was chosen in this study for ease of synthesis and since the active site of α-chymotrypsin is located close to the enzyme surface.[17]

A MOF (as depicted in Figure S1 in the Supporting Information) has additional advantages. Fluorescence of spiropyran is known to be greatly enhanced when coupled to a MOF,[18] to provide significantly improved sensitivity compared to the equivalent solution-based experiments. This is particularly important when using the small sample volumes required for subcellular-scale biological samples.[18a] A MOF also provides a potential platform for performing in vivo assays in confined and well-defined locations such as would be expected in potential in vivo sensing applications. Furthermore, the air holes within the MOF can be used to guide light to interact with both the attached molecules and the sample solution within. These voids simultaneously act as microsample chambers.

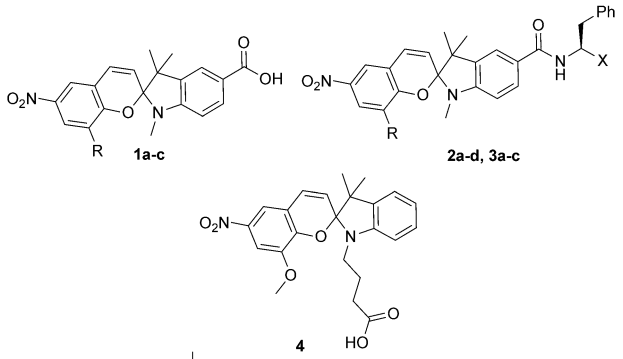
Synthesis of the inhibitors

The photoswitchable inhibitors prepared for this study are depicted in Figure 2. Compounds **2a–d** and **3a–c** contain a phenylalanine (Phe) analogue at the C terminus for binding to α-chymotrypsin. Derivatives **3a**/**3c** and **3b** also contain a C-terminal electrophilic warhead (an aldehyde and α-ketoester, respectively) that is capable of interacting with the protease active site once bound. A number of compounds were prepared with functionality for possible surface attachment, for example, a carboxylic acid (**1a–c**, **4**) for amide coupling, an azide (**1b**, **2c**, **3c**) for Huisgen cycloaddition, and an alcohol (**1c**, **2d**) for esterification. Compound **2d** provides a combination of the C-terminal Phe mimetic and a suitable C8′ tether for surface attachment. This compound also contains a chemically stable Weinreb amide at the C terminus, which is capable of hydrogen bonding with the enzyme's active site.

Compounds **1a–c** were prepared in three steps starting from 4-hydrazinobenzoic acid (Scheme 1). In particular, reaction with 2-methylbutan-2-one, under acidic conditions, gave indole **6**. This was subsequently N-alkylated on treatment with methyl iodide in toluene and acetonitrile to give **7** in 87% yield over two steps. Separate condensation of **7** with the hydroxyl nitro-benzaldehydes **8a–c**, under reflux, gave the desired spiropyran analogues **1a–c** in 45–60% yield.

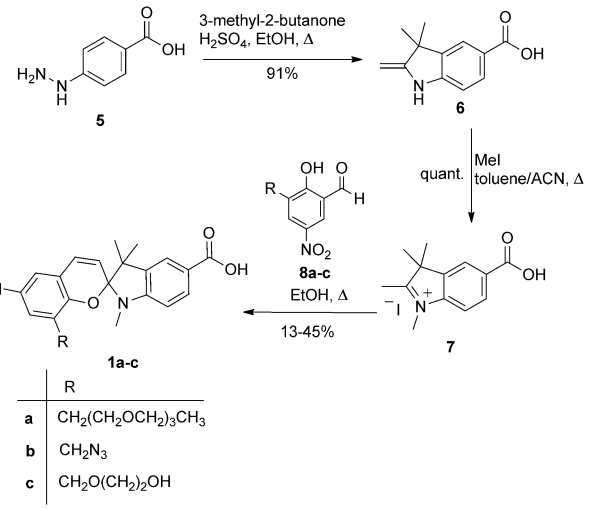
HATU-mediated coupling of **1a** and **1b** with either phenylalaninol (**9a**) or methyl-3-amino-2-hydroxy-4-phenylbutanoate (**9b**)[19] then gave **2a–c** (Scheme 2). The phenylalaninol groups of **2a** and **2c** were oxidized with Dess–Martin periodinane to give the corresponding aldehydes **3a** and **3c**, respectively. The α-ketoester **3b** was similarly obtained by Dess–Martin oxidation of the α-hydroxymethylester group of **2b**.

The spiropyran **2d** was prepared as shown in Scheme 3. BOP-mediated coupling of commercially available *N*,*O*-dimethylhydroxylamine hydrochloride with *N*-Boc-L-phenylalanine, followed by removal of the Boc group on treatment with TFA,



	R	X
1a	$(CH_2OCH_2)_3CH_3$	—
1b	CH_2N_3	—
1c	$CH_2O(CH_2)_2OH$	—
2a	$(CH_2OCH_2)_3CH_3$	CH_2OH
2b	$(CH_2OCH_2)_3CH_3$	$CHOHCOOCH_3$
2c	CH_2N_3	CH_2OH
2d	$CH_2O(CH_2)_2OH$	$CONCH_3OCH_3$
3a	$(CH_2OCH_2)_3CH_3$	CHO
3b	$(CH_2OCH_2)_3CH_3$	$COCOOCH_3$
3c	CH_2N_3	CHO

Figure 2. Structures of target spiropyrans.



	R
a	$CH_2(CH_2OCH_2)_3CH_3$
b	CH_2N_3
c	$CH_2O(CH_2)_2OH$

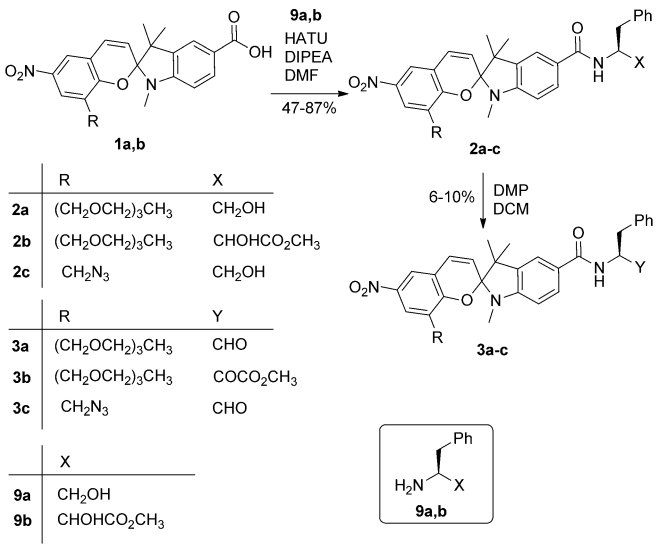
Scheme 1. Synthesis of the core spiropyran unit.

gave the key intermediate **11**. This was coupled with **1c**, in the presence of HATU, to give the spiropyran **2d**.

In vitro inhibition assay against α-chymotrypsin

The spiropyrans **1**–**4** were assayed against bovine α-chymotrypsin as described in the Supporting Information and the results are shown in Table 1. K_i values for all the inhibitors were determined graphically according to the Dixon methodology.[20]

Compounds **1a** and **1b**, which lack a hydrophobic phenylalanine mimic at their C termini, were weak inhibitors of α-chy-

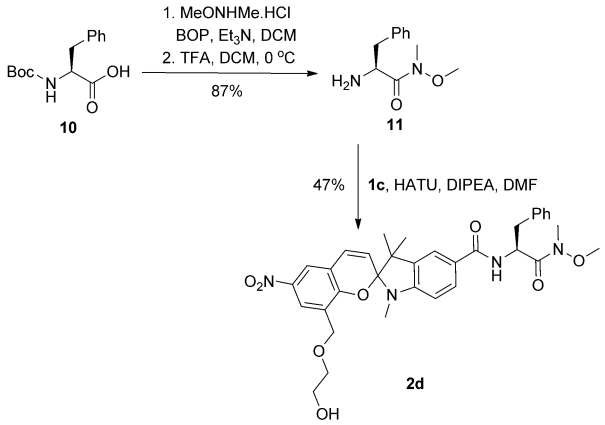


	R	X
2a	$(CH_2OCH_2)_3CH_3$	CH_2OH
2b	$(CH_2OCH_2)_3CH_3$	$CHOHCO_2CH_3$
2c	CH_2N_3	CH_2OH

	R	Y
3a	$(CH_2OCH_2)_3CH_3$	CHO
3b	$(CH_2OCH_2)_3CH_3$	$COCO_2CH_3$
3c	CH_2N_3	CHO

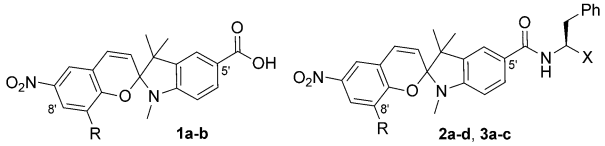
	X
9a	CH_2OH
9b	$CHOHCO_2CH_3$

Scheme 2. Preparation of C-terminal substituted spiropyrans.



Scheme 3. Synthesis of **2d**.

Table 1. In vitro inhibition of α-chymotrypsin by compounds **1a,b**, **2a–d**, **3a–c** and **4**.



	R	X	K_i [μM]
1a	$(CH_2OCH_2)_3CH_3$	–	305 ± 45
1b	CH_2N_3	–	170 ± 27
2a	$(CH_2OCH_2)_3CH_3$	CH_2OH	48 ± 6
2b	$(CH_2OCH_2)_3CH_3$	$CHOHCO_2CH_3$	75 ± 9
2c	CH_2N_3	CH_2OH	23 ± 4
2d	$CH_2O(CH_2)_2OH$	$CONCH_3CH_3$	86 ± 11
3a	$(CH_2OCH_2)_3CH_3$	CHO	1.7 ± 0.26
3b	$(CH_2OCH_2)_3CH_3$	$COCO_2CH_3$	0.12 ± 0.016
3c	CH_2N_3	CHO	1.8 ± 0.56
4	OCH_3	–	> 1000

motrypsin, with similar K_i values of 305 and 170 μM, respectively. Even weak inhibitory activity here is particularly interesting given that these compounds lack any significant peptide character normally associated[21] with protease inhibitors. The nature of the R group at the C8 of the benzopyran ring appears to have little influence on potency (c.f. **1a** and **1b**), an important observation given that this is a potential site for attachment to the MOF as discussed later. On the other hand, compound **4**, with a pentanoic acid linker on the indole nitrogen, was inactive against α-chymotrypsin at a concentration of 1000 μM. Thus, this is not a suitable site for surface attachment.

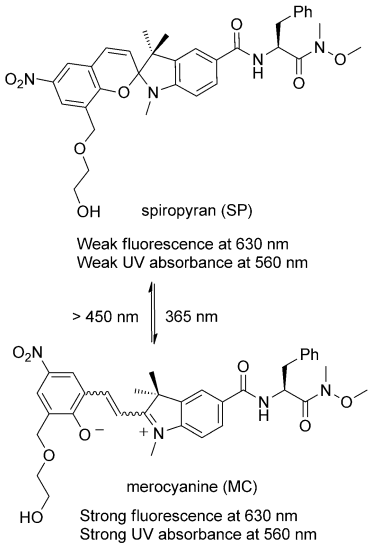
The incorporation of phenylalanine mimetic at C5 of the indole (**2a–c**) results in a significant improvement in potency, with K_i values of 48, 75 and 23 μM, respectively. This suggests that the substituted spiropyran-core can act as a surrogate of the backbone of a protease inhibitor or substrate. The introduction of a reactive C-terminal group as in the aldehyde of **3a** and **3c** further improves potency, with these compounds having K_i values of 1.7 and 1.8 μM, respectively. Compound **3b**, with a C-terminal α-ketoester as found in other serine protease inhibitors,[22] proved to be particularly potent with a K_i of 115 nM. This compound is >1000-fold more potent than **1a** that contains the spiropyran base unit only. Gratifyingly, the spiropyran **2d**, with an ethylene glycol substituent at C8 suitable for attachment to a MOF, was a reasonable inhibitor of α-chymotrypsin in solution with a $K_i = 86$ μM. This compound has the advantage that it lacks a reactive C-terminal warhead and as such was chosen for initial surface studies and the associated characterization. Importantly, compound **2d** was shown to be a competitive reversible inhibitor of α-chymotrypsin by Dixon plot and Cornish–Bowden plot analyses[20,23] (Figure S2 in the Supporting Information). It thus competes for substrate, an important observation if the spiropyran is to bind in the active site as a peptide backbone mimetic.

Solution-based photoisomerism of compound 2d

Studies on the photoswitching of spiropyran **2d** in solution were undertaken in order to compare the relative potencies of the two photoswitchable states against α-chymotrypsin. These two switchable isomers are depicted in Scheme 4 as ring-closed spiropyran (SP) and ring-opened merocyanine (MC).

A solution of **2d** in the assay buffer was irradiated with UV light at 365 nm, for 5 min, and the resulting sample was incubated with α-chymotrypsin in the dark to minimize any isomerization back to the SP isomer. This sample proved to be a very weak inhibitor of α-chymotrypsin, with an IC_{50} greater than 500 μM. Thus, the SP-based photostationary state is significantly more potent (>fivefold) than the equivalent MC-based state. This observation presumably reflects significant differences in polarity and conformation of the two isomers, and hence their abilities to bind to the protease.

The ability of **2d** to photoswitch between its two isomers was then studied. Specifically, a sample of **2d** was irradiated with visible light (>400 nm) and the resulting UV–visible (UV/Vis) absorption spectrum was measured as shown in Figure 3



Scheme 4. Photoisomerization of **2d**, where SP depicts the ring-closed non-fluorescent isomer and MC the ring-opened fluorescent isomer.

(red). The lack of absorption at 550 nm indicates that the sample consists predominantly of the SP isomer, where the MC isomer typically has strong absorption in this region.[24] The solution of **2d** in methanol was then irradiated with UV light (365 nm) for 15 min to induce isomerization to the MC isomer as evidenced by a significant and characteristic increase in absorption at 550 nm (Figure 3a, blue). The sample was further irradiated with visible light to give a spectrum essentially the same as the initial one (black and red, Figure 3a). Switching between the two isomers was apparent after multiple cycles of alternate irradiation with UV and visible light, without any evidence of photobleaching, as shown in Figure 3b and Figure S5 in the Supporting Information. A significant increase in fluorescence of **2d** was also observed upon UV irradiation and switching to the MC isomer, while a decrease in fluorescence was apparent upon visible light irradiation to switch the compound back to the SP isomer (Figure S3 in the Supporting Information). Consistent with the UV/Vis absorption experiments, the fluorescence experiments also demonstrate the ability of compound **2d** to reversibly photoisomerize between two isomers.

In silico docking

The SP and MC isomers of **2d** were separately docked into a structure of α-chymotrypsin (*Bos Taurus*, PDB ID. 1GGD)[25] using AutoDock4.2[26] in order to gain some insight into the potential mode of active site binding and associated differences in potency. Docking studies were carried out by performing 25 docking runs on each isomer using the Lamarckian Genetic Algorithm[27] and a maximum of 500000 energy evaluations. The results of these studies are showed in Figure 4. The SP isomer of **2d** docked with a binding energy of −7.04 kcal mol^{-1} and docking energy of −13.57 kcal mol^{-1} (Figure 4a) while the apparently less active MC isomer docked less well

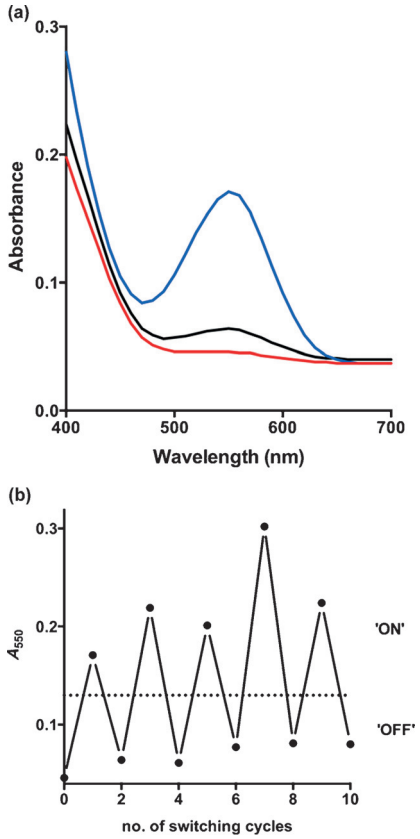


Figure 3. a) The UV/Vis absorption spectra of **2d** in MeOH under visible light (red), after irradiation with UV light (365 nm) for 15 min (blue). Compound **2d** was then isomerized by visible light irradiation again to produce spectrum in black. b) Photoswitching of **2d** induced by UV (365 nm) and visible light (>400 nm) irradiations. Cycle 0 represents the state before **2d** was irradiated with UV light. Cycles 1, 3, 5, 7 and 9 represent the state of **2d** after irradiated with UV light for 15 min. Cycles 2, 4, 6, 8, and 10 represent the state of **2d** after irradiated with visible light for 15 min.

with a binding energy of −0.61 and a docking energy of −8.93 kcal mol^{-1}, respectively (Figure 4b). Figure 4a reveals that the SP isomer sits within the active site, with the amide carbonyl near the key active site serine as would be expected for a competitive reversible inhibitor. In comparison, the MC isomer docks poorly at the periphery of the active site (Figure 4b).

Figure 4c depicts interactions of SP isomer of **2d** with key binding pockets within the enzyme's active site as observed in the docked structures. Interestingly, the benzyl group of Phe mimic resides in the S_2' binding pocket (highlighted in green). This pocket is known to bind favorably with aromatic amino acids,[16c] however this interaction has been little explored in inhibitor design. This then positions the spiropyran amide carbonyl of **2d** in close proximity to the active site serine, with the indole ring sitting on the outer edge of the S_1 binding pocket. The N-methyl group then projects into this pocket. The ethylene glycol linker on the benzopyran is positioned on the surface of the enzyme. The overall result is a somewhat unusual mode of binding to α-chymotrypsin, where the spiropyran provides a suitable scaffold for other key interactions to occur with the active site. It is noteworthy that our original

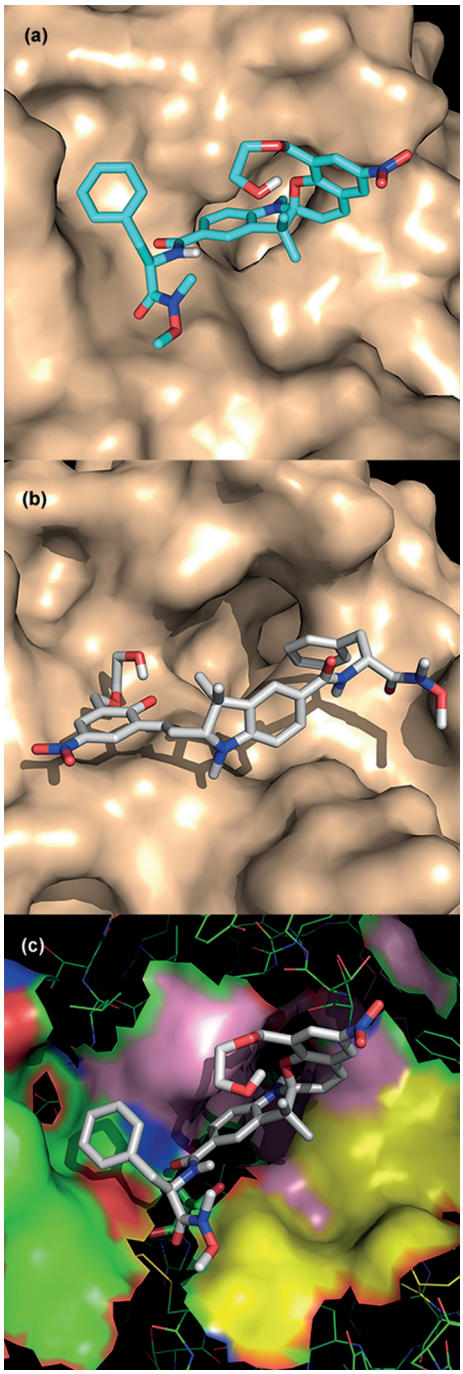


Figure 4. AutoDock4 results of **2d** in the active site of α-chymotrypsin (PDB ID: 1GGD): a) ring-closed SP **2d**; b) ring-opened MC **2d**, and c) key binding interactions of SP **2d** with S2′, S1, and S2 binding pockets highlighted in green, pink and yellow, respectively.

design had the Phe mimetic of **2d** interacting with the S_1 pocket rather than S_2' as is apparent from these studies. This difference is of little consequence to the current study, but it does present some intriguing future inhibitor design possibilities. It is also important to note that the ethylene glycol-based linker of **2d** is positioned toward the enzyme surface of the protease and is thus suitably located for attachment to the MOF.

For comparison, the SP isomers of **3a–c** and **4** were also docked into α-chymotrypsin (see the Supporting Information for detail). All these structures bind to the active site of the enzyme with similar binding energies and docking energies compared to the SP isomer of **2d**. Compounds **3b** and **3c** bind in essentially the same orientation as **2d**, while **3a** differs only in the orientation of the benzyl group (Supporting Information, Figures S6–S8).

Microstructured optical fiber-based experiments

Compound **2d** was next attached to a silica-based suspended-core MOF (Supporting Information, Figure S1) in order to probe its surface-based interactions with α-chymotrypsin as a step toward a functional sensor. All surface functionalization procedures were carried out under anhydrous conditions in a sealed metal chamber pressurized with N_2.[18a] The fiber was first treated with pirahna solution (3:1 sulfuric acid/ 30% H_2O_2 v/v) and the thus liberated free hydroxyl groups were reacted with 2.5% carboxyethylsilanetriol.[28] Steglich esterification[29] of the resulting carboxyl groups, with **2d** in the presence of DIC, HOBt, and DMAP, gave the functionalized fiber (**Fiber-2d**).

The ability of **2d** to photoswitch when attached to the MOF was studied using the setup depicted in Figure 5.

In particular, **Fiber-2d** (SP isomer) was filled with Mili-Q water by capillary action and irradiated for 10 ms using a 532 nm laser and the resulting fluorescence was measured as shown in Figure 6.[18] The functionalized fiber was next irradiated with UV light ($\lambda = 365$ nm) for an extended period of 5 min in order to switch the surface attached SP-**2d** to MC-**2d** as per the solution experiments. Subsequent excitation of this isomer with 532 nm laser (as before) resulted in an enhanced (sixfold) average fluorescence relative to the SP isomer. This is consistent with the fluorescence characteristics of the MC isomer, where this isomer is known to have enhanced fluorescence due to π-conjugation as reported in literature[13,24,30] (Figure 6, black, $\lambda_{em} \sim 630$ nm). Subsequent exposure of the **Fiber-2d** to visible light for 5 min decreased the fluorescence intensity, as expected for a photostationary state enriched in SP isomer. Thus, **2d** is able to be reversibly photoswitched on fiber as per in solution. This is an important observation for sensor development.

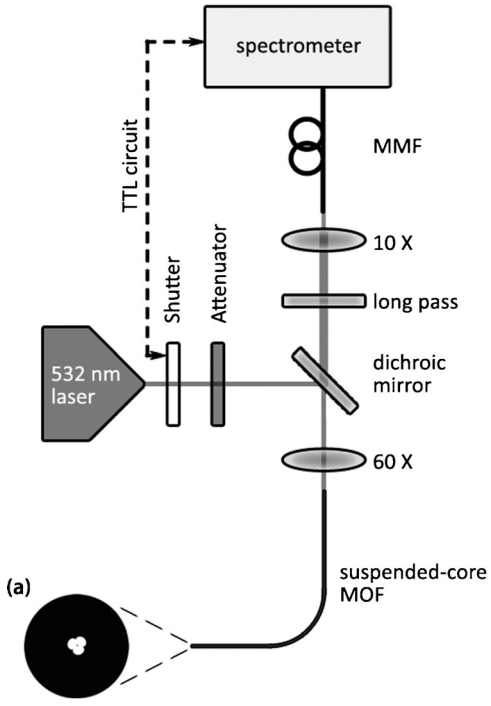


Figure 5. Optical set-up for measuring the fluorescence of a functionalized MOF. The area of illumination is shown by the dotted rectangle. (a) A SEM cross section of the silica suspended-core fiber.

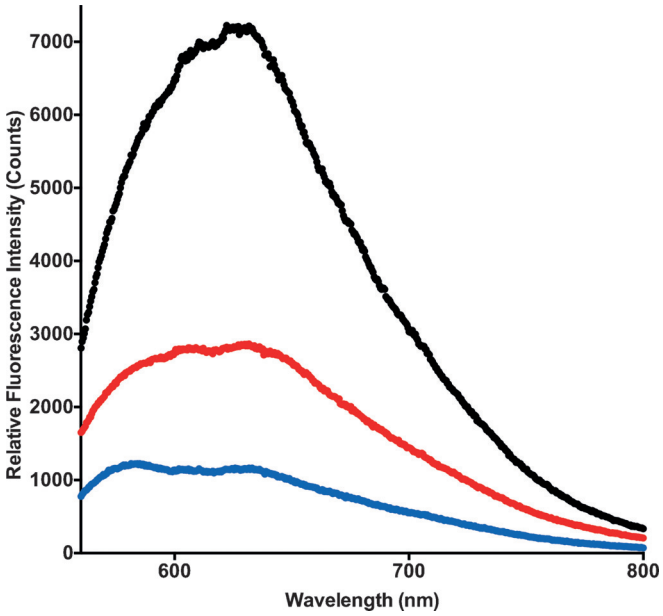


Figure 6. Fluorescence spectra of **Fiber-2d** (SP) in water (blue), fiber-bound **2d** in water irradiated with UV light ($\lambda = 365$ nm; black) and **Fiber-2d** subsequently irradiated with visible light (red). Fluorescence was induced by exposure of **Fiber-2d** to 10 cycles of the 532 nm laser irradiation 10 ms per exposure. The spectra are averages of 10 fluorescence measurements of each sample.

Next, the fluorescence of **Fiber-2d** (SP isomer) in the presence of α-chymotrypsin was measured to investigate enzyme binding. A new section of **Fiber-2d** was filled with a solution of α-chymotrypsin in water (1 μM) over 5 min and the fluorescence was measured at 0, 10, 25, and 85 min by excitation with a 532 nm laser (10×10 ms pulses) in each case. After 10 min the fluorescence was observed to be sixfold lower (c.f. Figure 7a, red and brown lines), which we suggest is the result of active site binding of the SP isomer of **2d** that would quench the inhibitor's fluorescence. A similar decrease in fluorescence was observed in the solution-based experiments with compound **2d** and α-chymotrypsin (see Figure S8 in the Supporting Information for detail). The fluorescence intensity increased gradually over the ensuring measurements (25, 85 min, see Figure 7a, blue and black curves, respectively).

This is consistent with slow but incomplete isomerism to the MC isomer in the dark,[31] with this isomer having an inherent

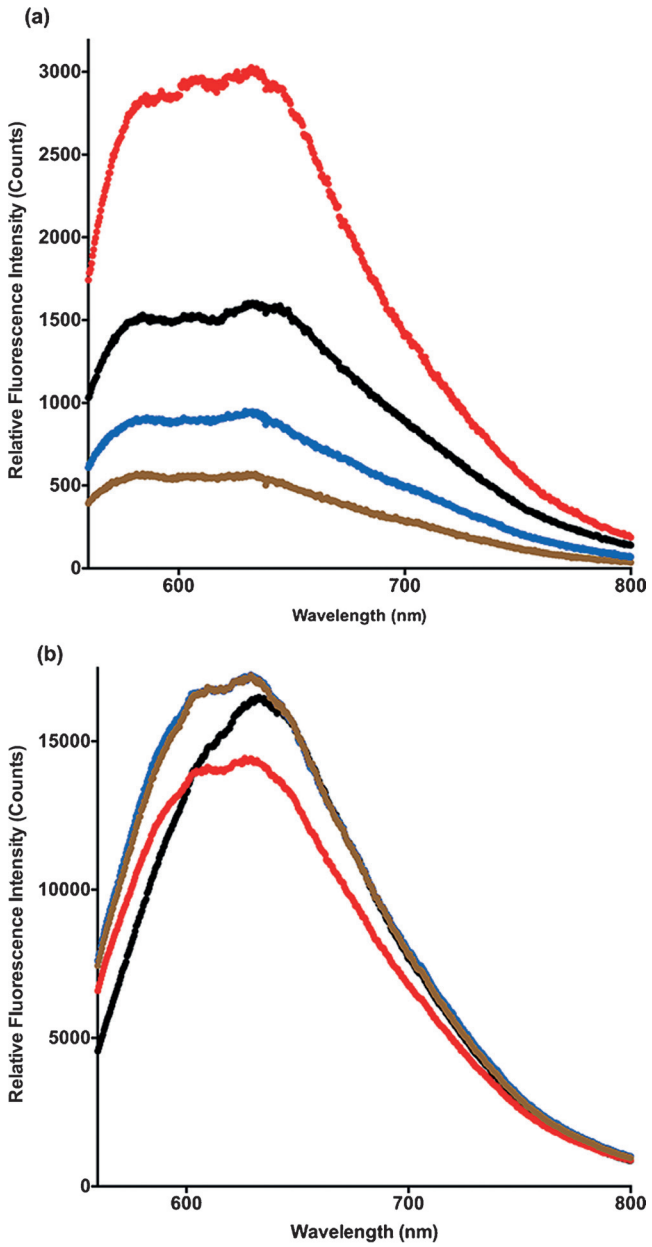


Figure 7. Fluorescent emissions of: a) SP of fiber-bound **2d** with α-chymotrypsin left at room temperature for 0 min (red), 10 min (brown), 25 min (blue), and 85 min (black). The fiber-bound compound was excited by 10 pulses of 532 nm laser irradiation before each measurement; and b) MC of fiber-bound **2d** (isomerized under UV irradiation; $\lambda = 365$ nm) with α-chymotrypsin left at room temperature for 0 (red), 10 (brown), 25 (blue), and 85 min (black).

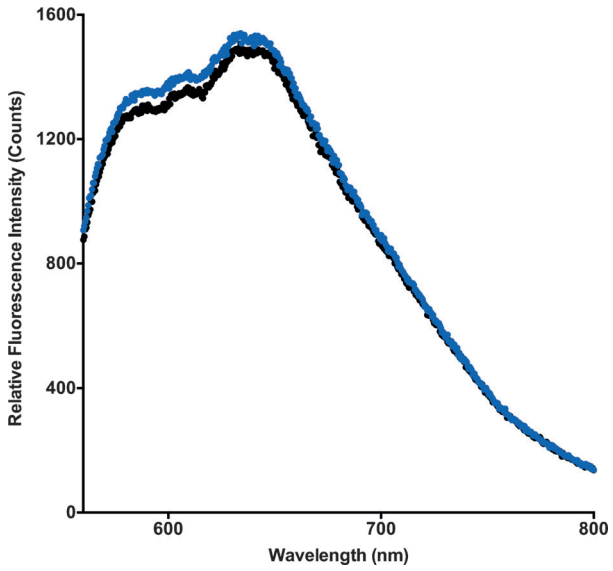


Figure 8. Fluorescence emissions of compound **2d** incubated in Mili-Q water for 0 (blue) and 85 min (black).

enhanced fluorescence while also dissociating from the enzyme due to its weaker binding affinity as demonstrated in the solution-based experiments. In support, it is known that α-chymotrypsin has a greater affinity for nonpolar inhibitors (e.g., the SP isomer) compared to polar inhibitors (e.g., MC isomer).[32] Repeated photoswitching between the SP and MC isomer produced similar changes in fluorescence intensities (see Figure S9 in the Supporting Information for detail), which demonstrates that MOF is a suitable platform for monitoring chymotrypsin activity reversibly. Finally, the experiment was repeated using water in place of α-chymotrypsin as a negative control. No significant change in fluorescence was apparent after 85 min (Figure 8), which confirms that the earlier changes in fluorescence on fiber were due to the enzyme binding to **2d**.

The binding of fiber-bound MC isomer with α-chymotrypsin was similarly investigated. The solution-filled section of the fiber was cleaved and discarded and the remaining section was infused with a fresh sample of α-chymotrypsin by capillary action over 5 min with concomitant UV (365 nm) irradiation in order to give a photostationary state enriched in the MC isomer. Fluorescence was measured at 0, 10, 25, and 85 min with laser irradiations (10×10 ms, 532 nm) as before, without any apparent significant change in fluorescence over the time course (Figure 7b). This is consistent with the earlier solution-based observation that the MC isomer has significantly reduced activity and does not effectively bind to the protease. Small variations in fluorescence are likely a result of incomplete isomerization from the SP isomer to the MC isomer. This demonstrates that attaching **2d** to fiber does not affect the relative binding affinities of the SP and MC isomers.

Conclusions

Here we report the development of the first examples of spiropyran-based protease inhibitors, which are able to undergo reversible photoisomerization between an active nonpolar SP isomer and a significantly less active polar MC isomer upon UV–visible light irradiations. The inhibitors reported here were specifically designed to target α-chymotrypsin as a first step toward a more modular approach to a range of proteases.

The spiropyrans **1a–c**, **2a–d** and **3a–c** were synthesized and assayed against α-chymotrypsin. The spiropyran cores alone (**1a–c**) were active against α-chymotrypsin. The incorporation of phenylalanyl mimetic at the C terminus (**2a–d**, **3a–c**) signifi-

cantly improved the binding affinity. Compound **3b** with an additional C-terminal α-ketoester, was found to be the most active inhibitor in this series, with a K_i of 115 nM.

The Weinreb amide **2d** was chosen as a stable analogue suitable for fiber functionalization while exhibiting reasonable activity against α-chymotrypsin (K_i = 86 μM). This compound was shown to bind as a competitive inhibitor by the Dixon plot and the in silico docking experiments. The UV irradiation of **2d** in solution gave a photostationary state enriched in the MC isomer that was sixfold less active against the enzyme than the SP isomer. This is consistent with the general observations that α-chymotrypsin has a greater affinity for nonpolar inhibitors compared to polar inhibitors. A sample of **2d** attached to a MOF (**Fiber-2d**) could be reversibly photoisomerized to the MC isomer on fiber as per in solution.

Quenching of fluorescence (sixfold) was observed for the fiber-bound SP isomer of **2d** after 10 min incubation with α-chymotrypsin, which is consistent with active site binding. No significant change in fluorescence was apparent over 85 min incubation of the MC fiber-bound isomer with α-chymotrypsin. This is consistent with the earlier solution-based observation that the MC isomer has significantly reduced binding affinity compared to the SP isomer.

In this work we report a new class of spiropyran-based protease inhibitor, the inhibitory activity of which can be controlled by irradiation with light of a specific wavelength, both in solution and on attachment to a MOF. This provides an important basis for probing and modulating enzyme activity in a confined and well-defined environment, and paves the way for potential real-time biosensing applications for in vivo disease diagnosis.

Experimental Section

General information

Unless otherwise indicated, all starting materials, enzymes, chemicals and anhydrous solvents were purchased from Sigma–Aldrich (NSW, Australia) and were used without further purification. UNIBOND C-18 reverse phase silica gel was purchased from Analtech Inc. (USA). 3-(Chloromethyl)-2-hydroxy-5-nitrobenzaldehyde and compound **4** were prepared as previously described.[18a] ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz and a Varian Inova 600 MHz instruments in the indicated solvents. Chemical shifts are reported in ppm (δ). Signals are reported as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet) or m (multiplet). High-resolution mass spectra were collected using an LTQ Orbitrap XL ETD with flow injection, with a flow rate of 5 μL min^{-1}. Where indicated compounds were analyzed and purified by reverse phase HPLC, using an HP 1100 LC system equipped with a Phenomenex C-18 column (250×4.6 mm) for analytical traces and a Gilson GX-Prep HPLC system equipped with a Phenomenex C18 column (250×21.2 mm). H_2O/TFA (100:0.1 by v/v) and MeCN/TFA (100:0.08 by v/v) solutions were used as aqueous and organic buffers. All graphs were generated using GraphPad Prism 6 software. A mercury lamp (365 nm) and a halogen lamp (>450 nm) were used as the UV and visible light sources in photoswitching experiments.

Synthesis and characterization

8-(2-(2-Ethoxyethoxy)ethoxy)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxylic acid (1a): To an ice-cooled solution of NaH (371 mg, 8.90 mmol) in anhydrous THF (15 mL) was added diethylene glycol monoethyl ether (747 mg, 5.57 mmol) dropwise under N_2. The mixture was stirred for 15 min with ice cooling and then a solution of 3-(chloromethyl)-2-hydroxy-5-nitrobenzaldehyde (1 g, 4.64 mmol) in anhydrous THF (5 mL) was added dropwise. The mixture was allowed to warm to room temperature with stirring for 18 h under N_2. The mixture was cooled in ice before H_2O (160 mL) was added slowly to quench the reaction. The mixture was washed with CH_2Cl_2 (2×90 mL). The aqueous layer was acidified to pH 1 with 1 M aqueous HCl and extracted with ethyl acetate (3×90 mL). The organic phase was combined, dried over Na_2SO_4 and concentrated in vacuo to give **8a** (1.12 g, 77%), which was used in the subsequent step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.02 (s, 1H), 8.62 (s, 1H), 8.49 (s, 1H), 4.70 (s, 2H), 3.90–3.54 (m, 10H), 1.26–1.19 ppm (t, J = 4.5 Hz, 3H); and MS: $[M]^+_{calcd}$ = 313.12, $[M]^+_{found}$ = 313.12.

To a solution of **8a** (181 mg, 0.58 mmol) and **7** (125 mg, 0.58 mmol) in anhydrous ethanol (17 mM) was added Et_3N (59 mg, 0.58 mmol). The mixture was refluxed for 3 h under N_2 and concentrated in vacuo. The crude mixture was purified using C-18 reverse phase silica gel (35% MeCN in H_2O) to give **1a** (80 mg, 27%). ^{1}H NMR (600 MHz, CD_3OD): δ = 8.14 (d, J = 2.6 Hz, 1H), 8.06 (d, J = 2.7 Hz, 1H), 7.91 (d, J = 8.2, 1H), 7.74 (s, 1H), 7.12 (d, J = 10.4 Hz, 1H), 6.63 (d, J = 8.2 Hz, 1H), 6.00 (d, J = 10.4 Hz, 1H), 4.37–4.25 (m, 2H), 3.59–3.38 (m, 10H), 2.79 (s, 3H), 1.32 (s, 3H), 1.20 (s, 3H), 1.14 ppm (t, J = 7.0 Hz, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 156.2, 151.5, 140.8, 136.1, 131.0, 128.5, 128.4, 125.8, 124.3, 122.9, 121.6, 120.4, 118.6, 106.5, 105.9, 70.2, 70.0, 69.4, 66.2, 51.4, 39.0, 27.6, 24.8, 18.7, 14.0 ppm; HRMS (ESI) found $[M+H]^+$ 513.2230, $C_{27}H_{33}N_2O_8^+$ requires 513.2237.

8-(Azidomethyl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxylic acid (1b): To a solution of sodium azide (166 mg, 2.60 mmol) in anhydrous DMSO (4.6 mL) was added 3-(chloromethyl)-2-hydroxy-5-nitrobenzaldehyde (500 mg, 2.30 mmol) in one portion. The mixture was stirred at room temperature under N_2 for 18 h. H_2O (5 mL) was added dropwise and the mixture was extracted with ethyl acetate (2×20 mL). The combined organic phase was washed with H_2O (20 mL), dried over Na_2SO_4 and concentrated in vacuo to yield **8b** as a pale yellow solid (389 mg, 76%), which was subsequently used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 12.01 (s, 1H), 10.02 (s, 1H), 8.55 (s, 1H), 8.48 (s, 1H), 5.30 (s, 1H), 4.56 (s, 2H), 1.55 ppm (s, 1H); and MS: $[M]^+_{calcd}$ = 222.04, $[M]^+_{found}$ = 222.04.

To a solution of **8b** (611 mg, 2.75 mmol) and **7** (593 mg, 2.75 mmol) in anhydrous ethanol (17 mM) was added Et_3N (278 mg, 2.75 mmol). The mixture was refluxed for 3 h under N_2 and then concentrated in vacuo. The mixture was purified using C-18 reverse phase silica gel (25% MeCN in H_2O) to give **1b** (150 mg, 13%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.26 (s, 1H), 8.17 (s, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.68 (s, 1H), 7.28 (d, J = 10.5 Hz, 1H), 6.68 (d, J = 8.1 Hz, 1H), 6.06 (d, J = 10.5 Hz, 1H), 4.27 (d, J = 13.6 Hz, 1H), 4.23 (d, J = 13.6 Hz, 1H), 2.75 (s, 3H), 1.27 (s, 3H), 1.15 ppm (s, 3H); ^{13}C NMR (151 MHz, [D$_6$]DMSO): δ = 167.7, 157.0, 151.0, 140.2, 135.8, 130.9, 128.5, 125.9, 123.1, 122.9, 121.1, 119.0, 106.7, 106.5, 51.6, 48.1, 28.5, 25.8, 19.6 ppm. HRMS (ESI) found $[M]^+$ 421.1381, $C_{21}H_{19}N_5O_5^+$ requires 421.1386.

8-((2-Hydroxyethoxy)methyl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxylic acid (1c): To an ice-cooled solution of NaH (63 mg, 1.59 mmol) in anhydrous THF (2.8 mL) was

added diethylene glycol monoethyl ether (99 mg, 1.59 mmol) dropwise at under N_2. The mixture was stirred for 15 min with ice cooling and then a solution of 3-(chloromethyl)-2-hydroxy-5-nitrobenzaldehyde (200 mg, 0.80 mmol) in anhydrous THF (0.7 mL) was added dropwise. The mixture was allowed to warmed to room temperature with stirring for 18 h under N_2. The mixture was cooled in ice before H_2O (35 mL mmol^{-1} aldehyde) was added slowly to quench the reaction. The mixture was washed with CH_2Cl_2 (2×20 mL). The aqueous layer was acidified to pH 1 with 1 M aqueous HCl and extracted with ethyl acetate (3×20 mL). The organic phase was combined, dried over Na_2SO_4 and concentrated in vacuo to give **8c** (115 mg, 60%), which was used in the subsequent step without purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.92 (s, 1H), 10.01 (s, 1H), 8.57 (s, 2H), 8.50 (s, 1H), 4.85 (s, 2H), 2.25–2.11 (m, 2H), 1.58 (s, 1H), 1.25 ppm (s, 1H); and MS: $[M]^+_{calcd}$ = 241.06, $[M]^+_{found}$ = 241.06.

To a solution of **8c** (115 mg, 0.47 mmol) and **7** (101 mg, 0.47 mmol) in anhydrous ethanol (17 mM) was added Et_3N (47 mg, 0.47 mmol). The mixture was refluxed for 3 h under N_2 and concentrated in vacuo. The crude mixture was purified using C-18 reverse phase silica gel (30% MeCN in H_2O) to give **1c** (93 mg, 45%). ^{1}H NMR (500 MHz, CD_3OD): δ = 8.19 (s, 1H), 8.07 (s, 1H), 7.92 (d, J = 8.2 Hz, 1H), 7.75 (s, 1H), 7.14 (d, J = 10.4 Hz, 1H), 6.64 (d, J = 8.2 Hz, 1H), 6.01 (d, J = 10.4 Hz, 1H), 4.57 (br s, 1H), 4.32 (s, 2H), 3.62–3.52 (m, 2H), 3.45–3.35 (m, 2H), 2.80 (s, 3H), 1.33 (s, 3H), 1.22 ppm (s, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 157.7, 152.8, 142.5, 137.6, 132.4, 130.1, 127.5, 125.7, 124.4, 123.1, 122.1, 120.2, 108.1, 107.4, 73.7, 67.7, 62.4, 40.6, 29.2, 26.3, 20.3 ppm; HRMS (ESI) found $[M]^+$ 440.1581, $C_{23}H_{24}N_2O_7^+$ requires 440.1584.

General procedure A for HATU-mediated peptide coupling

To a solution of carboxylic acid (1 equiv) in anhydrous DMF (40 mM) was sequentially added HATU (1 equiv) and the amine (1.2 equiv). The mixture was stirred under N_2 for 15 min, cooled in an ice bath, and DIPEA (4 equiv) was added dropwise. The mixture was allowed to warm to room temperature while stirring under N_2 for 18 h. Aqueous HCl (1 M; 10 mL mmol^{-1} of carboxylic acid) was added and the mixture was extracted with ethyl acetate (3× 50 mL mmol^{-1} of carboxylic acid). The combined organic phase was washed with 1 M aqueous HCl (10 mL mmol^{-1} of carboxylic acid), saturated $NaHCO_3$ (10 mL mmol^{-1} of carboxylic acid) and brine (20 mL mmol^{-1} of carboxylic acid). The organic phase was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified using C-18 reverse phase silica gel (30–50% MeCN in H_2O).

8-(2-(2-Ethoxyethoxy)ethoxy)-*N*-((*R*)-1-hydroxy-3-phenylpropan-2-yl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxamide (2a): Compound **2a** (96 mg, 62%) was prepared by coupling **1a** with L-phenylalaninol **9a** according to general procedure A. ^{1}H NMR (600 MHz, CD_3OD): δ = 8.19 (s, 1H), 8.08 (s, 1H), 7.79–7.61 (m, 1H), 7.51 (s, 1H), 7.33–7.21 (m, 2H), 7.19–7.15 (m, 1H), 7.13 (d, J = 10.4 Hz, 1H), 6.62 (d, J = 8.2 Hz, 1H), 6.00 (d, J = 10.4 Hz, 1H), 4.59 (br s, 1H), 4.41–4.23 (m, 3H), 3.75–3.59 (m, 2H), 3.55–3.37 (m, 10H), 3.13–2.95 (m, 1H), 2.95–2.83 (m, 1H), 2.78 (s, 3H), 2.65 (s, 2H), 1.37 (s, 3H), 1.22 (s, 3H), 1.14 ppm (t, J = 7.0 Hz, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 168.8, 156.2, 150.6, 140.8, 138.7, 136.3, 128.9, 128.5, 128.0, 127.9, 125.9, 125.8, 125.5, 124.2, 121.6, 120.7, 120.5, 118.6, 106.5, 106.0, 70.1, 70.0, 69.4, 66.2, 63.0, 53.4, 51.5, 39.0, 36.6, 27.60, 24.70, 18.7, 14.0 ppm; HRMS (ESI) found $[M]^+$ 645.3055, $C_{36}H_{43}N_3O_8^+$ requires 645.3050.

8-(2-(2-Ethoxyethoxy)ethoxy)-*N*-((2*R*,3*S*)-1-carbomethoxy-1-hydroxy-3-phenylpropan-2-yl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxamide (2b): Compound **2b** (54 mg, 47%) was prepared by coupling of compound **1a** with (2*R*,3*S*)-methyl 3-amino-2-hydroxy-4-phenylbutanoate (**9b**)[19] according to general procedure A. ^{1}H NMR (599 MHz, CD_3OD): δ = 8.17 (s, 1H), 8.08 (s, 1H), 7.90 (s, 1H), 7.72–7.62 (m, 1H), 7.53 (s, 1H), 7.40–7.27 (m, 4H), 7.26–7.18 (m, 1H), 7.15 (d, J = 10.4 Hz, 1H), 6.65 (d, J = 8.2 Hz, 1H), 6.01 (d, J = 10.4 Hz, 1H), 4.71 (m, 1H), 4.58 (br s, 1H), 4.32 (s, 2H), 4.22 (s, 1H), 3.68 (s, 3H), 3.58–3.39 (m, 10H), 3.15–2.94 (m, 2H), 2.78 (s, 3H), 1.34 (s, 3H), 1.22 (s, 3H), 1.15 ppm (t, J = 7.0 Hz, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 175.2, 170.2, 157.7, 152.3, 142.4, 139.7, 137.9, 130.6, 130.1, 129.7, 127.8, 127.4, 126.7, 125.8, 123.1, 122.3, 122.0, 120.2, 108.1, 107.6, 72.1, 71.7, 71.6, 71.0, 67.7, 55.6, 53.1, 52.8, 38.5, 29.2, 26.3, 20.2, 15.6 ppm; HRMS (ESI) found $[M]^+$ 703.3104, $C_{38}H_{45}N_3O_{10}^+$ requires 703.3105.

8-(Azidomethyl)-*N*-((*R*)-1-hydroxy-3-phenylpropan-2-yl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxamide (2c): Compound **2c** (120 mg, 87%) was prepared by coupling of compound **1b** with L-phenylalaninol **9a** according to general procedure A. ^{1}H NMR (500 MHz, CD_3OD): δ = 8.12 (s, 1H), 8.09 (s, 1H), 7.67 (d, J = 8.2 Hz, 1H), 7.56 (d, J = 6.0 Hz, 1H), 7.27 (m, 4H), 7.16 (m, 2H), 6.62 (d, J = 8.2 Hz, 1H), 6.02 (d, J = 10.4 Hz, 1H), 4.58 (s, 1H), 4.38–4.29 (m, 1H), 4.26 (d, J = 13.8 Hz, 1H), 4.18 (d, J = 13.8 Hz, 1H), 3.65 (d, J = 5.3 Hz, 2H), 3.10–2.85 (m, 2H), 2.81 (s, 3H), 1.34 (s, 3H), 1.21 ppm (s, 3H); ^{13}C NMR (126 MHz, CD_3OD): δ = 168.9, 156.8, 150.5, 140.7, 138.7, 136.01, 128.9, 128.4, 128.1, 127.9, 125.9, 125.6, 125.2, 123.0, 122.4, 120.7, 120.7, 119.2, 107.1, 106.0, 62.9, 53.4, 51.7, 36.6, 27.6, 24.9, 24.8, 18.7 ppm; HRMS (ESI) found $[M]^+$ 554.2278, $C_{30}H_{30}N_6O_5^+$ requires 554.2278.

8-((2-Hydroxyethoxy)methyl)-*N*-((*R*)-1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-carboxamide (2d): Compound **2d** (74 mg, 57%) was prepared by coupling of compound **1c** with compound **11** according to general procedure A. ^{1}H NMR (500 MHz, CD_3OD): δ = 8.19 (s, 1H), 8.07 (s, 1H), 7.69 (d, J = 7.5 Hz, 1H), 7.58 (s, 1H), 7.29 (d, J = 3.4 Hz, 4H), 7.21 (br s, 1H), 7.13 (d, J = 10.4 Hz, 1H), 6.63 (d, J = 8.4 Hz, 1H), 6.00 (d, J = 10.5 Hz, 1H), 5.35 (br s, 1H), 4.30 (s, 1H), 3.80 (s, 3H), 3.56 (t, J = 4.6 Hz, 2H), 3.35 (d, J = 4.5 Hz, 2H), 3.20 (s, 3H), 3.18–3.02 (m, 2H), 2.78 (s, 3H), 1.34 (s, 3H), 1.21 ppm (s, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 156.1, 150.9, 140.9, 136.3, 128.9, 128.6, 128.3, 128.0, 126.4, 125.8, 124.1, 121.5, 120.8, 120.4, 118.6, 106.5, 106.0, 72.1, 66.1, 60.8, 56.07, 51.5, 41.0, 29.4, 27.6, 23.0, 18.6, 16.0 ppm; HRMS (ESI) found $[M]^+$ 630.2694, $C_{34}H_{38}N_4O_8^+$ requires 630.2690.

General procedure B for Dess–Martin oxidation of alcohol

To a solution of alcohol (1 equiv) in anhydrous CH_2Cl_2 (7 mM) under N_2 was added Dess–Martin periodinane (3 equiv) in one portion. The mixture was stirred at room temperature under N_2 for 1.5 h. The mixture was diluted with CH_2Cl_2 (100 mL mmol^{-1} of alcohol) and then $Na_2S_2O_3$ in saturated $NaHCO_3$ (10% w/v, 10 mL mmol^{-1} of alcohol) was added. The mixture was stirred for 15 min and the aqueous phase was separated and extracted with CH_2Cl_2 (50 mL mmol^{-1} of alcohol). The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified using RP-HPLC.

8-((2-(2-Ethoxyethoxy)ethoxy)methyl)-1′,3′,3′-trimethyl-6-nitro-*N*-((*R*)-1-oxo-3-phenylpropan-2-yl)spiro[chromene-2,2′-indoline]-5′-carboxamide (3a): Compound **2a** was oxidized according to general procedure B to give compound **3a** (3 mg, 8%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.74 (s, 1H), 8.20 (s, 1H), 7.95 (d, J = 2.6 Hz, 1H), 7.54 (m, 2H), 7.40–7.18 (m, 5H), 6.96 (d, J = 10.4 Hz, 1H), 6.52 (d, J = 8.0 Hz, 1H), 5.84 (d, J = 10.2 Hz, 1H), 4.89 (s, 1H), 4.29 (s,

2H), 3.67–3.44 (m, 10H), 3.29 (m, 2H), 2.75 (s, 3H), 1.29 (s, 3H), 1.23–1.12 ppm (m, 6H); ^{13}C NMR (151 MHz, CD_3OD): δ = 168.9, 156.2, 150.6, 140.8, 139.2, 129.0, 128.9, 128.5, 128.1, 127.9, 127.3, 125.8, 125.8, 124.2, 121.6, 120.8, 118.6, 106.5, 105.9, 70.1, 70.0, 69.8, 66.1, 51.5, 39.0, 34.7, 34.6, 27.6, 24.7, 24.7, 18.7, 14.0 ppm; HRMS (ESI) found $[M]^+$ 643.2894, $C_{36}H_{41}N_3O_8^+$ requires 643.2898.

(3*R*)-Methyl 3-(8-((2-(2-ethoxyethoxy)ethoxy)methyl)-1′,3′,3′-trimethyl-6-nitrospiro[chromene-2,2′-indoline]-5′-ylcarboxamido)-2-oxo-4-phenylbutanoate (3b): To a solution of **2b** (33 mg, 0.047 mM) in anhydrous CH_2Cl_2 (2.4 mL) under N_2 was added Dess–Martin periodinane (20 mg, 0.047 mM) in one portion. The mixture was stirred at room temperature under N_2 for 3 h. The mixture was diluted with CH_2Cl_2 (4.7 mL) and $Na_2S_2O_3$ in saturated $NaHCO_3$ (10% w/v, 0.5 mL) was added. The mixture was stirred for 15 min and the aqueous phase was separated and extracted with CH_2Cl_2 (2.4 mL). The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo to give the crude product as a dark red oil, which was purified by RP-HPLC to give **3b** as a purple solid (2 mg, 6%). ^{1}H NMR (500 MHz, $[D_6]$DMSO): δ = 8.15 (s, 1H), 8.06 (s, 1H), 7.68 (d, J = 6.7 Hz, 1H), 7.56 (s, 1H), 7.28–7.20 (m, 5H), 7.12 (d, J = 10.4 Hz, 1H), 6.63 (d, J = 8.2 Hz, 1H), 5.99 (d, J = 10.3 Hz, 1H), 5.08 (s, 1H), 4.30 (s, 1H), 3.72 (s, 3H), 3.54–3.36 (m, 10H), 3.28–3.06 (m, 2H), 2.78 (s, 3H), 1.32 (s, 3H), 1.20 (s, 3H), 1.14 ppm (t, J = 7.0 Hz, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 173.0, 168.8, 156.2, 149.4, 140.8, 138.5, 136.2, 129.0, 129.0, 128.5, 128.1, 127.8, 127.8, 126.4, 125.8, 125.8, 124.9, 124.2, 121.6, 120.7, 120.4, 118.6, 106.0, 105.9, 70.2, 70.1, 70.0, 70.0, 69.5, 66.2, 56.1, 51.5, 33.5, 27.6, 24.7, 18.6, 14.0 ppm; HRMS (ESI) found $[M]^+$ 701.2932, $C_{38}H_{43}N_3O_{10}^+$ requires 701.2948.

8-(Azidomethyl)-1′,3′,3′-trimethyl-6-nitro-*N*-((*R*)-1-oxo-3-phenylpropan-2-yl)spiro[chromene-2,2′-indoline]-5′-carboxamide (3c): Compound **2c** was oxidized according to general procedure B to give **3c** (7 mg, 10%). ^{1}H NMR (500 MHz, $[D_6]$DMSO): δ = 9.57 (s, 1H), 8.64 (d, J = 6.1 Hz, 1H), 8.25 (s, 1H), 8.16 (s, 1H), 7.70 (d, J = 8.2 Hz, 1H), 7.62 (s, 1H), 7.24 (m, 5H), 6.67 (d, J = 8.3 Hz, 1H), 6.04 (d, J = 10.4 Hz, 1H), 4.41 (m, 1H), 4.26 (d, J = 13.3 Hz, 1H), 4.18 (d, J = 13.3 Hz, 1H), 3.32–3.23 (m, 1H), 2.99–2.84 (m, 1H), 2.72 (s, 3H), 1.25 (s, 3H), 1.13 ppm (s, 3H); ^{13}C NMR (151 MHz, CD_3OD): δ = 169.1, 156.8, 150.5, 140.7, 138.7, 136.0, 1290.0, 128.4, 128.1, 127.9, 127.2, 125.8, 125.2, 123.0, 122.4, 120.7, 119.1, 107.1, 106.0, 97.8, 97.5, 55.8, 55.6, 51.7, 34.7, 27.6, 24.8, 18.7 ppm; HRMS (ESI) found $[M]^+$ 552.2138, $C_{30}H_{28}N_6O_5^+$ requires 552.2121.

3,3-Dimethyl-2-methyleneindoline-5-carboxylic acid (6): To a solution of 4-hydrazinobenzoic acid (2.00 g, 13.2 mmol) and 3-methyl-2-butanone (1.26 g, 14.4 mmol) in anhydrous ethanol was added 98% H_2SO_4 (0.4 mL) slowly. The reaction mixture was refluxed under N_2 for 18 h. The mixture was allowed to cool to room temperature and the resulting precipitate was removed by vacuum filtration. The filtrate was basified with saturated $NaHCO_3$ and washed with CH_2Cl_2 (2×15 mL). The pH of the aqueous phase was adjusted to 4 with 1 M aqueous HCl and extracted with CH_2Cl_2 (2×30 mL). The combined organic phase was dried over Na_2SO_4 and concentrated in vacuo to give **6** as a dark red solid (2.43 g, 91%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.13 (d, J = 8.0 Hz, 1H), 8.04 (s, 1H), 7.63 (d, J = 8.5 Hz, 1H), 2.36 (s, 3H), 1.36 ppm (s, 6H).

5-Carboxy-1,2,3,3-tetramethyl-3*H*-indolium iodide (7): To a solution of **6** (1.12 g, 5.5 mmol) in a mixture of anhydrous toluene and MeCN (2:1, 30 mL) was added methyl iodide (782 mg, 5.5 mmol) dropwise. The mixture was refluxed for 18 h. After cooling to room temperature, the red precipitate was collected by vacuum filtration and washed with ethanol (5 mL) and hexane (30 mL) to yield **7** as a light grey solid (1.19 g, quant.). ^{1}H NMR (500 MHz, $[D_6]$DMSO): δ = 8.36 (s, 1H), 8.17 (d, J = 8.0 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 3.97 (s, 3H), 2.79 (s, 3H), 1.55 ppm (s, 6H).

(*S*)-2-Amino-*N*-methoxy-*N*-methyl-3-phenylpropanamide (11): To a solution of *N*-(*tert*-butoxycarbonyl)-L-phenylalanine (500 mg, 1.88 mmol) in anhydrous CH_2Cl_2 (8 mL) was added BOP (833 mg, 1.88 mmol) and Et_3N (188 mg, 1.87 mmol). A solution of CH_3ONHCH_3.HCl (220 mg, 2.3 mmol) and Et_3N (155 mg, 1.5 mmol) in anhydrous DCM (4.3 mL) was added to the mixture. The mixture was stirred under N_2 at room temperature for 21 h before quenched by 1 M aqueous HCl (20 mL). The mixture was diluted with DCM (50 mL) and the aqueous layer was separated and extracted with CH_2Cl_2 (2×50 mL). The combined organic phase was washed with 1 M aqueous HCl (50 mL), sat. $NaHCO_3$ (50 mL) and brine (50 mL), dried with Na_2SO_4 and concentrated in vacuo to give the crude product (630 mg), which was then dissolved in CH_2Cl_2 (24 mL). The solution was cooled on ice and then TFA (3 mL) was added. The mixture was stirred with ice cooling for 1 h. The volatiles were removed in vacuo to give the desired analogue **11** as a white wax (188 mg, quant.). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35–7.11 (m, 5H), 5.15 (s, 1H), 4.95 (s, 1H), 3.65 (s, 3H), 3.16 (s, 3H), 3.10–2.81 ppm (m, 2H).

Surface attachment of 2d to a MOF (Fiber-2d)

The MOFs used in this work were fabricated in-house. Details of the fiber and functionalization set-up have been reported previously.[18a] The fiber was sealed into a metal chamber with a positive pressure of 50 psi applied to force solutions through the fiber. The inner surfaces of the fiber was washed with piranha solution (3:1 sulfuric acid/30% H_2O_2 v/v, 1.5 mL) and carboxyethylsilanetriol in water (2.5% v/v, 9 mL) was forced through the fiber to functionalize the core surface by silanization.[28] A solution of **2d** (0.5 mM), DIC (0.5 mM), HOBt (0.5 mM) and DMAP (0.3 mM) in MeCN (10 mL) was then applied through the fiber, overnight. The functionalized fiber (**Fiber-2d**) was washed thoroughly with MeCN (10 mL) and water (10 mL) for 6 h to remove unreacted **2d** and other reagents.

Microstructured optical fiber (MOF) experiments

All MOF experiments were conducted in the dark unless stated otherwise.

Fiber-2d was first coupled to an optical set-up as previously reported by Heng et al.[18a] The fiber was filled with Mili-Q water for 5 min through capillary action. **Fiber-2d** was excited by 10 pulses of laser irradiation (532 nm, 10 ms per pulse) and the resulting fluorescence emission after each pulse was measured by a spectrometer. **Fiber-2d** was then irradiated with UV light (365 nm) generated from a mercury lamp for 5 min. **Fiber-2d** was again subjected to 10 pulses of laser irradiation (532 nm, 10 ms per pulse) with the resulting fluorescence emissions measured similarly. **Fiber-2d** was then irradiated with visible light generated from a halogen lamp (>450 nM) and was similarly subjected to laser irradiation with the fluorescence measured. The filled section of the fiber was cleaved and removed.

Fiber-2d (SP isomer) was then filled with a solution of α-chymotrypsin in Mili-Q water (1 μM) for 5 min and excited with 10 pulses of laser light (532 nm) as before for fluorescence measurements. **Fiber-2d** (SP isomer) with α-chymotrypsin solution was left at room temperature for a total of 85 min with fluorescence measured at 10, 25 and 85 min. Ten pulses of 532 nm laser light were used to induce fluorescence before each measurement. The solution-filled section of the fiber was cleaved and discarded and the remaining section of the fiber was filled with Mili-Q water over

5 min. **Fiber-2 d** was similarly excited with ten laser pulses and the resulting fluorescence emission was measured. **Fiber-2 d** was left at room temperature for 85 min before the fluorescence was measured again. The solution-filled section of the fiber was again cleaved and the remaining section was filled with α-chymotrypsin in Mili-Q water (1 μM) for 5 min. **Fiber-2 d** was concomitantly irradiated with UV light (365 nm) to allow isomerization to the corresponding MC isomer. **Fiber-2 d** (MC isomer) with α-chymotrypsin solution was left at room temperature for a total of 85 min with laser-induced fluorescence measured at 0, 10, 25 and 85 min. The solution filled section of the fiber was cleaved and discarded.

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