Synthesis and Characterization of the Novel Fluorescent Prolyl Oligopeptidase Inhibitor 4-Fluoresceinthiocarbamoyl-6-aminocaproyl-L-prolyl-2(*S*)-(Hydroxyacetyl)pyrrolidine

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Abstract: The synthesis and characterization of the first fluorescent prolyl oligopeptidase inhibitor 4-fluoresceinthiocarbamoyl-6-aminocaproyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine is described. This compound has an IC_{50} value of 0.83 nM and a dissociation half-life of 160 min, and its fluorescence signal is detectable using standard filters for fluorescein. These properties make this compound a suitable probe for visualizing prolyl oligopeptidase in various applications.

Prolyl oligopeptidase (POP, EC 3.4.21.26) is an 80 kDa enzyme that belongs to the prolyl oligopeptidase family of serine proteases, which is of ancient origin and unrelated to the trypsin and subtilisin families of serine proteases.[1,2] POP is a widely distributed enzyme among different organisms, and it hydrolyzes prolyl-containing oligopeptides at the carboxyl side of the proline residue.[3] Many biologically active peptides, such as substance P, arginine-vasopressin, neurotensin, and thyroliberin, are substrates of POP.[4−6]

POP has been implicated as a target in a wide range of disorders through different mechanisms. It has been suggested to be involved in cognitive performance because several of its substrates are known to enhance learning and memory.[7−9] Indeed, POP inhibitors have been shown to reverse scopolamine-induced amnesia in rats[10,11] and to enhance cognition in aged rats[12] and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated monkeys, which is a model for early Parkinsonism.[13] These effects are believed to be derived from the increase in the brain levels of several learning and memory-related neuropeptides.[4,6]

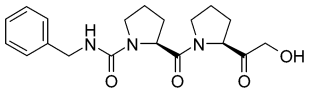
Recent studies have also shown that POP is linked to IP_3 formation by negatively regulating multiple inositol polyphosphate polyphosphatase (MInsPP), which generates IP_3 from IP_6, and as a consequence, POP inhibition increases IP_3 concentration.[14,15] This mechanism has been suggested to underlie the finding that POP inhibitors reverse the effect of three mood-stabilizing drugs: lithium, carbamazepine, and valproic acid.[16]

The unique proline-specific endopeptidase activity of POP has aroused considerable interest also in the celiac sprue research. Namely, it has been shown that POP is able to accelerate the breakdown of very stable immunogenic proline-rich gluten peptides in the gut lumen, offering a way to treat celiac sprue by oral administration of POP.[17−19]

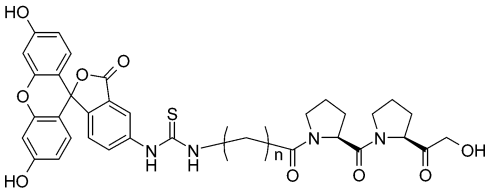
Furthermore, the POP from parasite *Trypanosoma cruzi*, which is the causative agent in Chagas' disease, has also been implicated as a therapeutic target. The POP of *T. cruzi* exhibits the unusual property of cleaving collagens of the extracellular matrix.[20] Inhibitors developed for the POP of *T. cruzi* might prevent the invasion phase in Chagas' disease.[21]

A variety of peptide substrates with cleavable fluoro- or chromophores, such as Suc-Gly-Pro-AMC and Z-Gly-Pro-AMC, have been developed to measure POP activity.[22,23] These substrates have been successfully used to determine POP activity from different sources and the potency of novel POP inhibitors. However, at the moment there are no fluorescent compounds available that could be used to directly label the POP enzyme. These types of compounds could be used in several applications, as is the case for fluorochrome-tagged inhibitors of caspases (FLICA), which have been used to detect the activation of caspases in apoptosis by fluorescence microscope, laser scanning cytometer, and fluorescence plate reader.[24] Other attractive uses for a fluorescent inhibitor include the study of the localization and distribution of its target enzyme in cells and tissues and detecting the protein in electrophoretic gels.

In the design of the fluorescent POP inhibitor, the well-reported POP inhibitor JTP-4819 was taken as the starting point. It is a selective, very potent, tight-binding POP inhibitor.[11,25] The objective was to incorporate a generally used fluorescent group into this structure without losing the high potency and tight-binding properties. The structure−activity relationships of previously reported POP inhibitors have shown that there is considerable freedom at the P3 site and beyond.



JTP-4819, IC_{50}=0.20 nM (porcine)



1: n=1, IC_{50}=180 nM (porcine)
2: n=5, IC_{50}=0.83 nM (porcine)

The fluorescein moiety was chosen as the fluorescent group, and it was linked to the inhibitor structure at the P3 position with glycyl and 6-aminocaproyl linkers, resulting in **1** and **2**, respectively. In the design of the compounds the ideas were tested by molecular docking before synthesis. The docking was carried out in a

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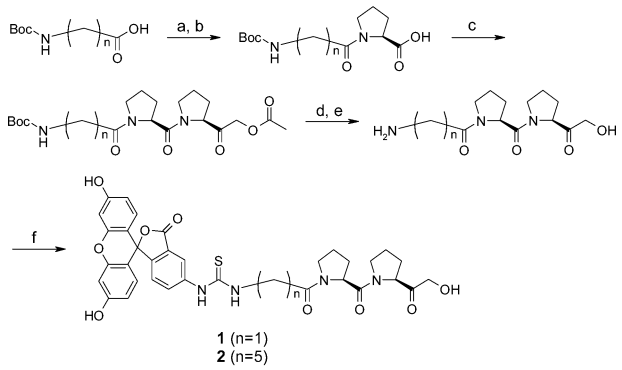
Scheme 1[a]



[a] Reagents: (a) (1) pivaloyl chloride, Et_3N/DCM; (2) L-proline methyl ester, Et_3N/DCM; (b) LiOH/MeOH, H_2O; (c) (1) pivaloyl chloride, Et_3N/DCM; (2) 2(*S*)-(hydroxyacetyl)pyrrolidine TFA salt,[27] Et_3N/DCM; (d) K_2CO_3/MeOH, H_2O; (e) TFA/DCM; (f) fluorescein isothiocyanate, DIPEA/DMF.

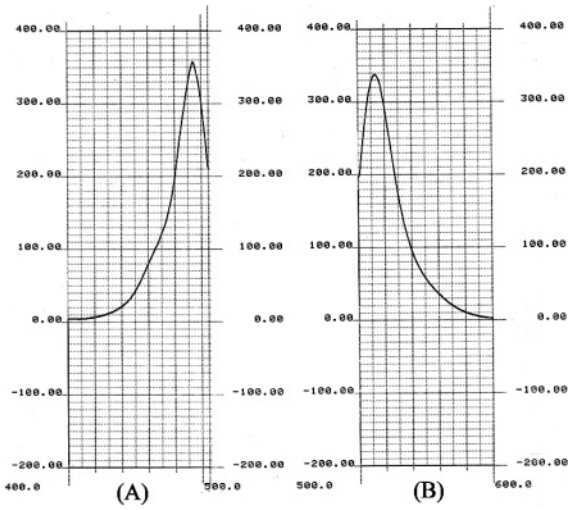


Figure 1. Excitation and emission spectra of **2**. Fluorescence was measured in 0.1 M Na-K-phosphate buffer, pH 7.0, containing 100 nM of **2**. The excitation spectrum (A) was measured at an emission wavelength of 515 nm, and the emission spectrum (B) was measured at an excitation wavelength of 485 nm.

similar fashion as in a recent article by our group.[26] Compounds **1** and **2** were synthesized according to Scheme 1.

The IC_{50} values (with 95% confidence intervals) against porcine POP for **1** and **2** were 180 nM (150−220 nM) and 0.83 nM (0.69−1.0 nM), respectively. The active site of POP was able to accommodate the relatively large fluorescein moiety very well. However, the result indicates that a long flexible tether is required to attach the fluorescein moiety to the POP inhibitor structure to maintain the high potency. Therefore, the more potent compound **2** was chosen for spectral and kinetic characterization.

The excitation and emission spectra of **2** are presented in Figure 1. The curves show that the fluorescence of **2** has the excitation and emission maximum at 492 and 512 nm, respectively. Hence, the fluorescence intensity is detectable using standard fluorometer filters for fluorescein, 470−495 nm for excitation and 510−540 nm for emission.

The binding of **2** into the active site of POP decreased the maximum fluorescence intensity by 30% but did not change the wavelength of the emission maximum (Figure 2A,B). The emission spectrum was constant from 30 to 60 min after the addition of the enzyme (data not shown). The inhibitor binding to the active site of POP was reversible because the addition of a large excess of tight-binding POP inhibitor Z-L-Pro-L-prolinal ($3000K_i$) resulted in a slow increase in fluorescence intensity due to the dissociation of the compound from the active site of POP (Figure 2C−E). The fluorescence intensity returned to the level of free inhibitor after 20 h of incubation.

The dissociation half-life of the enzyme−inhibitor complex was determined by following the disappearance of the bound inhibitor after the addition of a large excess of the tight-binding POP inhibitor Z-L-Pro-L-prolinal (Figure 3). The data were fitted into the one-phase exponential decay equation, and a dissociation rate constant of 0.004 25 ± 0.000 41 min^{-1} was obtained. This corresponds to a half-life of dissociation of 160 min with a 95% confidence interval of 140−210 min. This result is strongly supported by the experiment shown in Figure 2, where the increase in fluorescence was fitted into the one-phase exponential association equation (data points from 5 min to 20 h) and a dissociation half-life of 120 min was obtained. The long dissociation half-life can be increased even further by storing the sample on ice after the inhibitor binding (data not shown).

The presented fluorescent POP inhibitor 4-fluoresceinthiocarbamoyl-6-aminocaproyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine **2** has a high affinity, slow dissociation from the enzyme and spectral properties suitable for a

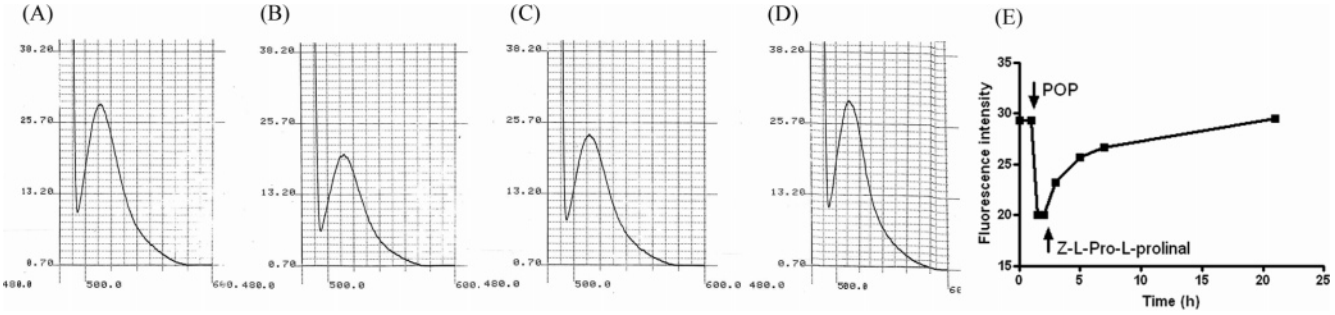


Figure 2. Change in the emission spectrum of **2** upon binding to POP. (A) Emission spectrum of 10 nM compound in 0.1 M Na-K-phosphate buffer, pH 7.0. Purified POP was added to give 10 nM, and the fluorescence was remeasured after 60 min of incubation at 23 °C. (B). Z-L-Pro-L-prolinal was added to give 1 μM, and the fluorescence was determined at different times after the addition. Emission spectra at 1 h (C) and 20 h (D) after the addition of Z-L-Pro-L-prolinal. (E) Graph of fluorescence intensity change (at 512 nm) during this experiment.

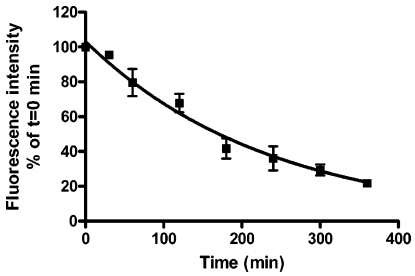


Figure 3. Dissociation of **2** from the active site of POP. A mixture of 10 nM inhibitor and 5 nM purified porcine POP was incubated for 30 min at 23 °C. Then, Z-L-Pro-L-prolinal was added to give 1 μM. Samples of 1 mL were taken at appropriate times, filtered, and washed once. The concentrate was dissolved in the assay buffer, and the fluorescence intensity was measured with a Victor2 fluorescence plate reader at 485 nm (excitation) and 535 nm (emission). Each data point represents the mean ± SEM of two to three independent measurements.

fluorescent probe. This compound is a new fluorescent scientific tool that is likely to have many applications in the expanding field of POP research.

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Supporting Information Available: Experimental details and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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