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# Inhibitors of Prolyl Oligopeptidases for the Therapy of Human Diseases: Defining Diseases and Inhibitors

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## Introduction

The activity and lifetime of proteins or peptides in living organisms are highly dependent on their processing by proteolytic enzymes also known as proteases. Proteases perform different tasks including post-translational modifications of proteins (e.g., cleavage of inactive zymogens to the corresponding active enzymes), regulation of peptide functions, and digestion of proteins into smaller peptides. Some proteases, the exopeptidases, can only cleave a few amino acids off the C-terminal or N-terminal ends of proteins, while others, the endopeptidases, can hydrolyze internal peptide bonds. Most proteases are highly specific and can only process a limited number of substrates with defined amino acid sequences. Similarly, some substrates are only processed by a very small number of proteases. For instance, many biologically active peptides are protected from general proteolytic degradation by evolutionarily conserved prolines.[1,2] Proline residues impose conformational constraints and kinks in the secondary structure and folding of peptides or proteins,[3] which in turn require very specific enzymes to process them. In fact, only a few proteolytic enzymes, referred to as proline-specific proteases, can accommodate the particular shape of proline-containing peptides in their active site and cleave off the amino acid chain adjacent to proline residues. Among these enzymes, prolyl oligopeptidase (POP,[a] EC 3.4.21.26) is a post-proline-endopeptidase, cleaving peptides on the carboxy side of proline residues located in the core of peptide chains.

Over the past 2 decades, researchers have linked abnormal mammalian POP activity to neurological disorders. To better understand and treat these different diseases and to minimize toxicity and side effects, inhibitors of POP must be potent and also selective for the target enzyme. Given the many seemingly similar enzymes grouped under proline-specific proteases, we aim to first highlight the similarities and differences between a few of the enzymes of this family. In a subsequent part of this Perspective, we will focus on one proline-specific peptidase, POP, and discuss the structure−activity relationship of inhibitors developed as potential therapeutic drugs treating neurological disorders. Furthermore, we will discuss two possible pharmacophores, one encompassing features to achieve selectivity for POP over other proline-specific proteases and another one ranking features to improve inhibitor potency. We believe that the data collected within this review will guide the future development of novel, selective POP inhibitors as more effective candidate drugs (CDs).

This Perspective focuses on a selection of reports that illustrate our discussion on POP inhibitors and their inhibitory potency and selectivity. In no way do we claim to detail an exhaustive review of all the literature in this domain. However, most of the key articles and inhibitors are covered and are representative of the literature and patents as of today. Männistö and co-workers recently reviewed several POP inhibitors evaluated in preclinical animal models, which will only be mentioned when appropriate.[4]

## Proline-Specific Exo- and Endopeptidases

**Proline-Specific Peptidases.** Researchers have found proline-specific proteases and peptidases in bacteria, protozoa, plants, and animals, including mammals. Despite close similarities between proline-specific peptidases in these different organisms (most of them belong to the serine−protease class of enzymes), these enzymes do not seem to share similar functions. For example, mammalian proline-specific peptidases can easily process small peptides (such as small peptide hormones) but can only cleave larger proteins once other proteases have processed them into smaller fragments, whereas proline-specific proteases from microorganisms can digest larger substrates.[5−7] The expression of proline-specific proteases and peptidases in almost all living organisms suggests that they play major roles in regulating biological functions via the processing of critical, biologically active peptides.

**Proline-Specific Exopeptidases: The Family of Pro Aminodipeptidases, CD26/DPPIV and FAP-α/Seprase.** Dipeptidyl peptidase IV (DPPIV/CD26, EC 3.4.14.5) is a dimeric type II integral membrane glycoprotein (MW = 220 kDa), mainly expressed by epithelia. DPPIV is a strict exopeptidase that releases X-Pro (or X-Ala) dipeptide from the free N-terminus of peptides. DPPIV is itself a target of great therapeutic interest, and inhibitors of DPPIV are clinically used for the treatment of type 2 diabetes.[8] Clearly, selectivity for POP

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[a] Abbreviations: POP, prolyl oligopeptidase; CD, candidate drug; DPP, dipeptidyl peptidase; FAP, fibroblast activation protein; CNS, central nervous system; APP, amyloid precursor protein; BBB, blood−brain barrier.



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**Figure 1.** Alignment of the primary POP sequences from various species (bovine, pig, mouse, rat, human, chicken). The residues in direct contact with the bound inhibitor **1** (see below) are highlighted in red.

over DPPIV is critical for the development of POP inhibitors as drugs.

Fibroblast activation protein-α (FAP-α/seprase)[9] is a dimeric type II integral membrane prolyl dipeptidase with a molecular weight and an enzymatic activity comparable to those of DPPIV but also displays endoproteolytic gelatinase and collagenase activities[8,10−16] comparable to those of POP. However, FAP-α/seprase may respond differently to some inhibitors developed against POP.[17] FAP-α/seprase is a cell surface antigen of reactive fibroblasts in cancer, such as melanoma or sarcoma,[1] found at remodeling stroma, in tumors and healing wounds,[18] and in serum.[17] In fact, Santos and co-workers recently found that they could reduce tumor sizes in mice by inhibiting FAP-α/seprase, demonstrating that FAP is a potential therapeutic target.[19] The DPPIV family of proline-specific exopeptidases will not be reviewed in detail here, since many recent reviews have been published.[20,21]

**Proline-Specific Endopeptidases: Prolyl Oligopeptidases (POPs).** Prolyl oligopeptidases have received different names such as post-proline cleaving enzyme (PPCE)[22,23] and proline endopeptidase (PEP)[24,25] before they were named prolyl oligopeptidases. The POP enzyme family evolved before the archae, prokaryota, and eukaryota and was highly conserved during evolution (Figure 1). In mammals, prolyl oligopeptidase (EC 3.4.21.26) is a post-proline-endopeptidase of 80 kDa, which belongs to the S9a subfamily.[1,2,12,26−28] Mammalian POP is widely expressed and

most highly in the brain.[26] Proline-specific endoproteases are also widely distributed in plants, bacteria, and fungi. POP may be secreted and is involved in the invasive properties of parasites.[29]

Proline-specific serine proteases, including POP, exhibit similarities in their catalytic behavior: preference for proline in the substrate's P1-position, similar rate constants, and a mechanism of substrate-assisted catalysis, which means that the interaction between enzyme and substrate promotes conformational changes in the substrate, a productive binding resulting in hydrolysis of the prolyl bond.[30] Given these similarities, selective POP inhibition over other proline-specific exo- and endopeptidases remains a challenge.

## POP and Other Proline-Specific Peptidases as Potential Therapeutic Targets

**POP as a Therapeutic Target.** Researchers have shown that POP participates in several aspects and functions of the central nervous system (CNS), including learning, memory, mood, hypertension, and eating, and in some neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Subsequently, POP has been identified as a potential target in cognitive function, memory, and neurodegenerative disorders such as amnesia, Alzheimer's disease, and depression (detailed reviews in refs 31−33). As a result, researchers have designed inhibitors such as Cbz-Pro-prolinal (**1**)[5] targeting POP for its role in neurological diseases. Although correlations and straightforward conclusions were not always obvious, preclinical studies suggested promising applications of POP inhibition to memory and learning disorders compared to other disorders. We have found little information on the potential of such molecules in infectious, oncological, or inflammatory disorders, and their possible therapeutic role in these diseases will not be discussed here.

**POP and Protein Aggregation.** In vitro, POP inhibitors suppressed the production of $\beta$-amyloid in cells.[34] However, POP activity may be responsible for generating $\beta$-amyloid, but this activity is inhibitor- or cell-dependent,[35] suggesting either that several proteases with comparable specificity are acting or that the enzymes are located in different cell compartments. In vivo, POP inhibitors have been mainly evaluated in animal models of Alzheimer's disease (models such as the brain of aged mouse or aged rat and transgenic mice expressing human amyloid precursor protein, APP), generally improving the deteriorated cognitive and memory functions, as well as decreasing the amyloid deposition.[36−41] Although seemingly linked with Alzheimer's disease, POP was not found to be associated with activated glial cells in amyloid plaques in the brains of people with the disease.[37] In addition, conflicting results have been reported in the literature. For example, Kohsaka and Nakajima showed that the POP inhibitors **2** (JTP-4819[34]) and **3** (Y-29794[38]) (Figure 2) abolished the formation of $\beta$-amyloid.[34,38] Others demonstrated that POP inhibitors had no effect on $\beta$-amyloid levels in certain cell types, indicating that other proteases are involved or that the enzymes producing the $\beta$-amyloid peptides, as well as the $\beta$-amyloid precursor, were located in different cell compartments than the $\beta$-amyloid peptides.[35] Researchers noted, through immunostaining, that POP and amyloid $\beta$-peptide were colocalized in the brain of age-accelerated mice,[42] although POP activity seems to be associated with neuronal damage rather than with $\beta$-amyloid accumulation.[43] In this latter report, only POP-like activity

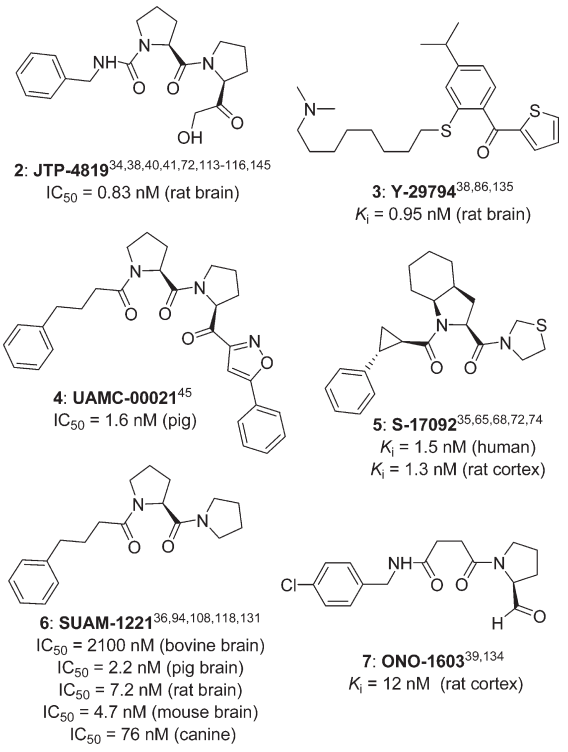


**Figure 2.** Selected POP inhibitors **1**−**7**.

was determined, without precise characterization of the exact enzyme.

Fragments of several other intracellular proteins of the CNS, which are also known to aggregate, are potential substrates for POP,[44] suggesting that POP inhibitors may have an effect on neurodegenerative disorders, slowing the aggregation of a number of proteins. In vitro recombinant POP accelerated the aggregation of α-synuclein,[45] a protein found in the Lewy body in the brains of people suffering from Parkinson's disease. The presence of POP caused α-synuclein to aggregate, without truncating α-synuclein. Interestingly, when POP inhibitors were added (for example, **1** and **4** (UAMC-00021[45])), α-synuclein aggregation was reversed. Whereas α-synuclein is not a substrate for POP, POP can hydrolyze fragments of α-synuclein at the Pro138−Asp139 bonds,[44] suggesting cooperation between several proteases.

**Role of POP in the Central Nervous System (CNS).** Männistö demonstrated that POP and/or POP-like activity is distributed throughout the CNS of humans and rats,[46,47] possibly indicating a role for POP in motor functions, and also at the cellular level in protein processing and secretion.[37,48] Männistö colocalized POP with components of the inositol phosphate pathways[46,47] and with several neuropeptides.[47,49] From these studies, the interaction of POP with the components of the inositol phosphate pathways has been postulated in neurological diseases. However, researchers have yet to confirm the exact mode of action and the peptide mediator(s) involved, postulating only that several neuropeptides may be involved.[32,50] In fact, the actual neuropeptide substrates of POP remain uncertain, although the enzyme has the ability to hydrolyze several peptide hormones and neuropeptides in vitro (Table 1).[1,8,12,27,51−53]

Neurons in the brain express POP, but the level of expression is different in various areas of the brain and is age-dependent.[37] POP is a synaptosomal membrane peptidase[27]

**Table 1.** Selected Potential Neuropeptide Substrates of POP[32,50]

| bioactive substrates of POP | peptide sequence | ref |
|---|---|---|
| angiotensin I | DRVYIHPFHL | 52, 54, 55 |
| bradykinin potentiating peptide | pGLUGGWPRPGPEIPP | 56 |
| bradykinin | RPPGFSPFR | 52, 57 |
| luliberin (LHRH[a]) | pEHWSYGLRPG$NH_2$ | 54, 58 |
| melantropin | SYSMEHFRWGKPV$NH_2$ | 59 |
| neurotensin | pELYENKPRRPYIL | 52, 60 |
| oxytocin | CYIQNCPLG$NH_2$ | 52, 61 |
| substance P | RPKPQQFFGLM | 52, 55 |
| thyroliberin | pEHP$NH_2$ | 54, 62, 63 |
| tuftsin | TKPR | 59 |
| vasopressin | CYFQNCPRG | 55 |

[a] LHRH: leuteinizing hormone releasing hormone.

localized intracellularly[48] mainly in the perinuclear space associated with the cytoskeletal tubulin in human neuroblastoma and glioma cells. POP inhibition does not change the intracellular localization of POP or its association with tubulin. Thus, association of POP with tubulin is independent of its peptidase activity. In glioma cells, antisense techniques or POP inhibition resulted in increased inositol 1,4,5-triphosphate concentrations with decreased POP activity, further supporting that POP is involved in this pathway. Substance P (one of the substrates of POP in vitro) may mediate this process, binding to its neurokinin-1 receptor, and may modulate cellular pathways important in learning and memory function.[64] Thus, the cellular localization of POP suggests functions such as intracellular trafficking, sorting, and secretion.

**POP Inhibition in Animal Models.** POP inhibitors showed beneficial effects, reducing cognitive deficits in monkeys having Parkinson's disease symptoms induced by the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT), killing neurons in specific regions of the brain.[65] Morain hypothesized that the inhibitors reduced neuropeptide degradation.[40,41,66−68]

In animal models from preclinical studies, POP inhibitors could reverse age-related or neurodegeneration-related memory loss. Alterations in the inositol pathway explain, at least in part, the effects of POP inhibitors for bipolar disorders in experimental models. Synthetic POP inhibitors increase concentrations of some neuropeptides, which are potential substrates of POP, supporting the role of POP in some neurological disorders. POP-like activity decreased in brain extracts of animals treated with these inhibitors. We note that the preclinical and clinical evaluations of POP inhibitors suffer from several problems. The role of POP in the brain may go beyond neuropeptide processing, including regulating intracellular pathways, neuroprotection, perhaps even acting as an antiapoptosis agent. In addition, most published information evaluated "POP-like" activity, without detailed characterization of the actual protease displaying the activity in order to confirm enzyme identity. Neuropeptides may have multiple hydrolysis sites and associated peptidases, in addition to a site for POP.

**Passage through the Blood−Brain Barrier (BBB).** Researchers have reported many synthetic POP inhibitors, of which most are substrate-like short pseudopeptides that have little potential of crossing the BBB, a very unfavorable pharmacokinetics characteristic. In most preclinical studies, researchers evaluated the inhibition of POP-like activity in animal brain extracts after the animals were treated with POP inhibitors. However, in their published papers, researchers did not definitely prove the mechanism of *transport* of POP inhibitors across the BBB. None measured the levels of the inhibitors in brain extracts and, more importantly, the distribution of the inhibitors between the brain compartments, in particular the brain vasculature forming the BBB, and the brain parenchyma. Some authors have used artificial membranes to evaluate permeability to membrane models, but this is still not representative of the BBB.[69,70]

The lone fact that these molecules have an effect on CNS pathologies does not prove that they were physically transported into the brain parenchyma: inhibitors may indirectly act in the blood, they may be trapped in the CNS vasculature where they exert their action, or they may in fact reach the brain parenchyma. Techniques to study these possibilities are established but not easy to perform: using (mostly radioactive but not always) inhibitors, animals are bled before their organs are removed, and the organs and the blood can be separately evaluated to determine whether the inhibitors are present (or absent) in each. To differentiate whether inhibitors are trapped in either the brain vasculature or the brain parenchyma, brain vessels are separated from brain parenchyma, and again, researchers can properly determine whether the inhibitors and enzyme are present (or absent) in the two compartments. To the best of our knowledge and according to the published papers, researchers have not systematically separated the organs from the blood or separated brain vessels from brain parenchyma nor have they thoroughly characterized and analyzed the contents of each; therefore, none of them have definitely proved inhibitor transport across the BBB. The vascular density of the brain is too high for researchers to perform autoradiography to localize radioactive molecules. Although fluorescent reporters could be attached to inhibitors, these fluorescent tags may alter their biodisposition.

In order for researchers to successfully develop efficient treatments for the diseases of the CNS, therapeutic agents must be transported across the specialized vascular system of the brain, the blood−brain barrier (BBB), a huge obstacle in the development of therapies of the CNS, preventing the brain from taking up most (>98%) small molecules and all large molecules using transvascular routes after the inhibitors are administered intravenously.[71] The BBB is a system of vascular cellular structures mainly represented by tight junctions between endothelial cells and an ensemble of enzymes, receptors, efflux pumps, and transporter systems that all control and limit the access of molecules to the brain, by either para-cellular or transcellular pathways. The presence of tight junctions between cells, precluding molecules of any size from diffusing para-cellularily, the lack of fenestrae, and the low occurrence of pinocytic vesicles differentiate endothelial cells of the BBB from endothelial cells of the rest of the body. Numerous detoxifying enzymes as well as drug efflux systems such as the ATP-driven efflux pumps of the multidrug resistance pathways can expel many hydrophilic as well as hydrophobic agents. Furthermore, a basement membrane of perivascular cells and the astrocytes' extended processes, the astrocyte end-feet that cover the vast majority of the abluminal surface of the BBB capillaries and that contact the endothelial cells, also help to seal the interstitial space of the brain from the circulating plasma. Therefore, all the structures forming the BBB constitute a diffusion barrier not only for large molecules but

also for small molecules. Also, under normal conditions, the lack of pinocytosis of endothelial cells of the BBB limits transcellular transport, which alone can move therapeutic agents across the BBB.

The BBB is naturally breached in a few sites along the midline of the brain, known as the circumventricular organs. Here, the capillaries are fenestrated, allowing relatively free exchange between the blood and the brain. Besides these few sites, the BBB is the bottleneck of the development of drugs for CNS diseases. Transport across the BBB requires transport across the luminal and abluminal membranes of the capillaries and the associated cells forming the BBB and across the basement membrane surrounding these cells. Whereas passive diffusion allows a few lipid-soluble molecules to pass freely from the blood to the interstitium of the brain, ionic solutes are unable to cross. And even if the brain cerebral endothelium takes them up, these solutes will be trapped in the endothelium or efflux pumps will extrude them toward the blood. These ionic solutes will not reach the brain parenchyma. Therefore, most therapeutic drugs do not cross the BBB, unless invasive processes or chemical modifications of drugs with recognition and transcytosis ligands open the BBB (for a more detailed review, see ref 71). Families of influx transporters expressed at the BBB include the carrier-mediated transporters of small molecules, the receptor-mediated transporters, or adsorptive-mediated endocytosis. However, the drugs must structurally resemble the normal transporter substrates in order to be recognized. A small molecule that is an inhibitor of POP but also resembling a natural BBB transporter substrate would have to be designed in order for the POP inhibitor to cross the BBB in this way.

**Clinical Trials.** The role of proline-specific peptidase activity has been almost exclusively studied in preclinical models of neurological diseases, and clinical data of POP inhibitors are scarce. Among the very few inhibitors investigated in humans, **5** (S-17092[72]) and **2** are the most widely studied (and reported) POP inhibitors. **2** was orally administered to young healthy male volunteers as a single daily dose, or three times per day, for 1 week.[73] In this study, the plasma and urine concentration of **2** were determined; standard plasma assays were performed, and the plasma level of several neuropeptides was quantified. Overall, researchers observed acceptable pharmacodynamics and pharmacokinetics profiles with the only abnormal finding being a transient elevation in plasma cholinesterase in the multiple-dose study. They observed no strong evidence of changes in plasma neuropeptide levels, whereas in a preclinical study in rats, the researchers noted brain neuropeptide levels increased in animals treated with **2**.[40]

In another preclinical study, the Morain group found that 60 min after oral administration of **5**, POP-like activity was reduced in all brain regions whereas levels of substance P and α-melanocyte-stimulating hormone increased.[68,72] This study in rodents was followed by studies in humans. A short (1 week) human phase I exploratory trial with **5** (Figure 2) at single and repeated doses in healthy elderly volunteers showed that circulating POP-like activity was inhibited but circulating levels of neuropeptides were not measured.[72,74] The inhibitor was rapidly absorbed after oral administration (within 1 h), and the inhibition of POP-like activity in the blood lasted from 0.5−1 h (peak) to 12 h. However, the researchers did not characterize and identify the enzyme responsible for this POP-like activity in the blood; therefore, we cannot be sure that this decreased enzyme activity was due to POP inhibition and not inhibition of an enzyme with a similar function. The percentage of enzyme inhibition was not obviously different between doses, but the duration of inhibition was dose-dependent. The inhibitor was well tolerated, and no abnormalities in standard laboratory parameters were detected. The Morain group used electroencephalography (EEG) to measure drug penetration into the human brain, drug efficacy, and the duration of the drug's effects. Increased α band in quantitative EEG and improved cognitive memory tests were observed in the volunteers, but these effects were not dose-dependent.

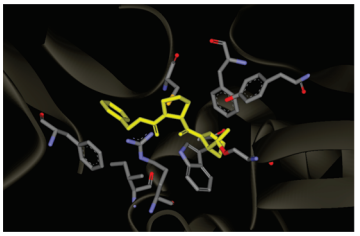


**Figure 3.** Crystal structure of inhibitor **1** bound to porcine POP (left). Phe173, Cys255, Tyr473, Phe476, catalytic Ser554, Ile591, Trp595, and Arg643 are shown. Structure and affinities for POP from various species for inhibitor **1** (right).

## POP Structural Information

A number of crystal structures of POP either unbound (PDB codes 1vz2,[30] 1h2w[75]) or bound to various ligands (1h2y,[75] 1o6g[76]) have been reported, but the catalytic serine was mutated to alanine for many of these structures (1uoq,[77] 1uoo,[77] 1h2z[75]). Two structures include **1** covalently bound to porcine POP (1qfs,[28] Figure 3, porcine muscle POP, resolution 2.00 Å, and 1h2y,[75] porcine brain POP Y473F, resolution 1.78 Å) and another includes Cbz-Ala-prolinal covalently bound to microbial POP (2bkl,[78] *Myxococcus xanthus* POP, resolution 1.50 Å), resulting in information on the binding mode of covalent inhibitors. Structures of POP cocrystallized with noncovalently bound inhibitors (3eq9) have been reported as well.[79]

The crystal structure of purified porcine brain POP shows a cylindrical structure (60 Å × 50 Å) consisting of an α/β-peptidase domain, where the central tunnel of an unusual β-propeller covers the catalytic triad (Ser554, His680, and Asp641).[28] The crystal structure of POP in complex with the inhibitor **1**[28] suggests conformational changes consistent with the observation that the rate-limiting step of catalysis involves a conformational change.[80] In this early report, reagents (such as *N*-ethylmaleimide) react with an unpaired cysteine (Cys255) on the propeller domain of the enzyme, impairing important conformational changes and inhibiting the enzyme. Interestingly, when crystallized, the rigid seven-bladed propeller acts as a gate that appears to be too narrow to allow passage of the substrate, thus controlling the selectivity of the enzyme. In addition, the active site entry is narrower than the average diameter of most peptides and small proteins, thus preventing longer sequences from uncontrolled degradation. The lack of an apparent entrance for the substrate further supports substantial conformational changes of POP prior to substrate binding, involving flexible regions of the protein. A small tunnel between the flexible N-terminal segment of the peptidase domain and the facing hydrophilic loop of the propeller domain was identified as a possible entry passage for the substrate.

## POP Inhibition

**Targeting Proline-Specific Proteases.** Researchers have linked proline-specific protease activity with several human diseases, such as cancer, neurodegenerative, or immunological/inflammatory disorders. Thus, modulating the activity of proline-specific proteases may be a relevant therapeutic approach. Substrates for proline-specific proteases include autocrine peptides (produced by the cells expressing the proteases) or paracrine peptides (produced by cells other than the cells expressing the proteases). Therefore, inhibitors may be developed for the inhibition of extracellular proteases, either secreted or whose catalytic site is inserted in the outer cell membrane, or for the inhibition of intracellular proteases. While drugs developed for inhibiting extracellular protease activity have been very successful, targeting intracellular proteases and evaluating inhibitors in intact cells has been much more difficult.[81]

Since comparable proline-specific peptidase activities are found in many locations in and outside cells, the exact site, mode of action, and biological target(s) of these inhibitors cannot necessarily be attributed to inhibition of one enzyme. To avoid side effects and toxicity of inhibitors, researchers must develop compounds that selectively inhibit proline-specific protease activity of one enzyme.

**Potent and Selective Inhibition.** With data suggesting that POP inhibitors can modulate memory and other neurological disorders, interest in mammalian POP and POP inhibitors increased. To this end, hundreds of natural or synthetic compounds have been tested.[82] Many of them are substrate analogues, of which **1**, **2**, **5**, **6** (SUAM-1221[36]) and **7** (ONO1603[39]) are the most studied. However, the reported inhibitors were evaluated on POP enzymes from several species, including microorganisms and mammals. For instance, testing **1** on *Flavobacterium meningosepticum*,[5] as well as rabbit,[83] mouse,[84] and bovine origin[5] revealed species-dependent $K_i$ values (Figure 3). Therefore, comparing inhibitory potency of molecules between species is not recommended as a screening strategy, and published information must carefully address this. In this article, we will therefore focus our discussion on mammalian POP inhibitors. All the available biological data, and the species that these data apply to, are given.

**Inhibitors of POP in Human Diseases.** The S1 site of POP typically accommodates a proline residue. However, POP sometimes can also accommodate an alanine residue, although the rate of hydrolysis at this residue is much lower than with proline. The S1′ site is typically occupied by a hydrophobic residue, located on the right side of the scissile bond (Figure 4), although arginine may also be at that location, as in neurotensin, a substrate of POP.

As reviewed above, brain cell-expressed prolyl oligopeptidases (POPs) have been linked to a number of neurological disorders. The selective inhibition of POPs over other peptide-cleaving enzymes as a therapeutic approach has been explored, mainly with the development of peptidic and pseudopeptidic inhibitors, as well as a few nonpeptidic molecules. To develop more potent and selective POP inhibitors, medicinal chemists need to better understand the key inhibitor sites for selectivity and potency. By highlighting and comparing the beneficial and detrimental changes to the sites of POP inhibitors, we will define an optimal pharmacophore of POP inhibitors. In this Perspective, we will develop this pharmacophore by grouping known

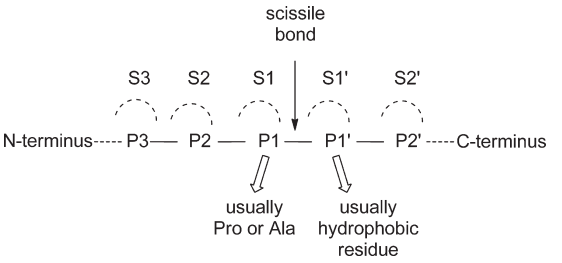


**Figure 4.** Interaction sites (S$x$) of POP active site with portions (P$x$) of substrate or inhibitor.

inhibitors according to their key sites (P3, P2, P1), in relation to mammalian POP. For the following sections of this article, we will review all structural changes to each of these key inhibitor sites (P3, P2, P1). When necessary, we will mention recent (from 2000 to present), relevant patents, since De Nanteuil, Portevin, and Lepagnol provided an extensive review of inhibitors patented prior to 1998.[82] Furthermore, we have noted that many of the patented compounds closely resemble compounds previously published in journals. Among several examples, a series of compounds (structures not disclosed) very similar to **3** were patented as POP inhibitors in 2009.[85]

## Peptidic, Pseudopeptidic, and Peptidomimetic POP Inhibitors

From as early as 1983, with the discovery by the Wilk group that **1** selectively inhibited POP from rabbit brain ($K_i$ = 14 nM) over a number of other peptidases such as papain, trypsin, and chymotrpysin,[83] researchers have thoroughly investigated the effects of structural changes at P1, P2, and P3 of peptidic, pseudopeptidic, and peptidomimetic inhibitors. Early efforts came from academia with groups in the U.S.,[22,56,86] Japan,[5,22,23,87−99] Belgium,[100,101] France,[102−106] and Hungary,[79,107] as well as from industrial research groups (e.g., Zeria Pharmaceuticals,[108,109] Ajinomoto,[110] Meiji Seika Kaisha,[111,112] Japan Tobacco,[40,41,76,113−116] Pfizer,[117] and Servier[36,72]). Most of the research efforts, from academia and the pharmaceutical industry, focused on developing pseudopeptidic and peptidomimetic POP inhibitors. Recently, a group from Finland has further developed a number of pseudopeptidic inhibitors,[118−128] as did industrial efforts from Genentech[129] and GlaxoSmithKline.[130] Interestingly, to our knowledge, all the reported inhibitors bind to the S3, S2, S1 sites of the catalytic site while only one of these reported small molecule inhibitors (**4**)[45] may fill the S1′, S2′, and S3′ pockets of POP.

**Modification of P3 for Optimal Binding.** We selected inhibitors with representative variations at P3 and depicted them in Figure 5. Tight binding of inhibitors to POP often requires a hydrophobic group at P3 (e.g., Cbz group of **1**) to interact with the hydrophobic S3 pocket lined with several nonpolar residues such as Phe173, Met235, Cys255, Ile591, and Ala594.[28] In fact, most of the known substrates feature a polar or hydrophobic residue but no charged residues at this position. When the Cbz group was replaced with a phenylacyl group of varying chain length (**6**,[36,108,131] Figure 2), a chain length of three carbon atoms (phenylbutanoyl) was found to be optimal for low nanomolar inhibition of POP originating from various species. The obtained potency of **6** on bovine brain POP was comparable to the $IC_{50}$ of **8** having a shorter Cbz group at P3.[94] Clearly, the substitution of an oxygen by two methylene groups is reasonable.[94,131]

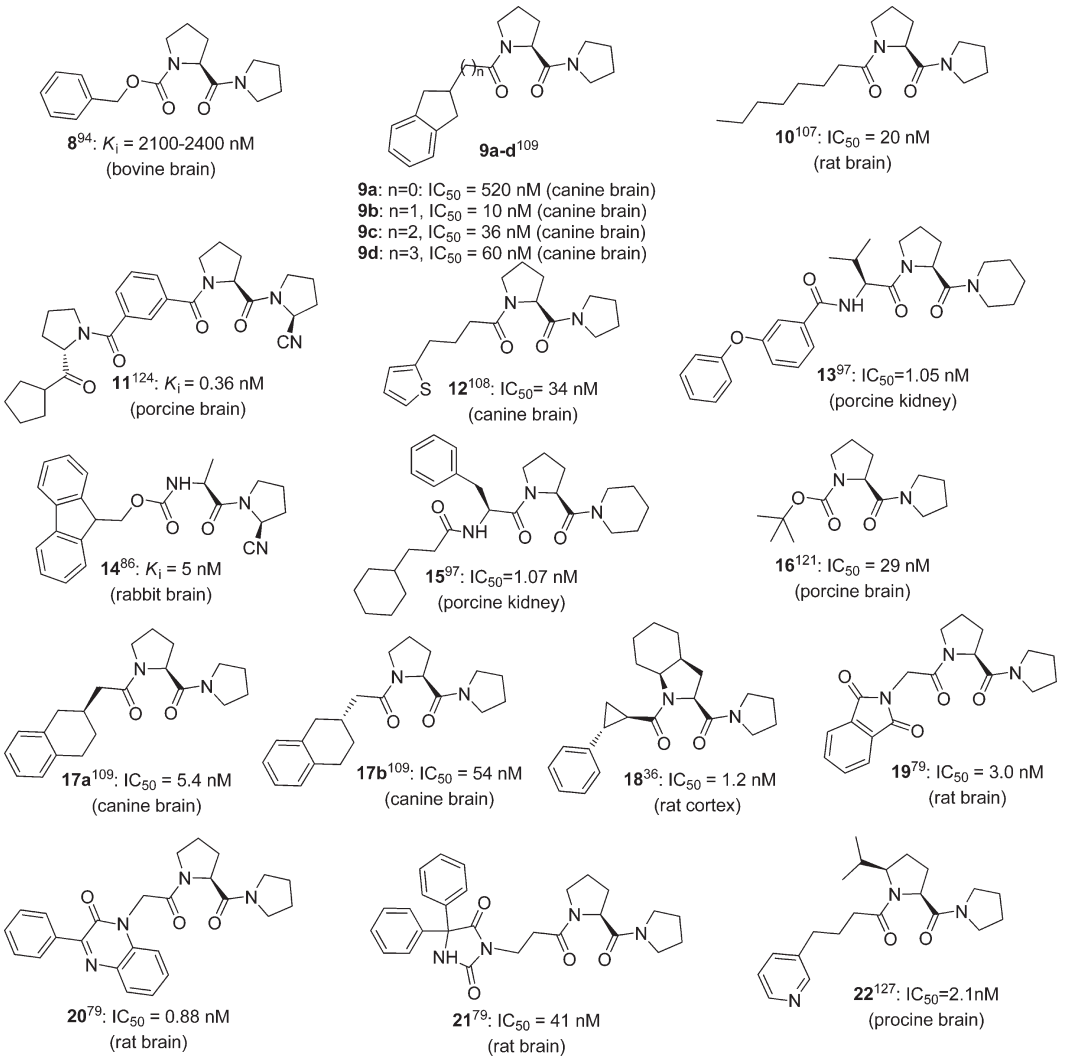


**Figure 5.** P3 substitutions.

When the floppy chain of **6** was constrained (**9a**−**d**),[109] the observed $IC_{50}$ on canine brain POP was improved (by 1−2 orders of magnitude). Further investigation of the P3 site with acyl and alkenyl groups of varying chain length led to the conclusion that octanoyl (**10**) was the longest acyl group that could be tolerated at P3 to favorably fill and interact with the S3 pocket of POP.[107] Longer alkyl chains lead not only to poor solubility but also probably to steric clashes in the S3 pocket and large entropy penalties upon binding, resulting in less potent inhibitors.[107] Although most of the reported investigations on P3 inhibitor−enzyme interaction recommend flexible linkers to properly fill the S3 site of POP and to avoid any detrimental clashes with the protein, long and rigid moieties have also been successfully introduced (**11**). However, additional changes to these molecules were also required to achieve potent inhibition, rendering their direct comparison with other inhibitors difficult.[124] Similarly, the large and bulky fluorescein tag was best tolerated at P3 if a five-carbon linker was employed, although high nanomolar activities were still recorded with a single carbon.[125]

The Toide group successfully replaced the oxygen of the Cbz group with a nitrogen (as in **2**).[113,114] This demonstrates that a hydrogen bond donor (**2**), a hydrogen bond acceptor (**1**), and a methylene group (**6**) are tolerated at position P3.

Researchers also modified the phenyl of the Cbz group of **1**. SAR data show that the phenyl ring could successfully be replaced with isosteric heterocycles (**12**),[108] large, more rigid groups such as 3-phenoxybenzoyl (**13**)[97] or Fmoc (**14**),[86] nonaromatic groups such as 3-cyclohexylpropionyl (**15**)[97] and *tert*-butyl (**16**),[121] and also constrained (i.e., cyclized) moieties such as tetrahydronaphthylacetyl (**9a**−**d**) and indanoylacetyl (**17a**,**b**).[109] Similarly, Portevin et al. successfully introduced (2-phenylcyclopropyl)carbonyl (**18**) as a rigidified version of 4-phenylbutanoyl, yielding an even more potent inhibitor ($IC_{50}$ of 1.2 nM on POP from rat brain).[36] More recently, Kanai et al. explored different highly rigid aromatic groups at P3, preparing phthalimido (**19**), quinoxalinone (**20**), and the bulkier hydantoinyl (**21**) derivatives,[79] while Jarho et al. focused on pyridine (**22**), all with varying acyl chain lengths.[127] The introduction of an ionizable group such as pyridine is expected to increase the water solubility of the otherwise hydrophobic phenyl rings. In fact, Jarho and co-workers noted that the log *P* of the inhibitor dropped significantly while the inhibitor potency was maintained ($IC_{50}$ of 2.1 nM on POP from pig brain) when they substituted the phenyl ring by a pyridine ring.[127] The introduction at P3 of diaminophosphinyl substituents has also been investigated as surrogates for the Cbz group of **1** and analogues.[132]

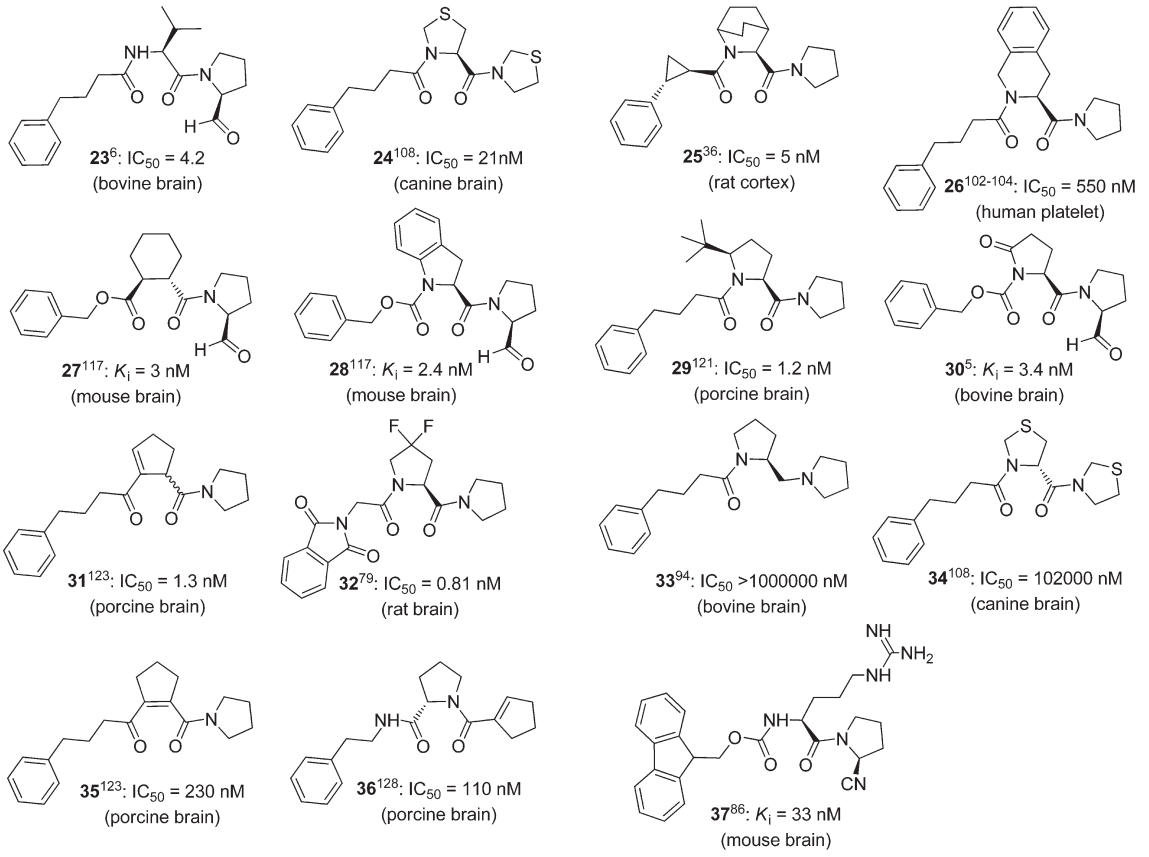


**Figure 6.** P2 substitutions.

Although size (length, bulk, etc.) has been thoroughly investigated, only a few reports detail stereochemical requirements of the P3 subsite (**17a**,**b**).[95,109] These studies demonstrate that configuration does not have a major impact on potency (1 order of magnitude for **17a** vs **17b**).

**Modification of P2 for Optimal Binding.** The S2 pocket of POP has no distinguishing feature that would entail substrate or inhibitor selectivity at this site, although a few residues (Asn534, Tyr453, and Arg618) may be key, as they are conserved in members of the POP family.[78] A selection of inhibitors highlighting some interesting P2 modifications is given in Figure 6. In most cases, P2 was kept as a proline, a proline derivative, or a proline mimic. In only a few examples, researchers investigated replacing the proline residue at P2 with other amino acid residues (such as valine in **23**).[6,133]The Yoshimoto group substituted the proline at P2 by thiazolidine *S*-oxide, increasing inhibitor potency.[94] The potency further increased when thiazolidine was used (**24**).[93,94,108] The combined data indicate that subtle changes either steric or electronic in nature affect the inhibitor binding.[93,94,100,107−109] A few groups searched for a suitable replacement of the P2 amino acid. A few groups have successfully replaced proline with some non-natural amino acids containing perhydroindole (**18** and **5**), azabicyclo-[2.2.2]octane (**25**), and azabicyclo[2.2.1]heptane.[36] Similarly, tetrahydroisoquinoline (**26**),[102−104] cyclohexyl (**27**),[117] and indoline (**28**)[117] based POP inhibitors were reported. Wallén et al. functionalized the proline at P2 with either methyl or *tert*-butyl at position 5 of the ring, testing both (*R*)- and (*S*)-configurations (**29**). Although the substitution did not lead to drastic increase or loss of potency, they found that the (*R*)-stereochemistry was optimal.[121] These results along with the results for compound **30**[5] clearly indicate that there is some unexplored space in the S2 pocket of the POP binding site that could be filled to improve inhibitor binding. Wallén et al. later substituted a cyclopent-2-enecarbonyl (**31**) for the proline at P2, yielding inhibitors equipotent to **6** but also more lipophilic which could improve cell permeability.[123] However, the cyclopent-2-enecarbonyl unit of **31** could act as a Michael acceptor to any nucleophilic species (with, for example, the thiol of a cysteine side chain), forming a covalent bond upon binding and leading to undesired side effects. Very recently, researchers showed that substituting the $\gamma$-$CH_2$ of proline with a $\gamma$-$CF_2$-group (**32**) could enhance the inhibitory activity by adding an extra hydrogen bond with the enzyme but also by inducing a slight shift in the bound pose.[79] The opening of the ring and the use of a succinic acid core led to the development of **7**.[134]

The carbonyl group between the two pyrrolidine rings of **1** has been shown to interact with the protein binding site (PDB code 1h2y). In fact, removal of this oxygen led to a complete loss of potency (**33**).[94]

The stereochemistry at P2 has also been investigated through a variety of inhibitor structures. The Arai group showed that the natural proline stereochemistry is crucial for optimal activity (**34** vs **24**).[108] Replacing the asymmetric carbon by a flat $sp^2$ carbon or amide nitrogen also led to a significant loss of potency (**31** vs **35**, **36**).[123,128]

To date, most of the modifications at P2 are designed to mimic the proline residue of **1** and significant changes should be made if we aim to develop more "druglike" molecules. In this context, phenyl rings have been used successfully.[85,135] In fact, a closer look at the natural substrates (Table 1) reveals that a number of other residues can be tolerated at

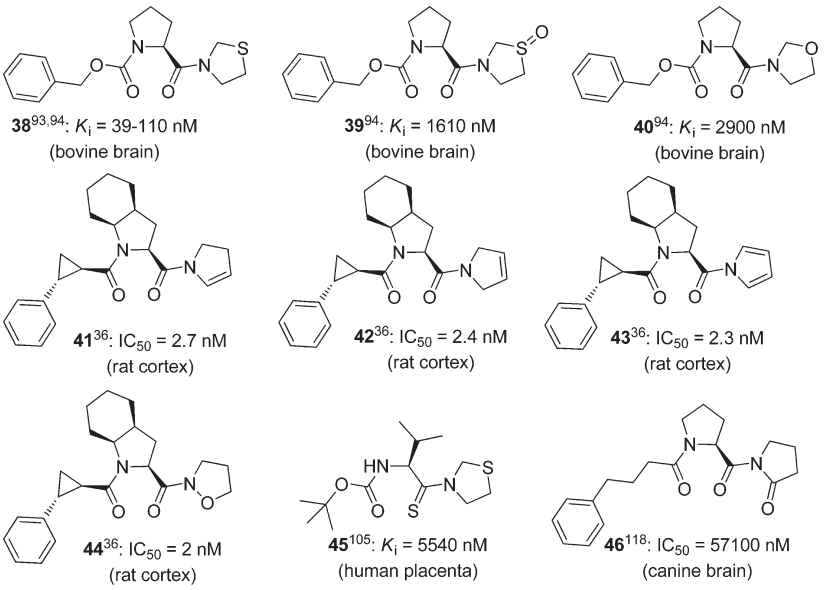


**Figure 7.** P1 substitutions.

this position and should guide the design of novel inhibitors. In addition, the activity of a series of Fmoc-aminoacylpyrrolidine-2-nitriles such as **14** and **37** revealed that an alanine or a charged arginine residue can suitably replace the P2 proline of **1** with only a small loss of binding affinity.[86]

**Modification of P1 for Optimal Binding.** The S1 pocket is lined with several hydrophobic residues (Trp595, Phe476, Val644, Val580, and Tyr599) allowing the proline ring of its substrate to fit tightly and stack with the indole ring of Trp595 and rendering POP very specific for substrates having residues that can tightly fit within this pocket.[28] A selection of P1 modifications are given in Figure 7. The chemical nature of the P1 substituent governs whether the designed inhibitor will act covalently or noncovalently. If P1 is a pyrrolidine, the inhibitor acts in a noncovalent fashion (for example, **6**). The pyrrolidine mimics the right-hand-side ring in **1**. When noncovalent inhibitors are compared, compounds with a thiazolidine (**38**) at P1 were found to be more potent than pyrrolidine- (**8**) thiazolidine *S*-oxide- (**39**), and oxazolidine- (**40**) containing compounds.[93,94,104] Portevin et al. further explored nonreactive heterocycles at P1 finding comparable potency with 2- and 3-pyrrolines (**41**, **42**), pyrroles (**43**), and isoxazolidine (**44**).[36] The amide bond was also converted into the thioxo amide bond (**45**) but with significant loss of potency.[105] Addition of an oxygen to **6** led to **46** which demonstrated significantly lower potency.

Once more, the structural modifications at P1 reported to date focused on proline mimics. However, as we can see from the structures of the substrates, POP is very selective for proline residues (alanine residues are tolerated but with a significant decrease in protease activity) and drastic changes at P1 are expected to correlate with a loss of inhibitory potency.[108] In fact, the oxygen of the P1 ring in **40** is most likely facing the catalytic serine oxygen (which reacts with the carbonyl in **1**), leading to severe electrostatic repulsions.

**P1′ Substitution, Covalent versus Noncovalent Inhibitors.** If the C-terminus at P1 ends with a reactive functional group, such as an aldehyde, hydroxyacetyl, or nitrile, then the inhibitor will most likely covalently bind with the catalytic serine of the active site of the enzyme (Figure 8).[136] Researchers introduced phosphonate esters at P1 (structure not shown), which they claimed to induce a covalent bond with the active site serine, resulting in irreversible inhibition of the enzyme.[137] The formation of a covalent bond has been hypothesized based on kinetic studies and further confirmed by X-ray crystallography of POP cocrystallized with **1** (Figure 3). Investigation of active-site specific, covalent inhibitors of POP can be traced back as early as 1977 with chloromethyl ketone derivatives of a few dipeptides (Figure 8, **47**[22]) and then the discovery of **1** in 1983.[5,83] Later, SAR studies confirmed that covalent inhibitors were more potent than noncovalent inhibitors (**48** vs **1**[5,100]).[121,124] Extensive SAR has shown that aldehydes (**49**) could be replaced with α-keto heterocycles like in **50**, with a concomitant increase in inhibitory potency.[106,111,112] Other reactive groups, such as terminal boronates (**51**)[129] and hydroxymethyl ketones (**2**),[116] were also shown to inhibit POP with improved potency over the corresponding non covalently acting structures. Acetal derivatives of **1** and thiazolidine analogues were also prepared as exemplified by **52** (ZTTA[138]) but were less potent, exhibiting high nanomolar inhibitory activity.[100] Further studies showed that α-keto esters[111] exhibited nanomolar inhibitory activity.

Vendeville and co-workers closely examined the impact of a reactive group attached to **26**, showing that aldehydes and nitriles can be advantageously replaced by α-chloromethyl ketones and α-hydroxymethyl ketones.[104] However, kinetic studies of analogues of **11** revealed that the association and dissociation were faster for the nitrile derivative (**53**, KYP-2047[126]) than for the hydroxymethyl ketone and aldehyde.[122] As a result, the half-life of these covalent inhibitors was controlled by slow dissociation.[122,126] Similar observations were made with **9**, **54**, and **55**.[109]

## Nonpeptidic POP Inhibitors

Thus far, very few nonpeptidic POP inhibitors have been reported. From a library of traditional Chinese medicine, the natural product berberine (Figure 9, **56**) was identified as a weak POP inhibitor ($IC_{50}$ found to be 145 000 nM).[139] In particular, berberine was efficient for the treatment of patients with bipolar affective disorders, as they present increased

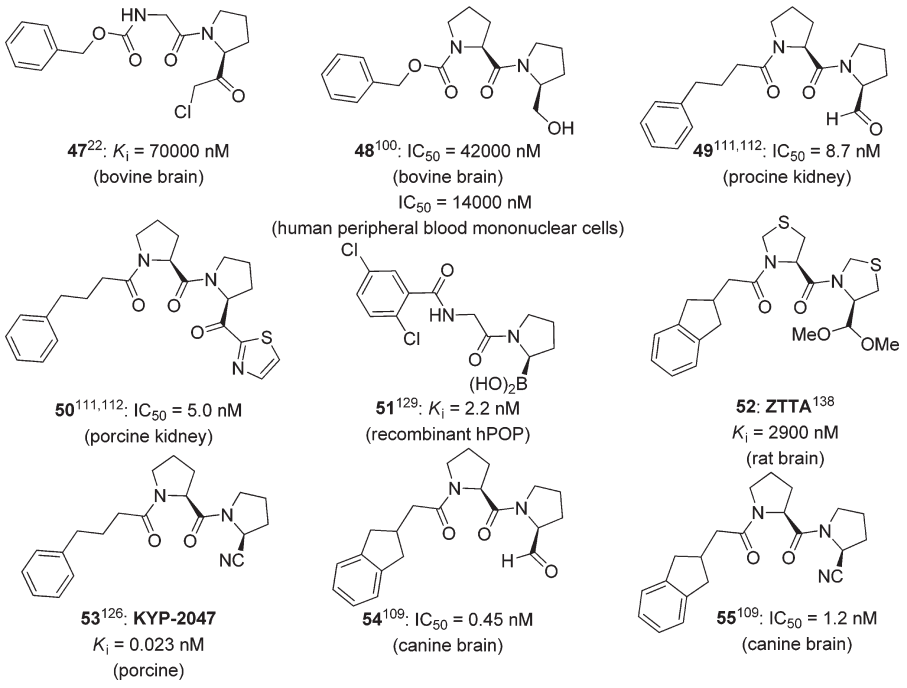


**Figure 8.** Reactive functional groups.

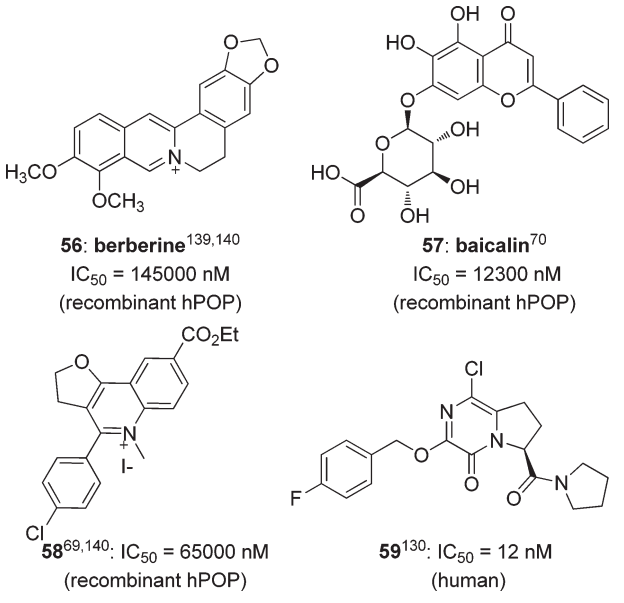


**Figure 9.** Examples of nonpeptidic POP inhibitors.

levels of POP activity in serum.[140] Later on, further screening of plant extracts led to the discovery of baicalin (**57**) from *Scuttellaria baicalensis*. Baicalin was found to act as a prodrug with the sugar moiety being hydrolyzed in the gut. Rat gut $\beta$-glucuronidase cleaves baicalin, releasing the active polyphenol portion (baicalein) which can potentially cross the blood−brain barrier and other biological barriers, according to parallel artificial membrane permeability assay data, and inhibit POP in the brain. Baicalin showed POP selectivity, exhibiting significantly less inhibition of DPPIV.[70] The major advantage of compounds like berberine and baicalin is that they have been used in humans for years without noticeable side effects. This is a clear advantage over novel synthetic inhibitors with a priori unknown pharmacokinetic and toxicokinetic profiles.

Inspired by the structure of berberine, isoquinolium derivatives, like **58**, were prepared as potential POP inhibitors and other analogues (structures not disclosed) were patented as POP inhibitors in 2008.[140] In addition to being POP inhibitors, these compounds also crossed parallel lipid artificial membranes.[69] Very recently, Haffner chose to step away from peptidic POP inhibitors by constraining them into pyrrolidinylpyridone and pyrazinone analogues. All the reported analogues exhibited nanomolar inhibitory potency for POP, and **59** could be cocrystallized with the enzyme, showing the key interactions formed in the bound complex.[130] To our knowledge **3** (Figure 2) is the only druglike POP inhibitor that has been discovered by screening a collection of molecules and not through rational design starting from **1**.[135]

Compounds **3**, **56**, and **57** are structurally different from the other inhibitors described above. In fact, the authors do not give details on whether these bind to the active site of POP or an allosteric site.

**Selectivity of POP Inhibitors.** There are three levels of selectivity important in the design of POP inhibitors. The first is that the inhibitors are selective for POP over all other proteases and peptidases. Yoshimoto et al. verified that their covalent inhibitor (**47**) was selective for POP over other serine proteases like trypsin, chymotrypsin, elastase, and papain (Figure 10).[22] Second, the inhibitors must bind selectively to POP and must not bind to other enzymes cleaving peptides at sites adjacent to proline residues, such as dipeptidyl aminopeptidase 2 and IV (DPP2 and DPPIV), aminopeptidase P, and others. For example, selectivity of **2** for POP over DPPIV, proline iminopeptidase, APP, prolidase, and prolyl-carboxypeptidase was noted, although no explanation for this observation was given.[113] Similarly, synthesized Fmoc-aminoacylpyrrolidine-2-nitriles (**14**) selectively inhibited POP over DPPIV (no DPPIV inhibition detected at 5 $\mu$M inhibitor), but no rationalization was given.[86] Poor selectivity for FAP-$\alpha$/seprase and POP against DPPIV was achieved with synthesized boronate inhibitors (**60**, **61**) but better selectivity for POP versus DPPIV with decreased FAP-$\alpha$/seprase

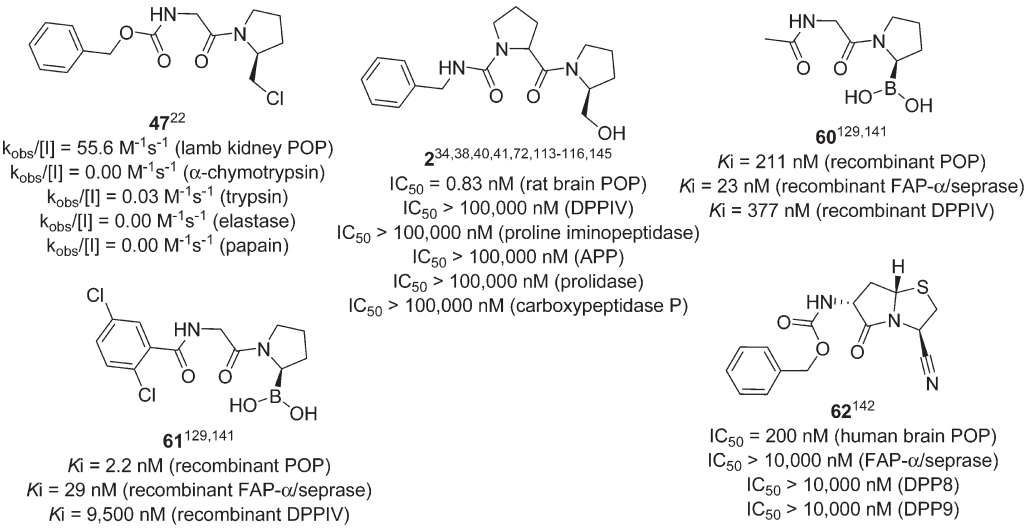


**Figure 10.** Selective POP inhibitors.

inhibitory activity as demonstrated by Wolf and co-workers.[129,141] Lawandi et al. successfully developed a series of bicyclic scaffold-based inhibitors (like **62**) to selectively inhibit POP activity over other proline-specific peptidase activity.[142] Finally, as for the third level of selectivity, inhibitors should be able to discriminate between POP from different species (not discussed here).

## Structure-Based Design of POP Inhibitors

The available structural information was further exploited to understand the ligand binding process. For instance, docking inhibitors to the crystal structure 1qfs using GOLD in combination with CoMSIA analysis of the ligands shed light on the key interactions between the ligands and the protein binding site[124] while docking inhibitors to the crystal structure 1h2w (porcine POP) with AutoDock provided hints on the binding of benzimidazolium derivatives.[69] Using the linear interaction energy method (LIE),[143,144] Kánai et al. have attempted to derive binding free energies from computations.[79] Applied to six different ligands, this molecular-dynamics-based method not only identified the weakest binder (no inhibition at 0.1 $\mu$M) and the strongest binder within the set but also predicted the $IC_{50}$ within 2 orders of magnitude (binding free energies within 2.5 kcal). More recently, Lawandi et al. have reported the successful development of novel, potent, and selective inhibitors (**62**), guided by docking with the program FITTED, which considers covalently bound inhibitors.[142]

## Conclusions and Prospects

**POP and POP-like Inhibition.** Researchers focused on developing POP inhibitors because they hypothesized that compounds able to positively modulate the brain levels of neuropeptides, which are important in cognitive process, neurodegeneration, or age-related cognitive decline, may be of clinical and therapeutic interest. Researchers have demonstrated some of these positive effects, in vivo, in preclinical animal models of these diseases of the CNS and in the few reported phase 1 clinical trials, and none of the inhibitors showed any toxicological or safety problems. Toward this goal of treating diseases of the CNS, researchers have studied behavioral pharmacology in animals, evaluated enzyme inhibition, either in human or in animal blood, and in animal brain extracts, and for some studies, researchers even quantified how POP inhibitors affected brain levels of neurotransmitters. Most studies extracted the whole brain from animals and then measured POP activity without thoroughly characterizing the enzyme affected. Furthermore, in many cases of potentially effective POP inhibitors, no in vivo testing has been reported for their potential application in other diseases where in vitro cell models suggested a role for POP in infectious, oncological, or inflammatory disorders. In this Perspective, we restricted our evaluation of their possible clinical interest to CNS disorders.

Despite promising preclinical and clinical results, researchers have yet to evaluate POP inhibitors in large human clinical trials for memory disorders. Furthermore, several aspects of the physiology of POP and its inhibitors are missing and must be addressed before their therapeutic involvement can be clearly evaluated:

1. Where is (are) the exact target enzyme(s) of POP inhibitors located?
2. Are these enzymes intracellular or extracellular?
3. Which are the exact biological substrates of POP and POP-like enzymes?
4. Do these POP inhibitors pass the BBB? And, if yes, how?
5. Are these inhibitors trapped in the cerebral vasculature, where they may exert their effects?
6. What is the role and the impact of other POP-like activities in the evaluation of inhibitors in biological models?

A number of biological processes and diseases are linked to POP and will most likely motivate more research on this enzyme toward the development of POP inhibitors. POP activity may lead to both quantitative and qualitative changes in the signaling potential of bioactive peptides, and inhibitors of POP activity may be developed as valuable chemotherapeutic agents for neurological disorders. For instance, the use of **2** has shown promising applications for the treatment of sensorimotor dysfunctions caused by brain trauma.[145] A combination of physical therapy with drug treatment with **2** induced enhanced functional recovery in rats with focal forebrain ischemia.[145] However, the observed

effects may originate from families of proline-specific enzymes having comparable activity but having either related or unrelated sequence and structure homology. Researchers have yet to pinpoint the exact origin of the observed effects of these inhibitors. The published information on neurological diseases reviewed here suggests multiple targets for the inhibitors of the proline-specific proteases and peptidases. The effects of the reported inhibitors may result from an indirect effect regulating other pathways than those initially targeted.

Given the expression of these proline-specific enzymatic activities in most tissues, if not in most cells, an approach to consider for future development includes the preparation of prodrugs, combining, for example, a POP inhibitor with a cell-targeting agent (for examples, see refs 71 and 146) in order to improve the delivery of these potential therapeutic agents to the cells to be treated.

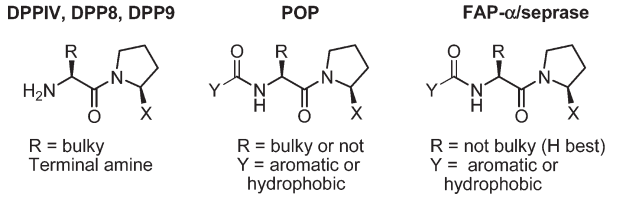


**Figure 11.** General considerations to design inhibitors of DPPIV, DPP8, DPP9, POP, or FAP-α/seprase.

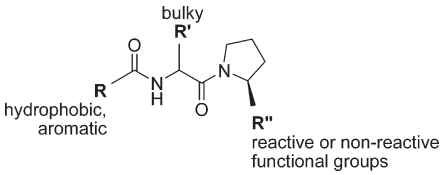


**Figure 12.** Achieving selectivity for POP over DPPIV, DPP8, DPP9, and FAP-α/seprase.

Both natural and synthetic compounds have been evaluated for their effects on proline-specific endoproteases. However, in most cases, enzymes from different species, either from microorganisms or from mammals, have been used to evaluate many of the reported inhibitors. Translating inhibitor potentials measured on POP from one species to a different species must be done very carefully, knowing that differences exist between proline-specific endoproteases of different origin. Furthermore, the therapeutic potential of the known proline-specific endoprotease inhibitors and of future generations of inhibitors can only be exploited if we can develop an inhibitor exhibiting a high level of selectivity for an enzyme of one species.

**Selective and Potent Inhibitors.** In order to guide the design of novel selective inhibitors, we summarized the necessary SAR data in Figures 11, 12, and 13. We can combine the information from Figure 11 into a first pharmacophore (Figure 12) to develop inhibitors selective for POP over other proline-specific proteases including FAP-α/seprase, DPPIV, DPP8, and DPP9.[129,147,148] We have also combined all the information from the structure−activity relationship data into a schematic representation of the functional/structural requirements of a peptidomimetic POP inhibitor that can be visualized and used for the design of future generations of potent, selective inhibitors.

Covalent inhibitors are more potent than the noncovalent inhibitors developed thus far, although subnanomolar noncovalent inhibitors have been discovered (**20**). This is not surprising given the trends in drug design toward incorporating covalent modifiers highlighted in a few recent reviews.[149,150]

Generally, there is a need to direct future drug design toward more druglike, nonpeptidic compounds. Very few studies on druglike molecules have been reported. In fact, only **37**, analogues of **37**, and **3**[135] have been derived from a hit discovered by screening while most of the other inhibitors mimic **1**. Given the availability of crystal structures,[28,76] we were surprised to find that only a few potent POP inhibitors

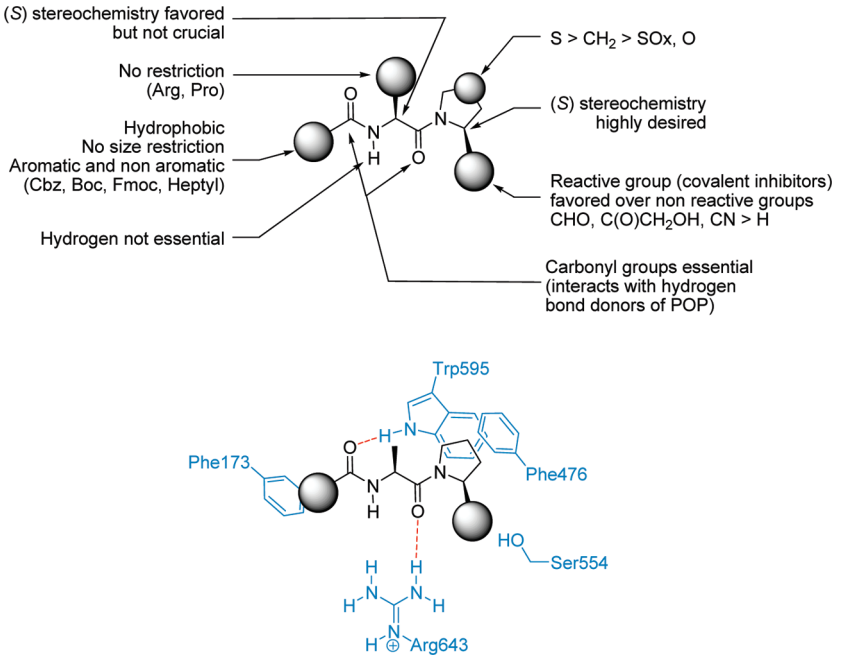


**Figure 13.** Optimal pharmacophore for achieving potency for POP (top) and key interactions between inhibitors and POP (bottom).

were developed using computational methods based on these structures. For example, druglike candidates could be obtained by virtual screening, a strategy we are currently exploring in our laboratories.

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## Biographies

**Janice Lawandi** studied biochemistry at Concordia University, Montreal, Canada. As a member of the Institute for Cooperative Education, she gained work experience in such fields as bioorganic chemistry, biophysical chemistry, and surgical research. After obtaining her B.Sc. Honors with distinction in 2004, she chose to further expand her knowledge, joining McGill University's Department of Chemistry, Canada. As a Ph.D. student under the supervision of Prof. Nicolas Moitessier, she works on two projects, a carbohydrate chemistry project, on selective glycosylation of sugars using directing/protecting groups, and a medicinal chemistry project, designing and synthesizing POP inhibitors.

**Sandrine Gerber-Lemaire** studied at the Ecole Nationale Supérieure de Chimie de Paris and obtained her Diploma and her M.Sc. in Organic Chemistry in 1993. She obtained a Ph.D. degree in 1996 and moved to the University of Lausanne, Switzerland, for a postdoctoral experience. In 1998, she was appointed as First Assistant. In 2001, she joined the Institute of Chemical Sciences and Engineering (ISIC) at the Ecole Polytechnique Fédérale de Lausanne where she received a tenured research position in 2006 as Senior Scientist. Since 2007, she acts as Deputy Director of the ISIC. Her main research interests focus on the development of synthetic pathways towards complex natural products and biologically relevant molecules. She is involved in several teachings of organic chemistry at the Bachelor and Master levels.

**Lucienne Juillerat-Jeanneret** obtained her M.Sc. in Biochemistry in 1971, her Ph.D. in 1983, and her Privat Docent in 2001. After postdoctoral experiences in the Department of Biochemistry of the University of Geneva, Switzerland, and in the Division of Hypertension of the University Hospital of Lausanne, Switzerland, she joined in 1990 the University Institute of Pathology of Lausanne and received in 1993 a tenured research position as an independent senior group leader. She is mainly involved in teaching the interface between biomedicine and chemistry or materials. Her main research interests are focused at defining, evaluating, and validating new potential therapeutic targets, new molecules as drug leads, and innovative materials to selectively deliver drugs to the targeted tissues, including nanotechonologies and proteolytic and glycolytic pathways.

**Nicolas Moitessier** completed his Ph.D. in 1998 from Université Henri Poincaré-Nancy I, France, under the supervision of Dr. Yves Chapleur. After postdoctoral studies with Prof. Stephen Hanessian in Montreal, Canada, he moved back to University of Nancy to start an academic career. In 2003 he decided to return to Canada and was appointed as an Assistant Professor at McGill University, Montreal, Canada, and then as an Associate Professor in 2009. His current interests integrate computational chemistry and organic/medicinal chemistry, spanning from the development of software for computer-aided molecular design to the design and synthesis of directing/protecting groups for carbohydrates and development of POP inhibitors. He has published nearly 50 peer-reviewed papers.

## References

(1) Rosenblum, J. S.; Kozarich, J. W. Prolyl peptidases: a serine protease subfamily with high potential for drug discovery. *Curr. Opin. Chem. Biol.* **2003**, *7*, 496–504.
(2) Polgár, L. The prolyl oligopeptidase family. *Cell. Mol. Life Sci.* **2002**, *59*, 349–362.
(3) Yaron, A.; Naider, F. Proline-dependent structural and biological properties of peptides and proteins. *Crit. Rev. Biochem. Mol. Biol.* **1993**, *28*, 31–81.
(4) Männistö, P. T.; Venäläinen, J.; Jalkanen, A.; García-Horsman, J. A. Prolyl oligopeptidase: a potential target for the treatment of cognitive disorders. *Drug News Perspect.* **2007**, *20*, 293–306.
(5) Yoshimoto, T.; Kawahara, K.; Matsubara, F.; Kado, K.; Tsuru, D. Comparison of inhibitory effects of prolinal-containing peptide derivatives on prolyl endopeptidases from bovine brain and Flavobacterium. *J. Biochem.* **1985**, *98*, 975–979.
(6) Saito, M.; Hashimoto, M.; Kawaguchi, N.; Fukami, H.; Tanaka, T.; Higuchi, N. Synthesis and inhibitory activity of acyl-peptidyl-prolinal derivatives toward post-proline cleaving enzyme as nootropic agents. *J. Enzyme Inhib.* **1990**, *3*, 163–178.
(7) Santana, J. M.; Grellier, P.; Schrével, J.; Teixeira, A. R. L. A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. *Biochem. J.* **1997**, *325*, 129–137.
(8) Mentlein, R. Therapeutic assessment of glucagon-like peptide-1 agonists compared with dipeptidyl peptidase IV inhibitors as potential antidiabetic drugs. *Expert Opin. Invest. Drugs* **2005**, *14*, 57–64.
(9) Park, J. E.; Lenter, M. C.; Zimmermann, R. N.; Garin-Chesa, P.; Old, L. J.; Rettig, W. J. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J. Biol. Chem.* **1999**, *274*, 36505–36512.
(10) Busek, P.; Malik, R.; Sedo, A. Dipeptidyl peptidase IV activity and/or structure homologues (DASH) and their substrates in cancer. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 408–21.
(11) Mentlein, R. Dipeptidyl-peptidase IV (CD26)—role in the inactivation of regulatory peptides. *Regul. Pept.* **1999**, *85*, 9–24.
(12) Cunningham, D. F.; O'Connor, B. Proline specific peptidases. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1997**, *1343*, 160–186.
(13) Šedo, A.; Malík, R. Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2001**, *1550*, 107–116.
(14) Kelly, T. Evaluation of seprase activity. *Clin. Exp. Metastasis* **1999**, *17*, 57–62.
(15) Gorrell, M. D. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin. Sci.* **2005**, *108*, 277–292.
(16) Chen, W. T.; Kelly, T.; Ghersi, G. DPPIV, seprase, and related serine peptidases in multiple cellular functions. *Curr. Top. Dev. Biol.* **2003**, *54*, 207–232.
(17) Collins, P. J.; McMahon, G.; O'Brien, P.; O'Connor, B. Purification, identification and characterisation of seprase from bovine serum. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 2320–2333.
(18) Aimes, R. T.; Zijlstra, A.; Hooper, J. D.; Ogbourne, S. M.; Sit, M. L.; Fuchs, S.; Gotley, D. C.; Quigley, J. P.; Antalis, T. M. Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis. *Thromb. Haemostasis* **2003**, *89*, 561–572.
(19) Santos, A. M.; Jung, J.; Aziz, N.; Kissil, J. L.; Puré, E. Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J. Clin. Invest.* **2009**, *119*, 3613–3625.
(20) Juillerat-Jeanneret, L.; Gerber-Lemaire, S. The prolyl-aminodipeptidases and their inhibitors as therapeutic targets for fibrogenic disorders. *Mini-Rev. Med. Chem.* **2009**, *9*, 215–226.
(21) Juillerat-Jeanneret, L. Prolyl-specific peptidases and their inhibitors in biological processes. *Curr. Chem. Biol.* **2008**, *2*, 97–109.
(22) Yoshimoto, T.; Orlowski, R. C.; Walter, R. Postproline cleaving enzyme: identification as serine protease using active site specific inhibitors. *Biochemistry* **1977**, *16*, 2942–2948.
(23) Yokosawa, H.; Nishikata, M.; Ishii, S. *N*-Benzyloxycarbonyl-valyl-prolinal, a potent inhibitor of post-proline cleaving enzyme. *J. Biochem.* **1984**, *95*, 1819–1821.
(24) Friedman, T. C.; Orlowski, M.; Wilk, S. Prolyl endopeptidase: inhibition in vivo by *N*-benzyloxycarbonyl-prolyl-prolinal. *J. Neurochem.* **1984**, *42*, 237–241.
(25) Wilk, S. Prolyl endopeptidase. *Life Sci.* **1983**, *33*, 2149–2157.
(26) Irazusta, J.; Larrinaga, G.; González-Maeso, J.; Gil, J.; Meana, J. J.; Casis, L. Distribution of prolyl endopeptidase activities in rat and human brain. *Neurochem. Int.* **2002**, *40*, 337–345.
(27) O'Leary, R. M.; Gallagher, S. P.; O'Connor, B. Purification and characterization of a novel membrane-bound form of prolyl

endopeptidase from bovine brain. *Int. J. Biochem. Cell Biol.* **1996**, *28*, 441–449.
(28) Fülöp, V.; Böcskei, Z.; Polgar, L. Prolyl oligopeptidase: an unusual beta-propeller domain regulates proteolysis. *Cell* **1998**, *94*, 161–70.
(29) Bastos, I. M. D.; Grellier, P.; Martins, N. F.; Cadavid-Restrepo, G.; De Souza-Ault, M. R.; Augustyns, K.; Teixeira, A. R. L.; Schrével, J.; Maigret, B.; Da Silveira, J. F.; Santana, J. M. Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells. *Biochem. J.* **2005**, *388*, 29–38.
(30) Szeltner, Z.; Rea, D.; Juhasz, T.; Renner, V.; Fulop, V.; Polgar, L. Concerted structural changes in the peptidase and the propeller domains of prolyl oligopeptidase are required for substrate binding. *J. Mol. Biol.* **2004**, *340*, 627–37.
(31) Mantle, D.; Falkous, G.; Ishiura, S.; Blanchard, P. J.; Perry, E. K. Comparison of proline endopeptidase activity in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease. *Clin. Chim. Acta* **1996**, *249*, 129–139.
(32) García-Horsman, J. A.; Männistö, P. T.; Venäläinen, J. I. On the role of prolyl oligopeptidase in health and disease. *Neuropeptides* **2007**, *41*, 1–24.
(33) Williams, R. S. B. Prolyl oligopeptidase and bipolar disorder. *Clin. Neurosci. Res.* **2004**, *4*, 233–242.
(34) Shinoda, M.; Toide, K.; Ohsawa, I.; Kohsaka, S. Specific inhibitor for prolyl endopeptidase suppresses the generation of amyloid $\beta$ protein in NG108-15 cells. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 641–645.
(35) Petit, A.; Barelli, H.; Morain, P.; Checler, F. Novel proline endopeptidase inhibitors do not modify A$\beta$40/42 formation and degradation by human cells expressing wild-type and Swedish mutated $\beta$-amyloid precursor protein. *Br. J. Pharmacol.* **2000**, *130*, 1613–1617.
(36) Portevin, B.; Benoist, A.; Rémond, G.; Herve, Y.; Vincent, M.; Lepagnol, J.; De Nanteuil, G. New prolyl endopeptidase inhibitors: in vitro and in vivo activities of azabicyclo[2.2.2]octane, azabicyclo[2.2.1]heptane, and perhydroindole derivatives. *J. Med. Chem.* **1996**, *39*, 2379–2391.
(37) Rossner, S.; Schulz, I.; Zeitschel, U.; Schliebs, R.; Bigl, V.; Demuth, H. U. Brain prolyl endopeptidase expression in aging, APP transgenic mice and Alzheimer's disease. *Neurochem. Res.* **2005**, *30*, 695–702.
(38) Kato, A.; Fukunari, A.; Sakai, Y.; Nakajima, T. Prevention of amyloid-like deposition by a selective prolyl endopeptidase inhibitor, Y-29794, in senescence-accelerated mouse. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 328–335.
(39) Katsube, N.; Sunaga, K.; Aishita, H.; Chuang, D. M.; Ishitani, R. ONO-1603, a potential antidementia drug, delays age-induced apoptosis and suppresses overexpression of glyceraldehyde-3-phosphate dehydrogenase in cultured central nervous system neurons. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 6–13.
(40) Toide, K.; Shinoda, M.; Fujiwara, T.; Iwamoto, Y. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on spatial memory and central cholinergic neurons in aged rats. *Pharmacol., Biochem. Behav.* **1997**, *56*, 427–434.
(41) Toide, K.; Shinoda, M.; Iwamoto, Y.; Fujiwara, T.; Okamiya, K.; Uemura, A. A novel propyl endopeptidase inhibitor, JTP-4819, with potential for treating Alzheimer's disease. *Behav. Brain Res.* **1997**, *83*, 147–151.
(42) Fukunari, A.; Kato, A.; Sakai, Y.; Yoshimoto, T.; Ishiura, S.; Suzuki, K.; Nakajima, T. Colocalization of prolyl endopeptidase and amyloid P-peptide in brains of senescence-accelerated mouse. *Neurosci. Lett.* **1994**, *176*, 201–204.
(43) Laitinen, K. S. M.; Van Groen, T.; Tanila, H.; Venäläinen, J.; Männistö, P. T.; Alafuzoff, I. Brain prolyl oligopeptidase activity is associated with neuronal damage rather than $\beta$-amyloid accumulation. *NeuroReport* **2001**, *12*, 3309–3312.
(44) Brandt, I.; Vriendt, K. D.; Devreese, B.; Beeumen, J. V.; Dongen, W. V.; Augustyns, K.; Meester, I. D.; Scharpé, S.; Lambeir, A. M. Search for substrates for prolyl oligopeptidase in porcine brain. *Peptides* **2005**, *26*, 2536–2546.
(45) Brandt, I.; Gérard, M.; Sergeant, K.; Devreese, B.; Baekelandt, V.; Augustyns, K.; Scharpé, S.; Engelborghs, Y.; Lambeir, A. M. Prolyl oligopeptidase stimulates the aggregation of $\alpha$-synuclein. *Peptides* **2008**, *29*, 1472–1478.
(46) Myöhänen, T. T.; Venäläinen, J. I.; Tupala, E.; Garcia-Horsman, J. A.; Miettinen, R.; Männistö, P. T. Distribution of immunoreactive prolyl oligopeptidase in human and rat brain. *Neurochem. Res.* **2007**, *32*, 1365–1374.
(47) Myöhänen, T. T.; Venäläinen, J. I.; Garcia-Horsman, J. A.; Männistö, P. T. Spatial association of prolyl oligopeptidase, inositol 1,4,5-triphosphate type 1 receptor, substance P and its neurokinin-1 receptor in the rat brain: an immunohistochemical colocalization study. *Neuroscience* **2008**, *153*, 1177–1189.
(48) Schulz, I.; Zeitschel, U.; Rudolph, T.; Ruiz-Carrillo, D.; Rahfeld, J. U.; Gerhartz, B.; Bigl, V.; Demuth, H. U.; Rossner, S. Subcellular localization suggests novel functions for prolyl endopeptidase in protein secretion. *J. Neurochem.* **2005**, *94*, 970–979.
(49) Myöhänen, T. T.; Venäläinen, J. I.; Garcia-Horsman, J. A.; Piltonen, M.; Männistö, P. T. Cellular and subcellular distribution of rat brain prolyl oligopeptidase and its association with specific neuronal neurotransmitters. *J. Comp. Neurol.* **2008**, *507*, 1694–1708.
(50) Brandt, I.; Scharpé, S.; Lambeir, A. M. Suggested functions for prolyl oligopeptidase: a puzzling paradox. *Clin. Chim. Acta* **2007**, *377*, 50–61.
(51) Polgár, L. Oligopeptidase B: a new type of serine peptidase with a unique substrate-dependent temperature sensitivity. *Biochemistry* **1999**, *38*, 15548–15555.
(52) Orlowski, M.; Wilk, E.; Pearce, S.; Wilk, S. Purification and properties of a prolyl endopeptidase from rabbit brain. *J. Neurochem.* **1979**, *33*, 461–469.
(53) Weber, A. E. Dipeptidyl peptidase IV inhibitors for the treatment of diabetes. *J. Med. Chem.* **2004**, *47*, 4135–4141.
(54) Knisatschek, H.; Bauer, K. Characterization of "thyroliberin-deamidating enzyme" as a post-proline-cleaving enzyme: partial purification and enzyme-chemical analysis of the enzyme from anterior pituitary tissue. *J. Biol. Chem.* **1979**, *254*, 10936–10943.
(55) Moriyama, A.; Nakanishi, M.; Sasaki, M. Porcine muscle prolyl endopeptidase and its endogenous substrates. *J. Biochem.* **1988**, *104*, 112–7.
(56) Koida, M.; Walter, R. Post proline cleaving enzyme. Purification of this endopeptidase by affinity chromatography. *J. Biol. Chem.* **1976**, *251*, 7593–7599.
(57) Greene, L. J.; Spadaro, A. C. C.; Martins, A. R. Brain endo-oligopeptidase B: a post-proline cleaving enzyme that inactivates angiotensin I and II. *Hypertension* **1982**, *4*, 178–184.
(58) Mendez, M.; Cruz, C.; Joseph-Bravo, P.; Wilk, S.; Charli, J. L. Evaluation of the role of prolyl endopeptidase and pyroglutamyl peptidase I in the metabolism of LHRH and TRH in brain. *Neuropeptides* **1990**, *17*, 55–62.
(59) Tate, S. S. Purification and properties of a bovine brain thyrotropin-releasing-factor deamidase. *Eur. J. Biochem.* **1981**, *118*, 17–23.
(60) Camargo, A. C. M.; Almeida, M. L. C.; Emson, P. C. Involvement of endo-oligopeptidases A and B in the degradation of neurotensin by rabbit brain. *J. Neurochem.* **1984**, *42*, 1758–1761.
(61) Walter, R.; Shlank, H.; Glass, J. D.; Schwartz, I. L.; Kerenyi, T. D. Leucylglycinamide released from oxytocin by human uterine enzyme. *Science* **1971**, *173*, 827–829.
(62) Hersh, L. B.; McKelvy, J. F. Enzymes involved in the degradation of thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LH-RH) in bovine brain. *Brain Res.* **1979**, *168*, 553–564.
(63) Bøler, J.; Enzmann, F.; Folkers, K.; Bowers, C. Y.; Schally, A. V. The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem. Biophys. Res. Commun.* **1969**, *37*, 705–710.
(64) Schulz, I.; Gerhartz, B.; Neubauer, A.; Holloschi, A.; Heiser, U.; Hafner, M.; Demuth, H. U. Modulation of inositol 1,4,5-triphosphate concentration by prolyl endopeptidase inhibition. *Eur. J. Biochem.* **2002**, *269*, 5813–5820.
(65) Schneider, J. S.; Giardiniere, M.; Morain, P. Effects of the prolyl endopeptidase inhibitor S 17092 on cognitive deficits in chronic low dose MPTP-treated monkeys. *Neuropsychopharmacology* **2002**, *26*, 176–182.
(66) Kovács, G. L.; De Wied, D. Peptidergic modulation of learning and memory processes. *Pharamcol. Rev.* **1994**, *46*, 269–291.
(67) Barelli, H.; Petit, A.; Hirsch, E.; Wilk, S.; De Nanteuil, G.; Morain, P.; Checler, F. S 17092-1, a highly potent, specific and cell permeant inhibitor of human proline endopeptidase. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 657–661.
(68) Bellemére, G.; Morain, P.; Vaudry, H.; Jégou, S. Effect of S 17092, a novel prolyl endopeptidase inhibitor, on substance P and $\alpha$-melanocyte-stimulating hormone breakdown in the rat brain. *J. Neurochem.* **2003**, *84*, 919–929.
(69) Tarragó, T.; Masdeu, C.; Gómez, E.; Isambert, N.; Lavilla, R.; Giralt, E. Benzimidazolium salts as small, nonpeptidic and BBB-permeable human prolyl oligopeptidase inhibitors. *ChemMedChem* **2008**, *3*, 1558–1565.

(70) Tarragó, T.; Kichik, N.; Claasen, B.; Prades, R.; Teixidó, M.; Giralt, E. Baicalin, a prodrug able to reach the CNS, is a prolyl oligopeptidase inhibitor. *Bioorg. Med. Chem.* **2008**, *16*, 7516–7524.
(71) Juillerat-Jeanneret, L. The targeted delivery of cancer drugs across the blood−brain barrier: chemical modifications of drugs or drug-nanoparticles? *Drug Discovery Today* **2008**, *13*, 1099–1106.
(72) Morain, P.; Lestage, P.; De Nanteuil, G.; Jochemsen, R.; Robin, J. L.; Guez, D.; Boyer, P. A. S 17092: a prolyl endopeptidase inhibitor as a potential therapeutic drug for memory impairment. Preclinical and clinical studies. *CNS Drug Rev.* **2002**, *8*, 31–52.
(73) Umemura, K.; Kondo, K.; Ikeda, Y.; Kobayashi, T.; Urata, Y.; Nakashima, M. Pharmacokinetics and safety of JTP-4819, a novel specific orally active prolyl endopeptidase inhibitor, in healthy male volunteers. *Br. J. Clin. Pharmacol.* **1997**, *43*, 613–618.
(74) Morain, P.; Robin, J. L.; De Nanteuil, G.; Jochemsen, R.; Heidet, V.; Guez, D. Pharmacodynamic and pharmacokinetic profile of S 17092, a new orally active prolyl endopeptidase inhibitor, in elderly healthy volunteers. A phase I study. *Br. J. Clin. Pharmacol.* **2000**, *50*, 350–359.
(75) Szeltner, Z.; Rea, D.; Renner, V.; Fulop, V.; Polgar, L. Electrostatic effects and binding determinants in the catalysis of prolyl oligopeptidase. Site specific mutagenesis at the oxyanion binding site. *J. Biol. Chem.* **2002**, *277*, 42613–42622.
(76) Szeltner, Z.; Rea, D.; Juhasz, T.; Renner, V.; Mucsi, Z.; Orosz, G.; Fulop, V.; Polgar, L. Substrate-dependent competency of the catalytic triad of prolyl oligopeptidase. *J. Biol. Chem.* **2002**, *277*, 44597–605.
(77) Szeltner, Z.; Rea, D.; Renner, V.; Juliano, L.; Fulop, V.; Polgar, L. Electrostatic environment at the active site of prolyl oligopeptidase is highly influential during substrate binding. *J. Biol. Chem.* **2003**, *278*, 48786–48793.
(78) Shan, L.; Mathews, I. I.; Khosla, C. Structural and mechanistic analysis of two prolyl endopeptidases: role of interdomain dynamics in catalysis and specificity. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3599–604.
(79) Kánai, K.; Arányi, P.; Böcskei, Z.; Ferenczy, G.; Harmat, V.; Simon, K.; Bátori, S.; Náray-Szabó, G.; Hermecz, I. Prolyl oligopeptidase inhibition by *N*-acyl-pro-pyrrolidine-type molecules. *J. Med. Chem.* **2008**, *51*, 7514–7522.
(80) Polgár, L. Prolyl endopeptidase catalysis. A physical rather than a chemical step is rate-limiting. *Biochem. J.* **1992**, *283*, 647–648.
(81) Berger, Y.; Bernasconi, C. C.; Schmitt, F.; Neier, R.; Juillerat-Jeanneret, L. Determination of intracellular prolyl/glycyl proteases in intact living human cells and protoporphyrin IX production as a reporter system. *Chem. Biol.* **2005**, *12*, 867–872.
(82) De Nanteuil, G.; Portevin, B.; Lepagnol, J. Prolyl endopeptidase inhibitors: a new class of memory enhancing drugs. *Drugs Future* **1998**, *23*, 167–179.
(83) Wilk, S.; Orlowski, M. Inhibition of rabbit brain prolyl endopeptidase by *N*-benzyloxycarbonyl-prolyl-prolinal, a transition state aldehyde inhibitor. *J. Neurochem.* **1983**, *41*, 69–75.
(84) Bakker, A. V.; Jung, S.; Spencer, R. W.; Vinick, F. J.; Faraci, W. S. Slow tight-binding inhibition of prolyl endopeptidase by benzyloxycarbonyl-prolyl-prolinal. *Biochem. J.* **1990**, *271*, 559–562.
(85) Niestroj, A. J.; Heiser, U.; Demuth, H. U.; Aust, S. Inhibitors of Prolyl Endopeptidase. US 7,592,467 B2, **2009**.
(86) Li, J.; Wilk, E.; Wilk, S. Inhibition of prolyl oligopeptidase by Fmoc-aminoacylpyrrolidine-2-nitriles. *J. Neurochem.* **1996**, *66*, 2105–2112.
(87) Yoshimoto, T.; Fischl, M.; Orlowski, R. C.; Walter, R. Post-proline cleaving enzyme and post-proline dipeptidyl aminopeptidase. Comparison of two peptidases with high specificity for proline residues. *J. Biol. Chem.* **1978**, *253*, 3708–3716.
(88) Yoshimoto, T.; Ogita, K.; Walter, R.; Koida, M.; Tsuru, D. Post-proline cleaving enzyme. Synthesis of a new fluorogenic substrate and distribution of the endopeptidase in rat tissues and body fluids of man. *Biochim. Biophys. Acta* **1979**, *569*, 184–192.
(89) Yoshimoto, T.; Simmons, W. H.; Kita, T.; Tsuru, D. Post-proline cleaving enzyme from lamb brain. *J. Biochem.* **1981**, *90*, 325–334.
(90) Yoshimoto, T.; Tsukumo, K.; Takatsuka, N.; Tsuru, D. An inhibitor for post-proline cleaving enzyme; distribution and partial purification from porcine pancreas. *J. Pharmacobio-Dyn.* **1982**, *5*, 734–740.
(91) Yoshimoto, T.; Nishimura, T.; Kita, T.; Tsuru, D. Post-proline cleaving enzyme (prolyl endopeptidase) from bovine brain. *J. Biochem.* **1983**, *94*, 1179–1190.
(92) Yoshimoto, T.; Kado, K.; Matsubara, F.; Koriyama, N.; Kaneto, H.; Tsuru, D. Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. *J. Pharmacobio-Dyn.* **1987**, *10*, 730–735.
(93) Tsuru, D.; Yoshimoto, T.; Koriyama, N.; Furukawa, S. Thiazolidine derivatives as potent inhibitors specific for prolyl endopeptidase. *J. Biochem.* **1988**, *104*, 580–586.
(94) Yoshimoto, T.; Tsuru, D.; Yamamoto, N.; Ikezawa, R.; Furukawa, S. Structure activity relationship of inhibitors specific for prolyl endopeptidase. *Agric. Biol. Chem.* **1991**, *55*, 37–43.
(95) Nishikata, M.; Yokosawa, H.; Ishii, S. Synthesis and structure of prolinal-containing peptides, and their use as specific inhibitors of prolyl endopeptidases. *Chem. Pharm. Bull.* **1986**, *34*, 2931–2936.
(96) Tsuda, M.; Muraoka, Y.; Nagai, M.; Aoya, T.; Takeuchi, T. Poststatin, a new inhibitor of prolyl endopeptidase. VIII. Endopeptidase inhibitory activity of non-peptidyl poststatin analogues. *J. Antibiot.* **1996**, *49*, 1022–1030.
(97) Tsuda, M.; Muraoka, Y.; Nagai, M.; Aoyagi, T.; Takeuchi, T. Poststatin, a new inhibitor of prolyl endopeptidase. VII. *N*-Cycloalkylamide analogues. *J. Antibiot.* **1996**, *49*, 909–920.
(98) Tsuda, M.; Muraoka, Y.; Nagai, M.; Aoyagi, T.; Takeuchi, T. Poststatin, a new inhibitor of prolyl endopeptidase. V. Endopeptidase inhibitory activity of poststatin analogues. *J. Antibiot.* **1996**, *49*, 890–899.
(99) Tsuda, M.; Muraoka, Y.; Someno, T.; Nagai, M.; Aoyagi, T.; Takeuchi, T. Poststatin, a new inhibitor of prolyl endopeptidase. VI. Endopeptidase inhibitory activity of poststatin analogues containing pyrrolidine ring. *J. Antibiot.* **1996**, *49*, 900–908.
(100) Augustyns, K.; Borloo, M.; Belyaev, A.; Rajan, P.; Goossens, F.; Hendriks, D.; Scharpe, S.; Haemers, A. Synthesis of peptidyl acetals as inhibitors of prolyl endopeptidase. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1265–1270.
(101) Bal, G.; Van Der Veken, P.; Antonov, D.; Lambeir, A. M.; Grellier, P.; Croft, S. L.; Augustyns, K.; Haemers, A. Prolylisoxazoles: potent inhibitors of prolyloligopeptidase with antitrypanosomal activity. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2875–2878.
(102) Vendeville, S.; Bourel, L.; Davioud-Charvet, E.; Grellier, P.; Deprez, B.; Sergheraert, C. Automated parallel synthesis of a tetrahydroisoquinolin-based library: potential prolyl endopeptidase inhibitors. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 437–442.
(103) Vendeville, S.; Buisine, E.; Williard, X.; Schrevel, J.; Grellier, P.; Santana, J.; Sergheraert, C. Identification of inhibitors of an 80 kDa protease from *Trypanosoma cruzi* through the screening of a combinatorial peptide library. *Chem. Pharm. Bull.* **1999**, *47*, 194–198.
(104) Vendeville, S.; Goossens, F.; Debreu-Fontaine, M. A.; Landry, V.; Davioud-Charvet, E.; Grellier, P.; Scharpe, S.; Sergheraert, C. Comparison of the inhibition of human and *Trypanosoma cruzi* prolyl endopeptidases. *Bioorg. Med. Chem.* **2002**, *10*, 1719–1729.
(105) Stöckel-Maschek, A.; Mrestani-Klaus, C.; Stiebitz, B.; Demuth, H. U.; Neubert, K. Thioxo amino acid pyrrolidides and thiazolidides: new inhibitors of proline specific peptidases. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2000**, *1479*, 15–31.
(106) Joyeau, R.; Maoulida, C.; Guillet, C.; Frappier, F.; Teixeira, A. R. L.; Schrével, J.; Santana, J.; Grellier, P. Synthesis and activity of pyrrolidinyl- and thiazolidinyl-dipeptide derivatives as inhibitors of the Tc80 prolyl oligopeptidase from *Trypanosoma cruzi*. *Eur. J. Med. Chem.* **2000**, *35*, 257–266.
(107) Károly, K.; Sándor, E.; Edit, S.; Miklós, F.; Judit, S.; Benjámin, P.; Andrea, S.; István, H. Prolyl endopeptidase inhibitors: *N*-acyl derivatives of L-thioproline-pyrrolidine. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1701–1704.
(108) Arai, H.; Nishioka, H.; Niwa, S.; Yamanaka, T.; Tanaka, Y.; Yoshinaga, K.; Kobayashi, N.; Miura, N.; Ikeda, Y. Synthesis of prolyl endopeptidase inhibitors and evaluation of their structure−activity relationships: in vitro inhibition of prolyl endopeptidase from canine brain. *Chem. Pharm. Bull.* **1993**, *41*, 1583–1588.
(109) Tanaka, Y.; Niwa, S.; Nishioka, H.; Yamanaka, T.; Torizuka, M.; Yoshinaga, K.; Kobayashi, N.; Ikeda, Y.; Arai, H. New potent prolyl endopeptidase inhibitors: synthesis and structure−activity relationships of indan and tetralin derivatives and their analogues. *J. Med. Chem.* **1994**, *37*, 2071–2078.
(110) Asano, M.; Nio, N.; Ariyoshi, Y. Inhibition of prolyl endopeptidase by synthetic beta-casein peptides and their derivatives with a C-terminal prolinol or prolinal. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 976–977.
(111) Tsutsumi, S.; Okonogi, T.; Shibahara, S.; Ohuchi, S.; Hatsushiba, E.; Patchett, A. A.; Christensen, B. G. Synthesis and structure−activity relationships of peptidyl α-keto heterocycles as novel inhibitors of prolyl endopeptidase. *J. Med. Chem.* **1994**, *37*, 3492–3502.
(112) Tsutsumi, S.; Okonogi, T.; Shibahara, S.; Patchett, A. A.; Christensen, B. G. Alpha-ketothiazole inhibitors of prolyl endopeptidase. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 831–834.

(113) Toide, K.; Iwamoto, Y.; Fujiwara, T.; Abe, H. JTP-4819: a novel prolyl endopeptidase inhibitor with potential as a cognitive enhancer. *J. Pharmacol. Exp. Ther.* **1995**, *274*, 1370–1378.
(114) Toide, K.; Okamiya, K.; Iwamoto, Y.; Kato, T. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on prolyl endopeptidase activity and substance P- and arginine-vasopressin-like immunoreactivity in the brains of aged rats. *J. Neurochem.* **1995**, *65*, 234–240.
(115) Toide, K.; Fujiwara, T.; Iwamoto, Y.; Shinoda, M.; Okamiya, K.; Kato, T. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on neuropeptide metabolism in the rat brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1996**, *353*, 355–362.
(116) Toide, K.; Shinoda, M.; Iwamoto, Y.; Fujiwara, T.; Abe, H.; Uchida, I. A novel prolyl endopeptidase inhibitor, JTP-4819, for the treatment of Alzheimer's disease: review of preclinical pharmacology. *CNS Drug Rev.* **1996**, *2*, 343–362.
(117) Bakker, A. V.; Daffeh, J.; Jung, S.; Vincent, L. A.; Nagel, A. A.; Spencer, R. W.; Vinick, F. J.; Faraci, W. S. Novel in vitro and in vivo inhibitors of prolyl endopeptidase. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 585–590.
(118) Wallén, E. A. A.; Christiaans, J. A. M.; Saario, S. M.; Forsberg, M. M.; Venäläinen, J. I.; Paso, H. M.; Männistö, P. T.; Gynther, J. 4-Phenylbutanoyl-2(*S*)-acylpyrrolidines and 4-phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines as prolyl oligopeptidase inhibitors. *Bioorg. Med. Chem.* **2002**, *10*, 2199–2206.
(119) Wallén, E. A. A.; Christiaans, J. A. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J. Dicarboxylic acid bis(L-prolyl-pyrrolidine) amides as prolyl oligopeptidase inhibitors. *J. Med. Chem.* **2002**, *45*, 4581–4584.
(120) Wallén, E. A. A.; Christiaans, J. A. M.; Jarho, E. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J. New prolyl oligopeptidase inhibitors developed from dicarboxylic acid bis(L-prolyl-pyrrolidine)amides. *J. Med. Chem.* **2003**, *46*, 4543–4551.
(121) Wallén, E. A. A.; Christiaans, J. A. M.; Saarinen, T. J.; Jarho, E. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J. Conformationally rigid *N*-acyl-5-alkyl-L-prolyl-pyrrolidines as prolyl oligopeptidase inhibitors. *Bioorg. Med. Chem.* **2003**, *11*, 3611–3619.
(122) Venäläinen, J. I.; Juvonen, R. O.; Garcia-Horsman, J. A.; Wallén, E. A. A.; Christiaans, J. A. M.; Jarho, E. M.; Gynther, J.; Männistö, P. T. Slow-binding inhibitors of prolyl oligopeptidase with different functional groups at the P1 site. *Biochem. J.* **2004**, *382*, 1003–1008.
(123) Jarho, E. M.; Venöläinen, J. I.; Huuskonen, J.; Christiaans, J. A. M.; Garcia-Horsman, J. A.; Forsberg, M. M.; Järvinen, T.; Gynther, J.; Männistö, P. T.; Wallén, E. A. A. A cyclopent-2-enecarbonyl group mimics proline at the P2 position of prolyl oligopeptidase inhibitors. *J. Med. Chem.* **2004**, *47*, 5605–5607.
(124) Jarho, E. M.; Wallén, E. A. A.; Christiaans, J. A. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J.; Poso, A. Dicarboxylic acid azacycle L-prolyl-pyrrolidine amides as prolyl oligopeptidase inhibitors and three-dimensional quantitative structure−activity relationship of the enzyme−inhibitor interactions. *J. Med. Chem.* **2005**, *48*, 4772–4782.
(125) Venäläinen, J. I.; Wallén, E. A. A.; Poso, A.; Garcia-Horsman, J. A.; Männistö, P. T. Synthesis and characterization of the novel fluorescent prolyl oligopeptidase inhibitor 4-fluoresceinthiocarbamoyl-6-aminocaproyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine. *J. Med. Chem.* **2005**, *48*, 7093–7095.
(126) Venäläinen, J. I.; Garcia-Horsman, J. A.; Forsberg, M. M.; Jalkanen, A.; Wallén, E. A. A.; Jarho, E. M.; Christiaans, J. A. M.; Gynther, J.; Männistö, P. T. Binding kinetics and duration of in vivo action of novel prolyl oligopeptidase inhibitors. *Biochem. Pharmacol.* **2006**, *71*, 683–692.
(127) Jarho, E. M.; Venäläinen, J. I.; Juntunen, J.; Yli-Kokko, A. L.; Vepsäläinen, J.; Christiaans, J. A. M.; Forsberg, M. M.; Järvinen, T.; Männistö, P. T.; Wallén, E. A. A. An introduction of a pyridine group into the structure of prolyl oligopeptidase inhibitors. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5590–5593.
(128) Jarho, E. M.; Venäläinen, J. I.; Poutiainen, S.; Leskinen, H.; Vepsäläinen, J.; Christiaans, J. A. M.; Forsberg, M. M.; Männistö, P. T.; Wallén, E. A. A. 2(*S*)-(Cycloalk-1-enecarbonyl)-1-(4-phenyl-butanoyl)pyrrolidines and 2(*S*)-(aroyl)-1-(4-phenyl-butanoyl)pyrrolidines as prolyl oligopeptidase inhibitors. *Bioorg. Med. Chem.* **2007**, *15*, 2024–2031.
(129) Tran, T.; Quan, C.; Edosada, C. Y.; Mayeda, M.; Wiesmann, C.; Sutherlin, D.; Wolf, B. B. Synthesis and structure−activity relationship of N-acyl-Gly-, N-acyl-Sar- and N-blocked-boroPro inhibitors of FAP, DPP4, and POP. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1438–1442.
(130) Haffner, C. D.; Diaz, C. J.; Miller, A. B.; Reid, R. A.; Madauss, K. P.; Hassell, A.; Hanlon, M. H.; Porter, D. J. T.; Becherer, J. D.; Carter, L. H. Pyrrolidinyl pyridone and pyrazinone analogues as potent inhibitors of prolyl oligopeptidase (POP). *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4360–4363.
(131) Atack, J. R.; Suman-Chauhan, N.; Dawson, G.; Kulagowski, J. J. In vitro and in vivo inhibition of prolyl endopeptidase. *Eur. J. Pharmacol.* **1991**, *205*, 157–163.
(132) Abe, M.; Muraoka, Y.; Kojima, F. Prolyl Oligopeptidase Inhibitor. WO 2005/027934, **2005**.
(133) Saito, M.; Hashimoto, M.; Kawaguchi, N.; Shibata, H.; Fukami, H.; Tanaka, T.; Higuchi, N. Synthesis and inhibitory activity of acyl-peptidyl-pyrrolidine derivatives toward post-proline cleaving enzyme; a study of subsite specificity. *J. Enzyme Inhib.* **1991**, *5*, 51–75.
(134) Katsube, N.; Ishitani, R. A review of the neurotrophic and neuroprotective properties of ONO-1603: comparison with those of tetrahydroaminoacridine, an antidementia drug. *CNS Drug Rev.* **2000**, *6*, 21–34.
(135) Nakajima, T.; Ono, Y.; Kato, A.; Maeda, J.; Ohe, T. Y-29794—a non-peptide prolyl endopeptidase inhibitor that can penetrate into the brain. *Neurosci. Lett.* **1992**, *141*, 156–160.
(136) Kahyaoglu, A.; Haghjoo, K.; Kraicsovits, F.; Jordan, F.; Polgar, L. Benzyloxycarbonylprolylprolinal, a transition-state analogue for prolyl oligopeptidase, forms a tetrahedral adduct with catalytic serine, not a reactive cysteine. *Biochem. J.* **1997**, *322*, 839–843.
(137) Augustyns, K.; Vanhoof, G.; Borloo, M.; de Meester, I.; Goossens, F.; Haemers, A. Tetrahydroisoquinoline 3-Carboxamide Derivatives. WO 95/34538, **1995**.
(138) Shishido, Y.; Furushiro, M.; Tanabe, S.; Nishiyama, S.; Hashimoto, S.; Ohno, M.; Yamamoto, T.; Watanabe, S. ZTTA, a postproline cleaving enzyme inhibitor, improves cerebral ischemia-induced deficits in a three-panel runway task in rats. *Pharmacol., Biochem. Behav.* **1996**, *55*, 333–338.
(139) Tarragó, T.; Kichik, N.; Segu, J.; Giralt, E. The natural product berberine is a human prolyl oligopeptidase inhibitor. *ChemMedChem* **2007**, *2*, 354–359.
(140) Giralt Lledó, E.; Tarragó Clua, M. T. Therapeutic Agent for Treatment of Bipolar Affective Disorder in Mammals. WO 2008/077978, **2008**.
(141) Edosada, C. Y.; Quan, C.; Wiesmann, C.; Tran, T.; Sutherlin, D.; Reynolds, M.; Elliott, J. M.; Raab, H.; Fairbrother, W.; Wolf, B. B. Selective inhibition of fibroblast activation protein protease based on dipeptide substrate specificity. *J. Biol. Chem.* **2006**, *281*, 7437–44.
(142) Lawandi, J.; Toumieux, S.; Seyer, V.; Campbell, P.; Thielges, S.; Juillerat-Jeanneret, L.; Moitessier, N. Constrained peptidomimetics reveal detailed geometric requirements of covalent prolyl oligopeptidase inhibitors. *J. Med. Chem.* **2009**, *52*, 6672–6684.
(143) Aqvist, J.; Luzhkov, V. B.; Brandsdal, B. O. Ligand binding affinities from MD simulations. *Acc. Chem. Res.* **2002**, *35*, 358–365.
(144) Aqvist, J.; Medina, C.; Samuelsson, J. E. A new method for predicting binding affinity in computer-aided drug design. *Protein Eng.* **1994**, *7*, 385–391.
(145) Sirviö, J.; Lehtimäki, T. Prolyl Oligopeptidase Inhibitors Ameliorating Recovery from Brain Trauma. WO 2005/002624, **2005**.
(146) Juillerat-Jeanneret, L.; Schmitt, F. Chemical modification of therapeutic drugs or drug vector systems to achieve targeted therapy: looking for the grail. *Med. Res. Rev.* **2007**, *27*, 574–590.
(147) Edosada, C. Y.; Quan, C.; Tran, T.; Pham, V.; Wiesmann, C.; Fairbrother, W.; Wolf, B. B. Peptide substrate profiling defines fibroblast activation protein as an endopeptidase of strict Gly(2)-Pro(1)-cleaving specificity. *FEBS Lett.* **2006**, *580*, 1581–1586.
(148) Van der Veken, P.; De Meester, I.; Dubois, V.; Soroka, A.; Van Goethem, S.; Maes, M. B.; Brandt, I.; Lambeir, A. M.; Chen, X.; Haemers, A.; Scharpé, S.; Augustyns, K. Inhibitors of dipeptidyl peptidase 8 and dipeptidyl peptidase 9. Part 1: Identification of dipeptide derived leads. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4154–4158.
(149) Potashman, M. H.; Duggan, M. E. Covalent modifiers: an orthogonal approach to drug design. *J. Med. Chem.* **2009**, *52*, 1231–1246.
(150) Smith, A. J.; Zhang, X.; Leach, A. G.; Houk, K. N. Beyond picomolar affinities: quantitative aspects of noncovalent and covalent binding of drugs to proteins. *J. Med. Chem.* **2009**, *52*, 225–233.