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Benzimidazolium Salts as Small, Nonpeptidic and BBB-Permeable Human Prolyl Oligopeptidase Inhibitors

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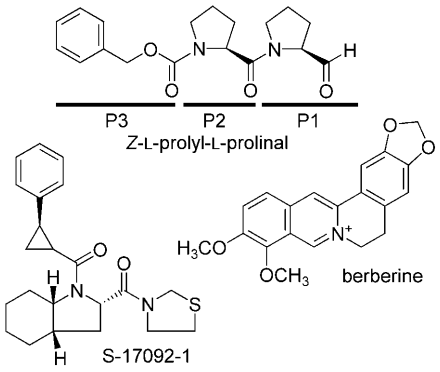
Prolyl oligopeptidase (POP) is a cytosolic serine peptidase that hydrolyzes proline-containing peptides at the carboxy terminus of proline residues. This peptidase has gained importance as a target for the treatment of cognitive disturbances of patients with neuropsychiatric diseases. Our research addresses the identification of POP inhibitors from a small focused library of polar heterocyclic compounds arising from multicomponent reactions. Two selective POP-specific inhibitors were identified on the basis of their inhibition of dipeptidyl peptidase IV. The most active compounds were evaluated for their in vitro transport through the blood–brain barrier (BBB) using a parallel artificial membrane permeability assay. Our results show for the first time that benzimidazolium salts are new POP-inhibitory scaffolds with properties of solubility, specificity, and lipophilicity that may allow them to cross the BBB by passive diffusion. These findings constitute an excellent starting point to synthesize new POP inhibitors with enhanced properties.

Introduction

The protease prolyl oligopeptidase (POP; EC 3.4.21.26) is a cytosolic serine protease that hydrolyses small proline-containing peptides at the carboxy terminus of proline-residues.[1,2] Many bioactive peptides, such as substance P, thyroliberin, β-endorphin, and arginine-vasopressin, are POP substrates. However, the in vivo involvement of POP in the metabolism of these substrates is not yet clearly established.[3] POP was recently cloned by our research group from human brain RNA expressed in *E. coli*, and a homology model based on the X-ray structure of porcine POP was obtained.[4]

POP inhibitors might be valuable compounds for the treatment of a variety of clinical conditions of the brain, such as the cognitive disturbances present in schizophrenia (SZ), bipolar affective disorder (BD), and Alzheimer's disease, as shown by the neuroprotective and cognition-enhancing effects of POP inhibitors in experimental animals.[3,5–7] In addition, the POP inhibitor S-17092-1 has been tested in phase I clinical trials for its capacity to enhance cognition.[8,9] However, the mechanisms by which POP affects the psychological state are still obscure.[3] Recently, the involvement of POP in the metabolism of inositol-1,4,5-triphosphate (IP_3) was reported. IP_3 is a key molecule in the transduction cascade of neuropeptide signaling. Neuropeptides induce an increase in IP_3 levels, which in turn binds to its receptor in the membrane of the endoplasmic reticulum and stimulates the release of Ca^{2+}, which is believed to play a crucial role in learning and memory.[10] Recent findings have demonstrated that POP inhibition increases the concentration of IP_3.[11,12] The IP_3 signaling pathway participates in the therapeutic action of several mood-stabilizing drugs (lithium, carbamazepine, and valproic acid)[13] and defects in the mechanisms that regulate IP_3 signaling may underlie BD. This observation also indicates that other small molecule inhibitors of POP may be useful in the treatment of BD. Moreover, the mood-stabilizing drug commonly used to treat BD, valproic acid, directly inhibits recombinant POP activity.[14]

Several low-molecular-weight POP inhibitors have been reported. Most of these have an acyl-L-prolyl-pyrrolidine motif in common, wherein a lipophilic acyl end group has been shown



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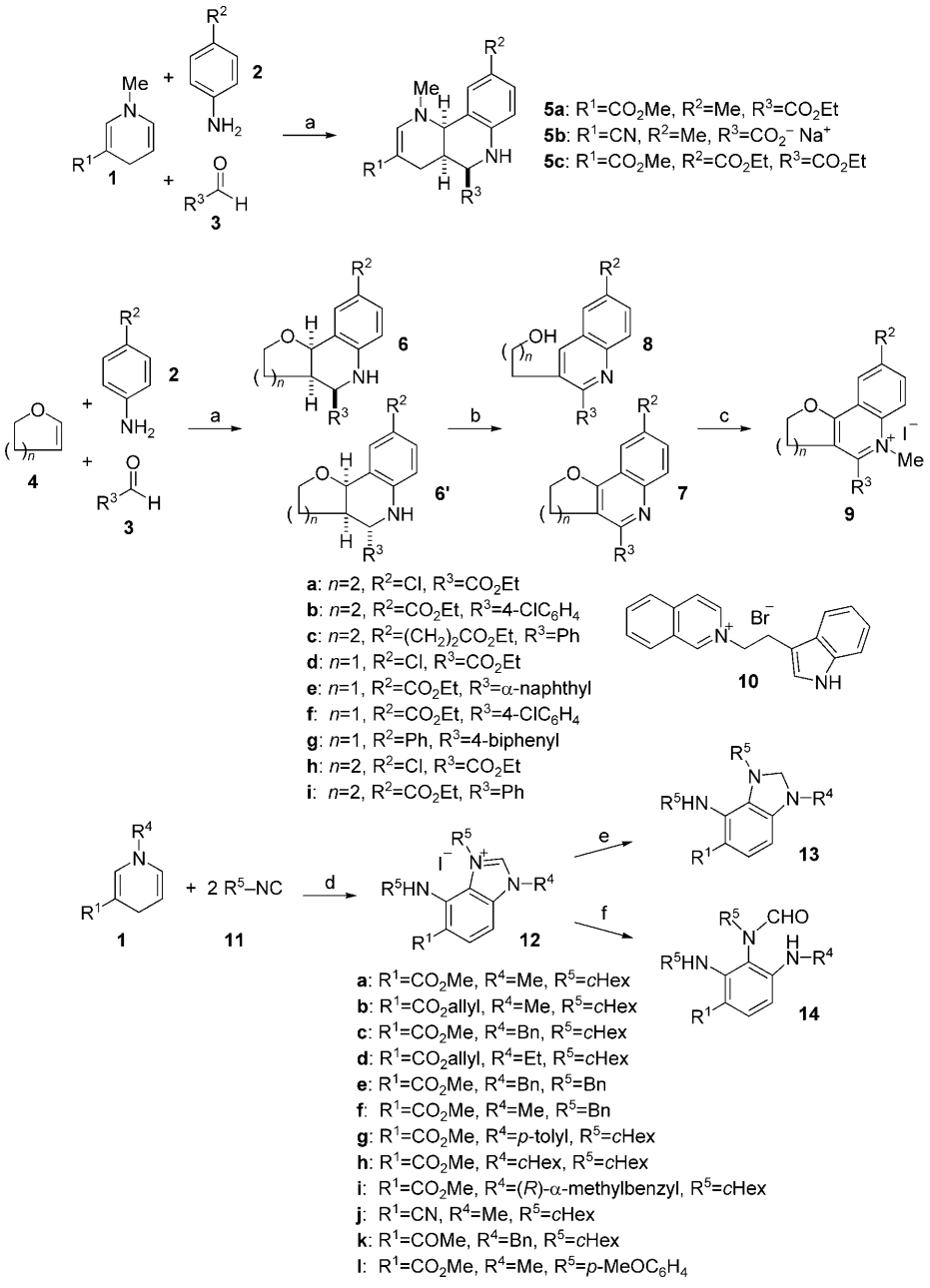
Supporting information (synthetic procedures and characterization data for intermediates and final compounds) for this article is available on the WWW under http://www.chemmedchem.org or from the author.

to be important for high inhibitory activity.[15–17] One extensively studied POP inhibitor is *Z*-L-prolyl-L-prolinal (ZPP),[18] whose crystal structure within the enzyme active site has been published.[19] Enlargement of the pyrrolidine ring or addition of an oxo substituent to the 2-position of the pyrrolidine ring in P1 position decreases the inhibitory activity.[16,20] The L-prolyl moiety at the P2 site has been very difficult to replace. Only a few successful substitutions that increased potency have been published.[21–23] Several substitutions at the P1 pyrrolidine group that increase the inhibitory activity have been reported including strong electrophilic groups such as formyl,[18] cyano,[24] and hydroxyacetyl[25] that interact with the serine at the active site of the enzyme. In a previous study, we used ^{19}F NMR to search for new POP inhibitors from a library of plant extracts used in traditional Chinese medicine, and identified several as powerful inhibitors of this peptidase. The alkaloid berberine was isolated as the POP inhibitory molecule in *Rhizoma coptidis* extract.[26]

In the present work we report our results on a new class of POP inhibitors that share a benzimidazolium moiety that recalls the isoquinolinium structure present in berberine. We have prepared a small compound library of potential POP inhibitors applying recent results on the development of new multicomponent reactions (MCRs)[27] which allowed straightforward access to a variety of diversely substituted heteroaromatic structures (benzonaphthyridines, pyranoquinolines, and benzimidazolium salts).

Results and Discussion

In the first stage of the study, compounds available from previous projects were selected and their capacity to inhibit human POP was measured. Two methodologies were considered for the synthesis of the compounds to be screened. The Povarov reaction (the condensation of an electron-rich alkene, an aniline, and an aldehyde)[28] was used for the preparation of compounds **5** and **6**. Thus, interaction of 1,4-dihydropyridines **1** with anilines **2** and ethyl glyoxalate or aromatic aldehydes **3** under $Sc(OTf)_3$ catalysis nicely afforded the benzonaphthyridine adducts **5**.[29] Furthermore, under similar conditions, a related condensation of cyclic enol ethers **4** afforded adducts **6** and **6′**, which were DDQ-oxidized to the quinoline derivatives **7**.[30] During this event, small amounts of open chain quinolyl alcohols **8** were isolated in some cases. N-alkylation of the quinoline derivatives **7** with methyl iodide gave the corresponding quinolinium salts **9**. The *N*-tryptophyl isoquinolinium salt **10**[31] was prepared to compare its performance with that of berberine. Benzimidazolium iodides **12** were synthesized in just one step using a recently described reaction where dihydropyridines **1** react with commercially available



Scheme 1. Synthesis and structures of the library members. Reagents and conditions: a) $Sc(OTf)_3$, CH_3CN, RT; b) DDQ; c) MeI; d) I_2, THF, −78 °C→RT; e) $NaBH_4$, MeOH; f) KOH, H_2O.

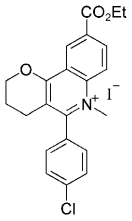
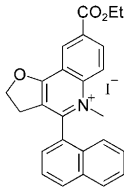
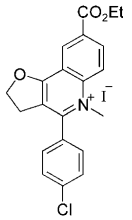
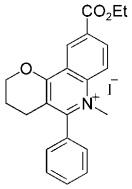
isocyanides **11** in the presence of iodine.[32] The imidazolium moiety present in **12a** was further modified by $NaBH_4$ reduction to afford quantitatively the benzimidazoline **13a**, in a reversible manner, as the latter compound is spontaneously converted into the former by exposure to atmospheric oxygen. Hydrolytic treatment of the salt **12a** (KOH, H_2O) took place regioselectively to give the anilinoformamide **14a** (Scheme 1).

In this context, we studied the inhibitory activity of a library of 36 compounds with purities higher than 95% against POP. Pure compounds were evaluated fluorimetrically using human POP and *Z*-Gly-Pro-AMC as substrate. The first evaluation of the molecules was performed at a final concentration of 200 μM. The molecules that inhibited more than 50% of POP activity were then selected and the concentrations required for half-maximal inhibition (IC_{50}) were calculated. Moderate inhibition was observed for structures **7c** and **5a**. However, as these constitute a new family of POP inhibitory compounds, these results were very promising. Speculating with a putative structural analogy with berberine, the role of a positive charge in the activity was studied, and a cationic center was introduced in the selected compounds. Thus, the second generation was screened against POP following the same methodology and the IC_{50} values of the compounds which showed greatest inhibitory properties was calculated (Table 1). The beneficial effect of a cationic center was proposed, as indicated in the enhanced inhibition by compounds **9b**, **9f**, **9i**, and **9e** in comparison with their neutral quinoline counterparts, which were inactive. Interestingly, the tryptophylquinolinium salt **10** was inactive, which suggests specificity in the mode of action. At this point, we decided to test a family of recently described benzimidazolium salts **12** (Table 2). Again, inhibition was observed for almost all the compounds displaying the benzimidazolium core: **12a**–**l**. Interestingly, the structure–activity relationship revealed a pattern where the highest potency was linked to the presence of a carbonyl moiety (methyl ketone or methyl ester) at the benzene ring, whereas for the rest of the substituents the data suggest that the best combination consisted of two adjacent cyclohexyl groups (one in the imidazolium ring, and the other attached to the aniline nitrogen), although aromatic rings or benzyl groups behaved similarly. The remaining nitrogen substituent is best accommodated by a methyl or benzyl group. Given the expected reactivity for these compounds, and their prospective metabolism, we analyzed the behavior of benzimidazoline **13a** and the formamidoaniline **14a**, (the products of reduction and hydrolysis of the parent benzimidazolium salt **12a**) to determine their lack of activity (Table 2).

The IC_{50} values of the most inhibitory compounds with a benzimidazolium core were noticeably better than the IC_{50} value of 145 μM previously reported for berberine.[26] Regarding the potency of our best inhibitors when compared with typical reference compounds such as ZPP,[18] it should be considered that the latter is a covalent inhibitor that reacts with the serine in the enzyme active site. These kinds of highly reactive, covalent inhibitors may be inadequate for clinical use because of stability problems and serious side effects, in addition to limitations in accessing the central nervous system (CNS), as brain entry is hindered by the blood–brain barrier (BBB).[33] In contrast, the mechanism of reaction of benzimidazolium salts is probably a noncovalent interaction with the POP active site. Nevertheless, the mechanism of interaction is currently under study. Another important feature is the low reactivity of benzimidazolium salts when compared with formyl or other previously described covalent inhibitors, which could contribute to decreasing clinical side effects. However, when the potency of our best inhibitors is compared with typical noncovalent reference compounds, such as S-17092-1 (IC_{50} value in rat cortical extract: 8.3 nM),[34] SUAM 1221 (IC_{50} value in bovine brain extract: 190 nM),[35] it is clear that the potency of these new scaffolds needs to be improved.

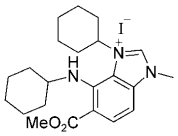
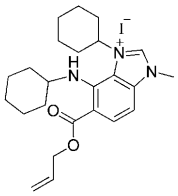
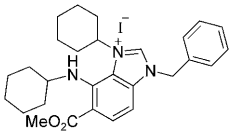
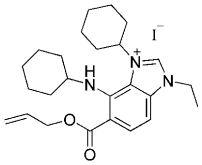
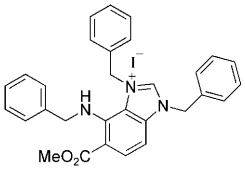
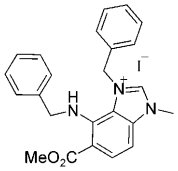
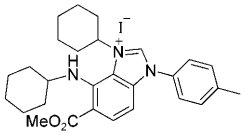
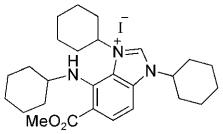
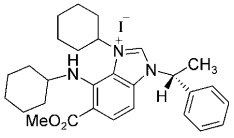
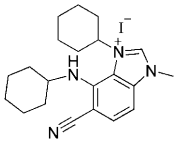
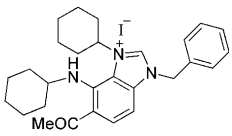
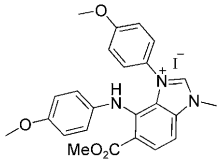
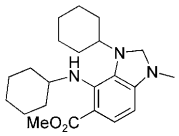
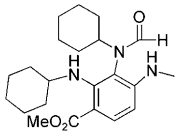
The inhibition specificity of the most potent compounds was studied using the protease dipeptidyl peptidase IV (DPPIV). The molecules were evaluated fluorimetrically using recombinant human DPPIV and Gly-Pro-AMC as substrate and the IC_{50} values was calculated (Table 3). Compounds **12a** and **12k** showed specificity for POP, **12e** inhibition of this enzyme was slightly greater to that for DPPIV whereas compounds **12l** and **9e** were not specific.

Table 1. IC_{50} values of inhibitors derived from Povarov reactions against POP.

Compd[a]	Structure	IC_{50} [μM]
9b		124 ± 13
9e		75 ± 5
9f		133 ± 5
9i		135 ± 12

[a] Compounds **5a** and **7c** showed moderate activity at 200 μM, whereas **5b**, **5c**, **6a**, **6′a**, **6b**, **6d**, **6′d**, **7a**–**b**, **7d**–**f**, **7i**, **7c**, and **10** were not active at 200 μM.

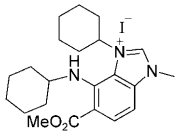
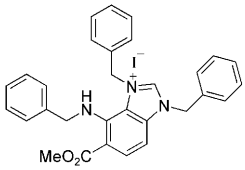
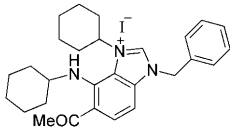
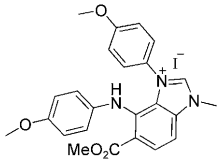
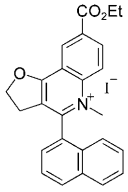
Table 2. IC_{50} values of benzimidazolium salts **12** and derivatives **13** and **14** against POP.

Compd	Structure	IC_{50} [μM]
12a		72 ± 9
12b		140 ± 1
12c		122 ± 3
12d		129 ± 20
12e		51 ± 8
12f		147 ± 4
12g		194 ± 5
12h		214 ± 3
12i		169 ± 15
12j		201 ± 25
12k		37 ± 12
12l		99 ± 7
13a		n.a.[a]
14a		n.a.[a]

[a] Not active at 200 μM.

Unlike many small-molecule drugs, typical POP inhibitors are neutral nonionizable compounds. A permanent positive charge, such as the one present in the reported compounds, may improve water solubility and allow the formation of different salts by anion exchange. These features may prove useful

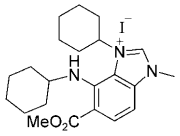
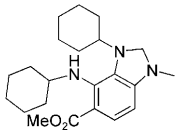
Table 3. Specificity against dipeptidyl peptidase IV.

Compd	Structure	IC_{50} [μM]
12a		n.a.[a]
12e		133 ± 5
12k		n.a.[a]
12l		100 ± 5
9e		65 ± 12

[a] Not active at 200 μM.

in future formulations of a putative drug based on this structural type, although it may sometimes be detrimental for crossing the highly lipophilic BBB. Delivery to the brain is a complex process as compounds must cross a natural defense mechanism, the BBB, designed to keep harmful substances out of this organ.[36] The parallel artificial membrane permeability assay (PAMPA) uses an artificial membrane in the form of filter-supported phospholipid bilayers. To mimic the BBB, a porcine polar lipid extract is used to coat the filter. The phospholipid membrane mimics the cell membrane but has no means for active or paracellular transport of drug molecules and is therefore a convenient tool to evaluate the transport of compounds by passive diffusion.[37] To further study the absorption and transport mechanisms of the new POP inhibitory compounds, we selected benzimidazolium salt **12a** and its reduced counterpart **13a** and evaluated their transport in vitro through the BBB using PAMPA. We hypothesized that the reduced compound is more permeable through the BBB than the oxidized one. However, our results showed that the BBB permeability values of **12a** and **13a** were similar (Table 4). As **13a** showed a

Table 4. Compound permeability (*Pe*) through the BBB as determined by PAMPA.

Compd	Structure	*Pe* [10^{-6} cm s^{-1}]	Log *Pe*
12a		1.9 ± 0.22	−5.6 ± 0.08
13a		2 ± 0.36	−5.7 ± 0.08

strong tendency to oxidize in aqueous medium, we performed the same assay in the presence of 10 mM reduced glutathione in the medium to prevent its spontaneous in situ transformation into **12a**. However, we observed the same results. PAMPA showed that both molecules, the reduced form **13a** and the oxidized form **12a**, were able to potentially cross BBB by passive diffusion although low BBB permeability was predicted.

To provide a structural explanation for our observations, we performed preliminary computational docking studies using the structure of porcine POP (PDB code: 1H2W).[38] Some of the most active compounds have a benzimidazolium core in common containing three carbocyclic substituents whereas other compounds presenting good inhibitory potencies have only two carbocyclic substituents. For these reasons, docking of compounds **12a**, **12b**, and **12k** was studied. All of them were successfully docked into the binding cavity of the POP active site (Figure 1). In the three cases, the positive charge was located in a proximal area which is between 7.5 Å (for **12a**) and 9.2 Å (for **12b**) away from the hydroxy group of the active-site serine, subsequently the possibility of a nucleophilic attack of serine might be excluded. The three compounds

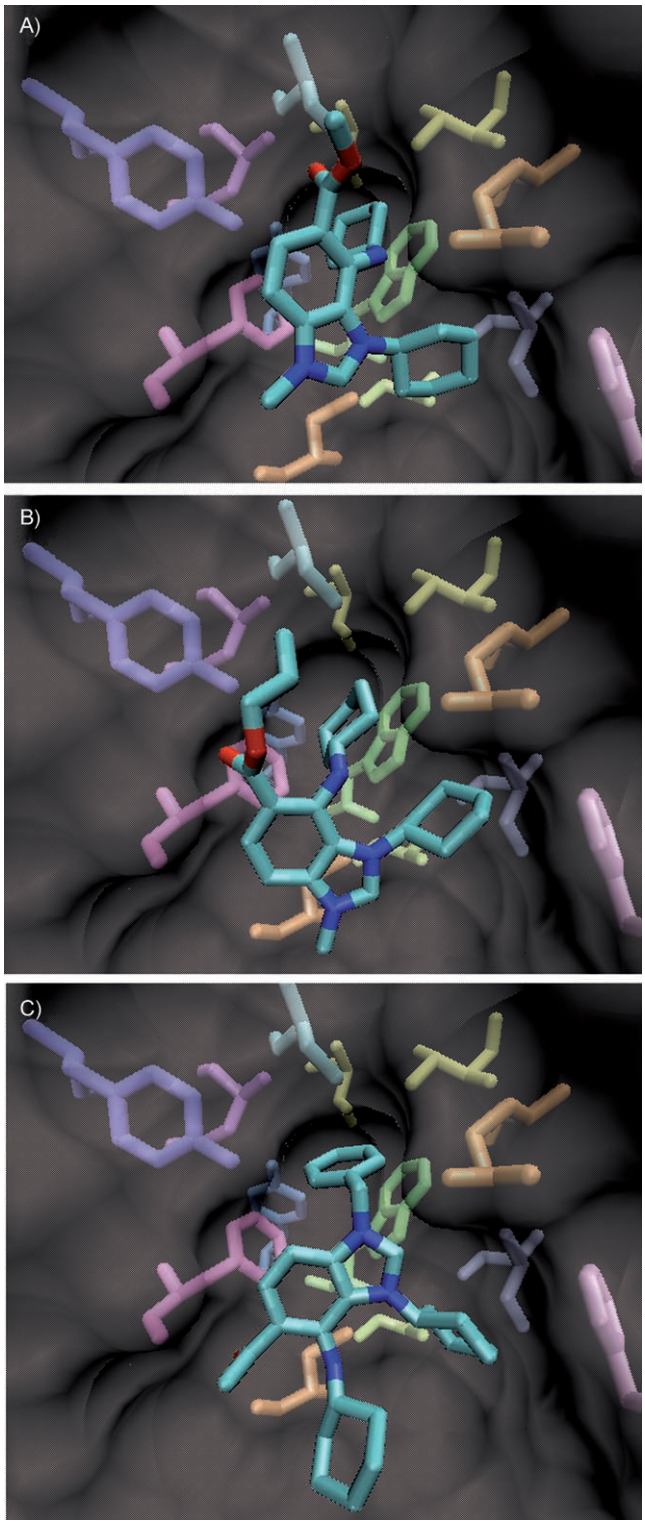


Figure 1. Docking of compounds A) **12a**, B) **12b**, and C) **12k**.

adopted similar binding strategies: they addressed their hydrophobic substituents towards the S1 and S3 pockets. For compounds **12a** and **12b**, the cyclohexyl substituent group was buried in the hydrophobic environment of the S1 pocket and the second *N*-cyclohexyl substituent was close to the S3 site. In contrast, compound **12k** addressed its benzyl group to-

wards the S1 site and its *N*-cyclohexyl towards S3. Consequently, compared with **12a** and **12b**, compound **12k** was turned 180° and its methyl ketone group was placed on the opposite site, far away from the nucleophilic serine of the active site. Moreover, the degree of burial of the hydrophobic groups in the S1 site is correlated with inhibitory activity; compounds with the most deeply buried groups are the most powerful. In the three cases a cyclohexyl group occupied the nonpolar S3 site, which is in agreement with the preference for hydrophobic residues previously reported in this site.[19]

Conclusions

Here we have identified a new family of POP inhibitors through the systematic screening of a small library that includes compounds arising from MCRs using heterocycles (dihydropyridines and cyclic enol ethers). Among the most potent compounds, pyranoquinolinium and benzimidazolium salts display properties of interest, such as solubility, specificity, and lipophilicity, which might allow them to cross the BBB by passive diffusion and therefore reach the CNS. A preliminary analysis of these newly identified scaffolds has been made and the results suggest that they would be an excellent starting point to synthesize new POP inhibitors with enhanced properties.

Experimental Section

Z-Gly-Pro-AMC and Gly-Pro-AMC were obtained from Bachem (Bubendorf, Switzerland). Other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany). Fluorescence was measured using a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments, Vermont, USA). Unless stated otherwise, all reactions were carried out under argon atmosphere in dried glassware. Commercially available reactants were used without further purification. Reaction temperatures were controlled by an IKA temperature modulator. Thin-layer chromatography was conducted with Merck silica gel 60 F_{254} sheets and visualized by UV and $KMnO_4$ solutions. Silica gel (particle size 35–70 mm) was used for flash column chromatography. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 (at 400 and 100 MHz, respectively). Unless otherwise stated, NMR spectra were recorded in $CDCl_3$ solution with TMS as an internal reference. Data for ^1H NMR spectra are reported as follows: chemical shift (δ in ppm), multiplicity, integration, and coupling constant (J in Hz). Data for ^{13}C NMR spectra are reported in terms of chemical shift (δ). IR spectra were recorded on a Thermo Nicolet Nexus spectrometer and are reported in frequency of absorption (cm^{-1}). HPLC–MS data were obtained with a Waters HPLC with a Waters Symmetry C_{18} column (3.9×150 mm, 5 mm) using a flow rate of 1 mL min^{-1} and a ramp of 0→100% B (A: H_2O with 0.1% HCO_2H; B: CH_3CN with 0.1% HCO_2H) over 10 min with visualization at 220 nm and 254 nm coupled with a Waters Micromass ZQ spectrometer (see Supporting Information).

Preparation of starting materials

The starting *N*-alkyl-1,4-dihydropyridines **1** were prepared by reduction of the corresponding pyridinium salts with sodium dithionite, as described.[39] The following *N*-substituted-1,4-dihydropyridines (*N*-*p*-tolyl, *N*-*c*Hex, and *N*-α-methylbenzyl)[40] were prepared following a general method.[41]

General procedure for the synthesis of benzonaphthyridines 5. $Sc(OTf)_3$ (0.2 mmol) was added to a solution of aldehyde **3** (1 mmol) and aniline **2** (1 mmol) in dry CH_3CN (4 mL), and the mixture was stirred at room temperature. A solution of dihydropyridine **1** (1 mmol) in dry CH_3CN (3 mL) was then added and the resulting suspension was stirred at room temperature under N_2 atmosphere for 12 h. A saturated aqueous $NaHCO_3$ solution was added and the resulting mixture was extracted with EtOAc. The organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, hexanes/EtOAc) to give the desired product **5**. For experimental details see ref. [29].

General procedure for the synthesis of fused tetrahydroquinolines 6 and 6′. $Sc(OTf)_3$ (0.2 mmol) was added to a solution of aldehyde **3** (1 mmol) and aniline **2** (1 mmol) in dry CH_3CN (4 mL), and the mixture was stirred at room temperature. A solution of cyclic enol ether **4** (1 mmol) in dry CH_3CN (3 mL) was then added and the resulting suspension was stirred at room temperature (unless otherwise specified) under N_2 atmosphere for 12 h. A saturated aqueous $NaHCO_3$ solution was added and the resulting mixture was extracted with EtOAc. The organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, hexanes/EtOAc) to give the desired products **6** and **6′**.

General procedure for the oxidation of tetrahydroquinolines 6 and 6′. A modification of the method of Borrione et al.[30a] was used. DDQ (2 mmol) was added to a solution of a mixture of tetrahydroquinolines **6** and **6′** (1 mmol) in dichloromethane (10 mL), and the resulting mixture was stirred at room temperature until the starting material had disappeared (TLC control, typically 24–48 h). An aqueous saturated solution of Na_2CO_3 (50 mL) was added, the phases were separated, and the aqueous layer was extracted with dichloromethane (3×10 mL). Then the organic phases were reunited, dried (anhydrous Na_2SO_4), and filtered. The solvent was removed under reduced pressure and the residue was subjected to column chromatography (SiO_2, hexanes/EtOAc) to yield pure quinolines **7**. In one case, small amounts of the hydroxyalkylquinoline **8** were isolated.

General procedure for the alkylation of quinolines 7. Methyl iodide (10 equiv) was added to a solution of isoquinoline **7** (1 equiv) in a mixture of acetone/Et_2O (1:1) and the mixture was stirred at room temperature until the starting material had disappeared (approximately 72 h). The precipitate was filtered and washed with Et_2O to yield pure quinolinium salt **9**.

Preparation of isoquinolinium salt 10. This compound was prepared following the method described by J. W. Huffman.[31]

General procedure for the synthesis of benzimidazolium salts 12. The corresponding isocyanide **11** (0.96 mmol) and a solution of I_2 (248 mg, 0.98 mmol) in anhydrous CH_2Cl_2 (10 mL) were added to a solution of a dihydropyridine **1** (0.98 mmol) in anhydrous dichloromethane (5 mL) kept under an inert atmosphere at −78°C (dry ice–acetone bath). The reaction mixture was stirred for 20 h, gradually reaching room temperature (no more dry ice was added). H_2O (20 mL) was added and the mixture was extracted twice with CH_2Cl_2. The combined organic extracts were washed with an aqueous $Na_2S_2O_3$ solution (5%, 2×10 mL) and brine (25 mL), and then dried (Na_2SO_4) and filtered. The solvent was removed under reduced pressure and the residue was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH) to yield the pure benzimidazolium salt **12** as a brown powder. For experimental details, see ref. [32].

Reduction of benzimidazolium salt 12a. $NaBH_4$ (152 mg, 4.02 mmol) was added to a solution of **12a** (200 mg, 0.40 mmol) in MeOH (5 mL) and the resulting mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue partitioned with CH_2Cl_2 and H_2O (40 mL, 1:1). The phases were separated, the aqueous layer was extracted with CH_2Cl_2 (2×10 mL), and the combined organic extracts were dried (Na_2SO_4) and filtered. The solvent was removed under reduced pressure to yield essentially pure benzimidazoline **13a** (129 mg, 87%) as a slightly unstable foam.

Hydrolysis of benzimidazolium salt 12a. KOH (20 mg, 0.36 mmol) was added to a solution of **12a** (90 mg, 0.18 mmol) in MeOH (1.5 mL) and the resulting mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the residue was partitioned with CH_2Cl_2 and H_2O (40 mL, 1:1). The phases were separated and the aqueous layer was extracted with CH_2Cl_2 (2×10 mL). The combined organic extracts were then dried (Na_2SO_4) and filtered. The solvent was removed under reduced pressure to yield the anilinoformamide **14a** (53 mg, 76%).

Expression and purification of POP. POP was obtained by expression in *E. coli* and affinity purification using a His tail fusion, as reported previously.[42]

POP inhibition assay

POP activity was determined following the method described by Toide et al.[5] The reactions were performed in 96-well microtiter plates, which allowed simultaneous monitoring of multiple reactions. For each reaction, activity buffer (131 μL, 100 mM Na/K phosphate buffer, pH 8.0) was preincubated for 15 min at 37 °C with POP (7 nM) and the corresponding inhibitor solution (3 μL). A stock solution of inhibitor was prepared in [D_6]DMSO (100 mM), and dilutions with [D_6]DMSO were prepared from this stock solution. A control with the same concentration was also prepared in [D_6]DMSO. After pre-incubation (15 min at 37 °C), ZGP-AMC (10 μL, 3 mM in 40% 1,4-dioxane) was added, and the reaction was incubated for 1 h at 37 °C. The reaction was stopped with sodium acetate (150 μL, 1 M, pH 4) and the formation of AMC was measured fluorimetrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. The IC_{50} value was defined as the concentration of compound required to inhibit 50% of POP activity.

DPPIV inhibition assay

DPPIV activity was determined following the method described by Checler et al.[43] The reactions were performed in a 96-well microtiter plate. For each well, the reaction mixture contained activity buffer (131 μL, 100 mM Na/K phosphate buffer, pH 8.0), 0.7 nM DPPIV, and 3 μL of the corresponding inhibitor solution (in [D_6]DMSO). After pre-incubation (15 min at 37 °C), GP-AMC (10 μL, 3 mM in 40% 1,4-dioxane) was added, and the reaction was incubated for 1 h at 37 °C. Finally, the reaction was stopped with sodium acetate (150 μL, 1 M, pH 4) and the formation of AMC was measured fluorimetrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. The IC_{50} value was defined as the concentration of compound required to inhibit 50% of DPPIV activity.

Parallel artificial membrane permeability assay (PAMPA)

PAMPA assays were carried out in a 96-well plate (pION, Inc). Donor and acceptor wells were separated by a polyvinylidenefluoride (PVDF) membrane coated with polar brain lipid extract porcine (PBLEP) (Avanti Polar Lipids, Inc.) which contained phosphatidylcholine (12.6%), phosphatidylethanolamine (33.1%), phosphatidylserine (18.5%), phosphatidylinositol (4.1%), phosphatidic acid (0.8%), and cerebrosides and pigments (30.9%). The PVDF membrane was coated with PBLEP in an amount equivalent to 300 bilayers. All the donor and receptor wells were at pH 7.4. Donor wells were filled with the compound at 200 μM and acceptor wells were filled with system solution (pION, Inc), (195 μL, pH 7.4). All PAMPA measures were done in triplicate. The assays were performed in a Gut-Box device (pION, Inc). The PAMPA plate was incubated for 4 h in a humidity-saturated atmosphere. The stirring rate was equivalent to 25 μm thickness of unstirred water layer (UWL). After 4 h, the donor and acceptor wells were analyzed by HPLC in a gradient of 0→100% MeCN over 15 min, and the compounds were detected between 210 and 315 nm. The compounds were identified on the basis of their retention times and UV spectra. After HPLC analysis, the effective permeability (*Pe*) values were calculated by the following expression: $Pe = (-218.3/t)\ \mathrm{Log}[1-(2C_A(t)/C_D(0)]\ 10^{-6}\ \mathrm{cm\,s^{-1}}$ in which t is the experiment time (incubation), $C_A(t)$ is the concentration of the compound in the acceptor well at the end of the assay, and $C_D(0)$ is the concentration of the compound in the donor well at the beginning of the assay (time zero).

Docking simulations

Docking calculations with AutoDock4[44] were carried out using the X-ray structure of porcine POP, (PDB code: 1H2W).[38] The protonation state of the histidines was considered neutral. A grid map of 60×60×60 points with a grid-point spacing of 0.375 Å was prepared using AutoDock Tools. The grid maps were calculated using AutoGrid, version 4. Docking calculations were performed by defining flexibility of the ligands. For each ligand, 100 dockings were performed with the Lamarckian Genetic Algorithm using a population size of 50 individuals with a total of 2×10^6 energy evaluations. Default parameter values of selection pressure, crossover, and mutation weights were selected.

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