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Bioorganic & Medicinal Chemistry Letters 13 (2003) 2875–2878

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Prolylisoxazoles: Potent Inhibitors of Prolyloligopeptidase with Antitrypanosomal Activity

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Received 24 March 2003; revised 3 June 2003; accepted 3 June 2003

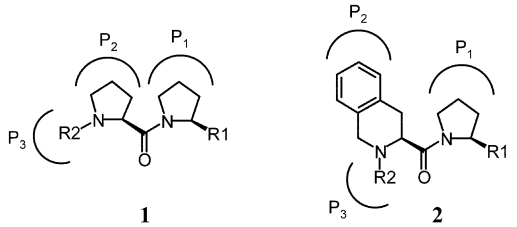
Abstract—Prolylprolylisoxazoles and prolylprolylisoxazolines were synthesized through a 1,3-dipolar cycloaddition reaction. These compounds are potent inhibitors of human and trypanosomal prolyloligopeptidase. They were shown to inhibit *Trypanosoma cruzi* and *Trypanosoma b. brucei* in in vitro systems with ED_{50}'s in the lower μM range.

Prolyl oligopeptidase (PO, POP, prolylendopeptidase, PEP, EC 3.4.21.26) is an endopeptidase with high substrate specificity for proline residues in peptides. It is a serine protease, cleaving peptide bonds on the C-terminal side of prolyl residues within peptides. PO is widely distributed between organisms and has been isolated from several sources, including plants, micro-organisms, invertebrates and various mammalian tissues and body fluids.[1]

An 80-kDa proteinase (Tc80) secreted by the infective trypomastigotes of *Trypanosoma cruzi* was characterized as a member of the PO family.[2] This enzyme hydrolyses collagen (types I and IV) and might be involved in the invasion of the parasite in the mammalian cells by degrading extracellular matrix components. Hence PO inhibitors may prevent the invasion of *T. cruzi* trypomastigotes into mammalian cells. This protozoan parasite is the causative agent of Chagas' disease. Several inhibitors have been synthesized[3] and were found to effectively inhibit this invasion in vitro.

Inhibition of human and *T. cruzi* PO has been compared.[3d] Trypanosomal PO is usually more sensitive to inhibitors but selectivity is limited and the maximum selectivity index (IC_{50} human PO/IC_{50} *T. cruzi* PO) reported is around 80.

Most PO inhibitors are substrate analogues. Some important inhibitors are illustrated in Figure 1. They are often slow binding inhibitors, with transition state mimicking properties. They are characterized by a proline residue where the carboxylic group is omitted or substituted for an electrophilic group such as a carbonitrile, an aldehyde or a ketone. The latter groups scavenge the serine alcoholate in the active site of the enzyme. Sulphonyl-activated Michael substrates are also used as an electrophilic group. The P2 position is mostly proline (**1**) or a cyclic amino acid such as Tic (**2**) and the P3 position a carbamate (e.g., benzyloxycarbonyl) or amide [e.g., 4-(phenyl)butanoyl] group.



R1: -H, -CN, -CHO, -$COCH_2OH$, -CO-2-thiazol
R2: -benzyloxycarbonyl, -4-phenylbutanoyl

Figure 1. PO inhibitors.

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doi:10.1016/S0960-894X(03)00579-1

Several reports have described the use of electron-withdrawing heterocycles to activate the reactive P1 carbonyl group in the development of protease (e.g., chymase and human neutrophil elastase) inhibitors.[4]

Tsutsumi et al.[5] synthesized PO inhibitors in which α-ketoheterocycles are introduced at the C-terminal end of peptides with PO substrate activity. Many of these products exhibit IC_{50} values at nM levels against PO. A thiazole as illustrated in Figure 1 shows an IC_{50} of 8.5 nM against human PO. Structure–activity studies of the C-terminal heterocyclic groups indicate the importance of an sp^2 nitrogen atom at the β-position of the adjoining carbonyl group. This heterocyclic nitrogen atom would provide a critical hydrogen-bond interaction with the histidine residue of the catalytic triad in PO.[5] This hydrogen bond was also observed in the X-ray crystal structure of porcine pancreatic elastase with a peptidyl α-ketobenzoxazole.[4a] Other ketoheterocycles[4c] used in serine protease inhibitor design are pyrroles, oxazoles, imidazoles, tetrazoles, pyridines, pyrimidines, benzimidazoles, benzothiazoles and oxazolopyridines.

We were particularly interested in the 3-acylisoxazole group as inhibitory moiety in PO inhibitor design. This heterocycle was until now unexplored in this field. Moreover, it is well known that 3-acylisoxazoles undergo a ring-opening upon reaction with alcoholates, giving rise to formylacetonitrile and the corresponding ester.[6] We assumed that the same reaction could happen in the active site of the enzyme, where the catalytic serine acts as a nucleophile.

We were also interested in the corresponding isoxazolines. We prepared a set (Table 1) of substituted prolylisoxazoles **3a–3e** and prolylisoxazolines **3f–3i** as potential PO inhibiting compounds.

Z-Proline or *N*-(4-(phenyl)butanoyl)proline was chosen for the P2–P3 position. These *N*-substitutions have been shown to fit well in the non-polar environment of the S_3 site of the enzyme.[7]

Table 1. PO inhibitory activity of prolylisoxazoles **3a–3e** and prolylisoxazolines **3f–3i**

	R1	R2	R3	PO human[d]	PO Tc80[d]
3a	–H	–H	*Z*	36	72
3b	$-CH_2OCH_2C_6H_5$	–H	*Z*	4	0.28
3c	$-COOCH_3$	$-COOCH_3$	*Z*	—[e]	—[e]
3d	$-C_6H_5$	–H	*Z*	15	1.87
3e	$-Si(CH_3)_3$	–H	*Z*	19	0.45
3f	$-CH_2OCH_2C_6H_5$	–H	*X*[c]	0.34	NT
3g1[a]	$-CH_2OCH_2C_6H_5$	–H	*Z*	6	1.25
3g2[a]	$-CH_2OCH_2C_6H_5$	–H	*Z*	NT[f]	3.30
3h	–CN	–H	*Z*	38	7.50
3i	$-C_6H_5$	–H	*Z*	10	0.21
Ref[b]	—	—	—	16	0.26

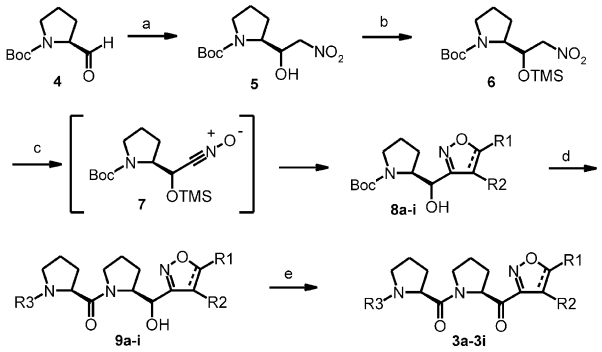
[a]Diastereomers.
[b]2-(*Z*-Prolyl-prolyl)thiazole.
[c]4-(Phenyl)butanoyl.
[d]K_i (nM).
[e]Moderately active: $IC_{50} > 1$ μM.
[f]Not tested.

A typical approach to synthesize five-membered rings, is a 1,3-dipolar cycloaddition. A [3 + 2] cycloaddition[8] of a nitrile oxide to a triple bond will provide an isoxazole ring. The corresponding isoxazolines are readily prepared by the same [3 + 2] cycloaddition with the corresponding alkene.

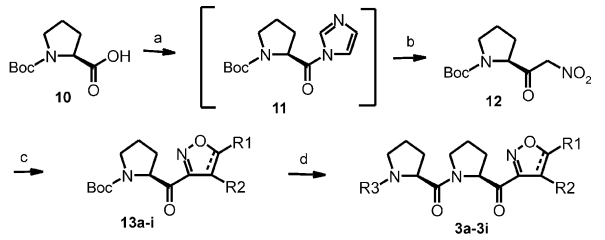
The key compound in this synthesis is a nitrile oxide. Nitrile oxides however are not stable and are generally formed in situ from nitro compounds.[9] Either prolyl nitro-alcohols or prolyl nitroketones can be used to prepare the nitrile oxide.

In a first approach nitro-alcohol (**5**) was synthesized by a Henry reaction[10] between Boc-prolinal (**4**)[11] and nitromethane using tetrabutylammonium fluoride as a base.[12] The nitro-alcohol was protected by a trimethylsilyl group with trimethylsilylchloride and triethylamine.[13] This TMS-protected 2-nitro-alcohol (**6**) was dehydrated in situ by phenylisocyanate and a catalytic amount of triethylamine in refluxing benzene to the desired nitrile oxide (**7**) which immediately reacted further with the alkyne or alkene to the isoxazole or isoxazoline (**8a–i**).[8,13] Starting materials were commercially available or were prepared by benzylation of the commercial propargylalcohol and allylalcohol. Deprotection with trifluoroacetic acid (TFA/H_2O) and coupling with *Z*-Pro or *N*-phenylbutanoyl-Pro with TBTU resulted in the alcohols **9a–i.** Final compounds **3a–i** were obtained by a Swern oxidation (Scheme 1).[8,14]

In a second approach the [3 + 2] cycloaddition was performed on a nitroketone. Boc-proline (10) was activated as carbonylimidazole (**11**) using 1,1′-carbonyldiimidazole (CDI) in dry tetrahydrofurane (THF).[15] The activated proline was not isolated but immediately used in the next step. Reaction with nitromethane and NaH in dry THF resulted in the desired 2-nitroketone (**12**). As described before, dehydration of this nitroketone using phenylisocyanate and a catalytic amount of triethylamine, followed by a [3 + 2] cycloaddition with the corresponding alkyne/alkene, gave the isoxazole/isoxazolines (**13a–i**). Deprotection with TFA/dichloromethane (DCM) and coupling with *Z*-Pro or *N*-phenylbutanoyl-Pro with TBTU resulted in the final compounds **3a–i**. The advantage of this pathway is the reduction of the number of steps from nine to five but



Scheme 1. (a) CH_3NO_2, $N(Bu)_4^+F^-$; (b) TMSCl, Et_3N; (c) (i) PhNCO, Et_3N, alkyne/alkene; (ii) $AcOH/H_2O$; (d) (i) TFA/H_2O; (ii) *Z*-ProOH or *N*-4-(phenyl)butanoyl-ProOH, TBTU, Et_3N; (e) $(COCl)_2$, DMSO.



Scheme 2. (a) CDI/THF; (b) NaH, CH_3NO_2; (c) PhCNO, alkyne/alkene; (f) (i) TFA/DCM; (ii) *Z*-ProOH or *N*-(4-(phenyl)butanoyl)-ProOH, TBTU, Et_3N.

the overall yield is lower due to the low yields of the cycloaddition and of the final coupling step (max 37%) (Scheme 2).

During the formation of the heterocycle two regioisomers can be formed. Indeed, the substituent on the isoxazole/isoxazoline ring can be in position 4 or 5 depending on the regioselectivity of the 1,3-dipolar cycloaddition. We could isolate only one regioisomer for the isoxazoles as well as for the isoxazolines. ^{1}H NMR data proved that the substituents were located on position 5 for both the isoxazoles and isoxazolines.[16,17]

This regioselectivity is in good correlation with the literature. Monosubstituted olefins and acetylenes show high regioselectivity and give 5-substituted derivatives for both electron donating and electron withdrawing groups. Only very strong electron withdrawing groups, such as $-SO_2R$, give predominantly 4-substituted derivatives.[18] This selectivity can be explained by a Frontier orbital model of the 1,3-dipolar cycloaddition and the application of the perturbation theory.[19]

This cycloaddition introduces chirality at position 5 of the heterocycle. For compound **3g** the two diastereomers (**3g^1** and **3g^2**) were separated by column chromatography but not characterized.

The peptidyl α-ketoisoxazoles/-isooxazolines have been tested as potential inhibitors of human PO[20] as well as for PO Tc 80 from *T. cruzi*.[2] Table 1 shows the inhibitory potencies of the tested compounds on both enzymes. 2-(*Z*-Prolyl-prolyl)thiazole (Fig. 1)[5] was used as reference compound.

All compounds were active against PO in the nM range. Allowing for some variations for the assays used in different laboratories were more effective against *T. cruzi* PO than against human PO (selectivity about 50).

Even with the small number of compounds tested in this study, it is clear that the type of substitutions on the isoxazole/isoxazoline ring is important. Compounds such as **3c** with an electron withdrawing substituent were less active. There is only a slight difference in activity between the isoxazoles and the isoxazolines. Hence, the alcoholate sensitivity of ketoisoxazoles appears not to be predictive for the inhibitory potency.

The most active product against trypanosomal PO is the 5-phenyl isoxazoline **3i.**

The keto function is essential for this high potency. The corresponding alcohol of compound **3b** (**9b**) gave a 100-fold reduction. This confirmed the necessity of an electrophilic moiety at the P_1 position.

These results prompted us to test some promising compounds such as **3d** and **3f** for in vitro antitrypanosomal activity against *Trypanosoma b. rhodesiense*, *T. cruzi* and *Leishmania donovani*.[21]

The results are summarized in Table 2. Both compounds show ED_{50} values in the low μM range. *T. cruzi* and *T. b. rhodesiense* appears the most sensitive organism and **3f** the most interesting compound. Indeed the toxicity (ED_{50}) of this compound against KB cells (nasopharyngeal cancer cell line) is >300 μg/mL.

We can conclude that these prolylisoxazole derived PO inhibitors are very interesting compounds in the search for new antitrypanosomal compounds. It was already known that PO inhibitors decrease the ability of *T. cruzi* trypomastigotes to invade in mammalian cells.[3e] These ketoisoxazoles show also substantial antitrypanosomal activity against *L. donovani*, *T. cruzi* and *T. b. rhodesiense* in vitro and low toxicity against KB cells. Further research will be required to investigate the detailed mechanism of action of these compounds and to establish a structure–activity relationship. Although **3f** appears non-toxic against KB cells, the compounds

Table 2. Antiprotozoal activity of compounds **3d** and **3f**

	Parasite	% Inhibition (μg/mL)				ED_{50} (μg/mL)	Toxicity[a] ED_{50} (μg/mL)
		30	10	3	1		
3d	*L. donovani*	100 (T)	1.8	0	—	13.5	
	T. cruzi	86.7 (T)	59.9	29	10.8	6.7	23
	T. b. rhodesiense	99.9	99.8	83.4	7.7	2.2	
3f	*L. donovani*[b]	100 (T)[e]	6.9	0	—	13.1	
	T. cruzi[c]	86.7(T)	69.4	42.6	16	4.7	>300
	T. b. rhodesiense[d]	99.9	95.5	6.9	0.9	5.5	

[a]KB cell toxicity—control drug: podophyllotoxin—ED_{50}: 0.008 μg/mL.
[b]*L. donovani* L82—control drug: pentostam—ED_{50}: 9.7 μg/mL.
[c]*T. cruzi* Lac Z—control drug: benznidazol—ED_{50}: 0.23 μg/mL.
[d]*T. b. rhodesiense* STIB900—control drug: pentamidine–ED_{50}: 0.00125 μg/mL.
[e](T): toxic to host macrophages.

described show only limited selectivity against human PO. PO degrades proline-containing neuropeptides such as vasopressin, substance P and thyrotropin-releasing hormone, which are involved in the process of learning and memory[22] and PO inhibitors are therefore considered as potential cognition-enhancing drugs. To prevent side effects due to interference with PO function in mammals compounds with pronounced selectivity will be necessary.

Acknowledgements

This work received support from The Fund for Scientific Research, Flanders, Belgium (FWO). G.B. is research assistant with the FWO. P.V.d.V. is a fellow of the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT). S.L.C. received financial support from UNDP/World/Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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16. In the unsubstituted isoxazole (**3a**), the chemical shifts of the isoxazole-protons were determined at δ 6.73–6.75 ppm and δ 8.45–8.47 ppm. Because of the electron-withdrawing oxygen next to the C-5 proton, the doublets at δ 8.45–8.47 ppm were attributed to the C-5 proton. Based on this knowledge we were able to assign the proton signals for the other isoxazoles. The proton signals were all situated in the range δ 6.63–6.88 ppm. The isoxazolines showed always two protons in the area δ 3.08–3.85 ppm, corresponding with the C-4 protons, and only one proton at δ 5.22–6.00 ppm corresponding with the C-5 proton. These chemical shift values are in accordance with examples from literature.[17]
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