6572 J. Med. Chem. 2010, 53, 6572–6583
DOI: 10.1021/jm1002556

Journal of Medicinal Chemistry Article

Substituted 4-Carboxymethylpyroglutamic Acid Diamides as Potent and Selective Inhibitors of Fibroblast Activation Protein

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Received February 26, 2010

Fibroblast activation protein (FAP) belongs to the prolyl peptidase family. FAP inhibition is expected to become a new antitumor target. Most known FAP inhibitors often resemble the dipeptide cleavage products, with a boroproline at the P1 site; however, these inhibitors also inhibit DPP-IV, DPP-II, DPP8, and DPP9. Potent and selective FAP inhibitor is needed in evaluating that FAP as a therapeutic target. Therefore, it is important to develop selective FAP inhibitors for the use of target validation. To achieve this, optimization of the nonselective DPP-IV inhibitor **8** led to the discovery of a new class of substituted 4-carboxymethylpyroglutamic acid diamides as FAP inhibitors. SAR studies resulted in a number of FAP inhibitors having IC_{50} of <100 nM with excellent selectivity over DPP-IV, DPP-II, DPP8, and DPP9 ($IC_{50} > 100\ \mu M$). Compounds **18a**, **18b**, and **19** are the only known potent and selective FAP inhibitors, which prompts us to further study the physiological role of FAP.

Introduction

Fibroblast activation protein (FAP[a]) is a type II transmembrane serine protease belonging to the prolyl peptidase family, which comprises serine proteases that cleave bioactive peptides preferentially after proline residues. Members of this family include dipeptidyl peptidase-IV (DPP-IV), DPP-II (DPP7), DPP8, and DPP9, and this family has been implicated in several diseases, including diabetes, cancer, and mood disorder.[1,2] FAP is expressed on reactive stromal fibroblasts in over 90% of common human epithelial cancers,[3] in granulation tissue of healing wounds, and in bone and soft tissue sarcomas.[4,5] It has been suggested that FAP promotes tumorigenesis and that FAP inhibition may attenuate tumor growth.[6−8] The development of potent and specific inhibitors for each of these DPP enzymes can be an important tool to study the physiological function and to validate their potential as a therapeutic target. Specific inhibitors for DPP-IV,[9−11] DPP-II,[12,13] and DPP8/9[14] have been identified, and investigations of selective inhibitors for FAP have only started recently.[15−17]

Dipeptidyl peptidase IV (DPP-IV, also known as CD26) (EC 3.4.14.5) is a drug target for type II diabetes. The active form of glucagon-like peptide-1 (GLP-1) stimulates insulin secretion, inhibits glucagons release,[18,19] and slows gastric emptying,[20−22] each a benefit in the control of glucose homeostasis in patients with type 2 diabetes.[23,24] Therefore, inhibition of DPP-IV prolongs the action of GLP-1, which offers a new strategy for treating type 2 diabetes. Antidiabetic efficacy has been demonstrated clinically with DPP-IV inhibitors; sitagliptin **1** (MK-0431) was approved by the U.S. Food and Drug Administration for the treatment of type 2 diabetes,[10] and vildagliptin **2** (LAF237) was approved for use in the European market (Figure 1).[9]

Dipeptidyl peptidase II (DPP-II, EC 3.4.14.2) is believed to be involved in the physiological breakdown of some proline-containing neuropeptides and in the degradation of collagen and substance P.[25,26] Using 1-[(*S*)-2,4-diaminobutanoyl]piperidine as lead compound, Senten et al. developed a series of potent and selective DPP-II inhibitors.[12,13] The representative DPP-II inhibitor **3** (Figure 1) showed $IC_{50} = 0.23$ nM and a high selectivity toward DPP-IV ($IC_{50} = 345\ \mu M$, Table 1).[13] Our studies found that compound **3** was inactive toward FAP, DPP8, and DPP9 (Table 1). These selective DPP-II inhibitors are outstanding tools to determine the physiological function of DPP-II and the therapeutic potential of DPP-II inhibitors.

Dipeptidyl peptidase 8/9 (DPP8/9) are cytoplasmic proteases with a 51% homology in amino acid level with DPP-IV.[27] Previously, the administration of selective DPP8/9 inhibitor **4** (Figure 1) may be associated with profound toxicities in preclinical species, which included alopecia, thrombocytopenia, anemia, enlarged spleen, multiple histological pathologies, and animal mortality shown in rats.[28] Highly specific and potent DPP8/9 inhibitors were also developed by Jiaang et al. The representative DPP8/9 inhibitor **5** (also called 1G-244, Figure 1) had IC_{50} values of 14 and 53 nM against DPP8 and DPP9, respectively (Table 1).[14] It did not inhibit DPP-IV, FAP, or DPP-II, with IC_{50} values greater than 100 μM. Recently, by using this potent and selective DPP8/9 inhibitor,

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[a] Abbreviations: FAP, fibroblast activation protein; DPP, dipeptidyl peptidase; GLP-1, glucagon-like peptide-1; LiHMDS, lithium hexamethyldisilazide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TFA, trifluoroacetic acid; LC−MS, liquid chromatography coupled mass spectrometry; SAR, structure−activity relationship; HOBt, *N*-hydroxybenzotrizole.

Wu et al. show that the inhibition is not associated with any animal toxicity.[29]

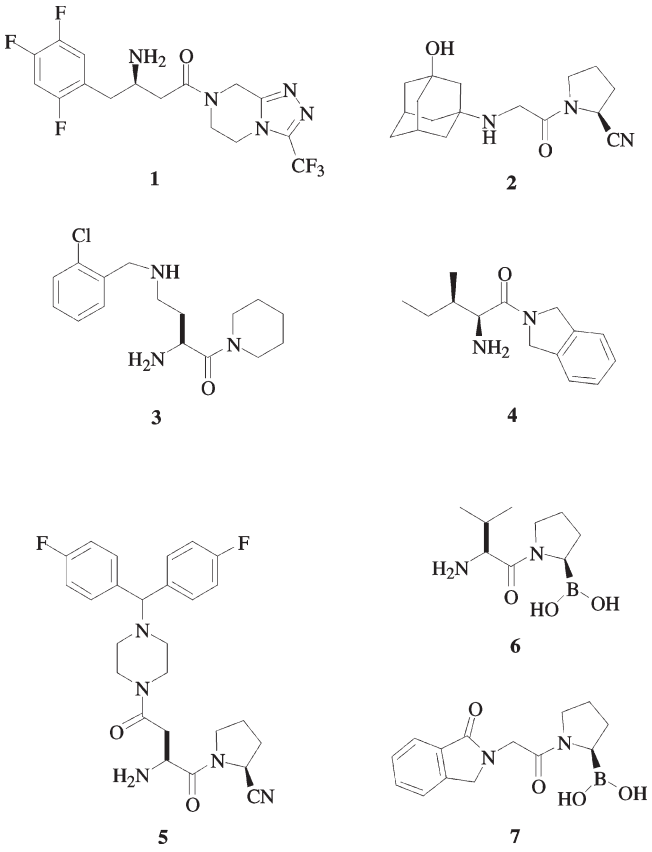


Figure 1. DPP-IV inhibitors **1** and **2**, DPP-II inhibitor **3**, DPP8/9 inhibitors **4** and **5**, nonselective inhibitor **6**, and FAP inhibitor **7**.

Previously, N- and αC-substituted Gly-boro-Pro derivatives have been developed, and these compounds strongly inhibited DPP-IV, DPP7 (DPP-II), and FAP. In this class, a representative compound is Val-boroPro **6** (Figure 1), having IC_{50} of < 40 nM against all the three DPPs (Table 1).[15] On the basis of FAP's preference for Gly-Pro-based endopeptidase substrates, Tran et al. developed a series of *N*-acyl-Gly-, *N*-acyl-Sar- (sarcosine), and *N*-blocked-boroPro derivatives. Representative compound in this class was inhibitor **7** (Figure 1), which preferentially inhibited FAP versus DPP-IV, but the selectivity against DPP-II, DPP8, and DPP9 was not reported (Table 1).[16] In our studies, this compound had weak inhibitory activity against DPP8 and DPP9 (11 and 6.5 μM, respectively) and was inactive toward DPP-II (Table 1).

In this paper, we report a systematic search for potent and selective FAP inhibitors. A structure−activity relationship was investigated starting from L-homoglutamic acid **8** (Figure 2), a dual DPP-V and FAP inhibitor (IC_{50} = 10 and 54 nM, respectively), as a lead compound (Table 1).[30] To develop selective FAP inhibitors, introduction of ring constraint in the P2 portion of lead **8** and modification of the P2 site secondary amine to amide were done as depicted in Figure 2. The replacement of secondary amine with amide is based on the fact that FAP acts as both prolin-specific endopeptidase and dipeptidyl peptidase, but DPP-IV prefers to display the latter activity.[8,16,31] Further exploration of the 2-position pyrrolidine derivatives (P1 site) and the 4-position amine substituents at the carbonylmethyl group (P2 site) led to the discovery of potent pyroglutamic acid-based FAP inhibitors with a high selectivity for FAP over DPP-IV, DPP-II, DPP8, and DPP9.

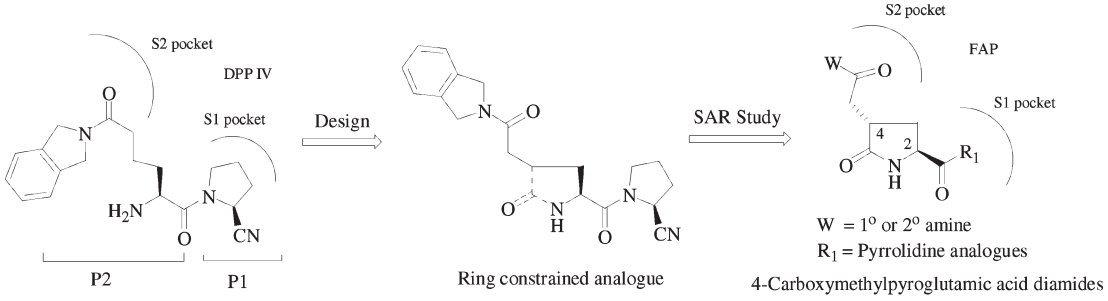
Chemistry

Reference compounds *N*-acyl-Gly-boroPro **9** and *N*-acyl-Gly-cyanoPro **10** were prepared according to the literature

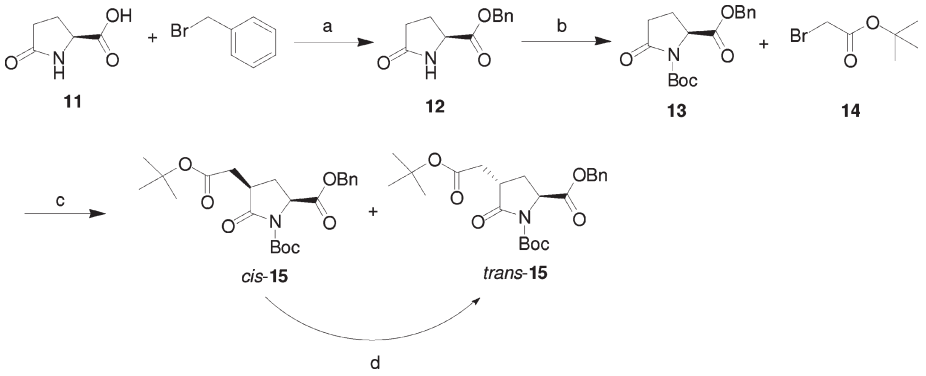
Table 1. Potency and Selectivity of Compounds **1**−**8**

		IC_{50} (μM)[a]					
compd	name	FAP	DPP8	DPP9	DPP-II	DPP-IV	reference[b]
1	sitagliptin	> 100	> 100	> 100	> 100	0.023	this study
2	vildagliptin	> 100	14	1.2	> 50	0.056	this study
3	DPP-II selective	ND	ND	ND	0.00023	345	13
		> 100	> 100	> 100	0.005	> 100	this study
4	DPP8/9 selective	> 100	0.15	0.24	> 100	> 100	this study
5	1G-244	> 100	0.014	0.053	> 100	> 100	this study
6	nonselective	0.011	ND	ND	0.038	0.0003	15
7	FAP selective	0.008	ND	ND	ND	23	16
		0.12	11	6.5	> 100	8.0	this study
8	1G-409	0.054	0.36	0.089	2.6	0.010	this study

[a] Mean values of at least three experiments; standard deviations are ±20%. ND, no data. [b] "This study" means in-house data.

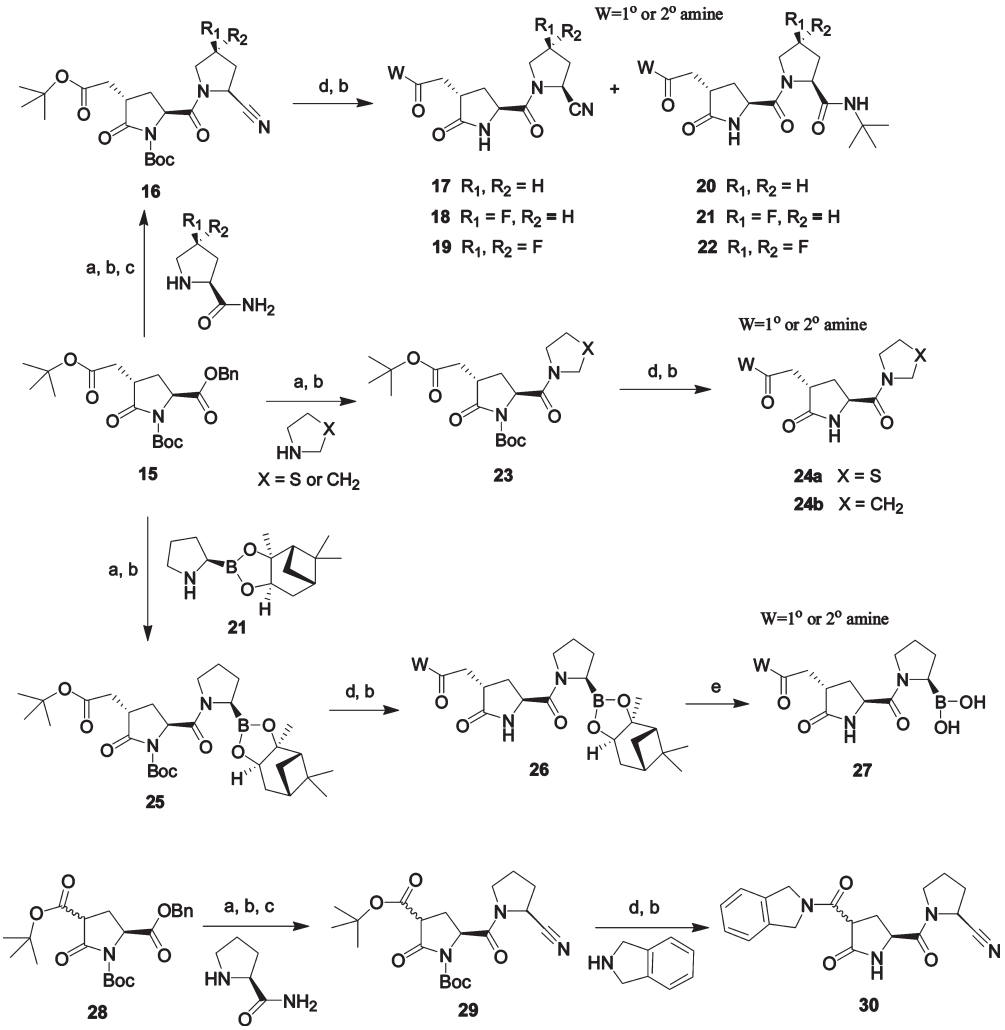


Scheme 1. Synthesis of C(4)-Substituted Pyroglutamate Ester Urethanes[a]



[a] Reagents: (a) DIEPA, CH_2Cl_2; (b) $(Boc)_2O$, 4-DMAP, CH_2Cl_2; (c) LHMDS, THF, −78 °C; (d) DBU, CH_2Cl_2, 0 °C to room temp.

Scheme 2. Synthesis of FAP Inhibitors **17**−**22**, **24**, **27**, and **30**[a]

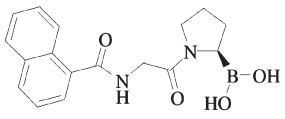


[a] Reagents: (a) H_2, Pd/C, MeOH; (b) EDC,HOBt, CH_2Cl_2/1,4-dioxane, various amines; (c) $POCl_3$, imidazole, pyridine; (d) TFA, CH_3CN, 0 °C to room temp; (e) BCl_3.

procedures with some modifications.[9,16] (2*S*)-Cyanopyrrolidine analogues **17**−**19**, **24**, **27**, **30**, and **31** were prepared as described in Schemes 1 and 2 and are listed in Tables 2 and 3. The synthesis (Scheme 1) proceeded in good yield following the procedure of Young and co-workers to afford the fully protected pyroglutamate **13**.[32] The stereoselective alkylation using

moacetate **14** gave compound **15** in 70% yield with a *cis*/*trans* ratio of 3:1. The *cis* isomer **15** was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in methylene chloride for 24 h to give inverted product in a 1:3 ratio and high yield. The assignment of the *cis*/*trans* configuration was based on the results reported in the literature.[33,34] By use of the building block of C(4)-

Table 2. Inhibition of FAP, DPP-IV, DPP8, DPP9, and DPP-II by Compounds **9**, **10**, **17**, and **31**



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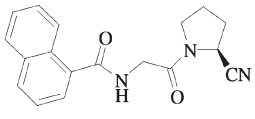

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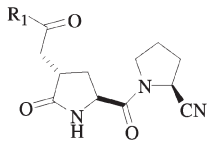
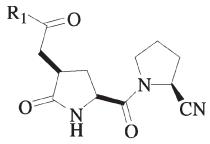


17



31

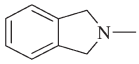
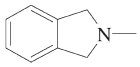
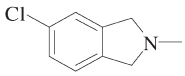
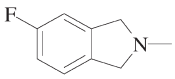
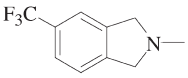
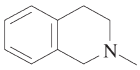
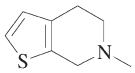
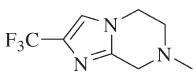
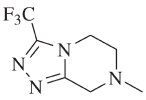
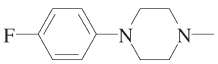
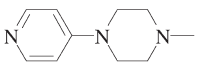
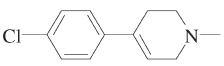
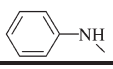
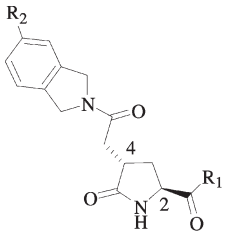
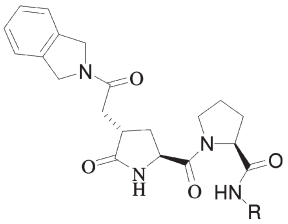
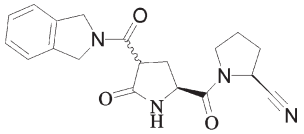
Compd	R_1	IC_{50} (μM)[a]				
		FAP	DPP-IV	DPP8	DPP9	DPP-II
9		0.019	>20	3.7	2.7	>100
10		0.61	>100	>100	>100	>100
17a		0.079	>100	>100	>50	>100
31		0.21	>100	>100	>100	>100
17b		0.066	>100	>100	>50	>100
17c		0.059	>100	>100	>100	>100
17d		0.11	>100	>100	>50	>100
17e		0.088	>100	>100	>100	>100
17f		0.25	>100	>100	>20	>100
17g		0.65	>100	>100	>20	>100
17h		15	>100	>100	>20	>100
17i		0.51	>100	>100	>100	>100
17j		3.2	>100	>100	>100	>100
17k		0.20	>100	>100	>100	>100
17l		0.073	>100	>100	>100	>100
17m		0.73	>100	>100	>100	>100
17n		>20	>100	>100	>100	>100

Table 3. Inhibition of FAP, DPP-IV, DPP8, DPP9, and DPP-II by Compounds **17**−**20**, **24**, **27**, **30**, and **32**

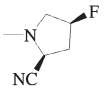
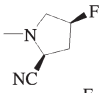
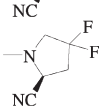
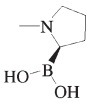




20 R = *t*-butyl
32 R = H



30

Compd	R_1	R_2	IC_{50} (μM)[a]				
			FAP	DPP-IV	DPP8	DPP9	DPP-II
17a		H	0.079	>100	>100	>50	>100
18a		H	0.020	>100	>100	>50	>100
18b		Cl	0.063	>100	>100	>100	>100
19		H	0.022	>100	>100	>100	>100
24a		H	>50	>100	>100	>100	>100
24b		H	>100	>100	>100	>100	>100
27		H	0.031	0.57	0.50	0.52	0.40
20			>100	>100	>100	>100	>100
32			>100	>100	>100	>100	>100
30			>20	>100	>100	>50	>100

[a] Mean values of at least three experiments; standard deviations are ±20%.

pyroglutamic acid-based FAP inhibitors were synthesized. As shown in Scheme 2, the fully protected pyroglutamate *trans*-**15** was deprotected by standard hydrogenation condition and was 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-coupled with pyrrolidine derivatives to give *tert*-butyl esters **23** and **25**[17] or followed by dehydration of the amides to give **16**.[9,35] Removal of the *tert*-butyl protecting group of **16**, **23**, and **25** with trifluoroacetic acid followed by EDC coupling with primary or secondary amines provided the desired (2*S*)-cyanopyrrolidine analogues **17**−**19**, thiazolidine **24**, and boronic ester **26**, respectively. The free boronic acid **27** was obtained through acid catalyzed transesterification of boronic ester **26** with boron trichloride.[17] The synthesis of *cis*-**31** from *cis*-**15** was identical to that of *trans*-**17** from *trans*-**15**. The proposed mechanism for the formation of byproducts **20**−**22** is that the nitrile group of **17**−**19** trapped the *tert*-butyl carbocation followed by hydrolysis to produce amide compounds **20**−**22**, respectively. Therefore, the search for scavengers of alkylating agents is required. When the thioanisole ability to suppress the formation of **20**−**22** was not effective. The more promising result was observed when acetonitrile (CH_3CN) was applied as cosolvent with trifluoroacetic acid (TFA), and thus, the nitrile group of CH_3CN participated in the reaction by acting as acceptor of the leaving *tert*-butyl cation. As expected, this approach slashed the yield of **20**−**22** and produced another byproduct *N*-*tert*-butylacetamide (M_w = 115, detected by LC−MS). Compound **30** was prepared in five steps from the known building block **28**, which was synthesized according to literature report (*cis* or *trans* configuration is not assigned).[36]

Results and Discussion

To establish an optimized P2 site for FAP inhibition, C(4)-substituted pyroglutamic acid based inhibitors with various amines were explored, including bicyclic ring system (**17a**−**h**), piperazine ring system (**17i**,**j**), monocyclic system (**17l**,**m**), and phenylamine (**17n**). These derivatives described above were tested for inhibition of FAP, DPP-IV, DPP8, DPP9, and DPP-II, and

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