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Synthesis and enzymatic evaluation of novel partially fluorinated thiol dual ACE/NEP inhibitors

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ABSTRACT

A novel family of peptidomimetics incorporating fluoroalkyl groups, mainly a trifluoromethyl, in α-position to a zinc(II)-binding thiol function, was synthesized in solution as well as in solid-phase. These compounds showed inhibitory potency in the nanomolar range against both angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP), whereas no inhibition of endothelin-converting enzyme-1 (ECE-1) was observed. The trifluoromethyl-derivatives were more potent than the parent unfluorinated analogues in the case of ACE, and less potent in the case of NEP.

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Angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin-converting enzyme-1 (ECE-1) are zinc metallopeptidases located on the outer membrane of various cell types (ectoenzymes). They have an important role in the metabolism of a number of regulatory peptides of human nervous, cardiovascular, inflammatory and immune system. ACE is a dipeptidyl carboxypeptidase which converts angiotensin I into angiotensin II and degrades kinins, while NEP catalyses the degradation of a variety of renal and CNS-active peptides including substance P, bradykinin, enkephalins, atrial natriuresis.[1a]

ECE-1 cleaves its biologically inactive native substrate, big endothelin-1, to generate a powerful vasoactive 21-amino acid peptide, endothelin-1.[1b] Inhibition of ACE has been a highly successful program in the pharmaceutical industry resulting in a number of effective antihypertensive drugs.[2] Elegant studies relying on structure-based design have laid a strong foundation for early pioneering efforts in the development of Captopril[3] and its variants.[4] On the basis of these positive results in a range of cardiovascular diseases, including hypertension, diabetes and renal disease, several investigators have started to study the potentially added beneficial effects by blocking other endogenous enzymes involved in peptide activation or degradation. In various experimental models, combined ACE/NEP inhibition led to more potent and synergistic hemodynamic and renal effects than selective inhibitors of the individual enzymes.[5] The synergistic effect of combined ACE and NEP inhibition is based on similar modes of action, including blockade of angiotensin synthesis and simultaneously unmasking and potentiation of the effect of peptides such as ANP, BNP, and bradykinin (by preventing their degradation), in turn inducing vasodilatation and diuresis and improving myocardial function. However, the earliest dual metalloprotease inhibitors had limitations due to low potency, short duration of action, or limited oral bioavailability.

Inhibitory potencies in the nanomolar range for the NEP enzyme were achieved with pseudo-dipeptides and tripeptides designed from the structure of thiorphan, of general formula HS–CH(R^1)–CH(R^2)–CONH–CH(R^3)–COOH and HS–CH(R^1)–CH(R^2)–CONH–CH(R^3)–CONH–CH(R^4)–COOH, respectively,[6] interacting with S1′ and S2′ subsites of the enzymes and bearing a thiol group as zinc-chelating moiety. Additional interactions with the S1 binding subsite probably account for the subnanomolar inhibitory potencies for the ACE enzyme, as evidenced by the construction of a hypothetical model of the ACE and NEP active sites (Fig. 1).[7]

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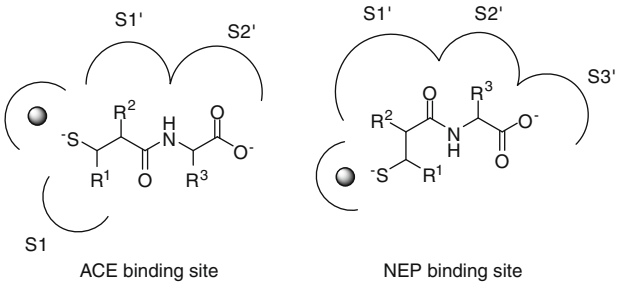


Figure 1. Cartoon outlining the ACE/NEP binding sites.

Selective incorporation of one or more fluorine atoms in specific points of an organic molecule can be an effective strategy for improving and modifying its biological activity, for example by inducing a local modification of charge or polarity or conformation, which in turn can have a profound impact in terms of binding to the receptor site. Alternatively introduction of fluorine can be used to increase the metabolic stability of a bioactive molecule, for example by preventing oxidative or hydrolytic metabolism.[8] In particular, the trifluoromethyl group is recognized in medicinal chemistry as a substituent of distinctive qualities. It is, in fact, at the same time highly hydrophobic, electron-withdrawing (EW), and rather sterically demanding, moreover it can provide high in vivo stability, and can behave as a good mimic of several naturally occurring residues such as ethyl, isopropyl, phenyl, etc.[9] For example, we previously reported that the trifluoromethyl group can replace and mimic an isobutyl group in peptidomimetic inhibitors of proteases.[9e]

Within the framework of a project aimed at the investigation of the effect of fluoroalkyl groups on the binding process to metalloproteases,[10] we designed novel thiol-containing pseudodi- and tripeptides analogues **3a–e** and **4**, respectively, in which the isobutyl substituent of known inhibitors **1** and **2** is replaced by a fluoroalkyl group (Fig. 2).

This was done with the hope of (1) better understanding the importance of the S1 pocket in the active site of both peptidases, (2) increasing both the ACE and NEP affinity of such inhibitors as compared to the known inhibitors **1** and **2**, thus generating new hit compounds for further development by introducing suitable substituents in P1′ position, (3) deeply modifying the physicochemical properties of the inhibitors, such as local hydrophobicity, acidity and nucleophilicity of the neighboring thiol group, preferred conformation (inducing conformational constrictions due to the sterically demanding fluoroalkyl groups) and (4) provide the basis for a spectroscopic study of the binding process through ^{19}F NMR.[11]

For the synthesis of the target compounds, we decided to exploit the high Michael-type reactivity toward nucleophiles of C=C bonds activated by an EW fluoroalkyl group in β position.[12] Thus, trifluorocrotonic acid **6a** and *trans*-β-pentafluoroethyl acrylic acid **6b** (Scheme 1) were prepared as described in the literature.[13] The intermediate fluorocrotonoyl chlorides were obtained in good yields by distillation of the refluxing mixture of the acids **6a**,**b** and phthaloyl dichloride, and subsequently condensed with different α-aminoacid methylesters, such as H-Phe-OMe, H-*tert*-BuO-Tyr-OMe, H-4-F-Phe-OMe, and H-Trp-OMe affording the Michael acceptors **7a–e**, respectively.

The Michael addition of thiolacetic acid, which is a rather poor nucleophile, to **7a–e** afforded pseudo-dipeptides **8a–e** (Scheme 1) as equimolecular mixtures of the two diastereoisomers in very good yields (70–89%) when performed in polar solvents such as methanol and when the fluoroalkyl substituent is the highly EW trifluoromethyl group. The yield dropped (33%) when the less EW, and thus less activating, pentafluoroethyl group was present instead. Before the final step, derivative **8b** (R^1 = 4-*tert*-BuO–Ph–CH_2–) was treated with TFA in DCM in order to deprotect the phenolic hydroxy group. Finally, hydrolysis of both the ester and thioester functions was accomplished in quantitative yields with a degassed 2 N methanolic solution of NaOH, in order to avoid air oxidation to the corresponding disulfides.

To synthesize the pseudo-tripeptide **4** (Scheme 2), the acylchloride derivative of **6a** was coupled with H-Phe-O*tert*-Bu affording the Michael acceptor **9** in good yield (Scheme 2). The *tert*-butyl ester was then hydrolyzed (TFA/DCM), and the resulting carboxylic acid was coupled with H-(4-*tert*-Bu)Tyr-OMe using EDC/HOBt providing the Michael acceptor **10**.

Michael addition of thiolacetic acid in MeOH led to the formation of the adduct **11** in very good yield, as an equimolecular mixture of two diastereoisomers. The protected compound **11** was then converted into the target pseudo-tripeptide **4** by cleavage of the *tert*-butyl protecting group followed by hydrolysis of methyl- and thiolesters under standard conditions (see above).

In order to skip the chromatographic purification of the intermediates, we decided to apply the same synthetic strategy also in solid phase. The free aminic function of resin-bound phenylalanine **12** (Scheme 3) was coupled with a slight excess of acylchloride derivative of **6a** leading to the solid-supported Michael acceptor **13**. The latter was reacted with thiolacetic acid in the presence of TEA in EtOH for three days, affording the Michael adduct **14**. Both steps could be efficiently monitored by FT-IR spectroscopy following the appearance of the typical bands belonging to the amide and thioester carbonyls, respectively. Finally, by treating resin **14** with a degassed 2 N methanolic solution of NaOH we obtained, after filtration, acidification and extraction with organic

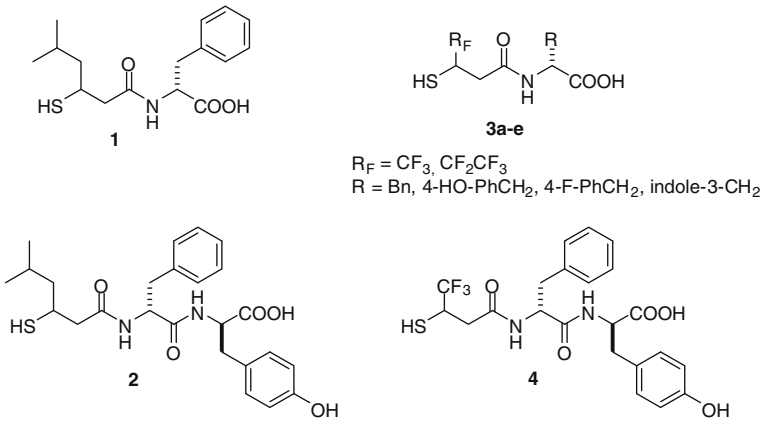
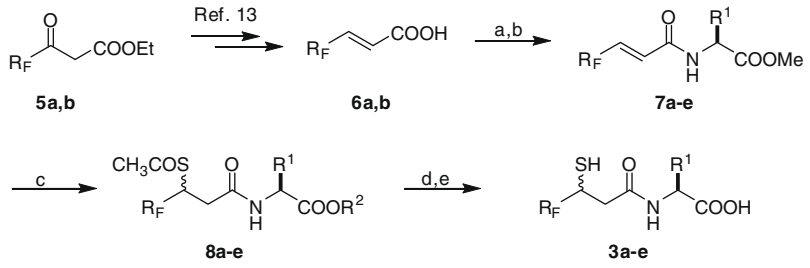
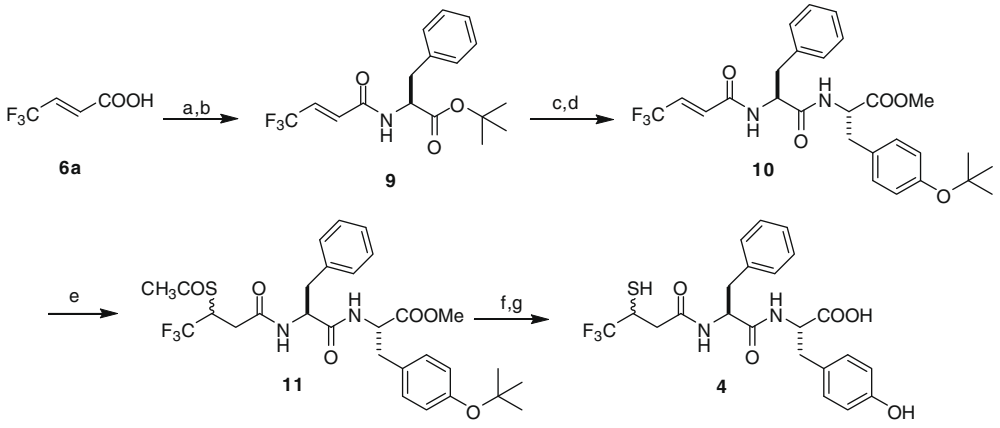


Figure 2. Fluorinated analogues of dual ACE/NEP inhibitors.



Scheme 1. Typical synthesis of fluorinated inhibitors. Reagents and conditions: (a) phthaloyl dichloride, 180 °C, (70–77%); (b) H-AA-OMe, TEA, DCM, 0 °C (73–96%); (c) AcSH, EtOH, rt (rd = 1:1, 30–89%); (d) for R^1 = 4-*tert*-BuO–Ph–CH_2–, 20% TFA, DCM, rt, 1 h (98%); (e) degassed 2 N NaOH in MeOH, 0 °C, 1 h (quantitative).



Scheme 2. Synthesis of pseudo-tripeptide **4**. Reagents and conditions: (a) phtaloyl dichloride, 180 °C, (77%); (b) H-Phe-O*tert*-Bu, TEA, DCM, 0 °C (83%); (c) 20% TFA, DCM, rt, 1 h (98%); (d) H-(4-*tert*-Bu)Tyr-OMe, HOBt, EDC, TMP, DCM, rt, 3 h (77%); (e) AcSH, EtOH, rt (r.d. = 1:1, 75%); (f) 20% TFA, DCM, rt, 1 h (98%); (g) degassed 2 N NaOH in MeOH, 0 °C, 1 h (quantitative).

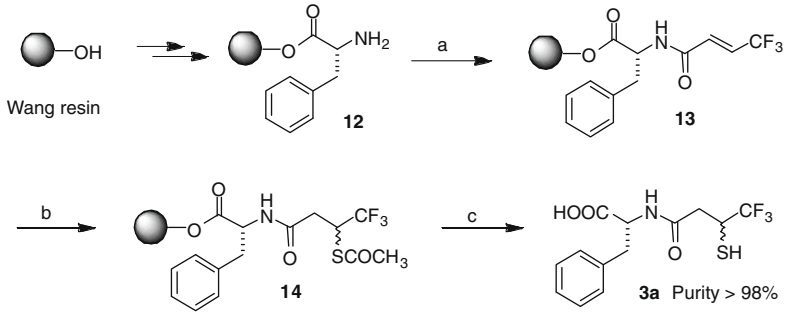
solvent, the final pseudo-dipeptide **3a** in high purity (>98%) without any further purification.

All the partially fluorinated pseudo-peptides above were assayed for their capacity to inhibit ACE, NEP, and ECE (Table 1).[14] The enzyme inhibitory assays were carried out according to the general procedures reported in the literature,[6b] and validated through the IC_{50} determination of the following reference inhibitor compounds: thiorphan, captopril, and phosphoramidon, for ACE, NEP, and ECE, respectively.[15,1b] Moreover, an IC_{50} value of 41 nM was extrapolated from the experimental curve obtained with compound **1** synthesized in our laboratories. This result was equivalent to that previously reported by Gomez-Monterrey et al. of 37 nM.[6a]

All the compounds showed inhibitory activity in the nanomolar range towards both NEP and ACE. In contrast, no inhibitory activity was evidenced on ECE-1. Among the assayed compounds, **3b**, bearing a Tyrosine terminal group, was the most potent, showing an inhibitory activity towards NEP similar to those of the known reference compounds **1** and **2**.

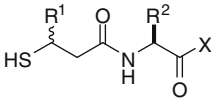
In general, replacement of the isobutyl group with a trifluoromethyl group as the R^1 substituent (see Fig. 1) produced a significant increase of the ACE inhibitor activity of the thiol compounds **3,4**, as compared with the previously reported data on the non-fluorinated analogues, such as **1,2**.[6a] This effect is flanked by a clear-cut reduction of selectivity, because these CF_3-compounds showed also a slight decrease of potency towards NEP. Replacement of trifluoromethyl by pentafluoroethyl group (as in **3e**) brought about a reduction of the inhibitory capacity towards both NEP and ACE.

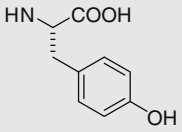
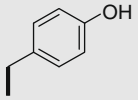
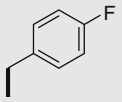
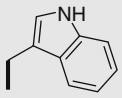
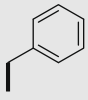
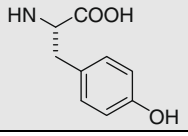
In conclusion, considering the ACE/NEP dual nanomolar inhibitory activity of these novel trifluoromethyl substituted peptidomimetics, these results should pave the way for further biological



Scheme 3. Solid phase synthesis of **3a**. Reagents and conditions: (a) *trans*-CF_3–C=C–COCl (1.5 equiv), TMP, DCM, rt, 3 h; (b) AcSH, EtOH, rt, 3 days; (c) degassed 2 N NaOH in MeOH, 0 °C, 1 h.

Table 1
IC_{50} values for compounds **1**, **2**, **3a–e**, and **4**



Compounds	R^1	R^2	X	IC_{50} (nM)		
				ACE	NEP	ECE-1
1			OH	>10,000 (>10,000)[a]	41 (37)[a]	>10,000
2[a]				2800	50	n.d.[b]
3a	CF_3		OH	830	300	>10,000
3b	CF_3		OH	160	80	>10,000
3c	CF_3		OH	300	230	>10,000
3d	CF_3		OH	400	580	>10,000
3e	CF_2CF_3		OH	1010	1150	2420
4	CF_3			280	430	>10,000

[a] IC_{50} values taken from the ref. Gomez-Monterrey et al.[6a]
[b] Not determined.

and pharmaceutical investigations on possible applications of these derivatives, that is, for the treatment of congestive heart failure and chronic hypertension.[16–18] Furthermore, these results confirm that a trifluoromethyl group can be used as a replacement of an isobutyl group in protease inhibitors, without affecting the affinity for the active site, but producing a significant overall physico-chemical and electronic perturbation due to the strong electronic difference (EW character, polarity, charge, etc.) between these two groups.[9e] We are currently investigating structural and electronic factors that are responsible for the influence of different fluoroalkyl groups, particularly the trifluoromethyl, on the inhibitory activity and binding affinity of these novel fluorinated metalloprotease inhibitors.

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Supplementary data

Supplementary data (experimental procedures and ^{1}H NMR for the final compuonds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.064.

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14. All the enzyme inhibitory assays were carried out with a Victor3 Fluorometer (PerkinElmer) according to the general procedures reported in Ref. 6b. *ACE Activity***:** Human recombinant Angiotensin-converting Enzyme-1 (ACE) was purchased from Calbiochem (Darmstadt, Germany), specific fluorescent substrate o-aminobenzoic acid-Phe-Arg-Lys(2,4-dinitrophenyl)-Pro-OH [Abz-FRK(Dnp)-P] was obtained from Biomol (PA, USA). A concentration of Abz-FRK(Dnp)-P of 10 μM (K_m determined value) was adopted and the fluorescence was measured at λ_{ex} = 320 nm and λ_{em} = 420 nm. *NEP Activity:* The assay was carried out with commercial purified NEP from porcine kidney (Calbiochem, Germany), specific fluorescent substrate *N*-Dansyl-D-Ala-Gly-p-nitro-Phe-Gly (DAGNPG) purchased from Sigma–Aldrich (St. Louis, MO). The assays were performed at λ_{ex} = 340 nm and λ_{em} = 530 nm using a DAGNPG concentration of 58 μM (Km determined value). *ECE-1 Activity:* Human recombinant ECE-1 and fluorescent specific substrate 7-methoxycoumarin-4-acetyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(2,4-dinitrophenyl)-OH [Mca-RPPGFSAFK(Dnp)-OH] was purchased from R&D Systems (Minneapolis, MN). The assays were carried out at λ_{ex} = 340 nm and λ_{em} = 405 nm with a Mca-RPPGFSAFK(Dnp)-OH concentration of 16 μM (K_m determined value).
15. The IC_{50} values of the reference compounds we obtained were tiorphan IC_{50} of 6.2 nM versus NEP, captopril IC_{50} of 0.12 nM versus ACE and phosphoramidon IC_{50} of 17.0 nM versus ECE. All the determined values were in good agreement with previously reported data: (a) Roques, B. P.; Fournié-Zaluski, M. C.; Soroca, E.; Lecomte, J. M.; Malfroy, B.; Llorens, C.; Schwartz, J. C. *Nature* **1980**, *288*, 286; (b) Binevski, P. V.; Sizova, E. A.; Pozdnev, V. F.; Kost, O. A. *FEBS Lett.* **2003**, *550*, 84.
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