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Fluorinated peptidomimetics: synthesis, conformational and biological features

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Abstract

Peptides modified with fluoroalkyl functions in key backbone positions have been scarcely studied so far. Thus, little is known about their synthesis, their structural and physico-chemical properties, and their biological features. Our interest in this field of research led to the development of stereocontrolled synthetic protocols, both in solution and in solid phase, for many different fluoroalkyl peptidomimetics, some of which are overviewed in this paper: (a) ψ[CH(CF_3)NH]-peptide mimics holding a great potential as hybrids between natural peptides and hydrolytic transition state analogs; (b) trifluoromethyl (Tfm) malic peptidomimetics as micromolar inhibitors of some matrix metalloproteinases; (c) bis-Tfm analogs of Pepstatin A, that are nanomolar and selective inhibitors of the protozoal aspartyl protease Plasmepsin II.

Keywords: Trifluoromethyl; Peptidomimetics; Matrix metalloproteinases; Plasmepsins

1. Introduction

Fluoroorganic substances have found a number of uses in modern life, for many of which they are unrivalled [1]. In medicine, fluorocarbons are used as inhalation anesthetics, aerosol propellants, vascular implants, breathing liquids for damaged or immature lungs, and components in blood substitutes. In biotechnology, fluorocarbon liquids are used to transport respiratory gases in cell culture systems. Fluorine is present in a large and growing number of agrochemicals and pharmaceuticals, typically as a single fluoro or trifluoromethyl substituent on a hydrocarbon-derived molecule [2]. In the realm of peptides, incorporation of tailored fluorinated functions has been used to replace and/or mimic critical peptide bonds [3]. For example, a fluorovinyl group has been demonstrated to be a hydrolytically stable replacement for a peptide bond and a much better electronic mimic than the unfluorinated vinyl function. Another important application consists in the incorporation of a CF_3CO [4], or $COCF_2CO$ function [5] in a key backbone position in order to achieve inhibition of proteolytic enzymes, in particular serine proteases, thanks to the ability of the fluoroalkyl group to stabilize the gem-diolic form, that is able to mimic the hydrolitic transition state. Recently, fluorinated amino acids (trifluoroleucine, trifluorovaline, trifluoromethionine, etc.) have been incorporated into protein structures, resulting in a deep modification of properties such as enhanced thermal and chemical stability, affinity for lipid bilayer membranes, stronger self-association, and so on [6–9]. In another interesting application, 4-fluoroproline has been demonstrated to bring about peculiar conformational features to collagen-like structures, depending on the stereochemistry of the fluorinated stereogenic center [10].

Abbreviations: DCM, dichloromethane; TMP, *sym*-collidine (2,4,6-trimethylpyridine); DCC, dicyclohexylcarbodiimide; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; CAN, ceric ammonium nitrate; GABOB, γ-amino-β-hydroxybutyric acid; PMP, *p*-methoxyphenyl; DABCO, 1,4-diazabicyclo[2,2,2]octane; TFA, trifluoroacetic acid; TEA, triethylamine

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However, very little is known about the synthesis and the properties of backbone-modified peptides with fluoroalkyl functions incorporated in critical positions. With this in mind, about five years ago we undertook a study aimed at a better understanding and rationalization of the 'fluorine-effect' in peptidomimetic structures.

2. Ψ[CH(CF$_3$)NH]Gly-peptides

Recently, within the frame of a broad project aimed at the investigation of the 'fluorine-effect' in peptides, we have described the synthesis and some structural and conformational features of a new class of fluorinated retropeptides, namely partially modified retro (PMR)-ψ[(NHCH(CF$_3$)]Gly-peptides (Fig. 1, R = H), having a [CH(CF$_3$)NH] unit instead of the natural [CONH] peptide bond [11–14]. This unit is a sort of hybrid between a peptide bond mimic and a proteolytic transition state analog, as it combines some of the properties of a peptidyl –CONH– group (low NH basicity, CH(CF$_3$)–NH–CH backbone angle close to 120°, C–CF$_3$ bond substantially isopolar with the C=O) with some others of the tetrahedral intermediate (Fig. 1) involved in the protease-mediated hydrolysis reaction of a peptide bond (high electron density on the trifluoromethyl group, tetrahedral backbone carbon). Moreover, the presence of the bulky CF$_3$ group is probably the driving force for the high stability of turn-like conformations of appropriately configured PMR-ψ[(NHCH(CF$_3$)]Gly-peptides both in low-polarity organic solvents solutions and in the solid state. A significant advancement in the development of backbone-modified peptidomimetics having a [CH(CF$_3$)NH] module as a replacement of a peptide bond [CONH], is represented by ψ[CH(CF$_3$)NH]Gly-peptides **1** (Fig. 1) [15], which are much closer to natural peptides than PMR-ψ[NHCH(CF$_3$)]Gly-peptides. In analogy with the latter compounds, the [CH(CF$_3$)NH] unit is expected to behave as a sort of hybrid between a [CONH] mimic and a proteolytic transition state analog.

The stereocontrolled synthesis of these brand new peptidomimetics is based on a key aza-Michael reaction

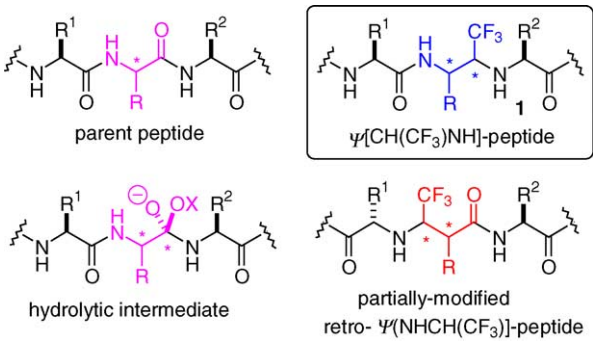
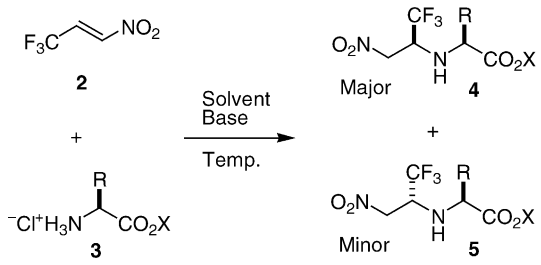


Fig. 1. Structure of natural peptides, PMR-ψ[NHCH(CF$_3$)]Gly-peptides and ψ[CH(CF$_3$)NH]Gly-peptides 1.



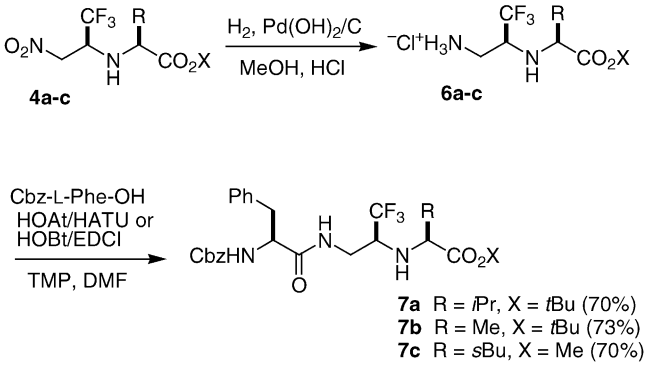
Scheme 1. The aza-Michael reaction to give the ψ[NHCH(CF$_3$)]Gly-peptide backbone.

involving 3,3,3-trifluoro-1-nitropropene **2** (Scheme 1) and an array of α-amino esters, generated in situ from the hydrochlorides **3** with a base. The reactions took place almost instantaneously at rt, affording the diastereomeric α′-Tfm-β′-nitro α-amino esters **4** (major) and **5** (minor) under kinetic control.

The diastereoselectivity of the process was studied in detail. We found that it depends mainly on four reaction parameters: (1) base, (2) solvent, (3) stoichiometry of the base, and (4) R side chain of **3**. Concerning the base, the best stereocontrol (63% d.e. using L-Val esters as nucleophiles) was achieved with DIPEA, whereas NaHCO$_3$, TMP and DABCO gave modest results. Low-polarity solvents provided remarkably higher diastereocontrol. Thus, toluene afforded 84% d.e., whereas DCM and THF afforded modest d.e.s. Quite surprisingly, intermediate results were observed using a polar CCl$_4$. Even more surprisingly, also the stoichiometry of DIPEA was found to have a profound effect on the stereocontrol. The optimum amount was found to be 1.1 equivalent (as used in the experiments cited above). In the absence of free DIPEA the d.e. dropped dramatically. Accordingly, a progressive decrease of stereoselectivity was observed by increasing the amount of DIPEA from 1.1 to 1.7 equivalent, whereas little variation occurred beyond this quantity. Other bases, such as TMP and NaHCO$_3$, did not feature the same 'stoichiometry-effect' affording comparable d.e.s upon changing the number of equivalents used. The effect of the R side chain of **3** was in line with the expectations. In fact, the highest d.e.s were observed with bulky R groups (*iso*-Pr, *sec*-Bu) whereas lower degrees of stereocontrol were observed with R = Me, Bn, etc.

Room temperature was found to be essential in order to achieve high yields of **4** and **5**, whereas at lower temperatures (for example −40 or −70 °C) very complex mixtures of products were obtained. All the experimental evidences above suggest that these aza-Michael reactions occur through a tight, polar, termolecular transition state (TS), involving **2**, **3** and DIPEA, which appears to play a fundamental catalytic role. Polar solvents, as well as the presence of more than one molecule of DIPEA, may disrupt these TS, thus lowering the stereocontrol.

Elaboration of the major adducts **4a–c** into the target ψ[CH(CF$_3$)NH]Gly-peptides **7a–c** is shown in Scheme 2. The nitro group of **4a–c** was hydrogenated to amino group



Scheme 2. Elaboration of the aza-Michael adducts **4** into ψ[CH(CF_3)NH]Gly-peptides **7**.

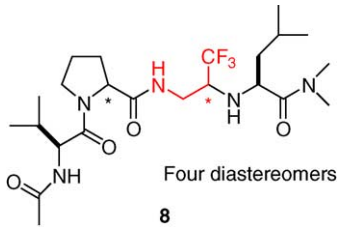


Fig. 2. Complex model ψ[CH(CF_3)NH]Gly-tetrapeptides.

using the Pearlman's catalyst, and the resulting diamino compounds were trapped as hydrochlorides **6a–c** and submitted without purification to coupling with Cbz-L-Phe-OH affording the ψ[CH(CF_3)NH]Gly-tripeptides **7a–c** in good overall yields.

In order to proof the value of this synthetic methodology in the preparation of more complex ψ[CH(CF_3)NH]Gly-peptides, we have recently completed the synthesis of all of the four diastereomers of tetrapeptide **8** (Fig. 2) [16]. The latter is an interesting model for conformational studies, because some of the analogs incorporating a natural Gly have been shown to assume very stable and highly populated β-hairpin conformations, depending on the configuration of the Pro residue [17]. A detailed study of the conformational features of the stereoisomers **8** is currently in progress.

3. Trifluoro-analogs of peptidomimetic metalloproteinase inhibitors

Matrix metalloproteinases (MMPs) are zinc (II)-dependent proteolytic enzymes involved in the degradation of the extracellular matrix [18–21]. More than 25 human MMPs have been identified so far. Loss in the regulation of their activity can result in the pathological destruction of connective tissue, a process associated with a number of severe diseases, such as cancer and arthritis. The inhibition of various MMPs has been envisaged as a strategy for the therapeutic intervention against such pathologies. To date, however, a number of drawbacks have hampered the

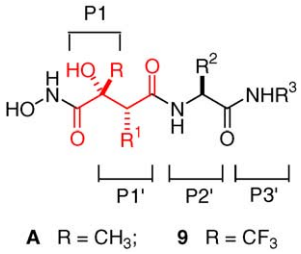


Fig. 3. The DuPont Merck's MMP inhibitors (**A**) and their CF_3-analogs (**9**).

successful exploitation of MMPs as pharmacological targets. In particular, the toxicity demonstrated by many MMPs' inhibitors in clinical trials has been ascribed to nonspecific inhibition.

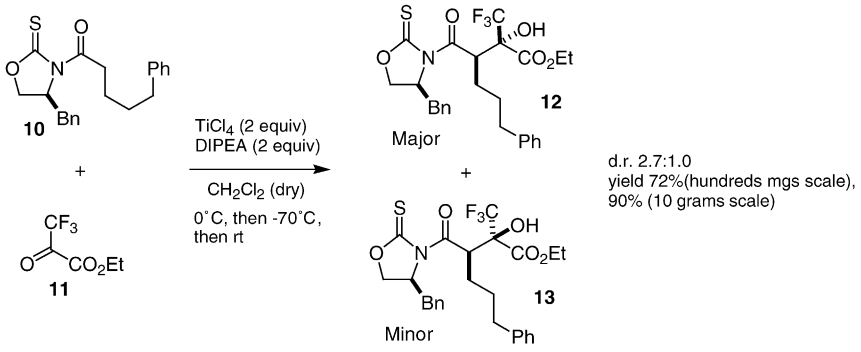
Recently, Jacobson and coworkers described a new family of potent peptidomimetic hydroxamate inhibitors **A** (Fig. 3) of MMP-1, -3 and -9, bearing a quaternary α-methyl-alcoholic moiety at P1 position, and several different R^1 groups at P1′ [22]. Interestingly, the other stereoisomers, including the epimers at the quaternary carbinol function, showed much lower activity, as the authors demonstrated that the hydroxamic binding function was moved away from the catalytic Zn^{2+} center.

We hypothesized that incorporation of fluoroalkyl substituents on the P1 quaternary position of the inhibitors **A** could be an effective strategy for optimising and tuning their binding properties, thus increasing the selectivity toward the different MMPs. For this reason we accomplished the synthesis of the Tfm-analogs **9** (Fig. 3) of **A**, and the effect of the replacement of the α-CH_3 group with a CF_3 on the inhibition of MMP-9 [23].

We decided to concentrate our efforts on the substrates **9** having $R^1 = (CH_2)_3Ph$, since their analogs **A** were reported to be very active. First attempts to synthesize the α-Tfm-malic unit of **9** via titanium (IV) catalyzed aldol reaction of trifluoropyruvic esters with enantiopure *N*-acyl oxazolidin-2-ones gave disappointing results [24]. Although this reaction was per se satisfactory (61–87% yields, d.r. up to 8:1 depending on the *N*-acyl group), the subsequent exocyclic cleavage of the oxazolidin-2-one auxiliary could not be performed, despite intensive efforts. We therefore turned our attention to oxazolidin-2-thiones [25], whose cleavage was reported to occur much more smoothly [26].

The $TiCl_4$ catalyzed reaction of the *N*-acyl-oxazolidin-2-thione **10** (Scheme 3) with ethyl trifluoropyruvate **11** afforded the two diastereomeric adducts **12** and **13**, out of four possible, in low diastereomeric ratio. The reaction featured a favourable scale-up effect, affording ca. 70% yield on a 100-mg scale, and 90% on a 10-g scale. A number of alternative conditions were explored, but neither significant improvement nor switch of the diastereocontrol could be achieved.

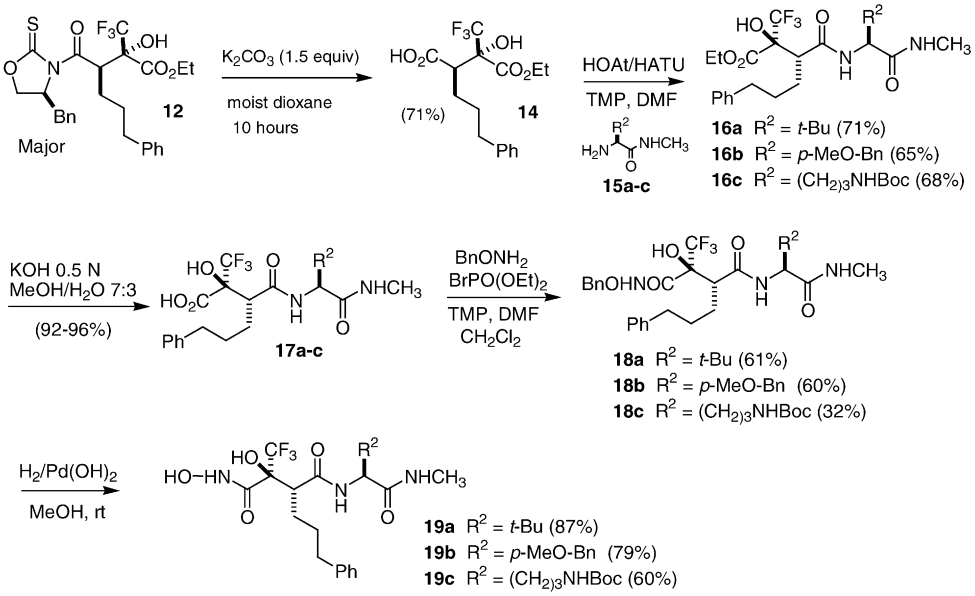
The synthesis of the major diastereomers **19a–c** (Scheme 4) was developed first. Under the standard conditions reported in the literature (BnOH, catalytic DMAP, DCM, rt)



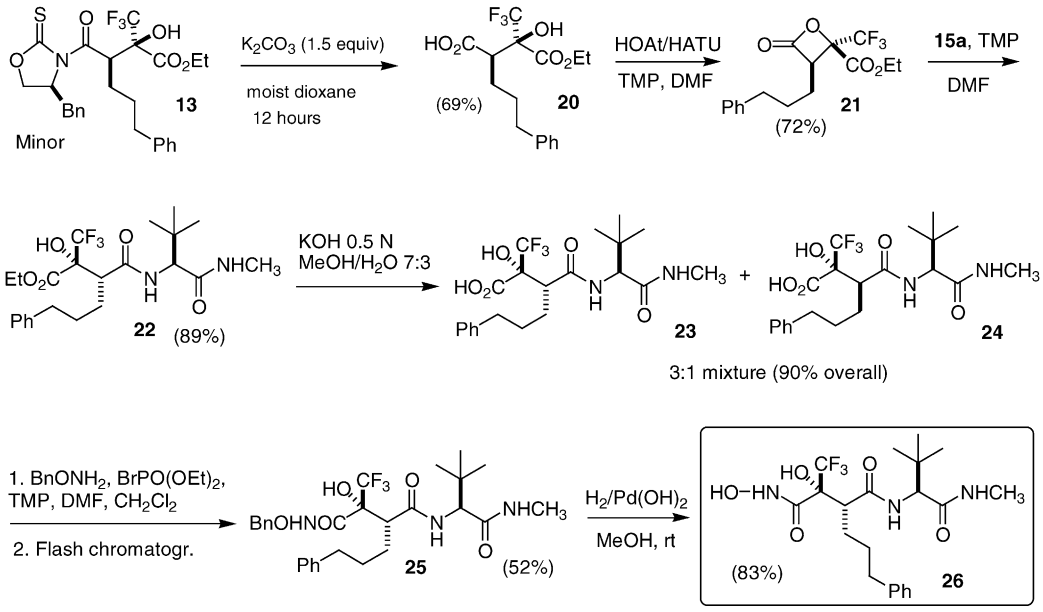
Scheme 3. Stereocontrolled synthesis of the Tfm-malic framework.

the exocyclic cleavage of the oxazolidinethione on **12** was very slow, affording modest conversion to the corresponding Bn-ester and partial epimerization of the secondary stereocenter. However, we found that solid K_2CO_3 in moist dioxane (rt, 10–12 h), was able to produce directly the key carboxylic acid intermediate **14** in satisfactory yields and with very low α-epimerization (2%). Coupling of the acid **14** with α-amino acid amides **15a–c** was achieved in good yields with the HOAt/HATU system [27]. The resulting peptidomimetic esters **16a–c** were submitted to saponification, affording the acids **17a–c** in high yields. The subsequent coupling of **17a–c** with *O*-Bn hydroxylamine proved to be extremely challenging, owing to the low reactivity and high steric hindrance of the carboxylic group bound to the quaternary α-Tfm carbinolic center. A number of 'conventional' coupling agents for peptides were unsuccessfully tested, but finally we found that freshly prepared $BrPO(OEt)_2$ was able to promote the coupling in reasonable yields (32–61%). With **18a–c** in hand we addressed the final *O*-Bn cleavage by hydrogenolysis that provided the targeted hydroxamates **19a–c** in good yields.

Since **19a–c** are the 'wrong' diastereomers with respect to **A**, we deemed necessary to synthesize at least one analog having the correct stereochemistry, in order to have a complete set of biological data on the effect of the introduction of the Tfm group. However, a tailored synthetic protocol had to be developed ex novo, because the minor diastereomer **13** (Scheme 5) featured a dramatically different reactivity in the key steps of the synthesis. Since we noticed that the coupling of **13** and **15a** with HATU/HOAt gave rise to relevant amounts of the β-lactone **21** (which had to be processed separately) besides the expected coupling product **22**, we decided to prepare first the intermediate **21**. The latter was reacted with free **15a**, affording the desired molecule **22** in high yields. Basic hydrolysis of the ester **22** occurred effectively, although a partial epimerization of the $[Ph(CH_2)_3]$-stereocenter occurred, affording a 3:1 mixture of diastereomers **23** and



Scheme 4. Total synthesis of the hydroxamates derived from the major diastereomer.



Scheme 5. Total synthesis of the hydroxamate derived from the minor diastereomer.

24 under optimized conditions, that were subjected together to coupling with $BnONH_2$. The resulting diastereomeric *O*-Bn hydroxamates could be separated by FC, affording pure **25**, that was hydrogenated to the target free hydroxamate **26**.

The hydroxamates **19a–c** and **26** were tested for their ability to inhibit MMP-2, MMP-3 and MMP-9 activity using zymographic analysis. The IC_{50} values (μM) portrayed in Table 1 show that diastereomers **19a–c** displayed low inhibitory activity, in line with the parent CH_3-compounds. Disappointingly, **26** showed a much lower activity than the exact CH_3-analog **A** that was reported to be a low nanomolar inhibitor of MMP-3 and -9 (K_i = 13 nM toward MMP-3 and <1 nM toward MMP-9). It is also worth noting that **19a** and **26** showed little selectivity, whereas **19b** and **19c** showed a fairly better affinity for MMP-9, in comparison with MMP-2 and -3. A possible explanation for the drop of activity upon replacement of the quaternary methyl with a CF_3, is that **26**, biased by the bulky CF_3-group, is unable to assume the crucial binding conformation of the CH_3-analog **A**. Alternatively, one can hypothesize that the bulky and highly electron-rich CF_3 group is unable to fit the S1 pocket of the hitherto tested MMPs.

Current work is actively pursuing the synthesis of novel fluorinated analogs of MMPs inhibitors having improved pharmacological properties.

Table 1
IC_{50} (μM) of the target Tfm-hydroxamates

Compound	MMP-2	MMP-3	MMP-9
19a	156	>1000	121
19b	407	>1000	84
19c	722	>1000	23
26	23	43	15

4. Bis-trifluoromethyl Pepstatin A as nanomolar inhibitor of aspartyl proteases

Pepstatin A (Iva-Val-Val-Sta-Ala-Sta) is a subnanomolar inhibitor of many aspartyl proteases, with the notable exceptions of HIV-protease and renin [28]. The two statine units are known to play a key role, particularly the central one occupying the P1 and P1′ portions (Fig. 4). Many structural modifications of Pepstatin A, including the statine isobutyl side chain [29,30], have been investigated, but the effect of incorporation of fluoroalkyl functions had not been described previously.

We therefore decided to undertake the synthesis of bis-Tfm-Pepstatin **27** [31,32]. In 1998 we published the synthesis of enantiomerically pure γ-Tfm GABOB, a statine mimic with the isobutyl side chain replaced by a Tfm group [33]. We deemed interesting such structural modification, because the Tfm group has been often described as sterically very similar to an isopropyl group. However, the volume of the Tfm group is known to be larger, and intermediate between that of isopropyl and isobutyl groups [34]. Thus, we decided to assess whether the Tfm could behave as a substitute and mimic of the isobutyl group, within the frame of peptidomimetic structures.

Lithium sulfoxide **28** (Scheme 6), prepared in situ from (*R*)-*p*-tolyl γ-butenyl sulfoxide, was treated with a THF solution of trifluoro imine **29** at −70 °C. The reaction afforded with overwhelming preference two diastereomeric *N*-PMP β-amino sulfoxides (2*R*,3*S*,R_S)-**30** and (2*S*,3*R*,R_S)-**31** out of four possible, in 1.0/2.75 d.r. and nearly quantitative overall isolated yields. Attempts to improve the stereocontrol were made, but relatively little changes of diastereoselectivity were generally recorded.

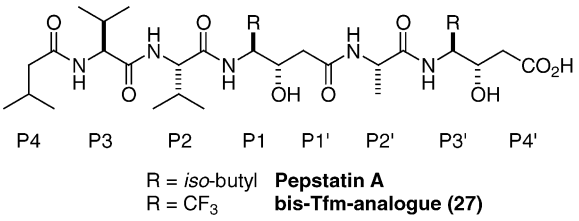
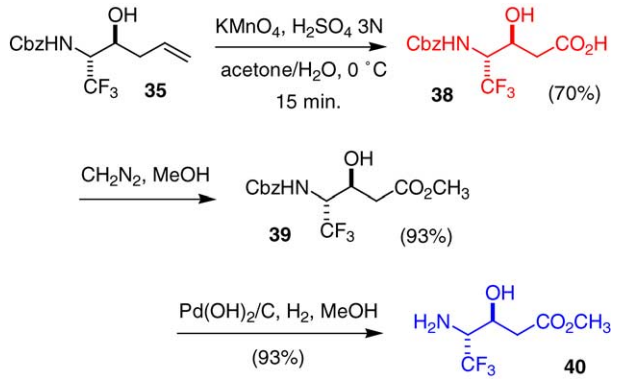


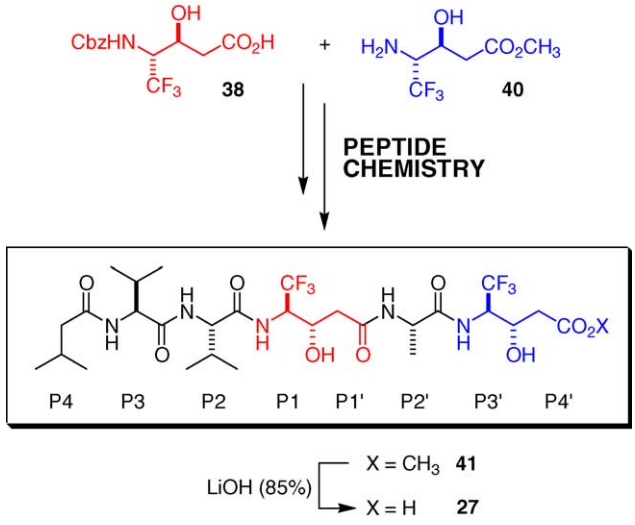
Fig. 4. Pepstatin A and its bis-Tfm-analog.

The major sulfoxide **31** was treated with CAN to cleave the *N*-PMP group, providing the free amino sulfoxide **32**, that was reprotected as *N*-Cbz derivative **33** and finally submitted to the 'non-oxidative' Pummerer reaction (NOPR) [35]. Treatment of **33** with trifluoroacetic anhydride and *sym*-collidine triggered a S_N2-type displacement of the sulfinyl by a trifluoroacetoxy group, leading to the intermediate sulfenamide **34**. One-pot treatment with aqueous K_2CO_3 up to pH 7 and finally with an excess of $NaBH_4$, provided the β-amino alcohol (2*R*,3*S*)-**35** in a very clean manner, with overall stereoselectivity >98/2 (the other diastereomer was not detected). Conversion of (2*R*,3*S*)-**35** into the corresponding *O*-benzoate **36**, and oxidative cleavage of the double bond with $KMnO_4$ delivered the targeted enantiopure γ-Tfm-GABOB (−)-(3*S*,4*R*)-**37**. Attempts to employ the latter compound for the synthesis of the target **27** were unsuccessful. We therefore turned our attention to the synthesis of the orthogonally protected derivatives **38** and **40** (Scheme 7) from **35**. The former was prepared by oxidative cleavage with $KMnO_4$, which occurred with excellent chemoselectivity. Next, the carboxylic acid **38** was treated with diazomethane, then the Cbz group of the resulting ester **39**, was hydrogenolyzed providing **40**.

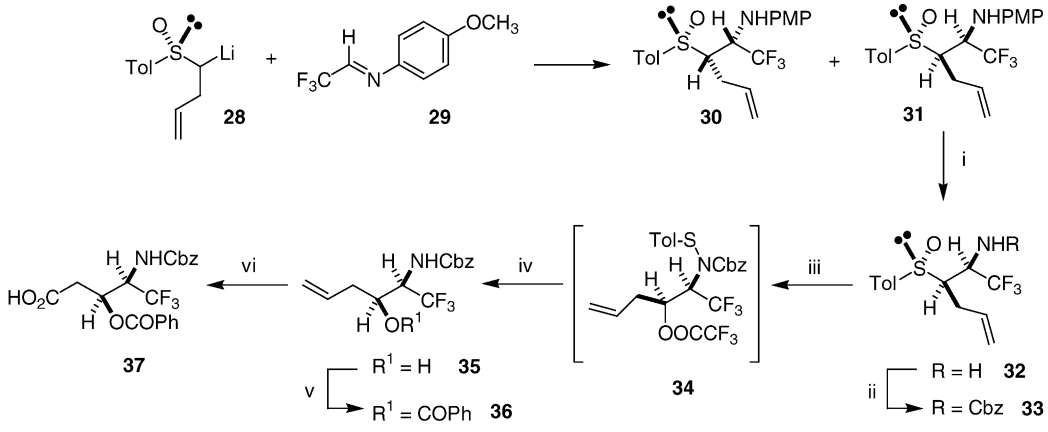
With the orthogonally protected Tfm-statines **38** and **40** in hand, the peptide sequence of the bis-Tfm analog of Pepstatin **27** was assembled (Scheme 8). Following several sequences of coupling, the final step was the hydrolysis of



Scheme 7. Synthesis of orthogonally protected Tfm statines **38** and **40**.



Scheme 8. Final steps of the synthesis of bis-Tfm Pepstatin **27** and its methyl ester **41**.



Scheme 6. Key: (i) CAN, acetonitrile, H_2O (66%). (ii) $ClCO_2Bn$, K_2CO_3 50%, dioxane (>98%). (iii) Trifluorocetic anhydride, *sym*-collidine, acetonitrile. (iv) a. K_2CO_3/H_2O up to pH 7; b. $NaBH_4$, THF/H_2O, 0 °C (94%). (v) $PhCO_2H$, DCC, DMAP (catalyst), CH_2Cl_2 (98%). (vi) $KMnO_4$, $H_2SO_4$3N (89%), acetone/H_2O.

Table 2
Inhibition tests for Pepstatin A and its bis-Tfm-analogs

	IC_{50} (nM)			
	HIV-Protease	Plasmepsin II	Plasmepsin IV	Cathepsin D
Pepstatin A	2500	0.44	0.61	0.64
41	Not determined	2.4	96	2900
27	>1 50 000	1.3	23	120

the methyl ester **41** by means of LiOH, to afford **27** in good overall yield.

Bis-Tfm-Pepstatin **27** and its methyl ester **41** were assayed for their ability to inhibit several aspartyl proteinases, including HIV-protease, Plasmepsins II and IV, and human Cathepsin D (Table 2). Native Pepstatin A is not a good inhibitor of HIV-protease, therefore we were not surprised to find that up to a concentration of 150 μM, compound **27** did not show any inhibition of the proteolytic activity.

Much stronger inhibitory activity was found toward Plasmepsin II, an aspartic protease of *Plasmodium falciparum*, the protozoal that causes the most serious forms of malaria [36]. Plasmepsin II is regarded as a very promising target in malaria therapy, therefore there is a strong interest in effective inhibitors of this proteolytic enzyme [37]. Compound **27** was found to be a low nanomolar inhibitor of Plasmepsin II, having nearly the same potency of Pepstatin A. However, **27** is considerably more selective toward closely related enzymes, such as Plasmepsin IV and, in particular, Cathepsin D. The selectivity toward the latter enzyme is a very attractive feature for a Plasmepsin II inhibitor, because of the toxicity that could arise from a non-selective inhibition of both protozoal Plasmepsin II and human Cathepsin D [38]. Approximatively the same inhibitory potency versus Plasmepsin II was measured for the methyl ester **41**. Interestingly, this compound demonstrated even better selectivity toward Cathepsin D.

In order to gain a deeper insight into the mechanism of action of bis-Tfm-Pepstatins **27** and **41**, and assess whether the Tfm group was actually a mimic of the iso-butyl side-chains of Pepstatin A, we undertook a collaboration with the laboratory of protein crystallography of the pharmaceutical company Actelion (Allschwil, Switzerland), in order to obtain the crystal structure of **27** and **41** complexed with Plasmepsin II (see Fig. 5).

Both structures were successfully solved at a resolution of 2.4 and 2.8 Å, respectively. In addition, the complex Pepstatin A/Plasmepsin II was solved at a 1.7 Å resolution [39]. It is apparent that the Plasmepsin II backbones in the three complexes are very similar, showing a close proximity in the binding modes to the three inhibitors. A closer view of

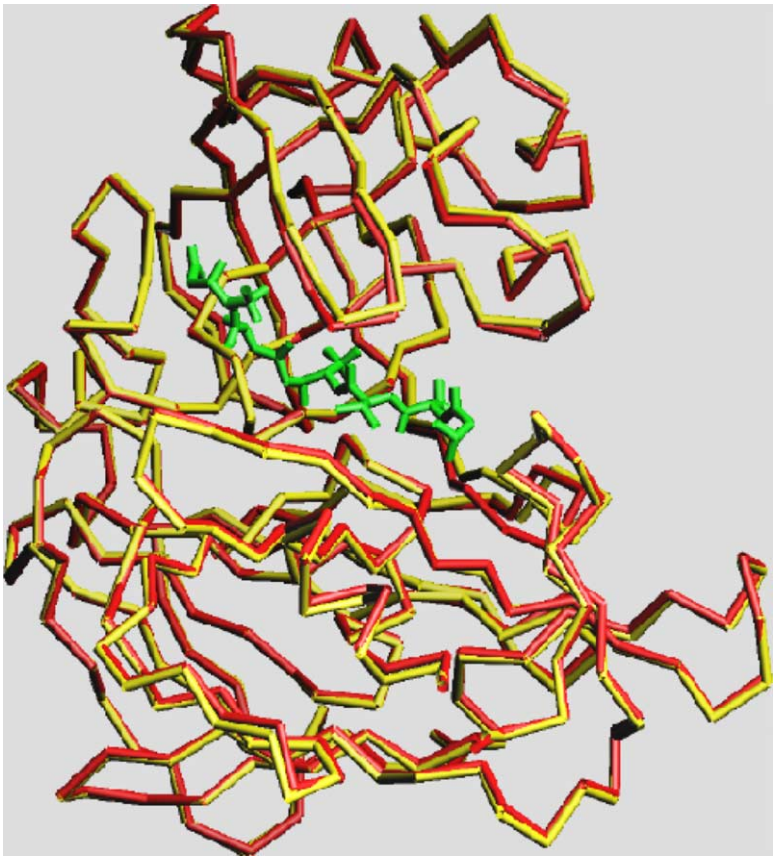


Fig. 5. Inhibitor **27** bound to the active site of Plasmepsin II. Key: green, fluorinated inhibitor **27**; red, backbone of Plasmepsin II complexed with Pepstatin A; yellow, backbone of Plasmepsin II complexed with **41**.

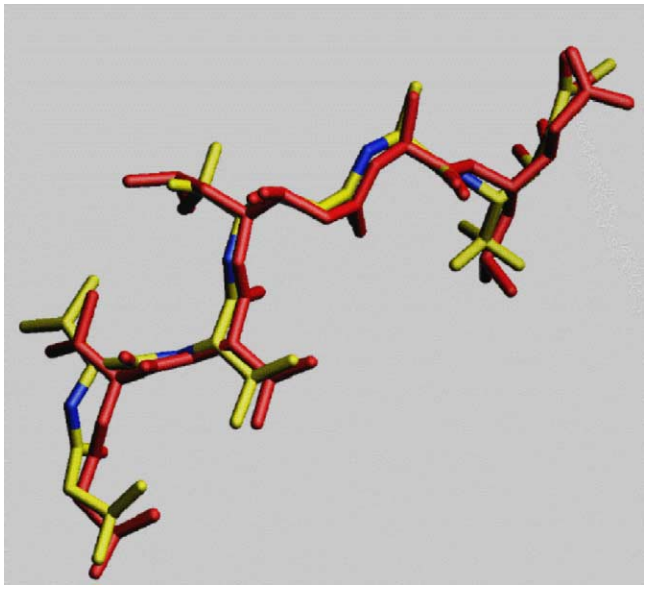


Fig. 6. Conformations of Pepstatin A (red) and fluorinated analog **27** (yellow/blue) in Plasmepsin II active site.

Pepstatin A (red) (Fig. 6) and bis-Tfm-Pepstatin **27** in the Plasmepsin II binding site also shows a surprising almost identical conformation, with **27** that adopts a sort of backbone-stretching in order fill the S1 and S3' enzyme pockets with the Tfm groups, which are 'shorter' than the native isobutyl substituents.

The crystal structures above, together with the biological results, demonstrate for the first time that a Tfm group should be regarded as a very effective mimic of an isobutyl, at least within the realm of peptidomimetic structures.

Unfortunately, neither **27** nor **41** showed detectable activity in erythrocyte based anti-malarial tests, confirming the inappropriate pharmacological profile of Pepstatin A analogs [40].

5. Conclusions

In conclusion, we have shown that a wide range of enantiomerically pure Tfm-containing peptides and pseudopeptides can be synthesized in a stereocontrolled manner both in solution and in solid phase. The work carried out so far is expected to open up the route to further classes and combinatorial libraries of fluorinated peptidomimetics, allowing for a systematic study of their hitherto largely unknown biological, conformational and structural properties, which are likely to be extremely interesting and peculiar owing to the presence of fluorine. Further research will hopefully contribute to shed light on the chemistry and the biology of backbone-modified fluorinated peptidomimetics, which have typically been difficult to address owing to the complexity of their synthesis in stereo-defined manner. It is easy to predict that the field of fluorine-containing peptides and mimics will see important and exciting developments in the next future.

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