PERSPECTIVE

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Trifluoromethyl group: an effective xenobiotic function for peptide backbone modification†

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Received (in Montpellier, France) 20th April 2004, Accepted 24th June 2004
First published as an Advance Article on the web 12th November 2004

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 the solid phase, for many different fluoroalkyl peptidomimetics, which are reviewed in this paper: (a) bis-trifluoromethyl (Tfm) analogues of Pepstatin A, which are nanomolar and selective inhibitors of the protozoal aspartyl protease Plasmepsin II; (b) Tfm-malic peptidomimetics that are micromolar inhibitors of some matrix metalloproteinases; (c) partially modified retro (PMR) and retro-inverso (PMRI) ψ[CH(CF_3)NH] peptides with a strong proclivity to assume turn-like conformations; (d) ψ[CH(CF_3)NH] peptide mimics having a great potential as hybrids between natural peptides and hydrolytic transition state analogues; (e) the first PMR peptides incorporating a trifluoroalanine surrogate. These novel classes of fluorinated peptide mimics are likely to represent just the top of an iceberg formed by new peptidomimetic structures with unique biomedicinal and pharmaceutical properties.

Introduction

Fluorine is a magic element: with its small steric size, it is able to bring about dramatic, and often unexpected, changes in physico-chemical properties, reactivity and biological features of organic molecules.[1] Fluorine is not at all a rare element on the earth's crust, in fact it ranks 13th on the abundance list.[2] However, only about ten natural monofluorinated organic molecules have been hitherto described, moreover in a very limited amount.[3] Thus, one can safely state that fluoroorganic molecules are nearly exclusively man-made. A notable exception to this rule stems from the groundbreaking work of O'Hagan *et al.*, who recently discovered the first fluorinating enzyme from the microorganism *Streptomyces cattleya*,[4] and resolved its structure by X-ray diffraction, providing an exciting insight into the mechanism of biocatalyzed organofluorination with inorganic fluoride.[5]

In bioorganic and medicinal chemistry, judicious introduction of fluorine atoms or appropriate fluorinated functions into a molecule has become a method of choice in order to modify and tune its biological properties.[6] Thus, for example, a fluorine atom has been used with great success as a replacement for a hydrogen atom or a hydroxy group, and a CF_2 has been used as a mimic for an oxygen atom. In particular, the xenobiotic trifluoromethyl (Tfm) group is recognized in medicinal chemistry as a substituent of distinctive qualities. It is, in fact, at the same time highly hydrophobic, electron-rich and sterically demanding; moreover, it can provide high *in vivo* stability and features a good mimicry with several naturally occurring residues such as methyl, isopropyl, phenyl, *etc.*[7]

In the realm of peptides, incorporation of tailored fluorinated functions has been used to replace and/or mimic critical

† Key to the abbreviations and acronyms used in this paper: DCM = dichloromethane; TMP = *sym*-collidine (2,4,6-trimethylpyridine); TFAA = trifluoroacetic anhydride; DCC = dicyclohexylcarbodiimide; DMAP = 4-(*N*,*N*-dimethylamino)pyridine; EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt = 1-hydroxybenzotriazole; DIC = diisopropylcarbodiimide; 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; DIPEA = diisopropylethylamine; Teoc = trimethylsilylethoxycarbonyl.

Dr. Matteo Zanda was born in 1966 in Alzano Lombardo (Bergamo). He received the "Laurea" degree in chemistry at the Università degli Studi di Milano under the supervision of Prof. C. Scolastico and Prof. G. Poli in 1991. After his civil service he joined the group of Prof. P. Bravo in the chemistry department of Politecnico di Milano and obtained a Ph.D. in industrial chemistry in 1998. He next spent one year as a postdoctoral fellow at ULP Strasbourg (France) under the supervision of Dr. C. Mioskowski. After 3 years as a junior researcher at Politecnico, in December 2001 he was appointed senior researcher of the National Research Council (CNR). He currently works at the Institute of Chemistry of Molecular Recognition (ICRM) and leads a group of 15 researchers working at the interface of organic chemistry and biomedicine. The main scientific interests of his group are (1) fluorinated peptidomimetics as protease inhibitors and (2) new molecules for the targeting of protein markers of angiogenesis. He is coauthor of about 95 papers and regional editor of Letters in Organic Chemistry (Bentham).

DOI: 10.1039/b405955g

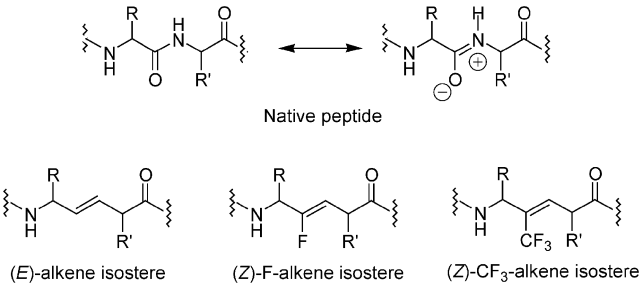


Fig. 1 Alkene and fluoroalkene peptide isosteres.

peptide bonds (Fig. 1). For example, fluorovinyl[8] and trifluoromethylvinyl[9] groups have been demonstrated to be hydrolytically stable replacements for a peptide bond and much better electronic mimics than the unfluorinated vinyl function.

Another important application consists in the incorporation of a CF_3CO or $COCF_2CO$ function (Fig. 2) 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, which is able to mimic the hydrolitic transition state.[10]

Myers *et al.* recently described the synthesis of fluorinated analogues of Indinavir,[11] bearing a fluorine atom on the central hydroxyethylene dipeptide isostere. The inhibitory activity toward HIV-protease was found to be strongly dependent on the stereochemistry of the core fluorinated unit: the syn,syn diastereomer portrayed in Fig. 3 was the most potent inhibitor, essentially equipotent to Indinavir, whereas its anti epimer at the fluorinated stereocenter was about 14 times less potent, as a likely consequence of a higher population of a conformation having lower affinity for the enzyme binding site.

Young and coworkers have incorporated (2*S*,4*S*)-5-fluoroleucine in the hydrophobic core of ubiquitin,[12] as well as into dihydrofolate reductase,[13] to study protein folding using ^{19}F NMR.

In another interesting application of fluoro amino acids, 4-fluoroproline has been demonstrated to bring about peculiar conformational features to collagen-like structures, depending on the stereochemistry of the fluorinated stereogenic centre.[14]

Recently, trifluoro amino acids have been incorporated into protein structures, resulting in a strong modification of their properties and leading to enhanced stability, affinity for lipid bilayer membranes, stronger self-association, and so on. Tirrell *et al.* successfully incorporated trifluoroisoleucine by recombinant methods into coiled-coil proteins that turned out to display enhanced thermal and chemical stability.[15] Trifluoromethionine[16] and 5,5,5-trifluoroisoleucine[17] could also be successfully incorporated by recombinant methods into target proteins. In contrast, much less success was achieved in the incorporation of hexafluoroleucine, of 6,6,6-trifluoro-2-aminohexanoic acid and of an isomeric trifluoroisoleucine (3-Tfm-pentanoic acid). The group of Tirrell also reported incorporation of trifluoroleucine into the amphiphilic peptide Melittin by synthetic methods.[18] The group of Kumar was able to incorporate by synthetic methods four 5,5,5-trifluoroleucine and

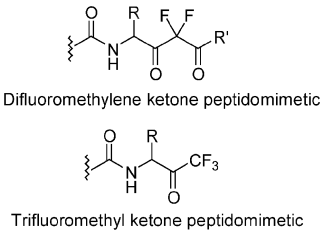


Fig. 2 Fluorine-containing protease inhibitors based on the difluoromethylene and trifluoromethyl ketone functions.

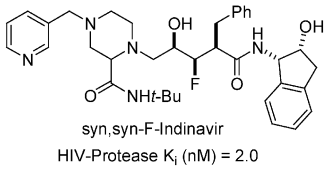


Fig. 3 Myers' fluoro-Saquinavir analogue.

three 4,4,4-trifluoronorvaline residues instead of the natural leucines and valines, respectively, achieving a more stable coiled-coil structure with a "fluorous core", owing to the sequestering of the more hydrophobic Tfrm groups from aqueous solvent.[19] In line with the findings above, Horng and Raleigh have shown that two small proteins (56 residues) having single 4,4,4-trifluorovaline for valine substitutions in positions that are largely buried in the native protein brought about an impressive stabilization of both trifluoro protein variants.[20] Arai, Nishino and coworkers studied *de novo* designed amphiphilic peptides incorporating six or nine 2-(2,2,2)-trifluoroethyl)glycines, which assumed random conformation in water, whereas the parent unfluorinated peptides assume a helical conformation.[21] However, they took a helical conformation upon addition of >20% volume of trifluoroethanol, confirming the hydrophobic nature of these fluoro amino acids.

In spite of this important progress in trifluoro polypeptides and proteins, very little remains known about the synthesis and the properties of small peptides backbone-modified with Tfrm 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, which is reviewed in this perspective article.

Bis-trifluoromethyl Pepstatin A as a 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.[22] 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,[23] have been investigated, but the effect of incorporation of fluoroalkyl functions has not been described previously.

We therefore decided to undertake the synthesis of bis-Tfm-Pepstatin **1**.[24] 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.[25] We deemed such a structural modification interesting because the Tfm group has often been 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 the isopropyl and isobutyl groups.[26] Thus, we decided to assess whether Tfm could behave as a substitute and mimic of the isobutyl group, within the frame of peptidomimetic structures.

Lithium sulfoxide **2** (Scheme 1), prepared *in situ* from (*R*)-*p*-tolyl γ-butenyl sulfoxide, was treated with a THF solution of trifluoro imine **3** at −70 °C. The reaction afforded, with over-

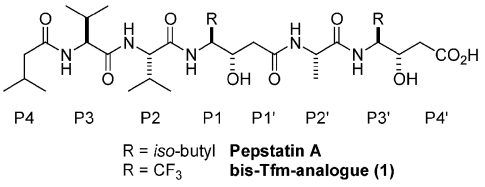
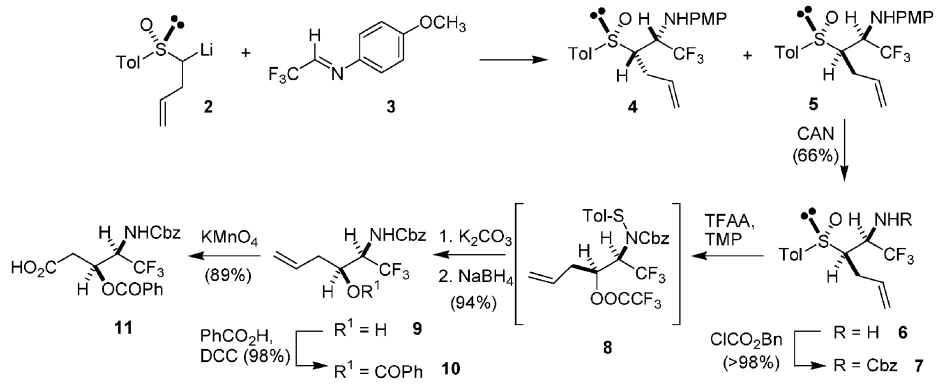


Fig. 4 Natural Pepstatin A and its bis-trifluoromethyl analogue **1**.



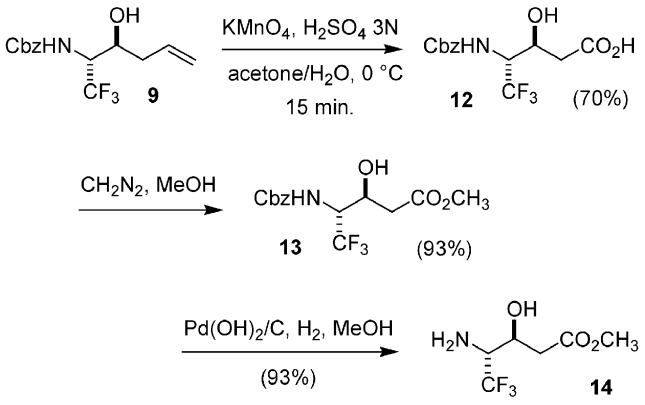
Scheme 1 Stereocontrolled synthesis of the trifluoromethyl-statine framework.

whelming preference, two diastereomeric *N*-PMP β-aminosulfoxides (2*R*,3*S*,R_S)-**4** and (2*S*,3*R*,R_S)-**5** out of four possible, in a 1.0 : 2.75 diastereomeric ratio (d.r.) and nearly quantitative overall isolated yields. Attempts to improve the stereocontrol were made, but relatively little changes of diastereoselectivity were generally recorded.

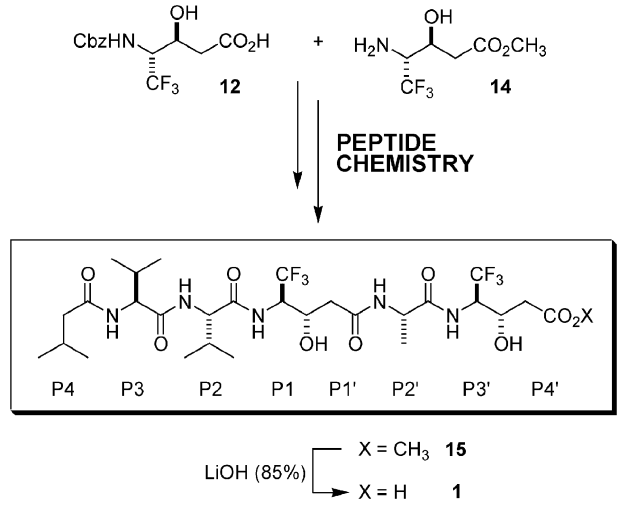
The major sulfoxide **5** was treated with CAN to cleave the *N*-PMP group, providing the free amino sulfoxide **6**, which was reprotected as the *N*-Cbz derivative **7** and finally submitted to the "non-oxidative" Pummerer reaction (NOPR).[27] Treatment of **7** with TFAA and TMP triggered an S_N2-type displacement of the sulfinyl by a trifluoroacetoxy group, leading to the intermediate sulfenamide **8**. 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*)-**9** in a very clean manner, with overall stereoselectivity >98 : 2 (the other diastereomer was not detected). Conversion of (2*R*,3*S*)-**9** into the corresponding *O*-benzoate **10**, followed by oxidative cleavage of the double bond with $KMnO_4$, delivered the targeted enantiopure γ-Tfm-GABOB, (−)-(3*S*,4*R*)-**11**. Attempts to employ the latter compound for the synthesis of the target **1** were unsuccessful. We therefore turned our attention to the synthesis of the orthogonally protected derivatives **12** and **14** (Scheme 2) from **9**. The former was prepared by oxidative cleavage with $KMnO_4$. Next, the carboxylic acid **12** was treated with diazomethane, then the Cbz group of the resulting ester **13** was hydrogenolyzed, providing **14**.

With the orthogonally protected Tfm-statines **12** and **14** in hand, the peptide sequence of the bis-Tfm analogue of Pepstatin **1** was assembled (Scheme 3). Following several coupling sequences, the final step was the hydrolysis of the methyl ester **15** by means of LiOH, to afford **1** in good overall yield.

Bis-Tfm-Pepstatin **1** and its methyl ester **15** were assayed for their ability to inhibit several aspartyl proteinases, including HIV-protease, Plasmepsins II and IV, and human Cathepsin D



Scheme 2 Synthesis of orthogonally protected Tfm-statines **12** and **14**.



Scheme 3 Final steps of the synthesis of bis-Tfm Pepstatin **1** and its methyl ester **15**.

(Table 1). 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 **1** 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.[28] 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.[29] Within the framework of a collaboration with the pharmaceutical company Actelion (Allschwil, Switzerland), compound **1** was found to be a low nanomolar inhibitor of Plasmepsin II. Moreover, **1** is remarkably 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

Table 1 Biological activity of natural and fluorinated Pepstatins

	IC_{50}/nM			
	HIV-protease	Plasmepsin II	Plasmepsin IV	Cathepsin D
Pepstatin A	2500	0.44	0.61	0.64
15	Not determined	2.4	96	2900
1	>150000	1.3	23	120

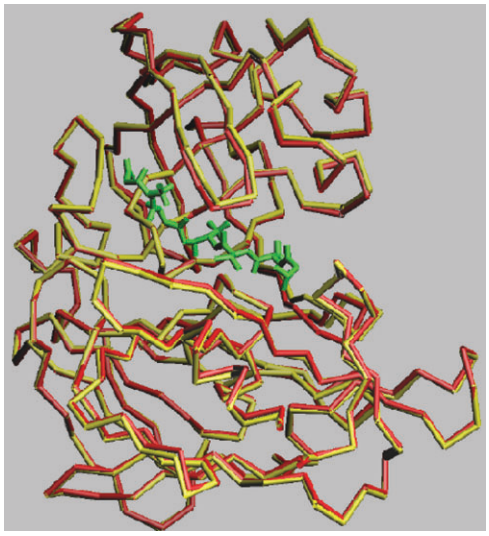


Fig. 5 Inhibitor **1** bound to the active site of Plasmepsin II. Key: (in green) fluorinated inhibitor **1**; (in red) backbone of Plasmepsin II complexed with Pepstatin A; (in yellow) backbone of Plasmepsin II complexed with **15**.

and human Cathepsin D.[30] Approximately the same inhibitory potency *vs.* Plasmepsin II was measured for the methyl ester **15**. 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 **1** and **15**, and assess whether the Tfm group is actually a mimic of the isobutyl side-chains of Pepstatin A, we undertook a collaboration with the laboratory of protein crystallography of Actelion, in order to obtain the crystal structure of **1** and **15** complexed with Plasmepsin II.

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 (Fig. 5).[31] 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 Pepstatin A (in red in Fig. 6) and bis-Tfm-Pepstatin **1** in the Plasmepsin II binding site shows a surprisingly almost identical conformation, with **1** 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 can be regarded as a very effective mimic of an isobutyl, at least within the realm of peptidomimetic structures.

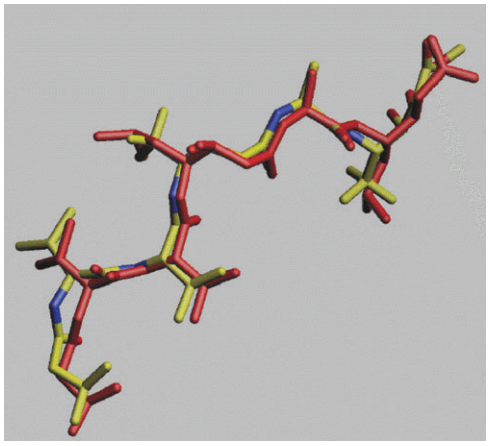


Fig. 6 Conformations of Pepstatin A (red) and its fluorinated analogue **1** (yellow and blue) in the Plasmepsin II active site.

Unfortunately, neither **1** nor **15** showed any detectable activity in erythrocyte-based anti-malarial tests, confirming the inappropriate pharmacological profile of Pepstatin A analogues.[32]

Trifluoro analogues of peptidomimetic metalloproteinase inhibitors

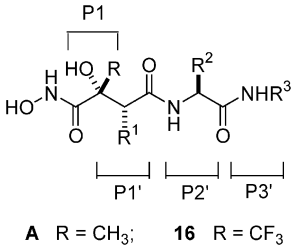
Matrix metalloproteinases (MMPs) are zinc(II)-dependent proteolytic enzymes involved in the degradation of the extracellular matrix.[33] More than 25 human MMPs have been identified so far. Loss in the regulation of their activity can result in the pathological destruction of connective tissue, a process 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 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. 7) of MMP-1, MMP-3 and MMP-9, bearing a quaternary α-methyl alcohol moiety at the P1 position, and several different R^1 groups at P1′.[34] 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+} centre.

We hypothesized that incorporation of fluoroalkyl substituents on the P1 quaternary position of the inhibitors **A** could be an effective strategy for changing and tuning their binding properties. In fact, *albeit* the α-Tfm group was expected to diminish to some extent the chelating properties of the hydroxamate function, this could lead to increased selectivity toward the different MMPs. For this reason we accomplished the synthesis of the Tfm-analogues **16** (Fig. 7) of **A**, and studied the effect of the replacement of the α-CH_3 group with a Tfm on the inhibition of MMP-9.[35]

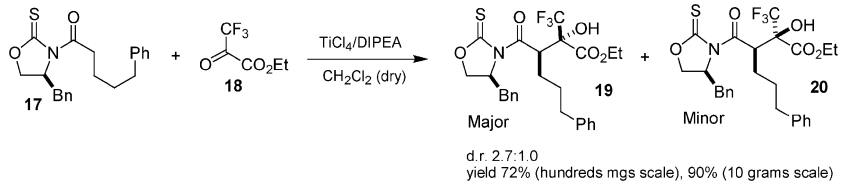
We decided to concentrate our efforts on the substrates **16** having $R^1 = (CH_2)_3Ph$, since their analogues **A** were reported to be very active. First attempts to synthesize the α-Tfm-malic unit of **16** *via* a titanium(IV) catalyzed aldol reaction of trifluoropyruvic esters with enantiopure *N*-acyl oxazolidin-2-ones gave disappointing results.[36] 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 decided to use oxazolidin-2-thiones,[37] whose cleavage was reported to occur much more easily.[38]

The $TiCl_4$ catalyzed reaction of *N*-acyl-oxazolidin-2-thione **17** (Scheme 4) with ethyl trifluoropyruvate **18** afforded the two diastereomeric adducts **19** and **20**, out of four possible, in low diastereomeric ratio. The reaction featured a favourable scale-up effect, affording *ca.* 70% yield on a hundreds of milligrams



A R = CH_3; **16** R = CF_3

Fig. 7 DuPont Merck's MMP inhibitors (**A**) and their CF_3 analogues (**16**).



Scheme 4 Stereocontrolled synthesis of the Tfm-malic framework.

scale, and 90% on a ten gram scale. A number of alternative conditions were explored, but neither significant improvement nor switching of the diastereocontrol could be achieved.

The synthesis of the major diastereomers **26a–c** (Scheme 5) was developed first. Under the standard conditions reported in the literature (BnOH, cat. DMAP, DCM, r.t.) the exocyclic cleavage of the oxazolidinethione on **19** was very slow, affording modest conversion to the corresponding Bn-ester and partial epimerization of the secondary stereocentre. 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 **21** in satisfactory yields and with very low α-epimerization (2%). Coupling of the acid **21** with α-amino acid amides **22a–c** was achieved in good yields with the HOAt/HATU system.[39] The resulting peptidomimetic esters **23a–c** were submitted to saponification, affording the acids **24a–c** in high yields. The subsequent coupling of **24a–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 centre. 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 **25a–c** in hand we addressed the final *O*-Bn cleavage by hydrogenolysis, which provided the targeted hydroxamates **26a–c** in good yields.

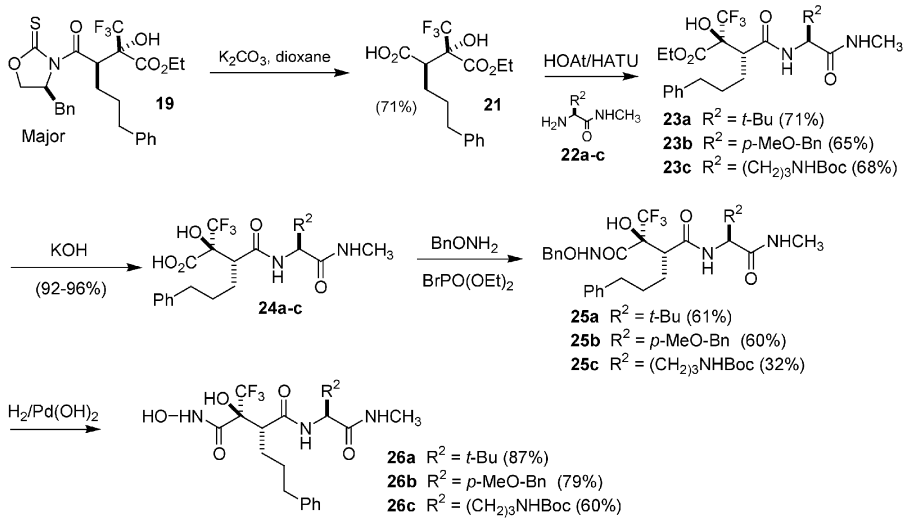
Since **26a–c** are the "wrong" diastereomers with respect to **A**, we deemed it necessary to synthesize at least one analogue having the correct stereochemistry, in order to have a complete set of biological data on the effect of the introduction of the Tfm group. However, a tailored synthetic protocol had to be developed *ex novo*, because the minor diastereomer **20** (Scheme 6) featured a dramatically different reactivity in the key steps of the synthesis. Since we noticed that the coupling of **20** and **22a** with HATU/HOAt gave rise to relevant amounts of the β-lactone **28** (which had to be processed separately), besides the expected coupling product **29**, we decided to prepare first the intermediate **28**. The latter was reacted with free **22a**, affording the desired **29** in high yields. Basic hydrolysis of the ester **29** occurred effectively, although a partial epimerization of the $[Ph(CH_2)_3]$ stereocentre occurred, affording a 3 : 1 mixture of diastereomers **30** and **31** under optimized conditions; these were subjected together to coupling with $BnONH_2$. The resulting diastereomeric *O*-Bn hydroxamates could be separated by flash chromatography, affording pure **32**, which was hydrogenated to the targeted free hydroxamate **33**.

The hydroxamates **26a–c** and **33** were tested for their ability to inhibit the activity of MMP-2, MMP-3 and MMP-9 using zymographic analysis. The IC_{50} values listed in Table 2 show that diastereomers **26a–c** displayed a low inhibitory activity, in line with the parent CH_3 compounds. Disappointingly, **33** showed a much lower activity than the CH_3 analogue **A**, which was reported to be a low nanomolar inhibitor of MMP-3 and MMP-9 (K_i = 13 nM toward MMP-3 and <1 nM toward MMP-9). It is also worth noting that **26a** and **33** showed little selectivity, whereas **26b** and **26c** showed a fairly better affinity for MMP-9, in comparison with MMP-2 and MMP-3. A possible explanation for the dramatic drop of activity upon replacement of the quaternary methyl with a Tfm is that **33**, hindered by the bulky Tfm group, is unable to assume the crucial binding conformation of the CH_3 analogue **A**. Alternatively, one can hypothesize that the bulky and highly electron-rich Tfm group is unable to fit the S1 pocket of the hitherto tested MMPs.

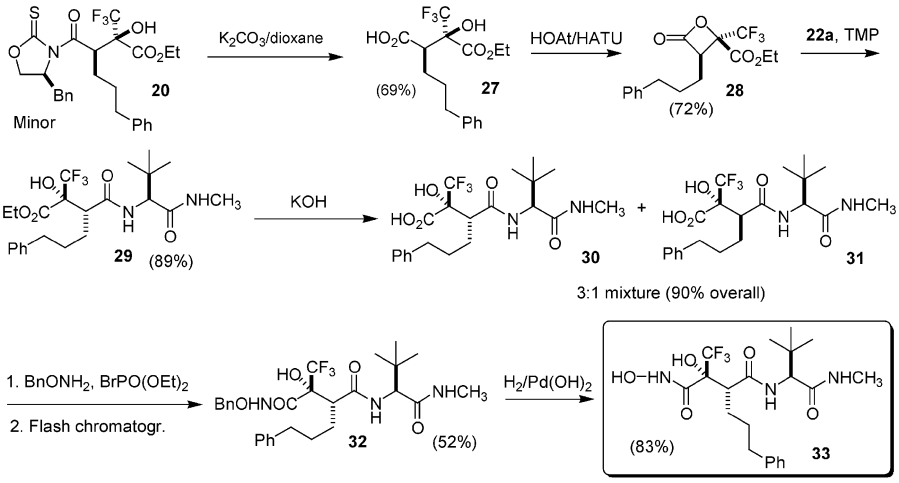
Current work is actively pursuing the synthesis of novel fluorinated analogues of MMPs inhibitors having improved pharmacological properties.

Partially modified retro and retro-inverso ψ[NHCH(CF$_3$)]gly peptides

The need for drug-like molecules to retain both the activity and potency of the parent peptides, while being much more stable and orally active,[40] has been a major driving -force for the development of a variety of peptide mimics. Two very attractive ways of generating bioactive peptide mimics with improved biostability are: (a) to replace a peptide bond with a surrogate unit X, which is usually symbolized as ψ(X);[41] (b) to



Scheme 5 Total synthesis of the hydroxamates derived from the major diastereomer.



Scheme 6 Total synthesis of the hydroxamate derived from the minor diastereomer.

reverse all or some of the peptide bonds (NH–CO instead of CO–NH) giving rise to the so called retro- or partially modified retro (PMR) peptides, respectively.[42] When the stereochemistry of one or more amino acids of the reversed segment is inverted, the resulting pseudo-peptide is termed as retro-inverso. A malonic unit is classically incorporated to provide partially modified retro-peptides, while the direction can be restored by incorporating an additional *gem*-diaminoalkyl unit. Recently, we proposed a new strategy for generating peptidomimetic sequences, based on the idea of combining both the "surrogate unit" and the "direction reversal" strategies in a novel class of pseudo-peptides having a ψ[NHCH(CF_3)] instead of the ψ(NHCO) unit featured by retro-peptides (Fig. 8).[43] A first intuitive consequence of this structural modification is that the symmetry of conventional malonyl PMR peptides is broken, resulting in the generation of two families of regioisomeric PMR ψ[NHCH(CF_3)] and PMR ψ[CH(CF_3)NH] peptides, each one existing in two epimeric forms at the CF_3-substituted carbon. In this way a great diversity of structurally related, potentially bioactive molecules can be created. Further important biological and physicochemical features are expected to arise from the presence of the [CH(CF_3)NH] group. For example, the ψ[NHCH(CF_3)] surrogate should be very stable toward proteolytic degradation. Furthermore, the stereo-electronically demanding Tfm group is expected to constrain the molecule, limiting the number of accessible conformational states and leading to peptidomimetics displaying well-defined conformational motifs. Last but not least, it should modify the binding properties of the parent peptide, changing the hydrogen bonding or coordinative features of the ligand in the putative receptor sites.

Our synthetic approach to the ψ[NHCH(CF_3)]-containing dipeptide units involved an aza-Michael reaction of a series of structurally varied L-α-amino esters **34** (Scheme 7) with the enantiomerically and geometrically pure Michael acceptors (*S*)-(*E*)-**35**.

Diastereomeric pseudo-dipeptides **36** (major) and **37** (minor) were formed in excellent yields at room temperature in 16–88 h, under kinetic control. The solvent has a strong influence on

Table 2 IC_{50} (in μM) of the target Tfm-hydroxamates

Compound	MMP-2	MMP-3	MMP-9
26a	156	>1000	121
26b	407	>1000	84
26c	722	>1000	23
33	23	43	15

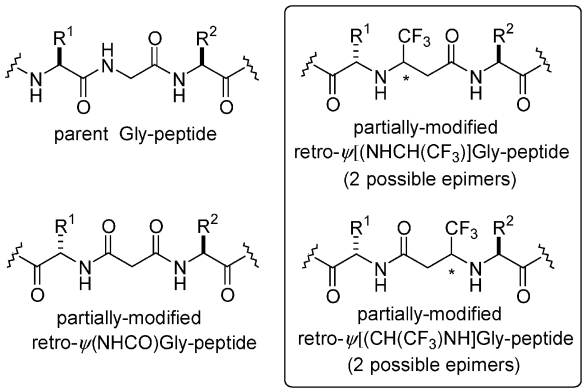
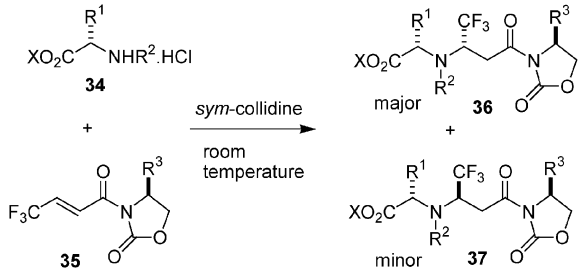
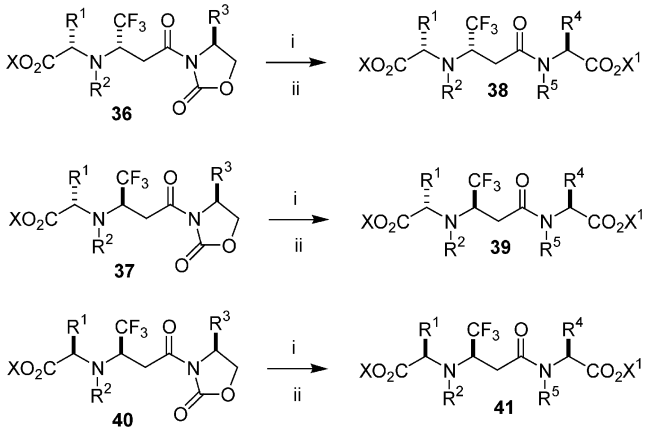


Fig. 8 Different isomers of ψ[NHCH(CF_3)]Gly and ψ[CH(CF_3)NH]Gly retropeptides.

stereoselectivity and the best results were obtained in DCM (50% diastereomeric excess, d.e.), while a substantial drop of d.e. was observed with more polar solvents such as ethanol, acetonitrile, THF, DMF or mixtures. The base also plays an active role, as demonstrated by the fact that the use of DABCO, instead of TMP, accelerates the reaction but slightly lowers the d.e. of the products. In the absence of a base, namely pre-generating the free α-amino ester from the hydrochloride by treatment with $NaHCO_3$, the diastereoselectivity was similar to that achieved with TMP. In light of these results, DCM and TMP were used as, respectively, the solvent and the base of choice for the preparation of a number of pseudo-dipeptides **36**. We could also demonstrate that the facial diastereoselectivity of these reactions is mainly controlled by the nucleophiles **34** rather than by the acceptors **35**. The degree of diastereoselectivity followed the trend R^1 = iPr > iBu > Me > Bn > H (d.e. up to 78%). Modest d.e. was obtained with the cyclic



Scheme 7 Aza-Michael reaction with aminoester nucleophiles **1**.



Scheme 8 (i) LiOH, H_2O_2; (ii) HATU/HOAt, *sym*-collidine, DMF, α-amino ester.

α-amino ester L-Pro-OBn, but the reaction occurred also in this case with very good yield. On the other hand, the R^3 substituent on the oxazolidinone stereocentre had a lower effect on the stereoselectivity. No meaningful effect was exerted by the X group of the nucleophile. These results can be rationalized if one considers that in the absence of chelating agents, *N*-(*E*)-enoyloxazolidin-2-ones **35** exist in a transoid conformation (as portrayed in Schemes 7 and 8), with the R^3 substituent pointing away from the C=C bond, thus exerting little control of the facial selectivity. In contrast, the R^1 side-chain of α-amino esters **34** should be spatially close to the forming stereogenic centre in the transition state; therefore, its influence is much more important. Chelation with Lewis acids for biasing the Tfm-oxazolidin-2-ones **35** in the cisoid conformation was tried with little success, probably due to their poor basic character.

The method is viable for preparing longer PMR and PMRI ψ[NHCH(CF_3)]Gly peptides by using unprotected N-terminal peptides as nucleophiles in the aza-Michael reactions with **35**, which occur with excellent yields and good stereocontrol.

The chemoselective cleavage of the oxazolidinone auxiliary was achieved in 55–82% yields upon treatment of the oxazolidinone pseudo-peptides **36**, **37** and **40** (Scheme 8) with LiOH/H_2O_2.[44] The resulting pseudo-peptides having a terminal CO_2H group were coupled with another α-amino ester. The final diastereomeric PMR tripeptides **38**, **39** and PMRI tripeptides **40**, orthogonally protected at the carboxy end groups (except **9e**) and therefore suitable for further selective elongation, were obtained in quantitative yields, often as solid materials.

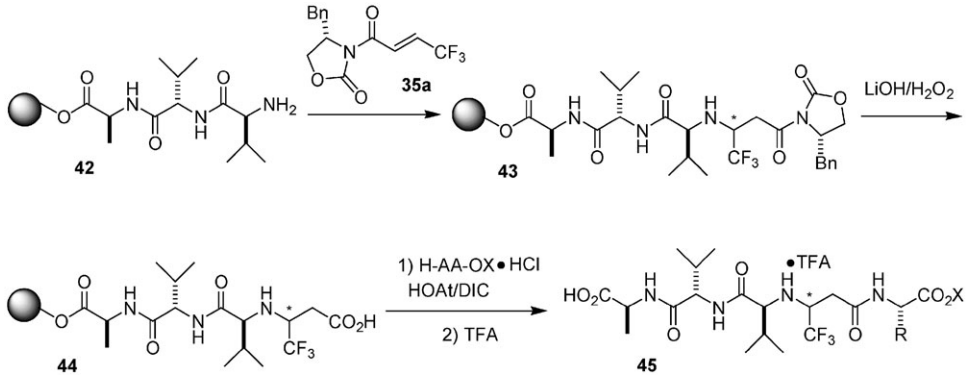
A very interesting feature of PMR ψ[NHCH(CF_3)]Gly peptides like **36** is their weakly basic nature, which is due to the presence of the strongly electron-withdrawing CF_3 in the α-position to the amino group. These compounds do not form stable salts with trifluoroacetic acid or 2 N hydrochloric acid. One can therefore say that, in terms of basicity, the [NHCH(CF_3)] moiety resembles more closely a retropeptide unit [NHCO] than a conventional secondary amine moiety.

The parallel solid-phase synthesis of small libraries of PMR ψ[NHCH(CF_3)]Gly tri-, tetra-, and pentapeptides, which in perspective should be applicable in the preparation of wider libraries of PMR ψ[NHCH(CF_3)] polypeptides for high-throughput biological screening, was also developed.[45] As an example, in Scheme 9 the synthesis of PMR ψ[NHCH(CF_3)]Gly pentapeptides is described. The tripeptide resin H–Val–Val–Ala–OWang **42** was prepared by Fmoc chemistry and subjected to conjugate addition with **35a**, taking place very effectively in 3 days at r.t. A 10 : 1 mixture of diastereoisomers **43** was formed. In general these aza-Michael reactions in the solid phase occurred with diastereocontrol comparable with those in solution (see above). Interestingly, in the case of tripeptides as polymer-supported nucleophiles, we observed the highest diastereoselectivity as compared with the conjugate additions of H–Val–OWang and H–Val–Gly–OWang (7 : 1 and 4.5 : 1 mixtures of diastereoisomers, respectively). This shows that additional stereocentres, even in remote positions of the nucleophile, can exert a strong influence on the stereochemical outcome of the conjugate additions.

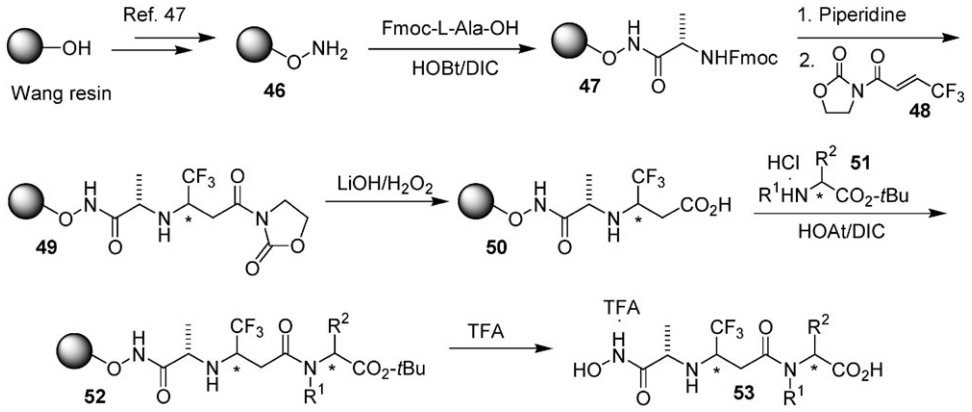
The resin **43** was chemoselectively hydrolyzed at the C-terminus by treatment with lithium hydroperoxide generated *in situ*. The resulting pseudo-tetrapeptide resin **44** was coupled (HOAt/DIC) with different α-amino acid esters to generate, after release from the resin, the PMR ψ[NHCH(CF_3)]Gly pentapeptides **45** with very good overall yields and purity.

This method was also adapted to the solution and solid-phase synthesis of PMR and PMRI ψ[NHCH(CF_3)]-Gly peptidyl hydroxamates,[46] which are of interest since the HON-HCO end group is very effective in coordinating the Zn^{2+} cofactor of MMPs, as seen in the previous section. In the solid-phase approach, the hydroxylamine resin **46** (Scheme 10) was prepared in two steps from commercial Wang resin, according to the method of Floyd *et al.*,[47] and coupled to an excess of L-Fmoc–Ala to give the protected alanine polymer **47**, from which the Fmoc group was cleaved with 20% piperidine in DMF. The resulting resin-bound α-amino hydroxamate was submitted to 1,4-conjugate addition with the achiral oxazolidin-2-one **48**. As a general trend, low diastereoselectivity was observed in these aza-Michael reactions involving hydroxamates as nucleophiles. Thus, in analogy with the synthesis in solution, the adducts **49** were formed as nearly equimolar mixtures of epimers at the Tfm-substituted centre. Treatment of **49** with lithium hydroperoxide cleaved the oxazolidin-2-one with excellent chemoselectivity. Coupling of **50** to α-amino esters **51** afforded the tripeptidyl resins **52**, from which the retro- and retro-inverso hydroxamates **53** were released in good yields and purity upon treatment with TFA.

Tetrapeptidyl hydroxamates and a tripeptidyl hydroxamate having a methylamide terminus, which is often encountered in MMPs inhibitors, were prepared as well by the same method, thus demonstrating its wide scope.



Scheme 9 Solid-phase synthesis of PMR ψ[NHCH(CF_3)]Gly peptides.



Scheme 10 Solid-phase synthesis of PMR ψ[NHCH(CF_3)]Gly peptidyl hydroxamates.

The conformational features of PMR ψ[NHCH(CF_3)]Gly peptides were studied in detail. It has been shown that very simple PMR peptides, such as the triamide **54** (Fig. 9), adopt turn-like nine-membered folding patterns with an intramolecular N–H···O=C hydrogen bond in the solid state, as well as in low-polarity solvent solutions.[48] Racemic ψ[NHCH(CF_3)] diamide **55** (Fig. 9) was synthesized in good overall yield by means of the standard protocol (see Schemes 7 and 8). ^{1}H NMR spectroscopy, supported by molecular dynamics calculations, showed the stability of the turn-like conformations for **55** in a weakly hydrogen-bonding solvent, such as $CDCl_3$, comparable to that of parent malonyl-based retropeptides **54**. Similar turn-like conformations were found in the solid state, as demonstrated by X-ray diffraction studies of several ψ[NHCH(CF_3)]Gly peptides, such as **56**.

This very interesting conformational behaviour is a likely consequence of two main factors: (1) severe torsional restrictions about the sp^3 bonds in the [CO–CH_2–CH(CF_3)–NH–CH(R)–CO] module, which is biased by the stereoelectronically demanding Tfm group and the R side-chain; (2) formation of nine-membered intramolecularly hydrogen-bonded rings, which have been clearly detected both in $CHCl_3$ solution and in some crystal structures. The former factor seems to be more important, as turn-like conformations were found in the solid state even in the absence of intramolecular hydrogen bonding. The relative configuration of the –C*H(CF_3)NHC*H(R)– stereogenic centres was found to have a major effect on the stability of the turn-like conformation, which seems to require a syn stereochemistry, such as **38** and **56**, whereas the diastereomers **39** investigated so far did not show the same conformational properties.

This could represent a new general concept for the rational design of linear peptidomimetics incorporating a turn-like secondary structure.

X-Ray diffraction and *ab initio* computational studies showed that the [–CH(CF_3)NH–] group can be seen as a sort of hybrid between a peptide bond mimic and a proteolytic transition state analogue, as it combines some of the properties of a peptidyl –CONH– group [low NH basicity, CH(CF_3)–NH–CH backbone angle close to 120d, C–CF_3 bond substantially isopolar with the C=O] with some properties (high electron density on the Tfm group, tetrahedral backbone carbon) of the tetrahedral intermediate [–C(OX)(O^-)NH–] involved in the protease-mediated hydrolysis reaction of a peptide bond.

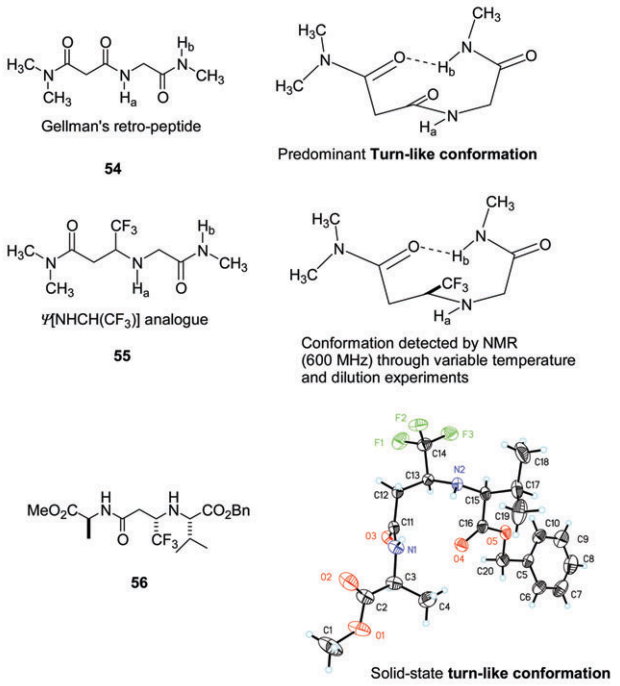


Fig. 9 Solution and solid-state conformations of some PMR ψ[NHCH(CF_3)]Gly peptides.

ψ[CH(CF_3)NH]Gly peptides

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 **57** (Fig. 10),[49] 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 analogue.

The stereocontrolled synthesis of these brand new peptidomimetics is based on another key aza-Michael reaction involving 3,3,3-trifluoro-1-nitropropene **58** (Scheme 11) and an array of α-amino esters, generated *in situ* from the hydrochlorides **59** with a base. The reactions took place almost instanta-

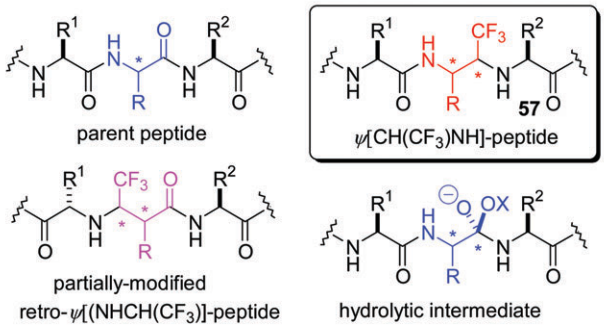
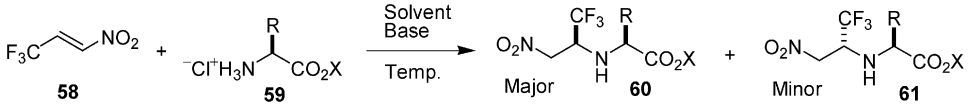


Fig. 10 Structure of natural and ψ[CH(CF_3)NH]Gly peptides.



58 59 60 61

Scheme 11 The aza-Michael reaction to give the ψ[NHCH(CF_3)]Gly peptide backbone.

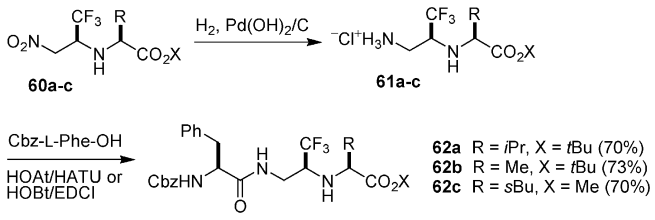
neously at r.t., affording the diastereomeric α′-Tfm-β′-nitro-α-amino esters **60** (major) and **61** (minor) under kinetic control.

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, (4) R side-chain of **59**. 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. As observed for other aza-Michael reactions (see above), 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 apolar CCl_4. Even more surprisingly, the stoichiometry of DIPEA was also found to have a profound effect on the stereocontrol. The optimum amount was found to be 1.1 equiv. (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 equiv., whereas little variation occurred beyond this quantity. Other bases, such as TMP and $NaHCO_3$, did not exhibit the same stoichiometry effect, affording comparable d.e.'s upon changing the number of equivalents used. The effect of the R side-chain of **59** was in line with expectations. In fact, the highest d.e.'s were observed with bulky R groups (iPr, *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 **60** and **61**, whereas at lower temperatures (for example −40 or −70 °C) very complex mixtures of products were obtained. All the experimental evidence above suggests that these aza-Michael reactions occur through a tight, polar, termolecular transition state (TS), involving **58**, **59** 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 this TS, thus lowering the stereocontrol.

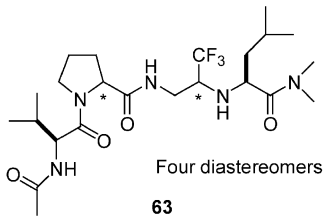
Elaboration of the major adducts **60a–c** into the target ψ[CH(CF_3)NH]Gly peptides **62a–c** is shown in Scheme 12. The nitro group of **60a–c** was hydrogenated to an amino group using Pearlman's catalyst, and the resulting diamino compounds were trapped as hydrochlorides **61a–c** and submitted without purification to coupling with Cbz-L-Phe–OH, affording the ψ[CH(CF_3)NH]Gly tripeptides **62a–c** in good overall yields.

In order to prove 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 four diastereomers of tetrapeptide **63** (Fig. 11).[50] The latter is an interesting model for conformational studies, because some of the analogues incorporating a natural Gly have been shown to



60a-c 61a-c

Scheme 12 Elaboration of the aza-Michael adducts **60** into ψ[CH(CF_3)NH]Gly peptides **62**.



63

Fig. 11 Complex model ψ[CH(CF_3)NH]Gly tetrapeptides.

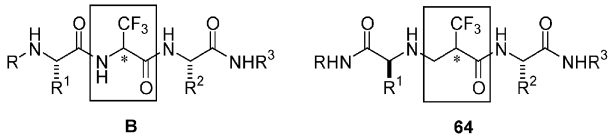
assume very stable and highly populated β-hairpin conformations, depending on the configuration of the Pro residue.[51] A detailed study of the conformational features of the stereoisomers **63** is currently in progress.

Partially modified retro-peptide mimics incorporating a trifluoroalanine surrogate

Incorporation of trifluoro amino acids,[52] and particularly of α-Tfm α-amino acids,[53] into peptide sequences has been the object of growing interest in the last decade, because it represents an effective strategy to retard proteolytic degradation and to stabilize and modify secondary structures. In the case of α-Tfm α-amino acids, this synthetic task is quite challenging because of (1) the strong electronegative influence of the α-Tfm group on both the amino and the carboxylic groups and (2) the steric hindrance of the α-Tfm group. Thus, the common strategies for achieving peptide coupling are not applicable to C-terminal introduction of α-Tfm α-amino acids into peptides, whereas N-terminal introduction is more accessible. C-Terminal introduction has been achieved, for example, by Burger *et al. via in situ* deprotection of *N*-Teoc derivatives, followed by coupling with Fmoc-amino acid fluorides,[54] and by Dal Pozzo *et al.* by coupling with amino acid bromides.[55] N-Terminal introduction has been achieved both by chemical methods[56] and by enzymatic ones.[57]

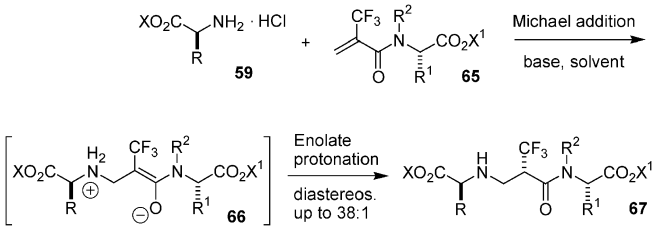
Incorporation of 3,3,3-trifluoroalanine (TF-Ala) into a peptide sequence **B** (Fig. 12) is an even more challenging endeavour, owing to its low chemical and configurational stability at pH > 6.[58] On the other hand, no mimics of TF-Ala-containing peptides had been described in the literature. For this reason, we undertook the synthesis of PMR ψ[$NHCH_2$] peptide mimics **64**, incorporating a chemically stable and stereo-defined [$CH_2CH(CF_3)CO$] surrogate of TF-Ala.[59]

Retrosynthetic analysis of the problem suggested that asymmetric aza-Michael N-addition of α-amino esters to *N*-(α-trifluoromethyl)acryloyl-α-amino esters could represent a viable entry to the target structures **64**. Michael acceptors **65** (Scheme 11; obtained upon reacting the appropriate α-amino ester H–AA–OX[1] with α-Tfm-acryloyl chloride) were reacted with α-amino esters (generated *in situ* from **59** with a tertiary amine), affording an array of PMR tripeptides **67** in 75–98% yields. In this case also the diastereoselectivity of the process



B 64

Fig. 12 A peptide incorporating trifluoroalanine (**B**) and a PMR ψ[$NHCH_2$] peptide (**64**) incorporating a trifluoroalanine mimic.



Scheme 13 Tandem reaction producing PMR ψ[$NHCH_2$] tripeptides **67**.

was studied in detail. We found that it depends mainly on four reaction parameters: (1) base, (2) solvent, (3) R and R^1 side-chains of **59** and **65**, respectively, (4) the relative stereochemistry of **59** and **65**. Concerning the base, the best diastereocontrol was achieved with DABCO, whereas lower d.e.'s were achieved with TMA, $NaHCO_3$, DIPEA, TEA, DMAP, TMP (in this order). Interestingly, chiral bases such as quinidine and cinchonine gave the worst diastereocontrol. The solvent is another key factor in this reaction, with low-polarity or apolar solvents providing much higher diastereocontrol. Thus, the best stereocontrol was achieved with CCl_4, followed in order by toluene, THF, CH_2Cl_2 and acetonitrile.

The R side-chain of **59** was proven to have a strong influence: the d.e. of the products **67** increased in the sense R = H < iBu < Bn < Me < *s*-Bu < iPr, therefore the best stereocontrol was achieved with the bulkiest R groups. Also the R^1 side-chain belonging to the acceptors **65** had a strong influence; its effect on the d.e. substantially followed the same trend observed for R. The configuration of the reaction partners **59** and **65** had also a profound effect, as demonstrated by the use of matched/mismatched pairs of reactants. In general, the *like* combination (D/D or L/L) provided remarkably higher stereoselectivity. In the best case, when L-Val esters **59** were reacted with L-Val derived acceptors **65** in CCl_4, with DABCO as base, a d.e. as high as 95% was achieved.

Although detailed kinetic and computational studies will be necessary in order to draw a reliable mechanistic picture of this process, the body of experimental evidence discussed above suggests that amino ester **59**, Michael acceptor **65** and DABCO might form a tight termolecular ion-pair TS, which is energetically favoured and not disrupted in non-polar solvents, during the critical stereogenic intramolecular proton transfer from the intermediate zwitterion **66** (Scheme 13) to the final product **67**.

An efficient stereocontrolled synthesis of PMR ψ[$NHCH_2$] tripeptide mimics was developed on the solid phase as well.[60] *N*-Fmoc α-amino acids loaded on Wang resin, **68** (Scheme 14), were deprotected to **69** upon treatment with piperidine. Next, the resins **69** were reacted with an excess of 2-trifluoromethylpropenoyl chloride. This process provided the trifluoromethylated resins **70**, functionalized as chiral Michael acceptors. The crucial aza-Michael reaction was performed by addition of 3 equiv. of the appropriate α-amino ester to a suspension of resin **70** in the appropriate solvent, in the presence of 6 equiv. of base, producing the desired resins **71** in a very effective manner.

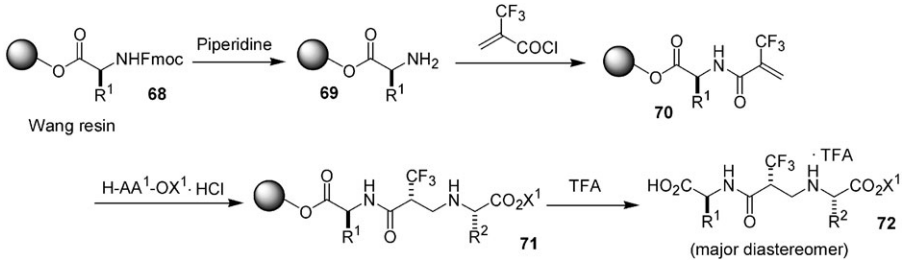
Release of the PMR peptides **72** from the solid support was achieved upon treatment of **71** with TFA in DCM. The target compounds **72** were invariably obtained with good-to-excellent chemical purity. In general, one can say that the main features of the solution-phase process were retained in the solid-phase version. In fact, the stereocontrol could be strongly improved (up to 15 : 1) by using apolar solvents like carbon tetrachloride and DABCO as base. The main difference was that the solid-phase reactions were less diastereoselective than those in solution. This could be due to the fact that the polymeric support biases the reaction partners in a different transition state with respect to that assumed in the highly stereoselective solution-phase process. However, this drawback is counterbalanced by the much greater potential of this solid-phase version for a fast, automated generation of large arrays of PMR ψ[$NHCH_2$] peptides **72** for high throughput assays, and for the synthesis of polypeptide mimetics as well.

Future and outlook

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. Several questions already arise: will we be able to propose rationale predictive models for the biological and conformational properties of fluorinated peptidomimetics? How general is the bioisosteric replacement of alkyl side-chains (isobutyl, isopropyl, methyl, *etc.*) with fluoroalkyl (CF_3, C_2F_5, *etc.*) groups? Are there other effective bioisosteric replacements of native peptide functions with fluorinated groups? Can we exploit fluorinated substituents to improve the pharmacological profile of peptides? Further research will hopefully contribute to shed light on these and other intriguing questions, which have typically been difficult to address owing to the complexity of the synthesis of fluorinated peptidomimetics in a stereodefined 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.

Acknowledgements

The research discussed herein is the result of the enthusiastic work of the coauthors of our publications cited within the references: my deepest thanks are directed to them. Special thanks are dedicated to Prof. Pierfrancesco Bravo, Dr. Alessandro Volonterio, Monica Sani, Dr. Fiorenza Viani and Dr. Giorgio Colombo. Many thanks also to Prof. Santos Fustero (University of Valencia, Spain), Dr. Christoph Binkert, Dr. Lars Prade and Dr. Daniel Bur (Actelion, Swizterland), Dr. Stefano Bellosta (University of Milan, Italy), Gabriele Candiani and Dr. Francoise Pecker (Creteil, France), Dr.



Scheme 14 Solid-phase synthesis of PMR ψ[$NHCH_2$] peptides **72** incorporating a 3,3,3-trifluoroalanine mimic.

Raffaella Giavazzi (Istituto Mario Negri, Bergamo, Italy) and Prof. Dario Neri (ETH-Zurich, Switzerland). I am also grateful to the CNR, Politecnico di Milano, MIUR (Cofin 2002, Project "Peptidi Sintetici Bioattivi"), and European Commission (IHP Network grant "FLUOR MMPI" HPRN-CT-2002-00181) for financial support.

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