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Total synthesis of a pepstatin analog incorporating two trifluoromethyl hydroxymethylene isosteres (Tfm-GABOB) and evaluation of Tfm-GABOB containing peptides as inhibitors of HIV-1 protease and MMP-9

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Abstract—We describe the asymmetric total synthesis of a trifluoromethyl (Tfm) analogue of the aspartate protease inhibitor pepstatin incorporating two γ-Tfm-γ-amino-β-hydroxybutyric acid (γ-Tfm-GABOB) units instead of the natural statine units. The title compound as well as several Tfm-substituted precursors were tested as inhibitors of HIV-1 protease and Gelatinase B (MMP-9) © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Pepstatin (Iva-Val-Val-Sta-Ala-Sta)[1] (Fig. 1) is a naturally occurring inhibitor for aspartic proteases which contains two units of the unusual amino-acid (4*S*,3*S*)-statine. Its binding is often characterized by dissociation constants in the range of 0.1–1 nM.[2] Exceptions are the mammalian aspartic protease renin[3] and the retroviral proteases such as HIV-1 protease,[4] for which pepstatin is less inhibitory (IC_{50}=0.32 and 2.5 μM, respectively). Kinetic studies have shown that the (3*S*)-hydroxyl group of the central statine is important for tight binding of pepstatin,[5] and its stereochemistry has a large effect on protease inhibition, a *syn* diastereomeric relationship between the amine and hydroxyl groups being required.[2b] Analysis of the crystal structures of native endothiapepsin and endothiapepsin complexed with pepstatin indicates that this hydroxyl displaces the catalytic water of the enzyme.[6] Much effort has been directed toward the synthesis of pepstatin mimetics in order to discover analogues having improved properties,[2b,7] including a few fluorinated derivatives incorporating difluorostatine and difluorostatone units, which have been reported to be potent inhibitors of penicillopepsin.[8] Replacement of the statine isobutyl residue in the P1 position with other groups is a strategy that has sometimes led to analogues with improved properties.[9] However, its replacement with a fluoroalkyl residue has never been reported, despite the fact that fluoroalkyl groups are known to deeply modify physical–chemical properties

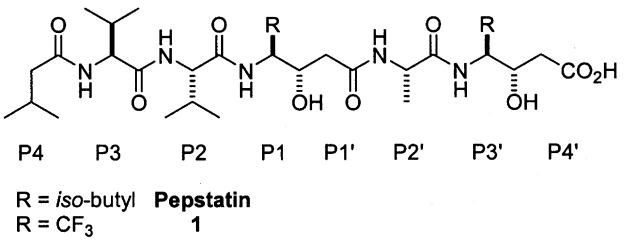

Figure 1.

Keywords: asymmetric synthesis; peptide isosteres; HIV; metalloproteinase; trifluoromethyl group.

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Abbreviations: GABOB, γ-amino-β-hydroxybutyric acid; EDCI, *N*-Ethyl-*N'*-(3-dimethyl-aminopropyl)carbodiimide hydrochloride; HOBt, 1-Hydroxybenzotriazole; HATU, *N*,*N*,*N'*,*N'*-Tetramethyl-*O*-(7-azabenzo-triazol-1-yl)uronium hexafluorophosphate; HOAt, 1-Hydroxy-7-azabenzotriazole; TMP, 2,4,6-Trimethylpyridine (*sym*-collidine); NMM, *N*-Methyl-morpholine. EDTA, Ethylenediaminetetraacetic acid; DTT, Dithiothreitol; MES, 4-Morpholineethanesulfonic acid; Abz, 2-aminobenzoic acid; DMEM, Dulbecco Modified Eagle's medium; BSA, Bovine serum albumin; PMSF, Phenylmethylsulfonylfluoride; SDS, Sodium dodecyl sulfate.

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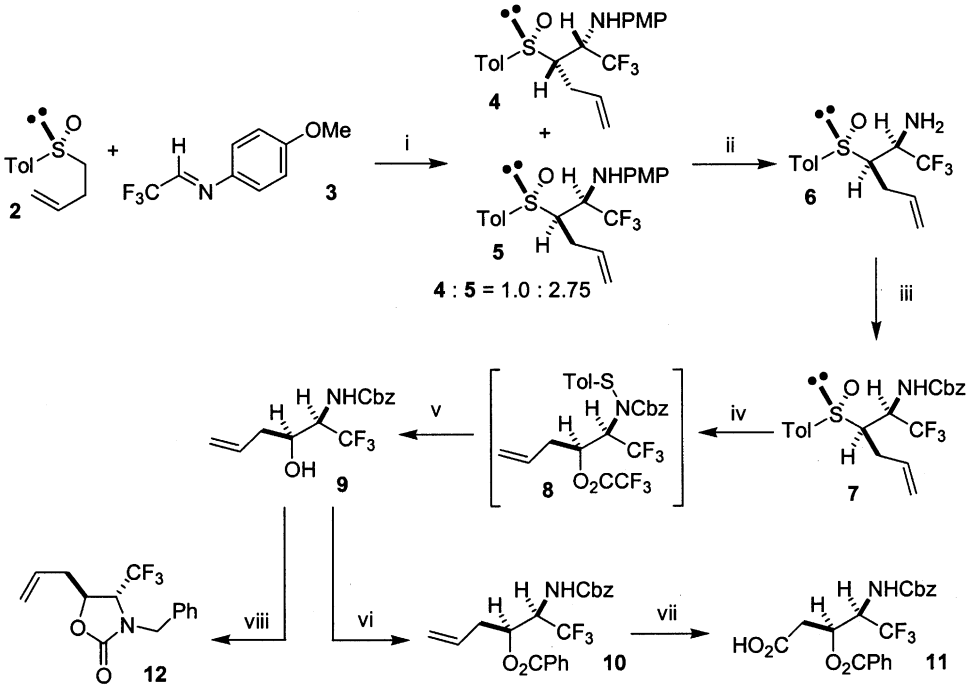


Scheme 1. (i) LDA, THF, −70°C. (ii) CAN, CH_3CN, H_2O, then FC (66%). (iii) $ClCO_2CH_2Ph$, 50% aq. K_2CO_3, dioxane (>98%). (iv) $(CF_3CO)_2O$, *sym*-collidine, CH_3CN. (v) $NaBH_4$, THF/H_2O, 0°C (94%). (vi) $PhCO_2H$, DCC, DMAP (cat.), CH_2Cl_2 (98%). (vii) $KMnO_4$, H_2SO_4 3N, acetone/H_2O (89%). (viii) 1. NaH; 2. $PhCH_2Br$ (93%).

such as local hydrophobicity, acidity/basicity, nucleophilicity, preferred conformation (inducing conformational constrictions in the case of sterically demanding groups such as CF_3), and that useful spectroscopic data on the binding process might be obtained by ^{19}F NMR.[10]

This gap could be due to the fact that the requisite stereodefined γ-fluoroalkyl, γ-amino, β-hydroxy acid units[11] have been hitherto synthetically unavailable. Moreover, incorporation of α-fluoroalkyl amino moieties into peptidic sequences via amide bond formation is a challenging endeavour, due to the low nucleophilicity of the NH_2 function. This is particularly true for α-trifluoromethyl (Tfm) amino derivatives,[12a] due to the strong electron-withdrawing effect of Tfm. In this paper we describe in full account the total solution-phase synthesis of the pepstatin analog **1** (Fig. 1),[13] having two γ-Tfm-γ-amino-β-hydroxybutyric (GABOB) units[14] in place of the natural *syn*-(3*S*,4*S*)-statines in the P1 and P3′ positions. Moreover, the evaluation of **1** and several precursors as inhibitors of HIV-1 protease and the matrix metalloproteinase Gelatinase-B (MMP-9) has been carried out,[15] as a part of a project aiming at the investigation of the effect of fluoroalkyl groups on the binding process to proteases.

2. Results

2.1. Synthesis of γ-Tfm-GABOB

Our first goal toward the synthesis of **1** was the development of a multigram stereocontrolled preparation of the orthogonally protected enantio- and diasteromerically pure (3*S*,4*R*)-Tfm-analog of statine, namely the γ-Tfm-GABOB **11** (Scheme 1). We envisaged an approach to **11** which takes advantage of a synergistic combination of two methodologies recently developed in our laboratories: (1) the chiral sulfoxide stereocontrolled additions of carbon nucleophiles to fluorinated imines[16] and (2) the 'non-oxidative' Pummerer reaction,[17] that allows for a one-pot S_N2-type displacement of the sulfinyl auxiliary by a hydroxyl. Thus, easily available α-lithium sulfoxides[18] can be used as chiral α-hydroxyalkyl anion equivalents for preparing β-amino alcohols. In this work, lithiated (*R*)-*p*-tolyl γ-butenyl

Table 1. Influence of some reaction parameters on the diastereoselectivity

Run	Conditions	Diast. ratio (5/4)	Yield (%)
1	LDA, THF, −70°C	2.75/1.0	>98
2	LDA, THF, −70–0°C, then addition at −70°C	2.2/1.0	77
3	LTMP, THF, −70°C	2.25/1.0	Not det.
4	LDA, Toluene, −70°C	2.8/1.0	95
5	LHMDS, THF, −70°C	1.0/2.0	>98
6	LDA, HMPA (2 equiv.), THF, −70°C	1.0/2.25[a]	Not det.

[a] A third diastereomeric β-sulfinyl amine, which formed only in minute amounts in runs 1–5, was also produced as the 30.4% of the whole diastereomeric mixture.

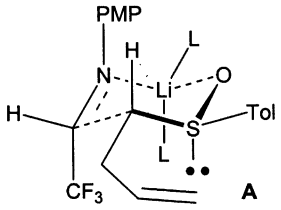


Figure 2.

sulfoxide **2** was used as chiral α-hydroxy-γ-butenyl anion equivalent with the *N*-*p*-methoxyphenyl (*N*-PMP) imine of fluoral **3**, to achieve the synthesis of a *syn* (Tfm)GABOB unit.

A THF solution of lithium sulfoxide **2** (Scheme 1), prepared from (*R*)-*p*-tolyl γ-butenyl sulfoxide with 1.2 equiv. of LDA, was treated with a THF solution of imine **3** at −70°C. The reaction afforded, with overwhelming preference, two diastereomeric *N*-PMP-β-amino sulfoxides (2*R*,3*S*,R_S)-**4** and (2*S*,3*R*,R_S)-**5** out of four theoretically possible, in a ratio of 1.0/2.75 and nearly quantitative overall isolated yields (Table 1, run 1). Attempts to improve the stereocontrol were made, but relatively little changes of diastereoselectivity were generally recorded and the best overall result remained that of run 1 (Table 1). A little effect of reaction temperature on the stereochemical outcome was observed (run 2), in fact when the THF solution of lithiated **2** (LDA, −70°C) was allowed to warm up to 0°C for 15 min, then rapidly cooled again to −70°C and treated with the imine **2**, only a little change in diastereoselectivity was observed (**5**/**4**=2.2/1.0, 77%). Analogously, the use of more bulky bases, like lithium tetramethylpiperidide (run 3), or the use of toluene as solvent (run 4) provided little or no changes of the reaction outcome. The only exception was encountered when lithium hexamethyldisilazane (LiHMDS) was used as base (THF, −78°C) (run 5). In that case we observed reversal of diastereoselectivity in favor of **4**, as a likely result of the formation of a structurally different lithiated sulfoxide **2**.

The preferential formation of the diastereomer **5** under the optimized conditions (run 1) might be explained by the fact that the lithiated butenyl sulfoxide **2** reacts mainly in the *anti*-geometry, having *p*-tolyl and allyl groups *trans* with respect to the plane defined by the O–S–C–Li bonds,[19] through a Zimmerman–Traxler (aldol-type) transition state **A** (Fig. 2).[18b,20] This mechanistic rationale is supported by an additional experiment (run 6). Performing the reaction of α-lithium **2** with **3** in the presence of 2 equiv. of hexamethylphosphoramide (HMPA), which is known to coordinate very well the lithium atom and therefore is supposed to inhibit the formation of lithium-mediated transition state **A**, we observed a dramatic change of diastereoselectivity with respect to run 1. In fact a 1.00:2.25:1.42 mixture of **4**, **5** and a third diastereomeric β-sulfinyl amine, respectively. The latter was formed only in traces in the previous experiments (runs 1–5), thus confirming that the lithium cation is involved in the formation of a chelated TS of type **A**.

The mixture of diastereomeric sulfoxides **4** and **5** was treated with ceric ammonium nitrate (CAN) (5 equiv.) in acetonitrile/water to cleave the *N*-PMP group, providing the free amino sulfoxide **6** in diastereomerically pure form after flash chromatography (FC). The absolute stereochemistry of **6** was determined by X-ray diffraction (Fig. 3).[21] This finding allowed us to assess the stereochemistry of both diastereomeric sulfoxides **4** and **5**. In fact, a mixture of **5** (which must have the same stereochemistry of **6**) and **4** were deoxygenated with Me_3SiCl/NaI providing the corresponding sulfides, which were found to be enantiomers by ^1H NMR analysis (a single set of signals was detected). This proved that **4** has (2*R*,3*S*,R_S)-stereochemistry.

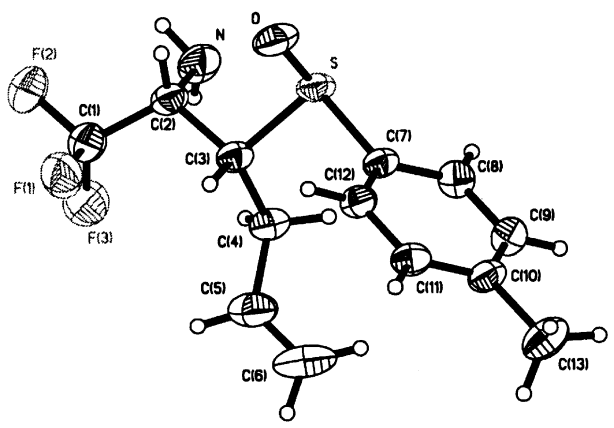
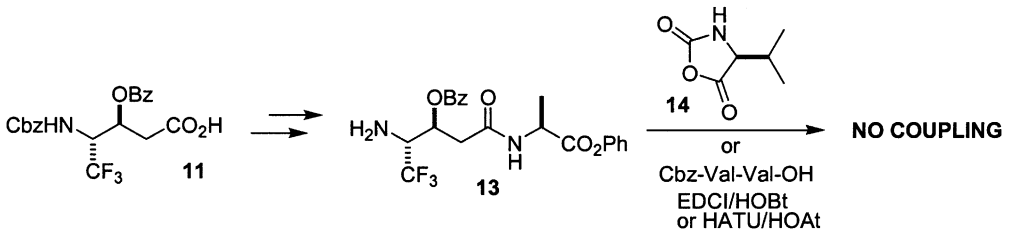
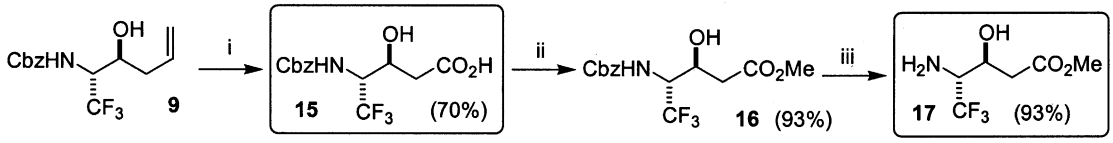


Figure 3. ORTEP view of **6** showing the atomic labelling scheme; 30% thermal ellipsoid for non-hydrogen atoms are shown.

Compound **6** was reprotected as *N*-Cbz derivative **7**, then submitted to the 'non-oxidative' Pummerer reaction protocol.[22] As expected, treatment of **7** with trifluoroacetic anhydride (5 equiv.) and *sym*-collidine (3 equiv.) triggered a S_N2 displacement of the sulfinyl by a trifluoroacetoxy group, leading to the intermediate sulfenamide **8**. Treatment of **8** with an excess of $NaBH_4$, provided the β-amino alcohol (2*R*,3*S*)-**9** (Scheme 1) in a very clean manner (94%), with overall stereoselectivity >98/2 (the other diastereomer was not detected). In order to confirm the stereochemistry of **9**, this compound was treated with NaH and $PhCH_2Br$



Scheme 2.



Scheme 3. (i) $KMnO_4$, H_2SO_4 3N, acetone/H_2O, 0°C, 15 min. (ii) CH_2N_2, MeOH. (iii) $Pd(OH)_2/C$, H_2, MeOH.

affording the oxazolidinone **12**, whose J_{2-3}=3.6 Hz strongly suggests a *trans* disposition of the substituents, in full agreement with the expected stereochemistry. Conversion of (2*R*,3*S*)-**9** into the corresponding *O*-benzoate **10**, and oxidative cleavage of the double bond with $KMnO_4$ delivered the targeted enantiomerically pure γ-Tfm-GABOB (−)-(3*S*,4*R*)-**11**, orthogonally protected and potentially suitable for preparing novel fluorinated peptide analogs via coupling to other amino acids.

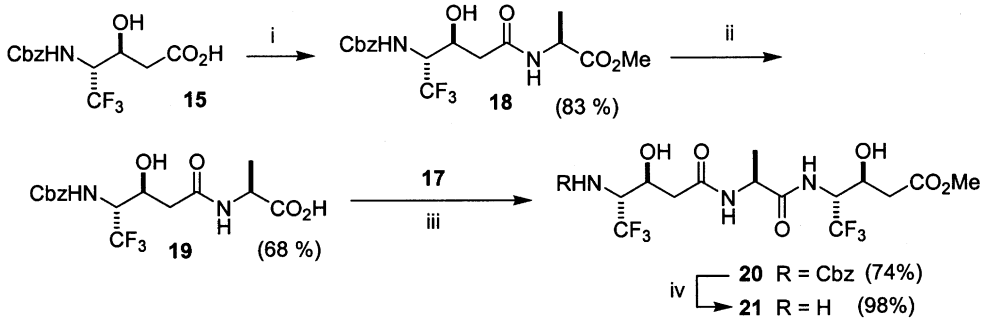
2.2. Synthesis of Tfm-pepstatin

First, we investigated the *N*-coupling of (Tfm)GABOB, since this step was suspected to be a difficult one, owing to the poor nucleophilicity of the amino group in **11** (Scheme 2). In fact, the dipeptide **13**, prepared by standard coupling of **11** to L-Ala-OPh, failed to undergo coupling with Cbz-Val-Val-OH under a variey of conditions (for example EDCI/HOBt or HATU/HOAt[23] in DMF–TMP at 0°C) and also with Val derived Leuchs anhydride (**14**).[24] However, a partial migration of the *O*-Bz group to the amino function was occasionally observed under coupling conditions. This suggested us that an unprotected β-OH group might favor the coupling.[25]

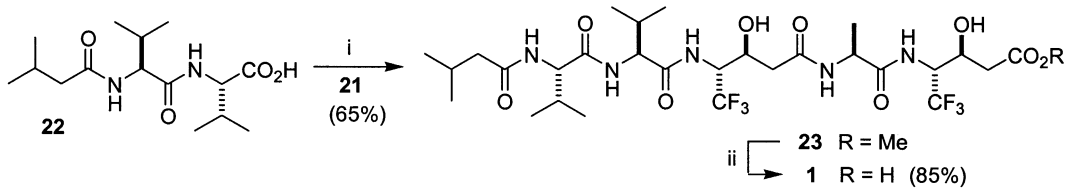
Therefore, we decided to re-undertake the synthesis of **1** starting from *O*-unprotected statine isosteres **15** and **17** (Scheme 3). The former was prepared from the olefin **9** by oxidative cleavage with $KMnO_4$, which occurred with excellent chemoselectivity. Next, the carboxylic acid **15** was treated with diazomethane, then the Cbz group of the resulting ester **16**, was hydrogenolyzed providing **17**.

As a strategy to accomplish the synthesis of **1**, we planned the assembling of the tripeptide fragment H-(Tfm)GABOB-Ala-(Tfm)GABOB with Iva-Val-Val-OH, therefore the synthesis of the fluorinated tripeptide **21** was undertaken first (Scheme 4). Coupling of **15** with H-Ala-OMe (HATU/HOAt, DMF–TMP) afforded **18**, which was hydrolyzed with LiOH to the corresponding acid **19**. The key N-coupling of **17** to **19** occurred efficiently, affording good yields of **20**, which was hydrogenolyzed to the H_2N-tripeptide **21**.

The final assembling of **21** with Iva-Val-Val-OH (**22**), prepared from commercial H-Val-Val-OH by standard solution-phase technique (82%), proved to be unexpectedly challenging (Scheme 5). In fact, under a variety of conditions (for example HATU/HOAt both in DMF and AcOEt, or *iso*-$BuCO_2Cl$/NMM in DMF) epimerization of the second Val unit took place, affording the target **23** as a mixture of diastereomers. This problem was solved by using the exact conditions reported by Bartlett for the synthesis of a phosphorus-containing analog of pepstatin (*iso*-$BuCO_2Cl$/NMM in AcOEt)[26] which provided the stereochemically pure pentapeptide **23**, that was hydrolyzed with LiOH in excellent yield to the final target pepstatin analog **1**.



Scheme 4. (i) H-Ala-OMe, HATU–HOAt, DMF–TMP. (ii) $LiOH.H_2O$, THF/H_2O. (iii) HATU–HOAt, DMF–TMP. (iv) $Pd(OH)_2/C$, H_2, MeOH.



Scheme 5. (i) *iso*-$BuCO_2Cl$, NMM, AcOEt, 4 days. (ii) DMSO–H_2O, $LiOH.H_2O$.

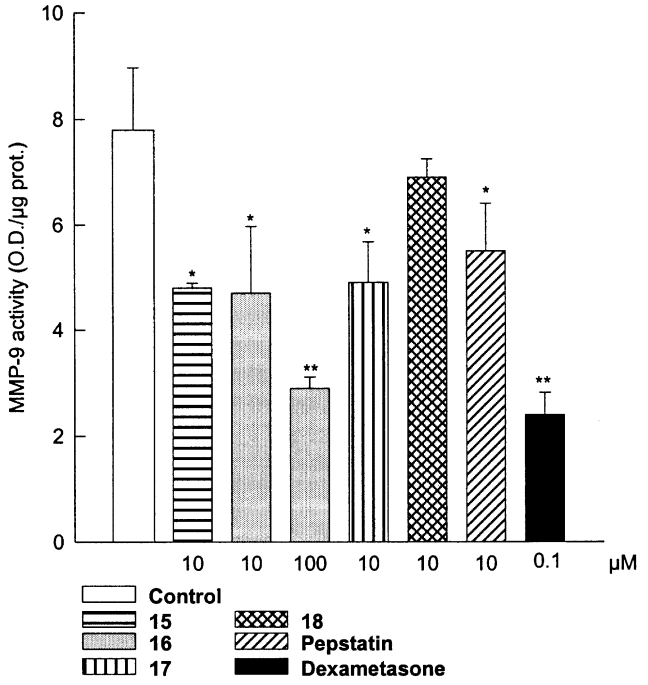


Figure 4. Effect of compounds on MMP-9 activity in human macrophages (Student's *t*-test: $^{*}p<0.05$; $^{**}p<0.01$ vs control).

3. Biological tests

3.1. Assays on HIV-1 protease

The target **1** was evaluated for its capacity to inhibit HIV-1 protease. This evaluation was carried out by measuring the effect of **1** on the rate of the proteolytic reaction in a fluorimetric assay using a recombinant HIV-1 protease and the fluorogenic substrate Abz-NF*-6 [Abz-Thr-Ile-Nle-Phe-(NO_2)-Gln-Arg-NH_2].[27] However, up to a concentration of 150 μM, compound **1** did not show any inhibition of the proteolytic activity.[28]

3.2. Assays on MMP-9

To study the effect on MMP-9 (gelatinase B) expression, we incubated human monocyte-derived macrophages for 24 h with the different compounds. The conditioned media were then collected and analyzed by gelatin-zymography to evaluate the potential gelatinolytic capacity of MMP-9. As shown in Fig. 4, several compounds inhibited MMP-9 gelatinolytic capacity at the concentrations tested. The analysis of the data obtained in three different experiments showed that the addition of natural pepstatin and the compounds **15**, **16**, **17** at 10 μM significantly inhibited MMP-9 gelatinolytic capacity at a similar level (about 40% inhibition). We tested compound **16** also at the concentration of 100 μM, and the data show a reduction of gelatinase B activity up to 60%.

To check if the inhibitory effect of the compounds was also due to a direct interference with the activation process of MMP-9, aliquots of conditioned media obtained after

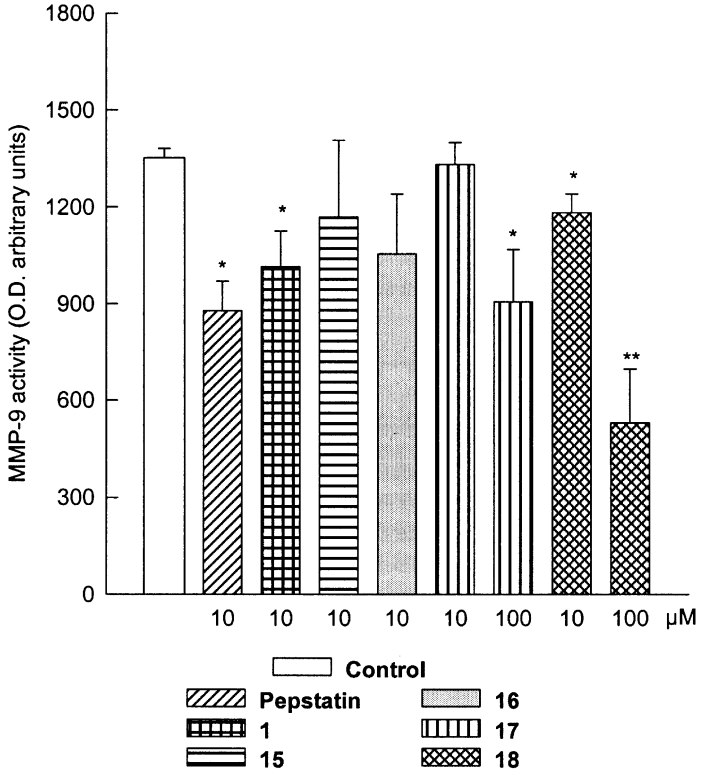


Figure 5. Effect of compounds on activity of secreted MMP-9 (Student's *t*-test: $^{*}p<0.05$; $^{**}p<0.01$ vs control).

incubation of human macrophages with DMEM alone were electrophoresed on gelatin containing gels. Then, the gels were cut in strips and the compounds added during the overnight activation step. The data reported in Fig. 5 show that pepstatin and compounds **1**, **17** and **18** display a statistically significant inhibitory effect in these experimental conditions, suggesting a direct interaction with the proteinase, affecting its activation. This demonstrates that the in vitro incubation of human macrophages with pepstatin and the Tfm-GABOB derivatives reduced the activity of MMP-9 (gelatinase B). The experiments showed that pepstatin and some of the Tfm-GABOB derivatives reduced MMP-9 total potential gelatinolytic capacity in gelatin-zymography. This effect is probably due to an inhibitory effect of the compounds on proteinase release from cells. However, our data on the secreted proteinase suggest that part of this effect is consequent to a direct inhibition of proteinase activity.

4. Conclusions

The challenging incorporation of an α-Tfm-amino β-hydroxy peptide isostere into a complex peptidic sequence has been successfully accomplished, producing the Tfm-pepstatin analogue **1** on a hundreds of milligrams scale and very good overall yield. The title compound, in which the P1 and P3′ *iso*-butyl residues of natural pepstatin are replaced by two Tfm groups, did not inhibit HIV-1 protease. The reasons for this drop of activity are presently unknown, but future work addressed to the preparation of further fluorinated derivatives of pepstatin might clarify this issue. However, the title compound **1**, as well as its precursors **15**–**18** were able to reduce the total potential gelatinolytic capacity of Gelatinase B (MMP-9) in a way comparable to that of pepstatin.

5. Experimental

5.1. General

Chemical shifts (δ) are reported in parts per million (ppm) of the applied field. Coupling constants (J) are reported in hertz. Me_4Si was used as internal standard (δ_H and δ_C=0.00) for ^1H and ^{13}C nuclei, while C_6F_6 was used as external standard (δ_F=162.90) for ^{19}F nuclei. Peak multiplicities are abbreviated as: singlet, s; doublet, d; triplet, t; quartet, q; multiplet, m; etc. Anhydrous solvents were obtained by distillation from sodium (THF, benzene) or from calcium hydride (dichloromethane, diisopropylamine). In all other cases commercially available reagent-grade solvents were employed without purification. Grignard reagents were purchased from Sigma/Aldrich/Fluka Company. Reactions performed in dry solvents were carried out under nitrogen atmosphere. Melting points are uncorrected and were obtained on a capillary apparatus. Analytical thin-layer chromatography (TLC) was routinely used to monitor reactions. Plates precoated with E. Merck silica gel 60 F_{254} of 0.25 mm thickness were used. Merck silica gel 60 (230–400 ASTM mesh) was employed for flash chromatography (FC).

5.2. Crystal data

$C_{13}H_{16}F_3NOS$, FW 291.33, Monoclinic, space group $P2_1$, a=10.575(x) Å, b=6.872(x) Å, c=10.754(x) Å, β=109.600(x), V=736.3(7) Å^3, Z=2, D_c=1.314 g cm^{-3}, μ=2.206 mm^{-1}, F(000)=304.

5.3. Data collection

X-Ray diffraction data were collected from a colourless prismatic crystal of **6** (size 0.2×0.2×0.1), with graphite monochromated CuKα radiation (λ=1.5418 Å) on a Siemens P4 diffractometer (θ–2θ scan technique). 1899 Reflections were collected ($4.35<\theta<58.03$; $+h,+k,+l$ and $-h$, $-k$, $-l$), 1611 unique; three standard reflections, measured every 100 reflections, showed no significant decay. Data were corrected for Lorentz and polarization effects and an empirical absorption correction was applied.

5.4. Structure analysis and refinement

The structure was solved by direct methods using SIR92,[29] and refined by full-matrix least squares on F2 using SHELXL-97.[30] Non-hydrogen atoms were refined anisotropically. The amine hydrogens were located by difference-Fourier technique and refined, while all the others have been included at calculated positions and refined with group temperature factors. Final values of the residual $R1$ for reflections with $I>2\sigma$ and for all reflections were 0.062 and 0.065, respectively. The highest peak and hole in the final difference-Fourier map were 0.346 and −0.352 e Å^3. The refined value of Flack's parameter was −0.03 (4).[31a]

Bond lengths and angles fall in the expected range.[31b] The hydrogen atoms found by Fourier-difference map indicate a tetrahedral geometry on N, as expected for amine compounds. An intermolecular hydrogen bonding is found, involving one of the amminic hydrogens and the oxygen atom (O–HA 2.246(1) Å, O–HA–N 151.2(3)°) but also a weak H···F interaction (F2···H13 2.596(1) Å, F2···H132–C13 164.2(2)°) may play a significant role in the crystal packing. One hydrogen atom on C4 is eclipsed with C6, which is consistent with the sp^2 hybridization on C5. The high thermal parameter and the corresponding elongated thermal ellipsoid of atom C6 suggest disorder involving this atom. In the crystal, the molecules of **6** pack arranging the aromatic rings of adjacent molecules with a favourable herring bone interaction geometry.

5.4.1. Synthesis of 3-[(4-methylphenyl)sulfinyl]-1,1,1-trifluoro-2-(*N*-4-methylphenyl)-hex-5-enyl-2-amines 4 and 5. To a stirred solution of LDA (1.2 mmol) in dry THF (1 mL) and cooled to −70°C, a solution of (*R*)-*p*-tolyl sulfoxide **2**[16] (1.0 mmol, 194 mg) in 2.5 mL of dry THF was added dropwise at the same temperature under nitrogen atmosphere. Then, a solution of *N*-(2,2,2-trifluoroethylidene)-*p*-methoxyaniline **3**[16] (1.2 mmol, 244 mg) in 2.5 mL of dry THF was added dropwise. After 5 min, TLC monitoring (*n*-Hex/AcOEt 8:2) showed that the reaction was complete. The reaction was quenched at −70°C with aqueous NH_4Cl, extracted with AcOEt and the collected organic phases were dried over anhydrous Na_2SO_4, filtered and the solvent was removed in vacuo.

After FC (*n*-Hex/AcOEt 9:1) **4** and **5** were isolated as a mixture (yield 98%). The diastereoselectivity of the reaction was determined from ^{1}H- and ^{19}F NMR analyses of the crude reaction product and gave 2.75:1.0 ratio of diastereomers **5**:**4**, plus traces (<5%) of a third diastereomer, whose configuration was not assessed.

(2*S*,3*R*,*R*$_S$)-5: R_f 0.35; ^{1}H NMR ($CDCl_3$) δ: 2.2–2.5 (2H, m), 2.40 (3H, br s), 3.09 (1H, dt, J=2.5 and 5.6 Hz), 3.74 (3H, br s), 4.14 (1H, d, J=9.0 Hz), 4.79 (1H, ddq, J=9.0, 2.5 and 7.9 Hz), 4.99 and 5.01 (2H, m), 5.54 (1H, m), 6.78, 6.85, 7.31 and 7.59 (8H, m), ^{19}F NMR ($CDCl_3$) δ: −72.73 (br d, J=7.4 Hz); ^{13}C NMR ($CDCl_3$) δ: 21.5, 28.4, 55.5 (q, $J_{C,F}$=28.5 Hz), 55.6, 64.7, 114.8, 116.5, 118.5, 126.0 (q, $J_{C,F}$=286.4 Hz), 126.0, 130.2, 138.7, 139.8, 143.0, 153.6, 133.6.

(2*R*,3*S*,*R*$_S$)-4: (a diastereomerically pure sample could be purified) R_f 0.35; $[\alpha]_D^{20}$=+53.3 (*c* 1.2, $CHCl_3$); mp 134–135°C (diisopropylether); FT-IR (film) (cm^{-1}) 3342, 2934, 1516, 1241, 1170, 1124, 1037; ^{1}H NMR ($CDCl_3$) δ: 2.37 (3H, br s), 2.61 and 2.73 (2H, m), 3.25 (1H, dt, J=3.2 and 6.1 Hz), 3.76 (3H, s), 3.80 (1H, d, J=9.0 Hz), 4.33 (1H, ddq, J=9.0, 3.2 and 7.4 Hz), 5.22 and 5.24 (2H, m), 5.93 (1H, ddt, J=17.0, 10.2 and 6.9 Hz), 6.48, 6.75, 7.16 and 7.38 (8H, m); ^{19}F NMR ($CDCl_3$) δ: −73.23 (br d, J=7.4 Hz); ^{13}C NMR ($CDCl_3$) δ: 21.4, 27.6, 53.7 (q, $J_{C,F}$=29.0 Hz), 55.6, 63.4, 114.6, 115.3, 118.9, 125.1, 125.7 (q, $J_{C,F}$=285.8 Hz), 129.8, 134.0, 136.8, 139.0, 142.3, 153.3. MS (DIS EI 70 eV) *m*/*z* (%): 398 [$(M+H)^+$] (25), 397 [$(M)^{+\cdot}$] (100), 258 [$C_{13}H_{15}NOF_3^+$] (22), 257 [$C_{13}H_{14}NOF_3^{+\cdot}$] (92). Anal calcd for $C_{20}H_{22}F_3NO_2S$: C, 60.44; H, 5.58; N, 3.52. Found: C, 60.40; H, 5.60; N, 3.49.

5.4.2. Synthesis of 3-[(4-methylphenyl)sulfinyl]-1,1,1-trifluoro-hex-5-enyl-2-amine (6). A solution of **4** and **5** mixture (1.29 mmol, 514 mg) in acetonitrile (27 mL) was cooled to 0°C and a solution of CAN (2.09 mmol, 115 mg) in water (2 mL) was added dropwise. After 45 min at 0°C (TLC monitoring), a 5% aqueous $NaHCO_3$ solution was added at the same temperature until pH 5, and the resulting mixture was stirred at rt for 15 min. Then, solid Na_2SO_3 was added portionwise until the yellow slurry became light brown. The mixture was diluted with water (50 mL) and extracted with AcOEt (3×25 mL). The combined organics were dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated in vacuo. After FC purification (CH_2Cl_2/diisopropylether 7:3 and CH_2Cl_2/diisopropylether/TEA 7:3:1) **6** was isolated in 66% overall yield (from **2** and **3**).

(2*S*,3*R*,*R*$_S$)-6: R_f 0.35; $[\alpha]_D^{20}$=+162.8 (*c* 0.5, $CHCl_3$); mp 73–74°C (diisopropylether); FT-IR (nujol) (cm^{-1}): 3380, 2855, 2925, 1640, 1460, 1378, 1260; ^{1}H NMR ($CDCl_3$) δ: 2.43 (3H, br s), 2.61 (2H, dd, J=7.0 and 6.6 Hz), 2.77 (2H, br signal), 2.89 (1H, dt, J=1.8 and 6.6 Hz), 3.95 (1H, dq, J=1.8 and 8.3 Hz), 5.13 and 5.16 (2H, m), 5.75 (1H, m), 7.37 and 7.53 (4H, m); ^{19}F NMR ($CDCl_3$) δ: −75.38 (br d, J=8.3 Hz); ^{13}C NMR ($CDCl_3$) δ: 21.4, 28.1, 51.2 (q, $J_{C,F}$=30.4 Hz); 62.6, 118.9, 125.2, 125.4 (q, $J_{C,F}$=282.5 Hz), 130.2, 133.3, 138.0, 142.5; MS (DIS EI 70 eV) *m*/*z* (%): 292 [$(M+H)^+$] (50), 291 [$(M)^{+\cdot}$] (28), 192 [$C_{11}H_{12}SO^+$] (100). Anal calcd for $C_{13}H_{16}F_3NOS$: C, 53.60; H, 5.54; N, 4.81. Found: C, 53.57; H, 5.52; N, 4.84. A crystalline sample of (2*S*,3*R*,*R*$_S$)-**6** was used for the determination of the absolute stereochemistry by X-ray diffraction.

5.4.3. Synthesis of 3-[(4-methylphenyl)sulfinyl]-1,1,1-trifluoro-2-(*N*-carbobenzoxy)-hex-5-enyl-2-amine (7). To a solution of **6** (0.447 mmol, 130 mg) in dioxane (7.5 mL) a 50 wt% solution of K_2CO_3 (130 μL) was added, followed by dropwise addition of neat benzyl chloroformiate (127 μL) under stirring. The mixture was heated to 50°C and stirring was continued at the same temperature for 40 min (TLC monitoring). Then the reaction mixture was diluted with water (10 mL) and extracted with AcOEt (3×10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated in vacuo. After FC purification (*n*-Hex/AcOEt 75:25) (2*S*,3*R*,*R*$_S$)-**7** was isolated in 98% yield: R_f 0.35; $[\alpha]_D^{20}$=+154.1 (*c* 1.1, $CHCl_3$); mp 86–88°C (diisopropylether); FT-IR (KBr) (cm^{-1}): 3273, 3041, 2953, 1730, 1539, 1446, 1311, 1287, 1237; ^{1}H NMR ($CDCl_3$) δ: 2.27 and 2.33 (2H, m), 2.43 (3H, br s), 2.89 (1H, dt, J=2.5 and 7.2 Hz), 4.98 (1H, ddq, J=10.0, 2.5 and 8.5 Hz), 5.13 and 5.15 (2H, m), 5.13 and 5.18 (2H, brd, J=12.1 Hz), 5.57 (1H, m), 6.26 (1H, d, J=10.0 Hz), 7.2–7.7 (9H, m); ^{19}F NMR ($CDCl_3$) δ: −72.32 (3F, brd, J=8.5 Hz); ^{13}C NMR ($CDCl_3$) δ: 21.6, 29.3, 51.7 (q, $J_{C,F}$=31.0 Hz); 63.4, 67.6, 119.8, 124.9 (q, $J_{C,F}$=282.5 Hz); 125.7, 128.3, 128.3, 128.6, 130.3, 135.9, 138.2, 143.2, 132.6, 144.4; MS (DIS EI 70 eV) *m*/*z* (%): 426 [$(M+H)^+$] (22), 425 [M^+] (12), 286 [$C_{14}H_{15}NO_2F_3^+$] (58), 214 [$C_{14}H_{14}O_2^{+\cdot}$] (17), 194 [$C_{11}H_{14}SO^{+\cdot}$] (22). Anal calcd for $C_{20}H_{22}F_3NO_3S$: C, 58.10; H, 5.36; N, 3.39. Found: C, 58.13; H, 5.33; N, 3.38.

5.4.4. Synthesis of 2-(*N*-carbobenzoxy)amino-1,1,1-trifluoro-hex-5-en-3-ol (9). To a stirred solution of **7** (0.325 mmol, 138 mg) and *sym*-collidine (0.974 mmol, 130 μL) in acetonitrile (10 mL) stirred under nitrogen atmosphere at 0°C, neat trifluoroacetic anhydride (1.623 mmol, 325 μL) was added dropwise. The reaction mixture was stirred at 0°C and after 10 min the reaction was quenched with water and the organics were extracted with AcOEt (3×10 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and the solvent was removed in vacuo. The residue containing the intermediate sulfenamide **8** was dissolved in a 4:1 vol. THF/H_2O solution (47 mL), cooled to 0°C and $NaBH_4$ (1.944 mmol, 74 mg) was added portionwise at the same temperature. Then the mixture was allowed to warm to rt and, after 15 min, the reaction was quenched with a saturated aqueous NH_4Cl solution, extracted with AcOEt and the combined organic layers dried over anhydrous Na_2SO_4, filtered and the solvent was removed in vacuo. FC (*n*-Hex/AcOEt 9:1) afforded (2*R*,3*S*)-**9** in 94% yield: R_f 0.35; $[\alpha]_D^{20}$=−22.4 (*c* 1.0, $CHCl_3$); oil; FT-IR (film) (cm^{-1}): 3434, 2927, 1717, 1517, 1288; ^{1}H NMR ($CDCl_3$) δ: 2.2–2.4 (3H, m), 4.13 (1H, dt, J=1.2 and 6.9 Hz), 4.25 (1H, ddq, J=10.2, 1.2 and 8.0 Hz), 5.13 and 5.17 (2H, m), 5.14 (2H, brs), 5.65 (1H, d, J=10.2 Hz), 5.74 (1H, m), 7.2–7.5 (5H, m); ^{19}F NMR ($CDCl_3$) δ: −75.25 (brd, J=8.0 Hz); ^{13}C NMR ($CDCl_3$) δ: 35.6, 54.7 (q, $J_{C,F}$=29.5 Hz); 67.0, 67.6, 119.6, 124.9 (q, $J_{C,F}$=283.0 Hz), 128.1, 128.4, 128.6, 132.5, 135.8, 156.2; MS (DIS EI 70 eV) *m*/*z* (%): 304 [$(M+H)^+$] (40), 303 [$M^{+\cdot}$] (15), 233 [$C_{10}H_{10}NO_2F_3^{+\cdot}$] (4). Anal calcd

for $C_{14}H_{16}F_3NO_3$: C, 55.44; H, 5.32; N, 4.62. Found: C, 55.40; H, 5.33; N, 4.60.

5.4.5. Synthesis of 3-[2-*N*-(carbobenzoxy)amino-1,1,1-trifluoro-hex-5-enyl] benzoate (10). To a solution of **9** (0.33 mmol, 100 mg) in CH_2Cl_2 (2.5 mL), neat benzoic acid (0.363 mmol, 44 mg) was added, followed by DCC (0.363 mmol, 75 mg) and DMAP (0.033 mmol, 4 mg). The white slurry was stirred at 0°C for 21 h, then the dicyclohexylurea was filtered off on a Celite pad, the filtrate was evaporated in vacuo and the crude was purified by FC (*n*-Hex/AcOEt 95:5) to give (2*R*,3*S*)-**10** in 98% yield: R_f 0.35; $[\alpha]_D^{20}=-54.4$ (*c* 1.3, $CHCl_3$); oil; FT-IR (film) (cm^{-1}): 3339, 2926, 2855, 1730, 1530, 1454; ^1H NMR ($CDCl_3$) δ: 2.45 and 2.53 (2H, m), 4.63 (1H, ddq, *J*=10.3, 2.2 and 7.8 Hz), 5.12 and 5.15 (2H, m), 5.14 (2H, brs), 5.45 (1H, d, *J*=10.3 Hz), 5.62 (1H, dt, *J*=2.2 and 6.9 Hz), 5.78 (1H, m), 7.2–8.1 (10H, m); ^{19}F NMR ($CDCl_3$) δ: −75.32 (brd, *J*=7.8 Hz); ^{13}C NMR ($CDCl_3$) δ: 35.8, 53.7 (q, $J_{C,F}$=31.5 Hz); 67.9, 69.4, 120.2, 124.2 (q, $J_{C,F}$= 284.0 Hz), 128.3, 128.5, 128.6, 128.8, 129.2, 129.6, 131.1, 135.6, 133.4, 155.9, 164.9; MS (DIS EI 70 eV) *m/z* (%): 408 $[(M+H)^+]$ (18), 407 $[M^{+\cdot}]$ (10), 286 $[C_{14}H_{15}NO_2F_3^+]$ (8), 285 $[C_{14}H_{14}NO_2F_3^{+\cdot}]$ (15), 196 $[C_7H_9NO_2F_3^+]$ (16). Anal calcd for $C_{21}H_{20}F_3NO_4$: C, 61.91; H, 4.95; N, 3.44. Found: C, 61.85; H, 4.93; N, 3.44.

5.4.6. Synthesis of 4-(*N*-carbobenzoxy)amino-3-(*O*-benzoyl)hydroxy-5,5,5-trifluoropentanoic acid (11). To a solution of **10** (0.196 mmol, 80 mg) in acetone (3.1 mL), cooled to 0°C, a solution of $KMnO_4$ (1.238 mmol, 196 mg) in water (6 mL) was added dropwise. The reaction mixture was stirred at the same temperature for 30 min, then solid $NaHSO_3$ was added portionwise until the solution became clear and brown MnO_2 precipitated. After filtration to remove the precipitate, the filtrate was extracted with AcOEt (3×5 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue was purified by FC ($CHCl_3$/AcOEt/CH_3CO_2H 8:2:0.1 to give (3*S*,4*R*)-**11** in 89% yield: R_f 0.35; $[\alpha]_D^{20}=-37.4$ (*c* 1.2, $CHCl_3$); oil; FT-IR (film) (cm^{-1}): 3318, 2963, 1729, 1524, 1409, 1287, 1147, 1093, 775, 718; ^1H NMR ($CDCl_3$) δ: 2.81 (1H, dd, *J*=17.0 and 7.7 Hz), 2.88 (1H, dd, *J*=17.0 and 5.9 Hz), 4.82 (1H, ddq, *J*=10.5, 2.3 and 7.4 Hz), 5.15 and 5.18 (2H, brd, *J*= 11.9 Hz), 5.32 (1H, d, *J*=10.5 Hz), 5.89 (1H, ddd, *J*=7.7, 5.9 and 2.3 Hz), 7.2–7.5 (5H, m), 7.41, 7.58 and 7.95 (15H, m), 9.10 (1H, br signal); ^{19}F NMR ($CDCl_3$) δ: −74.83 (m); ^{13}C NMR ($CDCl_3$) δ: 35.3, 54.3 (q, $J_{C,F}$=30.8 Hz); 66.7, 68.2, 124.0 (q, $J_{C,F}$=283.0 Hz), 128.4, 128.6, 128.6, 128.7, 128.8, 129.7, 133.7, 135.3, 156.2, 164.8, 173.8; MS (DIS EI 70 eV) *m/z* (%): 426 $[(M+H)^+]$ (22), 382 $[C_{19}H_{19}NO_4F_3^+]$ (10), 318 $[C_{13}H_{11}NO_5F_3^+]$ (6), 214 $[C_{14}H_{14}O3^{+\cdot}]$ (38). Anal calcd for $C_{21}H_{18}F_3NO_6$: C, 57.67; H, 4.15; N, 3.20. Found: C, 57.70; H, 4.13; N, 3.22.

5.4.7. Synthesis of 3-(*N*-benzyl)-5-(2-propenyl)-4-trifluoromethyl oxazolidinone (12). A solution of (2*R*,3*S*)-**9** (0.33 mmol, 100 mg) in dry THF (3 mL) and DMF (1.5 mL) was added dropwise to a slurry of NaH (0.561 mmol, 17 mg—80% in *n*-hexane) in dry THF (1 mL) cooled to 0°C. Neat benzylbromide (0.66 mmol, 78 μL) was added by syringe at the same temperature, then the slurry was stirred at 0°C for 20 min, then the reaction mixture was diluted with water (0.5 mL) and the organics were extracted with AcOEt (3×1 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the solvent was removed in vacuo. The residue was purified by FC (*n*-Hex/AcOEt 9:1) to give (4*R*,5*S*)-**12** in 93% yield: R_f 0.35; $[\alpha]_D^{20}=-70.6$ (*c* 0.9, $CHCl_3$); mp=56–57°C (diisopropylether); FT-IR (KBr) (cm^{-1}): 3423, 2930, 2364, 1748, 1414, 1282; ^1H NMR ($CDCl_3$) δ: 2.27 and 2.33 (2H, m), 3.58 (1H, dq, *J*=3.5 and 6.5 Hz), 4.06 and 5.06 (2H, brd, *J*=14.8 Hz), 4.55 (1H, ddd, *J*=6.5, 5.5 and 3.5 Hz), 4.91 and 5.02 (2H, m), 5.50 (1H, m), 7.2–7.5 (5H, m); ^{19}F NMR ($CDCl_3$) δ: −76.49 (br d, *J*=6.5 Hz); ^{13}C NMR ($CDCl_3$) δ: 38.6, 47.3, 57.9 (q, $J_{C,F}$=31.7 Hz), 72.2, 121.2, 128.9, 124.3 (q, $J_{C,F}$= 283.5 Hz), 128.7, 128.8, 129.1, 134.4, 157.0; MS (DIS EI 70 eV) *m/z* (%): 286 $[(M+H)^+]$ (44), 285 $[M^{+\cdot}]$ (40), 174 $[C_7H_6NO_2F_2^+]$ (5), 149 $[C_8H_7NO_2^{+\cdot}]$ (10), 104 $[C_7H_6N^+]$ (28), 91 $[C_7H_7^+]$ (100). Anal calcd for $C_{14}H_{14}F_3NO_2$: C, 58.95; H, 4.95; N, 4.91. Found: C, 58.99; H, 4.93; N, 4.90.

5.4.8. Synthesis of 4-(*N*-carbobenzoxy)amino-3-hydroxy-5,5,5-trifluoropentanoic acid (15). A solution of $KMnO_4$ (156 mg, 0.99 mmol) in H_2O (4.8 mL) was added dropwise to a stirred and cooled (0°C) solution of 2-(*N*-carbobenzyloxy)amino-1,1,1-trifluoro-hex-5-en-3-ol (**9**) (50 mg, 0.165 mmol) and H_2SO_4 (328 μl, $3N_{aq.}$ solution) in acetone (2.7 mL). The reaction was monitored by TLC ($CHCl_3$/AcOEt/CH_3CO_2H=7:3:0.1) and, after 5 min, neat $NaHSO_3$ was added up to total disappearance of the colour, then the organics were extracted with ethyl acetate (3×5 mL). The combined extracts were dried over anhydrous Na_2SO_4, filtered, then the solvent was removed in vacuo to give a residue which was purified by FC ($CHCl_3$/AcOEt/CH_3CO_2H 5:5:0.1) to give (3*S*,4*R*)-**7** in 70% yield: R_f 0.35; $[\alpha]_D^{20}=-18.0$ (*c* 0.9, MeOH); mp=141–142°C (AcOEt); FT-IR (KBr) (cm^{-1}): 3436, 2487, 1721, 1658, 1459, 1342, 1265; ^1H NMR (CD_3OD+D_2O) δ: 2.50 (2H, d, *J*=6.8 Hz), 4.40 (1H, dq, *J*=1.7 and 8.0 Hz), 4.48 (1H, dt, *J*=1.7 and 6.8 Hz), 5.12 and 5.17 (2H, brd, *J*=12.8 Hz), 7.2–7.5 (5H, m); ^{19}F NMR (CD_3OD) δ: −71.93 (3F, brd, *J*=8.0 Hz); ^{13}C NMR (CD_3OD), δ: 39.6, 56.9 (q, $J_{C,F}$=29.0 Hz), 65.7, 68.2, 125.7 (q, $J_{C,F}$=281.1 Hz), 128.9, 129.2, 129.5, 137.9, 158.7, 174.2; MS (DIS EI 70 eV) *m/z* (%): 322 $[(M+H)^+]$ (22), 321 $[M^{+\cdot}]$ (21), 278 $[C_{12}H_{15}NO_3F_3^+]$ (23), 233 $[C_{10}H_{10}NO_2F_3^{+\cdot}]$ (12), 172 $[C_5H_7O_3F_3^{+\cdot}]$ (13), 91 $[C_7H_7^+]$ (100). Anal calcd for $C_{13}H_{14}F_3NO_5$: C, 48.60; H, 4.39; N, 4.36. Found: C, 48.55; H, 4.40; N, 4.39.

5.4.9. Synthesis of Cbz-(Tfm)GABOB-OMe (16). An ethereal solution of diazomethane (30 mL, about 0.5 N) was added dropwise to a cooled (0°C) solution of Cbz-(Tfm)GABOB-OH (**15**) (800 mg, 2.49 mmol) in methanol (20 mL). The reaction was monitored by TLC (*n*-Hex/AcOEt=4:6) and after 10 min, some drops of neat CH_3CO_2H were added to the reaction mixture until the solution became colourless. The solvents were evaporated to dryness (CH_3CO_2H stripped with benzene in vacuo) and the residue was purified by FC (Hex/AcOET 7:3) to give Cbz-(Tfm)GABOB-OMe (**16**) (774 mg, 93% yield): R_f 0.35 (*n*-Hex/AcOEt=7:3); $[\alpha]_D^{20}=-28.9$ (*c* 1.1 in $CHCl_3$); mp 143–144°C (diisopropylether); ^1H NMR ($CDCl_3$) δ: 2.51 (dd, *J*=16.9 and 3.7 Hz, 1H), 2.61 (dd, *J*=16.9 and 9.5 Hz, 1H), 3.32 (q, *J*=3.5 Hz, 1H), 3.71 (s, 3H), 4.23

(ddq, J=10.1, 1.5 and 7.7 Hz, 1H), 4.57 (dddd, J=9.5, 3.7, 3.5 and 1.5 Hz, 1H), 5.16 (br s, 2H), 5.60 (d, J= 10.1 Hz, 1H), 7.3–7.5 (m, 5H); ^{19}F NMR ($CDCl_3$) δ: −75.06 (br d, J=7.7 Hz); ^{13}C NMR ($CDCl_3$) δ: 37.9, 52.2, 55.4 (q, $J_{C,F}$=29.5 Hz); 64.4, 67.7, 124.4 (q, $J_{C,F}$= 283.0 Hz); 128.2, 128.5, 128.7, 135.7, 156.2, 172.3; FT-IR (KBr) (cm^{-1}): 1732, 1691, 1524, 1442, 1321, 1294; MS (DIS EI, 70 eV) m/z (%): 336 [$(M+H)^+$, 100], 292 [$(M+H-CO_2)^+$, 22], 181 ($C_6H_6F_3NO_2^{+\cdot}$, 20).

5.4.10. Synthesis of H-(Tfm)GABOB-OMe (17). Solid $Pd(OH)_2$/C (ca. 50 mg) was added to a stirred solution of Cbz-(Tfm)GABOB-OMe (**16**) (897 mg, 2.68 mmol) in MeOH (36 mL) and the reaction mixture was kept under stirring and H_2 atmosphere at rt for 45 min. After TLC monitoring (*n*-Hex/AcOEt=1:1), the catalyst was filtered off, the filtrate was evaporated in vacuo and the white residue was purified by fractional crystallization (diisopropylether) to give H-(Tfm)GABOB-OMe (**17**) (454 mg, 93% yield) in pure form: R_f 0.35 (*n*-Hex/AcOEt 1:1); $[\alpha]_D^{20}$= −0.6 (*c* 1.0 in $CHCl_3$); $[\alpha]_D^{20}$=+4.1 (*c* 1.0 in CH_3OH); mp 65–67°C (diisopropylether); ^{1}H NMR ($CDCl_3$) δ: 2.00 (br signal, 3H), 2.59 (dd, J=16.5 and 3.9 Hz, 1H), 2.79 (dd, J=16.5 and 8.8 Hz, 1H), 3.14 (dq, J=3.0 and 7.7 Hz, 1H), 3.73 (q, 3H), 4.36 (ddd, J=8.8, 3.9 and 3.0 Hz, 1H); ^{19}F NMR ($CDCl_3$) δ: −76.70 (br d, J=7.7 Hz); ^{13}C NMR ($CDCl_3$) δ: 38.4, 52.0, 56.5 (q, $J_{C,F}$=27.8 Hz), 65.1, 125.9 (q, $J_{C,F}$=281.8 Hz), 172.6; FT-IR (KBr) (cm^{-1}): 3408, 3318, 3124, 1731, 1603, 1443, 1343, 1294; MS (DIS EI, 70 eV) m/z (%): 202 [$(M+H)^+$, 27], 183 [$(M-H_2O)^{+\cdot}$, 8], 170 [$(M+H-CH_3OH)^+$, 15], 152 ($C_5H_5F_3NO^+$, 42), 128 ($C_3H_5F_3NO^+$, 18), 79 ($C_2H_3F_2N^{+\cdot}$, 100).

5.4.11. Synthesis of Cbz-(Tfm)GABOB-Ala-OMe (18). TMP (4.67 mmol, 620 μL) was added to a stirred solution of H-Ala-OMe.HCl (1.56 mmol, 220 mg) and Cbz-(Tfm)GABOB-OH (**15**) (1.56 mmol, 500 mg) in DMF (stored overnight over 3 Å molecular sieves—13 mL). The reaction mixture was cooled to 0°C and HOAt (1.56 mmol, 210 mg) and HATU (1.56 mmol, 600 mg) were added. The temperature was allowed to raise to rt and the stirring was continued for 2 h. After TLC monitoring (*n*-Hex/AcOEt 4:6), a 1N aqueous HCl solution was added, the organics were extracted with AcOEt (3×20 mL) and the combined organic layers were washed with a 5% aqueous $NaHCO_3$ solution, a saturated aqueous NaCl solution and dried over anhydrous Na_2SO_4. After filtration and solvent removal in vacuo, the residue was purified by fractional crystallization (diisopropylether) to give pure Cbz-(Tfm)GABOB-Ala-OMe (**18**) (530 mg, 83% yield): R_f 0.35 (*n*-Hex/AcOEt 1:1); $[\alpha]_D^{20}$=−19.3 (*c* 0.7 in $CHCl_3$); mp 138–139°C (diisopropylether); ^{1}H NMR ($CDCl_3$) δ: 1.39 (d, J=7.3 Hz, 3H), 2.38 (dd, J=15.2 and 4.0 Hz, 1H), 2.48 (dd, J=15.2 and 8.8 Hz, 1H), 3.75 (s, 3H), 4.28 (ddq, J=10.1, 1.5 and 7.8 Hz, 1H), 4.48 (br s, 1H), 4.53 (ddd, J=8.8, 4.0 and 1.5 Hz, 1H), 4.55 (dq, J=7.4 and 7.3 Hz, 1H), 5.16 (br s, 2H), 5.72 (d, J=10.1 Hz, 1H), 6.62 (d, J=7.4 Hz, 1H), 7.3–7.5 (m, 5H); ^{19}F NMR ($CDCl_3$) δ: −74.79 (br d, J=7.8 Hz); ^{13}C NMR ($CDCl_3$) δ: 17.9, 39.2, 48.2, 52.7, 55.7 (q, $J_{C,F}$=29.6 Hz), 65.1, 67.8, 124.4 (q, $J_{C,F}$=282.3 Hz), 128.1, 128.3, 128.6, 135.8, 156.5, 170. 9, 173.0; IR (KBr) (cm^{-1}): 3307, 1737, 1699, 1626, 1536, 1251; MS (DIS EI, 70 eV) m/z (%): 406 ($M^{+\cdot}$, 10), 388 [$(M-H_2O)^{+\cdot}$, 4], 347 [$(M-COOCH_3)^+$, 12], 299 ($C_{10}H_{14}F_3N_2O_5^+$, 18). Anal. Calcd for $C_{16}H_{21}F_3N_2O_6$: C, 48.73; H, 5.37; N, 7.10. Found: C, 48.76; H, 5.33; N, 7.06.

5.4.12. Synthesis of Cbz-(Tfm)GABOB-Ala-OH (19). A solution of LiOH (0.862 mmol, 20 mg) in water (3 mL) was added to a stirred solution of Cbz-(Tfm)GABOB-Ala-OMe (**18**) (0.493 mmol, 200 mg) in a THF/H_2O=5:1 mixture (18 mL). The reaction mixture was stirred at rt for 30 min, then monitored by TLC (*n*-Hex/AcOEt 4:6), diluted with water, added with a 1N aqueous HCl solution up to pH 1 and the organics were extracted with AcOEt (3×10 mL). The combined organic layers were dried over anhydrous Na_2SO_4. After filtration and solvent removal in vacuo, the residue was purified by FC (cyclohexane/AcOEt/CH_3CO_2H=2:8:0.1) to give pure Cbz-(Tfm)GABOB-Ala-OH (**19**) (50 mg, 68% yield): R_f 0.35 (cyclohexane/AcOEt/CH_3CO_2H=2:8:0.1); $[\alpha]_D^{20}$=−35.4 (*c* 0.8 in MeOH); mp 155–156°C (AcOEt); ^{1}H NMR (CD_3OD) δ: 1.38 (d, J=7.3 Hz, 3H), 2.40 (dd, J=14.6 and 5.4 Hz, 1H), 2.45 (dd, J=14.6 and 7.9 Hz, 1H), 4.38 (q, J=7.3 Hz, 1H), 4.39 (dq, J=1.5 and 8.1 Hz, 1H), 4.49 (ddd, J=7.9, 5.4 and 1.5 Hz, 1H), 5.14 (br s, 2H), 7.2–7.5 (m, 5H); ^{19}F NMR (CD_3OD) δ: −72.08 (br d, J=8.1 Hz); ^{13}C NMR (CD_3OD) δ: 17.6, 41.3, 49.4, 57.2 (q, $J_{C,F}$=28.7 Hz), 66.2, 68.4, 126.4 (q, $J_{C,F}$=282.0 Hz), 129.0, 129.2, 129.5, 137.9, 158.8, 172.6, 176.0; FT-IR (KBr) (cm^{-1}): 3415, 3350, 1749, 1666, 1541.8, 1459, 1400, 1341, 1273; MS (DIS EI, 70 eV) m/z (%): 393 [$(M+H)^+$, 63], 392 ($M^{+\cdot}$, 2), 374 [$(M-H_2O)^{+\cdot}$, 9], 349 [$(M+H-CO_2)^+$, 32], 285 ($C_9H_{12}F_3N_2O_5^+$, 21), 91 ($C_7H_7^+$, 100). Anal. Calcd for $C_{15}H_{23}F_3N_2O_6$: C, 46.87; H, 6.03; N, 7.29. Found: C, 46.86; H, 6.05; N, 7.29.

5.4.13. Synthesis of Cbz-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (20). To a solution of **19** (600 mg, 1.53 mmol) and **17** (308 mg, 1.53 mmol) in DMF (13 mL, stored overnight over 3 Å molecular sieves), TMP (371 mg, 0.406 mL, 3.06 mmol) was added and the resulting mixture was cooled with an ice-water bath. HATU (582 mg, 1.53 mmol) and HOAt (208 mg, 1.53 mmol) were added and the mixture was stirred for 25 min. A 1 M solution of HCl was added until pH 1–2 was reached, and the reaction mixture was extracted with AcOEt (3×10 mL). The collected organic layers were washed with a 5% aqueous $NaHCO_3$ solution, and then with brine. After drying over anhydrous Na_2SO_4, and filtration, the solvent was removed in vacuo, and the residue was purified by FC (*n*-Hex/AcOEt 1/1) to give Cbz-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (**20**) (650 mg, 74% yield), which was crystallized from *n*-Hex/AcOEt 1/1: R_f 0.35 (*n*-Hex/AcOEt=1:1); $[\alpha]_D^{20}$=−52.0 (*c* 1.0 in MeOH); mp 205–206°C (*n*-Hex/AcOEt=1:1); ^{1}H NMR (acetone-d_6) δ: 1.33 (d, J=7.2 Hz, 3H), 2.49 (d, J=6.6 Hz, 2H), 2.51 (d, J=6.7 Hz, 2H), 3.62 (s, 3H), 4.45 (ddq, J=9.9, 1.6 and 8.2 Hz, 1H), 4.48 (m, 1H), 4.50 (dq, J=6.6 and 7.2 Hz, 1H), 4.57 (m, 1H); 4.70 (ddq, J=9.9, 1.6 and 8.2 Hz, 1H), 4.76 (d, J=5.8 Hz, 1H), 4.86 (d, J=4.9 Hz, 1H), 5.13 (br d, J=12.5 Hz, 1H), 5.16 (br d, J=12.5 Hz, 1H), 6.72 (d, J=9.9 Hz, 1H), 7.25–7.50 (m, 5H), 7.56 (d, J=9.9 Hz, 1H), 7.65 (d, J=6.6 Hz, 1H); ^{19}F NMR (acetone-d_6) δ: −71.72 (br d, J=8.2 Hz, 3F), −71.41 (br d, J=8.2 Hz, 3F); ^{13}C NMR (acetone-d_6) δ: 18.1, 39.1, 40.1, 50.3, 51.8, 53.8 (q, $J_{C,F}$=28.7 Hz), 56.6 (q, $J_{C,F}$=28.7 Hz),

65.3, 66.0, 67.6, 126.0 (q, $J_{C,F}$=282.6 Hz), 126.1 (q, $J_{C,F}$=282.3 Hz), 128.6, 128.8, 129.3, 137.7, 157.5, 171.7, 171.8, 173.9; FT-IR (KBr): 3424, 3310, 1702, 1670, 1608, 1542, 1265; MS (DIS EI, 70 eV) *m/z* (%): 576 [(M+H)$^+$, 7], 575 (M$^{+\cdot}$, 9), 557 [(M–H_2O)$^{+\cdot}$, 11], 347 ($C_{15}H_{18}F_3N_2O_4^+$, 27), 325 ($C_{12}H_{16}F_3N_2O_5^+$, 10), 303 ($C_{13}H_{12}F_3NO_4^+$, 9), 202 ($C_8H_{14}N_2O_4^{+\cdot}$, 5), 170 ($C_5H_7F_3NO_2^+$, 4). Anal. Calcd for $C_{22}H_{27}F_6N_3O_8$: C, 45.92; H, 4.73; N, 7.30. Found: C, 45.96; H, 4.75; N, 7.26.

5.4.14. Synthesis of H-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (21). Palladium hydroxide [$Pd(OH)_2$/C—50 mg] was added neat to a stirred solution of Z-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (**20**) (1.043 mmol, 600 mg) in MeOH (45 mL) and the reaction mixture was kept vigorously stirred under hydrogen atmosphere at rt for 30 min. After TLC monitoring (*n*-Hex/AcOEt 1:9), the palladium powder was filtered over a Celite pad, washing the black solid with MeOH (3×30 mL). The solvent was removed in vacuo and the residue was purified by FC with AcOEt to give H-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (**21**) (450 mg, 98% yield): R_f 0.35 (AcOEt); $[\alpha]_D^{20}$=−43.6 (*c* 1.0 in MeOH); mp 198–199°C (AcOEt); ^{1}H NMR (CD_3OD+D_2O) δ: 1.39 (d, *J*=7.2 Hz, 3H), 2.26 (d, *J*=7.0 Hz, 2H), 2.55 (dd, *J*=14.7 and 7.0 Hz, 1H), 2.58 (dd, *J*=14.7 and 7.0 Hz, 1H), 3.25 (dq, *J*=2.1 and 8.2 Hz, 1H), 3.68 (s, 3H), 4.38 (dt, *J*=2.1 and 7.0 Hz, 1H), 4.42 (q, *J*=7.2 Hz, 1H), 4.52 (dt, *J*=1.4 and 7.0 Hz, 1H), 4.61 (dq, *J*=1.4 and 8.2 Hz, 1H); ^{19}F NMR (CD_3OD) δ: −73.82 (br d, *J*=8.2 Hz, 3F), −71.71 (br d, *J*=8.2 Hz, 3F); ^{13}C NMR (CD_3OD) δ: 18.0, 39.6, 41.3, 50.7, 52.3, 54.4 (q, $J_{C,F}$=282.0 Hz), 57.0 (q, $J_{C,F}$=282.0 Hz), 65.3, 66.4, 126.2 (q, $J_{C,F}$=27.1 Hz), 127.7 (q, $J_{C,F}$=29.0 Hz), 172.7, 173.3, 176.0; FT-IR (KBr) (cm^{-1}): 3500, 3332, 1729, 1648, 1528, 1446, 1393, 1299; MS (DIS EI, 70 eV) *m/z* (%): 441 (M$^{+\cdot}$, 4), 440 [(M−H)$^+$, 12], 213 ($C_7H_{12}F_3N_2O_2^+$, 4), 212 ($C_7H_{11}F_3N_2O_2^{+\cdot}$, 5). Anal. Calcd for $C_{14}H_{21}F_6N_3O_6$: C, 38.10; H, 4.80; N, 9.52. Found: C, 38.06; H, 4.85; N, 9.56.

5.4.15. Synthesis of Iva-Val-Val-OH (22). *iso*-Valeroyl chloride (0.925 mmol, 112 mg) and a 4N NaOH solution (0.462 mL) were added dropwise and simultaneously to a stirred solution of commercial H-Val-Val-OH (0.925 mmol, 200 mg) in a 4N NaOH solution (0.462 mL). After few minutes, a white precipitate was formed and after 30 min a 1N aqueous HCl solution was added up to pH 1 together with MeOH (5 mL) to dissolve the solid. The solvents were evaporated in vacuo and the residue was purified by FC in $CHCl_3$/MeOH/CH_3CO_2H=97:3:1 to give Iva-Val-Val-OH (**22**) (170 mg, 82% yield): R_f 0.35 ($CHCl_3$/MeOH/AcOH=97:3:1); $[\alpha]_D^{20}$=−48.1 (*c* 1.1 in MeOH); mp 166–167°C (diisopropylether); ^{1}H NMR (CD_3OD+D_2O) δ: 0.8–1.0 (m, 18H), 1.9–2.3 (m, 5H), 4.23 (d, *J*=8.1 Hz, 1H), 4.32 (d, *J*=5.7 Hz, 1H); ^{13}C NMR (CD_3OD) δ: 18.3, 19.1, 19.6, 19.8, 22.8, 27.5, 31.7, 31.8; 46.0, 58.9, 60.2, 174.1, 174.6, 175.6; FT-IR (KBr) (cm^{-1}): 3299, 2965, 1719, 1638, 1546, 1467, 1215; MS (DIS EI, 70 eV) *m/z* (%): 301 [(M+H)$^+$, 12], 184 ($C_{10}H_{19}NO_2^{+\cdot}$, 4). Anal. Calcd for $C_{15}H_{28}N_2O_4$: C, 59.98; H, 9.39; N, 9.33. Found: C, 59.96; H, 9.35; N, 9.30.

5.4.16. Synthesis of Iva-Val-Val-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (23). Coupling between **21** and **22**. A suspension of **22** (76 mg) in 3.6 mL of dry AcOEt was treated with 1.2 equiv. of NMM, cooled to −10°C, then 1.2 equiv. of *iso*-BuO_2CCl were added under stirring. After 5 min. a suspension of **21** (1 equiv.) in 2.5 mL of AcOEt was added. The mixture was stirred for 4 days at rt, then centrifuged and the solid was washed several times with AcOEt, MeOH and finally *n*-Hex to provide 100 mg (65%) of Iva-Val-Val-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (**23**) as an amorphous solid: mp 265–270°C (dec.); $[\alpha]_D^{20}$=−32.2 (*c* 0.36, DMSO); ^{1}H NMR (DMSO-d_6) δ 8.08 (d, *J*=7.0 Hz, 1H), 8.06 (d, *J*=9.5 Hz, 1H), 8.00 (d, *J*=9.5 Hz, 1H), 7.90 (d, *J*=9.0 Hz, 1H), 7.79 (d, *J*=9.0 Hz, 1H), 5.35 (br signal, 2H), 4.65–4.50 (m, 2H), 4.44 (dq, *J*=7.0 and 7.1 Hz, 1H), 4.31 (dd, *J*=9.0 and 7.2 Hz, 1H), 4.35–4.25 (m, 2H), 4.20 (dd, *J*=9.0 and 7.1 Hz, 1H), 2.31 and 2.28 (m, 2H), 2.25 (dd, *J*=14.9 and 8.5 Hz, 1H), 2.19 (dd, *J*=14.9 and 4.4 Hz, 1H), 2.10–1.85 (m, 5H), 1.24 (d, *J*=7.1 Hz, 3H), 0.90–0.80 (m, 18H); ^{19}F NMR (DMSO-d_6) δ −71.20 (br d, *J*=8.3 Hz), −71.08 (br d, *J*=8.2 Hz); ^{13}C NMR (DMSO-d_6) δ 173.3, 171.8, 171.62, 171.59, 171.3, 169.5, 124.9 (q, *J*=284.3 Hz, 2C), 64.2, 63.8, 57.8, 57.7, 52.9 (q, *J*=26.9 Hz), 52.4 (q, *J*=27.7 Hz), 48.2, 44.4, 39.5, 38.5, 30.1, 29.9, 25.6, 22.2, 19.19, 19.17, 18.23, 18.18, 17.7. MS (DIS EI, 70 e V) *m/z* (%): 710 [(M+H)$^+$, 4], 692 [(M+H−H_2O)$^+$, 6]. Anal. Calcd for $C_{29}H_{47}F_6N_5O_9$: C, 48.13; H, 6.55; N, 9.68. Found: C, 48.16; H, 6.55; N, 9.66.

5.4.17. Synthesis of Iva-Val-Val-(Tfm)GABOB-Ala-(Tfm)GABOB-OH (1). A solution of LiOH.H_2O (1.19 mmol, 50 mg) in water (1 mL) was added to a stirred solution of Iva-Val-Val-(Tfm)GABOB-Ala-(Tfm)GABOB-OMe (**23**) (0.097 mmol, 70 mg) in DMSO (12 mL). The reaction mixture was stirred at rt for 6 h, monitored by TLC (AcOEt/MeOH=9:1), diluted with water, added with a 1N aqueous HCl solution up to pH 1 and the organics were extracted with AcOEt (3×10 mL). The combined organic layers were dried in vacuo and a grey residue was obtained and washed several times with water, AcOEt, MeOH and finally acetone to give pure Iva-Val-Val-(Tfm)GABOB-Ala-(Tfm)GABOB-OH (**1**) (43 mg, 85% yield): $[\alpha]_D^{20}$=−27.6 (*c* 0.4 in DMSO); mp 254–260°C (acetone); ^{1}H NMR (DMSO-d_6) δ: 0.80–0.90 (m, 18H), 1.24 (d, *J*=7.1 Hz, 3H), 1.85–2.10 (m, 5H), 2.19 (dd, *J*=14.9 and 4.4 Hz, 1H), 2.25 (dd, *J*=14.9 and 8.5 Hz, 1H), 2.28 and 2.31 (m, 2H), 4.20 (dd, *J*=9.0 and 7.1 Hz, 1H), 4.25–4.35 (m, 2H), 4.31 (dd, *J*=9.0 and 7.2 Hz, 1H), 4.44 (dq, *J*=7.0 and 7.1 Hz, 1H), 4.50–4.65 (m, 2H), 5.35 (br signal, 2H), 7.79 (d, *J*=9.0 Hz, 1H), 7.90 (d, *J*=9.0 Hz, 1H), 8.00 (d, *J*=9.5 Hz, 1H), 8.06 (d, *J*=6.5 Hz, 1H), 8.08 (d, *J*=9.8 Hz, 1H); ^{19}F NMR (DMSO-d_6), δ: −71.20 (br d, *J*=8.3 Hz, 3F), −71.80 (br d, *J*=8.2 Hz, 3F); ^{13}C NMR (DMSO-d_6) δ: 17.7, 18.2, 18.2, 19.2, 19.2, 22.2, 25.6, 29.9, 30.1, 38.5, 39.5, 44.4, 48.2, 52.4 (q, $J_{C,F}$=27.7 Hz), 52.9 (q, $J_{C,F}$=26.9 Hz), 57.7, 57.8, 63.8, 64.2, 124.9 (q, $J_{C,F}$=284.3 Hz), 169.5, 171.3, 171.6, 171.6, 171.8, 173.3; FT-IR (KBr) (cm^{-1}): 3432, 3297, 1638, 1542, 1388, 1270; MS (DIS EI, 70 eV) *m/z* (%): 710 [(M+H)$^+$, 4], 692 [(M+H−H_2O)$^+$, 6], 495 ($C_{22}H_{38}F_3N_4O_5^+$, 4), 493 ($C_{22}H_{36}F_3N_4O_5^+$, 7), 312 ($C_{12}H_{21}F_3N_3O_3^+$, 20). Anal. Calcd for $C_{28}H_{45}F_6N_5O_9$: C, 47.39; H, 6.39; N, 9.87. Found: C, 47.36; H, 6.35; N, 9.89.

5.5. Assays on HIV-1 protease

The assays were carried out at 25°C with 10 nM HIV-1 Protease and the fluorogenic substrate Abz-NF*-6 (30 μM, diluted from a stock solution in DMSO), in 100 mM MES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% (vol/vol) DMSO, pH 5.5. Excitation and emission wavelenghts were 325 nm and 420 nm, respectively. Compound **1** was dissolved in DMSO and added to the assay solution at a final concentration of 150–0.15 μM, 1 min after the addition of the enzyme.

5.6. Tests on MMP-9. Cell culture

Circulating human monocytes were isolated from blood of healthy donors as previously described.[33] The monocytes were collected, washed, resuspended in serum free Dulbecco Modified Eagle's medium (GIBCO BRL, Life Technologies, Italy) and plated at a density of 3×10^6 cells in 35 mm dish. After 2 h, cell monolayers were washed twice and the adherent cells were incubated for 10–14 days with DMEM containing 10% human AB serum and insulin 8 μg mL^{-1}, to allow for differentiation in macrophages. To generate the conditioned media, cells were incubated for 24 h at 37°C with DMEM, supplemented with 0.2% bovine serum albumin (BSA; Sigma) and the indicated concentrations of compounds. At the end of the incubation, the conditioned media were collected and the gelatinolytic capacity of secreted MMP-9 analyzed by zymography.[32] Cellular protein content was measured according to Lowry.[33]

SDS Page Zymography. Samples (5 μl of conditioned medium per lane) underwent electrophoresis at 4°C on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg mL^{-1}) under non-reducing conditions and without boiling. After electrophoresis, SDS was removed from gels in two washes with 2.5% Triton X-100 (Sigma) at room temperature. After washes, the gels were incubated overnight at 37°C with gentle shaking in TRIS 50 mM pH 7.5 containing NaCl 150 mM, $CaCl_2$ 10 mM, $ZnCl_2$ 1 μM, to activate the metalloproteinase ability to digest the substrate. For inhibition studies and to confirm the identity of MMP-9, identical gels have been incubated in the above buffer containing either EDTA 20 mM, an inhibitor of MMPs, or PMSF 1 mM, an inhibitor of serine proteases. The addition of PMSF did not alter the MMP-9 gelatinolytic capacity, while the treatment with EDTA completely abolished it (data not shown). At the end of the incubation, the gels were stained with a solution of 0.1% Coomassie brilliant blue R-250 (Sigma) in 25% methanol and 7% acetic acid. Clear zones against the blue background indicated the presence of proteinolytic activity.

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