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Potential peptidic proteasome inhibitors by incorporation of an electrophilic trap based on amino acid derived α-substituted sulfonyl fluorides

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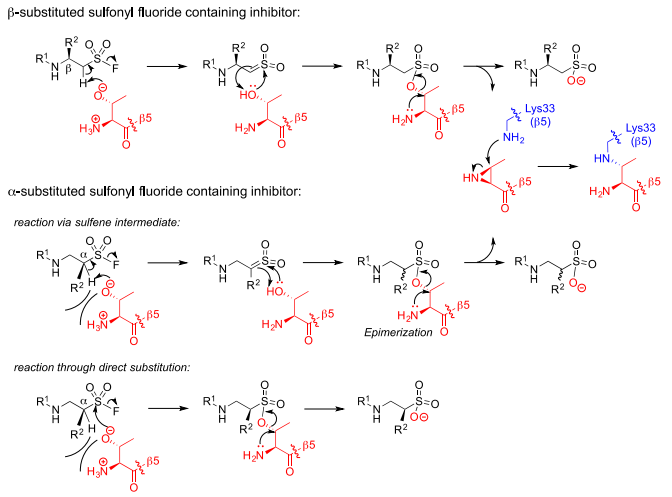
ABSTRACT

1. Introduction

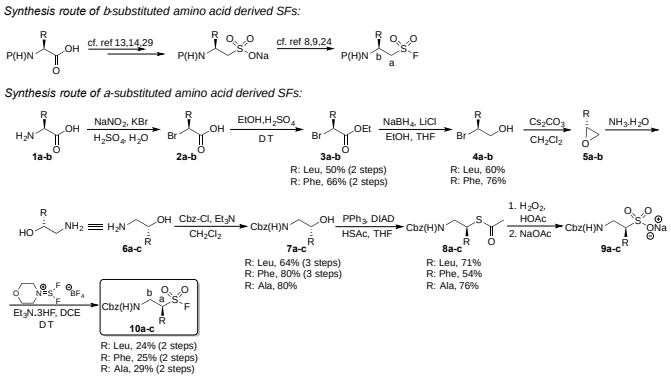
The proteasome is responsible for the majority of intracellular protein turnover in eukaryotic cells, *via* the ubiquitin-proteasome pathway, including the degradation of many critical proteins.[1] Identification of this enzyme complex as an effective therapeutic anticancer target has led to the development of numerous proteasome inhibitors, which are typically peptide-based and contain an electrophilic trap that reacts with the active *N*-terminal threonine of the proteolytic β1, β2 and β5-subunits.[2,3] While sharing the same catalytic mechanism these subunits differ in their substrate preference and cleave near acidic, basic and hydrophobic amino acid residues, respectively.[4] Although achievement of subunit specificity is mainly governed by the peptide backbone of the inhibitors, the target specificity and biological stability are determined by the electrophilic trap.[5] The most common electrophilic traps used for covalent proteasome inhibition are aldehydes, boronates, vinyl sulfones α', β'-epoxyketones and β-lactones. The main focus in the current design of proteasome inhibitors is avoiding "off-target" interactions and the development of resistance.[6]

Presently, a dominant electrophilic trap incorporated in proteasome inhibitors is the α,β unsaturated sulfone Michael acceptor. In fact, Michael acceptors are nowadays the most often used "warheads" in covalent enzyme inhibition, including kinase[7] and cysteine protease[8] inhibition. Although vinyl sulfones inhibit both the proteasome and cysteine proteases, high selectivity has been achieved through manipulation of the peptidic portion.[9] Examples of this are the selective inhibition of the caspase-like activity of the proteasome,[10] selective labelling of proteasomes,[11] and proteasome subunit specific probes.[12]

Adjustment of the chemical environment of the warhead plays a key role in inhibitor design since it enables the tuning of its reactivity and thereby its selectivity and stability. As a promising alternative electrophilic trap, we have described previously the incorporation of amino acid derived sulfonyl fluorides[13] into peptide backbones leading to peptido sulfonyl fluorides (PSF) as a new class of powerful proteasome inhibitors, showing a high specificity for the β5-subunit.[14] Several of these compounds could also effectively suppress malaria parasitic activity.[15] In addition, compounds containing the sulfonyl fluoride electrophile have found chemical biology applications as reactive probes, and have recently been highlighted as privileged warheads.[16] Structural analysis of the molecular mechanism of action of PSF inhibitors in the proteasome revealed the formation of an O-sulfonate adduct with the active site threonine, which may occur by direct nucleophilic substitution of the Fluor-atom or involve formation of a sulfene intermediate after proton abstraction (**Scheme 1**).[17] We have indications favoring the latter pathway from deuterium exchange experiments (unpublished data). However, we cannot completely rule out reaction of the SF through direct substitution, since (partial) epimerization,



Scheme 1 Proteasome inhibition by a β-substituted SF and proposed inhibition by an α-substituted SF. Although indicated as "direct substitution" the mechanism probably involves a trigonal bipyramidal intermediate.[18]



Scheme 2 General synthesis of β-substituted SFs and α-substituted SFs

indicative of the sulfene mechanism, may already have taken place before substitution.

For a sulfene-like mechanism, it is believed that two factors are important for the reactivity of the SF warhead: (1) ease of formation of the sulfene intermediate by deprotonation at the α-position; (2) steric hindrance: a more hindered SF will be less accessible for a nucleophile present in the active site of the proteasome. The latter factor may also play a role in the direct substitution mechanism.

In this research we present attempts towards tuning the reactivity of the sulfonyl fluoride (SF) warhead by changing the position of the substituent from the β to the α-position that is adjacent to the SF-moiety.

Although, alike our present β-substituted SFs, α-substituted SFs can still be conveniently derived from α-amino acids (**Scheme 2**), in the synthetic route towards **α-substituted** SFs further manipulation is required in order to shift the side chain to the desired α-position. This could be achieved by preparation of suitable epoxide derivative from an α-amino acid, followed by ring opening to re-introduce the amino functionality at the least hindered epoxide-carbon atom (**Scheme 2)**.

2. Results and Discussion

\Synthesis and reactivity of α-substituted amino sulfonyl fluorides (SFs)

Leucine (**1a**) and Phenylalanine (**1b**) were converted into the corresponding α-bromo acids **2a-b** with retention of configuration in a diazotisation reaction in the presence of KBr. These amino acids were selected because side chains derived from Leucine and Phenylalanine are present at the C-terminus of several proteasome inhibitors. Esterification led to ethylesters **3a** and **3b** with an optical purity in agreement with the literature.[19] After reduction with *in situ* prepared $LiBH_4$, bromo-alcohols **4a** and **4b** were obtained in acceptable yields. Next, epoxides **5a** and **5b** were prepared with inversion of configuration by treatment with base (Cs_2CO_3),[20]

which were immediately ring-opened by aqueous ammonia,[21] followed by protection of the amine with a Cbz-group[22] affording **7a** and **7b** in good (over 3 steps) yields of 64 and 80%, respectively. Alanine derived Cbz-protected amino alcohol **7c** was obtained upon protection of the commercially available (R)-1-amino-2-propanol. Introduction of the thioacetate moiety was performed by a Mitsunobu reaction,[23] which should take place with inversion and thereby a net retention of configuration of bromo alcohols **4a**,**4b**. Purification of the resulting thioacetates **8a-8c** turned out to be difficult and reduced the yields considerably. Oxidation using aqueous hydrogen peroxide (30% w/w) and acetic acid afforded sulfonates **9a-c**, after which the desired α-substituted sulfonyl fluorides **10a-c** were obtained in modest yields (24-29%)[a].

At this stage we wished to obtain a general idea of the reactivity of α-substituted SF warhead containing compounds with respect to nucleophiles and whether this behavior was different from our earlier β-substituted SF warhead containing compounds.

For this α-substituted SFs **10a** and **10b** were compared with β-substituted SF **11** (Figure 1, Table 1). It was found that α-substituted SFs did not differ from the β-substituted SFs with respect to their reactivity toward different nucleophiles.[b] Both categories of SFs do not react with thiols, even not in the presence of base (DiPEA). In fact, the relatively low reactivity of the sulfonyl fluoride group has been synthetically exploited for selective modifications in molecules containing other electrophilic moieties.[24]

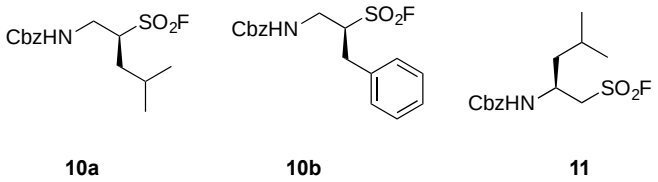
In addition, both SFs do react with amine nucleophiles such as piperidine and benzylamine. However, α-substituted SFs gave rise to more sulfonamide product formation after 24h, possibly indicative of a higher reactivity of α-substituted SFs (Table 1). This cannot be attributed to formation of a sulfene intermediate since abstraction of the α-proton in **10a** is probably more difficult

[a] The low yields are probably partly due to impurities present in the sulfonate salts, which are difficult to remove by chromatographic approaches.

[b] The results for the reactivity of these substituted fluorides are in agreement with our earlier observed reactivity with unsubstituted sulfonyl fluorides.[24]

than in **11**. Nevertheless, an explanation may be found assuming a trigonal bipyramidal intermediate in the "direct substitution"[c]

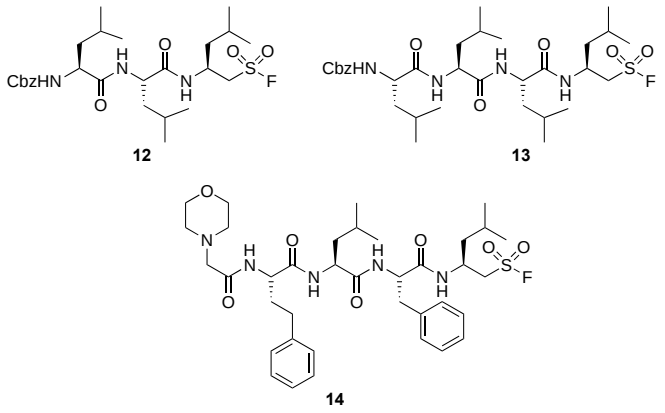
Incorporation of α-substituted SFs towards potential proteasome inhibitors



10a **10b** **11**

Figure 1. Structures of α-substituted-SFs **10a** and **10b** as well as β-substituted-SF **11**.

To evaluate the inhibitory potential of α-substituted SFs they were incorporated into peptide sequences derived from our earlier peptido sulfonyl fluoride (PSF) proteasome inhibitors **12**, **13** and **14**. We showed that these PSFs were very powerful proteasome inhibitors[14,17] and therefore their structural characteristics are suitable for further structure-activity studies.



12 **13**

14

Figure 1 Earlier developed PSF-proteasome inhibitors **12-14** containing a β-substituted SF derivative.

Cbz-Leu$_2$OH **17** and Cbz-Leu$_3$OH **20** were synthesized in solution in a few steps starting from methyl ester **15** as was described previously[28]. Carboxylic acids **17** and **20** were obtained (95% yield) after saponification of methyl esters **16** and **19** with Tesser's base (dioxane/methanol/4N NaOH 4:5:1) (Scheme 3).
Morpholino-hPhe-Leu-Phe-OH **23** was prepared by solid phase synthesis starting from chloro-trityl resin **21** using Fmoc-chemistry and cleavage from the resin using hexafluoro-iso propanol (HFIP).

Completion of the inhibitor synthesis was achieved by first deprotecting SFs **10a**,**b** by HBr in acetic acid and ion-exchange to the HCl-salt, followed by a DCC-coupling in the presence of 6-chloro-HOBt (HOBt-Cl). (Scheme 4) Liberation of the hydrochloride salt using Zn powder in THF[26] and the DCC/HOBt-Cl coupling method provided essentially neutral conditions to prevent further decomposition of the SF-warhead and minimize possible racemization of the warhead.

Entry	HN	H_2N	HS	HS OH
10a	72%	25%	n.r.[a,b]	n.r.[a,b]
10b	70%	30%[c]	n.r.[a,b]	n.r.[a,b]
11	40%	10%[c]	n.r.[a,b]	n.r.[a,b]

Table 1 Reactivity of sulfonyl fluorides towards different nucleophiles. Reaction were performed using 2.2 eq of the nucleophile in DCM over 24h.

[a] n.r.: no reaction; in the absence of base

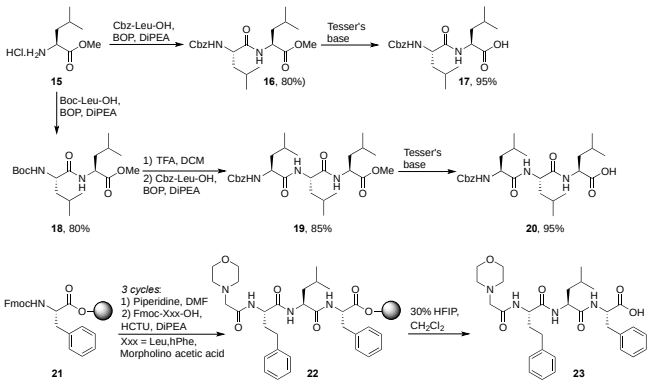
[b] in the presence of 2.2 eq of DiPEA

[c] based on starting material recovery

PSF-inhibitors **25a,b - 27a,b** were obtained after preparative reverse phase HPLC purification as diasteroisomeric mixtures in rather low yields (10-21%). The diastereomeric ratios were identical to those observed after the introduction of the SF under more basic coupling conditions during control experiments. Therefore, it was concluded that racemisation had occurred during the synthesis of the warhead, possibly after introduction of the sulfonyl moiety, which increases the acidity of the α-proton significantly. All attempts to separate the diastereomers by reverse phase HPLC failed.

Hydrolytic stability and biological activity

Scheme 3 Synthesis in solution of Cbz-Leu$_2$OH **17** and Cbz-Leu$_3$OH **20** and Solid phase synthesis of Morph-hPhe-Leu-Phe-OH **23**.



15 **16**, 80%) **17**, 95%

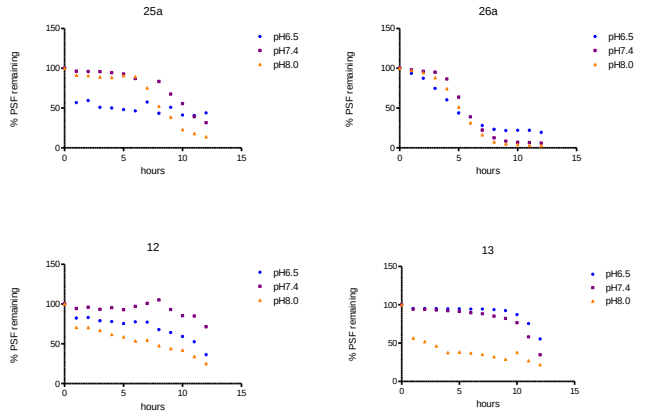
18, 80% **19**, 85% **20**, 95%

21 **22** **23**

[c] a trigonal bipyramidal intermediate (TBPI) may offer relieve of strain going from ca. 109º angles in starting material to ca 120º in the TBPI mechanism (Scheme 1) allowing "steric acceleration".[25]

The behavior of α-PSFs **25a**, **26a** and the β-PSF inhibitors **12** and **13** in an aqueous buffer at different pHs was studied (Figure 3). Although all tested PSFs showed a considerable aqueous stability, the α-PSFs **25a**, **26a** appeared to be more prone to undergo hydrolysis than their β-PSF counterparts **12** and **13**. Surprisingly, hydrolysis of **25a** at acidic pH was initially fast and then remained unchanged. The half-life of α-PSFs **25a** and **26a** at the different pH's varied between ca. 5-7 hours, whereas the β-PSF inhibitors **12** and **13** were more stable, and depending on the pH their half-life varied between ca 10-12 hours.

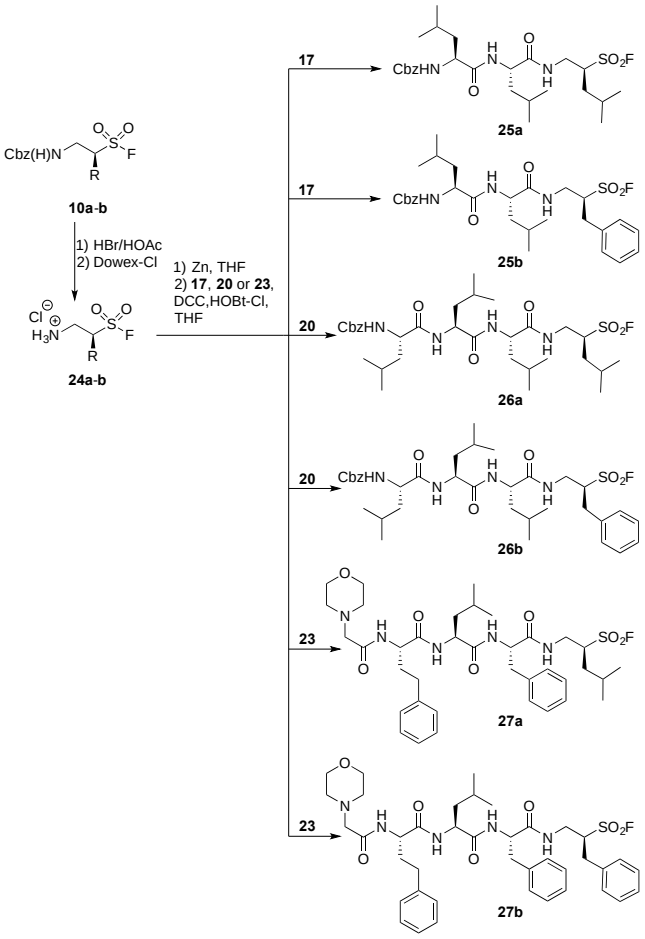
Figure 3 Buffer stability studies of compounds **25a**, **26a**, **12** and **13** at pH 6.5, 7.4 and 8.0



It was attempted to determine inhibition of the proteasome enzymatic activity by monitoring inhibition of the hydrolysis of the fluorogenic substrate Suc-LLVY-AMC[27] Unexpectedly, our inhibitors showed no proteasome inhibition in the used concentration range from 0.002 μM - 400 μM. However, strangely, it was impossible to obtain the normal sigmoid inhibition curves for any of the α-PSFs **25-27ab**, where it was possible to produce in the same assay sigmoid inhibition curves for β-PSF inhibitors **12** and **13**. (see supporting information, page S116). Possible explanations for this anomalous behavior include a complete devoid of activity of the compounds in the above used concentration range. However, this concentration range may not have been actually realized, since at a concentration of ca. 75 μM, compounds were starting to precipitate from the solution. With respect to this it is also noteworthy that at low concentrations (between ca. 0.1 - 1 μM, see supporting information, page S112) of a few inhibitors a start of a sigmoidal curve could be discerned, possibly indicating that a these concentrations the inhibitors are still soluble and capable of proteasome inhibition.

Increasing the DMSO concentration to 20% v/v (this is the maximal concentration tolerated by the proteasome) did not remedy this situation. Unfortunately, at this point we are unable to draw definitive conclusions about the proteasome inhibitory activity of the α-PSFs **25-27ab** as compared to the β-PSF inhibitors **12** and **13**.

3. Conclusions



Several new amino acid derived SFs have been synthesised containing the substituent in the α-position with respect to the SF moiety. The preparation of these αSFs was achieved using α-amino acids as starting materials leading to epoxides as synthons for shifting the side chain to the α-position with respect to the SF-moiety. An important future issue might be exact determination of the racemization causes and in which stage separation of the diastereoisomers should take place.

Scheme 4 Incorporation of the α-substituted SFs to yield proteasome inhibitors **25a,b**, **26a,b** and **27a,b**.

Although αSFs seemed to be slightly more reactive than a βSFs it is not clear as yet if and how this is translated to the bio-activity of the corresponding α-PSFs probably because of very poor solubility of α-PSFs **25-27ab**. Evidently, our near future research efforts will involve trying to improve the solubility of α-PSFs for example by modifying or removing the N-terminal protecting group.

4. Experimental part

All reagents were obtained from commercial sources and used without further purification unless specified otherwise. Air and/or moisture sensitive reactions were performed under an atmosphere of nitrogen in flame dried apparatus. Tetrahydrofuran (THF) and dichloromethane (CH_2Cl_2) were purified using a Pure-Solv™ 500 Solvent Purification System. Petroleum ether (PE) used for reactions and column chromatography was the 40–60 °C fraction. Thin layer chromatography (TLC) was performed using Merck silica gel 60 glass plates F_{254}. TLC plates were visualized under UV light at λ = 254 nm and stained using the most appropriated solution (ninhydrin, anisaldehyde, bromocresol green or

potassium permanganate). Flash column chromatography was performed with Silicaflash P60 gel (40–63 µm) from Silicycle (Canada) as solid support. All ^{1}H NMR spectra were recorded on Bruker Avance III 400 MHz and 500 MHz spectrometers at ambient temperature. Data are reported as follows: chemical shifts in ppm relative to TMS (0.0) on the δ scale, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, app. = apparent or a combination of these), coupling constant(s) in *J* (Hz), integration and assignment. All ^{13}C NMR spectra were recorded on Bruker Avance III 400 MHz and 500 MHz spectrometers at 101 MHz and 126 MHz at ambient temperature and assignments were carried out and/or confirmed using 2D spectra (HSQC, COSY) and DEPT. Data are reported as follows: chemical shift in ppm relative to $CDCl_3$ (77.0) on the δ scale and assignment. All ^{19}F NMR spectra were recorded on Bruker Avance III 500 MHz spectrometer at 471 MHz at ambient temperature. Chemical shifts are reported in ppm. Optical rotations were recorded using an automatic polarimeter Autopol V. High resolution mass spectra (HRMS) were recorded using positive chemical ionization (CI+) and electron impact (EI+) on Jeol MStation JMS-700 instrument and positive or negative ion electrospray (ESI+/ESI-) techniques on a Bruker micrOTOF-Q instrument. Analytical HPLC was performed on a Shimadzu Prominence instrument with a UV-detector operating at λ = 214 and 254 nm, using a C4 column (5 µm, 250 x 4.60 mm) or a C18 column (5 µm, 250 x 4.60 mm) at a flow rate of 1 mL/min. The mobile phase was water/CH_3CN/TFA (95/5/0.1, v/v/v, buffer A) and water/CH_3CN/TFA (5/95/0.1, v/v/v, buffer B). Samples were dissolved in buffer A/B (1/2 or 1/3). Preparative HPLC was performed on an Agilent 1260 Infinity instrument using a Phenomenex column (Gemini C18, 10 µm, 250 x 21.2 mm) at a flow rate of 12.5 mL/min, using the same buffers and sample preparation as described for the analytical HLPC. Analytical LC-MS was performed on a Thermo Scientific Dionex Ultimate 3000 LC system coupled to a Thermo Scientific LCQ Fleet^{TM} Ion trap mass spectrometer using a Dr. Maisch column (Reprosil Gold 120, C18, 3 µm, 150 x 4mm) with a linear gradient of 1 mL/min. The mobile phase was water/CH_3CN/TFA (95/5/0.1, v/v/v, buffer A) and water/CH_3CN/TFA (5/95/0.1, v/v/v, buffer B). Samples were dissolved in buffer A/B (1/1 or 1/2). The UV absorption was monitored at λ = 214 and 254 nm over 10, 40 or 60 min. Proteasome Enzymatic Assays were performed using the Enzo Life Sciences® 20S Proteasome Assay Kit for Drug Discovery (Enzo Life Science, USA). Fluorescence measurements were performed with a Clariostar microplate reader (BMG LABTECH, Germany). Diastereomeric ratios of the final inhibitors were determined from integration values of separated signals in ^{1}H NMR spectra.

Cbz-sulfonate salt 9a

Thioacetate **8a** (1.6 g, 4.7 mmol) was dissolved in acetic acid (15 mL) and an aqueous 30% H_2O_2 solution (5 mL) was added. The reaction was stirred at RT overnight. NaOAc (425 mg, 5.2 mmol) was added and the mixture was stirred at RT for 1 h. Acetic acid was then removed *in vacuo* and DMF was added (50 mL). Co-evaporation with DMF was repeated using the same amount until the excess of hydrogen peroxide/peracetic acid was removed (checked with a starch iodide paper). The crude mixture was then diluted with more water and lyophilised resulting in the desired sulfonate salt. The crude sulfonate was used as such in the fluorination step.

HRMS (ESI positive) calcd for $C_{14}H_{20}NNaO_5S$ $[M+Na]^+$ 360.0852, found 360.0843.

Cbz-sulfonate salt 9b

Thioacetate **8b** (4.2 g, 13.6 mmol) was dissolved in acetic acid (50 mL) and an aqueous 30% H_2O_2 solution (18 mL) was added. The reaction was stirred at RT overnight. NaOAc (1.2 g, 15 mmol) was added and the mixture was stirred at RT for 1 h. Work-up was carried out as was described for **9a**.

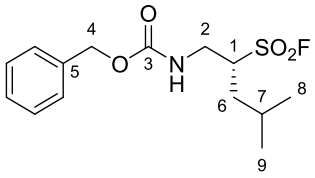
HRMS (ESI positive) calcd for $C_7H_{18}NNaO_5S$ $[M+Na]^+$ 394.0696, found 394.0693.

Cbz-sulfonate salt 9c

Thioacetate **8c** (2.16 g, 8.1 mmol) was dissolved in acetic acid (25 mL) and an aqueous 30% H_2O_2 solution (9 mL) was added. The reaction was stirred at RT overnight. NaOAc (730 mg, 8.9 mmol) was added and the mixture was stirred at RT for 1 h. Work-up was carried out as was described for **9a**.

HRMS (ESI negative) calcd for $C_{11}H_{14}NO_5S$ $[M-H]^-$ 272.0598, found 272.0578.

Cbz-sulfonyl fluoride 10a



The crude sulfonate salt **9a** (300 mg, 0.94 mmol) was dissolved in dry DCE (45 mL). XtalFluor-M (353 mg, 1.45 mmol) and $NEt_3{\cdot}3HF$ (6 µL, 0.03 mmol) were added and the reaction was stirred under nitrogen atmosphere at reflux overnight. Evaporation of the solvent *in vacuo* and purification of the crude product by silica gel column chromatography (EtOAc/*n*-hex, 0/10→2/8, v/v) afforded the desired sulfonyl fluoride (60 mg, 0.19 mmol, 24%) as a colourless oil.

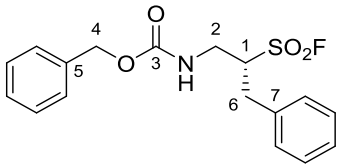
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 – 7.15 (m, 5H, CH-Ph), 5.26 (br s, 1H, NH), 5.03 (m, 2H, CH_2-C4), 3.74 – 3.65 (ddd, *J* = 12.4, 6.9, 2.2 Hz, 1H, CH_2-C2a), 3.50 (m, 2H, CH_2-C2b, CH-C1), 1.86 – 1.77 (m, 1H, CH-C7), 1.73 (m, 1H, CH_2-C6a), 1.59 – 1.50 (ddd, *J* = 14.2, 8.8, 5.4 Hz, 1H, CH_2-C6b), 0.89 (d, *J* = 6.5 Hz, 3H, CH_3-C8), 0.88 (d, *J* = 6.5 Hz, 3H, CH_3-C9).

^{13}C NMR (126 MHz, $CDCl_3$) δ 156.4 (C-C3), 136.1 (C-C5), 128.7 (CH-Ph), 128.4 (CH-Ph), 128.2 (CH-Ph), 67.3 (CH_2-C4), 61.6 (d, *J* = 9.4 Hz, CH-C1), 40.1 (CH_2-C2), 35.6 (CH-C6), 25.3 (CH-C7), 22.8 (CH_3-C8), 21.5 (CH_3-C9).

^{19}F NMR (471 MHz, $CDCl_3$) δ 48.38.

HRMS (ESI positive) calcd for $C_{14}H_{20}FNO_4S$ $[M+H]^+$ 317.1097, found 317.1101.

Cbz-sulfonyl fluoride 10b



The crude sulfonate salt **9b** (300 mg, 0.85 mmol) was dissolved in dry DCE (45 mL). XtalFluor-M (353 mg, 1.45 mmol) and $NEt_3{\cdot}3HF$ (6 µL, 0.03 mmol) were added and the reaction was stirred under nitrogen atmosphere at reflux overnight.

Evaporation of the solvent *in vacuo* and purification of the crude product by silica gel column chromatography (EtOAc/*n*-hex, 0/10→2/8, v/v) afforded the desired sulfonyl fluoride (70 mg, 0.20 mmol, 25%) as white crystals.

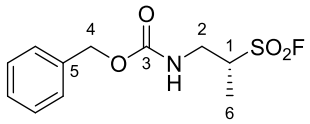
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.06 (m, 10H, CH-Ph), 5.17 (br s, 1H, NH), 5.06 – 4.94 (m, 2H, CH_2-C4), 3.81 (m, 1H, CH-C1), 3.65 (ddd, *J* = 14.7, 6.2, 3.0 Hz, 1H, CH_2-C2a), 3.52 (app dt, *J* = 14.7, 6.9 Hz, 1H, CH_2-C2b), 3.34 (dd, *J* = 14.4, 4.6 Hz, 1H, CH_2-C6a), 2.93 (dd, *J* = 14.4, 9.5 Hz, 1H, CH_2-C6b).

^{13}C NMR (126 MHz, $CDCl_3$) δ 156.0 (C-C3), 135.9, 134.5 (C-C5, C7), 129.1 (CH-Ph), 129.0 (CH-Ph), 128.6 (CH-Ph), 128.3 (CH-Ph), 128.1 (CH-Ph), 127.8 (CH-Ph), 67.2 (CH_2-C4), 63.9 (d, *J* = 8.8 Hz, CH-C1), 39.5 (CH_2-C2), 33.1 (CH_2-C6).

^{19}F NMR (471 MHz, $CDCl_3$) δ 50.56.

HRMS (ESI positive) calcd for $C_{17}H_{18}FNNaO_4S$ $[M+Na]^+$ 374.0833, found 374.0828.

Cbz-sulfonyl fluoride 10c



The crude sulfonate salt **9c** (1.4 g, 5 mmol) was dissolved in dry DCE (200 mL). XtalFluor-M (2.1 g, 8.5 mmol) and $NEt_3{\cdot}3HF$ (59 µL, 0.4 mmol) were added and the reaction was stirred under nitrogen atmosphere at reflux overnight. Evaporation of the solvent *in vacuo* and purification of the crude product by silica column chromatography (EtOAc/*n*-hex, 4/6, v/v) afforded the desired sulfonyl fluoride (230 mg, 0.89 mmol, 29%) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 – 7.16 (m, 5H, CH-Ph), 5.30 – 5.18 (br s, 1H, NH), 5.11 – 5.00 (m, 2H, CH_2-C4), 3.72 – 3.55 (m, 3H, CH-C1, CH_2-C2), 1.47 (d, *J* = 6.9 Hz, 3H, CH_3-C6).

^{13}C NMR (126 MHz, $CDCl_3$) δ 156.4 (C-C3), 136.0 (C-C5), 128.7 (CH-Ph), 128.5 (CH-Ph), 128.2 (CH-Ph), 67.4 (CH_2-C4), 58.3 (d, *J* = 11.7 Hz, CH-C1), 41.6 (CH_2-C2), 12.8 (CH_3-C6).

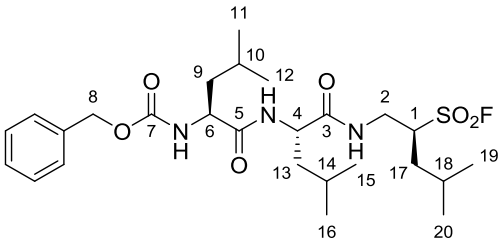
^{19}F NMR (471 MHz, $CDCl_3$) δ 46.10.

HRMS (ESI positive) calcd for $C_{11}H_{14}FNNaO_4S$ $[M+Na]^+$ 298.0520, found 298.0506.

General procedure for the coupling of α-substituted amino sulfonyl fluorides

Cbz-protected sulfonyl fluoride **10a,b** or **c** was treated with a 1:1 mixture of HBr/HOAc solution : CH_2Cl_2 for 1 h. After evaporation of the solvents, the crude product was dissolved in H_2O, stirred with Dowex-Cl resin (60 mg/0.1 mmol crude product) for 10 min, filtered and freeze dried, resulting in the corresponding hydrochloride salt. To generate the free amine in situ the salt was dissolved in THF (1mL/0.1 mmol) and treated with Zn powder (2 eq) for 30 min. After filtration, the amine was added to the reaction mixture of the peptide backbone (1 eq), DCC (1.1 eq) and HOBt-Cl (1.1 eq) in THF (1mL/0.1 mmol), which had been previously pre-activated for 10 min. The reaction was then stirred overnight. After concentration by evaporation of the volatiles, the crude product was dissolved in a 1:3 mixture of buffer A and buffer B and purified by preparative HPLC affording the desired compound.

Cbz-Leu-Leu-Leu-SO_2F 25a



The general procedure was followed on a 0.157 mmol scale delivering the desired product (15 mg, 0.027 mmol, 18%) as a white solid. Diastereomeric ratio 2.5:1, NMR-shifts of the diastereomer are indicated by a '*'

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.30 (m, 7H, CH-Ph, CH-Ph*), 7.13 (s, 1H, NH), 6.95 (s, 0.4H, NH*), 6.47 (d, *J* = 8.0 Hz, 0.4H, NH*), 6.41 (d, *J* = 8.0 Hz, 1H, NH), 5.18 – 5.08 (m, 4.2H, NH, NH*, CH_2-C8, CH_2-C8*), 4.50 – 4.35 (m, 1.4H, CH-C4, CH-C4*), 4.18 – 4.09 (m, 1.4H, CH-C6, CH-C6*), 3.83 – 3.69 (m, 2.4H, CH_2-C2a, CH_2-C2a*, CH-C1), 3.65 – 3.48 (m, 1.8H, CH_2-C2b, CH_2-C2b*, CH-C1*), 1.97 – 1.43 (m, 12.6H, CH_2-C9, C13, C17, CH-C10 C14, C18, CH_2-C9*, C13*, C17*, CH-C10*, C14*, C18*), 1.05 – 0.84 (m, 25.2H, CH_3-C11, C12, C15, C16, C19, C20, CH_3-C11*, C12*, C15*, C16*, C19*, C20*).

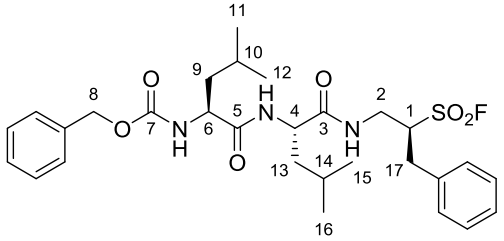
^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 172.4, 172.3 (C-C5, C3, C-C5*, C3*), 156.8 (C-C7, C-C7*), 135.9 (C-Ph*), 135.8 (C-Ph), 128.6, 128.4, 128.3, 128.2, 128.1 (CH-Ph, CH-Ph*)), 67.6 (CH_2-C8), 67.4 (CH_2-C8*), 61.0 (d, *J* = 9.4 Hz, CH-C1*), 60.5 (d, *J* = 9.7 Hz, CH-C1) 54.2 (CH-6), 53.9 (CH-C6*), 52.0 (CH-C4*), 51.7 (CH-C4), 40.7, 40.2, 40.1 (CH_2-C9, C13, CH_2-C9*, C13*), 38.7 (CH_2-C2, C2*), 35.9(CH_2-C17, CH_2-C17*), 25.3, 24.9, 24.8, 24.7 (CH-C10, C14, C18, CH-C10*, C14*, C18*), 22.9, 22.7, 21.7, 21.6, 21.5, 21.4 (CH_3-C11, C12, C15, C16, C19, C20).

^{19}F NMR (377 MHz, $CDCl_3$) δ 48.8, 48.4.

HRMS (ESI positive) calcd for $C_{26}H_{42}FN_3NaO_6S$ $[M+Na]^+$ 566.2671, found 566.2647.

t_R = 53.8 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 0B to 100B in 80 min)

Cbz-Leu-Leu-Phe-SO_2F 25b



The general procedure was followed on a 0.14 mmol scale delivering the desired product (10 mg, 0.017 mmol, 12%) as a white solid. Diastereomeric ratio 5:1,NMR-shifts of the diastereomer are indicated by a '*'.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 – 7.22 (m, 12.2H, CH-Ph, CH-Ph*, NH*), 7.08 – 7.00 (br s, 1H, NH), 6.80 (br s, 0.2H,

NH*), 6.30 (d, *J* = 8.0 Hz, 1.2H, NH, NH*), 5.17 – 5.09 (m, 2.4H, CH_2-C8, CH_2-C8*), 5.08 – 5.03 (br s, 1H, NH), 4.36 (m, 1.2H,CH-C4, CH-C4*), 4.09 (m, 2H, CH-C6, CH-C1), 3.91 (m, 0.4H, CH-C6*, C1*), 3.73 (m, 1.2H, CH_2-C2a, CH_2-C2a*), 3.56 (app dt, *J* = 14.6, 7.0 Hz, 1.2H, CH_2-C2b, CH_2-C2b*), 3.39 (dd, *J* = 14.5, 4.9 Hz, 1.2H, CH_2-C17a, CH_2-C17a*), 2.99 (dd, *J* = 14.5, 9.3 Hz, 1.2H, CH_2-C17b, CH_2-C17b*), 1.79 – 1.42 (m, 7.2H, CH_2-C9, C13, CH-C10, C14, CH_2-C9*, C13*, CH-C10*, C14*), 0.97 – 0.85 (m, 14.4H, CH_3-C11, C12, C15, C16, CH_3-C11*, C12*, C15*, C16*).

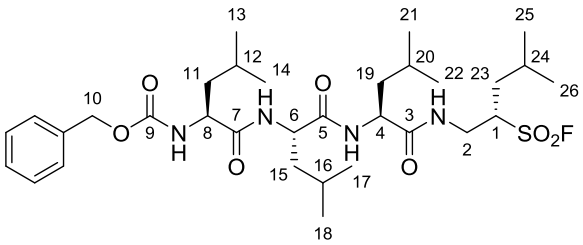
^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 172.2 (C-C4, C6, C-C4*, C6*), 156.8 (C-C7, C-C7*), 135.8, 135.7 , 134.6 (C-Ph, C-Ph*), 129.1, 129.0, 128.6, 128.4, 128.2, 128.1, 127.7 (CH-Ph, CH-Ph*), 67.6 (CH_2-C8), 67.4 (CH_2-C8*), 63.4 (CH-C1*), 62.8 (d, *J* = 8.6 Hz, CH-C1), 54.2 (CH-C6, CH-C6*), 51.9 (CH-C4*), 51.7 (CH-C4), 40.6, 40.2, 40.0 (CH_2-C9, C13, CH_2-C9*, C13*), 38.2 (CH_2-C2), 37.9 (CH_2-C2*), 33.4 (CH_2-C17, CH_2-C17*), 24.9, 24.8, 24.7 (CH-C10, C14, CH-C10*, C14*), 22.9, 21.7, 21.5 (CH_3-C11, C12, C15, C16, CH_3-C11*, C12*, C15*, C16*).

^{19}F NMR (377 MHz, $CDCl_3$) δ 51.31, 50.76.

HRMS (ESI positive) calcd for $C_{29}H_{40}FN_3NaO_6S$ $[M+Na]^+$ 600.2514, found 600.2492

t_R = 44.0 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 10B to 100B in 80 min)

Cbz-Leu-Leu-Leu-Leu-SO_2F 26a



The general procedure was followed on a 0.17 mmol scale delivering the desired product (15 mg, 0.023 mmol, 14%) as a white solid. Diastereomeric ratio 5:1, NMR-shifts of the diastereomer are indicated by a '*'.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 – 7.28 (m, 7.2H, CH-Ph, NH, CH-Ph*, NH*), 7.17 (br s, 0.2H, NH*), 6.95 (d, *J* = 7.7 Hz, 1.2H, NH, NH*), 6.46 (br s, 1H, NH), 5.21 – 5.05 (m, 3.6H, NH, CH_2-C10, NH*, CH_2-C10*), 4.45 – 4.37 (m, 1.2H, CH-C6, CH-C6*), 4.29 – 4.22 (m, 1.2H, CH-C4, CH-C4*), 4.12 (app dt, *J* = 17.0, 8.5 Hz, 1.2H, CH-C8, CH-C8*), 3.82-3.71 (m, 2.4H, CH_2-C2a, CH-C1, CH_2-C2a*, CH-C1*), 3.69-3.63 (m, 0.2H, CH_2-C2b *), 3.58 – 3.49 (m, 1H, CH_2-C2b), 1.98 – 1.44 (m, 14.4H, CH_2-C11, C15, C19, C23, CH-C12, C16, C20, C24, CH_2-C11, C15, C19, C23, CH-C12*, C16*, C20*, C24*), 1.03 – 0.81 (m, 28.8H, CH_3-C13, C14, C17, C18, C21, C22, C25, C26, CH_3-C13*, C14*, C17*, C18*, C21*, C22*, C25*, C26*).

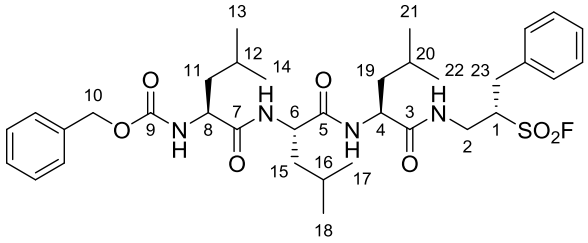
^{13}C NMR (126 MHz, $CDCl_3$) δ 173.4, 173.1, 171.8 (C-C7, C5, C3, C-C7*, C5*, C3*), 156.9 (C-C9, C-C9*) , 135.6 (C-Ph, C-Ph*), 128.7, 128.6, 128.0 (CH-Ph, CH-Ph*), 67.6 (CH_2-C10, CH_2-C10*), 60.2 (d, *J* = 8.1 Hz, CH-C1), 54.7 (CH-C8, CH-C8*), 53.4 (CH-C4, CH-C4*), 52.1, 51.9 (CH-C6, CH-C6*), 40.6, 39.9, 39.8, (CH_2-C11, C15, C19, CH_2-C11*, C15*, C19*), 39.1 (CH_2-C2, CH_2-C2*), 36.1 (CH_2-C23, CH_2-C23*), 25.4, 25.3, 25.01, 24.9, 24.8 (CH-C12, C16, C20, C24, CH-C12*, C16*, C20*, C24*), 23.2, 23.0, 22.9, 22.6, 21.6, 21.5, 21.1 (CH_3-C13, C14, C17, C18, C21, C22, C25, C26, CH_3-C13*, C14*, C17*, C18*, C21*, C22*, C25*, C26*).

^{19}F NMR (471 MHz, $CDCl_3$) δ 49.75, 48.68.

HRMS (ESI positive) calcd for $C_{32}H_{53}FN_4NaO_7S$ $[M+Na]^+$ 679.3511, found 679.3474.

t_R = 26.3 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 30B to 100B in 80 min)

Cbz-Leu-Leu-Leu-Phe-SO_2F 26b



The general procedure was followed on a 0.19 mmol scale delivering the desired product (13 mg, 0.019 mmol, 10%) as a white solid. Diastereomeric ratio: 3.3:1, NMR-shifts of the diastereomer are indicated by a '*'.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.16 (m, 14.3H, CH-Ph, NH, CH-Ph*, NH*), 7.03 (br s, 1.3H, NH, NH*), 6.54 (br s, 1.6H, NH, NH*, NH*), 5.19 (d, *J* = 7.4 Hz, 1H, NH), 5.17-5.10 (s, 2.6H, CH_2-C10, CH_2-C10*), 4.46-4.35 (m, 1.3H, CH-C6, CH-C6*), 4.30-4.23 (m, 1.3H, CH-C4, CH-C4*), 4.17-4.12 (m, 1.3H, CH-C8, CH-C8*), 4.12-4.07 (m, 1H, CH-C1), 4.05-3.99 (m, 0.3H, CH-C1*), 3.74-3.67 (m, 1.3H, CH_2-C2a, CH_2-C2a*), 3.63 – 3.53 (m, 1.3H, CH_2-C2b, CH_2-C2b*), 3.35 (dd, *J* = 14.5, 5.3 Hz, 1.3H, CH_2-C23a, CH_2-C23a*), 3.06 (dd, *J* = 14.5, 8.3 Hz, 1H, CH_2-C23b), 2.99 (m, 0.3H, CH_2-C23b*), 1.92 – 1.39 (m, 11.7H, CH_2-C11, C15, C19, CH-C12, C16, C20, CH_2-C11*, C15*, C19*, CH-C12*, C16*, C20*), 0.95 – 0.83 (m, 23.4H, CH_3-C13, C14, C17, C18, C21, C22, CH_3-C13*, C14*, C17*, C18*, C21*, C22*).

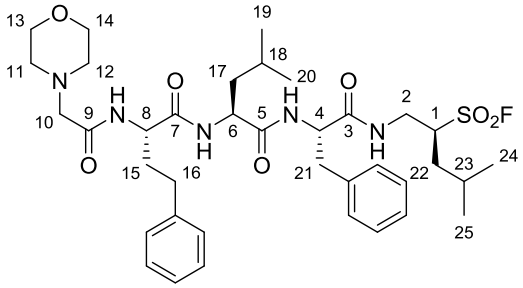
^{13}C NMR (126 MHz, $CDCl_3$) δ 173.4, 173.1, 171.9 (C-C7, C5, C3, C-C7*, C5*, C3*), 156.8 (C-C9, C-C9*), 135.6, 134.9 (C-Ph, C-Ph*), 129.2, 128.9, 128.7, 128.6, 128.0, 127.5 (CH-Ph, CH-Ph*), 67.5 (CH_2-C10, CH_2-C10*), 62.7 (d, *J* = 7.7 Hz, CH-C1), 54.6 (CH-C8, CH-C8*), 53.0 (CH-C4, CH-C4*), 52.0 (CH-C6, CH-C6*), 40.8, 40.0, 39.8 (CH_2-C11, C15, C19, CH_2-C11*, C15*, C19*), 38.6 (CH_2-C2, CH_2-C2*), 33.4 (CH_2-C23, CH_2-C23*), 25.0, 24.9, 24.8 (CH-C12, C16, C20, CH-C12*, C16*, C20*), 23.2, 22.9, 22.8, 21.8, 21.6, 21.2 (CH_3-C13, C14, C17, C18, C21, C22, CH_3-C13*, C14*, C17*, C18*, C21*, C22*).

^{19}F NMR (471 MHz, $CDCl_3$) δ 52.54, 51.60.

HRMS (ESI positive) calcd for $C_{35}H_{51}FN_4NaO_7S$ $[M+Na]^+$ 713.3355, found 713.3322.

t_R = 27.8 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 30B to 100B in 80 min)

Morph-hPhe-Leu-Phe-Leu-SO_2F 27a



The general procedure was followed on a 0.13 mmol scale delivering the desired product (20 mg, 0.027 mmol, 21%) as a white solid.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.69 (br s, 3H, NH), 7.36 – 6.89 (m, 10H, CH-Ph), 4.78 (m, 3H, CH- C4, C6, C8), 3.93 – 3.45 (m, 9H, CH_2-C13, C14, CH_2-C10, CH_2-C2, CH-C1), 3.28 – 2.89 (m, 6H, CH_2-C11, C12, CH_2-C21), 2.55 (s, 2H, CH_2-C16), 2.17 – 1.71 (m, 5H, CH_2-C15, CH_2-C22, CH-C23), 1.57 (m, 3H, CH_2-C17, CH-C18), 1.01 – 0.89 (m, 6H, CH_3-C24, C25), 0.82 (dd, *J* = 19.9, 5.6 Hz, 6H, CH_3-C19, C20).

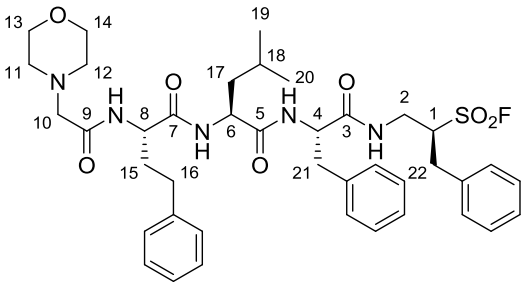
^{13}C NMR (151 MHz, $CDCl_3$) δ 172.7, 172.1, 171.7, 165.3 (C-C3, C5, C7, C9) , 140.6, 136.6 (C-Ph), 129.3, 128.8, 128.6, 128.5, 128.3, 127.1, 126.5 (CH-Ph), 64.0 (CH_2-C13, C14), 61.0 (d, *J* = 8.1 Hz, CH-C1), 58.2 (CH_2-C10), 54.6, 53.7 (CH-C4, C6), 53.6 (CH_2-C11, C12), 52.3 (CH-C8), 41.7(CH_2-C17) , 38.9 (CH_2-C2), 38.1 (CH_2-C21), 36.3 (CH_2-C22), 33.4 (CH_2-C15), 32.2 (CH_2-C16), 25.5, 25.0, (CH-C18, C23), 22.8, 22.6, 22.4, 21.6 (CH_3-C19, C20, C24, C25).

^{19}F NMR (471 MHz, $CDCl_3$) δ 49.75

HRMS (ESI positive) calcd for $C_{37}H_{55}FN_5O_7S$ $[M+H]^+$ 732.3801, found 732.3765.

t_R = 26.8 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 30B to 100B in 80 min)

Morph-hPhe-Leu-Phe-Phe-SO_2F 27b



The general procedure was followed on a 0.12 mmol scale delivering the desired product (10 mg, 0.013 mmol, 11%) as a white solid.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37 – 6.99 (m, 15H, CH-Ph), 4.66 (br s, 2H, CH-C8,) 4.42 (br s, 1H, CH-C4), 3.98 (br s, 1H, CH-C1), 3.83 (m, 8H, CH_2-C13, C14, CH_2-C10, CH_2-C2) 3.33 – 2.87 (m, 8H, CH_2-C22, CH_2-C11, C12, CH_2-C21), 2.65 – 2.59 (m, 2H, CH_2-C16), 2.05 (dd, *J* = 13.2, 6.3 Hz, 1H, CH_2-C15b), 1.96 (p, *J* = 7.5, 7.0 Hz, 1H, CH_2-C15b), 1.48 (br s, 3H, CH_2-C17, CH-C18), 0.83 (d, *J* = 5.0 Hz, 3H, CH_3-C19/C20), 0.79 (d, *J* = 5.0 Hz, 3H, CH_3-C19/C20).

^{13}C NMR (151 MHz, $CDCl_3$) δ 173.0, 172.8, 172.1, 172.0 (C-C3, C5, C7, C9), 140.5, 136.7, 136.6, 134.8 (C-Ph), 129.42, 129.3, 129.2, 129.2, 129.1, 128.8, 128.7, 128.5, 128.4, 127.9, 127.1, 126.5 (CH-Ph), 64.6, 64.4(CH2-C13, C14), 63.1 (d, *J* = 8.2 Hz, CH-C1), 59.0 (CH_2-C10), 54.5, 53.8 (CH-C4, C6), 53.2 (CH_2-C11, C12), 52.9 (CH-C8), 40.8 (CH_2-C17), 38.4 (CH_2-C2), 37.4 (CH_2-C21), 33.70 (CH_2-C22), 33.4 (CH_2-C15), 32.2 (CH_2-C16), 24.9 (CH-C18), 22.7, 21.9 (CH_3-C19,C20)

^{19}F NMR (471 MHz, $CDCl_3$) δ 51.73.

HRMS (ESI positive) calcd for $C_{40}H_{53}FN_5O_7S$ $[M+H]^+$ 766.3644, found 766.3608.

t_R = 27.8 min (Gemini column C18, 10 µm, 250 x 21.2 mm, 30B to 100B in 80 min)

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Supplementary Material

Supplementary material includes synthesis of the precursors of the SF-warheads, reactivity studies of the SFs, synthesis of the precursors of PSF inhibitors, NMR spectra, LC-MS-spectra, proteasome activity evaluation and buffer stability studies.