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Article

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Potent and highly selective Inhibitors of the Proteasome Trypsin-like site by Incorporation of Basic Side Chain containing Amino Acid derived Sulfonyl Fluorides

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ABSTRACT

A unique category of basic side chain containing amino acid derived sulfonyl fluorides (SFs) has been synthesized for incorporation into new proteasome inhibitors targeting the trypsin-like site of the 20S proteasome. Masking the former α-amino functionality of the amino acid starting derivatives as an azido functionality, allowed an elegant conversion to the corresponding amino acid derived sulfonyl fluorides. The inclusion of different SFs at the P1 site of a proteasome inhibitor resulted in 14 different peptido sulfonyl fluorides (PSFs) having a high potency and an excellent selectivity for the proteolytic activity of the β2 subunit over that of the β5 subunit. The results of this study strongly indicate that a free N-terminus of PSFs inhibitors is crucial for high selectivity towards the trypsin-like site of the

20S proteasome. Nevertheless, all compounds are slightly more selective for inhibition of the constitutive over the immunoproteasome.

INTRODUCTION

The ubiquitin-proteasome pathway (UPS) comprises the main machinery for degrading damaged, misfolded, pathogen derived and abnormal proteins in the cell.[1] Therefore, the proteasome plays a crucial role in the regulation of many cell cycle processes especially involving antigen processing and apoptosis after protein quality control.[2] Proteolysis of the designated proteins is achieved by the 20S proteasome which consists of 4 stacked rings comprising 28 subunits assembled in two outer α-rings and two inner β-rings. Within the proteolytic β-rings of the 20S constitutive proteasome the β1c, β2c and β5c subunits are found to show catalytic activity referred to as caspase-like activity (β1c), trypsin-like activity (β2c) and chymotrypsin-like activity (β5c). Upon exposure to interferon gamma (IFNγ) and/or tumor necrosis factor-α (TNFα) these subunits are substituted by β1i (LMP2), β2i (MECL-1) and β5i (LMP7), respectively, resulting in the so-called immunoproteasome.[3] Selective targeting of either constitutive or immunoproteasome subunits is a particular challenge and opens up further possibilities for the development of anti-cancer and anti-inflammatory therapeutic agents. This may be even extended to development of parasite-selective proteasome inhibitors.[4] The development of proteasome inhibitors has been an outstanding case showing that irreversible inhibitors may provide unique advantages by forming long-lived ties with their target.[5]

Since the approval of Bortezomib **1** in 2003[6] and Carfilzomib **2** in 2012[7] (Figure 1) for the treatment of multiple myeloma many peptide based inhibitors containing different

electrophilic traps have been reported.[8] These next generations of inhibitors like ixazomib[9,10] oprozomib[11] (currently in clinical trials) and LU-102 (**3**)[12,13] (in preclinical testing) clearly demonstrated that other "warheads" or electrophilic traps within a peptide based inhibitor can provide attractive alternatives.

We have initiated the exploration of the sulfonyl fluoride warhead for incorporation into proteasome inhibitors and other proteases inhibitors[14] leading to peptido sulfonyl fluorides (PSFs).[15,16] Since then this electrophilic trap has undergone considerable development as it is presently denoted as a "privileged warhead" in chemical biology, partly due to its considerable aqueous stability and chemical reactivity in the respective target enzyme.[a,17] Apart from obtaining potent proteasome inhibitors, in particular Cbz-Leu_4-SF **4** with an IC_{50}-value of 7 nM for the β 5c subunit of the constitutive proteasome,[15] it was found that the SF-warhead also endowed proteasome inhibitor **5** with a 25 fold higher selectivity for inhibition of the β5i subunit of the immunoproteasome over the β5c subunit although with loss of potency.[16] This showed clearly that SFs were not merely acting as a powerful electrophilic trap but also gave rise to higher selectivity by simply changing the electrophilic trap from an epoxyketone to a sulfonyl fluoride.

[a] Several transformations (e.g. oxidation, reduction, hydrolysis etc.) can be carried out in the presence of an aromatic SF moiety, which is indicative of the difference in stability of aliphatic and aromatic SFs. [18]

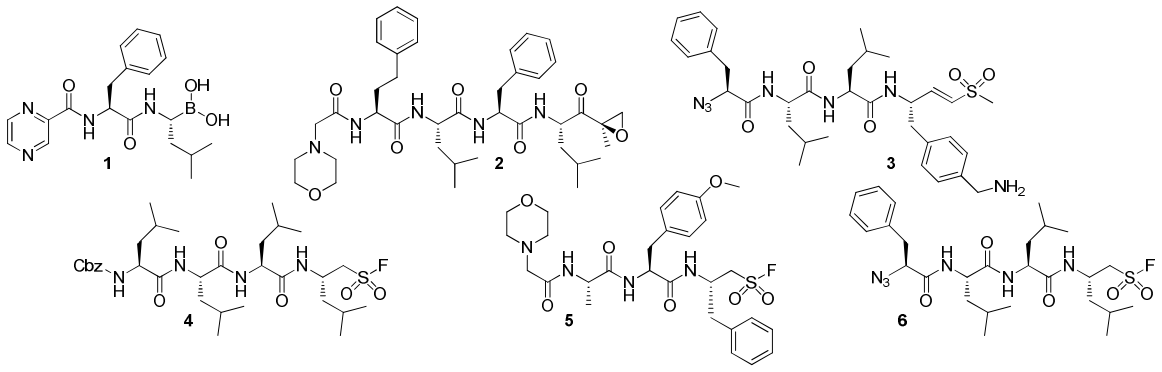


Figure 1. Structures of Bortezomib (**1**), Carfilzomib (**2**), LU-102 (**3**), Cbz-Leu$_4$-SF (**4**), β5i immunoproteasome selective PSF (**5**) and N_3-Phe-Leu$_3$-SF (**6**).

Although this was an important finding, we think that selectivity for a particular proteasome subunit or in general a protease is largely determined by the character and relative position of the P1 side chain with respect to the SF warhead.[b] Therefore, we focused our efforts on inhibitors with basic side chains at the P1 position to evaluate whether this would be sufficient to confer selectivity of the resulting inhibitors for the proteasome trypsin-like site (β2) over the chymotrypsin-like site (β5). Moreover, development of β2 selective inhibitors will contribute to overcoming resistance against existing (β5) inhibitors.[12,13]

Recently, we found that PSF **6** was a very potent proteasome inhibitor (IC_{50} 110 nM for β5c).[15] The known inhibitor LU-102 **3** (IC_{50} 3.8 nM for β2)[19] has largely the same backbone sequence but also contains a basic non-natural amino acid residue. This latter sequence was used here for incorporation of an SF warhead leading to development of the synthesis and investigation of the selectivity and potency of basic side chain containing PSF proteasome inhibitors. The required development of amino acid derived sulfonyl fluorides containing a basic side chain is described in this paper and their incorporation in PSF proteasome

[b] Recently it was found in peptido vinyl sulfonyl fluorides (PVSFs) that although a PVSF may be more reactive than a peptido sulfonyl fluoride (PSF), the sulfonyl fluoride warhead part may occupy a less favorable P1' position because it is further positioned from the P1 side chain, leading to a reduced inhibition.[20]

inhibitors led to both potent and highly selective inhibitors of the proteasome's trypsin-like (β 2) activity.

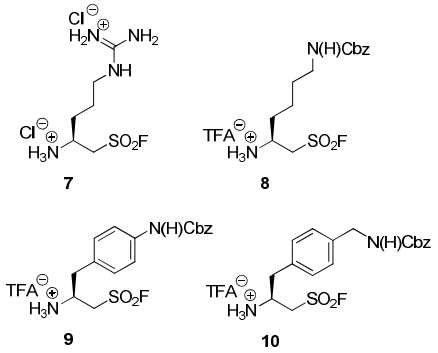


Figure 2. Basic side chain containing amino acid derived SFs **7** - **10**.

RESULTS AND DISCUSSION

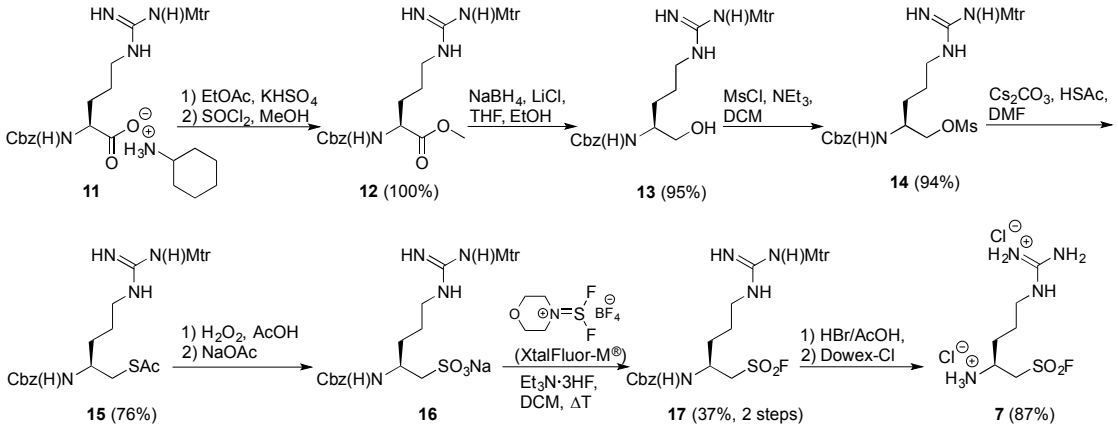
Synthesis of basic side chain containing amino acid derived sulfonyl fluorides

Considerations: Synthesis of an amino acid derived sulfonyl fluoride (SF) containing a basic side chain, which upon incorporation in the remainder of the proteasome inhibitor sequence should endow the resulting molecular construct with β 2-selectivity, was a significant challenge. Because of the simultaneous presence of an electrophilic site - the sulfonyl fluoride moiety - and two nucleophilic sites, that is, the former α -amino group and the basic side chain, a suitable protecting group strategy was necessary. It was known from our previous work that as long as the α -amino group is protected or protonated, it is possible to leave the SF-moiety intact and ultimately incorporate it in an inhibitor construct.[15] The desired amino acid derived SFs with a basic side chain for inhibitor construction are shown in Figure 2. Based on the pKa-values of the side chain and the relative position of the amino functionality with respect to the SF-electrophile, it was expected that the guanidine functionality will always remain protonated. Therefore, it will not react with an SF-moiety not even after the final deprotection step in the synthesis of the arginine building block **7**

(Scheme 1) and incorporation in PSFs **53**-**58** by peptide coupling reactions. The aromatic and benzylic amino groups obtained by deprotection after incorporation of **9** and **10**, respectively, will not be available for an intra-molecular reaction. This is probably also the case for the lysine derived amino group obtained by deprotection after incorporation of **8**, which apart from being protonated at physiological pH can only give rise to the formation of an 8-membered ring. Nevertheless, there are possibilities for intermolecular reactions and/or (slow) hydrolysis (see stability experiments, Figure 5) and therefore amino groups of amino SF derivatives **8**-**10** remained protected including the coupling steps leading to PSFs **59**-**67**.

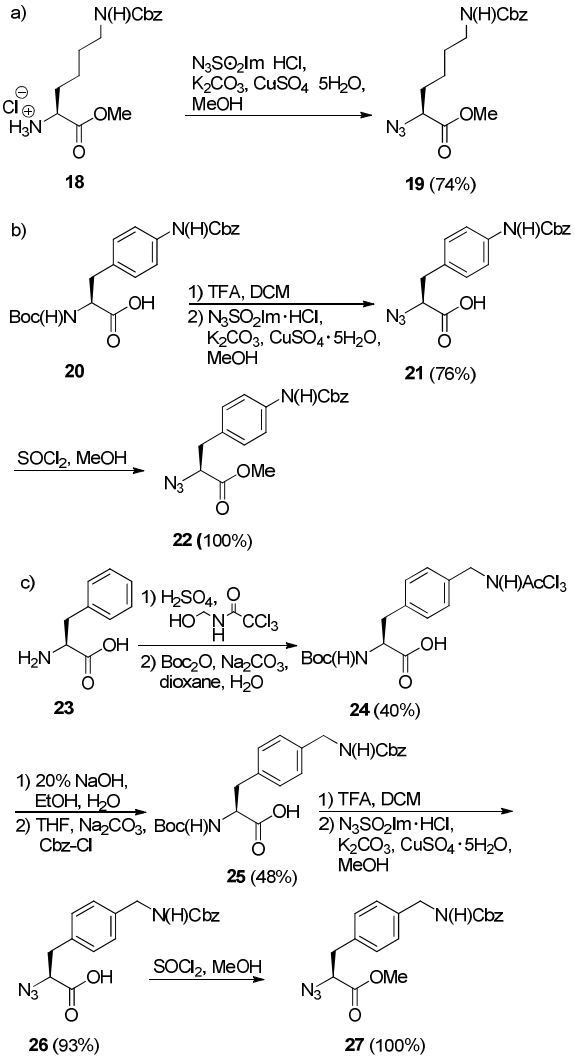
Synthesis: The syntheses of amino acid derived SFs **7**-**10** was carried out following our earlier described general strategy with modifications involving the fluoronating agent and in light of the considerations above (Schemes 1 - 3).[14] Starting from commercially available arginine compound **11**, which has protecting groups resistant to conditions used for the introduction of the SF warhead, it was first converted to methyl ester **12**. Reduction to alcohol **13**, preparation of mesylate **14** was followed by substitution to thioacetate **15**. After oxidation to the sulfonic acid derivative **16** the corresponding sulfonyl fluoride **17** was obtained using XtalFluor-M™.[21] Finally, simultaneous removal of the Cbz and Mtr protective groups afforded the arginine derived SF **7** ready for coupling to the remainder of the inhibitor sequence (Scheme 1)

Scheme 1. Synthesis of arginine derived sulfonyl fluoride (7).



For the synthesis of the lysine derived SF **8** and the amino phenylalanine derived SFs **9** and **10** a different synthetic strategy was necessary. To avoid manipulation of two orthogonal protecting groups present on both amino-functional groups during or at the end of synthesis, it was decided to mask the α-amino group as an azide functionality until the very end, that is, after completion of the synthesis of the SF-warhead. This strategy was successful and allowed a relatively straightforward synthesis of the side chain protected azido amino acid derived SFs **40**, **41** and **42**.

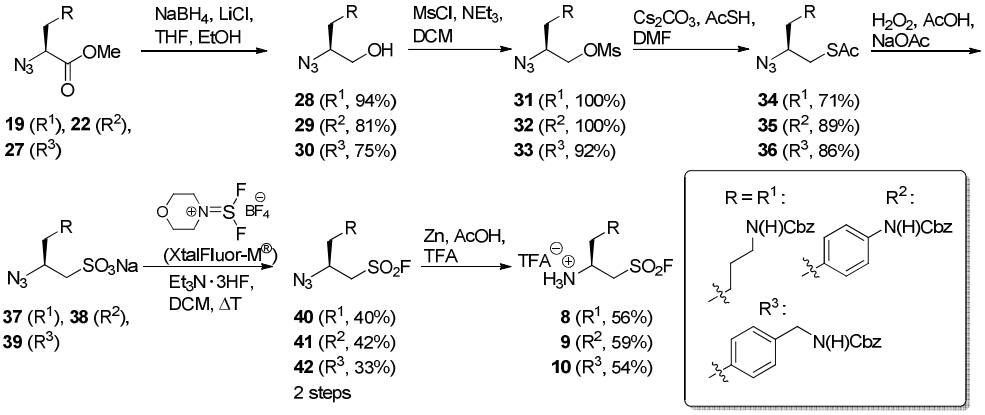
Scheme 2. Syntheses of azido precursor amino acids (19), (22) and (27).



Briefly, Lysine derivative **18** and amino phenyl alanine derivative **20** were converted to the corresponding α-azido derivatives **19** and **21** using the azido transfer reagent azido-sulfonyl-imidazole in the presence of cupric sulfate.[22] The required amino-methyl phenylalanine derivative **25** had to be prepared first in four steps from phenylalanine **23** similar to the synthesis of Geurink et al.[19] Compound **26** was then converted analogously to the required α-azido derivative **27** (Scheme 2). For these three α-azido derivatives **19**, **22** and **27** an identical series of synthetic steps was followed to obtain the desired substituted amino SFs **8** - **10** (Scheme 3). The steps for introduction of the sulfonic acid moiety comprised of reduction of the methyl ester to amino alcohol **28** - **30**, introduction of Ms-leaving group to mesylates

31 - **33**, followed by substitution to thioacetates **34** - **36** and finally oxidation to afford the sulfonates **37** - **39**, which were immediately converted to the corresponding SF-derivatives **40** - **42** using XtalFluor-M®. The combined oxidation - SF conversion is still a considerable hurdle. Nevertheless, still decent yields of 33 - 42% of over two steps (average 58 - 65% per step) were realized. Reduction of the azide group followed by protonation to the amino acid SF derivatives **8** - **10** was carried out using Zinc powder in 15% TFA in AcOH.[23] After purification by semi-preparative HPLC, SF derivatives were obtained as TFA-salts in moderate yields (54-59%). The overall yields of the SF warhead containing amino acid derivatives were quite satisfactory, i.e. 11% (7-steps) for TFA·H_2N-Lys(Cbz)ΨCH$_2$SO$_2$F (**8**), 7% (9-steps) for TFA·H_2N-Phe(4-N(H)Cbz)ΨCH$_2$SO$_2$F (**9**) and 2% (10 steps) for TFA·Phe(4-CH$_2$N(H)CbzΨCH$_2$SO$_2$F (**10**).

Scheme 3. Completion of the synthesis of basic side chain containing amino acid derived SFs 8 - 10.

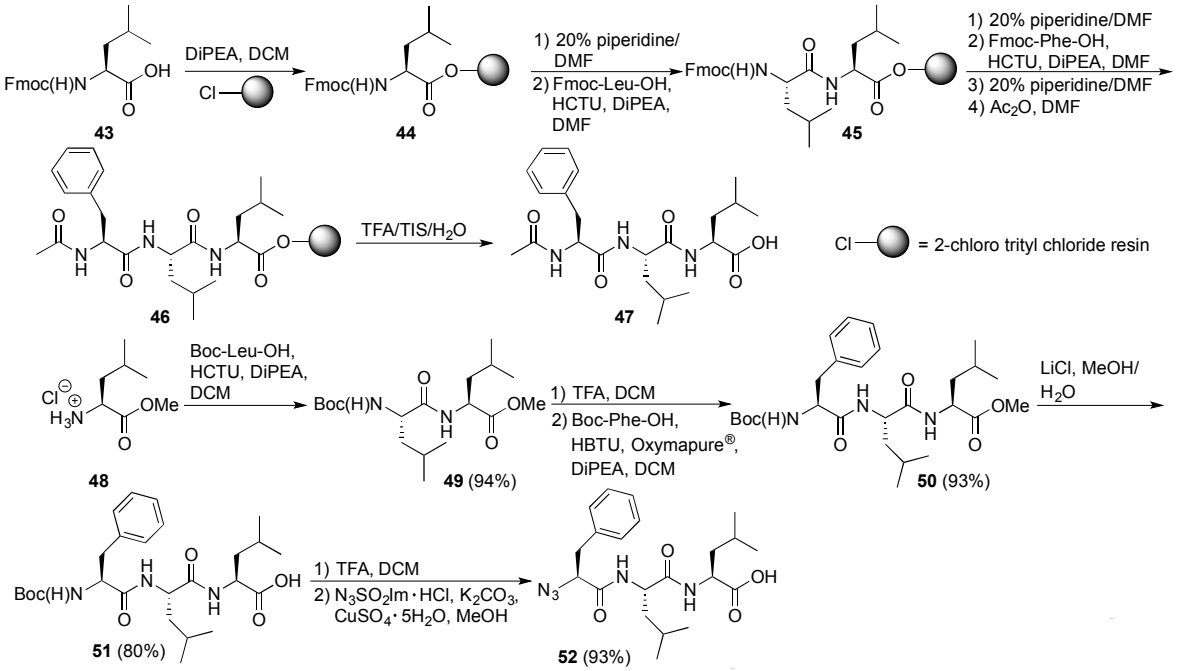


Incorporation of amino SF derivatives toward syntheses of peptido sulfonyl fluorides (PSFs)

For completion of the synthesis of the desired PSFs the amino acid derived SFs had to be incorporated into suitable peptide sequences i.e. **47** and **52**. These sequences were based on the earlier developed powerful PSF proteasome inhibitors.[15] Their syntheses are shown in Scheme 4. Assembly of Ac-Phe-Leu-Leu-OH **47** was carried out on a 2-chlorotrityl chloride resin to afford **46** using a SPPS protocol and preparation of Boc-Phe-Leu-Leu-OH **51** was carried out in solution.[c] To prevent racemization at the C-terminus, the methyl ester of **50** was saponified first before conversion of the amino terminus to an azide functionality in **52**. This azide containing precursor peptide was prepared, because the most active of our earlier described proteasome inhibitors[15] contained an azide functionality at the N-terminus of PSF **6**. As it was found that PSFs require at least a capped N-terminus for selectivity towards the immunoproteasome over the constitutive proteasome, also PSFs with an acetylated and unprotected N-terminus were synthesized to investigate if this was also crucial for β2 specificity.[16]

Scheme 4. Syntheses of the peptides necessary for incorporation of the amino acid derived SFs.

[c] There was no specific reason for synthesis of one tripeptide on the solid phase and the other tripeptide in solution. Either method can be used for both tripeptides.

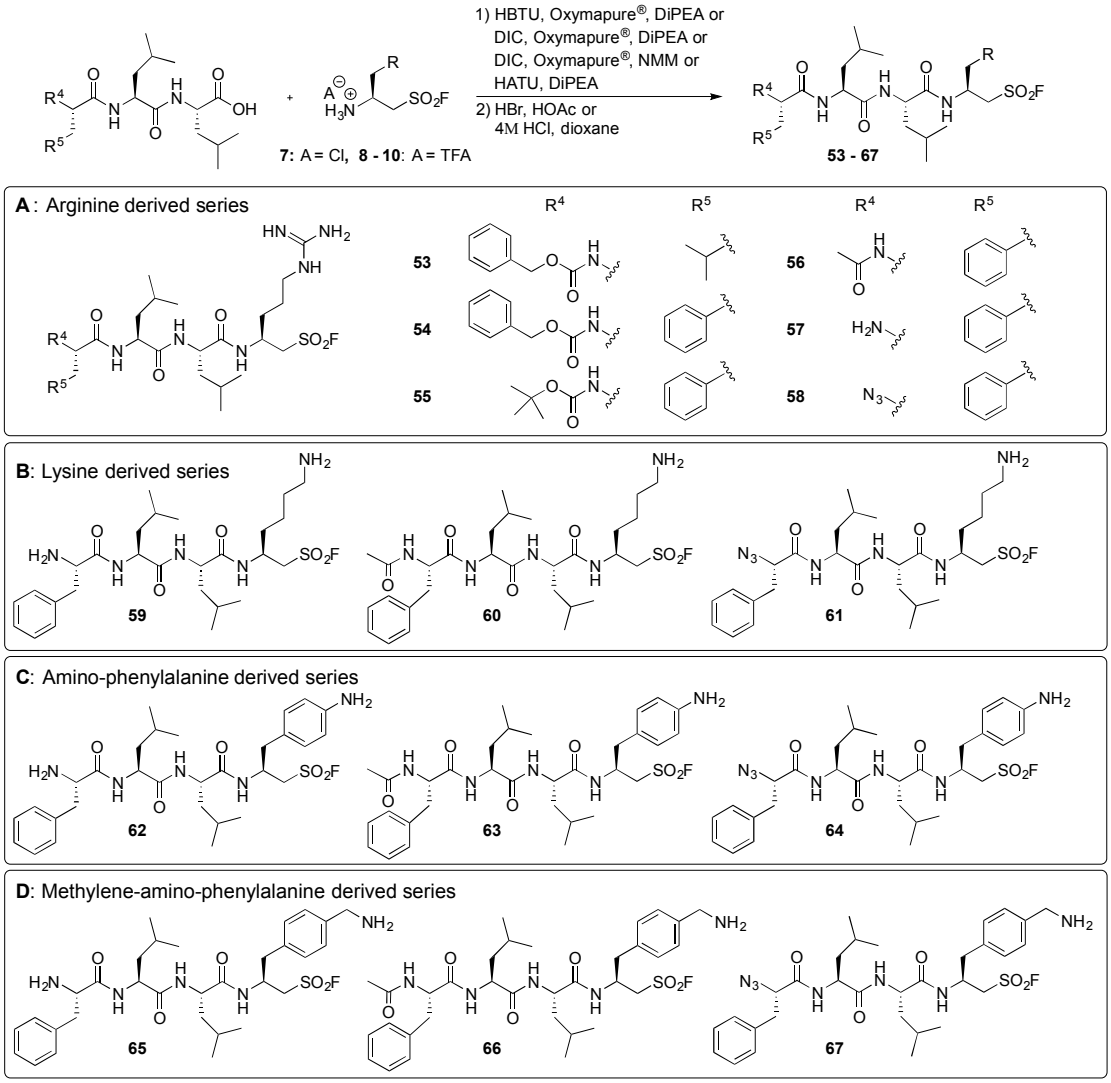


Introduction of SF warhead containing amino acid derivatives has always been viewed as one of the most challenging steps in the total synthesis of PSFs. First, the coupling conditions involving a nucleophilic amino group have to be selected in such a way that the SF electrophile stays as much as possible intact. Second, there is the possibility of racemization of the amino SF derivative in view of the electron-withdrawing character of the SF moiety. Therefore, several coupling reagents and conditions were attempted. These included HCTU, DIC/Oxymapure®, HBTU/Oxymapure®, BOP and HATU. Using a different base, for example NMM instead of DiPEA, during the coupling step did not affect the yields. Nevertheless, DIC/Oxymapure and HATU in combination with DiPEA as a base gave the best results in series in terms of yields and racemisation (see supporting information).[24,25]

For the final successful preparation of the basic side-chain containing proteasome containing inhibitors **53** - **67**, the Cbz and Boc protecting groups leading to PSFs **59, 60, 62, 63, 65** and **66** were removed by HBr in acetic acid. These deprotection conditions led to substitution of the azide functionality in compounds like N_3-Phe-Leu-Leu-Phe(4-NH_2)-SF **64** by bromide. Fortuitously, a 4 M HCl solution in dioxane led to the desired deprotected proteasome

inhibitor N_3-Phe-Leu-Leu-Phe(4-CH_2NH_2)-SF **67** and N_3-Phe-Leu-Leu-Lys-SF **61**. Unfortunately, this method was not successful to afford the inhibitor N_3-Phe-Leu-Leu-Phe(4-NH_2)-SF **64**. All PSF inhibitors containing an arginine at the P1 position were coupled successfully with unprotected arginine derived SF as it was anticipated that the guanidine moiety stays protonated during the reaction and therefore would not react.

Scheme 5. Synthesis of the peptide sulfonyl fluorides (PSFs) and overview of the structures of the inhibitors. Note: all amino and guanidine functionalities are protonated.



Biological evaluation of the PSFs

The IC_{50} values of the *in vitro* structure activity relationship studies (SAR) of the synthesized peptido sulfonyl fluorides were determined from the inhibitory curves in Figure 3 using constitutive human proteasome and are summarized in Table 1. The residual activity of the 20S proteasome activity was measured at time points using a fluorescent probe where a decrease in fluorescence corresponds to lower residual proteasome activity and therefore indicating a more potent inhibitor. In total, 14 basic amino acid derived peptido sulfonyl fluorides were tested of which compounds **53**, **54**, **57**, **62**, **65**, **66** and **67** showed IC_{50} values below 250 nM for the trypsin-like site. Particularly compounds **54**, **65**, **66** and **67** were the most potent inhibitors of this series with IC_{50} values of 150 nM for **54**, 140 nM for **65**, 119 nM for **66** and 130 nM for compound **67.** Although compounds **60** and **63** had IC_{50} values higher than 1 μM, all other compounds had IC_{50} values lower than 1 μM for inhibiting the trypsin-like proteasome activity. Compounds **59**, **65** and **67** could only be obtained as a diasteromeric mixtures in which the amino sulfonyl fluoride residue had probably partially racemised, nevertheless these PSFs still had relatively low IC_{50} values. In the arginine derived PSFs the Cbz-protecting group appeared to be beneficial for the potency when compound **54** is compared to compound **55**, **56**, **57** or **58**. For example, changing the Boc-group in PSF **55** to a Cbz protecting group in PSF **54** decreased the IC_{50} value by more than 500 nM. Due to the synthetic strategy of the PSFs **59** - **67**, the syntheses of Cbz N-termini protected analogs in this series of lysine, amino-phenylalanine and 4-aminomethyl-phenylalanine derived PSFs was not feasible at this point.[d] Comparing compounds **56** with **58** and **60** with **61**, it becomes apparent that an azido functionality does enhance the potency of

[d] The final deprotection step was removal of the side-chain Cbz-protecting group of the amino protected sulfonyl fluoride, which also would remove any terminal Cbz-protecting group

the inhibitor significantly (roughly a factor 2) compared to those of the acetyl capped counterparts. The lysine and amino-phenylalanine derived PSFs **59**, **60**, **61**, **62** and **63** showed in general the lowest potencies in this study indicating that the presence of stronger basic side chains in the P1 position was more favorable.

The key goal of this study was the development of β2 selective (trypsin-like) PSF inhibitors. It was shown that, in general, except for the aniline derived PSFs **62** and **63**, all inhibitors possessed a moderate to very high β2 selectivity. Outstanding β2-selectivity was shown by PSF inhibitors having a free terminus as in the arginine derived PSF **57** (~600 fold selectivity), the lysine derived PSF **59** (>1000 fold selectivity) and the methylene amino phenyl alanine derived PSF **65** (~900 fold selectivity), emphasizing the essentiality of free amino terminus with respect to this.

Originally, azide containing PSF-ligands[15] were developed to capture possibly formed ligand/proteasome covalent adducts using the copper(I) catalyzed azide-alkyne cyclo addition (click) reaction. However, later we found that formation of covalent adduct with the proteasome active site is followed by elimination of the ligand[16]. Nevertheless, PSF **6** having an azido N-terminus was uncovered as one of most active β5 proteasome inhibitors[15] and therefore azido containing PSF inhibitors were included in this study to evaluate whether this also would be the case for β2 inhibition. Although the azido containing inhibitors (**58**, **61** and **65**) were slightly more active or had a similar activity that N-terminally protected PSFs, as was mentioned above, a free-amino terminus, generally led to both the most potent and selective β2 compounds **57**, **59**, **62** and **65.**

Although the β5-inhibitory activity, that is inhibition of chymotrypsin activity, of all compounds was understandably poor, perhaps with the exception of **62** and **63** (both IC_{50}-values < 1 μM), this activity improves slightly by having a protected or masked (azide) N-terminus. This finding is in accordance with earlier findings that β5-selectivity (towards the

chymotrypsin-like site) requires at least a capped N-terminus of the PFSs.[15] Interestingly, compound **63** is the only one in this series, which gave rise to stronger inhibition of β5-activity as compared to inhibition of β2-activity. This might indicate that the hydrophobic character of 'aniline' side-chain plays a dominant role in determination of the selectivity towards β5-inhibition.

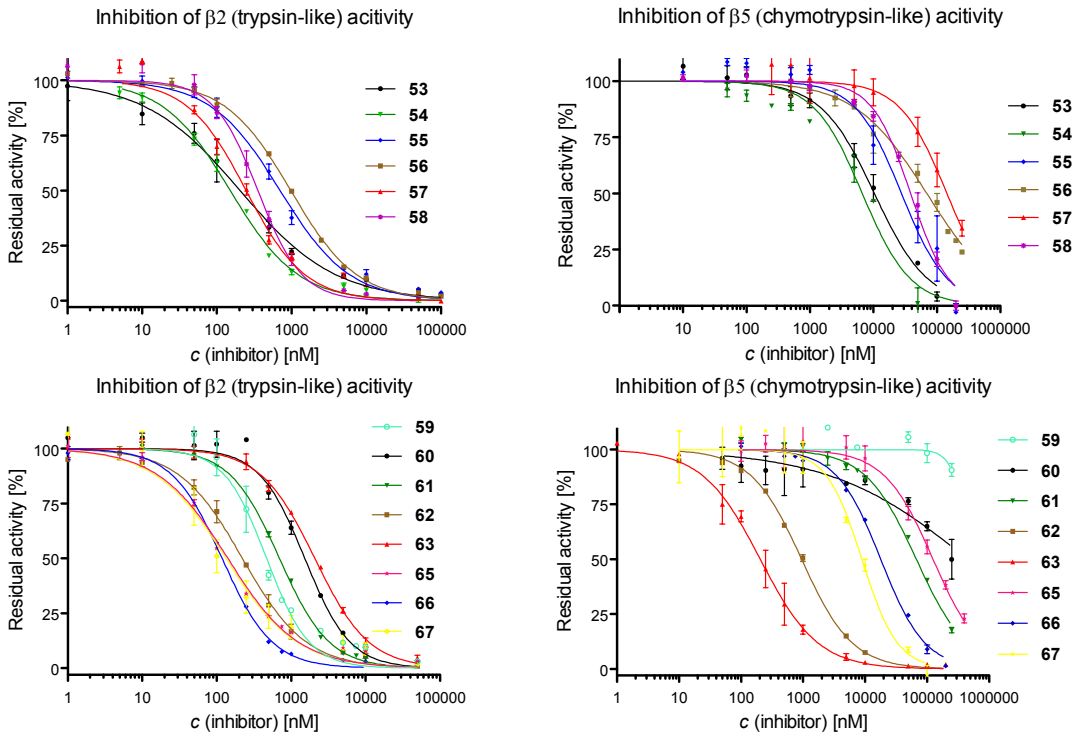
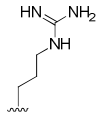
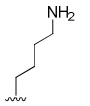
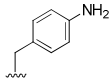
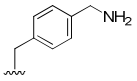


Figure 3. *In vitro* evaluation of PSFs using human constitutive 20S proteasome. Fluorogenic substrates were selective for the respective trypsin-like (Bz-VGR-AMC) or chymotrypsin-like (Suc-LLVY-AMC) subunit. Top: Arginine derived PSF´s. Bottom: Lysine, amino-phenylalanine and 4-aminomethyl-phenylalanine derived PSF. Left: Trypsin-like residual enzyme activity. Right: Chymotrypsin-like residual enzyme activity.

Table 1. Overview of the IC_{50} values of inhibition of the constitutive proteasome by synthesized PSFs.

Compound				IC_{50} [nM]		IC_{50} (β5)/ IC_{50} (β2)
	R^4	R^5	R	β2 (Trypsin-like)	β5 (Chymotrypsin-like)	
53	Cbz(H)N			200 ± 100	10,000 ± 3,400	50
54	Cbz(H)N			150 ± 30	6,700 ± 1,600	45
55	Boc(H)N			700 ± 200	27,800 ± 15,700	40
56	Ac(H)N			980 ± 10	69,000 ± 29,000	70
57	H_2N			250 ± 45	140,000 ± 12,100	560
58	N_3			350 ± 65	39,000 ± 8,000	110
59	H_2N			470 ± 110	>> 100 µM	> 1,000
60	Ac(H)N			1,500 ± 35	>> 100 µM	300
61	N_3			700 ± 45	67,000 ± 3,200	96
62	H_2N			220 ± 90	950 ± 65	4.3
63	Ac(H)N			2,100 ± 35	210 ± 80	0.1
64	N_3			n.d.	n.d.	n.d.
65	H_2N			140 ± 7	125,000 ± 26,000	900
66	Ac(H)N			119 ± 7	18,000 ± 380	150
67	N_3			130 ± 117	8,900± 360	66

Compounds **59**, **65** and **67** could only be obtained as a diasteromeric mixture in which the amino sulfonyl fluoride residue had probably partially racemised as was apparent from ^{1}H-NMR.

n.d.: not determined as compound **64** could not be synthesised.

A higher IC_{50} (β5)/ IC_{50} (β2) ratio indicates a higher β2 selectivity.

In this assay synthesized LU-102 showed IC_{50} values of 10.8 ± 2.4 nM(β2) and 2,600 ± 500 nM (β5) with an IC_{50} (β5)/ IC_{50} (β2) of 241, which is agreement with the literature[19]

Recently[16] we found that PSFs showed selective inhibition of the immuno proteasome. Therefore, a subset of the most powerful inhibitors were taken and evaluated in an immuno

proteasome assay as in shown in table 2 and figure 4. The data showed that the selectivity of β2 inhibition PSFs is maintained. However, in contrast to earlier developed PSFs[19] selectivity for inhibition of the immuno proteasome was virtually absent. This was also the case for Lu-102, which although a highly potent inhibitor, also showed selectivity for constitutive proteasome inhibition.

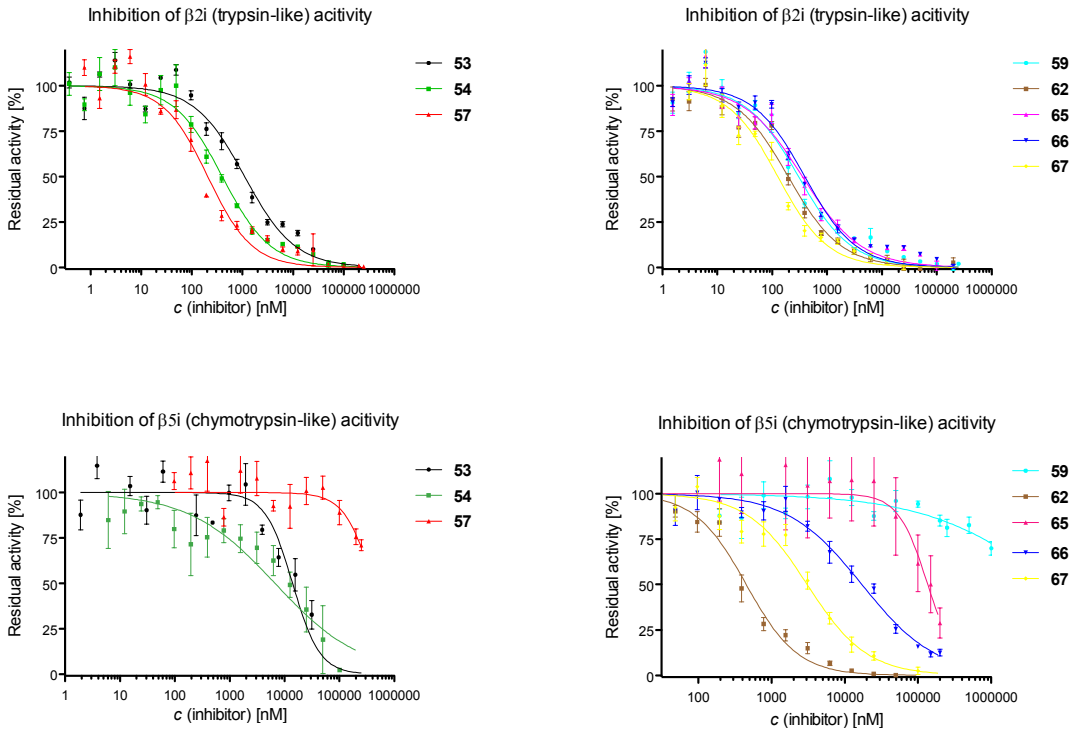
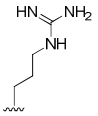
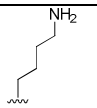
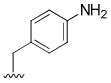
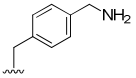


Figure 4. *In vitro* evaluation of PSFs using human immuno 20S proteasome. Fluorogenic substrates were selective for the respective trypsin-like (Bz-VGR-AMC) or chymotrypsin-like (Suc-LLVY-AMC) subunit. Top: Arginine derived PSF´s. Bottom: Lysine, amino-phenylalanine and 4-aminomethyl-phenylalanine derived PSF. Left: Trypsin-like residual enzyme activity. Right: Chymotrypsin-like residual enzyme activity.

Table 2. Overview of the IC_{50} values of inhibition of the immuno proteasome by selected PSFs.

Compound				IC_{50} [nM]		IC_{50} (β5)/ IC_{50} (β2)
	R^4	R^5	R	β2 (Trypsin-like)	β5 (Chymotrypsin-like)	
53	Cbz(H)N			1,110 ± 260	14,000± 7,300	13
54	Cbz(H)N			400± 130	6,700 ± 12,800	17
57	H_2N			210± 60	>> 100 µM	>1000
59	H_2N			300 ± 60	>> 100 µM	>1000
62	H_2N			200 ±60	450 ± 100	2.3
65	H_2N			360± 130	136,500 ± 68,600	380
66	Ac(H)N			395 ± 40	17,200 ± 6,700	44
67	N_3			130 ± 10	3,100 ± 1,500	24

Compounds **59**, **65** and **67** could only be obtained as a diasteromeric mixture in which the amino sulfonyl fluoride residue had probably partially racemised as was apparent from ^{1}H-NMR.

A higher IC_{50} (β5)/ IC_{50} (β2) ratio indicates a higher β2 selectivity.

In this assay synthesized LU-102 showed IC_{50} values of 27.5 ± 6.9 nM(β2) and 1,800 ± 2,400 nM (β5) with an IC_{50} (β5)/ IC_{50} (β2) of 64, which is agreement with the literature[19]

PSFs containing the SF-warhead comprise relatively new inhibitor ligands of which stability and reactivity properties have not been investigated as yet. In order to start determination of the former properties we have evaluated the degree of hydrolysis of PSFs in buffers of different pHs. Therefore, stability tests of several PSFs were carried out to study the rate of hydrolysis of the SF moiety at a physiological pH (7.4) as well as at pH 6.5 and pH 8.0. Acetyl capped PSFs were chosen to prevent a possible intermolecular reaction

involving the N-terminus and the SF-moiety. Of course intermolecular reactions involving the amino groups in **60**, **63** and **66** cannot be ruled out but are highly unlikely, because they are largely protonated at the studied pH's or poorly nucleophilic (**63**). An intermolecular reaction involving the guanidinium functionality in **56** is even less likely because it is virtually completely protonated at the studied pH range. So it was assumed that a possible decrease in stability of the SF-moiety is due to an increased inclination towards hydrolysis. In order to study only the hydrolytic stability and to exclude nucleophilic reactions of buffer components (for example tris(hydroxylmethyl)aminomethane in "Tris" buffer), phosphate buffered saline (PBS) was chosen as a buffer system.

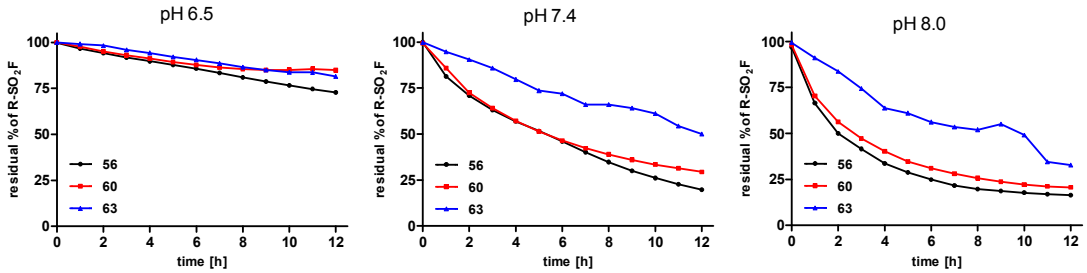


Figure 5. PBS buffer stability test at pH 6.5, 7.4 and 8.0. Hydrolysis of selected PSFs was measured via analytical HPLC over 12 h.

As anticipated, hydrolysis of PSFs was slower at slightly acidic pH (6.5) compared to physiological pH (7.4) and slightly basic pH (8.0). Even after 12 h about 75% of the initial used PSF remained unaffected at pH 6.5. After 1 h at pH 7.4 90% of the PSF was still intact. Thus, during an incubation time of one hour for the biological evaluation of a PSF, a large majority is still available for interaction with the proteasome. After 6 h at pH 7.4, $\geq$ 50% of the PSFs were still intact. However, rapid hydrolysis was observed at pH 8.0 at which 50% of the PSFs were hydrolyzed after 2 h (Figure 5).

CONCLUSIONS

We have shown that our general route for the preparation of amino acid derived SFs could be extended to SF derivatives containing nucleophilic groups in their side chains in the presence of the SF electrophilic trap. The route to the SF derived from arginine **7** was largely based on our earlier developed synthesis. The preparation of SF derivatives containing an amino group in their side chains was achieved using a judicious strategy masking the α-amino group as an azide functionality, thereby circumventing complicated (de)protection strategies. This resulted in the successful and convenient synthesis of lysine derived SF **8**, amino-phenylalanine **9** and methylene amino-phenylalanine derived SF **10** ready for incorporation in proteasome inhibitor sequences.

Although incorporation of the SF warhead into a peptide inhibitor sequence remains a challenging step in the total synthesis of PSFs, we have successfully synthesised several potent and highly selective PSFs capable of inhibiting the β2 (trypsin-like) activity over the β5 (chymotrypsin-like) activity for the first time.

In addition, we have established the crucial role of the N-terminus. PSFs with a free amino terminus gave rise to highly selective β2 proteasome inhibitors. Having an azide functionality as N-terminus did not lead to a tremendous enhanced potency as was found earlier with the β5-proteasome inhibitors. Although the described PSFs were less potent than e.g. LU-102 their selectivity for $\beta 2$ was similar. Both PSFs and LU-102 did not show selective inhibition of the immunoproteasome. Since PSF gave rise to permanent inhibition of the proteasome by ligand-induced crosslinking of the active site, it would be interesting to evaluate the significance of proteasome inhibition by irreversible inhibitors versus reversible inhibitors.

In view of the promising electrophilic trap properties leading to highly active and selective compounds, we embarked on initial stability studies in buffer of the SF-warhead. It was found that the stability at physiological pH was quite satisfactory. Clearly, this is just the start

toward gaining insights in the behaviour of these relatively novel aliphatic -amino acid derived- SFs and PSFs. Thus, there will be significant chemical challenges ahead with respect to modulating both reactivity and stability. This research will be guided by future studies directed towards investigations of cellular permeability and stability of the most promising PSFs described above.

EXPERIMENTAL PROCEDURES

General procedures. All starting materials, reagents and solvents were obtained from commercial sources and used as received. Dry solvents were obtained from a PureSolv^{TM} 500 MD solvent purification system. Reactions requiring dry conditions were performed in heat-gun dried glassware. All reactions were performed at ambient temperature unless stated otherwise. Reactions in solution were monitored by TLC analysis on Merck pre-coated silica gel 60 F_{254} (0.25 mm) glass backed plates. Spots were visualised by UV light (254 and 366 nm) and by heating plates after dipping in a ninhydrine or cerium/molybdenum solution. Column chromatography was performed on Siliaflash® P60 (40 - 63 μm) from Silicycle (Canada). Petroleum ether (40 - 60 °C fraction) and n-hexane were used for flash column chromatography. ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR spectra were recorded on a Bruker DPX 400 spectrometer or Bruker 500 spectrometer with chemical shift values reported in parts per million (ppm) relative to TMS (δ_H = 0.00 and δ_C = 0.0) or residual $CHCl_3$ (δ_H = 7.28 and δ_C = 77.16) or residual d_6-DMSO (δ_H = 2.50 and δ_C = 39.52) as standard. Assignments of ^{1}H and ^{13}C-NMR signals are based on two-dimensional COSY, HSQC, HMBC, DEPT and DEPTQ experiments, respectively. High-resolution electrospray ionization (ESI) mass spectra were measured on a Bruker micrOTOF-Q II in positive or negative mode and calibrated with an ESI tuning mix from Agilent Technologies. Infrared spectra were recorded using a Shimadzu FTIR 8400S apparatus. Optical rotations were

determined as solutions irradiating with the sodium D line (λ = 589 nm) using an Auto pol V polarimeter. $[\alpha]_D$ values are given in units 10^{-1} deg cm^2 g^{-1}. Semi-preparative high performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1260 Infinity system (12.5 $mLmin^{-1}$). Analytical HPLC chromatograms were recorded on a Shimadzu Prominence system (1 $mLmin^{-1}$). Buffers used for HPLC: buffer A (0.1% TFA in MeCN:H_2O v/v 5:95) and buffer B (0.1% TFA in MeCN:H_2O v/v 95:5). Semi-preparative runs started with an isocratic flow of buffer A (100% for 5 min), followed by a linear gradient of buffer B (in 60 min to X%). Subsequently, an isocratic flow of buffer B (100% for 5 min) was performed followed by a linear gradient to buffer A (in 5 min to 100%). Runs ended with an isocratic flow of buffer A (100% for 5 min). Used columns are mentioned in the supporting information. In addition, all HPLC chromatograms and retention times of all purified compounds are supplied in the supporting information. All compounds have purities $\geq$ 95%.

Proteasome inhibitory assays. Inhibition of the proteasome enzymatic activity was determined using the VIVAdetect™ 20S Assay Kit PLUS (Viva bioscience, UK) utilizing a Clariostar microplate reader (BMG LABTECH, Germany). All working solutions were freshly prepared for each measurement. Kinetic enzyme assays were performed using 96-wells Corning half area plates using 50 µL of total amount of liquid. Incubation of all measured inhibitors was at ambient temperature on a shaker for 60 min. Fluorescence measurements were carried out at λ_{ex} = 360 nm and λ_{ex} = 460 nm at 25 °C for 2 h. All assays were carried out in duplicate with three repetitions. Each well contained 35 µL VIVAdetect™ buffer. The final enzyme concentration in a well was 2.5 nM (5 µL of a 25 nM enzyme working solution in VIVAdetect™ buffer, prepared from 1 mg/mL 20S proteasome). Final substrate concentration was 100 µM (5 µL of a 1 mM substrate working solution in VIVAdetect™ buffer; Suc-LLVY-AMC for the chymotrypsin-like activity; Bz-

VGR-AMC for the trypsin-like activity). In order to use a final minimal concentration of DMSO stock solutions of each inhibitor were prepared: Arginine derived PSF´s were dissolved in 10% DMSO/H_2O, lysine derived PSF´s were dissolved in 30% DMSO/H_2O, 4-amino-phenylalanine and 4-methylene-amino-phenylalanine derived PSF´s were dissolved in 50% DMSO/H_2O. Dilution series of inhibitors were prepared using the appropriate stock solution. For the positive controls 5 µL of the stock DMSO-percentage solution was added instead of the respective inhibitor solution MG132 (Cbz-Leu-Leu-Leucinal) was used as a negative control with a final concentration of 5 µM (5 µL of a 50 µM working solution, supplied in the VIVAdetect^{TM} 20S Assay Kit PLUS). Final inhibitor concentrations were: 400 µM, 200 µM, 100 µM, 50 µM, 10 µM, 5 µM, 2.5 µM, 1 µM, 750 nM, 500 nM, 250 nM, 100 nM, 50 nM, 10 nM, 1 nM, 0.1 nM. The inhibitory activities of the compounds were expressed as IC_{50} values. IC_{50} values were obtained by plotting the residual percentage of enzymatic activity against the logarithm of the inhibitor concentrations. Experimental data were fitted to the equation %Residual Activity = 100/(1 + 10^((Log IC_{50}Logc(inhibitor)) * Hill Slope)) using GraphPad Prism software version 5.

Stability evaluation in buffer. Compounds **56**, **60** and **61** were dissolved in 90 µL DMSO and added to an aqueous 1 x PBS buffer solution (prepared from 10 x PBS buffer solution (Gibco) adjusted with aq. 2 M HCl or 2 M NaOH solution, respectively; pH 6.5 or 7.4 or 8.0, 910 µL) resulting in a final concentration of ~1 mM. The hydrolysis was monitored *via* analytical HPLC (C_{A3}) over 12 h. 0 h was measured directly after the addition of the PBS buffer solution and was taken as the reference peak.

Synthesis. The synthetic procedures and characterization data of crucial synthetic intermediates as well as those of the final PSFs are described below. All other synthetic procedures and characterization data are included in the supporting information, which also contains all NMR spectra, MS-data and HLPC traces of the final PSFs.

2HCl·H$_2$N-Arg-ϕ[CH$_2$SO$_2$]-F (7). Cbz-Arg(Mtr)-ϕ[CH$_2$SO$_2$]-F (**17**) (310 mg, 541 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (8 mL) and 33% HBr/AcOH solution (8 mL) was added. The reaction mixture was stirred for 1 h and the solvents removed *in vacuo*. H_2O (20 mL) was added and the aqueous layer extracted with EtOAc (20 mL) and treated with Dowex® 1×8 chloride form (777 mg) for 10 min. The mixture was filtered and freeze dried. The crude product was obtained as a yellowish solid (166 mg, 555 µmol, 100%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.63 (s, 3H, $NH_3\,\alpha$ C), 7.79 (t, J = 5.9 Hz, 1H, CN*H*CH$_2$), 4.48 (dt, J = 15.5, 6.0 Hz, 1H, CH$_a$SO$_2$F), 4.40 (dt, $^3J_{H,H,F}$ = 15.5, 5.5 Hz, 1H, CH$_b$SO$_2$F), 3.86 - 3.72 (m, 1H, H$_3$N α C*H*), 3.22 - 3.05 (m, 2H, α CNHC*H*$_2$), 1.87 - 1.72 (m, 2H, α CHC*H*$_2$), 1.72 - 1.55 (m, 2H, α CHCH$_2$C*H*$_2$). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_C (ppm) = 157.4 (NHCNH$_2$), 52.3 (d, $J_{C,S,F}$ = 14.8 Hz, CH$_2$SO$_2$F), 46.3 (α CH), 40.6 (α CNH<u>C</u>H$_2$), 29.3 (NH<u>C</u>H$_2$CH$_2$), 24.3 (α CNHCH$_2$<u>C</u>H$_2$). **^{19}F-NMR** (471 Hz, d_6-DMSO, 298 K): δ_F (ppm) = 60.3 (s, 1F, SO$_2$F). **HRMS** (ESI positive) calc. for $C_6H_{16}N_4O_2SF$ $[M+H]^+$ 227.0973, found 227.0971.

TFA·H-Lys(Cbz)-ψ[CH$_2$SO$_2$]-F (8). N$_3$-Lys(Cbz)-ψ[CH$_2$SO$_2$]-F (**40**) (300 mg, 837 µmol, 1.00 eq.) was dissolved in AcOH (12.5 mL). Next, zinc powder (547 mg, 8.37 mmol, 10.0 eq.) and TFA (1.9 mL) were added and the reaction mixture was stirred overnight at rt. The solvents were removed *in vacuo* and AcOH (10 mL) was added. The crude product was purified *via* semi-preparative HPLC (0 to 100% B, C_{P1}) and fractions containing the pure product were pooled and lyophilized. The pure product was obtained as a white solid (212 mg, 474 µmol, 57%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.39 (s, 3H, NH_3^+), 7.41 - 7.28 (m, 5H, Ar-H (Cbz)), 7.24 (t, J = 5.6 Hz, 1H, N*H*CH$_2$), 5.01 (s, 2H, CH$_2$ (Cbz)), 4.41 (dt, J = 15.6, 5.6 Hz, 1H, CH$_a$SO$_2$F), 4.25 (dt, J = 15.6, 5.7 Hz, 1H, CH$_b$SO$_2$F), 3.79 - 3.65 (m, 1H, NH$_3$C*H*), 3.04 - 2.92 (m, 2H, NHC*H*$_2$), 1.79 - 1.68 (m, 2H, αCHC*H*$_2$),

1.48 - 1.32 (m, 4H, $NHCH_2CH_2CH_2$). **^{13}C-NMR** (126 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 156.6 (C=O), 137.7 (C-Ar), 128.8, 128.2, 128.2 (CH-Ar), 65.6 (CH_2 (Cbz)), 52.3 (d, $J_{C,F}$ = 15.0 Hz, CH_2SO_2F), 46.6 (NH_3CH), 40.3 ($NHCH_2$), 31.8 ($NH_3CH\underline{C}H_2$), 29.3 (CH_2), 21.5 (CH_2). **^{19}F-NMR** (471 MHz, $CDCl_3$, 298 K): δ_F (ppm) = 62.7 (SO_2F). **HRMS** (ESI positive) calc. for $C_{14}H_{23}N_2O_4SF$ $[M+H]^+$ 333.1279, found 333.1260.

TFA·H-Phe(4-NHCbz)-ϕ [CH_2SO_2]-F (9). N_3-Phe(4-NHCbz)-ϕ [CH_2SO_2]-F (**41**) (314 mg, 799 µmol, 1.00 eq.) was dissolved in AcOH (12 mL). Next, zinc powder (522 mg, 7.99 mmol, 10.0 eq.) and TFA (1.73 mL) were added and the reaction mixture was stirred overnight at rt. The solvents were removed *in vacuo* and AcOH (10 mL) was added. The crude product was purified *via* semi-preparative HPLC (0 to 100% B, C_{P1}) and fractions containing the pure product were pooled and lyophilized. The pure product was obtained as a white solid (209 mg, 468 µmol, 59%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 9.84 (s, 1H, NH), 8.20 (br s, 3H, NH_3^+), 7.46 (d, J = 8.4 Hz, 2H, CH (Phe)), 7.44 - 7.32 (m, 5H, Ar-H (Cbz)), 7.23 (d, J = 8.4 Hz, 2H, CH (Phe)), 5.15 (s, 2H, CH_2 (Cbz)), 4.24 (app dt, J = 15.5, 5.3 Hz, 1H, CH_aSO_2F), 4.16 (app dt, J = 15.5, 6.0 Hz, 1H, CH_bSO_2F), 4.02 - 3.93 (m, 1H, NH_3^+ α C*H*), 3.03 - 2.92 (m, 2H, N_3 α $CHCH_2$). **^{13}C-NMR** (101 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 153.8 (C=O), 138.9 (C-Ar), 137.1 (C-Ar), 130.5 (CH-Ar (Phe)), 128.9 (CH-Ar (Cbz)), 128.6 (CH-Ar (Cbz)), 128.5 (CH-Ar (Cbz)), 128.0 (C-Ar), 119.0 (CH-Ar (Phe)), 66.2 (CH_2 (Cbz)), 52.2 (d, J = 15.7 Hz, CSO_2F), 47.8 (α CH), 37.3 (α CH $\underline{C}H_2$). **^{19}F-NMR** (377 MHz, $CDCl_3$, 298 K): δ_F (ppm) = 60.6 (t, J = 5.8 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{17}H_{19}N_2O_4SFNa$ $[M+Na]^+$ 389.0942, found 389.0926.

TFA·H_2N-Phe(4-CH_2NHCbz)-ϕ [CH_2SO_2]-F (10). N_3-Phe(4-CH_2NHCbz)-ϕ [CH_2SO_2]-F (**42**) (62 mg, 153 µmol, 1.00 eq.) was dissolved in AcOH (2.3 mL). Next, zinc powder (99.8 mg, 1.53 mmol, 10.0 eq.) and TFA (342 µL) were added and the reaction mixture was

stirred overnight at rt. Then, the solvents were removed *in vacuo* and AcOH (5 mL) was added. The crude product was purified *via* semi-preparative HPLC (0 to 100% B, C_{P1}) and fractions containing the pure product were pooled and lyophilized. The pure product was obtained as a white solid (40.9 mg, 82.7 µmol, 54%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.53 (br s, 3H, NH_3^+), 7.86 (t, *J* = 6.3 Hz, 1H, N*H*CH_2), 7.41 - 7.20 (m, 9H, Ar-H (Cbz), CH (Phe)), 5.05 (s, 2H, CH_2 (Cbz)), 4.31 - 4.16 (m, 4H, NHC*H*$_2$, CH_2SO_2F), 4.12 - 3.97 (m, 1H, NH_3C*H*), 3.17 - 2.97 (m, 2H, αCHC*H*$_2$Phe). **^{13}C-NMR** (101 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 156.9 (C=O), 139.5 (C-Ar), 137.6 (C-Ar), 133.3 (C-Ar), 130.0 (CH-Ar (Phe[4-CH_2NHCbz])), 128.8 (CH-Ar (Cbz)), 128.3 (CH-Ar (Phe[4-CH_2NHCbz])), 128.2 (CH-Ar (Cbz)), 127.9 (CH-Ar (Cbz)), 65.9 (CH_2 (Cbz)), 52.1 (d, *J* = 15.4 Hz, CH_2SO_2F), 47.7 (αCHCH_2Phe), 43.9 (CH_2NHCbz), 37.5 (αCHCH_2Phe). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 60.6 (s, *J* = 5.6 Hz, SO_2F). **HRMS** (ESI positive) calc. for $C_{18}H_{21}N_2O_4SFNa$ $[M+Na]^+$ 403.1098, found 403.1091.

Cbz-Arg(Mtr)-ϕ [CH_2O]-H (13). Sodium borohydride (1.81 g, 47.9 mmol, 2.50 eq.) was added to a mixture of N_α-*Z*-N_ω-(4-methoxy-2,3,6-trimethylbenzenesulfonyl)-L-arginine methyl ester **12** (10.2 g, 19.2 mmol, 1.00 eq.) and LiCl (2.03 g, 47.9 mmol, 2.50 eq.) in dry THF (40 mL) at rt and was stirred for 15 min. EtOH (55 mL) was added carefully and the resulting cloudy mixture stirred for 5 h (TLC controlled). The reaction mixture was cooled to 0 °C and quenched with saturated NH_4Cl (45 mL) and H_2O (13 mL). The mixture was extracted with EtOAc (3 × 100 mL) and the combined organic phases dried over $MgSO_4$. The solvent was removed *in vacuo* and the crude product purified *via* column chromatography (EA). The pure product was obtained as a white solid (9.19 g, 18.1 mmol, 95%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.29 - 7.14 (m, 5H, Ar-H (Cbz)), 6.42 (s, 1H, Ar-H (Mtr)), 6.26 - 6.03 (m, 3H, 3 × NH (guanidine)), 5.49 (d, *J* = 8.5 Hz, 1H, *H*NαCH), 4.96 (s,

2H, OCH_2Ph), 3.73 (s, 3H, OCH_3 (Mtr)), 3.61 - 3.41 (m, 3H, HN α C*H*, C*H*$_2$OH), 3.21 (br s, 1H, CH_2O*H*), 3.15 - 3.04 (m, 2H, NHC*H*$_2$), 2.57 (s, 3H, Ar-CH_3 (Mtr)), 2.50 (s, 3H, Ar-CH_3 (Mtr)), 2.03 (s, 3H, Ar-CH_3 (Mtr)), 1.52 - 1.32 (m, 4H, α CHC*H*$_2$C*H*$_2$). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_C(ppm) = 158.5 (C=O), 157.0 (C-guanidine), 156.4 (C-Ar), 138.5 (C-Ar), 136.4 (C-Ar (Cbz)), 133.3 (C-Ar), 128.5, 128.1, 128.0 (CH-Ar (Cbz)), 124.9 (C-Ar), 111.8 (CH-Ar (Mtr)), 66.8 (CH_2 (Cbz)), 64.7 (CH_2OH), 55.4 (OCH_3), 41.0 ($NHCH_2$), 28.5 (α CH<u>C</u>H$_2$CH$_2$), 25.6 (α CHCH$_2$<u>C</u>H$_2$), 24.1 CH_3 (Mtr)), 18.3 CH_3 (Mtr)), 11.9 (CH_3 (Mtr)). **HRMS** (ESI positive) calc. for $C_{24}H_{34}N_4O_3S$ $[M+Na]^+$ 529.2091, found 529.2073.

Cbz-Arg(Mtr)-ϕ [CH_2O]-Ms (14). Methanesulfonyl chloride (1.72 mL, 22.2 mmol, 1.30 eq.) was added dropwise to a solution of Cbz-Arg(Mtr)-ϕ [CH_2O]-H (**13**) (8.64 g, 17.1 mmol, 1.00 eq.) in dry CH2CL2 (200 mL) at 0 °C. NEt_3 (3.1 mL, 22.2 mmol, 1.30 eq.) was added dropwise and the reaction mixture was stirred for 2 h at rt (TLC controlled). After the reaction was finished the organic phase was washed with an aqueous 1 M $KHSO_4$ solution (250 mL), H_2O (250 mL) and brine (250 mL). The organic layer was dried over $MgSO_4$, the solvent was removed *in vacuo* and the crude product obtained as a white solid (9.36 g, 16.0 mmol, 94%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.31 - 7.20 (m, 5H, Ar-H (Cbz)), 6.44 (s, 1H, Ar-H (Mtr)), 6.11 (br s, 2H, 2 × NH (guanidine)), 5.99 (s, 1H, NH (guanidine)), 5.46 (d, *J* = 7.7 Hz, 1H, *H*N α CH), 5.01 (d, *J* = 12.2 Hz, 1H, OCH_aPh), 4.95 (d, *J* = 12.2 Hz, 1H, OCH_bPh), 4.11 (dd, *J* = 10.3, 4.3 Hz, 1H, C*H*$_a$OSO$_2$CH$_3$), 4.05 (dd, *J* = 9.9, 4.3 Hz, 1H, C*H*$_b$OSO$_2$CH$_3$), 3.85 - 3.76 (m, 1H, HN α C*H*), 3.74 (s, 3H, OCH_3 (Mtr)), 3.17 - 3.05 (m, 2H, HNC*H*$_2$), 2.86 (s, 3H, SO_2CH_3), 2.57 (s, 3H, Ar-CH_3(Mtr)), 2.50 (s, 3H, Ar-CH_3(Mtr)), 2.04 (s, 3H, Ar-CH_3 (Mtr)), 1.57 - 1.37 (m, 4H, α CHCH2CH2). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_C (ppm) = 158.6 (C=O), 156.4 (C-guanidino), 156.3 (C-Ar), 138.5 (C-Ar), 136.6 (C-Ar), 136.3 (C-Ar), 128.5, 128.2 , 128.0 (CH-Ar (Cbz)), 124.9

(C-Ar), 111.8 (CH-Ar (Mtr)), 71.1 ($\underline{C}H_2OSO_2CH_3$), 66.9 ($OCH_2$(Cbz)), 55.5 ($OCH_3$), 50.1 (α CH), 40.7 ($NHCH_2$), 37.2 (SO_2CH_3), 28.1 (α CH$\underline{C}H_2CH_2$), 25.5 (α CH$CH_2\underline{C}H_2$), 24.1 (CH_3 (Mtr)), 18.3 (CH_3 (Mtr)), 12.0 (CH_3 (Mtr)). **HRMS** (ESI positive) calc. for $C_{25}H_{36}N_4O_8S_2Na$ $[M+Na]^+$ 607.1867, found 607.1853

Cbz-Arg(Mtr)-ϕ [CH_2S]-Ac (15). Thioacetic acid (2.27 mL, 31.7 mmol, 2.00 eq.) was added to a suspension of Cs_2CO_3 (5.17 g, 15.9 mmol, 1.00 eq.) in DMF (10 mL) under argon atmosphere. Most of the Cs_2CO_3 was dissolved when added to a solution of Cbz-Arg(Mtr)-ϕ [CH_2O]-Ms (**14**) (9.28 g, 15.9 mmol, 1.00 eq.) in DMF (38 mL) under argon atmosphere. The reaction mixture was stirred overnight at rt with the flask covered in aluminium foil. EtOAc (160 mL) and H_2O (160 mL) were added to the reaction mixture and the organic layer washed with aq. solution of 1 M $KHSO_4$ (160 mL), aq. solution of 1 M $NaHCO_3$ (160 mL), brine (160 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* and the crude product purified *via* column chromatography (EA:Petrolium ether v/v 8:2 → EA). The pure product was obtained as a yellowish solid (6.85 g, 12.1 mmol, 76%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.30 - 7.15 (m, 5H, Ar-H (Cbz)), 6.43 (s, 1H, Ar-H (Mtr)), 6.27 - 5.97 (m, 3H, 3 × NH (guanidine)), 5.15 (d, *J* = 9.2 Hz, 1H, *H*N α CH), 5.00 (d, *J* = 12.4 Hz, 1H, OCH_aPh), 4.92 (d, *J* = 12.4 Hz, 1H, OCH_bPh), 3.72 (s, 3H, OCH_3 (Mtr)), 3.68 - 3.57 (m, 1H, HN α C*H*), 3.13 - 2.99 (m, 2H, HNCH_2), 2.89 (dd, *J* = 14.0, 4.7 Hz, 1H, $CH_aSC(O)CH_3$), 2.79 (dd, *J* = 14.0, 7.9 Hz, 1H, $CH_bSC(O)CH_3$), 2.58 (s, 3H, Ar-CH_3 (Mtr)), 2.51 (s, 3H, Ar-CH_3 (Mtr)), 2.19 (s, 3H, $SC(O)CH_3$), 2.03 (s, 3H, Ar-CH_3 (Mtr)), 1.51 - 1.28 (m, 4H, α CHCH_2CH_2). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_C (ppm) = 196.1 (S$\underline{C}$(O)CH_3), 158.4 (C=O), 156.6 (C-guanidino), 156.3 (C-Ar), 138.5 (C-Ar), 136.5 (C-Ar), 136.4 (C-Ar), 133.6 (C-Ar), 128.5, 128.1, 127.8 (CH-Ar (Cbz)), 124.8 (C-Ar), 111.7 (CH-Ar (Mtr)), 66.7 (OCH_2 (Cbz)), 55.4 (OCH_3), 51.1 (CH_2S), 40.8 ($NHCH_2$), 33.8 (NH α CH), 31.8 (α CH$\underline{C}H_2CH_2$),

30.5 (SC(O)$\underline{C}H_3$), 25.7 (αCHCH$_2\underline{C}H_2$), 24.1 (CH_3 (Mtr)), 18.3 (CH_3 (Mtr)), 12.0 (CH_3 (Mtr)). **HRMS** (ESI negative) calc. for $C_{26}H_{36}N_4O_6S_2$ $[M-H]^-$ 563.2003, found 563.1980.

Cbz-Arg(Mtr)-ϕ [CH_2SO_2]-F (17). Cbz-Arg(Mtr)-ϕ [CH_2S]-Ac (**15**) (6.63 g, 11.7 mmol, 1.00 eq.) was dissolved in AcOH (40 mL) and 30% H_2O_2 aq. solution (13.5 mL) was added. The reaction mixture was stirred for 48 h and additional H_2O_2 aq. solution (3.5 mL) was added. NaOAc (963 mg, 11.7 mmol, 1.00 eq.) was added and the mixture stirred for 1 h. DMF (20 mL) was added and the solution was concentrated *in vacuo* until a quarter of the volume. This procedure was repeated 3 more times and finally DMF removed completely. After co-evaporation with H_2O (2 × 100 mL) the crude product was lyophilized and **16** obtained as a white solid (6.82 g). The crude sodium salt **16** (600 mg, 1.01 mmol, 1.00 eq.) was dissolved in dry CH_2Cl_2 (25 mL) under argon atmosphere and XtalFluor-M® (442 mg, 1.82 mmol, 1.80 eq.) and 3HF·NEt_3 (7.1 µL, 43.6 µmol, 0.04 eq.) were added. The reaction mixture was heated to 40 °C and stirred overnight. Silica was added to quench the reaction and the solvent removed *in vacuo*. Column chromatography (EA) yielded the pure product as a white solid (320 mg, 559 µmol, 55% over two steps). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.37 - 7.24 (m, 5H, Ar-H (Cbz)), 6.51 (s, 1H, Ar-H (Mtr)), 6.36 - 6.04 (m, 3H, 3 × NH (guanidine)), 5.89 (br s, 1H, *H*NαCH), 5.13 - 4.99 (m, 2H, CH_2 (Cbz)), 4.20 - 4.00 (m, 1H, HNαC*H*), 3.80 (s, 3H, OCH_3 (Mtr)), 3.65 (dd, J = 15.2, 6.9 Hz, 1H, CH_aSO_2F), 3.52 - 3.39 (m, 1H, CH_bSO_2F), 3.15 (br s, 2H, HNCH_2), 2.63 (s, 3H, Ar-CH_3 (Mtr)), 2.56 (s, 3H, Ar-CH_3 (Mtr)), 2.10 (s, 3H, Ar-CH_3 (Mtr)), 1.79 - 1.38 (m, 4H, αCHCH_2CH_2). **^{13}C-NMR** (126 MHz, $CDCl_3$, 298 K): δ_C (ppm) = 158.7 (C=O), 156.3 (C-guanidino), 156.0 (C-Ar), 138.4 (C-Ar), 136.6 (C-Ar), 136.1 (C-Ar), 132.9 (C-Ar), 128.5 (CH-Ar (Cbz)), 128.2 (CH-Ar (Cbz)), 127.9 (CH-Ar (Cbz)), 125.0 (C-Ar), 111.8 (CH (Mtr)), 67.1 (CH_2 (Cbz)), 55.4 (OCH_3), 54.5 (d, $J_{C,F}$ = 13.2 Hz, CH_2SO_2F), 47.2 (αCH), 40.4 (αCHCH$_2$CH$_2\underline{C}H_2$), 30.5 (α

CH<u>C</u>H$_2$), 25.6 (α CHCH$_2$<u>C</u>H$_2$), 24.0 (CH$_3$ (Mtr)), 18.3 (CH$_3$ (Mtr)), 11.9 (CH$_3$ (Mtr)). **^{19}F-NMR** (377 MHz, CDCl$_3$, 298 K): δ_F (ppm) = 61.6 (s, 1F, SO$_2$F). **HRMS** (ESI positive) calc. for $C_{24}H_{33}FN_4O_7S_2$ $[M+Na]^+$ 595.1667, found 595.1670. $[\alpha]_D^{23}$ = +1.29 (c = 1.14, CDCl$_3$). **IR** (neat, cm^{-1}) = 1705, 1622, 1550, 1456 (SO$_2$), 1408, 1307, 1256, 1120, 731.

Boc-Phe(4-NHCbz)-OH (20). 4-Amino-(*N*-tert-butoxycarbonyl)-L-phenylalanine (9.95 g, 35.5 mmol, 1.00 eq.) was dissolved in a 1:1 mixture of H$_2$O/dioxane (200 mL). Benzyloxycarbonyl chloride (6.25 mL, 43.8 mmol, 1.23 eq.) was added and the pH adjusted to 8 by addition of sodium bicarbonate. The reaction mixture was stirred overnight. Dioxane was removed *in vacuo* and the aqueous layer was washed with EtOAc (2 × 200 mL). The aqueous layer was acidified with aq. solution of 2 m HCl and the pH adjusted to 1. The precipitate was extracted with EtOAc(3 × 200 mL), dried over MgSO$_4$ and filtered. The solvent was removed *in vacuo* and the crude product purified by flash column chromatography (EA/Hex v/v 1:1 + 1% AcOH). The pure product was obtained as a white solid (11.1 g, 26.8 mmol, 73%). **^{1}H-NMR** (400 MHz, CDCl$_3$, 298 K): δ_H (ppm) = 7.39 - 7.11 (m, 7H, Ar-H (Cbz), Ar-H (Phe)), 7.00 (d, *J* = 8.2 Hz, 2H, Ar-H (Phe)), 5.20 - 5.01 (m, 2H, CH$_2$ (Cbz)), 4.93 (d, *J* = 8.1 Hz, 1H, *H*N α CH), 4.61 - 4.45 (m, 1H, HN α C*H*), 3.10 - 2.96 (m, 2H, α CHC*H*$_2$), 1.33 (s, 9H, CH$_3$ (Boc)). **^{13}C-NMR** (126 MHz, CDCl$_3$, 298 K): δ_c (ppm) = 176.3 (CO$_2$H), 155.3 (C=O (Cbz)), 153.7 (C=O (Boc)), 136.9 (C-Ar), 136 (C-Ar), 130.8 (C-Ar), 130.1 (CH-Ar (Phe)), 128.6, 128.3, 128.3 (CH-Ar (Cbz)), 118.9 (CH-Ar (Phe)), 80.3 (<u>C</u>(CH$_3$)$_3$), 67.1 (CH$_2$ (Cbz)), 54.2 (α CH), 37.0 (α CH<u>C</u>H$_2$), 28.3 (C(<u>C</u>H$_3$)$_3$). **HRMS** (ESI positive) calc. for $C_{22}H_{26}N_2O_6Na$ $[M+Na]^+$ 437.1683, found 437.1666.

N$_3$-Phe(4-NHCbz)-OH (21). Boc-Phe(4-NHCbz)-OH (**20**) (11.1 g, 26.8 mmol, 1.00 eq.) was dissolved in CH$_2$Cl$_2$ (250 mL) and TFA (250 mL) was added and the reaction mixture stirred for 1 h at rt. The solvents were removed under reduced pressure and the residue co-

evaporated with toluene. The crude salt was obtained as a white solid. The crude TFA salt (11.5 g, 26.8 mmol, 1.00 eq.), $N_3SO_2Im{\cdot}HCl$ (6.74 g, 32.2 mmol, 1.20 eq.), $CuSO_4{\cdot}5H_2O$ (335 mg, 1.34 mmol, 0.05 eq.) and K_2CO_3 (9.26 g, 67.0 mmol, 2.50 eq.) were dissolved in MeOH (88 mL) and stirred at rt. After 18 h more $N_3SO_2Im{\cdot}HCl$ (5.62 g, 26.8 mmol, 1.00 eq.) was added and the reaction mixture was stirred for 2 days. The solvent was removed *in vacuo* and dissolved in H_2O (350 mL) and the aqueous mixture was acidified with aq. solution of 2 M HCl. The precipitate was extracted with EtOAc (3 × 200 mL) and the combined organic layers dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* and the crude product purified by flash column chromatography (EA/Hex v/v 7:3 + 1% AcOH) The pure product was obtained as a clear colourless oil (6.96 g, 20.5 mmol, 76%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 9.93 (s, 1H, CO_2H), 7.33 - 7.21 (m, 7H, CH (Phe), Ar-H (Cbz)), 7.13 - 7.05 (m, 2H, CH (Phe)), 6.86 (s, 1H, NH) 5.11 (s, 2H, CH_2 (Cbz)), 4.04 (dd, J = 8.0, 5.4 Hz, 1H, N_3 α CH), 3.07 (dd, J = 14.1, 5.4 Hz, 1H, α CHCH_a), 2.93 (dd, J = 14.1, 8.0 Hz, 1H, α CHCH_b). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 174.9 (CO_2H), 137.0 (C-Ar$_t$), 135.8 (C-Ar), 130.0 (C-Ar), 128.7 (CH (Phe)), 128.4 (CH (Cbz)), 128.3 (CH (Cbz)), 128.2 (CH (Cbz)), 119.0 (CH (Phe)), 67.3 (CH_2 (Cbz)), 63.0 (α CH), 36.9 (α CH<u>C</u>H$_2$). **HRMS** (ESI negative) calc. for $C_{17}H_{15}N_4O_4$ $[M-H]^-$ 339.1099, found 339.1092.

N_3-Phe(4-NHCbz)-OMe (22). N_3-Phe(4-NHCbz)-OH (**21**) (6.96 g, 20.5 mmol, 1.00 eq.) was dissolved in MeOH (60 mL) under N_2 atmosphere and cooled to -20 °C. Thionyl chloride (1.6 mL, 22.1 mmol, 1.05 eq.) was added and the mixture was allowed to warm up to rt and stirred overnight. The solvent was removed under reduced pressure and the residue co-evaporated with $CHCl_3$ (3 × 90 mL). The product was obtained as a yellow oil (7.26 g, 20.5 mmol, 100%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.44 - 7.30 (m, 7H, Ar-H (Cbz), Ar-H (Phe)), 7.16 (d, J = 8.5 Hz, 2H, Ar-H (Phe)), 6.74 (s, 1H, NH), 5.19 (s, 2H,

CH_2 (Cbz)), 4.04 (dd, *J* = 8.6, 5.5 Hz, 1H, N_3 α CH), 3.76 (s, 3H, CH_3), 3.12 (dd, *J* = 14.0, 5.5 Hz, 1H, α CHCH_a), 2.96 (dd, *J* = 14.0, 8.6 Hz, 1H, α CHCH_b). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 170.4 ($\underline{C}O_2CH_3$), 153.3 (C=O (Cbz)), 137.0 (C-Ar), 136.0 (C-Ar), 130.9 (C-Ar), 129.9 (CH-Ar (Phe)), 128.6 (CH-Ar (Cbz)), 128.4 (CH-Ar (Cbz)), 128.3 (CH-Ar (Cbz)), 118.9 (CH-Ar (Phe)), 67.1 (CH_2 (Cbz)), 63.3 (α CH), 52.7 (CH_3), 37.0 (α CH$\underline{C}H_2$). **HRMS** (ESI positive) calc. for $C_{18}H_{18}N_4O_4Na$ $[M+Na]^+$ 377.1220, found 377.1206.

N_3-Phe(4-CH_2NHCbz)-OH (26). Boc-Phe(4-CH_2NHCbz)-OH (**25**) (4.93 g, 11.5 mmol, 1.00 eq.) was dissolved in CH_2Cl_2 (60 mL) and TFA (22 mL) was added and the reaction mixture stirred for 45 min at rt. The solvents were removed *in vacuo* and the residue co-evaporated with toluene (3 × 100 mL) and $CHCl_3$ (3 × 100 mL). The crude was dissolved in MeOH (38 mL) and $CuSO_4 \cdot 5H_2O$ (144 mg, 0.58 mmol, 0.05 eq.), K_2CO_3 (3.98 g, 28.8 mmol, 2.50 eq.) and $N_3SO_2Im \cdot HCl$ (2.89 g, 13.8 mmol, 1.20 eq.) were added. The reaction mixture was stirred overnight at rt and the solvent removed *in vacuo*. H_2O (200 mL) was added and the pH adjusted to 1 with aq. solution of 1 m HCl solution. The aqueous layer was extracted with EtOAc (3 × 200 mL) and the combined organic layers dried over $MgSO_4$, filtered and the solvent removed *in vacuo*. The crude was purified *via* column chromatography (EA:petroleum ether v/v 1:1 + 1% AcOH) and the pure product was obtained as a yellowish oil (3.78 g, 10.7 mmol, 93%). **^{1}H-NMR** (500 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.33 - 7.19 (m, 5H, Ar-H (Cbz)), 7.18 - 7.07 (m, 4H, Phe), 5.09 (br s, 1H, CH_2N*H*Cbz), 5.06 (s, 1H, CH_2 (Cbz)), 4.28 (d, *J* = 5.9 Hz, 2H, CH_2NHCbz), 4.06 (dd, *J* = 8.5, 5.2 Hz, 1H, N_3 α CH), 3.12 (dd, *J* = 14.1, 5.2 Hz, 1H, N_3 α CHCH_a), 2.94 (dd, *J* = 14.1, 8.5 Hz, 1H, N_3 α CHCH_b). **^{13}C-NMR** (126 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 173.8 (C(O)OH), 156.7 (C=O), 137.4 (CH-Ar), 136.3 (CH-Ar), 135.1 (CH-Ar), 129.6 (CH-Ar (Phe)), 128.6 (CH-Ar (Cbz)), 128.3 (CH-Ar (Cbz)), 128.2 (CH-Ar (Cbz)), 127.9 (CH-Ar (Phe)), 67.1 (OCH_2Ph), 63.0 (α CH), 44.8

(CH_2NHCbz), 37.1 ($N_3\,\alpha\,CHCH_2$). **HRMS** (ESI positive) calc. for $C_{18}H_{18}N_4O_4Na$ $[M+Na]^+$ 377.1220, found 377.1212

N_3-Phe(4-CH_2NHCbz)-OMe (27). N_3-Phe(4-CH_2NHCbz)-OH (**26**) (3.78 g, 10.7 mmol, 1.00eq.) was dissolved in MeOH (50 mL) and cooled to -20 °C under nitrogen atmosphere. Thionyl chloride (813 µL, 11.2 mmol, 1.05 eq.) was added dropwise and the reaction mixture was allowed to warm up to rt and stirred overnight. The solvent was removed *in vacuo* and co-evapoarted with $CHCl_3$ (3 × 100 mL). The crude product was obtained as a white oil (3.92 g, 10.6 mmol, 99%). **^{1}H-NMR** (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.41 - 7.28 (m, 5H, Ar-H (Cbz)), 7.25 (d, J = 8.3 Hz, 2H, 2 × CH (Phe)), 7.19 (d, J = 8.0 Hz, 2H, 2 × CH (Phe)), 5.14 (s, 2H, CH_2 (Cbz)), 5.08 (br s, 1H, CH_2NHCbz), 4.37 (d, J = 6.0 Hz, 2H, CH_2NHCbz), 4.06 (dd, J = 8.8, 5.3 Hz, 1H, N_3αCH), 3.77 (s, 1H, OCH_3), 3.15 (dd, J = 14.0, 5.3 Hz, 1H, N_3αCHCH_a), 2.98 (dd, J = 14.0, 8.8 Hz, 1H N_3αCHCH_b). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 170.3 (C(O)OMe), 156.4 (C=O), 137.5 (C-Ar), 136.5 (C-Ar), 135.2 (C-Ar), 129.5 (CH-Ar (Phe)), 128.5 (CH-Ar (Cbz)), 128.2 (CH-Ar (Cbz)), 128.2 (CH-Ar (Cbz)), 127.9 (CH-Ar (Phe)), 66.9 (OCH_2Ph), 63.2 (αCH), 52.7 (OCH_3), 44.8 (CH_2NHCbz), 37.2 (N_3αCH$\underline{C}H_2$). **HRMS** (ESI positive) calc. for $C_{19}H_{20}N_4O_4Na$ $[M+Na]^+$ 391.1377, found 391.1368. **IR** (neat, cm^{-1}) = 2110 (N_3), 1742, 1720, 1516, 1244, 1043.

Cbz-Leu$_3$-Arg-ϕ [CH_2SO_2]-F (53). Cbz-Leu$_3$-OH[20] (78.3 mg, 159 µmol, 1.10 eq.) was dissolved in DMF (1.5 mL) and HBTU (60.4 mg, 159 µmol, 1.10 eq.), Oxymapure® (22.6 mg, 159 µmol, 1.10 eq.) and DiPEA (25.2 µL, 145 µmol, 1.00 eq.) were added and the reaction mixture was stirred for 5 min. Next, a solution of 2HCl·Arg-[CH_2SO_2]-F (**7**) (65.8 mg, 145 µmol, 1.00 eq.) in DMF (2 mL) was added to the reaction mixture followed by DiPEA (37.9 µL, 217 mmol, 1.50 eq.) and stirring was continued for 4 h. The solvent was removed *in vacuo* and the crude product purified by semi-preparative HPLC (0 to 100% B, C_{P1}). Fractions containing the product were pooled, lyophilized and the pure product was

obtained as a white solid (44.5 mg, 54.7 µmol, 37%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.14 (d, *J* = 8.6 Hz, 1H, N*H* α CH (Arg)), 7.98 (d, *J* = 8.1 Hz, 1H, N*H* α CH (Leu)), 7.82 (d, *J* = 8.0 Hz, 1H, N*H* α CH (Leu)), 7.57 (br s, 1H, N*H*CH_2 (Arg)), 7.44 (d, *J* = 8.1 Hz, 1H, N*H* α CH, (Leu)), 7.41 - 7.25 (m, 5H, Ar-H (Cbz)), 5.02 (s, 2H, CH_2 (Cbz)), 4.36 - 4.18 (m, 3H, 2 × NH α C*H* (Leu), NH α C*H* (Arg)), 4.15 (ddd, *J* = 15.0, 7.1, 3.4 Hz, 1H, CH_aSO_2F), 4.09 - 4.01 (m, 1H, NH α C*H* (Leu)), 3.93 (dd, *J* = 15.0, 9.2 Hz, 1H, CH_bSO_2F), 3.15 - 2.99 (m, 2H, NHCH_2 (Arg)), 1.70 - 1.54 (m, 3H, C*H*$(CH_3)_2$), 1.54 - 1.30 (m, 10H, 3 × CH_2 (Leu), NHCH$_2$CH_2CH_2 (Arg)), 0.95 - 0.73 (m, 18H, 6 × CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.7 (C=O), 171.1 (C=O), 171.0 (C=O), 156.1 (C (guanidine)), 155.3 (C=O (Cbz)), 136.4 (C_{quart} (Phe)), 127.7 (CH (Phe)), 127.2 (CH (Phe)), 127.0 (CH (Phe)), 64.8 (CH2 (Cbz)), 53.2 (d, *J* = 11.3 Hz, CH_2SO_2F), 52.5(CbzNH α C<u>H</u>), 50.5 (NH α C<u>H</u>), 50.3 (NH α C<u>H</u>), 43.6 (α CH (Arg)), 40.0 (CH_2 (Leu)), 39.9 (CH_2 (Leu)), 39.8 (CH_2 (Leu)), 39.7 ($NHCH_2$ (Arg)), 30.1 (α CH<u>C</u>H$_2$ (Arg)), 24.0 (α CHCH$_2$<u>C</u>H$_2$ (Arg)), 23.6 (CH (Leu)), 23.5 (CH (Leu)), 23.4 (CH (Leu)), 22.44 (CH_3), 22.42 (CH_3), 22.38 (CH_3), 21.0 (CH_3), 20.94 (CH_3), 20.88 (CH_3). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (s, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{32}H_{55}N_7O_7SF$ $[M+H]^+$ 700.3862, found 700.3841. **t_R** (0 to 100% B, 30 min, C_{A1}) = 23.0 min.

Cbz-Phe-Leu$_2$-Arg-ϕ [CH$_2$SO$_2$]-F (54). Cbz-Phe-Leu$_2$-OH (see supporting information) (112 mg, 213 µmol, 1.10 eq.) was dissolved in DMF (1.5 mL) and HBTU (80.7 mg, 213 µmol, 1.10 eq.), Oxyma (30.3 mg, 213 µmol, 1.10 eq.) and DiPEA (33.7 µL, 194 µmol, 1.00 eq.) were added and stirred for 5 min. Next, a solution of 2HCl·Arg-ϕ [CH$_2$SO$_2$]-F (**7**) (112 mg, 194 µmol, 1.00 eq.) in DMF (2.5 mL) was added to reaction mixture followed by DiPEA (50.6 µL, 290 mmol, 1.50 eq.) and stirring was continued for 6 h. The solvent was removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 100%

B, C_{P1}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (46.6 mg, 54.9 µmol, 28%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.16 (d, *J* = 8.6 Hz, 1H, N*H* α CH), 8.10 (d, *J* = 8.1 Hz, 1H, N*H* α CH), 7.94 (d, *J* = 8.1 Hz, 1H, N*H* α CH), 7.62 - 7.56 (m, 1H, N*H*CH_2 (Arg)), 7.49 (d, *J* = 8.5 Hz, 1H, N*H* α CH (Phe)), 7.37 - 7.15 (m, 10H, Ar-H (Phe, Cbz)), 4.94 (s, 2H, CH_2 (Cbz)), 4.37 - 4.20 (m, 4H, 2 × NH α C*H* (Leu), NH α C*H* (Arg), NH α C*H* (Phe)), 4.15 (ddd, *J* = 15.0, 6.9, 3.3 Hz, 1H, CH_aSO_2F), 3.93 (dd, *J* = 15.0, 9.1 Hz, 1H, CH_bSO_2F), 3.14 - 3.02 (m, 2H, NHC*H*$_2$ (Arg)), 2.98 (dd, *J* = 13.9, 3.8 Hz, 1H, CH_a (Phe)), 2.73 (dd, *J* = 13.9, 10.7 Hz, 1H, CH_b (Phe)), 1.66 - 1.56 (m, 2H, 2 × C*H*$(CH_3)_2$), 1.56 - 1.37 (m, 8H, 2 × CH_2 (Leu), NHCHC*H*$_2$C*H*$_2$ (Arg)), 0.92 - 0.80 (m, 12H, 4 × CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 172.19 (C=O), 172.17 (C=O), 172.0 (C=O), 157.2 (C (guanidine)), 156.3 (C=O (Cbz)), 138.5 (C-Ar), 137.4 (C-Ar), 129.6 (CH-Ar), 128.7 (CH-Ar), 128.5 (CH-Ar), 128.1 (CH-Ar), 127.9 (CH-Ar), 126.7 (CH-Ar), 65.7 (CH_2 (Cbz)), 56.5 (CbzNH α CH), 54.3 (d, *J* = 11.2 Hz, CH_2SO_2F), 51.6 (α CH (Leu)), 51.57 (α CH (Leu)), 44.6 (α CH (Arg)), 41.1 (CH_2 (Leu)), 40.9 (CH_2 (Leu)), 40.8 (CH_2NH (Arg)), 37.7 (CH_2 (Phe)), 31.2 (α CHCH_2 (Arg)), 25.1 (α CHCH_2CH_2 (Arg)), 24.6 (CH (Leu)), 24.5 (CH (Leu)), 23.50 (CH_3 (Leu)), 23.48 (CH_3 (Leu)), 22.2 (CH_3 (Leu)), 22.0 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (s, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{35}H_{53}N_7O_7SF$ $[M+H]^+$ 734.3706, found 734.3673. **t_R** (0 to 100% B, 30 min, C_{A1}) = 23.2 min

Boc-Phe-Leu$_2$-Arg-ϕ [CH_2SO_2]-F (55). Boc-Phe-Leu$_2$-OH (99.8 mg, 203 µmol, 1.10 eq.) was dissolved in DMF (2 mL) under nitrogen atmosphere and HBTU (77.0 mg, 203 µmol, 1.10 eq.), Oxyma (29 mg, 203 µmol, 1.10 eq.) and DiPEA (32.0 µL, 184 µmol, 1.00 eq.) were added and stirred for 5 min. Next, a solution of 2HCl·Arg-[CH_2SO_2]-F (**7**) (55.0 mg,

184 µmol, 1.00 eq.) in DMF (2 mL) was added to reaction mixture followed by DiPEA (48.0 µL, 276 mmol, 1.50 eq.) and stirring was continued for 2 h. The solvent was removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 100% B, C_{P1}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (58.5 mg, 71.9 µmol, 39%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.17 (d, *J* = 8.6 Hz, 1H, NH (Leu)), 7.99 - 7.92 (m, 2H, NH (Leu), BocNH), 7.54 (t, *J* = 5.8 Hz, 1H, N*H*CH_2 (Arg)), 7.34 - 7.16 (m, 5H, Ar-H (Phe)), 6.96 (d, *J* = 8.5 Hz, 1H, N*H* αCH (Arg)), 4.39 - 4.10 (m, 5H, BocNH αC*H*, 2 × NH αC*H* (Leu), NH αC*H* (Arg), CH_aSO_2F), 3.93 (ddd, *J* = 14.9, 9.2, 1.9 Hz, 1H, CH_bSO_2F), 3.14 - 3.00 (m, 2H, NHC*H*$_2$ (Arg)), 2.94 (dd, *J* = 13.8, 4.1 Hz, 1H, CH_a (Phe)), 2.72 (dd, *J* = 13.8, 10.4 Hz, 1H, CH_b (Phe)), 1.68 - 1.55 (m, 2H, 2 × C*H*$(CH_3)_2$), 1.54 - 1.34 (m, 8H, 2 × CH_2 (Leu), $NHCH_2CH_2CH_2$ (Arg)), 1.30 (s, 9H, $C(CH_3)_3$), 0.90 - 0.81 (m, 12H, 4 × CH_3 (Leu)). **^{13}C-NMR** (101 MHz, $CDCl_3$, 298 K): δ_c (ppm) = 172.19 (C=O), 172.16 (C=O), 172.02 (C=O), 157.1 (C.guanidino), 155.7 (C=O (Boc)), 138.6 (C-Ar), 129.6 (CH-Ar), 128.5 (CH-Ar), 126.6 (CH-Ar), 78.6 ($\underline{C}(CH_3)_3$), 56.1 (BocNHC), 54.3 (d, *J* = 10.8 Hz, CSO_2F), 51.6 (αCH (Leu)), 51.4 (αCH (Leu)), 44.6 (αCH (Arg)), 41.3 (CH_2 (Leu)), 40.9 (CH_2 (Leu)), 40.8 ($NHCH_2$), 37.5 (CH_2 (Phe)), 31.2 (αCH$\underline{C}H_2$ (Arg)), 28.6 ($C(\underline{C}H_3)_3$), 25.1 (αCHCH$_2\underline{C}H_2$ (Arg)), 24.5 (CH (Leu)), 24.45 (CH (Leu)), 23.6 (CH_3 (Leu)), 23.5 (CH_3 (Leu)), 22.1 (CH_3 (Leu)), 22.0 (CH_3 (Leu)). **^{19}F-NMR** (377 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (d, *J* = 7.2 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{32}H_{55}N_7O_7SF$ $[M+H]^+$ 642.3444, found 642.3414. **t_R** (0 to 100% B, 30 min, C_{A1}) = 23.1 min.

Ac-Phe-Leu$_2$-Arg-ϕ [CH_2SO_2]-F (56). Ac-Phe-Leu$_2$-OH (**47**) (88.0 mg, 203 µmol, 1.10 eq.) was dissolved in DMF (2 mL) under nitrogen atmosphere and HBTU (77.0 mg, 203 µmol, 1.10 eq.), Oxymapure® (29 mg, 203 µmol, 1.10 eq.) and DiPEA (32.0 µL, 184 µmol,

1.00 eq.) were added and the mixture stirred for 5 min. A solution of 2HCl·H_2N-Arg-ψ[CH_2SO_2]-F (**7**) (55.0 mg, 184 µmol, 1.00 eq.) in DMF (2 mL) was added to reaction mixture followed by DiPEA (48.0 µL, 276 mmol, 1.50 eq.) and stirring was continued for 2 h. The solvent was removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 100% B, C_{P1}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (44.6 mg, 59.0 µmol, 32%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.13 (d, *J* = 8.6 Hz, 1H, N*H*αCH (Arg)), 8.09 (d, *J* = 8.1 Hz, 1H, AcNH), 8.04 (d, *J* = 8.1 Hz, 1H, NH (Leu)), 7.88 (d, *J* = 8.0 Hz, 1H, NH (Leu)), 7.52 (t, *J* = 5.7 Hz, 1H, N*H*CH_2 (guanidine)), 7.29 - 7.15 (m, 5H, Ar-H (Phe)), 4.50 (ddd, *J* = 10.0, 8.1, 4.2 Hz, 1H, AcNHαC*H*), 4.33 - 4.19 (m, 3H, C*H*CH_2SO_2F, NHαC*H* (Leu^1), NHαC*H* (Leu^2)), 4.14 (ddd, *J* = 14.9, 6.8, 3.4 Hz, 1H, CH_bSO_2F), 3.93 (dd, *J* = 14.9, 9.2 Hz, 1H, CH_bSO_2F), 3.14 - 3.00 (m, 2H, NHC*H*$_2$ (guanidine)), 2.97 (dd, *J* = 14.0, 4.2 Hz, 1H, CH_b (Phe)), 2.72 (dd, *J* = 14.0, 10.0 Hz, 1H, CH_b (Phe)), 1.75 (s, 3H, $CH_3C(O)$), 1.66 - 1.37 (m, 10H, 2 × C*H*$(CH_3)_2$, 2 × CH_2 (Leu), NHCH_2C*H*$_2$C*H*$_2$ (Arg)), 0.89 (d, *J* = 2.6 Hz, 3H, CH_3), 0.87 (d, *J* = 2.7 Hz, 2H, CH_3), 0.84 (s, 3H, CH_3), 0.83 (s, 3H, CH_3). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 172.2 (C=O), 172.1 (C=O), 171.8 (C=O), 169.8 (C=O), 157.1 (C-guanidine), 138.4 (C-Ar), 129.6, 128.5, 126.7 (CH-Ar), 54.4 (AcNHC), 54.3 (d, *J* = 10.2 H, CH_2SO_2F), 51.6 (αCH (Leu)), 51.5 (αCH (Leu)), 44.6 (αCH (Arg)), 41.0 (CH_2 (Leu)), 40.9 (CH_2 (Leu)), 40.8 (CH_2 (Arg)), 37.7 (CH_2-Phe), 31.2 (αCH$\underline{C}H_2$ (Arg)), 25.1 (αCHC$H_2\underline{C}H_2$ (Arg)), 24.6 (CH (Leu)), 24.5 (CH (Leu)), 23.5 (CH_3 (Ac)), 22.9 (2 × CH_3 (Leu)), 22.1 (CH_3 (Leu)), 22.0 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (s, 1 F, SO_2F). **HRMS** (ESI positive) calc. for $C_{29}H_{49}N_7O_6SF$ $[M+H]^+$ 642.3444, found 642.3414. $\mathbf{t_R}$ (0 to 100% B, 50 min, C_{A1}) = 27.0 min.

H_2N-Phe-Leu_2-Arg-ϕ [CH_2SO_2]-F (57). Boc-Phe-Leu_2-Arg-ϕ [CH_2SO_2]-F (**55**) (49.7 mg, 61.1 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and TFA (5 mL) was added. The reaction mixture was stirred for 30 min at rt. The solvent was removed *in vacuo* and the crude purified by semi-preparative HPLC (0 to 100% B, C_{P1}). Fractions containing the product were pooled, lyophilized and the pure product obtained as a white solid (31.2 mg, 37.7 µmol, 62%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.63 (d, J = 8.4 Hz, 1H, NH (Leu)), 8.27 - 8.22 (m, 2H, NH (Leu), N*H* α CH (Arg)), 8.13 (s, 3H, NH_3^+), 7.83 - 7.72 (m, 1H, N*H*CH_2 (Arg)), 7.33 - 7.23 (m, 5H, Ar-H (Phe)), 4.44 - 4.38 (m, 1H, NH α C*H* (Leu)), 4.32 - 4.23 (m, 2H, NH α C*H* (Leu), NH α C*H* (Arg)), 4.15 (ddd, J = 14.9, 6.9, 3.3 Hz, 1H, CH_aSO_2F), 4.12 - 4.04 (m, 1H, NH_3^+C*H*), 3.94 (dd, J = 14.9, 9.3 Hz, 1H, CH_bSO_2F), 3.15 - 3.00 (m, 3H, NHC*H*$_2$ (Arg), CH_a (Phe)), 2.92 (dd, J = 14.2, 7.6 Hz, 1H, CH_a (Phe)), 1.67 - 1.57 (m, 2H, 2 × C*H*$(CH_3)_2$), 1.57 - 1.39 (m, 8H, 2 × CH_2 (Leu), NHCH$_2$C*H*$_2$C*H*$_2$ (Arg)), 0.92 - 0.88 (m, 6H, 2 × CH_3 (Leu)), 0.87 (d, J = 4.3 Hz, 3H, CH_3 (Leu)), 0.86 (d, J = 4.3 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.1 (C=O), 170.6 (C=O), 167.0 (C=O), 156.2 (C-guanidino), 134.1 (C-Ar), 129.0 (CH-Ar), 127.8 (CH-Ar), 126.5 (CH-Ar), 53.2 (d, J = 10.8 Hz, CSO_2F), 52.5 (NH_3^+C), 50.6 (α CH (Leu)), 50.4 (α CH (Leu)), 43.6 (α CH (Arg)), 40.4 (CH_2 (Leu)), 39.9 (CH_2 (Leu)), 39.7 (CH_2NH (Arg)), 36.3 (CH_2 (Phe)), 30.2 (α CH<u>C</u>H$_2$ (Arg)), 24.0 (α CHCH$_2$<u>C</u>H$_2$ (Arg)), 23.5 (CH (Leu)), 23.4 (CH (Leu)), 22.5 (CH_3 (Leu)), 22.4 (CH_3 (Leu)), 21.1 (CH_3 (Leu)), 21.0 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (s, 1 F, SO_2F). **HRMS** (ESI positive) calc. for $C_{27}H_{47}N_7O_5SF$ $[M+H]^+$ 600.3338, found 600.3321. $\mathbf{t_R}$ (0 to 100% B, 30 min, C_{A1}) = 17.1 min.

N_3-Phe-Leu_2-Arg-ϕ [CH_2SO_2]-F (58). N_3-Phe-Leu_2-OH (**52**) (81.5 mg, 195 µmol, 1.05 eq.) was dissolved in DMF (2 mL) under nitrogen atmosphere and cooled to 0 °C. HATU

(74.2 mg, 195 µmol, 1.05 e.q) was added and stirring was continued for 20 min. 2HCl·Arg-ψ[CH_2SO_2]-F (**7**) (55.6 mg, 186 µmol, 1.00 eq.) in DMF (2 mL) was added to the reaction mixture. After 30 min DiPEA (93.7 µL, 539 µmol, 2.90 eq.) was added and stirring was continued overnight at rt. The solvent was removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 70% B, C_{P3}). The obtained product (80 mg) required a second purification step by semi-preparative HPLC (0 to 80% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (16.9 mg, 22.8 µmol, 12%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.35 (d, *J* = 8.2 Hz, 1H, NH (Leu)), 8.18 (d, *J* = 8.6 Hz, 1H, N*H*αCH (Arg)), 8.10 (d, *J* = 8.1 Hz, 1H, NH (Leu)), 7.60 (t, *J* = 5.9 Hz, 1H, N*H*CH_2 (guanidine)), 7.33 - 7.22 (m, 5H, Ar-H (Phe)), 7.01 (br s, 3H), 4.38 (app q, *J* = 7.7 Hz, 1H, NHαC*H* (Leu)), 4.31 - 4.20 (m, 2H, C*H*CH_2SO_2F, NHαC*H* (Leu)), 4.15 (ddd, *J* = 14.0, 6.9, 3.4 Hz, 1H, CH_aSO_2F), 4.10 (dd, *J* = 9.1, 5.1 Hz, 1H, N_3CH), 3.93 (dd, *J* = 14.0, 9.5 Hz, 1H, CH_bSO_2F), 3.13 - 3.01 (m, 3H, NHC*H*$_2$ (guanidine), CH_a (Phe)), 2.89 (dd, *J* = 14.1, 9.1 Hz, 1H, CH_b (Phe)), 1.70 - 1.36 (m, 10H, 2 × C*H*$(CH_3)_2$, 2 × CH_2 (Leu), $NHCH_2CH_2CH_2$ (Arg)), 0.89 (d, *J* = 2.2 Hz, 3H, CH_3), 0.88 (d, *J* = 2.2 Hz, 3H, CH_3), 0.86 (d, *J* = 4.0 Hz, 3H, CH_3), 0.84 (d, *J* = 3.9 Hz, 3H, CH_3). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.1 (C=O), 170.7 (C=O), 168.2 (C=O), 156.1 (C-(guanidine), 136.3 (C-Ar), 128.4, 127.8, 126.1 (CH-Ar), 61.7 (N_3C), 53.2 (d, *J* = 11.1 Hz, CH_2SO_2F), 50.6 (αCH (Leu)), 50.5 (αCH (Leu)), 43.6 (αCH (Arg)) , 40.2 (CH_2 (Leu)), 39.8 (CH_2 (Leu)), 39.7 ($NHCH_2$ (Arg)), 36.2 (CH_2-Phe), 30.1 (αCH$\underline{C}H_2$ (Arg)), 24.0 ($NHCH_2\underline{C}H_2$ (Arg)), 23.5 (CH (Leu)), 23.5 (CH (Leu)), 22.4 (CH_3 (Leu)), 22.4 (CH_3 (Leu)), 21.0 (CH_3 (Leu)), 20.9 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (s, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{27}H_{45}N_9O_5SF$ $[M+H]^+$ 626.3243, found 626.3216. **t_R** (0 to 100% B, 50 min, C_{A3}) = 33.6 min.

2TFA·H_2N-Phe-Leu_2-Lys-ϕ [CH_2SO_2]-F (59). BocHN-Phe-Leu_2-Lys(Cbz)-ϕ [CH_2SO_2]-F (31.8 mg, 34.6 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and a 33% HBr/AcOH solution (5 mL) was added and the reaction mixture stirred for 45 min at rt. The solvent was removed *in vacuo* and the crude product purified by semi-preparative HPLC (0 to 50% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product obtained as a white solid (21.5 mg, 26.9 µmol, 78 %). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.66 - 8.60 (m, 1H, NH), 8.28 (d, J = 7.9 Hz, 1H, NH), 8.22 - 8.06 (m, 4H, NH, NH_3^+ (Phe)), 7.79 (br s, 3H, NH_3^+ (Lys)), 7.37 - 7.19 (m, 5H, CH-Ar), 4.42 (td, J = 8.7, 4.2 Hz, 1H, αCH (Leu)), 4.32 - 4.22 (m, 2H, αCH (Lys), α CH (Leu)), 4.14 (ddd, J = 15.0, 6.8, 3.3 Hz, 1H, CH_aSO_2F), 4.11 - 4.03 (m, 1H, αCH (Phe)), 3.90 (dd, J = 15.0, 9.1 Hz, 1H, CH_bSO_2F), 3.09 (dd, J = 14.2, 5.1 Hz, 1H, CH_aPhe), 2.92 (dd, J = 14.2, 7.7 Hz, 1H, CH_bPhe), 2.78 - 2.66 (m, 2H, C$H_2NH_3^+$), 1.69 - 1.38 (m, 10H, 2 × CH (Leu), CH_2 (Leu), CH_2 $CH_2CH_2CH_2NH_3^+$ (Lys)), 1.38 - 1.21 (m, 2H, C$H_2CH_2CH_2CH_2NH_3^+$ (Lys)), 0.96 - 0.79 (m, 12H, 4 × CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_C (ppm) = 172.0, 171.7, 168.0 (C=O), 135.2 (C-Ar), 130.0, 128.9, 127.6 (CH-Ar), 54.4 (d, J = 10.9 Hz, CSO_2F), 53.5 (αCH (Phe)), 51.6, 51.4 (αCH (Leu)), 44.5 (αCH (Lys)), 40.9, 40.8 (CH_2 (Leu)), 39.0 ($CH_2NH_3^+$), 37.4 (CH_2Phe), 33.4 ($\underline{C}H_2CH_2CH_2CH_2NH_3^+$ (Lys)), 26.8 ($\underline{C}H_2CH_2NH_3^+$ (Lys)), 24.5, 24.4 (CH (Leu)), 23.6, 23.5 (CH_3 (Leu)), 22.1 ($\underline{C}H_2CH_2CH_2NH_3^+$ (Lys)), 22.1, 22.0 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.6 (s, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{27}H_{47}N_5O_5SF$ $[M+H]^+$ 572.3276, found 572.3263. **t_R** (0 to 100% B, 50 min, C_{A3}) = 24.6 min.

TFA·Ac-Phe-Leu_2-Lys-ϕ [CH_2SO_2]-F (60). Ac-Phe-Leu_2-Lys(Cbz)-ϕ [CH_2SO_2]-F (20.0 mg, 23.2 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and 33% HBr/AcOH solution (5 mL) was added and the reaction mixture was stirred for 1 h at rt. The solvents were removed *in vacuo* and the crude was purified by semi-preparative HPLC (0 to 90% B, C_{P3}).

Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (11.1 mg, 15.2 µmol, 66%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.16 - 8.01 (m, 3H, 2 × N*H* α CH, N*H* α CH (Lys)), 7.93 (d, *J* = 8.1 Hz, 1H, N*H* α CH), 7.72 (br s, 3H, NH_3^+), 7.30 - 7.15 (m, 5H, Ar-H (Phe)), 4.50 (ddd, *J* = 10.0, 8.1, 4.2 Hz, 1H, AcNH α C*H*), 4.36 - 4.17 (m, 3H, 2 × NH α C*H*, NH α C*H* (Lys)), 4.12 (ddd, *J* = 14.8, 6.8, 3.4 Hz, 1H, CH_aSO_2F), 3.89 (dd, *J* = 14.8, 9.1 Hz, 1H, CH_bSO_2F), 2.97 (dd, *J* = 13.9, 4.2 Hz, 1H, CH_a (Phe)), 2.79 - 2.66 (m, 3H, CH_b (Phe), $NH_3^+CH_2$), 1.75 (s, 3H, CH_3 (AcNH)), 1.67 - 1.38 (m, 10H, 2 × C*H*$(CH_3)_2$ (Leu), 2 × CH_2 (Leu), $NH_3^+CH_2CH_2$, α CHC*H*$_2$ (Lys)), 1.36 - 1.22 (m, 2H, $NH_3^+CH_2CH_2CH_2$ (Lys)), 0.89 (d, *J* = 6.6 Hz, 3H, CH_3 (Leu)), 0.88 (d, *J* = 6.6 Hz, 3H, CH_3 (Leu)), 0.84 (d, *J* = 6.5 Hz, 3H, CH_3 (Leu)), 0.84 (d, *J* = 6.5 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (101 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.6 (C=O), 171.5 (C=O), 171.2 (C=O), 169.2 (C=O), 137.9 (C-Ar (Phe)), 129.0 (CH-Ar), 127.9 (CH-Ar), 126.1 (CH-Ar), 124.1 (CH-Ar), 53.8 (AcNHC), 53.7 (CSO_2F), 51.1 (α CH (Leu)), 50.9 (α CH (Leu)), 43.9 (α CH (Lys)), 40.5 (2 × CH_2 (Leu)), 37.2 (CH_2 (Phe)), 32.8 (α CH<u>C</u>H$_2$ (Lys)), 26.2 (NHCH$_2$<u>C</u>H$_2$ (Lys)), 24.0 (<u>C</u>H$(CH_3)_2$), 23.9 (<u>C</u>H$(CH_3)_2$), 23.0 (CH_3 (Leu)), 22.9 (CH_3 (Leu)), 22.3 (CH_3 (Ac)), 21.6 (α CHCH$_2$<u>C</u>H$_2$ (Lys)), 21.54 (CH_3 (Leu)), 21.47 (CH_3 (Leu)) **^{19}F-NMR** (377 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.6 (d, $J_{C,F}$ = 6.6 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{29}H_{48}N_5O_6SF$ $[M+H]^+$ 614.3382, found 614.3371. **t_R** (0 to 100% B, 50 min, C_{A3}) = 29.0 min.

TFA·N$_3$-Phe-Leu$_2$-Lys-ϕ [CH$_2$SO$_2$]-F (61). N$_3$-Phe-Leu$_2$-Lys(Cbz)-ϕ [CH_2SO_2]-F (20.0 mg, 23.6 µmol, 1.00 eq.) was set under nitrogen atmosphere and 4 m HCl solution in dioxane (10 mL) was added and the reaction mixture stirred overnight at rt. The solvent was removed *in vacuo* and the crude was purified by semi-preparative HPLC (0 to 100% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product was obtained

as a white solid (6.5 mg, 9.13 µmol, 39%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.33 (d, *J* = 8.2 Hz, 1H, N*H* α CH (Leu)), 8.13 - 8.06 (m, 2H, N*H* α CH (Leu), N*H* α CH (Lys)), 7.63 (br s, 3H, NH_3^+), 7.35 - 7.21 (m, 5H, Ar-H (Phe)), 4.38 (app q, *J* = 7.7 Hz, 1H, NH α C*H* (Leu)), 4.33 - 4.18 (m, 2H, NH α C*H* (Leu), NH α C*H* (Lys)), 4.17 - 4.07 (m, 2H, N_3 α C*H*, CH_aSO_2F), 3.89 (dd, *J* = 15.0, 9.1 Hz, 1H, CH_bSO_2F), 3.08 (dd, *J* = 14.1, 5.2 Hz, 1H, CH_a (Phe)), 2.88 (dd, *J* = 14.1, 9.1 Hz, 1H, CH_b (Phe)), 2.78 - 2.69 (m, 2H, $NHCH_2$ (Lys)), 1.67 - 1.39 (m, 4H, 2 × C*H*$(CH_3)_2$ (Leu), α CHC*H*$_2$ (Lys)), 1.33 - 1.19 (m, 8H, 2 × CH_2, $NHCH_2CH_2CH_2$ (Lys)), 0.95 - 0.77 (m, 12H, 4 × CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.7 (d, *J* = 6.8 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{27}H_{45}N_7O_5SF$ $[M+H]^+$ 598.3181, found 598.3157. **t_R** (0 to 100% B, 50 min, C_{A3}) = 30.3 min.

2TFA·H$_2$N-Phe-Leu$_2$-Phe(4-NH$_2$)-ψ [CH$_2$SO$_2$]-F (62). TFA·Boc-Phe-Leu$_2$-Phe(4-NHCbz)-ψ [CH_2SO_2]-F (29.7 mg, 31.1 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (10 mL) and a 33% HBr/AcOH solution (3 mL) was added and the reaction mixture stirred for 1.5 h at rt. The solvent was removed *in vacuo* and the crude was purified *via* semi-preparative HPLC (0 to 100% B, C_{P3}). The obtained product required a second purification step by semi-preparative HPLC (0 to 50% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product obtained as a white solid (20.6 mg, 24.7 µmol, 79%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.65 (d, *J* = 8.3 Hz, 1H, N*H* α CH (Leu)), 8.31 (d, *J* = 8.3 Hz, 1H, N*H* α CH (4-H_3N^+-Phe)), 8.20 (d, *J* = 8.2 Hz, 1H, N*H* α CH (Leu)), 7.34 - 7.23 (m, 5H, Ar-H (Phe)), 7.14 (d, *J* = 8.0 Hz, 2H, 2 × CH (4-H_3N^+-Phe)), 6.95 (d, *J* = 8.0 Hz, 2H, 2 × CH (4-H_3N^+-Phe)), 4.46 - 4.35 (m, 2H, NH α C*H* (4-H_3N^+-Phe), NH α C*H* (Leu)), 4.31 - 4.19 (m, 1H, NH α C*H* (Leu)), 4.11 - 4.02 (m, 2H, NH α C*H* (H_3N^+ α C*H*), CH_aSO_2F), 3.91 (dd, *J* = 15.4, 9.2 Hz, 1H, CH_bSO_2F), 3.10 (dd, *J* = 14.2, 5.0 Hz, 1H, CH_a

(Phe)), 2.92 (dd, J = 14.2, 7.7 Hz, 1H, CH_b (Phe)), 2.85 - 2.76 (m, 2H, α CHCH_2 (4-H_3N^+-Phe)), 1.68 - 1.60 (m, 1H, CH(CH_3)$_2$ (Leu)), 1.59 - 1.51 (m, 1H, CH(CH_3)$_2$ (Leu), 1.50 - 1.30 (m, 4H, 2 × CH_2 (Leu)), 0.91 (d, J = 6.6 Hz, 3H, CH_3 (Leu)), 0.89 - 0.85 (m, 6H, 2 × CH_3 (Leu)), 0.83 (d, J = 6.5 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.4 (C=O), 171.0 (C=O), 167.5 (C=O), 134.6 (C-Ar), 130.2 (CH-Ar (4-H_3N^+-Phe)), 129.5 (CH-Ar), 128.3 (CH-Ar), 127.0 (CH-Ar), 119.2 (CH-Ar (4-H_3N^+-Phe)), 53.0 (d, J = 12.3 Hz, CSO_2F), 52.96 (H_3N^+ α CH), 50.99 (α CH (Leu)), 50.95 (α CH (Leu)), 46.1 (α C<u>H</u>CH_2SO_2F), 41.0 (CH_2 (Leu)), 40.6 (CH_2 (Leu)), 38.3 (α CHC<u>H</u>$_2$ (4-H_3N^+-Phe)), 36.8 (CH_2 (Phe)), 24.0 (C<u>H</u>(CH_3)$_2$), 23.9 (C<u>H</u>(CH_3)$_2$), 23.0 (CH_3 (Leu)), 22.9 (CH_3 (Leu)), 21.6 (CH_3 (Leu)), 21.5 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, $CDCl_3$, 298 K): δ_F (ppm) = 59.7 (d, J = 6.6 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{30}H_{44}N_5O_5SFNa$ $[M+Na]^+$ 628.2939, found 628.2907. **t_R** (0 to 100% B, 30 min, C_{A3}) = 17.1 min.

TFA·Ac-Phe-Leu$_2$-Phe(4-NH$_2$)-ϕ[CH$_2$SO$_2$]-F (63). TFA·Ac-Phe-Leu$_2$-Phe(4-NHCbz)-ϕ[CH_2SO_2]-F (20 mg, 22.3 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and a 33% HBr/AcOH solution (5 mL) was added and the reaction mixture was stirred for 1 h at rt. The solvents were removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 100% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (8.7 mg, 11.4 µmol, 51%). **^{1}H-NMR** (400 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.21 (d, J = 8.3 Hz, 1H, NH α CH (4-H_3N^+-Phe)), 8.13 - 8.07 (m, 2H, NH α CH (Leu), AcNH α CH)), 7.81 (d, J = 8.3 Hz, 1H, NH α CH (Leu)), 7.28 - 7.22 (m, 4H, Ar-H (Phe)), 7.22 - 7.14 (m, 3H, Ar-H (Phe), Ar-H, 2 × CH (4-H_3N^+-Phe)), 7.01 (d, J = 7.8 Hz, 2H, , 2 × CH (4-H_3N^+-Phe)) 4.50 (ddd, J = 10.1, 8.2, 4.0 Hz, 1H, AcNH α CH), 4.45 - 4.33 (m, 1H, NH α CH (4-H_3N^+-Phe)), 4.29 (app q, J = 7.7 Hz, 1H, NH α CH (Leu)), 4.25 - 4.17 (m, 1H, NH α CH (Leu)), 4.07 (ddd, J = 14.9, 7.4, 3.1 Hz, 1H, CH_aSO_2F),

3.91 (dd, J = 14.9, 10.2 Hz, 1H, CH_bSO_2F), 2.97 (dd, J = 13.9, 4.0 Hz, 1H, CH_a (Phe)), 2.81 (d, J = 6.7 Hz, 2H, α CHCH_2 (4-H_3N^+-Phe)), 2.70 (dd, J = 13.9, 10.1 Hz, 1H, CH_b (Phe)), 1.75 (s, 3H, CH_3 (Ac)), 1.65 - 1.50 (m, 2H, 2 × CH(CH_3)$_2$ (Leu)), 1.49 - 1.27 (m, 4H, 2 × CH_2 (Leu)), 0.89 (d, J = 6.5 Hz, 3H, CH_3 (Leu)), 0.84 (d, J = 6.5 Hz, 6H, 2 × CH_3 (Leu)), 0.81 (d, J = 6.5 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (101 MHz, d_6-DMSO, 298 K): δ_c (ppm) = 171.38 (C=O), 171.35 (C=O), 171.30 (C=O), 169.2 (C=O), 137.9 (C-Ar), 130.3 (C-Ar), 130.3 (C-Ar), 129.0 (CH-Ar), 127.9 (CH-Ar), 126.1 (CH-Ar), 119.8 (CH-Ar (4-H_3N^+-Phe)), 53.8 (AcNHC), 53.0 (d, $J_{C,F}$ = 11.5 Hz, CSO_2F), 51.0 (α CH (Leu)), 50.9 (α CH (Leu)), 46.1 (α CH (4-H_3N^+-Phe)), 40.6 (C$\underline{H}$(CH_3)$_2$ (Leu)), 40.4 (C$\underline{H}$(CH_3)$_2$ (Leu)), 38.4 (α CH$\underline{C}H_2$ (4-H_3N^+-Phe)), 37.2 (CH_2 (Phe)), 24.0 (C$\underline{H}$(CH_3)$_2$ (Leu)), 23.9 (C$\underline{H}$(CH_3)$_2$ (Leu)), 23.0 (CH_3 (Leu)), 22.9 (CH_3 (Leu)), 22.3 (CH_3 (Ac)), 21.6 (CH_3 (Leu)), 21.5 (CH_3 (Leu)). **^{19}F-NMR** (377 MHz, $CDCl_3$, 298 K): δ_F (ppm) = 59.7 (d, J = 6.8 Hz, 1F, SO_2F). **HRMS** (ESI positive) calc. for $C_{32}H_{46}N_5O_6SFNa$ $[M+Na]^+$ 670.3045, found 670.3016. **t_R** (0 to 100% B, 50 min, C_{A3}) = 29.7 min.

TFA·N_3-Phe-Leu$_2$-Phe(4-NH_2)-ϕ [CH_2SO_2]-F (64). TFA·N_3-Phe-Leu$_2$-Phe(4-NHCbz)-ϕ [CH_2SO_2]-F (14.1 mg, 16.0 µmol, 1.00 eq.) was set under nitrogen atmosphere and 4 m HCl in dioxane (10 mL) was added and the reaction mixture stirred for 48 h at 60 °C. The solvent was removed *in vacuo* and the crude product purified by semi-preparative HPLC (0 to 100% B, C_{P3}). The starting material was recovered (13.0 mg) and no product could be isolated.

2TFA·H_2N-Phe-Leu$_2$-Phe(4-CH_2NH_2)-ϕ [CH_2SO_2]-F (65). TFA·BocHN-Phe-Leu$_2$-Phe(4-CH_2NH_2)-ϕ [CH_2SO_2]-F (12.7 mg, 13.1 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and 33% HBr/AcOH solution (5 mL) was added and the reaction mixture was stirred for 45 min at rt. The solvent was removed *in vacuo* and the crude product was purified by semi-preparative HPLC (0 to 60% B, C_{P2}). Fractions containing the product were pooled,

lyophilized and the pure product was obtained as a white solid (7.5 mg, 8.84 µmol, 68%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.68 (d, J = 8.0 Hz, 0.5H, NH rotamer), 8.62 (d, J = 8.3 Hz, 0.5H, NH rotamer), 8.39 - 8.30 (m, 2H, 2 × NH), 8.22 - 8.14 (m, 3H, NH_3^+), 8.09 (br s, 3H, NH_3^+), 7.40 - 7.21 (m, 9H, Ar-H), 4.51 - 4.38 (m, 2H, αCH (Leu), αCH ($PheCH_2NH_3^+$)), 4.31 - 4.12 (m, 2H αCH (Leu), CH_aSO_2F), 4.11 - 4.02 (m, 1H, αCH (Phe)), 4.02 - 3.90 (m, 3H, CH_bSO_2F, $CH_2NH_3^+$), 3.09 (dd, J = 14.3, 4.8 Hz, 1H, CH_aPhe), 2.98 - 2.78 (m, 3H, CH_bPhe, ($CH_2PheCH_2NH_3^+$)), 1.68 - 1.51 (m, 1H, $CH(CH_3)_2$), 1.50 - 1.39 (m, 3H, $CH(CH_3)_2$, CH_2 (Leu)), 1.38 - 1.28 (m, 1H, CH_2-a (Leu)), 1.29 - 1.14 (m, 1H, CH_2-b (Leu)), 0.93 - 0.74 (m, 12H, CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_C (ppm) = 171.9, 171.6, 168.2 (C=O), 137.9, 135.2, 132.8 (C-Ar), 130.0, 129.4, 129.1, 129.0, 127.6 (CH-Ar), 53.6 (d, J = 12.0 Hz, CSO_2F), 53.4 (NH_3^+αCH), 51.5, 51.4 (2 × αCH), 46.7 (αCH ($PheCH_2NH_3^+$)), 42.5 ($CH_3NH_3^+$), 41.5, 41.2 (2 × CH_2 (Leu)), 39.2 ($\underline{C}H_2PheCH_2NH_3^+$), 37.5 ($CH_2Phe$), 24.6, 24.5 (2 × CH (Leu)), 23.5, 23.4, 22.1, 22.0 (4 × CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (d, J = 5.9 Hz, SO_2F). **HRMS** (ESI positive) calc. for $C_{31}H_{47}N_5O_5SF$ $[M+H]^+$ 620.3276, found 620.3275. **t_R** (0 to 100% B, 50 min, C_{A1}) = 40.5 min.

TFA·Ac-Phe-Leu$_2$-Phe(4-CH$_2$NH$_2$)-ψ [CH$_2$SO$_2$]-F (66). TFA·Ac-Phe-Leu$_2$-Phe(4-CH_2NHCbz)-ψ [CH_2SO_2]-F (24.7 mg, 27.2 µmol, 1.00 eq.) was dissolved in CH_2Cl_2 (5 mL) and a 33% HBr/AcOH solution (5 mL) was added and the reaction mixture was stirred for 1 h at rt. The solvent was removed *in vacuo* and the crude product purified *via* semi-preparative HPLC (0 to 100% B, C_{P3}). The obtained product (15.5 mg) required a second purification step by semi- HPLC (0 to 50% B, C_{P3}). Fractions containing the product were pooled, lyophilized and the pure product was obtained as a white solid (6.2 mg, 8.0 µmol, 29%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.27 (d, J = 8.4 Hz, 1H, N$H\alpha$CH (4-$H_3N^+CH_2$-Phe)), 8.16 (br s, 3H, NH_3^+), 8.12 - 8.06 (m, 2H, AcN$H\alpha$CH, N$H\alpha$CH (Leu)),

7.78 (d, J = 8.3 Hz, 1H, N*H* α CH (Leu)), 7.37 (d, J = 7.9 Hz, 2H, Ar-H (4-$H_3N^+CH_2$-Phe)), 7.31 - 7.14 (m, 7H, 5 × Ar-H (Phe), 2 × Ar-H (4-$H_3N^+CH_2$-Phe)), 4.49 (ddd, J = 10.1, 8.1, 4.0 Hz, 1H, AcNH α C*H*), 4.47 - 4.39 (m, 1H, α C*H*CH_2SO_2F), 4.32 - 4.25 (m, 1H, NH α C*H* (Leu)), 4.22 (ddd, J = 10.2, 8.3, 5.0 Hz, 1H, NH α C*H* (Leu)), 4.08 - 3.98 (m, 3H, CH_aSO_2F, C*H*$_2NH_3^+$), 3.95 (dd, J = 14.1, 9.1 Hz, 1H, CH_bSO_2F), 2.97 (dd, J = 14.0, 4.0 Hz, 1H, CH_a (Phe)), 2.87 (d, J = 6.9 Hz, 2H, C*H*$_2$Phe(4-$CH_2NH_3^+$)), 2.70 (dd, J = 14.0, 10.1 Hz, 1H, CH_b (Phe)), 1.75 (s, 3H, CH_3 (Ac)), 1.63 - 1.51 (m, 2H, 2 × C*H*$(CH_3)_2$ (Leu)), 1.49 - 1.38 (m, 3H, CH_2 (Leu), CH_a (Leu)), 1.33 (ddd, J = 13.7, 9.0, 5.0 Hz, 1H, CH_b (Leu)), 0.89 (d, J = 6.6 Hz, 3H, CH_3 (Leu)), 0.85 (d, J = 6.5 Hz, 3H, CH_3 (Leu)), 0.84 (d, J = 6.5 Hz, 3H, CH_3 (Leu)), 0.81 (d, J = 6.5 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (101 MHz, d_6-DMSO, 298 K): δ_C (ppm) = 171.98 (C=O), 171.95 (C=O), 171.88 (C=O), 169.8 (C=O), 138.5 (C-Ar), 137.9 (C-Ar), 132.7 (C-Ar), 130.0 (CH-Ar), 129.6 (CH-Ar), 129.3 (CH-Ar), 128.5 (CH-Ar), 126.7 (CH-Ar)), 54.4 (AcNH α C), 53.8 (d, $J_{C,F}$ = 11.7 Hz, CSO_2F), 51.6 (α CH (Leu)), 51.4 (α CH (Leu)), 46.6 (α CHCH_2SO_2F), 42.5 ($CH_2NH_3^+$), 41.2 (CH_2 (Leu)), 41.0 (CH_2 (Leu)), 39.3 (CH_2Phe), 37.7 (CH_2Phe[4-$CH_2NH_3^+$]), 24.6 (C$H(CH_3)_2$), 24.4 (C$H(CH_3)_2$), 23.6 (CH_3 (Leu)), 23.5 (CH_3 (Leu)), 22.9 (CH_3 (Ac)), 22.1 (CH_3 (Leu)), 22.0 (CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (d, J = 6.0 Hz, SO_2F). **HRMS** (ESI positive) calc. for $C_{33}H_{49}N_5O_6SF$ $[M+H]^+$ 662.3382, found 662.3370. **t_R** (0 to 100% B, 50 min, C_{A3}) = 29.6 min.

TFA·N_3-Phe-Leu$_2$-Phe(4-CH_2NH_2)-ϕ [CH_2SO_2]-F (67). TFA·N_3-Phe-Leu$_2$-Phe(4-CH_2NHCbz)-ϕ [CH_2SO_2]-F (21.0 mg, 23.5 µmol, 1.00 eq.) was placed under nitrogen atmosphere and 4 M HCl solution in dioxane (10 mL) was added and the reaction mixture was stirred for 30 h at 50 °C. The solvent was removed *in vacuo* and the crude product was purified *via* semi-preparative HPLC (0 to 50% B, C_{P3}). Fractions containing the product were

pooled, lyophilizedMand the pure product was obtained as a white solid (9.8 mg, 12.9 µmol, 55%). **^{1}H-NMR** (500 MHz, d_6-DMSO, 298 K): δ_H (ppm) = 8.37 (d, J = 8.3 Hz, 1H, N*H* α CH (Leu)), 8.30 (d, J = 8.4 Hz, 1H, N*H* α CH (Phe[4-$CH_2NH_3^+$])), 8.16 (br s, 3H, $CH_2NH_3^+$), 8.00 (d, J = 8.3 Hz, 1H, N*H* α CH (Leu)), 7.37 (d, J = 7.9 Hz, 2H, Ar-H (Phe[4-$CH_2NH_3^+$])), 7.33 - 7.21 (m, 7H, Ar-H (Phe), 2 × Ar-H (Phe[4-$CH_2NH_3^+$])), 4.49 - 4.41 (m, 1H, NH α C*H* (Phe[4-$CH_2NH_3^+$])), 4.40 - 4.33 (m, 1H, NH α C*H* (Leu)), 4 4.26 – 4.19 (m, 1H, NH α C*H* (Leu)), 4.10 (dd, J = 9.3, 5.0 Hz, 1H, N_3 α C*H*), 4.08 - 3.91 (m, 4H, C$H_2NH_3^+$, CH_2SO_2F), 3.08 (dd, J = 14.1, 5.0 Hz, 1H, N_3 α CHCH_a), 2.91 - 2.84 (m, 2H, N_3 α CHCH_b, CH_2Phe(4-$CH_2NH_3^+$)), 1.62 - 1.50 (m, 2H, 2 × C*H*$(CH_3)_2$ (Leu)), 1.50 - 1.27 (m, 4H, 2 × CH_2 (Leu)), 0.89 (d, J = 6.7 Hz, 3H, CH_3 (Leu)), 0.87 - 0.84 (m, 6H, 2 × CH_3 (Leu)), 0.82 (d, J = 6.5 Hz, 3H, CH_3 (Leu)). **^{13}C-NMR** (126 MHz, d_6-DMSO, 298 K): δ_C (ppm) = 171.4 (C=O), 171.0 (C=O), 168. 7 (C=O), 137.3 (C-Ar), 136.8 (C-Ar), 132.1 (C-Ar), 129.4 (CH-Ar), 128.9 (CH-Ar), 128.8 (CH-Ar), 128.3 (CH-Ar), 126.6 (CH-Ar), 62.2 (N_3 α C), 53.2 (d, $J_{C,F}$ = 12.4 Hz, CSO_2F), 51.0 (α CH (Leu)), 50.9 (α CH (Leu)), 46.1 (α C<u>H</u>CH_2SO_2F), 41.9 (<u>C</u>$H_2NH_3^+$), 40.64 (CH_2 (Leu)), 40.58 (CH_2 (Leu)), 38.7 (<u>C</u>H_2Phe[4-$CH_2NH_3^+$]), 36.7 (N_3 α CH<u>C</u>H_2), 24.0 (<u>C</u>H$(CH_3)_2$), 23.9 (<u>C</u>H$(CH_3)_2$), 23.0 (CH_3 (Leu), 22.9 (CH_3 (Leu), 21.5 (2 × CH_3 (Leu)). **^{19}F-NMR** (471 MHz, d_6-DMSO, 298 K): δ_F (ppm) = 59.8 (d, J = 5.9 Hz, SO_2F). **HRMS** (ESI positive) calc. for $C_{31}H_{45}N_7O_5SF$ $[M+H]^+$ 646.3181, found 646.3161. **t_R** (0 to 100% B, 50 min, C_{A1}) = 34.3 min, 34.6 min.

ASSOCIATED CONTENT

Supporting information

Analytical and preparative HPLC-columns; synthesis of peptides required for incorporation of amino sulfonyl fluoride derivatives; synthesis of precursors of amino sulfonyl fluorides

(**28 - 30**, **32 - 36**); synthesis of amino sulfonyl fluorides (**40 - 42**); synthesis of protected peptide sulfonyl fluorides; proton, carbon and fluoride NMR spectra; HPLC-traces of peptide sulfonyl fluorides.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS USED

BOP, (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; HCTU, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; THF, tetrahydrofuran , DMF, *N*,*N*-dimethylformamide, DiPEA, *N*,*N*-

diisopropylethylamine; Boc, *tert*-butyloxycarbonyl; Cbz, carboxybenzyl; EtOAc, ethyl acetate; Hex, n-hexane.

REFERENCES

1. Weissman, A. M.; Shabek, N.; Ciechanover, A. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat. Rev. Mol. Cell. Biol.* **2011**, *12*, 605-620.
2. Ferrington, D. A.; Gergerson, D. S. Immunoproteasomes: Structure, function, and antigen presentation. *Prog. Mol. Biol. Transl. Sci.* **2012**, *109*, 75-112.
3. Groettrup, M.; Kirk, C. J.; Basler, M. Proteasomes in immune cells: more than peptide producers? *Nat. Rev. Immunology* **2010**, *10*, 73-78.
4. Li, H.; O'Donoghue, A. J.; van der Linden, W. A.; Xie, S. C.; Yoo, E.; Foe, I. T.; Tilley, L.; Craik, C. S.; da Fonseca, P. C. A.; Bogyo, M. Structure- and function-based design of Plasmodium-selective proteasome inhibitors. *Nature* **2016**, *530*, 233-236.
5. Guterman, L. Covalent drugs form long-lived ties, *Chemical & Engineering News* **2011**, *89* (36), 19-26.
6. Richardson, P.; Hideshima, T.; Anderson, K. C. Bortezomib (PS-341): A novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers. *Cancer Control* **2003**, *10* (5), 361-369.
7. Demo, S. D.; Kirk, C. J; Aujay, M. A.; Buchholz, T. J.; Dajee, M.; Ho, M. N.; Jiang, J. Laidig, G. J.; Lewis, E. R.; Parlati, F.M; Shenk, K. D.; Smyth, M.S.; Sun, C. M.; Vallone, M. K.; Woo, T. M.; Molineaux, C: J.; Bennett, M. K. Antitumor Activity of PR-171, a Novel irreversible inhibitor of the proteasome. *Cancer Res.* **2007**, *67* (13), 6383-6391.

8. Ettari, R.; Previti, S.; Bitto, A.; Grasso, S.; Zapalà, M. Immunoproteasome-selective inhibitors: a promising strategy tot treat hematologic malignancies, autoimmune and inflammatory diseases. *Curr. Med. Chem.* **2016**, *23*, 1217-1238.
9. Kumar, S.; Shaji K. Kumar, Bensinger, W. I.; Zimmerman, T. M.; Reeder, C. B.; Berenson, J. R.; Berg, D.; Hui, A.-M.; Gupta, N.; Di Bacco, A.; Yu, J.; Shou, Y.; Niesvizky R. Phase 1 study of weekly dosing with the investigational oral proteasome inhibitor ixazomib in relapsed/refractory multiple myeloma. *Blood* **2014**, *124* (7) , 1047-1055.
10. Muz, B.; Ghazarian, R. N.; Ou, M.; Luderer, M. J.; Kusdono, H. D.; Azab, A. K. Spotlight on ixazomib: potential in the treatment of multiple myeloma. *Drug Design, Development and Therapy* **2016**, *10*, 217-226.
11. Infante, J. R.; Mendelson, D. S.; Burris, H. A.; Bendell, J. C.; Tolcher, A. W.; Gordon, M. S.; Gillenwater, H. H.; Arastu-Kapur, S.; Wong, H.S.; Papadopoulos, K. P. A first-in-human dose-escalation study of the oral proteasome inhibitor oprozomib in patients with advanced solid tumors. *Invest New Drugs* **2016**, *34*, 216-224.
12. Kraus, M.; Bader, J.; Geurink, P. P.; Weyburne, E. S.;Mirabella, A. C.; Silzle, T.; Shabaneh, T. B.; van der Linden, W. A.; de Bruin, G.; Haile, S. R.; van Rooden, E.; Appenzeller, C.; Li, N.; Kisselev, A. F.; Overkleeft, H.; Driessen, C. The novel β 2-selective proteasome inhibitor LU-102 synergizes with bortezomib and carfilzomib to overcome proteasome inhibitor resistance of myeloma cells. *Haematologica* **2015**, *100* (10), 1350-1360.
13. Kraus, J.; Kraus, M.; Liu, N.; Besse, L.; Bader, J.; Geurink, P. P.; de Bruin, G, A. F.; Overkleeft, H.; Driessen, C, The novel β 2-selective proteasome inhibitor LU-102 decreases phosphorylation of I kappa B and induces highly synergistic

cytotoxicity in combination with ibrutinib in multiple myeloma cells. *Cancer Chemother. Pharmacol.* **2015** *76*, 383-396.

14. a. Brouwer, A. J.; Ceylan, T.; Jonker, A. M.; van der Linden, T.; Liskamp, R. M. J. Synthesis and biological evaluation of novel irreversible serine protease inhibitors using amino acid based sulfonyl fluorides as an electrophilic trap. *Bioorg. Med. Chem.* **2011**, *19*, 2397-2406; b. Brouwer, A. J.; Ceylan, T.; Jonker, A. M.; van der Linden, T.; Liskamp, R. M. J. Synthesis of β-aminoethane sulfonyl fluorides or 2-substituted taurine sulfonyl fluorides as potential protease inhibitors. *Tetrahedron Lett.* **2009**, *50*, 3391-3393.
15. Brouwer, A. J.; Jonker, A.; Werkhoven, P.; Kuo, E.; Li, N.; Gallastegui, N.; Kemmink, J.; Florea, B. I.; Groll, M.; Overkleeft, H. S.; Liskamp,R. M. J. Peptido sulfonyl fluorides as new powerful proteasome inhibitors. *J. Med. Chem.* **2012**, *55*, 10995-11003.
16. Dubiella, C.; Cui, H.; Gersch, M.; Brouwer, A. J.; Sieber, S. A.; Krüger, A.; Liskamp, R. M. J.; Groll, M. Selective inhibition of the immunoproteasome by ligand-induced crosslinking of the active site. *Angew. Chem. Int. Ed.* **2014**, *53*, 11969-11973.
17. Narayanan, A.; Jones, L. H. Sulfonyl fluorides as privileged warheads in chemical biology. *Chem. Sci.* **2015***, 6,* 2650-2659.
18. Fadeyi, O.; Parikh, M. D.; Chen, M. Z.,; Kyne, R. E., Jr.; Taylor, A. P.; O'Doherty, I.; Kaiser, S. E.; Barbas, S.; Niessen, S.; Shi, M.; Weinrich, S. L.; Kath, J. C.; Jones, L. H.; Robinson, R. P. Chemoselective preparation of clickable aryl sulfonyl fluoride monomers: a toolbox of highly functionalized intermediates for chemical biology probe synthesis. *Chembiochem* **2016**, *17*, 1925-1930.

19. Geurink, P. P.; van der Linden, W. A.; Mirabella, A. C.; Gallastegui, N.;de Bruin, G. Blom, A. E. M.; Voges, M. J.; Mock, E. D.; Florea, B. I.; van der Marel, G. A.; Driessen, C.; van der Stelt, M.; Groll, M.; Overkleeft, H. S.; Kisselev, A. F. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **2013**, *56*, 1262-1275.

20. Brouwer, A. J.; Herrero Álvarez, N.; Ciaffoni, A.; van de Langemheen, H.; Liskamp, R. M. J. Proteasome inhibition by new dual warhead containing peptido vinyl sulfonyl fluorides. *Bioorg. Med. Chem.* **2016**, *24*, 3429-3435.

21. Gilfillan, L.; Artschwager, R.; Harkiss, A. H.; Liskamp, R. M. J.; Sutherland, A. Synthesis of pyrazole containing α-amino acids *via* a highly regio-selective condensation/aza-Michael reaction of β-aryl α,β-unsaturated ketones. *Org. Biomol. Chem.* **2015**, *13*, 4514-4523.

22. Goddard-Borger, E. D.; Stick, R. V. An efficient, inexpensive, and shelf-stable diazotransfer reagent: imidazole-1-sulfonyl azide hydrochloride. *Org. Lett.* **2007**, *9* (19), 3797-800.

23. Kirk, D. N.; Wilson, M. A. D-homo-steroids. Part III. The preparation of D-homoandrostane derivatives: a study of the reactions of steroidal C(17)-spiro-oxirans and their derivatives. *J. Chem. Soc.* **1971**, 414.

24. El-Faham, A.; Al Marhoon, Z.; Abdel-Megeed, A.; Albericio, F. OxymaPure/DIC: An efficient reagent for the synthesis of a novel Series of 4-[2-(2-acetylaminophenyl)-2-oxo-acetylamino] benzoyl amino acid ester derivatives. *Molecules* **2013**, *18*, 14747-14759.

25. El-Faham, A.; Albericio, F. Peptide coupling reagents, more than a letter soup. *Chem. Rev.* **2011**, *111*, 6557-6602.

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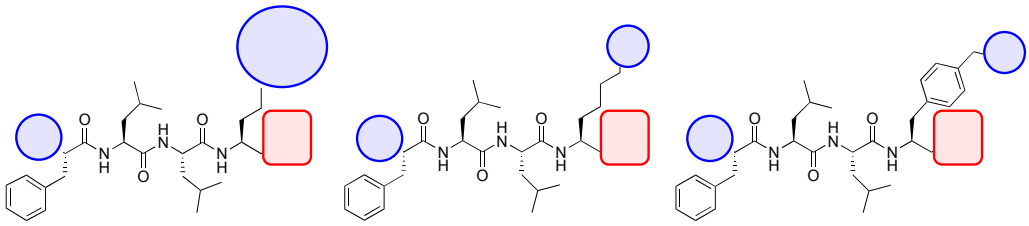

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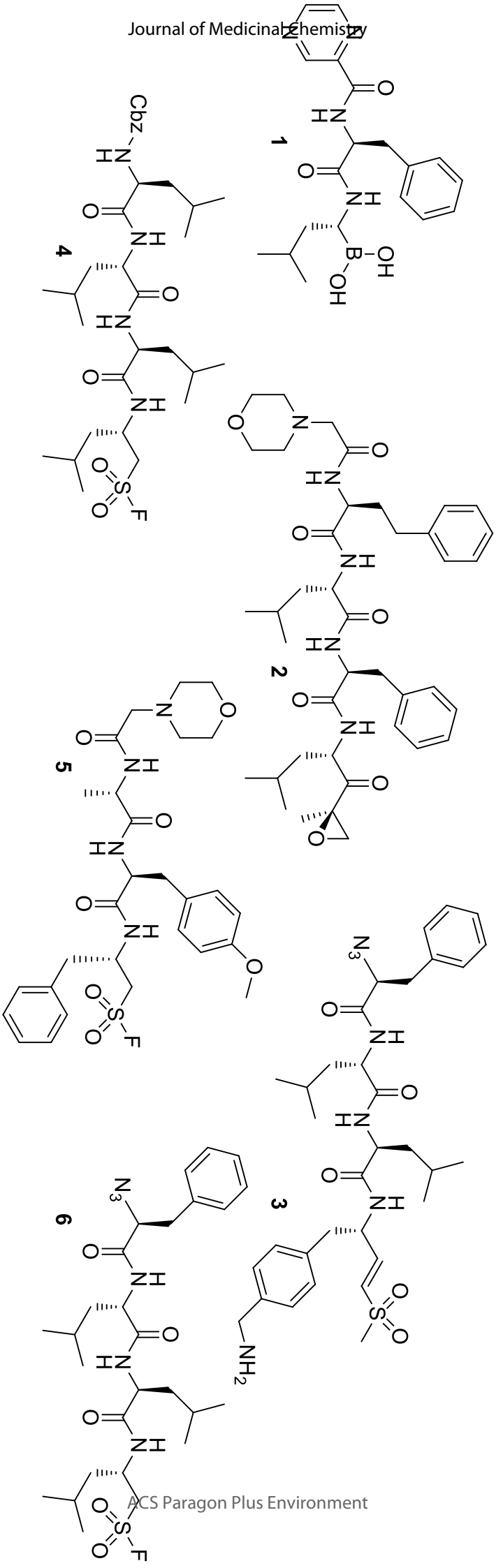
Selectivity over β5 inhibition:	>1000	>1000	380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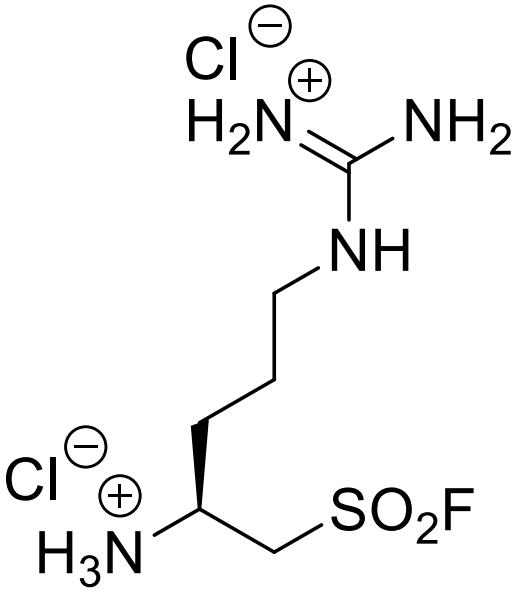
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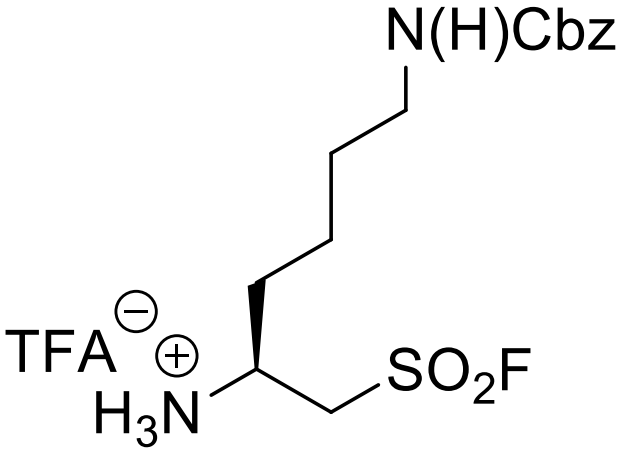
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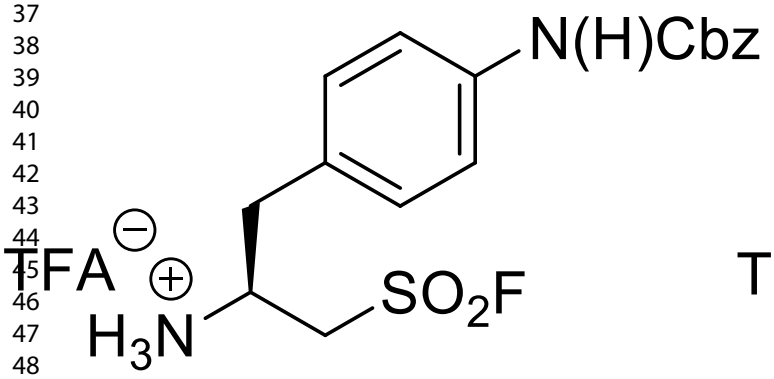
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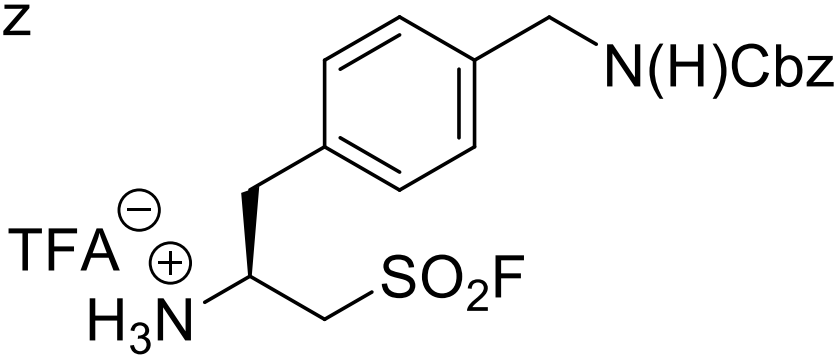
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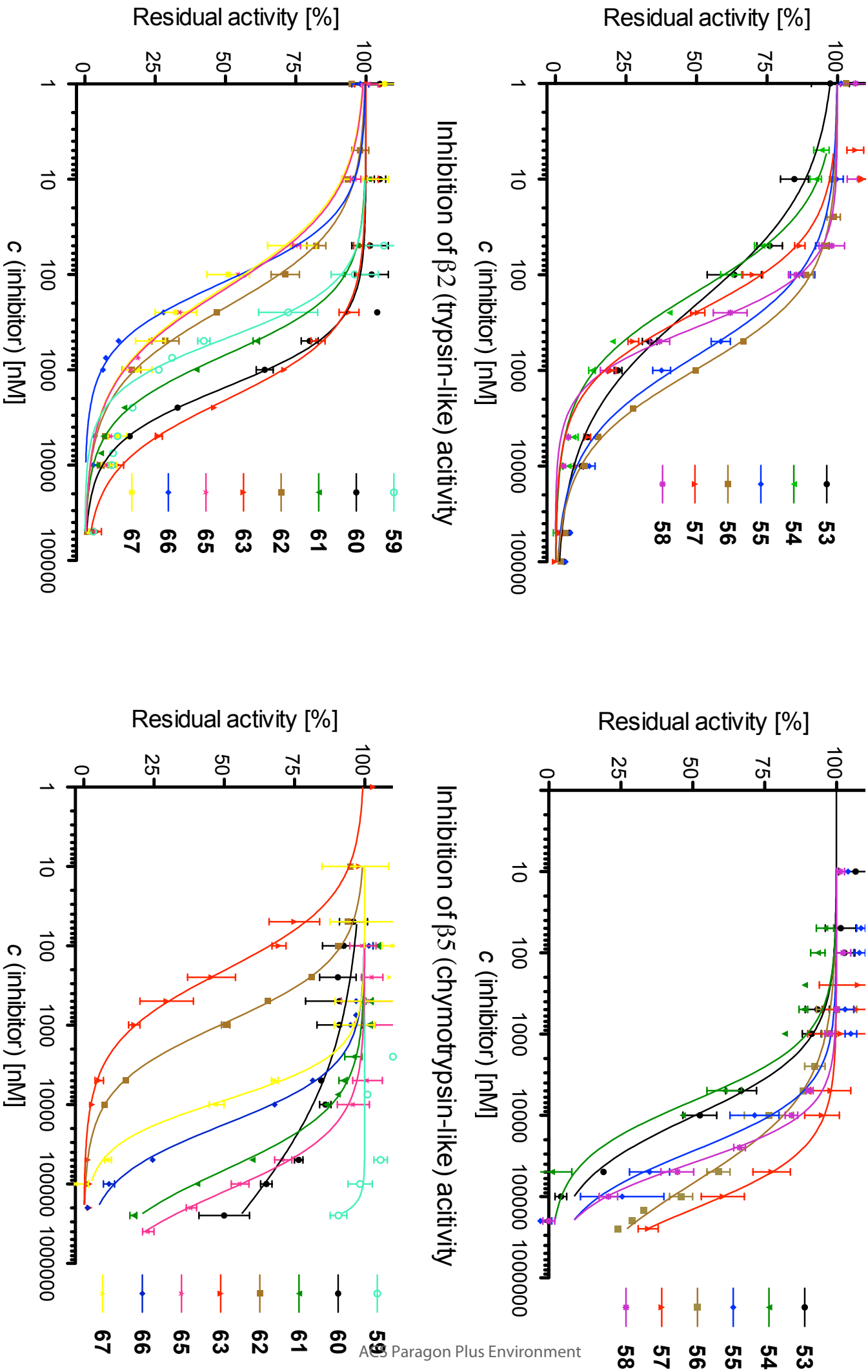
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Inhibition of β5 (chymotrypsin-like) acitivity
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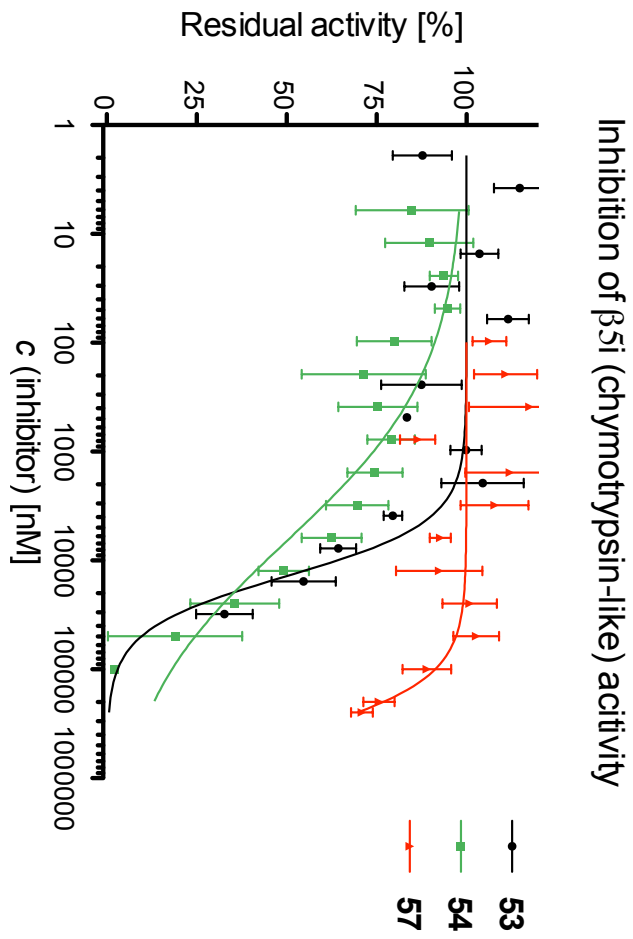
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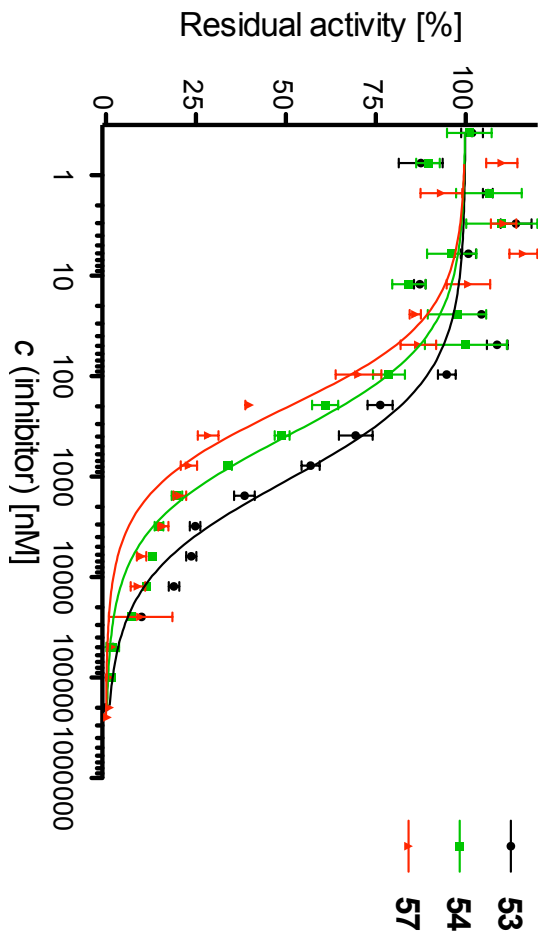
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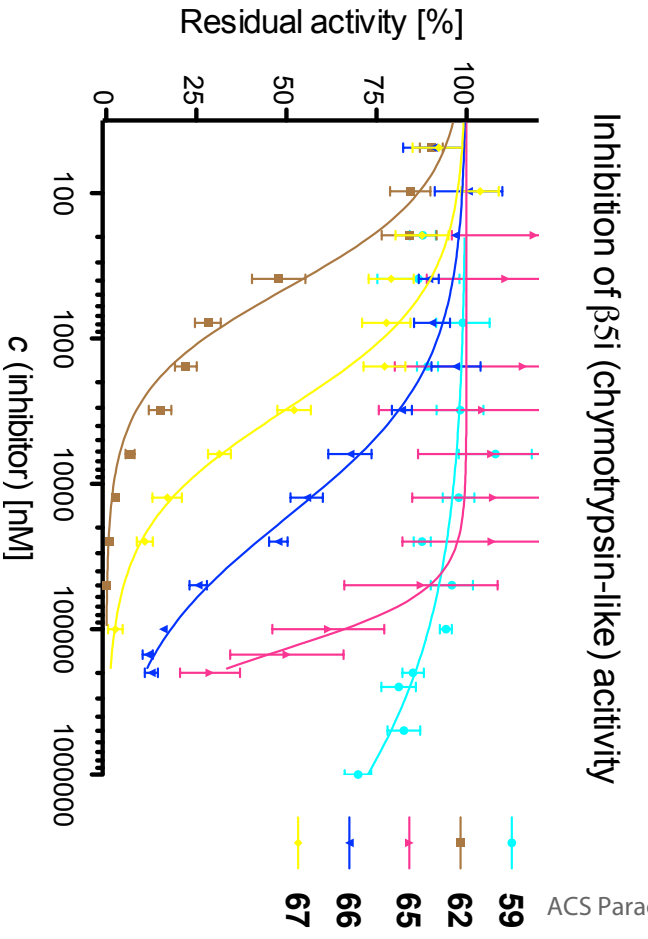
Residual activity [%]
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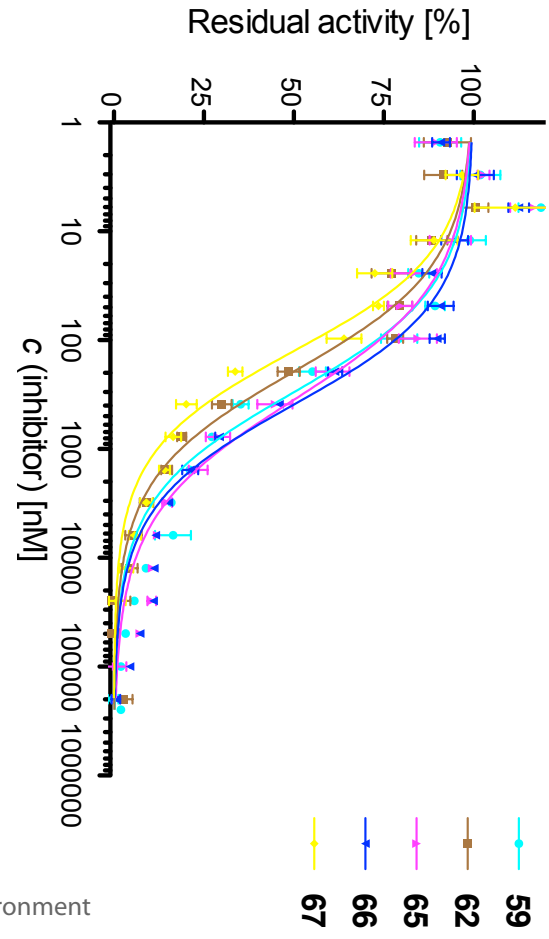
Residual activity [%]
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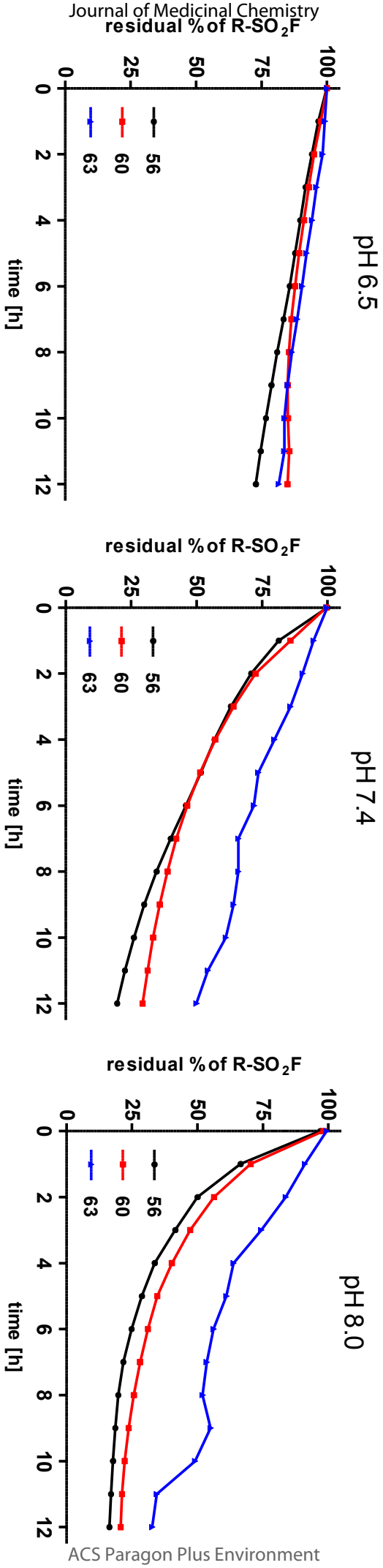
residual % of R-SO2F
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50
75
100
time [h]
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2
4
6
8
10
12
63
60
56
pH 6.5
residual % of R-SO2F
time [h]
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residual % of R-SO2F
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63
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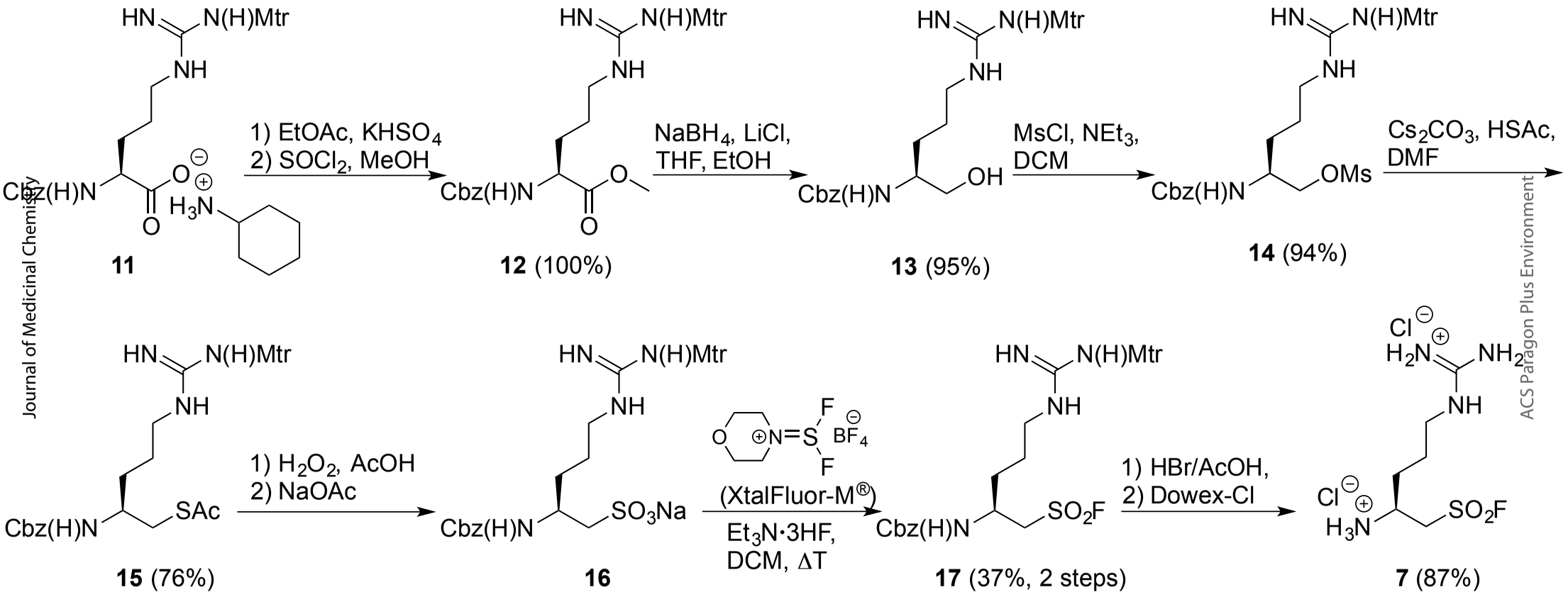

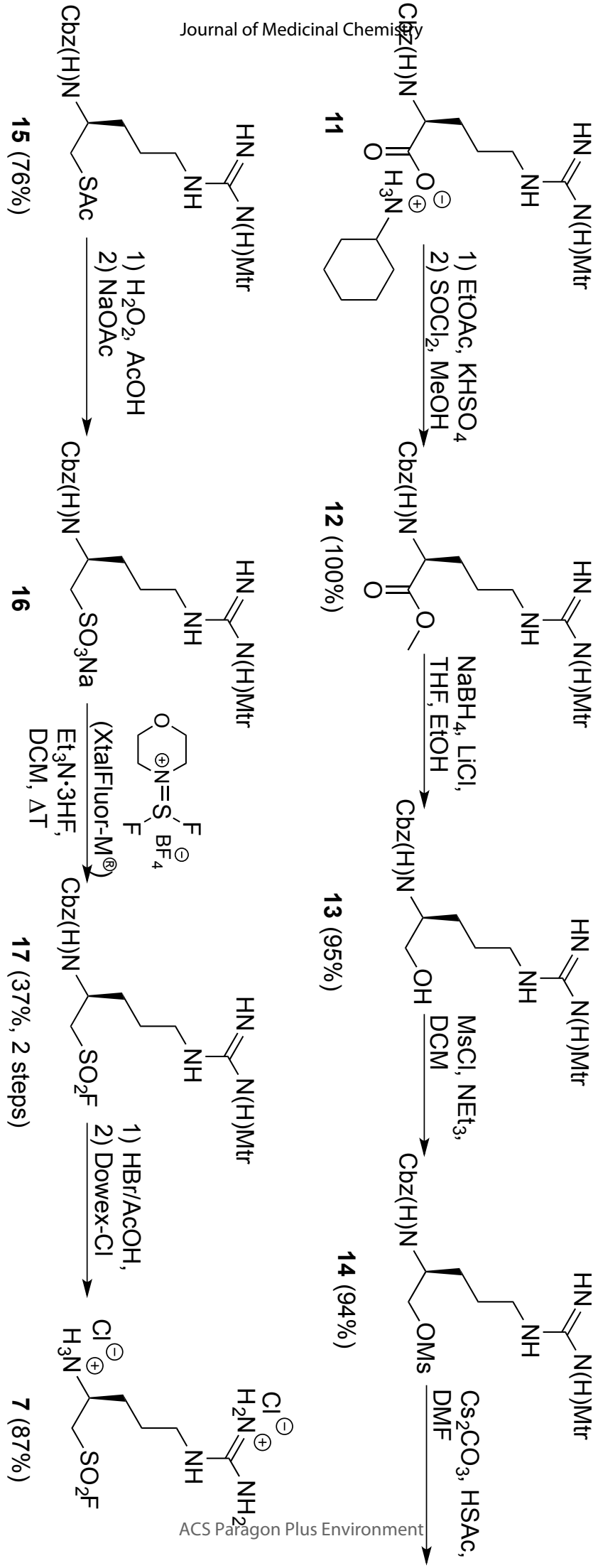
11
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2) SOCl2, MeOH
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NaBH4, LiCl,
THF, EtOH
13 (95%)
MsCl, NEt3,
DCM
14 (94%)
Cs2CO3, HSAc,
DMF
15 (76%)
1) H2O2, AcOH
2) NaOAc
16
(XtalFluor-M®)
Et3N·3HF,
DCM, ΔT
17 (37%, 2 steps)
1) HBr/AcOH,
2) Dowex-Cl
7 (87%)
Cbz(H)N
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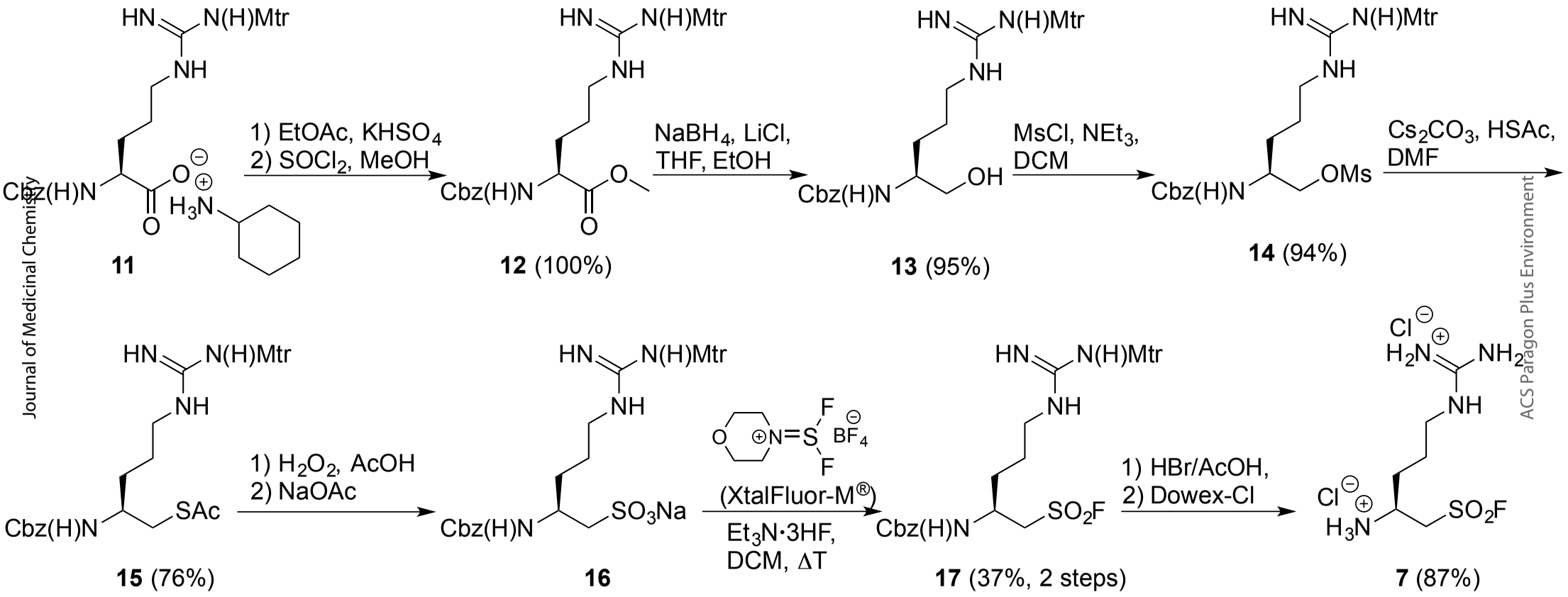
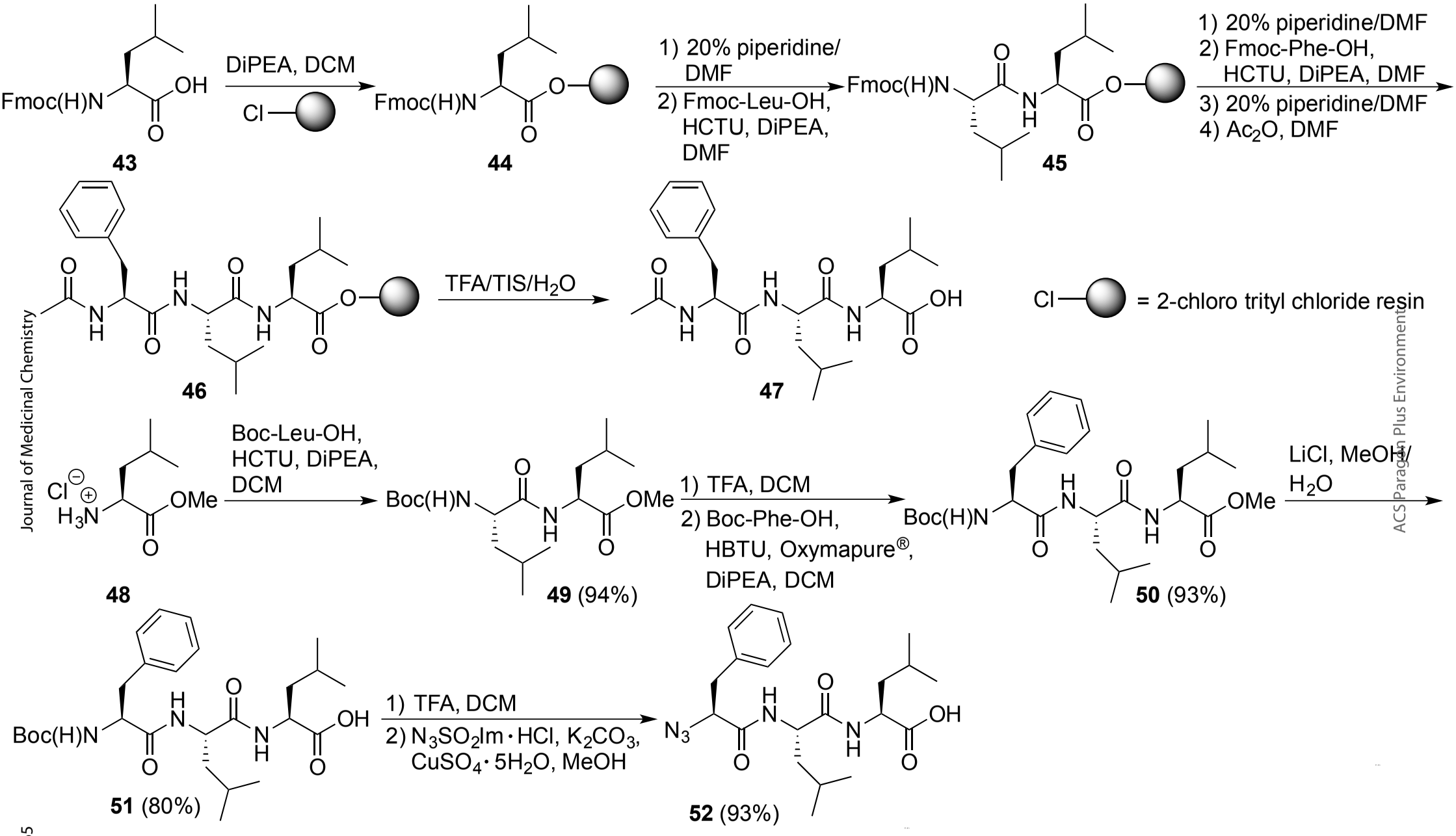
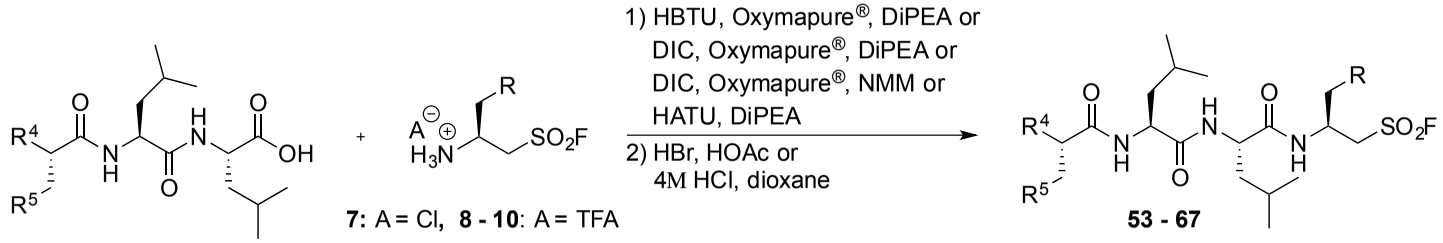
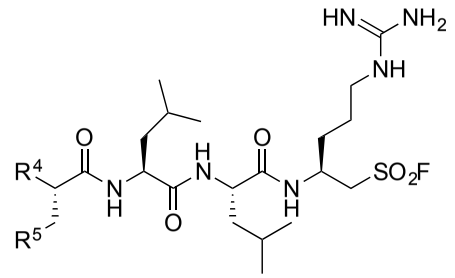
NH2

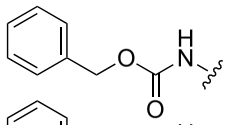
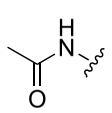
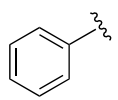
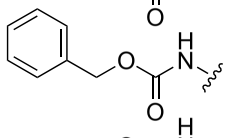
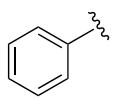
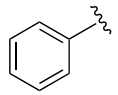
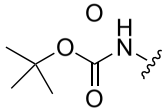
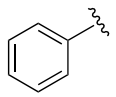
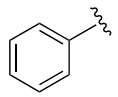




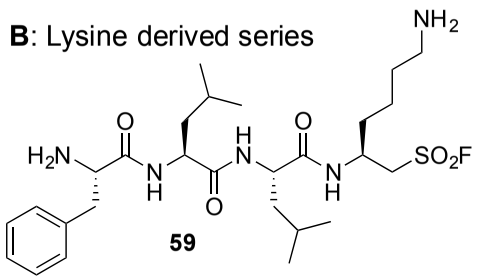


A: Arginine derived series



	R^4	R^5		R^4	R^5
53			**56**		
54			**57**	H_2N	
55			**58**	N_3	

B: Lysine derived series



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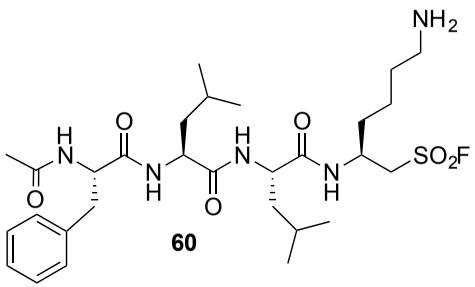

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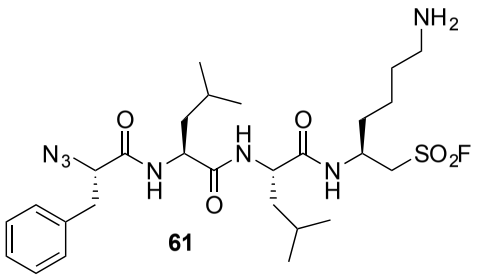
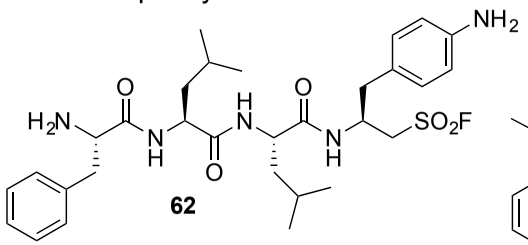


61

C: Amino-phenylalanine derived series



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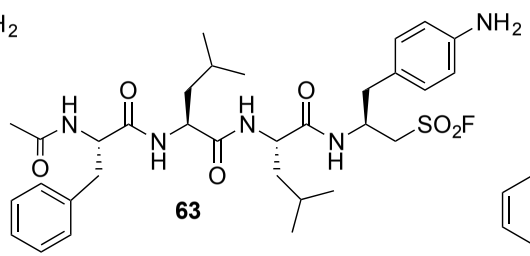

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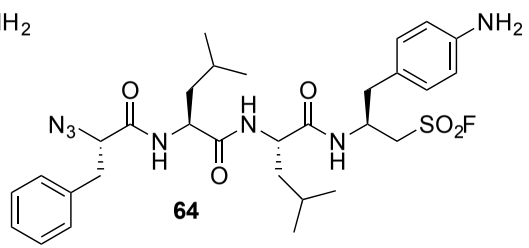
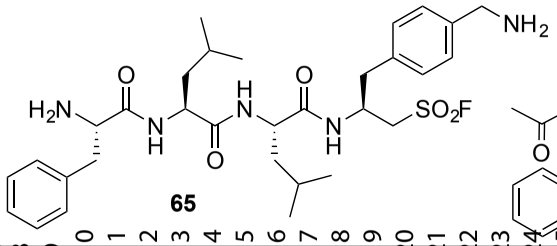


64

D: Methylene-amino-phenylalanine derived series



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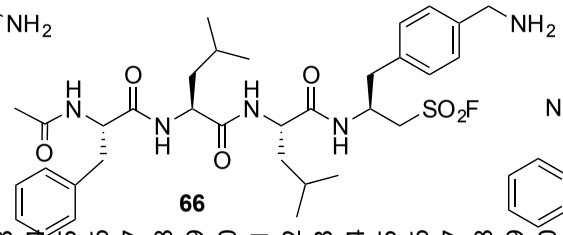

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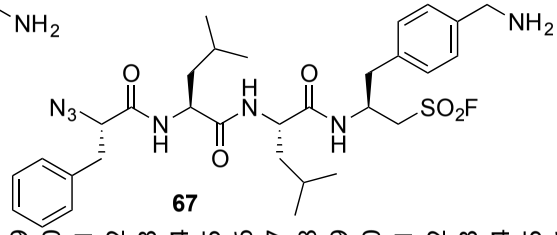
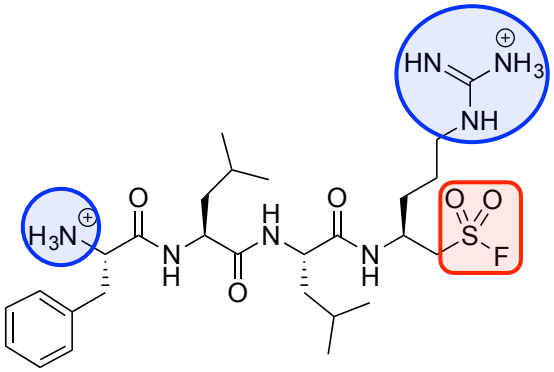
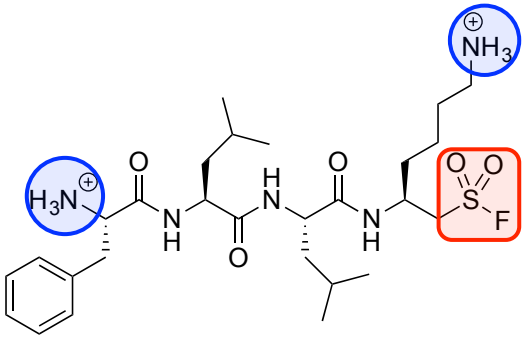
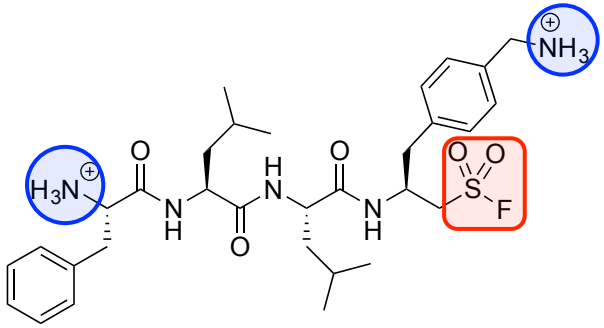


67

			
Inhibition β2 activity **constitutive proteasome, IC_{50}:**	250 nM	470 nM	140 nM
Selectivity over β5 inhibition:	560	>1000	900
Inhibition β2 activity **immuno proteasome, IC_{50}:**	210 nM	300 nM	360 nM
Selectivity over β5 inhibition:	>1000	>1000	380