1,2-Dimethylindole-3-sulfonyl (MIS), the Most Acid-Labile Sulfonyl-Protecting Group for the Side Chain of Arginine

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Abstract The protection of arginine (Arg) side chains is a crucial issue in peptide chemistry because of the propensity of the basic guanidinium group to produce side reactions. Currently, sulfonyl-type protecting groups, such as 2,2,5,7,8-pentamethylchroman (Pmc) and 2,2,4,6,7-pentamethyldihydrobenzofurane (Pbf), are the most widely used for this purpose. Nevertheless, Arg side chain protection remains problematic as a result of the acid stability of these two compounds. This issue is even more relevant in Arg-rich sequences, acid-sensitive peptides and large-scale syntheses. The 1,2-dimethylindole-3-sulfonyl (MIS) group is more acid-labile than Pmc and Pbf and can therefore be a better option for Arg side chain protection. In addition, MIS is compatible with tryptophan-containing peptides.

Keywords. Arginine protection. Peptide synthesis. 1,2-Dimethylindole-3-sulfonyl (MIS). Solid-Phase Synthesis. Guanidinium protection. Tryptophan side reactions.

Introduction.

Most peptides synthesized on solid-phase are prepared using the Fmoc/*tert*-butyl strategy.[1,2] Thus, α-amino temporary protection is achieved with the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group; amino acid side chains are protected by trifluoroacetic acid (TFA)-labile protecting groups, usually ^{t}Bu derivatives; and the *C*-terminal amino acid is anchored to the solid support through a TFA-labile linker/handle.

Nevertheless, *tert*-butyl-type protection of a number of amino acids is not the best option because of factors such as inefficiency at preventing side reactions or inadequate TFA lability. Among these amino acids, protection of the basic guanidinium group of Arginine (Arg) is possibly the most critical case.[3]

Currently, the most frequently used TFA-labile Arg-protecting groups are based on electron-rich benzene sulfonyl moieties. These groups are, by increasing order of acid lability: 4-methoxy-2,6-dimethylbenzenesulphonyl (Mds),[4] 4-methoxy-2,3,6-trimethylsulfonyl (Mtr),[4] 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc),[5] and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl

(Pbf)[6,7] (Figure 1). All of these mask the reactivity of the N^{ω}, are commercially available and have been extensively used in the Fmoc/^{t}Bu solid-phase strategy.[8] Nevertheless, side chain protection of Arg remains unsolved because even the Pbf group is too stable to TFA and its removal requires high TFA concentrations and long treatment times, which may not be appropriate for acid-sensitive peptides. The situation becomes increasingly more demanding when preparing multiple Arg-containing peptides, which show biological properties of great interest[9]. In addition, the preparation of Pmc and Pbf moieties is expensive.

The design of a new sulfonyl-based Arg-protecting group is not a straightforward process in the sense of simply adding electron-donating groups to an aromatic ring, because the planarity of the system, which is essential for TFA lability, is not easy conserved because of the presence of the sulfonyl group. Thus, trimethoxybenzenesulfonyl (Mtb), which contains more electron-rich substituents (3 MeO) is less acid-labile than Mds (1 MeO, 2 Me) and Mtr (1 MeO, 3 Me).[5] This characteristic is attributed to the loss of planarity caused by the presence of the two methoxy groups near the sulfonyl group. Furthermore, the sulfonyl derivative of the 3,4-ethylenedioxythiophene (EDOT), whose derived compounds are highly labile to TFA as carboxylic acid protectors,[10] is not labile as an Arg side-chain protector, possibly because of the same loss of planarity.[11] Common side-reactions associated with the use of these benzenesulfonyl-based protecting groups are arylation of sensitive residues, such as Trp,[12],or sulfonation of Trp and/or Arg residues themselves.[13] This side reactions are favored by the decomposition of the sulfonyl-protecting group in two moieties, the arylcarbocation and the sulfonyl.[13]

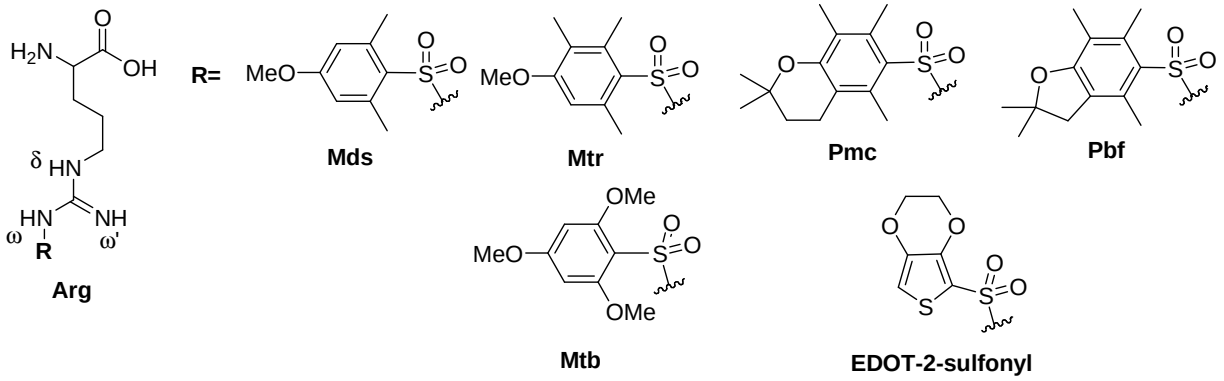


Figure 1. Arg protection

In an attempt to overcome the above mentioned drawbacks, here we describe a new more acid-labile Arg side chain-protecting group based on the indole system.

Results and Discussion.

General

A TFA-labile protecting group should be based on an electron-rich system. In this regard, *N*-alkylindole derivatives have been used as acid-labile amide linkers[14] and amide backbone protectors.[15] Taking this into account, we chose MIS (Figure 2) as guanidinium-protecting group. The extra methyl at position 2 should increase the acid lability of the protecting group and prevent electrophylic aromatic substitution. Furthermore, the 1,2-dimethylindole is commercially available.

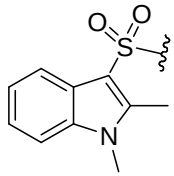


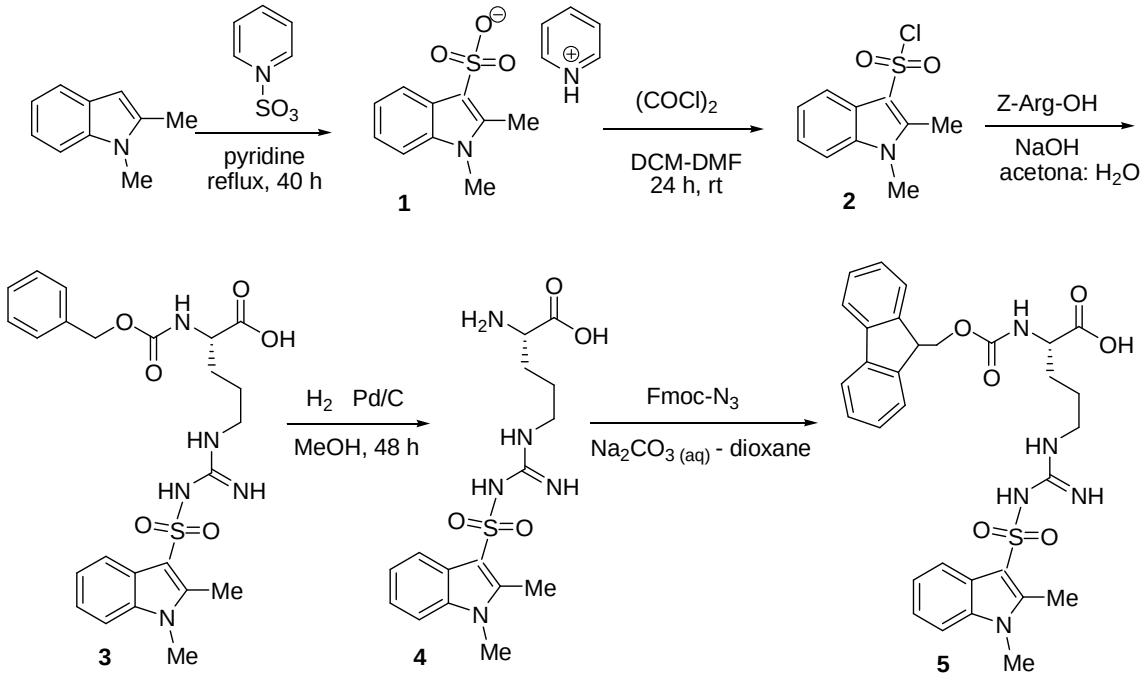
Figure 2. 1,2-Dimethylindole-3-sulfonyl (MIS)

Synthesis of the protecting group and Arg protection

As the 1,2-dimethylindole is prone to polymerize in strong acidic conditions, sulfonation of the indole ring must be carried out in neutral or basic media. Thus, chlorosulfonic acid, which is the reagent of choice for Pmc and Pbf sulfonylation, cannot be used in the case of 1,2-dimethylindole. Nevertheless, the use of sulphur trioxyde pyridine complex yielded the corresponding pyridinium sulfonate in good yield but in our hands longer time than that described in the literature was required.[16] Chlorination under mild conditions by treatment with oxalyl chloride yielded 1,2-dimethylindole-3-sulfonyl chloride (MIS-Cl). These conditions gave similar overall yields to those attained with Pbf and Pmc, with the advantage that 1,2-dimethylindole is commercially available.

We prepared Fmoc-Arg(MIS)-OH (Scheme 1) in a similar way to Pmc/Pbf derivatives[5,6], using Z-Arg-OH as starting material Z-Arg-OH was sulfonylated at the N^{ω} position with MIS-Cl and the Z

group was removed via catalytic hydrogenolysis. Final Fmoc protection was achieved via the azide method because the use of other more active Fmoc derivatives leads to the formation of dipeptides or other side reactions.[17,18]



Scheme 1. Synthesis of Fmoc-L-Arg(MIS)-OH

Synthesis of multiple arginine-containing peptides using MIS and Pbf protection

As Pbf removal is more complicated in multiple Arg-containing peptides, Ac-Phe-Arg-Arg-Arg-Arg-Val-NH_2 was chosen as a model peptide to compare the acid lability of MIS and Pbf. [19] The corresponding Pbf- and MIS-protected peptides were prepared using standard solid-phase peptide synthesis protocols on Sieber amide resin, which allows cleavage from the resin with small amounts of TFA (2%), thereby yielding the MIS- and Pbf-protected peptides respectively with excellent purity.

Removal assays

To compare the acid lability of the Pbf group, which is more acid-labile than the Pmc, with the one of the MIS group, protected peptide-bonded resins were treated with a range of concentrations of TFA in

DCM. These assays revealed that the MIS group is considerably more acid-labile than the Pbf one (Table 1).

Also, the MIS derivative generated in the removal process differs from the case of Pbf. For Pbf and Pmc, 2,2,5,7,8-pentamethylchroman and 2,2,4,6,7-pentamethyldihydrobenzofurane, respectively, are formed via a desulfonlylation mechanism[5] , while for MIS, the sulfonic acid (MIS-OH) was stable and was not desulfonated.

	MIS	Pbf
TFA–DCM–H_2O–TIS (**50**:45:2.5:2.5), t= 30 min	100 %	4%
TFA–DCM–H_2O–TIS (**50**:45:2.5:2.5), t= 1 h	100 %	38%

Table 1. Percentages of completely deprotected Ac-FRRRRV-NH_2

Optimization of the scavengers used in the removal:

As MIS-OH is a polar compound, it precipitates during the ether treatment after the cleavage step. Alternative scavengers to H_2O were tested to reduce the amounts of the strongly UV absorbant MIS-OH in order to facilitate purification. Among the scavengers tested, the most optimum were 10% of 3,4-dimethoxyphenol, 1,3,5-trimethoxybenzene (Tmb) or 3,5-dimethoxyphenol. The use of these scavengers reduced the amounts of MIS-OH more than 10 fold (40 times in the case of 1,3,5-trimethoxybenzene), thereby simplifying HPLC purification to yield the final product.

Synthesis of Trp-containing peptides:

To check the compatibility of the MIS group with Trp, we first synthesized the model peptides Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2 and Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 on a Sieber amide resin, which were obtained with an excellent HPLC purity.

Afterwards, both resins were treated with TFA-DCM-trimehtoxybenzene (50:40:10) to compare the purities of Trp-containing peptides after MIS and Pbf removal (Table 2). Trp alkylation or sulfonation was not detected in neither of the cases. The purity of the crude product was greater in the case of MIS and neither the MIS- protected peptides nor MIS-OH were detected by LC-MS. Nevertheless, in the case of the Pbf experiment, considerable amounts of the Pbf-protected peptide were detected (34% respect to unprotected peptide, HPLC, λ=220 nm).

Conclusions

MIS is the most acid-labile sulfonyl-type protecting group for Arg described to date. This feature makes it highly convenient for the synthesis of multiple Arg-containing peptides or peptides that contain acid-sensitive moieties. Furthermore, MIS is compatible with Trp-containing peptides.

Experimental Section

Synthesis of the protecting group and Arginine protection.

Pyridinium 1,2-dimethylindole-3-sulfonate (1)

1,2-Dimethylindole (19.7 g, 135.9 mmol) and sulphur trioxide pyridine complex (20.4 g, 128.3 mmol) were dissolved in pyridine (100 mL) under Ar atmosphere. The reaction mixture was refluxed for 40 h. It was then cooled to room temperature and H_2O was added (400 mL). The resulting solution was washed with diethyl ether (4 x 250 mL). The aqueous phase was evaporated to dryness and dried in the vacuum dessicator to render a red oil (37.6 g, 96 % yield). ^{1}H NMR (400 MHz, D_2O): δ= 8.44 (d, 2H, 2CH pyr., J= 5.8 Hz), 8.31 (m, 1H, CH pyr.), 7.75 (m, 2H, 2CH pyr), 7.67 (d, 1H, CH arom, J= 7.7 Hz), 7.14 (d, 1H, CH arom, J= 7.4 Hz), 7.05 (m, 2H, 2CH arom), 3.38 (s, 3H, CH_3), 2.41 (s, 3H, CH_3). ^{13}C NMR (100 MHz, D_2O): δ= 147.0 (CH), 140.9 (CH), 139.2 (C), 135.6 (C), 127.3 (CH), 124.1 (C), 122.0

(CH), 121.0 (CH), 119.2 (CH) 112.8 (C) 109.9 (CH), 29.2 (CH_3), 10.4 (CH_3). HRMS (CI): *m/z* calcd. for $C_{10}H_{10}NO_3S$ [M - H+] 224.0386, found 224.0388.

1,2-Dimethylindole-3-sulfonyl chloride (MIS-Cl) (2)

1 (16.4 g, 53.7 mmol) was suspended in dry DCM (120 mL) under N_2 atmosphere. The solution was cooled in an ice bath and oxalyl chloride (14 mL, 161 mmol) was slowly added. DMF (0.5 mL) was then slowly and carefully added and vigorous effervescence 7the starting material. The reaction mixture was stirred in an ice bath for a further 30 min until the effervescence ceased and was then stirred at room temperature. After 6 h, the solution was cooled in an ice bath and extra oxalyl chloride (4 mL, 46 mmol) and DMF (0.4 mL) were added and the reaction mixture was stirred at room temperature for further 15 h. A small aliquot (10 μL) was then treated with MeOH for 20 min and injected into the HPLC apparatus, which showed the presence of methyl 1,2-dimethylindole-3-sulfonate (94%) and starting material (6%). Therefore, additional oxalyl chloride (2 mL, 23 mmol) was added and after 4 h more at room temperature the HPLC assay showed that the reaction was completed. The reaction mixture was evaporated to dryness at room temperature, DCM (200 mL) was added, followed by H_2O (100 mL). The mixture was stirred for 5 min with care in order to remove the oxalyl chloride, the phases were separated and the organic phase was washed with H_2O (3x100 mL). The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness to give the target compound as a purple solid (10.2 g, 78% yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.82 (d, 1H, CH, J= 7.8 Hz), 7.36 (d, 1H, NH, J= 8.0 Hz), 7.08 (m, 2H, 2CH), 7.00 (m, 2H, 2CH), 3.63 (s, 3H, CH_3), 2.56 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO): δ= 137.2 (C), 135.9 (C), 125.5 (C), 121.4 (CH), 120.8 (CH), 120.1 (CH), 109.7 (CH), 30.0 (CH_3), 11.3 (CH_3). HRMS (CI): *m/z* calcd. for $C_{10}H_{10}NO_2S$ [M – Cl^-] 208.0426, found 224.0427.

Z-L-Arg(MIS)-OH (3)

Z-L-Arg-OH (2 g, 6.5 mmol) was dissolved in acetone (65 mL) and 3 N aqueous NaOH (18 mL, 54 mmol). The reaction was cooled in an ice bath and compound **2** (1.59 g, 6.5 mmol) dissolved in acetone (50 mL) was added over 10 min. The reaction mixture was stirred for 1 h at 0 ºC. Additional **2** (0.95 g, 3.9 mmol) in acetone (20 mL) was then added followed by 90 min of stirring at 0ºC. Finally, a final

amount of **2** (0.95 g. 3.9 mmol) in acetone (15 mL) was added and the reaction mixture was stirred for an additional 30 min at 0 ºC and 3 h at room temperature, until no **2** was observed by TLC (hexane–EtOAc 1:1). The pH of the reaction was neutralized with 10% aq. citric acid, the acetone was evaporated *in vacuo*, H_2O (100 mL) was added, the pH was acidified to 3 with 10% aqueous citric acid and the solution was extracted with EtOAc (3 x 100 mL). The organic phases were pooled, washed with H_2O (3 x 75 mL), dried with $MgSO_4$ and evaporated to dryness. The crude product obtained was purified twice by column chromatogrphy (DCM, MeOH, HOAc). The solvent of the pure fractions was removed *in vacuo* to yield an oil. The minimum amount of a mixture of EtOAc–DCM–MeOH was then added followed by addition of hexane until no further precipitated solid was observed. The solvent was decanted and the solid was washed 4 times with DCM–hexane (enough hexane to precipitate all the product) to remove the HOAc and was dried over $MgSO_4$ to give **3 (**0.61 g, 18 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.85 (d, 1H, CH, J= 7.6 Hz), 7.52 (d, 1H, NH, J= 8.0 Hz), 7.43 (d, 1H, CH, J= 8.0 Hz), 7.30 (m, 5H, 5CH Z), 7.10 (m, 2H, 2CH), 5.01 (s, 2H, CH_2), 3.87 (m, 1H, αCH), 3.66 (s, 3H, CH_3), 3.0 (m, 2H, CH_2), 2.60 (s, 3H, CH_3), 1.64 (m, 1H, CH_2), 1.49 (m, 1H, CH_2), 1.41 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO): δ= 174.4 (C), 157.0 (C), 156.8 (C), 139.4 (C), 137.7 (C), 135.9 (C), 129.0 (CH), 128.5 (CH), 128.4 (CH), 125.2 (C), 122.1 (CH), 121.1 (CH), 120.1 (CH), 110.4 (CH), 66.1 (CH_2), 54.3 (CH), 40.0 (CH_2), 30.2 (CH_3), 28.9 (CH_2), 26.4 (CH_2), 11.4 (CH_3). HRMS (CI): *m*/*z* calcd. for $C_{24}H_{30}N_5O_6S$ [M + H^+] 516.1911, found 516.1911.

H-L-Arg(MIS)-OH (4)

A mixture of **3 (**486 mg, 0.94 mmol) and 10 % Pd/C (110 mg) in MeOH (60 mL) was hydrogenated overnight at atmospheric pressure. After this time TLC (DCM–MeOH–HOAc, 90:9:1) still showed some starting material. More 10% Pd/C (100 mg) was added and the reaction was hydrogenated for 24 h more, after which TLC showed the absence of starting material. The reaction mixture was filtered over celite and evaporated to dryness to yield **4** (352 mg, 98 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.83 (d, 1H, CH, J= 7.6 Hz), 7.47 (d, 1H, NH, J= 8.1 Hz), 7.42 (d, 1H, CH, J= 8.1 Hz), 7.11 (m, 2H, 2CH),

3.65 (s, 3H, CH_3), 3.17 (m, 1H, CH), 3.00 (m, 2H, CH_2), 2.60 (s, 3H, CH_3), 1.65 (m, 1H, CH_2), 1.54 (m, 1H, CH_2), 1.42 (m, 2H, CH_2).

Fmoc-Arg(MIS)-OH (5)

Fmoc-Cl (84 mg, 0.32 mmol) was dissolved in 1,4-dioxane (0.5 mL). NaN_3 (25 mg, 0.39 mmol) in H_2O (0.4 mL) was added and the resulting emulsion was stirred for 2 h at room temperature. The emulsion was then slowly added to a solution of **4** (136 mg, 0.36 mmol) in H_2O–dioxane (1:1) at pH 9, controlled with 10% aqueous Na_2CO_3. The reaction mixture was stirred keeping the pH at 9 and when it was stabilized, it was left to stir overnight. After that, H_2O (30 mL) was added to the reaction mixture and it was washed with *tert*-butyl mehtyl ether (3 x 20 mL).The aqueous phase was acidified to pH 2-3 with 1N HCl and quickly extracted with EtOAc (3 x 30 mL). The organic phases were dried over $MgSO_4$ and evaporated to dryness to yield an oil (115 mg), which was dissolved in the minimum of acetone and aqueous Na_2CO_3 at pH 9 (20 mL) was added. The aqueous solution was washed with *tert*-butyl methyl ether (3 x 30 mL), acidified to pH 2-3 with 1N HCl, and extracted with EtOAc (3 x 20 mL), dried over $MgSO_4$ and evaporated to dryness to yield of the desired product (67.4 mg, 34.3 % yield). ^{1}H NMR (400 MHz, DMSO): δ= 7.86 (m, 3H, 2CH Fmoc, CH indole), 7.70 (d, 2H, 2CH Fmoc, J= 7.4 Hz), 7.59 (d, 1H, NH, J= 7.9 Hz), 7.42 (d, 1H, CH indole, J= 8.1 Hz), 7.39 (m, 2H, 2CH Fmoc), 7.30 (m, 2H, 2CH Fmoc), 7.10 (m, 2H, 2CH indole), 4.27 (m, 2H, CH_2 Fmoc), 4.20 (m, 1H, CH Fmoc), 3.86 (m, 1H, αCH), 3.66 (s, 3H, CH_3), 3.01 (m, 2H, CH_2), 2.61 (s, 3H, CH_3), 1.65 (m, 1H, CH_2), 1.52 (m, 1H, CH_2), 1.38 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO): δ= 174.4 (C), 157.0 (C), 156.8 (C), 144.5 (C), 141.4 (C), 139.4 (C) , 135.9 (C), 128.3 (CH), 127.8 (CH), 126.0 (CH), 125.2 (C), 122.1 (CH), 121.1 (CH), 120.8 (CH), 120.1 (CH), 110.4 (CH), 66.3 (CH_2), 55.6 (CH), 47.3 (CH), 40.0 (CH_2), 30.2 (CH_3), 28.8 (CH_2), 26.5 (CH_2) 11.4 (CH_3). HRMS (CI): *m*/*z* calcd. for $C_{31}H_{34}N_5O_6S$ [M + H^+] 602.2224, found 602.2222.

Synthesis of multiple arginine-containing peptides using MIS and Pbf protection:

Ac-Phe-Arg(MIS)-Arg(MIS) -Arg(MIS)-Arg(MIS)-Val-NH_2 (peptide 1)

Sieber amide resin (25 mg, 0.42 mmol/g) was placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed by treatment with piperidine–DMF (2:8) (1 x 1 min, 2 x 10 min). Fmoc-L-Val-OH (14.3 mg, 42.1 μmol) was coupled using HOBt (5.7 mg, 42.1 μmol) and DIC (6.7 μL, 42,1 μmol) in DMF, t = 90 min. The Fmoc group was removed in the usual way and Fmoc-L-Arg(MIS)-OH (15.8 mg, 26.3 μmol) was coupled using PyBOP (13.7 mg, 26.3 μmol) HOAt (3.6 mg, 26.3 μmol) and DIPEA (13.4 μL, 78.9 μmol) in DMF for 90 min. The resin was acetylated by treatment with Ac_2O (50 eq) and DIPEA (50 eq) in DMF for 25 min. The Fmoc group was removed and the same procedure was repeated three more times, acetylating the resin before each Fmoc removal. After the last Fmoc removal, Fmoc-L-Phe (13.6 mg, 35 μmol) was coupled using PyBOP (18.3 mg, 35 μmol) HOAt (4.8 mg, 35 μmol) and DIPEA (17.9 μL, 105.2 μmol) in DMF for 90 min. The Fmoc group was removed and the resulting free amino group was acetylated as before. The resin was washed with DMF, DCM and diethyl ether, dried *in vacuo*, and divided into five aliquots. One of these was swollen with DCM, and treated with 1.5 mL of TFA–DCM–TIS–H_2O (2:93:2.5:2.5) for 20 min in order to cleave the protected peptide from the resin. The resin was filtered and the collected solution was diluted with DCM and neutralised adding DIPEA (80 μL, 1.2 eq per eq of TFA). The solvent was then removed *in vacuo*, and H_2O and AcCN were added and the solution was frozen and lyophilized. The product obtained was characterised by LC-MS and HRMS (CI): *m*/*z* calcd. for $C_{80}H_{107}N_{23}O_{15}S_4$ [M + Na^+] 1780.7092, found 1780.7152.

Ac-Phe-Arg(Pbf) -Arg(Pbf) -Arg(Pbf)-Arg(Pbf)-Val-NH_2 (peptide 2)

The same procedure as for the synthesis of peptide 1 was used but replacing Fmoc-L-Arg(MIS)-OH by Fmoc-L-Arg(Pbf)-OH (17.1 mg, 26.3 μmol). The product obtained was characterised by LC-MS and HRMS (CI): *m*/*z* calcd. for $C_{92}H_{136}N_{19}O_{19}S_4$ [M + H^+] 1938.9137, found 1938.9202.

Removal assays:

General procedure: the resin (3 mg) was treated with cleavage solution (50 μL). After the cleavage time, the solution was poured into H_2O (4 mL), and TFA and DCM were evaporated. The resulting aqueous solution was washed with DCM (6 x 1 mL), frozen, lyophilized and analyzed by HPLC (λ= 220 nm) and ESMS or MALDI-TOF.

Optimization of the scavengers:

The same procedure as for the removal assays was followed. In all the experiments the resin was treated with TFA-DCM-scavenger (50:40:10) (50 μL) for 1 h. The scavengers tested were 3,4-dimethoxyphenol, 1,3,5-trimethoxybenzene (Tmb) or 3,5-dimethoxyphenol.

Synthesis of Trp-containing peptides:

Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2 and Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 :

Sieber amide resin (70 mg, 0.40 mmol/g) was placed in a 2-mL polypropylene syringe fitted with a polyethylene filter disk. The resin was swollen with DCM, washings with DCM and DMF were carried out and the Fmoc group was removed. Fmoc-L-Gly-OH (33.3 mg, 112 μmol), Fmoc-L-Ala-OH (34.9 mg, 112 μmol) and Fmoc-L-Trp(Boc)-OH (59.0 mg, 112 μmol) were sequentially coupled using PyBOP (58.3 mg, 112 μmol) HOAt (15.2 mg, 112 μmol) and DIPEA (57.4 μL, 336 μmol) in DMF, t= 1.5 h. The resin was divided into two equal parts.

Part 1 (Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2) : Z-Arg(MIS)-OH (28.9 mg, 56 μmol) was coupled using PyBOP (29.2 mg, 56 μmol) HOAt (7.6 mg, 56 μmol) and DIPEA (28.7 μL, 168 μmol) in DMF, t= 1.5 h. The resin was washed with DMF, DCM and diethyl ether, dried *in vacuo* and divided into 4-mg aliquots. One of these was swollen with DCM and treated with 1.5 mL of TFA–DCM–TIS–H_2O (2:93:2.5:2.5) for 20 min in order to cleave the protected peptide from the resin. The resin was filtered and the collected solution was diluted with DCM and neutralised by adding DIPEA (80 μL, 1.2 eq per

eq of TFA). The solvent was then removed *in vacuo*, and H_2O and AcCN were added and the solution was frozen and lyophilized. The product obtained was characterised by LC-MS (95 % purity).

Part 2 (Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2) : Fmoc-Arg(Pbf)-OH (36.3 mg, 56 μmol) was coupled using PyBOP (29.2 mg, 56 μmol) HOAt (7.6 mg, 56 μmol) and DIPEA (28.7 μL, 168 μmol) in DMF, t= 1.5 h. The Fmoc group was removed and the free amine was protected with the Z group by treatment with Z-OSu (14.0 mg, 56 μmol) and DIPEA (35.9 μL, 210 μmol). The resin was then washed with DMF, DCM and diethyl ether, dried *in vacuo*, divided into 4-mg aliquots, one of which was cleaved in the same way as for Part 1. The product obtained was characterised by LC-MS (96 % purity).

Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(MIS)-Trp(Boc)-Ala-Gly-NH_2 : Two aliquots from Part 1 were treated with TFA-DCM-1,3,5-trimehtoxybenzene (50:40:10) and TFA-DCM-H_2O (50:45:5) respectively for 1 h following the General Procedure for the removal assays described above. In the latter case, no DCM washings were performed. The two crude products resulting from these treatments were analyzed by LC-MS. No Trp alkylation or sulfonation nor MIS-protected peptide were observed.

Z-Arg-Trp-Ala-Gly-NH_2 from Z-Arg(Pbf)-Trp(Boc)-Ala-Gly-NH_2 : An aliquot from Part 2 was treated with TFA-DCM-trimehtoxybenzene (50:40:10) for 1 h following the General Procedure for the removal assays described above. The target peptide was analyzed by LC-MS (60% purity). 17% of Pbf- protected peptide was detected and no Trp alkylation or sulfonation was observed.

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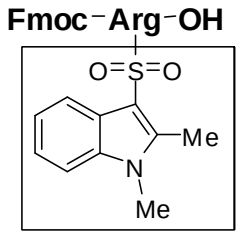
Supporting Information Available. NMR spectra and HPLC chromatograms of the prepared compounds.

TOC



MIS

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