1,2-Dimethylindole-3-sulfonyl (MIS) as protecting group for the side chain of arginine†

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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.

Introduction

Most peptides synthesized on a 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 in 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.[4] These groups are, in increasing order of acid lability: 4-methoxy-2,6-dimethylbenzenesulfonyl (Mds),[5] 4-methoxy-2,3,6-trimethylsulfonyl (Mtr),[5] 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc),[6,7] and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) (Fig. 1).[8,9]

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.[10] Nevertheless, the problem of the 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 conditions become increasingly more demanding when preparing multiple Arg-containing peptides, which show biological properties of great interest.[11]

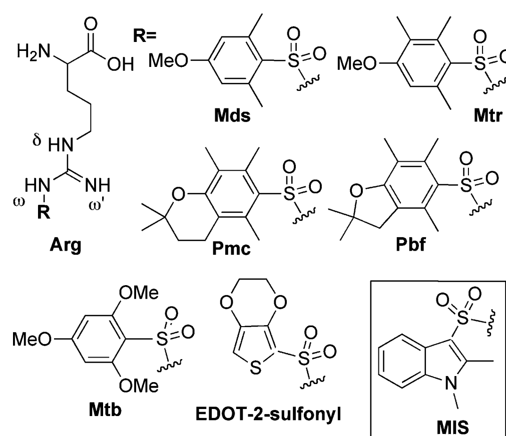

Fig. 1 Protecting groups for the side-chain of Arg.

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 easily 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).[6] 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 3,4-ethylenedioxythiophene (EDOT), whose derived compounds are highly labile to TFA as carboxylic acid protectors,[12] is not labile as an Arg side-chain protector, possibly because of the same loss of planarity.[13] Common side-reactions associated with the use of these benzenesulfonyl-based protecting groups are arylation of sensitive residues, such as Trp,[14]

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or sulfonation of Trp and/or Arg residues themselves.[15] These side reactions are favored by the decomposition of the sulfonyl-protecting group in two moieties, the arylcarbocation, which is an alkylating agent, and the sulfonyl part, which can cause addition of sulfur trioxide on the peptidic chain.[15]

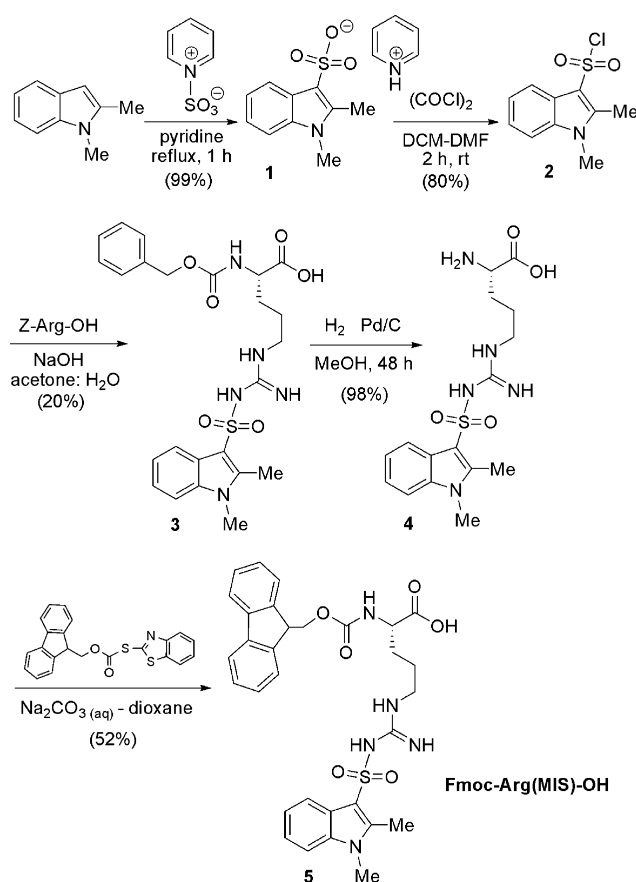
In an attempt to overcome the above mentioned drawbacks, we describe herein a new more acid-labile Arg side chain-protecting group based on the indole system.

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[16] and amide backbone protectors.[17] Taking this into account, we chose 1,2-dimethylindole-3-sulfonyl (MIS) as a guanidinium-protecting group (Fig. 1). The extra methyl at position 2 should increase the acid lability of the protecting group and prevent electrophilic aromatic substitution. Furthermore, the 1,2-dimethylindole is commercially available.

As the 1,2-dimethylindole is prone to polymerization 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 sulfur trioxide pyridine complex yielded the corresponding pyridinium sulfonate in good yield.[18] Chlorination under mild conditions by treatment with oxalyl chloride yielded 1,2-dimethylindole-3-sulfonyl chloride (MIS-Cl). These conditions gave much better overall yield (80%) to those attained with Pbf and Pmc (51% and 53% respectively),[6,8] with the advantage that 1,2-dimethylindole is commercially available (Scheme 1).



Scheme 1 Synthetic pathway for the preparation of Fmoc-Arg(MIS)-OH.

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

We prepared Fmoc-Arg(MIS)-OH in a similar way to Pmc/Pbf derivatives,[6,8] 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 by using Fmoc-2-mercaptobenzothiazole (Fmoc-2-MBT) because the use of other more active Fmoc derivatives leads to the formation of dipeptides or other side reactions.[19,20]

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.[8,21] 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 Pmc, with the MIS group, protected peptide-bonded resins were treated with **TFA**–CH_2Cl_2–H_2O–TIS (**50** : 45 : 2.5 : 2.5) for 30 (MIS, 100%; Pbf, 4%) and 60 min (MIS, 100%; Pbf, 38%). These assays revealed that the MIS group is considerably more acid-labile than the Pbf one.

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 desulfonylation mechanism,[6] while for MIS, the sulfonic acid (MIS-OH) was stable and was not desulfonated.

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 optimum were 10% 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 Tmb), thereby simplifying HPLC purification to yield the final product.

Synthesis of Trp-containing peptides

To check the compatibility of the MIS group with Trp,[22] 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-CH_2Cl_2-trimethoxybenzene (50 : 40 : 10) to compare the purities of

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Trp-containing peptides after MIS and Pbf removal. Trp alkylation or sulfonation was not detected in either case. 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% compared to unprotected peptide, HPLC, $\lambda = 220$ nm).

In summary, 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 (14.5 g, 99.8 mmol) and sulfur trioxide pyridine complex (19.1 g, 119.8 mmol) were dissolved in pyridine (70 mL) under an Ar atmosphere. The reaction mixture was refluxed for 1 h and HPLC indicated the reaction was complete (99.2% HPLC conversion, 254 nm). The reaction mixture was cooled to 60 °C and concentrated under vacuum to give a solid. The crude product was used directly for the next step.[23] ^{1}H NMR (400 MHz, D_2O): δ = 8.44 (d, 2H, 2CH, J = 5.8 Hz), 8.31 (m, 1H, CH), 7.75 (m, 2H, 2CH), 7.67 (d, 1H, CH, J = 7.7 Hz), 7.14 (d, 1H, CH, J = 7.4 Hz), 7.05 (m, 2H, 2CH), 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). All the crude product **1** obtained in the previous step was suspended in dry CH_2Cl_2 (200 mL) under Ar atmosphere. The suspension was cooled in an ice bath and oxalyl chloride (20.0 g, 158 mmol) was slowly added. DMF (0.5 mL) was then slowly and carefully added and vigorous effervescence was observed. 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 for 2 h. An aliquot (6 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 and an absence of starting material. CH_2Cl_2 (200 mL) was added to the reaction mixture and it was cooled to below 5 °C. H_2O (2–8 °C, 150 mL) was added and the mixture was stirred for 10 min. The organic phase was separated and washed with 2–8 °C H_2O (2 × 150 mL), and dried with anhydrous $MgSO_4$ (15 g). The solution was then concentrated to *ca.* 40 mL. The solid was filtered off and washed with CH_2Cl_2–*n*-hexane (1 : 1, 60 mL). The solid was dried under vacuum to give light pink solid (19.6 g, 80.4% yield, 98% HPLC purity, base on 1,2-dimethylindole). Mp = 67.7–73.5 °C. ^{1}H NMR (400 MHz, DMSO-*d*-6): 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-*d*-6): 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 208.0427.

Z-L-Arg(MIS)-OH (3). Z-L-Arg-OH (2.05 g, 6.7 mmol) was suspended in 3 N aqueous NaOH (6.7 mL, 20 mmol) and acetone (13.3 mL) was added to dissolve the product. The reaction was cooled in an ice bath and 3 N aqueous NaOH (6.7 mL) and a solution of compound **2** (3.69 g, 14.7 mmol) in acetone (13.3 mL) were simultaneously added over 10 min. The reaction mixture was stirred at 0 °C for 2 h and at room temperature for a further 2 h. After that time, starting material **2** was no longer detected by TLC (hexane–EtOAc, 1 : 1). H_2O (100 mL) was added and the suspension was washed with diethyl ether (3 × 80 mL). The aqueous phase was acidified to pH 2–3 by addition of 1 N HCl, the precipitate obtained was filtered, washed with acidic water (pH 2–3) and dried *in vacuo*. The crude product obtained was purified by column chromatography (CH_2Cl_2, MeOH, 1% HOAc). The solvent of the pure fractions was removed *in vacuo* to yield an oil. This process was repeated. Hexane and CH_2Cl_2 were then sequentially added and a precipitate appeared on scratching. The solvent was decanted and the solid was washed 4 times with CH_2Cl_2–hexane (enough hexane to precipitate all the product) to remove HOAc and give **3** (0.70 g, 20.4% yield).[24] Mp = 155.5–159.1 °C.^{1}H NMR (400 MHz, DMSO-*d*-6): 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-*d*-6): 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 Pd/C (10%) (110 mg) in MeOH (60 mL) was hydrogenated overnight at atmospheric pressure. After this time, TLC (CH_2Cl_2–MeOH–HOAc, 90:9:1) still showed some starting material. More 10% Pd/C (100 mg) was added and the mixture was hydrogenated for a further 24 h, 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). Mp = 153.2–155.0 °C.^{1}H NMR (400 MHz, DMSO-*d*-6): 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). HRMS (CI): m/z calcd. for $C_{16}H_{24}N_5O_4S$ $[M + H]^+$ 382.1544, found 382.1542.

Fmoc-Arg(MIS)-OH (5). H-Arg(MIS)-OH (250 mg, 0.658 mmol) was suspended in 1% aqueous Na_2CO_3 (2 mL). 1,4-dioxane (2 mL) was added and the product was dissolved. The pH was basified to 9–10 with saturated aqueous Na_2CO_3 (300 µL in our case). Fmoc-2-mercaptobenzotiazole (Fmoc-2-MBT) (256 mg, 0.658 mmol) in 1,4-dioxane (700 µL) was slowly added. The pH was kept between 9 and 10 with saturated aqueous Na_2CO_3 and the resulting suspension was stirred overnight. After 14 h of reaction, H_2O (9 mL) was added, the pH was neutralized with 1 N HCl and the solution was washed with *tert*-butylmethyl

ether (3 × 5 mL). The aqueous phase was acidified to pH 2–3 with 1 N HCl and extracted with EtOAc (3 × 7 mL). Note that to dissolve the precipitated product vigorous stirring is required. The organic phases were pooled, dried over dry $MgSO_4$, filtered and evaporated to dryness, thereby yielding a solid. Various co-evaporations with CH_2Cl_2 were performed to yield the desired product as a solid (207 mg, 52% yield). Mp = 137.2–146.4 °C. ^1H NMR (400 MHz, DMSO-*d*-6): δ = 7.86 (m, 3H, 3CH), 7.70 (d, 2H, 2CH, *J* = 7.4 Hz), 7.59 (d, 1H, NH, *J* = 7.9 Hz), 7.42 (d, 1H, CH, *J* = 8.1 Hz), 7.39 (m, 2H, 2CH), 7.30 (m, 2H, 2CH), 7.10 (m, 2H, 2CH), 4.27 (m, 2H, CH_2), 4.20 (m, 1H, CH), 3.86 (m, 1H, CH), 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-*d*-6): 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 $C_{31}H_{34}N_5O_6S$ $[M + H]^+$ 604.2224, found 604.2222.

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

Ac-Phe-Arg-Arg-Arg-Arg-Val-NH_2 (model peptide 1).

Ac-Phe-Arg(MIS)-Arg(MIS)-Arg(MIS)-Arg(MIS)-Val-NH_2. Sieber amide resin (25 mg, 0.42 mmol/g) was placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disc. The resin was swollen with CH_2Cl_2, washings with CH_2Cl_2 and DMF were carried out and the Fmoc group was removed by treatment with piperidine–DMF (2 : 8) (1 × 1 min, 2 × 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, CH_2Cl_2 and diethyl ether, dried *in vacuo*, and divided into five aliquots. One of these was swollen with CH_2Cl_2, and treated with 1.5 mL of TFA–CH_2Cl_2–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 solution collected was diluted with CH_2Cl_2 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 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. 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 CH_2Cl_2 were evaporated. The resulting aqueous solution was washed with CH_2Cl_2 (6 × 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-CH_2Cl_2-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.

Z-Arg-Trp-Ala-Gly-NH_2 (model peptide 2).

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 disc. The resin was swollen with CH_2Cl_2, washings with CH_2Cl_2 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, CH_2Cl_2 and diethyl ether, dried *in vacuo* and divided into 4 mg aliquots. One of them was swollen with CH_2Cl_2 and treated with 1.5 mL of TFA–CH_2Cl_2–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 CH_2Cl_2 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). HRMS (CI): *m/z* calcd. for $C_{45}H_{57}N_{10}O_{10}S$ $[M + H]^+$ 929.3974, found 929.3969.

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, CH_2Cl_2 and diethyl ether, dried *in vacuo*, and 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–CH_2Cl_2–1,3,5-trimehtoxybenzene (50 : 40 : 10) and TFA–CH_2Cl_2–H_2O (50 : 45 : 5) respectively for 1 h following the General procedure for the removal assays described above. In the latter case, no CH_2Cl_2 washings were performed. The two crude products resulting from these treatments were analyzed by LC-MS. No

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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-CH_2Cl_2-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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24 A similar procedure which avoids the column chromatography purification and may be more convenient for industrial scale has also been developed: Z-L-Arg-OH (5.0 g, 16.2 mmol) was dissolved in acetone (160 mL) and 3 N aqueous NaOH (45 mL, 135 mmol). The reaction was cooled in an ice bath and compound **2** (3.85 g, 15.8 mmol) dissolved in acetone (100 mL) was added over 10 min. The reaction mixture was stirred for 1 h at 0 °C. Additional, **2** (1.9 g, 7.8 mmol) in acetone (50 mL) was then added followed by 90 min of stirring at 0 °C. Finally, a last portion of compound **2** (1.9 g, 7.8 mmol) in acetone (50 mL) was added and the reaction mixture was stirred at 0 °C for additional 30 min and at room temperature for 3 h. At this point, species **2** was no longer observed by HPLC. Acetone, was distilled out under vacuum and H_2O (100 mL) was added (pH = 14). Then EtOAc (150 mL) was added and the pH adjusted to 3.0 with 50% aqueous citric acid. The solution became 3 phases. The above organic phase as well as the underneath oil phase were separated. H_2O (30 mL) was added to the oil phase, and the pH adjusted to 10. To make the solution more homogeneous, EtOAc (50 mL) was added and the pH adjusted to 3.0. The EtOAc phase was separated and combined with the EtOAc phase from the first extraction. The combining solution was concentrated to 100 mL. H_2O (100 mL) was added, the pH was adjusted firstly to 10 and finally to 3.0. At this point, the aqueous phase was discharged. This process was repeated and the EtOAc solution was concentrated to give a light yellow solid (2.9 g, yield: 24.5%, HPLC purity: 70.6%).