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ORIGINAL ARTICLE

A rapid and efficient method for the synthesis of selectively S-Trt or S-Mmt protected Cys-containing peptides

Panagiotis Stathopoulos · Serafim Papas · Marianna Sakka · Andreas G. Tzakos · Vassilios Tsikaris

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Abstract Selective removal of protecting groups under different cleavage mechanisms could be an asset in peptide synthesis, since it provides the feasibility to incorporate different functional groups in similar reactive centres. However, selective protection/deprotection of orthogonal protecting groups in peptides is still challenging, especially for Cys-containing peptides, where protection of the cysteine side-chain is mandatory since the nucleophilic thiol can be otherwise alkylated, acylated or oxidized. Herein, we established a protocol for the synthesis of Cys-selective S-Trt or S-Mmt protected Cys-containing peptides, in a rapid way. This was achieved by, simply fine-tuning the carbocation scavenger in the final acidolytic release of the peptide from the solid support in the classic SPPS.

Keywords Solid phase peptide synthesis · Cysteine · S-Trt · S-Mmt · TFA cleavage

Introduction

Cysteine is an exceptional amino acid, which can orchestrate numerous functions (Amand et al. 2012; Bauhuber et al. 2009; Papas et al. 2007; Stathopoulos et al. 2013; Torres and Gait 2012). In cyclotides, cysteine allows the formation of remarkably stable cyclic cystine-knotted topology structures (Grundemann et al. 2013). Furthermore, it can undertake regulatory roles in protein structure and function, in the form of functional redox switches (Cremers and Jakob 2013). Understanding its implication in deadly diseases, like cancer, can allow the shaping of new therapies (Hogg 2013). Peptide drugs rich in cysteines, like Linaclotide that is consisted of 3 disulfide bridges, require delicate strategies for the selection of distinct thiol protecting groups, to achieve the correct folding (Góngora-Benítez et al. 2011). Owing to this multifactorial importance, novel acid-labile Cys-protecting groups are constantly produced (Gongora-Benitez et al. 2012b), as also enzyme-labile protecting groups are starting to show up (Gongora-Benitez et al. 2012a).

TFA-mediated deprotection of side-chain protecting groups of sensitive amino acids is a crucial step in the Solid Phase Peptide Synthesis (SPPS), which results in numerous side reactions (Fields and Fields 1993; Giraud et al. 1999; King et al. 1990; Lundt et al. 1978; Stathopoulos et al. 2006, 2013; Stierandova et al. 1994). Carbocations, generated from the acidolytic release of the peptide from the solid support, are good electrophiles and thus capable to react with nucleophilic side-chain functionalities (Karlstrom et al. 2000). The stability of these carbocations is dependent both on steric and conjugative effects (Ramos-Tomillero et al. 2013). To trap these reactive species, and prevent their side reactions with nucleophilic residues, such as Cys and Trp, appropriate nucleophilic bases, termed carbocation scavengers, are used in the cleavage mixture (King et al. 1990; McCurdy 1989; Sieber 1987).

The most commonly utilized scavenger in the Fmoc SPPS is triisopropylsilane (TIS), which reduces the produced carbocations to alkanes (Mehta et al. 1992; Pearson et al. 1989). On the other hand, two of the most classic Cys-protecting groups are the Monomethoxytrityl (Mmt) and the Trityl group (Trt) (Barlos et al. 1996; McCurdy 1989). Mmt

P. Stathopoulos · S. Papas · M. Sakka · A. G. Tzakos (✉) · V. Tsikaris (✉)
Section of Organic Chemistry and Biochemistry, Department of Chemistry, University of Ioannina, 45110 Ioanina, Greece
e-mail: agtzakos@gmail.com

V. Tsikaris
e-mail: btsikari@cc.uoi.gr

can be removed in diluted TFA (1 % TFA in DCM), and due to its considerably more acid-labile character than the S-Trityl protecting group, it can be selectively removed in the presence of tBu, or even Trt-protecting groups. Using the classic acidolytic mixture (TFA/EDT/H_2O/TIS: 94/2.5/2.5/1; v/v/v/v) in S-Trt and/or S-Mmt Cys-protected peptides, both Mmt and Trt groups are effectively trapped by TIS, and the resulted peptide is free of protecting groups. A simple and rapid method is needed to selectively remove all amino acid protecting groups except those to cysteine residues. This method will allow for the creation of complex peptide architecture through control of disulfide bond formation (Góngora-Benítez et al. 2011; Gongora-Benitez et al. 2012b; McIntosh et al. 1994) after the deprotection of the other amino acid residues.

To achieve a rapid strategy for the synthesis of selective S-Trt or S-Mmt protected Cys-containing peptides that can be applied in the classic SPPS and to avoid tedious synthetic schemes for the development of novel cysteine-protecting groups, we explored the possibility to fine-tune the carbocation scavenger in the cleavage cocktail. Specifically, we focused on 1,3 dimethoxybenzene (DMB) which has been used in SPPS as a carbocation scavenger (Stathopoulos et al. 2006). Although, the DMB capacity to trap carbocations has been explored previously by us (Stathopoulos et al. 2006), its carbocation scavenging effect on sensitive amino acids, such as His and Cys, remained elusive. Herein, we explored the scavenging capacity of DMB in S-Trt and S-Mmt Cys-containing peptide models, and established a strategy to synthesize selectively Cys S-protected peptides independently of the location of Cys in the peptide sequence or the used polymeric resin support.

Materials and methods

Reagents

Fmoc amino acid derivatives, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-Hydroxybenzo-triazole (HOBt) were purchased from Neosystem Laboratoire (Strasbourg, France). 4-(2′,4′-Dimethoxy phenyl-Fmoc-aminomethyl)-phenoxy-linked polystyrene (Rink amide) and 4-(hydroxymethyl) phenoxy-methyl-linked polystyrene (Wang) resins were obtained from GL Biochem (Shanghai), while triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), *N*,*N*-diisopropyl ethyl amine (DIEA), trifluoroacetic acid (TFA), 1,3-dimethoxybenzene (DMB) and piperidine were Merck-Schuchardt (Darmstadt, Germany) products and used without further purification. *N*,*N*-Dimethylformamide (DMF) distillated over ninhydrin and stored under pre-activated molecular sieves 4E, dichloromethane and the gradient degree high performance liquid chromatography (HPLC) solvents acetonitrile and methanol were purchased from Labscan (Dublin, Ireland).

Peptide synthesis

All peptides presented in this work were synthesized manually, with standard Fmoc a-protection (Fields and Noble 1990) either on Rink Amide or Wang resin. Amino acids were introduced protected as Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH and Fmoc-Cys(Mmt)-OH (pbf: 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl, tBu: *tert*-butyl, Boc: *tert*-butyloxycarbonyl, OtBu: *tert*-butoxy). Fmoc deprotection steps were carried out with 20 % piperidine in DMF (v/v) for 15 min. Coupling reactions of Fmoc amino acids were performed in DMF, using a molar ratio of amino acid/HBTU/HOBt/DIEA/resin (3:3:3:6:1). Reactions were monitored with the color Kaiser test (Sarin et al. 1981). After, the dry peptide resin was placed into a rotating reaction vessel and the tested cleavage mixture TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v/v/v) or TFA/DMB (95/5; v/v) was added in a ratio of 20 mL g^{-1} peptide resin. After 3 h stirring, the resin was filtered and washed with TFA. The combined filtrates were concentrated under reduced pressure. Hexane was added and the resulted solution was reconcentrated. This procedure was performed twice. The peptide was precipitated with cold diethyl ether, filtered, dissolved in 2N acetic acid, and lyophilized.

LC/electrospray mass spectroscopy (ESI-MS) analysis

All LC/MS experiments were performed on a quadrupole ion-trap mass analyzer (Agilent Technologies, model MSD trap SL) retrofitted to a 1,100 binary HPLC system equipped with a degasser, autosampler, diode array detector and electrospray ionization source (Agilent Technologies, Karlsruhe, Germany). All hardware components were controlled by Agilent Chemstation Software.

The sample was dissolved in a mixture of H_2O/CH_3CN/0.1 % formic acid to the desired concentration. Separation was achieved on a reverse-phase 25 cm × 4.6 mm C18 analytical column, at a flow rate of 0.5 mL min^{-1}. Gradient elution was performed with the following solvents: A, H_2O/0.1 % formic acid and B, CH_3CN/0.1 % formic acid from 90 to 50 A for 30 min. The UV/Vis spectra were recorded in the range of 200–400 nm, and chromatograms were acquired at 214 and 280 nm. The ionization source conditions were as follows: capillary voltage, 3.5 kV; drying gas temperature, 350 °C; trap drive, 40–70 depending on the peptide analysis; skimmer, 40 V; nitrogen flow and pressure, 12 L min^{-1} and 50 psi, respectively. Maximum accumulation time of ion trap and the

Table 1 S-Trt or S-Mmt-protected peptide formation is depended on cleavage mixture conditions

Entry	Peptide analogues	Cleavage mixture (3 h)	Trt/Mmt-relative product
1	His(**Trt**)-Asn(**Trt**)-Asn(**Trt**)-Pro-Leu-Tyr(tBu)-Lys(Boc)-Glu(OtBu)-Ala-Rink Amide	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
2	His(**Trt**)-Asn(**Trt**)-Asn(**Trt**)-Pro-Leu-Tyr(tBu)-Lys(Boc)-Glu(OtBu)-Ala-Rink Amide	95 % TFA, 5 % DMB	–
3	Cys(**Trt**)-His(**Trt**)-Asn(**Trt**)-Asn(**Trt**)-Pro-Leu-Tyr(tBu)-Lys(Boc)-Glu(OtBu)-Ala-Rink Amide	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
4	Cys(**Trt**)-His(**Trt**)-Asn(**Trt**)-Asn(**Trt**)-Pro-Leu-Tyr(tBu)-Lys(Boc)-Glu(OtBu)-Ala-Rink Amide	95 % TFA, 5 % DMB	+
5	Ac-Ala-Arg(Pbf)-Cys(**Trt**)-Wang	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
6	Ac-Ala-Arg(Pbf)-Cys(**Trt**)-Wang	95 % TFA, 5 % DMB	+
7	Ac-Cys(**Trt**)-Arg(Pbf)-Ala-Wang	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
8	Ac-Cys(**Trt**)-Arg(Pbf)-Ala-Wang	95 % TFA, 5 % DMB	+
9	Ac-Cys(**Mmt**)-Arg(Pbf)-Ala-Wang	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
10	Ac-Cys(**Mmt**)-Arg(Pbf)-Ala-Wang	95 % TFA, 5 % DMB	+
11	Ac-Cys(**Mmt**)-Gly-Arg(Pbf)-Cys(**Trt**)-Ala-Wang	94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS	–
12	Ac-Cys(**Mmt**)-Gly-Arg(Pbf)-Cys(**Trt**)-Ala-Wang	95 % TFA, 5 % DMB	+

number of MS repetitions, to obtain the MS average spectra, were set at 30 and 3 ms, respectively.

Results and discussion

Exploring the carbocation scavenging properties of DMB in the absence of Cys

The carbocation scavenging properties of DMB have been partially studied on our former work (Stathopoulos et al. 2006). This work illustrated that DMB (when used in TFA/DMB 95/5; v/v) is able to trap Trt carbocations derived from Asn, tBu from Tyr, Ser, and OtBu from Glu, and Asp, as also Boc from Lys and Pbf from Arg. However, the effect of DMB as a carbocation scavenger in His and Cys-containing peptides remained unexplored.

To probe the efficacy of DMB to scavenge Trt carbocations not only from Asn, but also from His, we synthesized the His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide (entries **1**, **2** in Table 1). Using TFA/DMB (95/5; v/v) as the cleavage cocktail, an effective scavenging ability was observed for all resulted carbocations, even in the presence of His (Fig. 1). Thus, in the absence of Cys, DMB is an efficient scavenging agent that is able to block the effect of most carbocations resulted during the final acidic cleavage step.

Monitoring the carbocation scavenging capacity of DMB in the presence of Cys

To investigate the efficacy of DMB to selectively leave S-Trt protecting groups in Cys-containing peptides, we introduced a cysteine at the N-terminus of the His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide (Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2, see entries **3** and **4** in Table 1). Using the TFA/DMB (95/5; v/v) cleavage cocktail we observed, in the analytical RP-HPLC, in addition to the desired product (eluted at 6.5 min) the appearance of an additional peak with higher absorbance and longer retention time (eluted at 17 min) (Fig. 2). MS analysis of this peak uncovered the presence of two products. The first peak corresponded to the mass of the desired peptide (molecular ions $[M + 2H]^{2+} = 542.7$ and $[M + H]^{+} = 1084.4$) and the second peak to the mass of the desired peptide plus 242.0 amu (molecular ions $[M + 2H]^{2+} = 715.2$ and $[M + H]^{+} = 1429.4$). Since the molecular ion at *m/z* 242.9 could correspond to the Trt-H molecule produced under the ESI-MS conditions (high temperature and electric field intensity), we hypothesized that the second observed product could be due to a covalent coupling of the Trt group to the peptide. To further evaluate this hypothesis, the Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide resulted after its treatment with TFA/DMB (95/5; v/v), precipitation with cold diethyl ether, acidification with 2 N acetic acid and lyophilization was further incubated for 3 h in TFA/H_2O/TIS (95/2,5/2,5; v/v/v). Interestingly, LC-MS analysis of the resulted product illustrated an enhancement of the peak at 6.5 min, which corresponded to the desired (S-Trt free) peptide, and a complete suppression of the peak that had originally appeared at 17 min.

Based on the observed difference between the LC-MS of the His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide

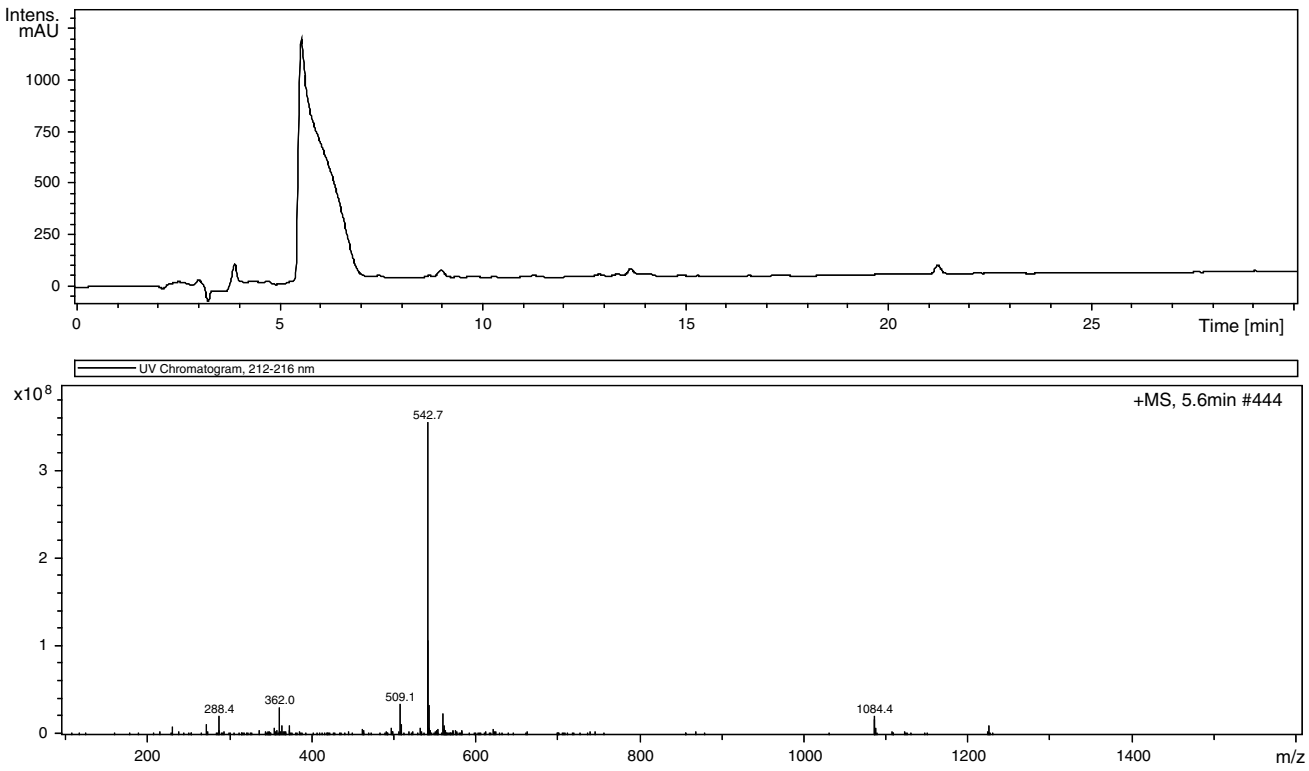

Fig. 1 LC/ESI-MS analysis of the crude His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide, using TFA/DMB (95/5; v/v) as the cleavage mixture. The mass spectrum is referred to the analytical RP-HPLC peak eluted at 5.6 min. The molecular ions $[M + 3H]^{3+} = 362.0$, $[M + 2H]^{2+} = 542.7$ and $[M + H]^{+} = 1084.4$ correspond to the MW of the desired His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptide. The asymmetric shape of the RP-HPLC peak is due to proline-mediated *cis-trans* isomerization phenomena

and the relevant Cys-containing analogue (Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2), we can conclude that in the DMB cleavage conditions (TFA/DMB 95/5; v/v), the Trt group either remains coupled or it is recaptured by Cys. This can be further confirmed, since upon the addition of TIS in the cleavage cocktail (TFA/H_2O/TIS 95/2.5/2.5; v/v/v), which scavenges mainly trityl cations; the same RP-HPLC profile is obtained both for the His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 and Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 peptides. This observation is additionally supported from the MS/MS analysis of the Ac-Cys(Trt)-Arg-Ala-OH peptide model that is reported in the next section.

DMB is a mild reagent that leaves S-Trt protecting groups in Cys-containing peptides independent of the Cys location in the peptide sequence or the used polymeric support

To explore the stability of trityl-protected cysteine residues in the presence of DMB, we synthesized model peptides with cysteine residues in different positions (N- and C-terminal). We first synthesized the Ac-Ala-Arg-Cys-OH peptide model (entries **5** and **6** in Table 1) in Wang resin in contrast to the former peptides (entries **1–4** in Table 1) that were synthesized on Rink amide resin. After the final acidolytic cleavage of the peptide from the solid support, using the classic TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v/v/v) cleavage cocktail, and RP-HPLC purification, no S-Trt related product was observed. In contrast, treatment of the Ac-Ala-Arg(Pbf)-Cys(Trt)-Wang peptide resin with the TFA/DMB (95/5; v/v) cleavage cocktail, resulted in a major product exhibiting an increased mass by 242.1 amu, with respect to the desired, free of S-protecting groups, peptide. In both cases, the fraction eluted at 10.6 min exhibited a UV absorbance at 280 nm, despite the fact that the studied peptide was depleted of aromatic residues. This corresponds to a *S-p*-hydroxybenzyl peptide side-product derived from the acidic Wang linker decomposition (Stathopoulos et al. 2013) (Fig. 3).

To evaluate whether the observed DMB effect is independent of the Cys location in the peptide sequence, the same cleavage conditions were repeated for the Ac-Cys(Trt)-Arg(Pbf)-Ala-Wang peptide resin that carries the Cys at its N-terminus (see entries **7** and **8** in Table 1). In accordance to the above results, after treatment of the Ac-Cys(Trt)-Arg(Pbf)-Ala-Wang peptide resin with the classic cleavage mixture TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v/v/

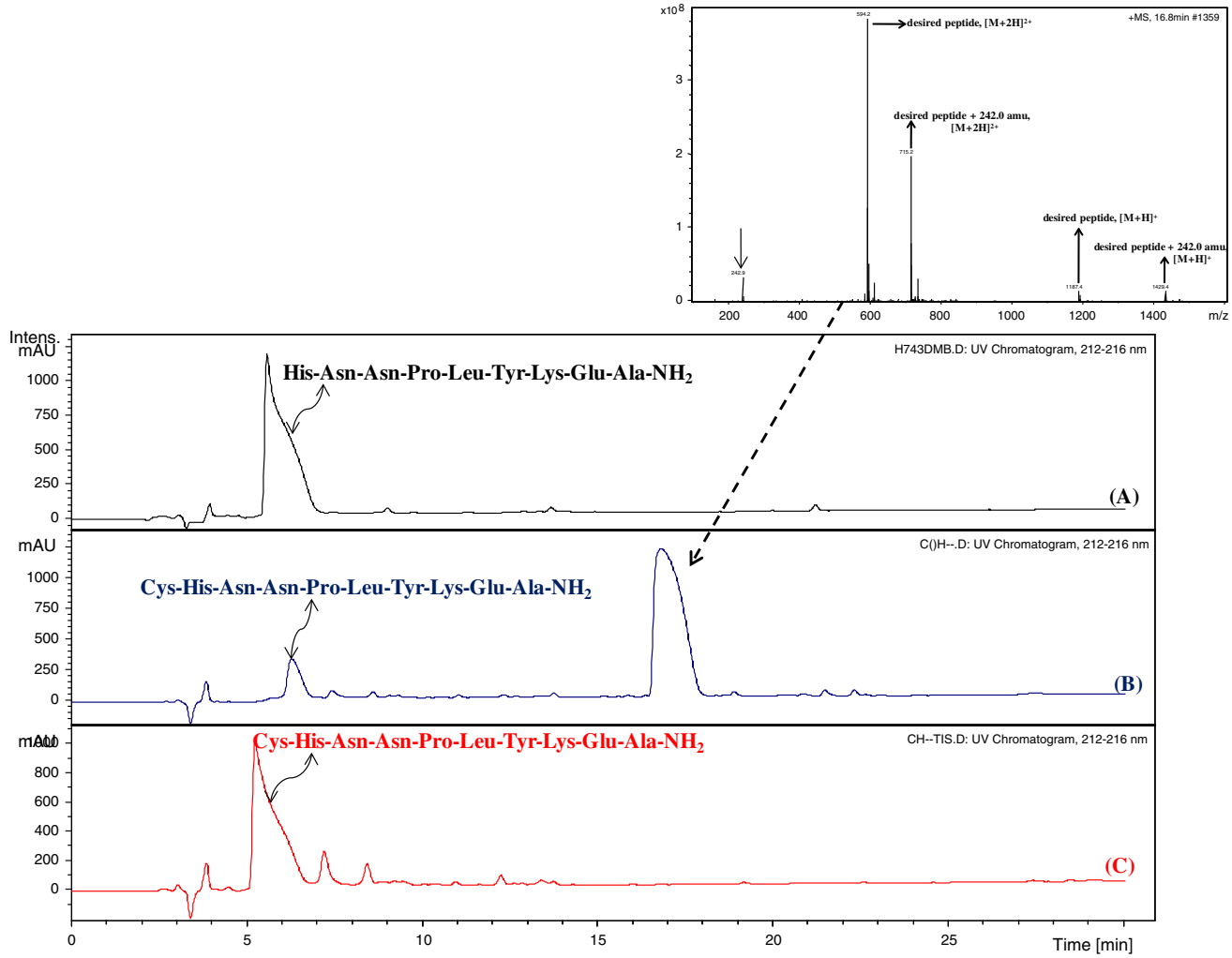

Fig. 2 Analytical RP-HPLC profiles at 214 nm of: **a** the His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 crude peptide, using TFA/DMB (95/5; v/v) as the cleavage cocktail (*colored in black*), **b** the Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 crude peptide, using TFA/DMB (95/5; v/v) as the cleavage cocktail (*colored in blue*); the ESI-mass spectrum is referring to the analytical RP-HPLC peak eluted at 5.6 min and **c** the Cys-His-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2 crude peptide, using TFA/DMB (95/5; v/v) as the primary cleavage mixture and after a further treatment with TFA/H_2O/TIS (95/2.5/2.5; v/v/v) for 3 h (illustrated in *red color*). The asymmetric shape of the RP-HPLC peak is due to proline-mediated *cis-trans* isomerization phenomena (color figure online)

v), no Trt-related product is observed. In contrast, using the TFA/DMB (95/5; v/v) cleavage cocktail, the major product (with retention time 26.5 min) corresponds to the desired peptide plus the molecular mass of the trityl group. In both cases, the fraction eluted at 11.4 min, corresponds to the *S-p*-hydroxybenzyl peptide side-product derived from the acidic Wang linker decomposition, as mentioned previously (Fig. 4).

The incorporation of the S-Trt group in the specific peptide sequence (Ac-Cys(Trt)-Arg-Ala-OH) was further confirmed by MS/MS analysis of the RP-HPLC isolated fraction at 26.5 min. The MS/MS analysis of the 633.1 molecular ion illustrated in the mass spectrum a single peak at *m/z* 242.9, which corresponds to the mass of the Trt-H molecule (Fig. 5).

These data indicate that for S-Trt protected Cys-containing peptides, utilization of DMB (in contrast to TIS) in the cleavage cocktail does not remove the acid-labile Trt groups. Thus, S-Trt protected Cys-peptides can be synthesized independently of the Cys position in the peptide sequence or the used polymer resin support (Wang and Rink Amide resins).

DMB-mediated cleavage leaves S-Mmt protecting groups in Cys-containing peptides

We next synthesized an S-Mmt Cys-containing peptide model, to monitor whether DMB is also able to leave unaltered the Mmt Cys-protecting group. To this, the Ac-Cys(Mmt)-Arg(Pbf)-Ala-Wang peptide resin (entries **9** and

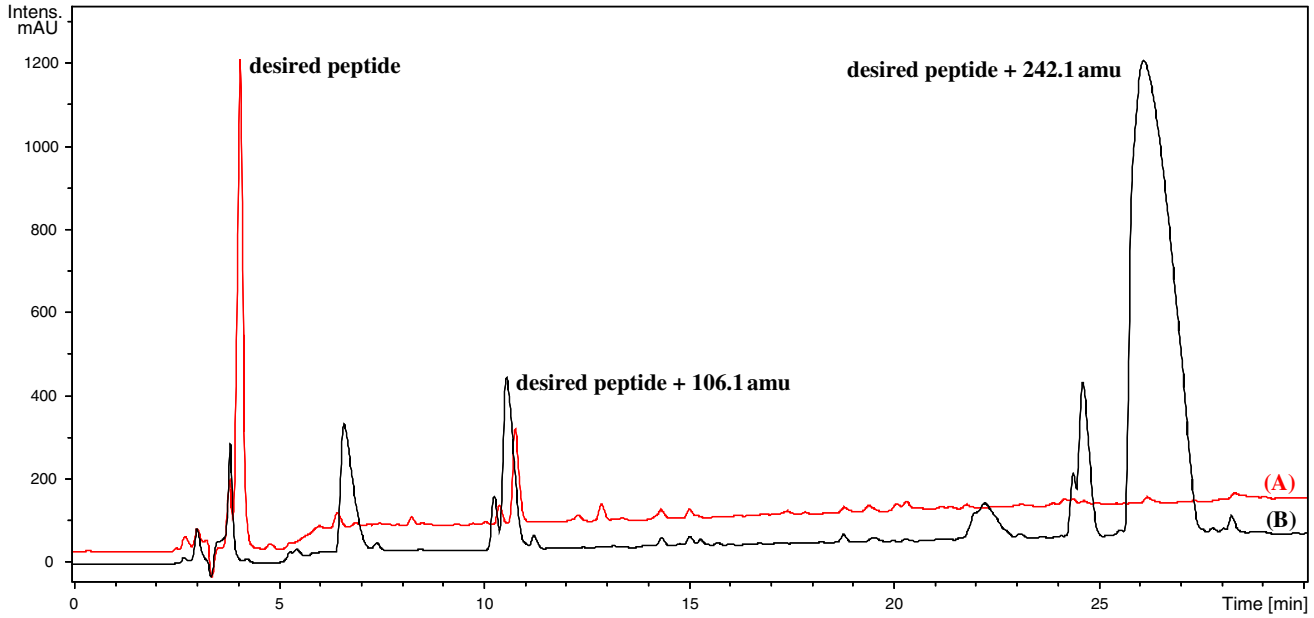

Fig. 3 LC/ESI-MS analysis of the crude Ac-Ala-Arg-Cys-OH peptide model carrying S-Trt protected Cys and using the cleavage mixture: **a** TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v/v/v) (*colored in red*) and **b** TFA/DMB (95/5; v/v) (*colored in black*) (color figure online)

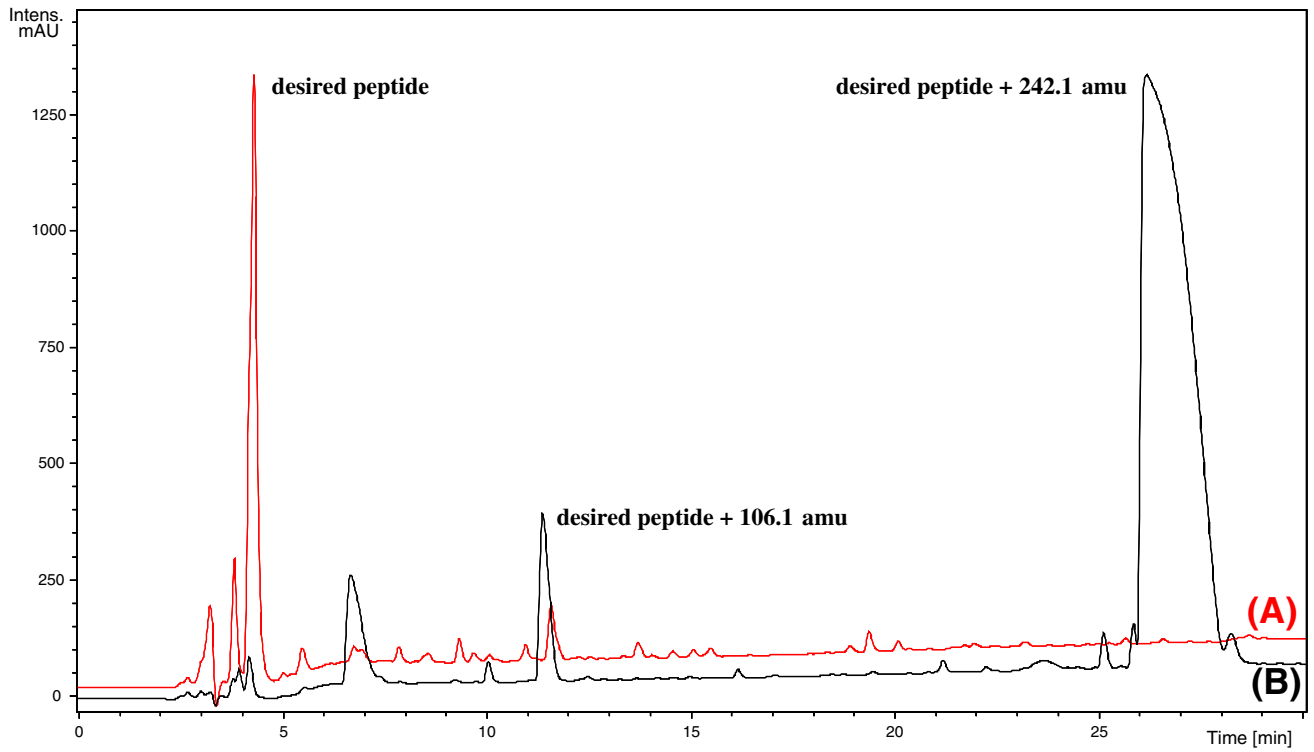

Fig. 4 LC/ESI-MS analysis of the crude Ac-Cys-Arg-Ala-OH peptide model using S-Trt protected Cys and as cleavage mixture: **a** TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v) (*colored in red*) and **b** TFA/DMB (95/5; v/v) (*colored in black*) (color figure online)

10 in Table 1) was synthesized carrying Mmt as the Cys-protecting group. The cation scavenging capacity for the Mmt carbocations was studied using both the classic TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v) and TFA/DMB (95/5; v/v) cleavage cocktails. LC-MS analysis of the cleavage products in the presence of TIS, revealed the absence of Mmt-related products. In contrast, in the DMB mediated cleavage, the major product (eluted at 26.5 min) exhibited an increased

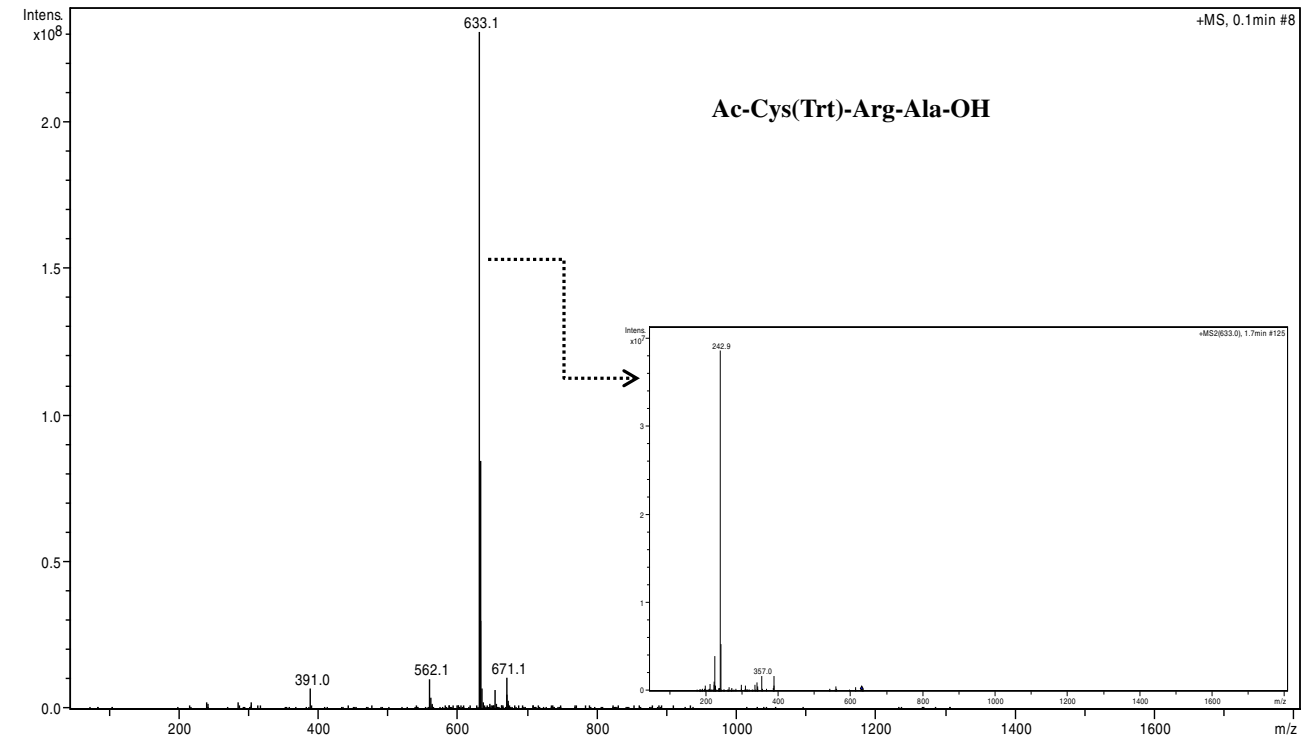

Fig. 5 ESI-MS/MS analysis of the RP-HPLC isolated Ac-Cys(Trt)-Arg-Ala-OH peptide

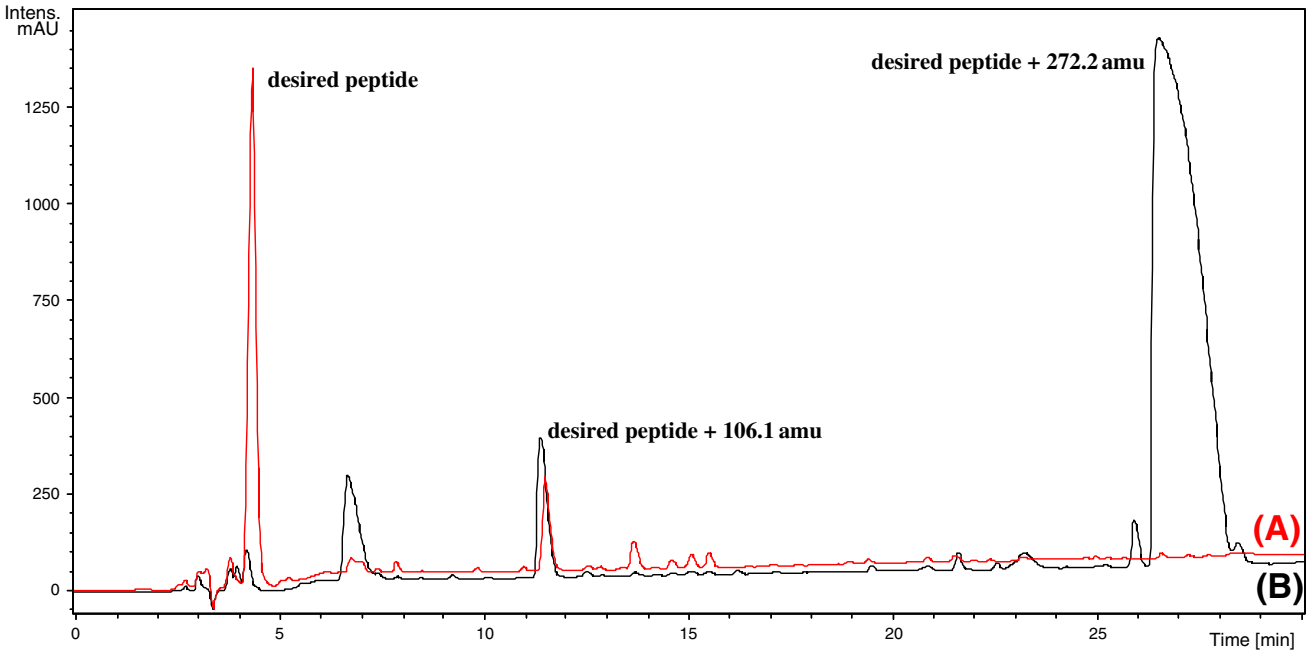

Fig. 6 LC/ESI-MS analysis of crude Ac-Cys-Arg-Ala-OH peptide model using S-Mmt protected Cys and cleavage mixtures: **a** TFA/EDT/H_2O/TIS (94/2.5/2.5/1; v/v/v/v) (*colored in red*) and **b** TFA/DMB (95/5; v/v) (*colored in black*) (color figure online)

mass by 272.2 amu, with respect to the target peptide, indicating the presence of S-Mmt on the cys-containing peptide. In both cases, the fraction eluted at 11.4 min, in the analytical RP-HPLC chromatogram, corresponded to an *S-p*-hydroxybenzyl peptide analogue derived from the acidic Wang linker decomposition (Fig. 6).

To further probe the existence of S-Mmt in this peptide, the fraction eluted at 26.5 min was isolated with RP-HPLC and

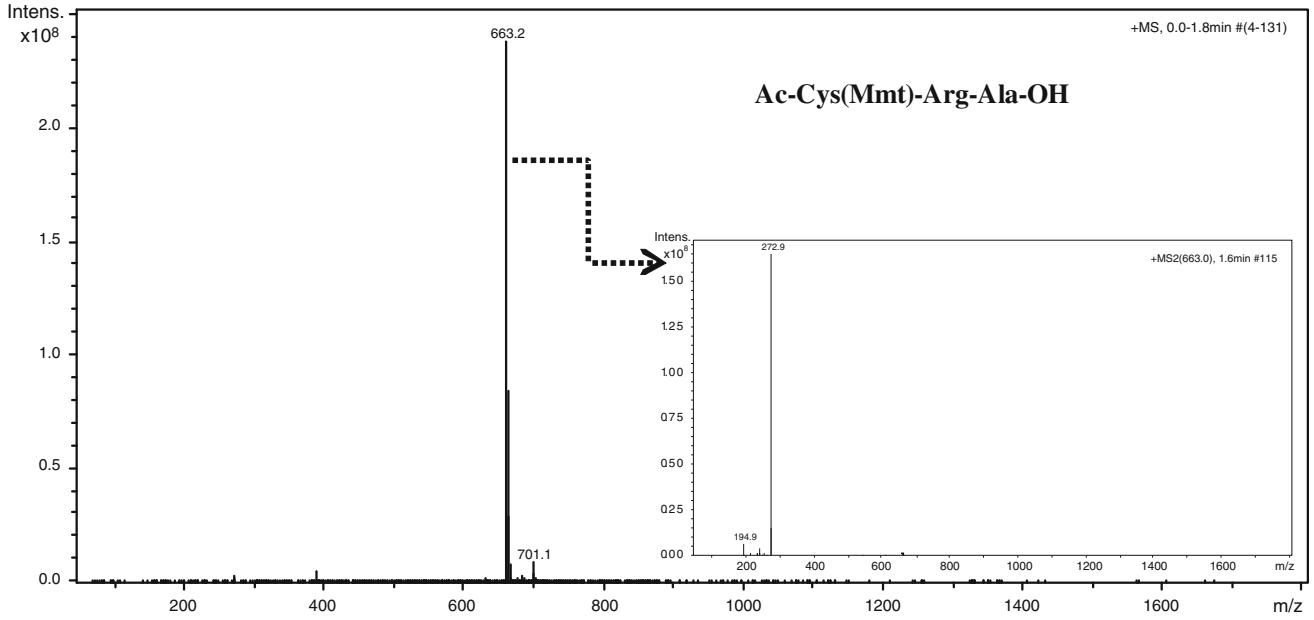

Fig. 7 ESI-MS/MS analysis of the isolated from RP-HPLC Ac-Cys(Mmt)-Arg-Ala-OH peptide analogue

analysed with MS/MS. MS/MS analysis of the 663.2 molecular ion illustrated a single peak at *m/z* 272.9, which corresponds to the mass of the Mmt-H molecule (Fig. 7). This observation further indicates the incorporation of S-Mmt in the peptide sequence (Ac-Cys(Mmt)-Arg-Ala-OH).

Exploring the mechanism of DMB in Trt and Mmt protecting group retention

Two different possible mechanisms could exist for the observed retention of the Trt/Mmt protecting groups on the Cys side chain upon utilization of the TFA/DMB (95/5; v/v) cleavage solution. Either the Trt/Mmt protecting group is not released or it is released but subsequently recaptured because the cysteine sulfhydryl group is a better scavenger than DMB. To assess these hypotheses, the Ac-Cys-Gly-Arg-Cys-Ala-OH peptide was synthesized incorporating two Cys with different orthogonal protecting groups (entries **11, 12** in Table 1). The Cys closer to the N-terminus was S-Mmt protected, and the Cys closer to the C-terminus was S-Trt protected. The Ac-Cys(Mmt)-Gly-Arg(Pbf)-Cys(Trt)-Ala-Wang peptide resin was treated with TFA/DMB (95/5; v/v) cleavage solution. If the mechanism responsible for the observed phenomenon is due to the higher affinity of the Trt/Mmt cations for Cys than DMB, we should expect, after the acidolytic treatment, the formation of a pool of different combinations for the Cys-protecting groups. Since, upon the acidic cleavage, both Cys residues will be available for carbocation attack, and since the mass of Mmt and Trt is different, we should expect to retrieve in the ESI-MS analysis of the crude peptide, every potential combination. These could range between the mass of the Ac-Cys-Gly-Arg-Cys-Ala-OH peptide and the variants carrying the S-Trt and/or S-Mmt protecting groups. Indeed, in the ESI-Mass Spectrum of the crude Ac-Cys-Gly-Arg-Cys-Ala-OH peptide, we observed three different mass combinations, corresponding to three different products: the MW of the peptide analogue plus 2 Mmt, the MW of the peptide analogue plus 2 Trt, and the MW of the peptide analogue plus 1 Mmt and 1 Trt, respectively (Fig. 8). This observation corroborate to the situation that Trt and Mmt are cleaved under the TFA cleavage conditions, though, DMB is a less effective scavenger for Trt and Mmt with respect to the thiol group of Cys. Thus, the resulted Trt and Mmt carbocations existing in the solution are recaptured by Cys.

Conclusions

In conclusion, we established a protocol for the selective synthesis of Cys-only S-Trt and/or S-Mmt protected Cys-containing peptides. This methodology is applicable in different polymeric resin supports and is independent of the location of Cys in the peptide sequence. It is simple, since it can be readily applied using commonly available Cys-protecting groups (Trt, Mmt); it does not require any tedious synthesis of novel Cys-protecting groups, or special modifications of the classic SPPS. This can be achieved by simply modifying, in the final acidolytic step, the strong carbocation scavengers, as TIS, by DMB. We illustrated that Trt/Mmt Cys-protecting groups, although are cleaved under the TFA cleavage conditions, the resulted carbocations are trapped

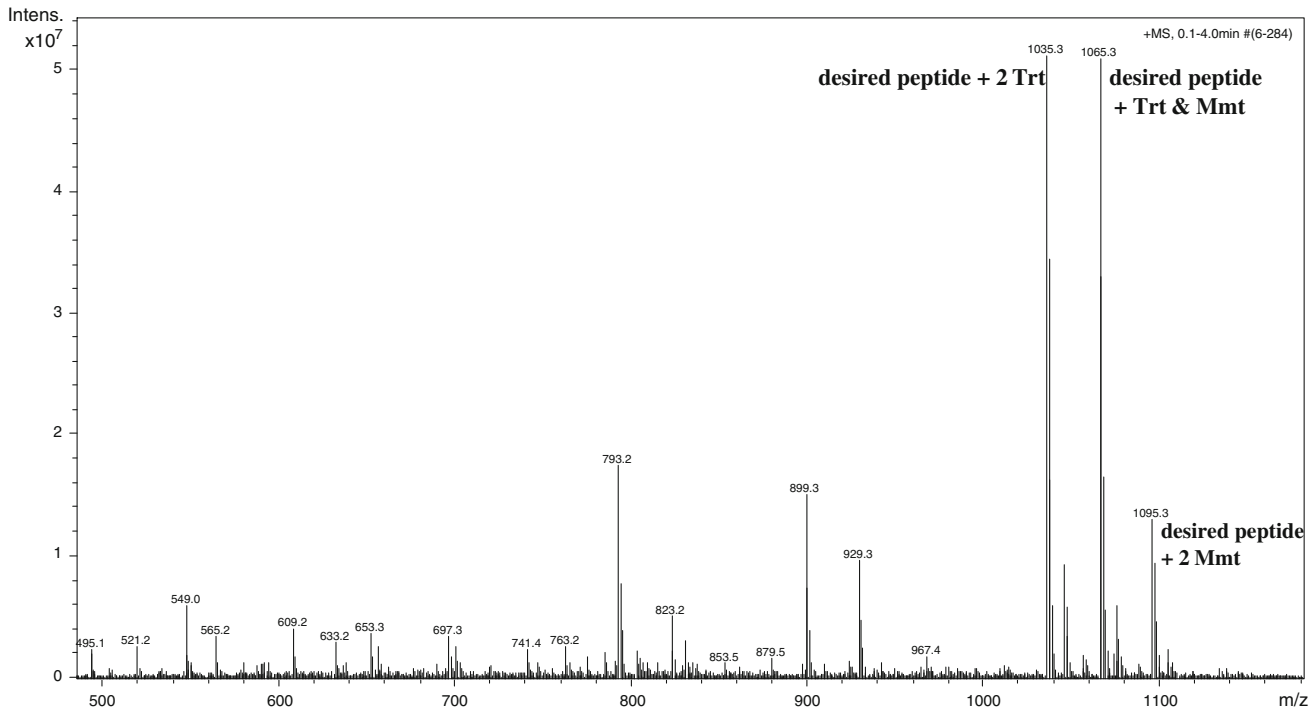

Fig. 8 ESI-Mass Spectrum analysis of Ac-Cys-Gly-Arg-Cys-Ala-OH peptide analogue. The molecular ions 1035.3, 1065.3 and 1095.3 correspond to $[M + H]^+$ of the desired peptide analogue plus 2 Trt group, plus 1 Trt and 1 Mmt group, and plus 2 Mmt groups, respectively

back to Cys, since DMB is a less effective Trt or Mmt cation scavenger than Cys. This synthetic strategy can pave the way towards the synthesis of biologically important peptides that are composed of complex Cys-related architectures, or allow the feasibility to load, in an orthogonal way, different biological cargoes (proteins, drugs etc.) in reactive centers of peptide vehicles, even in the presence of Cys.

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Conflict of interest The authors declare that they have no conflict of interest.

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