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ORIGINAL ARTICLE

Side reactions in the SPPS of Cys-containing peptides

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Abstract Alkylation of sensitive amino acids during synthesis of biologically important peptides is a common and well-documented problem in Fmoc-based strategy. Herein, we probed for the first time an unexpected S-alkylation of Cys-containing peptides that occur during the final TFA cleavage of peptides from the Wang solid support. Through a battery of approaches (NMR, UV and LC–MS) the formed by-product was assigned as the alkylation of the cysteine sulfydryl group by the *p*-hydroxyl benzyl group derived from the acidic Wang linker decomposition. Factors affecting this side reaction were monitored and a protocol that minimizes the presence of the by-product is reported.

Keywords Solid phase peptide synthesis · Wang resin decomposition · Cysteine · S-alkylation · TFA cleavage

Introduction

Decomposition of the resin linkers upon the TFA cleavage of the peptides in the Fmoc strategy is a common and well-documented phenomenon. This was observed to be the case both for the Rink amide resin (Fig. 1a), resulting in C-terminal alkylated amide by-products (Stathopoulos et al. 2006), as well as for the Wang resin (Fig. 1b), resulting in side chain alkylation of indole ring of Trp-containing peptides (Giraud et al. 1999; Mutulis et al. 2003; Atherton et al. 1988).

To our knowledge, such by-products have not been reported for cysteine-containing peptides, although they are prone to oxidation and alkylation by cations produced during the cleavage process. Cys-containing peptides are very important biomolecules due to the inherent reactivity of the thiol group, allowing them to participate in an array of processes ranging from redox reactions, metal ion binding, post-translational modifications, disulfide and thioether bond formation, to name some (Bauhuber et al. 2009; Papas et al. 2007; Torres and Gait 2012; Mand et al. 2012; Mann et al. 2010; Jancsó et al. 2011). In our laboratory, using the Wang resin for the synthesis of various Cys-containing peptides with the Fmoc strategy, we observed both the expected peptide and formation of a by-product (Fig. 2), similar to the case of Trp-containing peptides (Fig. 1b). The percentage of this formed by-product varied up to 35 % (data not shown).

This finding prompted us to explore the nature of this by-product, as also the influence of the position of Cys on the peptide sequence. A suggested mechanism for such by-product formation is also provided. The possibility of minimizing its formation, by changing resin substitution and composition of the cleavage mixture was explored.

Materials and methods

Reagents

Fmoc amino acid derivatives, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-Hydroxybenzotriazole (HOBt) were purchased from Neosystem Laboratoire (Strasbourg, France).

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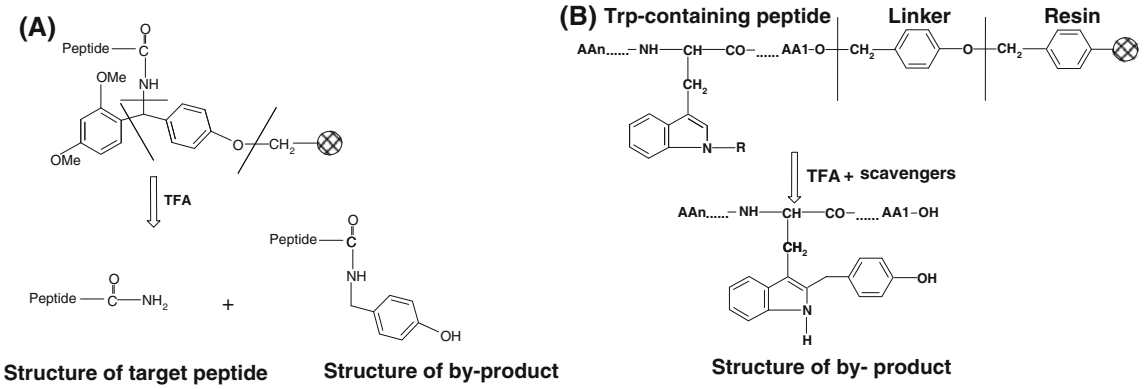

Fig. 1 Possible by-product formation in Rink Amide **a** and Wang **b** resins

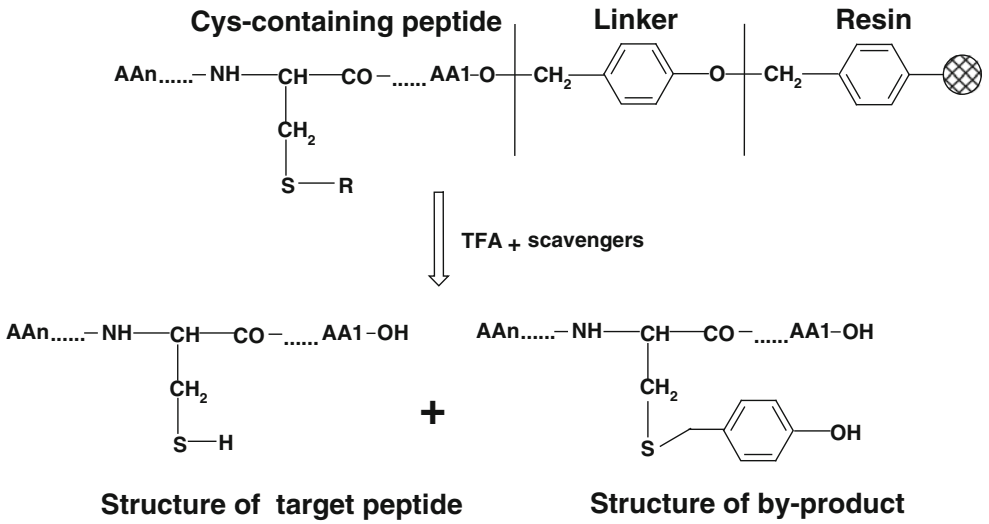

Fig. 2 TFA cleavage of a Cys-containing peptide from the Wang resin

4-(Hydroxymethyl) phenoxymethyl-linked polystyrene (Wang) resin and Fmoc-L-Ala-Wang resin were obtained from GL Biochem (Shanghai, China) and Neosystem Laboratoire (Strasbourg, France) respectively. TIS, EDT, *N,N*-diisopropylethylamine (DIEA), 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 preactivated 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

The peptides shown in Table 1 were synthesized manually with standard Fmoc α-protection (Fields and Noble 1990) first introduced by (Carpino and Han 1970, 1972). Amino acids were introduced protected as Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH and Fmoc-Trp(Boc)-OH. 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).

Cleavage and deprotection

The typical cleavage protocol included the following: aliquots of the dry peptide resin were placed into a rotating reaction vessel and the tested cleavage mixture was added in a ratio of 20 ml/g 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

Table 1 Percentage of side products (SP) depending on the cysteine position, resin substitution, and cleavage mixtures

Entry	Peptide analogues	Substitution	Cleavage mixture (3 h)	SP (%)[a]
1	Ac-Ala-Arg(Pbf)-**Cys(Trt)**-Wang	0.70	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**16.4**
2	Ac-Ala-Arg(Pbf)-**Cys(Trt)**-Wang	0.70	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS and 10 eq L-Trp	**14.3**
3	Ac-Ala-Arg(Pbf)-**Cys(Trt)**-Wang	0.70	80 %TFA, 18 %EDT, 1 %TIS, 1 %H_2O	**5.0**
4	Ac-Ala-Arg(Pbf)-**Cys(Trt)**-Wang	0.32	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**10.8**
5	Ac-Ala-Arg(Pbf)-**Cys(Trt)**-Wang	0.32	95 %TFA, 2.5 %TIS, 2.5 %H_2O	**61.6**
6	Ac-Arg(Pbf)-**Cys(Trt)**-Ala-Wang	0.60	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**9.2**
7	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.79	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**4.3**
8	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.79	95 %TFA, 2.5 %TIS, 2.5 %H_2O	**21.3**
9	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**3.9**
10	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	89 %TFA, 2.5 %H2O,2.5 %EDT, 1 %TIS, 5 %DMB	**2.0**
11	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	92.5 %TFA, 2.5 %TIS, 5 %DMB	**25.7**
12	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	90 %TFA, 2.5 %TIS, 2.5 %H_2O, 5 %DMB	**18.5**
13	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	95 %TFA, 2.5 %TIS, 2.5 %H_2O	**44.1**
14	Ac-**Cys(Trt)**-Arg(Pbf)-Ala-Wang	0.40	95 %TFA, 2.5 %TIS, 2.5 %EDT	**3.3**
15	Ac-**Trp(Boc)**-Arg(Pbf)-Ala-Wang	0.40	94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS	**2.2**
16	Ac-**Trp(Boc)**-Arg(Pbf)-Ala-Wang	0.40	95 %TFA, 2.5 %H_2O, 2.5 %TIS	**15.1**

[a] The percentages represent the relative peak intensities estimated from the related ESI–MS spectra

precipitated with cold diethyl ether, filtered, dissolved in 2 N acetic acid, and lyophilized.

NMR spectroscopy

All the NMR spectra were recorded on a Bruker AV-500 spectrometer equipped with a cryoprobe. The NMR samples were prepared by dissolving the solid material in DMSO-d6 at a concentration of 5 mM. 2D ^{1}H-^{1}H COSY, 2D ^{1}H-^{1}H TOCSY (80 ms mixing time), 2D ^{1}H-^{1}H ROESY, 2D ^{1}H-^{1}H NOESY (300 ms mixing time) experiments were performed on the isolated S-alkylated by-product of the Ac-Ala-Arg-Cys-OH at DMSO-d_6 and 298 K using standard Bruker pulse sequences. Diffusion ordered NMR spectroscopy measurements were recorded at 292 K using the bipolar pulse longitudinal eddy current delay (BPPLED) pulse sequence. More specific, 16 BPPLED spectra with 16 K data points were collected and the eddy current delay was set to 5ms. The pulse gradient was increased from 2 to 95 % of the maximum gradient strength using a linear ramp. After Fourier transformation and baseline correction, the diffusion dimension was treated with the Topspin 2.1 suite.

Electrospray mass spectroscopy (ESI–MS)

Electrospray mass spectra were obtained on a quadrupole ion-trap mass spectrometer (Agilent Technologies, model MSD trap SL). Samples were dissolved in the mixture $H_2O/CH_3CN/HCOOH$ (49:49:2) and injected into the ESI source (Agilent Technologies, Karlsruhe, Germany) at a flow rate of 6 µl/min. The ionization source conditions were as follows: capillary voltage, 3.5 kV; drying gas temperature, 325 °C; trap drive 50; skimmer, 40 V; nitrogen flow and pressure, 5 L min^{-1} and 15 psi, respectively. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 30 and 3 ms, respectively. All hardware components were controlled by Agilent Chemstation Software.

Results and discussion

Identification of by-product formation in a Cys-containing peptide model

To probe the nature of the Cys-containing by-products we synthesized the cysteine-containing peptide model Ac-Ala-Arg-Cys-OH. The synthesis occurred on Wang resin using the Fmoc based methodology. After the final TFA cleavage of peptide from the solid support using the standard cleavage mixture (94 % TFA, 2.5 % EDT, 2.5 % H_2O, 1 % TIS), and HPLC purification we isolated both the desired peptide and a by-product, which exhibited an increased mass by 106 Amu with respect to the target peptide (Figure S2). Moreover, this by-product exhibited an absorbance at 280 nm in the UV detector, despite the fact that the studied peptide sequence was depleted of aromatic residues (Fig. 3).

Using ESI–MS we found that the relative percentage of by-product (SP(%)) was ~16.4 (Table 1). To unambiguously determine the identity of the formed by-product we

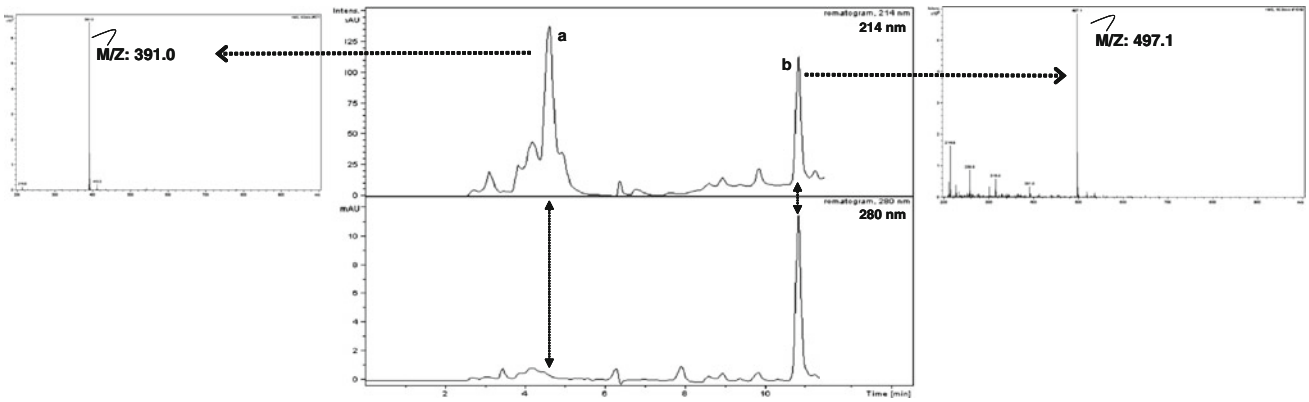

Fig. 3 ESI-MS spectra and HPLC profiles at 214 and 280 nm of the crude Ac-Ala-Arg-Cys-OH peptide (1) synthesized on the Wang resin. The numbers in the graph denote: the desired peptide (**a**) and the by-product with an increased weight of 106 Amu compared to the target molecule (**b**)

used NMR spectroscopy (Fig. 4). On the basis of the recorded ^{1}H-NMR spectrum a *p*-hydroxy benzyl group was found to be covalently attached on the thiol-group of Cys (Fig. 4), similarly to the case of Trp-containing peptides (Figs. 1b, 2). By lowering the temperature (from 298 to 292 K) a minor conformer could be observed (Figure S9). Diffusion-ordered spectroscopy (DOSY) is a sensitive NMR technique that allows the determination of self-diffusion coefficients and provides information about the given molecular species like shape, weight, size and assists the determination and detection of intermolecular interactions and conformational changes (Kontogianni et al. 2013; Primikyri et al. 2012). From the recorded DOSY spectrum (Figure S8 and +Figure S9) we found that the minor conformer presented the same diffusion coefficient to the major conformer. The minor conformer could have resulted possibly due to a cation-π interaction formed between the side-chain of arginine and the p-hydroxy benzyl ring. The accessibility of Cys containing peptides for alkylation was further verified by the synthesis of the Ac-Ala-Arg-OH peptide that did not resulted in any by-product formation (Figure S1).

Influence of cysteine position on the S-alkylated by-product formation

To evaluate the influence of cysteine position on the by-product formation we synthesized and studied the peptide variants Ac-Arg-Cys-Ala-OH and Ac-Cys-Arg-Ala-OH, altering the position of cysteine from the C- to the N-terminal end of the initial peptide model (Ac-Ala-Arg-Cys-OH). The experimental conditions used for the synthesis of the peptide analogues Ac-Arg-Cys-Ala-OH and Ac-Cys-Arg-Ala-OH were similar with those of the peptide model (Ac-Ala-Arg-Cys-OH). As showed in Table 1, alkylation of Cys-containing peptides with the p-hydroxy benzyl group seems to be sensitive to the position of Cys in the peptide sequence. Specifically, the highest tendency of by-product formation was observed when the cysteine residue occupied the C-terminus of the peptide (entries 1, 4 and 5).

Influence of the Wang resin substitution on the S-alkylated by-product formation

To probe the influence of the resin substitution on the by-product formation, cleavage experiments were performed for peptide analogues Ac-Ala-Arg-Cys-OH and Ac-Cys-Arg-Ala-OH, having cysteine residue at the C- and the N-terminus respectively,. In these experiments the cleavage mixture was kept constant (94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS), whereas the Wang resin substitution was varied. As illustrated in Table 1, when the resin substitution was increased, the yield of the S-alkylated by-product formation was also increased (entries 1, 4 and 7, 9 respectively).

Influence of the cleavage mixtures on the S-alkylated by-product formation

Evaluation of the cleavage conditions that could minimize the percentage of the S-alkylated by-product was estimated by using various cleavage mixtures. Table 1 summarized some of the results obtained from these experiments. From entries 9–14 of Table 1 we conclude that the presence of EDT in the cleavage mixture is crucial for suppression of S-alkylated by-product formation, while DMB and H_2O don't prevent significantly cysteine alkylation. The reduction of the by-product formation by EDT was confirmed both for higher resin substitution (entries 7, 8) as also for

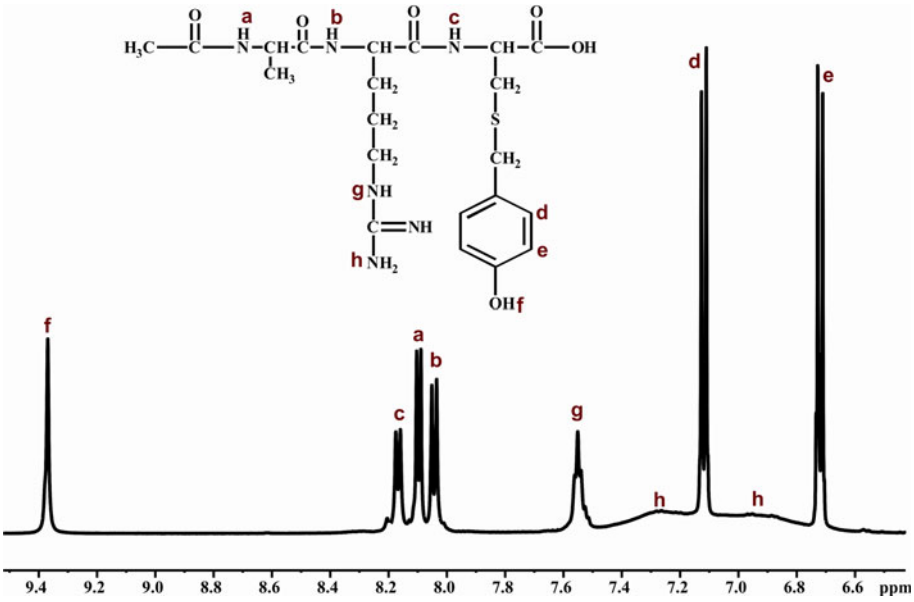

Fig. 4 Selected region of the 500 MHz ^{1}H-NMR spectrum of the isolated S-alkylated by-product of the Ac-Ala-Arg-Cys-OH at DMSO-d_6 at 298 K. The structure of the relevant compound is shown as inset and assignment of specific protons is illustrated on the spectrum. The full ^{1}H-NMR spectrum as also the 2D ^{1}H-^{1}H COSY, 2D ^{1}H-^{1}H TOCSY, 2D ^{1}H-^{1}H ROESY, 2D ^{1}H-^{1}H NOESY and DOSY NMR spectra can be found in Figures S3–S9

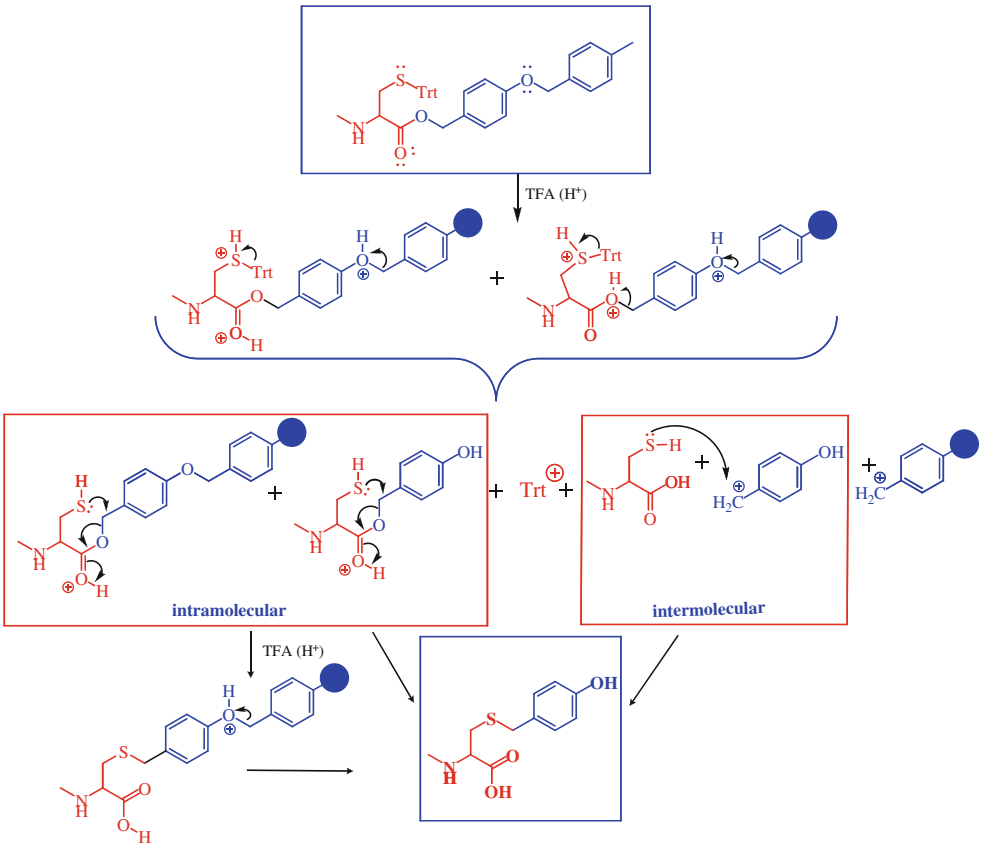

Fig. 5 Suggested intra-molecular and inter-molecular mechanisms for S-alkylated by-product formation, for the case that Cys is located in the C-terminus. A relevant inter-molecular mechanism could be suggested also for the case when Cys is not located on the C terminus (Figure S10). The Wang resin linker is illustrated in *blue color* and the peptide in *red color*. The polymer support is shown in blue sphere (color figure online)

different position of cysteine residue in the peptide sequence (entries 4, 5). These results emphasize the importance of EDT in the cleavage mixture towards cysteine alkylkation, that it is independent both on the location of cysteine, in the peptide sequence, as also on the resin substitution. A reduction of the byproduct formation to 14.3 % was also observed when in the cleavage mixture was added 10 eq. of L-Trp (94 %TFA, 2.5 %H_2O, 2.5 %EDT, 1 %TIS, 10 eq L-Trp), though the higher concentration of EDT provided better results (entries 2, 3).

Relevant reactivity of Cys- in respect to Trp-containing peptides for the alkylated by-product formation

To probe the relevant reactivity of Cys- in respect to Trp- containing peptides towards the alkylated by-product formation, the Ac-Cys(Trt)-Arg(Pbf)-Ala-Wang was compared to the Ac-Trp(Boc)-Arg(Pbf)-Ala-Wang peptide resin, using the same experimental cleavage conditions. Interestingly, the cysteine-containing peptide provided almost three times higher yield of the by-product in respect to the Trp-containing peptide, highlighting the higher sensitivity of cysteine in respect to the Trp residue (entries 13, 16). The beneficial role of EDT in the cleavage mixture was further confirmed also for the Trp-containing peptide. Absence of EDT from the cleavage mixture resulted in 15.1 % yield for the Trp alkylation in respect to 2.2 % obtained upon the presence of EDT (entries 15, 16).

Intermolecular versus intramolecular mechanism for S-alkylated by-product formation

As mentioned before, a higher tendency of S-alkylated by-product formation was observed when cysteine residue occupied the C terminus of the peptide sequence. Specifically, the relevant yields of the by-product for the Ac-Cys-Arg-Ala-OH, Ac-Arg-Cys-Ala-OH and Ac-Ala-Arg-Cys-OH peptides were determined as 4.3, 9.2 and 16.4 % respectively. Enhancement of the by-product abundance when cysteine was approaching the Wang linker triggered the hypothesis that an intramolecular mechanism could be postulated. Indeed, a closer spatial approximation of the thiol group of cysteine to the linker could be in favour of the performance of this side-reaction. To shed light on this hypothesis, and exclude the potential existence of an intermolecular mechanism, we incubated the purified peptides Ac-Ala-Arg-Cys-OH or Ac-Cys-Arg-Ala-OH with Fmoc-L-Ala-Wang in the presence of a standard cleavage mixture constituted of 94 %TFA, 2.5 %H_2O, 2.5 %EDT and 1 %TIS. After 3 h stirring, the TFA-scavengers solution was concentrated under reduced pressure and the peptide was precipitated with cold diethyl ether, filtered, dissolved in 2 N acetic acid, and lyophilized, as mentioned before. From ESI–MS spectra obtained for the two originally unmodified peptides Ac-Ala-Arg-Cys-OH or Ac-Cys-Arg-Ala-OH we detected the molecular ion both of the desired peptides (*M*/*Z*: 391) and of the corresponding S-alkylated side products (*M*/*Z*: 497). The percentage of these by-products were ~12 %. The above results indicate that a concomitant participation of both an intra- and an inter-molecular mechanism could co-exist in the formation of these by-products (Fig. 5).

Conclusions

Constructively, we exploited the potential formation of by-products for Cys containing peptides through Fmoc-based peptide synthesis on Wang solid support. The alkylation of Cys-containing peptides with the p-hydroxy benzyl group from the Wang resin linker decomposition was shown to be directly dependent of the position of Cys in the peptide sequence, resin substitution and cleavage mixture composition. The presence of EDT in the cleavage mixture is suitable for suppression of Cys alkylated peptides. Suggested intra- and inter-molecular mechanisms that could describe the formation and explain the relevant percentage of such side products are provided. It should be noted that although these observed Cys alkylated peptides can be thought of as a drawback in SPPS of Cys contained peptides in future it might be proved as a useful approach to sculpt ligands for novel drug targets (Janga and Tzakos 2009; Nagulapalli et al. 2012).

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

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