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Sequence-dependent modification of Trp by the Pmc protecting group of Arg during TFA deprotection

ALENA STIERANDOVÁ [1], NIKOLAI F. SEPETOV [1], GREGORY V. NIKIFOROVICH [2] and MICHAL LEBL [1]

[1] *Selectide Corporation, Department of Chemistry, Tucson, Arizona, USA,* [2] *Center for Molecular Design, Washington University, St. Louis, Missouri, USA*

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The extent of transfer of the Pmc protecting group from the guanidino group of arginine to the side chain of tryptophan depends on the spacial distance of these side chains. When these two amino acids are separated by one amino acid, the transfer of the Pmc protecting group is the most pronounced, and it cannot be completely prevented by the use of currently utilized scavenger mixtures. The extent of this side reaction also depends on the amino acid separating the arginine and tryptophan residues and position of tryptophan within the peptide chain as well as on the type of the solid-phase carrier. © Munksgaard 1994.

Key words: SPPS; side reactions; Pmc; Trp modification; scavenger mixtures; cotton; sequence dependence

One of the prerequisites for the successful application of drug discovery technologies based on synthetic peptide libraries is reliable synthesis of reasonably pure peptides in the mixtures. This requires on one hand complete coupling in every step of the synthesis and on the other hand complete deprotection of amino acid side chains at the end of the synthesis, without any consequent modifications of sensitive amino acid residues. Even though unintended side products may lead to the discovery of new biologically active peptides (1), analysis of millions of peptides in a peptide library for artifactual structures is not feasible. Therefore, in order to employ the synthetic peptide library approach in the peptide ligand discovery process, it was essential to first study the question of side reactions.

Side reactions in peptide chemistry have been investigated since the beginning of synthetic activity in this field. A number of side reactions were found to be a sequence-dependent phenomenon. As an example we can mention transpeptidation reactions of peptides with sequence Asp-Ser,† Asp-Gly (2), *N–O* shifts observed in peptides containing Ser (for a review see ref. 3), or rearrangements of the first two amino acid residues in peptides with Gly in position 3 (4, 5). However, side reactions involving protecting groups, which would be sequence-dependent, are very rare. One example of a side reaction involving a protecting group is the difficult deprotection of the trityl group in the case of an *N*-terminal asparagine (6).

Most of the groups used for the protection of amino acid side chains were designed for a particular synthetic strategy (Boc/Bzl, Fmoc/Bu^t). However, the protection of the side chain of arginine remains an unsolved challenge. The introduction of TFA-labile side-chain protection of arginine was an important improvement for the Fmoc strategy of SPPS. Commercially available and most commonly used derivatives of arginine for Fmoc synthesis are those containing 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) (7) or 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) (8) protecting groups. Since the Mtr protecting group on the guanidine function requires prolonged acidic treatment for its removal (several hours in TFA) (9), Pmc has become more popular among peptide chemists. Despite the advantage that relatively mild conditions and a short reaction time are all that is required to remove the Pmc group, the deprotection with TFA is accompanied by the same danger of modification of sensitive amino acids by highly reactive species as in the case of Mtr (10, 11). Most by-products have been found to be peptides in

† The one-letter notation of amino acid residues is used for description of peptide sequences. Derivatives of amino acids are represented by three-letter codes. The nomenclature and symbols obey the published recommendations (*Eur. J. Biochem.* **138**, 9, 1984). Other abbreviations used are as follows: AA, amino acid; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; FAB MS, fast atom bombardment mass spectroscopy; Fmoc, fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; RP HPLC, reversed-phase high-pressure liquid chromatography; SCAL, safety-catch amide linker; TFA, trifluoroacetic acid.

which the side chains of Tyr (12) or the indole ring of Trp has been modified (12–15), or Arg becomes sulfonated as a result of an incorrect cleavage of Pmc or Mtr (11).

We report that the relative sequence position of Trp and Arg, the cleavage conditions and the type of carrier may influence the extent of Trp modification which may occur when Pmc protecting group of Arg is cleaved during deprotection.

EXPERIMENTAL PROCEDURES

Materials

Fmoc protected amino acids used for the peptide synthesis were purchased from the following suppliers: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Tyr(Bu^t)-OH (Orpegen, Heidelberg, FRG), Fmoc-Trp-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Arg(Pmc)-OH (Propeptide, Vert-le-Petit, France) and D-amino acids (Advanced ChemTech, Louisville, KY). Protected amino acids were of the L-configuration unless stated otherwise. Supports used for the comparative synthesis were: Rink resin (16) (0.49 mmol/g; Advanced ChemTech, Louisville, KY), aminomethyl polystyrene resin (0.85 mmol/g; Rapp Polymere, Tubingen, FRG) and cotton strip (width 3 cm, substitution 0.14 mmol/g, VEB Bandtex Pulsnitz, FRG). The aminomethyl resin was modified with the SCAL (Safety-Catch Acid Labile) handle (17). The cotton strip, modified with Fmoc-Gly-OH via an ester bond (0.12 mmol/g) according to the described procedure (18), was coupled with 2-[(2′,4′-dimethoxy)-4-oxy-(9-fluorenylmethyloxycarbonyl)benzhydrylamino]acetic acid (19) (Bachem California, Torrance, CA). Further chemicals were obtained from the following companies and used without pretreatment: TFA (Halocarbon, North Augusta, SC), HOBt (Propeptide, Vert-le-Petit, France), DIC, piperidine (Aldrich, Milwaukee, WI).

Instrumentation

Small-scale solid-phase peptide syntheses were performed using a fully automatic peptide synthesizer, ACT 350 MPS (Advanced ChemTech, Louisville, KY). Fast atom bombardment (FAB) mass spectroscopy measurements were carried out on a ZAB EQ spectrometer (VG Analytical Ltd, Manchester, UK). ^{1}H NMR spectra were obtained on a General Electric QE 300 instrument. Sequencing by Edman degradation was performed on ABI 4778 protein sequencer (Applied Biosystems, Foster City, CA). Both analytical and preparative HPLC were carried out on a Waters 625 LC system with Waters 490E Programmable Multiwavelength Detector using a Vydac Peptide and Protein C18 analytical (0.46 × 250 mm, 5 μm, 1 mL/min) and preparative (10 × 250 mm, 10 μm, 3 mL/min) columns, respectively.

Synthesis and deprotection of sequences containing Trp and Pmc-protected Arg

Synthesis on resin. Syntheses were also carried out on two different types of resins using an automatic multiple peptide synthesizer model ACT 350 following the standard protocol (10 eq. excess of N^{α}-Fmoc amino acid/DIC/HOBt in DMF, 1 h coupling time, deprotection with 20% piperidine/DMF for 20 min). Supports used for the syntheses were Rink resin (40 mg/vessel; 0.47 mmol/g) and SCAL-modified TentaGel (50 mg/vessel; 0.34 mmol/g).

Synthesis on cotton. Sequences 1–5 were synthesized on five pieces of Gly-substituted cotton (0.2 g each; 0.14 mmol/g) modified with a TFA-labile handle (19). The synthesis was performed by Fmoc/Bu^t strategy in an arrangement between two glass plates (18). Each piece of the carrier was soaked with solutions of Fmoc-AA/DIC/HOBt (0.3 M) in DMF (0.5 mL) and allowed to react until bromophenol blue (BB) indicated the completed coupling (20). Piperidine/DMF (20%, 1 × 20 min) was used for deprotection.

Cleavage of peptides

Rink resin. Samples of peptide-resin (40 mg; 18.8 μmol) from the peptide synthesizer (*N*-terminal deprotected) were transferred into polypropylene syringes with Teflon frits, washed with MeOH and dried in a desiccator overnight. Samples were cleaved by shaking for 2 h with 2 mL of the following cleavage solutions: (a) neat TFA; (b) reagent K (82.5% TFA, 5% phenol, 5% water, 5% thiophenol, 2.5% ethanedithiol) (14) or (c) modified reagent B (TIBS) (21) (TFA/DCM/Bu^t_3SiH/phenol in ratio 85:10:5:5). Peptides were directly precipitated by injecting the cleavage solution into 20 mL of Et_2O and collected by centrifugation. Peptide pellets were washed with Et_2O (3 × 20 mL), dried, dissolved in 2 mL of 0.1% aqueous TFA and analyzed by RP HPLC. Peptide **19**, designed to preserve the side-chain protected arginine residue while being cleaved off the resin, was treated with 2 mL of TFA/DCM/Bu^t_3SiH (80:15:5) for 15 min, the cleavage solution was injected into 20 mL of Et_2O and fresh cleavage mixture was added for further 15 min. This treatment was repeated four times and each cleaved fraction was analyzed separately to identify and quantify the amount of protected peptide. The fully protected peptide was isolated from the first (15 min) cleavage and was deprotected in the presence of an equimolar amount of peptide **20** using each of the three cleavage solutions. The mixture containing 1 mg of **20** and 1.5 mg of **19** was treated with 100 μL of a cleavage solution for 2 h; peptides were isolated by Et_2O precipitation and worked up as described above. HPLC analysis of the mixed samples was compared with the standard peptides cleaved separately.

SCAL-TentaGel. *N*-Terminal deprotected peptides on the resin from the synthesizer were transferred into syringes, washed with MeOH and dried. The deprotection of side-chains and cleavage from the support were carried out in two separate steps. First, the three different cleavage solutions defined above were used for a continuous-flow side-chain deprotection performed in syringes with porous frits. The piece of peptide-cotton was placed into the syringe barrel, and cleavage solution was kept constantly flowing through by progressively adding additional solution. After 30 min of this extensive washing the syringe was closed by inserting the plunger and stoppering the outlet. The cleavage solution was exchanged every 20 min for the next 90 min. Reduction of SCAL was performed by shaking the peptide-carrier with a mixture of 1 M PPh_3/$(CH_3)_3SiCl$ in DCM for 1 h, and the final release of deprotected peptide-amides was carried out in TFA/DCM/Bu^i_3SiH (85:10:5). Isolation of the cleaved peptides was performed as described above.

Cotton. Small pieces of a peptide-cotton (ca. 60 mg; 8.4 μmol) were extensively washed with MeOH after the *N*-terminal deprotection and dried. The same cleavage protocol was followed as described above for Rink resin.

Evaluation of results summarized in Fig. 1 and Tables 1 and 2 is based on reversed-phase HPLC analyses using a linear gradient (0–60%) of 0.1% aqueous TFA and acetonitrile over 30 min (in the case of Fmoc protected peptides the gradient used was 20–100% over 40 min). The percentage of the Trp-modified side-product in a sample was estimated by integration of

TABLE 1

Extent of protecting group transfer in peptides 1–5 under various conditions

Peptide	Carrier	Cleavage reagent		
		TFA	TIBS	Mixture K
GAGAWR (1)	Rink	86	7	4
	SCAL-TG	75	16	12
	Cotton	42	8	3
GAGWAR (2)	Rink1	93	41	16
	SCAL-TG	91	61	46
	Cotton	81	30	13
GAWGAR (3)	Rink	91	32	15
	SCAL-TG	94	53	38
	Cotton	71	20	9
GWAGAR (4)	Rink	91	15	7
	SCAL-TG	91	34	23
	Cotton	60	8	4
WGAGAR (5)	Rink	62	4	1
	SCAL-TG	47	10	4
	Cotton	28	3	1

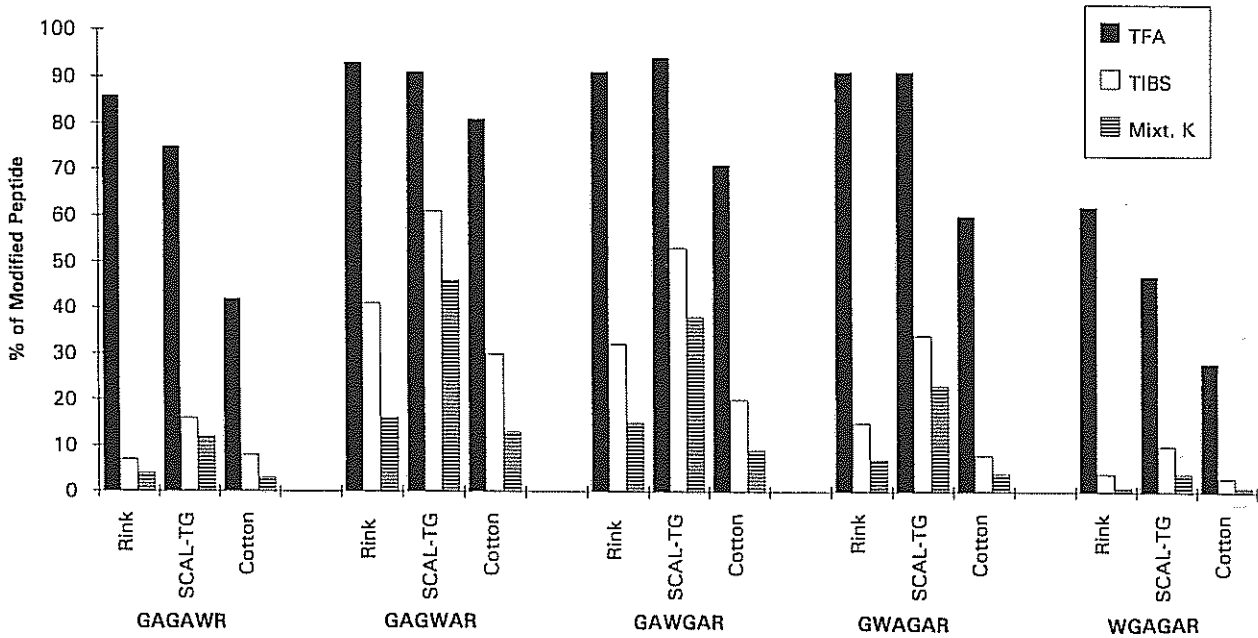

FIGURE 1

Extent of peptide modification by Pmc group transfer. Carriers (Rink, SCAL-TG, cotton) and cleavage conditions (TFA, TIBS, Mixture K) are defined in the experimental section.

TABLE 2

Extent of tryptophan modification in various sequences after cleavage with reagent K (lower case letters denote the D-configuration of the amino acid)

Peptide	Modified product (%)
GAGWaR-NH_2 (7)	< 1
GAGWPR-NH_2 (9)	< 1
GAGWpR-NH_2 (10)	< 1
GAGWGR-NH_2 (8)	7
GAGWAR-NH_2 (2)	16
GAGWSR-NH_2 (12)	21
GAGWYR-NH_2 (16)	22
GAGWFR-NH_2 (17)	22
GAGWLR-NH_2 (14)	24
GAGWKR-NH_2 (15)	26
GAGWDR-NH_2 (11)	29
GAGWVR-NH_2 (13)	32
WRAGA-NH_2 (21)	< 1
WARGA-NH_2 (22)	8
WAGRA-NH_2 (23)	4
WKRGA-NH_2 (24)	15
Fmoc-WKRGA-NH_2 (25)	8
WVRGA-NH_2 (26)	13
Fmoc-WVRGA-NH_2 (27)	4
WDRGA-NH_2 (28)	12

peak areas (280 nm) of a peptide with correct sequences and modified peptides. Minor impurities, if present, were excluded from integration. Both correct products and Trp-modified peptides were isolated by reversed-phase HPLC and characterized by FAB MS (Table 3). Localization of the Pmc group on Trp was determined by ^{1}H NMR measurement of the peptide **2** as a selected example. Other modified peptides characterized by FAB MS were cleaved repeatedly with reagent K for 1 h to exclude the possibility of incomplete Arg deprotection.

Energy calculation

Energy calculations for model peptides Ac-Trp-Arg-NHMe (**I**), Ac-Trp-Ala-Arg-NHMe (**II**), Ac-Trp-Ala-Gly-Arg-NHMe (**III**) and Ac-Trp-Ala-Gly-Ala-Arg-NHMe (**IV**) were performed for all combinations of backbone local minima, corresponding to β (two minima), $c^{eq}7$, αR, and αL minima on the Ramachandran map ($c^{ax}7$ and two minima opposed to β were added for Gly). The entire number of backbone conformations considered for peptide **I** was 25, for **II** was 125, for **III** was 5000 and for **IV** was 25000. All possible combinations of $\chi 1$ rotamers for Trp and Arg side chains (i.e. 9) were considered for peptides **I** and **II**. In all other cases the spacial arrangements of Trp and Arg side chains were optimized by a special algorithm for each backbone conformer before starting energy minimization (see details of the algorithm in ref. 22). The ECEPP potential force field was used for all energy calculations (23, 24).

TABLE 3

FAB MS data of prepared peptides

Peptide	Title sequence		Modified sequence	
	$(M+H)^+$ calc	$(M+H)^+$ found	$(M+H)^+$ calc	$(M+H)^+$ found
1–5	616.3	616	822.4	882
6	918.4	918	1184.5	1184
7	616.3	616	882.4	882
8	602.3	602.5	–	–
9, 10	642.3	642.6	–	–
11	660.3	660.3	926.4	926.2
12	632.3	632.5	898.4	898.2
13	644.4	644.5	910.5	910.3
14	658.4	658.4	924.5	924.5
15	673.4	673.5	939.5	939.5
16	708.4	708.5	974.5	974.5
17	692.4	692.4	958.5	958.6
19	430.3	430	–	–
20	460.2	460	725.3	725

RESULTS AND DISCUSSION

While synthesizing numerous short homologous sequences containing Trp and Arg in different positions we observed in several cases a high percentage of unplanned peptide by-product containing Trp modified by Pmc. The presence of Pmc in the modified peptides was identified by MS, and its localization on Trp in position 2 of the indole ring (Fig. 3) was determined by NMR spectral measurement. By comparing sequences containing the highest percentage of the modified by-product we observed a general trend: Trp was most 'sensitive' to Pmc group modification if the distance from Arg was one amino acid residue.

To study the influence of distance between Trp and Arg on the extent of Trp modification we synthesized a series of simple model hexapeptides with Trp and Arg in different relative positions. The influence of the support and cleavage conditions on the purity of cleaved sequences was also addressed.

Five peptides (**1–5**) were synthesized:
GAGAWR-NH_2 (**1**)
GAGWAR-NH_2 (**2**)
GAWGAR-NH_2 (**3**)
GWAGAR-NH_2 (**4**)
WGAGAR-NH_2 (**5**)

Each peptide was synthesized on three different supports: Rink resin (16), aminomethyl polystyrene resin modified with SCAL handle (17) and on cotton functionalized with Gly (18) and modified with an acid-labile handle (19). The use of Rink resin versus cotton modified with TFA-cleavable handle allowed us to compare the same attachment of the peptides to two supports which had very different composition and hydrophilic/hydrophobic characteristics. Synthesis on

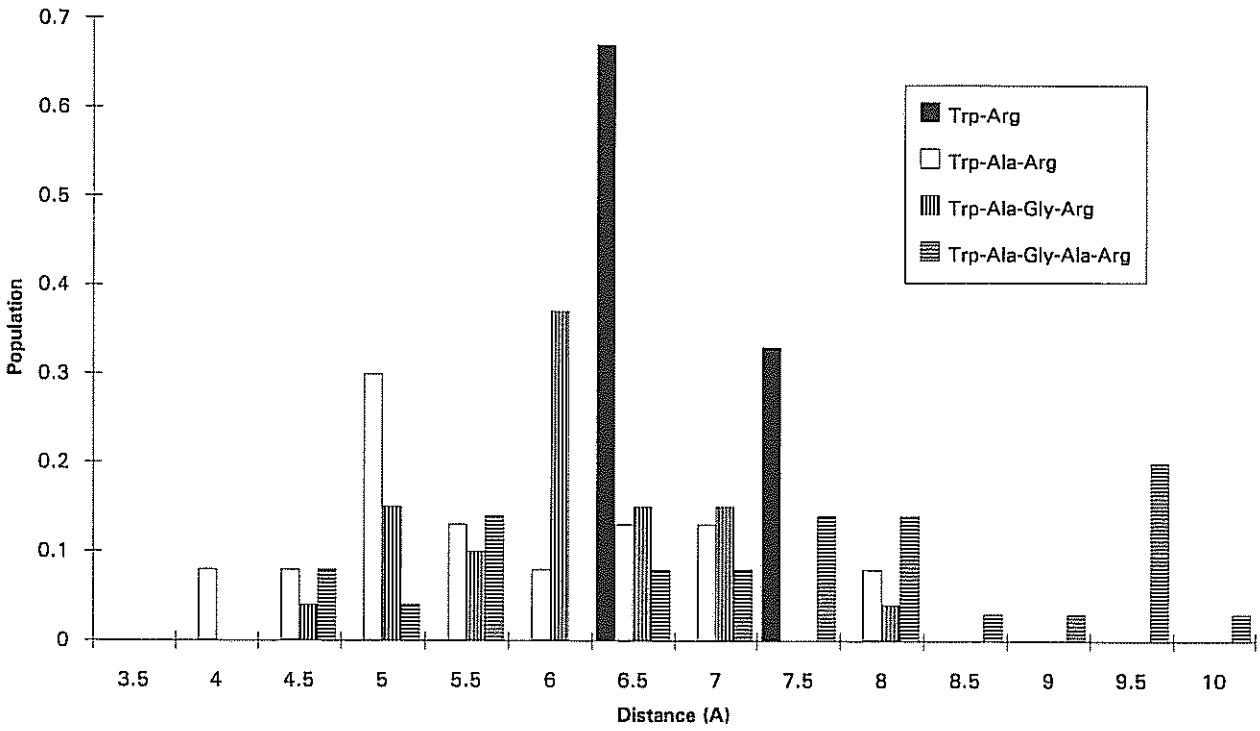

FIGURE 2

Histogram of Cγ atom of Trp and Cζ atom of Arg distance distribution for low-energy conformations of different model peptides.

aminomethyl resin modified with SCAL handle (stable in TFA in the oxidized form and labile in TFA after reduction to sulfide) allowed us to carry out the deprotection under conditions of continuous flow followed by the final cleavage of the deprotected peptide in a separate step. To compare the influence of a scavenger mixture on the purity of product each sequence was cleaved by three different solutions: (i) neat TFA, (ii) reagent K (15) and (iii) a cleavage mixture containing triisobutylsilane [modified mixture B (21)]. The purities of the cleaved peptides were checked by HPLC, main peaks were isolated and identified by MS, and by-products (Pmc-modified peptides) were characterized by MS and NMR measurements. Trp modification at the 2′ position was indicated by the singlet of the N–H proton of the indole ring observed at 11.83 ppm and the concomittant missing signal of C–H at the 2′ position of the indole ring.

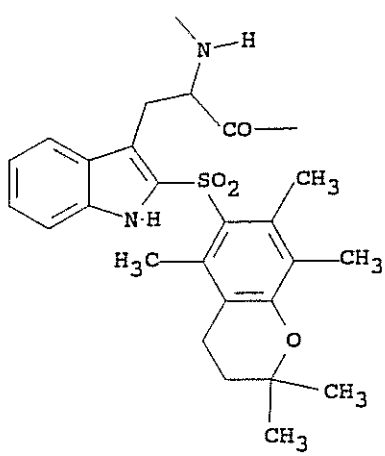

FIGURE 3

Structure of modified tryptophan side chain.

Fig. 1 compares the sequences, carriers and cleavage conditions. The amount of modified by-products for all of the different carriers and cleavage conditions was always the highest in cases when Trp was separated from Arg by one amino-acid residue, in agreement with our previous observations. With increasing distance the danger of modification becomes lesser and direct juxtaposition of Trp and Arg is also less deleterious. As expected, in neat TFA, the long lifetime of the highly reactive cations of the cleaved protecting groups favored the modification, and the amount of by-product was close to 100%. There was an exception in the case of cotton as a carrier, where even without any scavenger the percentage of correct peptide was relatively high. Reagent K turned out to be, in most cases, a better cleavage medium than the triisobutylsilane-containing cleavage mixture. Continuous flow deprotection, which was expected to prevent accumulation of high concen-

trations of dangerous cations and thus a lower probability of modification, did not produce any better results than common batch deprotection. These results suggest that both chemical reactions (acidolysis and aromatic substitution) are much faster than the diffusion of the reactants from the polymeric beads.

Based on the described observations, we decided to synthesize additional sequences to study further factors influencing the extent of modification. The sequence WWWWR-NH_2 (**6**) was synthesized on Rink resin and cleaved the same way as in all previous cases (neat TFA, reagent K, modified reagent B) to see how distant Trp would be modified by the Pmc group. A complex mixture of modified peptides was obtained by cleavage with neat TFA, but surprisingly the pure, correct product was obtained by the cleavage with reagent K (Fig. 4). The peptide cleaved with the Bu^t_3SiH-containing mixture was obtained in 60% purity. The presence of the Pmc group in the isolated by-product was proved by MS and a second cleavage of the peptide by mixture K was carried out to exclude incomplete deprotection of Arg. The position of the modified Trp in the sequence was determined by sequencing based on Edman degradation. A very small peak corresponding to Trp was detected in the third sequencing cycle, which indicated that Trp separated by one residue from Arg was modified.

To answer the question of how the spacial arrangement of Trp toward the Pmc protected Arg influenced the extent of modification, we have chosen the most sensitive sequence (GAGWAR-NH_2) and substituted L-Ala in the second position from the *C*-terminus by D-Ala (**7**). This substitution should change substantially the spacial arrangement within the 'critical' region. The sequence with D-Ala, synthesized on Rink resin, was again cleaved with all three different cleavage mixtures, and in the case of the mixtures containing scavengers the results were very positive. Both with reagent K and reagent B no modified by-product was detected by HPLC. The neat TFA cleavage gave 83% of modified product compared to 93% in the case of the L-Ala-containing sequence.

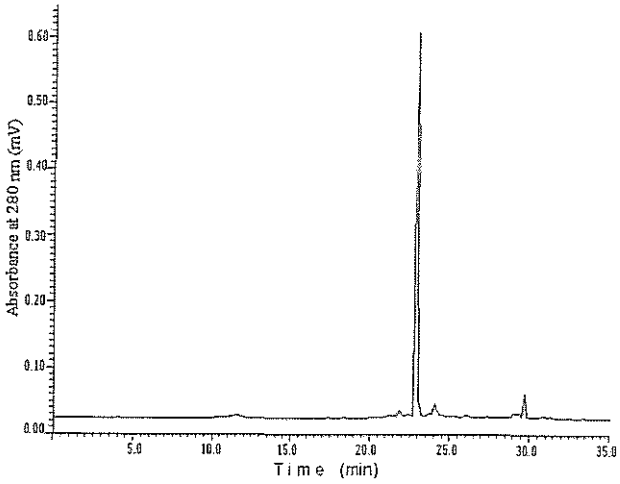

FIGURE 4

HPLC trace of crude peptide **6** cleaved with reagent K. Gradient 0–60% acetonitrile/water (0.1% TFA) in 30 min, 280 nm.

To study the influence the amino acid separating Trp and Arg had on the derivatization of Trp during the deprotection of the Pmc-protected Arg side chain, peptides **8–17** were synthesized.

GAGWXR-NH_2

X = Gly (**8**)	X = Val (**13**)
X = Pro (**9**)	X = Leu (**14**)
X = D-Pro (**10**)	X = Lys (**15**)
X = Asp (**11**)	X = Tyr (**16**)
X = Ser (**12**)	X = Phe (**17**)

The results obtained with these peptides are given in Table 3. A high degree of modification was observed for all amino acids with an L-configuration except in the case of Gly and Pro. In the case of Pro the peptide backbone is assumed to be less flexible, preventing the side chains of Trp and Arg from coming into close contact. This may account for the low degree of Trp modification also in the case of D-Pro. The very low percentage of modified Trp in the case of the Gly-containing peptide may be explained by a very high flexibility of the peptide backbone at this point, and hence no preference for 'modification-sensitive' or 'modification-preventing' conformation, but this is highly speculative.

To support the hypothesis that the Pmc group transfer to Trp was intramolecular, we synthesized the sequence GAGAR-NH_2 (**19**) in a protected form containing Arg(Pmc) and GAGWA-NH_2 (**20**). Cleavage was then carried out in solution using an equimolar mixture of peptides **19** and **20** treated with all three cleavage solutions at a high concentration (25 mg/mL) to promote the intermolecular modification of the Trp residue of peptide **20**. In the case of reagent K, practically no modified peptide **20** was detected. With reagent B only 8% of the Pmc-modified by-product was generated. However, in the neat TFA 68% of Trp modified peptide **20** was obtained, which is comparable with the TFA deprotection performed on a solid support. In the latter case (neat TFA) the Trp of peptide **20** served as the only scavenger present in the cleavage solution.

To find out if the steric arrangement of the molecule is the critical factor controlling the extent of this side reaction we performed energy calculations for the model peptides Ac-Trp-Arg-NHMe (**I**), Ac-Trp-Ala-Arg-NHMe (**II**), Ac-Trp-Ala-Gly-Arg-NHMe (**III**) and Ac-Trp-Ala-Gly-Ala-Arg-NHMe (**IV**). The sets of low-energy backbone conformers were found to be as follows: 20 conformers for peptide **I** (taking into account different rotamers of $\chi 1$, i.e. out of $25 \times 9 = 225$

conformers), 94 for peptide **II** (out of $125 \times 9 = 1125$ conformers), 129 for peptide **III**, and 175 for peptide **IV**. For each of these conformations the distances between the Cγ atom of Trp and the Cζ atom of Arg were calculated. Histograms of these distances for all conformers with relative energies of $\Delta E < (n + 1)$ kcal/mol, where n is the number of residues, are depicted in Fig. 2. As can be seen, in the peptide **I** with Trp adjacent to Arg there is a much lower probability that the side chains will be in close proximity compared to peptide **II**, in which these amino acids were separated by a single residue (the average distance between the Cγ atom of Trp residue and the Cζ atom of Arg residue is 6.96 Å in peptide **I** versus 5.99 Å in peptide **II**). Peptide **III**, with two amino acid residues between Arg and Trp, could still assume a conformation placing their side chains close enough for the intramolecular transfer of Pmc group (average distance 6.29 Å). Peptide **IV**, with three intercalating residues, could also assume a conformation with Arg and Trp side chains in close proximity, but the average distance is significantly higher (7.71 Å) and therefore the probability of the close contact is lower.

The results discussed above indicate that the reactive cation apparently has to come into close proximity with the aromatic nucleus for the modification of tryptophan to proceed, and that the predominant reaction is an intramolecular rearrangement. Therefore, introduction of a steric or electrorepulsive barrier close to the indole ring might prevent its modification. Additional sequences **21–28** were synthesized to study this possibility.

WRAGA-NH_2 (**21**)
WARGA-NH_2 (**22**)
WAGRA-NH_2 (**23**)
WKRGA-NH_2 (**24**)
Fmoc-WKRGA-NH_2 (**25**)
WVRGA-NH_2 (**26**)
Fmoc-WVRGA-NH_2 (**27**)
WDRGA-NH_2 (**28**)

These sequences contained either the free *N*-terminal amino group of tryptophan as the repulsive barrier (**21–24**, **26**, **28**), or this group combined with the side chain of an intervening lysine (**24**), or the bulky hydrophobic Fmoc group at the amino terminus (**25**, **27**). Deprotection of the arginine side chain was performed by mixture K under the same conditions described above. To be sure that the results were not influenced by minor changes in experimental details (temperature), one peptide studied earlier (**2**) was cleaved in parallel, giving the same result as previously. Results are given in Table 3. The Fmoc group was very efficient in decreasing the extent of tryptophan modification (**25** vs. **24** and **27** vs. **26**). The presence of a free amino group led to an approximately twofold decrease of the extent of modification independent of the side chain of an amino acid placed between the Arg and Trp moiety (**1** vs. **21**, **2** vs. **22**, **3** vs. **23**, **15** vs. **24**, **13** vs. **26**, **11** vs. **28**). This means that the steric arrangement of the C2′ carbon and the amino group of tryptophan caused an advantageous repulsion of the arginine side chain, such that the indole ring no longer assumed the critical position necessary for Trp modification during cleavage of the Pmc group.

CONCLUSIONS

We have demonstrated that synthesis of peptides containing both Trp and Pmc-protected Arg close in the sequence can give low yields of the correct peptides owing to Trp modification during TFA cleavage of the Pmc side-chain protection group. The extent of modification is at least partly predictable based on the distance separating the side chains of the two amino acids, the type of amino acids between them, and based on the position of Trp in the peptide chain relative to free or Fmoc protected amino terminus. We observed that the highest percentage of modification occured in cases where Trp was separated from Arg by one amino acid. In this situation the nature of the separating amino acids between Trp and Arg was important in order to create the proper milieu for the Pmc group transfer. Since spacial proximity appeared necessary for the transfer of Pmc group onto the tryptophan side chain, and since in a heterogenous mixture of two peptides containing Trp and the other Pmc-protected Arg there was practically no transfer of Pmc to Trp, it appears that the principle rearrangement is due to an intramolecular mechanism rather than an intermolecular reaction. This hypothesis is supported by the results of energy calculations for model peptides and by the observation that the modification was not prevented or even lowered when continuous flow deprotection was carried out.

The conclusion from our experiments is that the side reaction is not prevented completely by reagent K, supposed to be one of the most efficient scavenger mixtures, nor by the cleavage mixture based on triisobutylsilane, phenol and water as combination of scavengers. Furthermore, the continuous-flow deprotection in the case of synthesis on the safety-catch acid-cleavable linker did not prevent or even lower the extent of modification.

The considerably lower amount of by-products for the peptides cleaved from cotton, even in the case of neat TFA cleavage, supports an idea that free hydroxyls present on the cotton might have scavenging ability. In the view of our results it is even more attractive to consider the use of Arg(Boc_2) (25) as an alternative to Arg(Pmc) for Fmoc/Bu^t strategy of solid-phase peptide synthesis. Furthermore, if active peptides from a synthetic scheme employing Arg(Pmc) are identified which contain both Trp and Arg it is necessary to evaluate whether true active structure might be the Trp modified peptide.

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Address:

Michal Lebl, Ph.D., D.Sc.
Selectide Corp.
1580 E. Hanley Blvd.
Tucson, AZ 85737
USA