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C-terminal *N*-alkylated peptide amides resulting from the linker decomposition of the Rink amide resin. A new cleavage mixture prevents their formation

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Abstract: Decomposition of the resin linkers during TFA cleavage of the peptides in the Fmoc strategy leads to alkylation of sensitive amino acids. The *C*-terminal amide alkylation, reported for the first time, is shown to be a major problem in peptide amides synthesized on the Rink amide resin. This side reaction occurs as a result of the Rink amide linker decomposition under TFA treatment of the peptide resin. The use of 1,3-dimethoxybenzene in a cleavage cocktail prevents almost quantitatively formation of *C*-terminal *N*-alkylated peptide amides. Oxidized by-product in the tested Cys- and Met-containing peptides were not observed, even if thiols were not used in the cleavage mixture.

Keywords: cleavage scavengers; *C*-terminal *N*-alkylated amides; 1,3-dimethoxybenzene scavenger; linker decomposition; Reagent I; Rink amide resin; Trp alkylation

INTRODUCTION

Side reactions during the deprotection step of peptides from the resin in the Fmoc-based method with trifluoroacetic acid (TFA) are common and well-documented phenomena. The resulting carbocations alkylate the most sensitive amino acids such as Trp, Tyr, and Cys [1–6]. Acidolytic oxidation of Met and incomplete deprotection of Cys have also been reported [7–10]. A particular case of side reactions during TFA cleavage of peptides from the resin is represented by alkylation of the sensitive amino acids by carbocations resulting from decomposition of the resin linker. Alkylation of the indole nucleus of Trp-containing peptides during the TFA cleavage procedure as a result of Wang resin linker decomposition has been reported [11,12]. Martinez and coworkers have investigated this side reaction in details aiming at suppressing alkylation of Trp [12]. It was shown that this sequence-independent side reaction (except at the *C*-terminal position) occurs whatever the scavenger used.

Exploitation of the properties of the individual scavengers, namely, triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), thioanisole, etc. resulted in a variety of multiple scavenger cleavage mixtures that have been proposed and used in practice [1,13]. Reagent K (82.5% TFA: 5% phenol: 5% H_2O: 5% thioanisole: 2.5% EDT) [14], Reagent R (90% TFA: 5% thioanisole: 3% EDT: 2% anisole) [15], Reagent M (67% TFA: 2% 2-mercaptoethanol: 1% anisole: 30% dichloromethane) [16], Reagent B (88% TFA: 5% phenol: 5% H_2O: 2% TIS) [17], Reagent L (88% TFA: 2% TIS: 5% dithiothreitol: 5% H_2O) [18], and Reagent H (81% TFA: 5% phenol: 5% thioanisole: 2.5% EDT: 3% H_2O: 2% dimethylsulfide: 1.5% ammonium iodide) [19] are some of the cleavage mixtures used in order to minimize the occurrence of side reactions. In the case of sequences that do not contain sensitive amino acids, the cleavage mixture consisting of 95% TFA: 2.5% H_2O: 2.5% TIS is commonly used.

In our laboratory, using the Rink amide resin for the synthesis of various peptide amides with the Fmoc strategy, we observed, independently of the peptide sequence, the formation of a by-product exhibiting an increase in molecular weight of 106 a.m.u. as compared to the target molecule. The percentage of this by-product varied up to 35%. This finding prompted us to investigate its origin and the possibility of minimizing its formation.

In this work, we report on the identification of the *C*-terminal *N*-alkylated amide by-product and the use of 1,3-dimethoxybenzene (DMB) as a component of the scavenger cocktail consisting of 92.5% TFA: 2.5% TIS: 5% DMB (Reagent I) suitable for preventing its formation. It is demonstrated that formation of the *C*-terminal *N*-alkylated amide by-product result exclusively from decomposition of the Rink amide resin linker. Reagent I suppresses almost completely alkylation of the *C*-terminal amide group by preventing the linker decomposition during peptide cleavage from the resin. Numerous model peptides or peptides synthesized for other purposes were studied under various cleavage conditions. The optimal mixture of scavengers was tested in the presence of various side-chain protecting groups in order to estimate the limits of its application.

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MATERIALS AND METHODS

Reagents

Fmoc amino acid derivatives, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) were purchased from Neosystem Laboratoire (Strasbourg, France). 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-linked polystyrene (Rink amide) and 4-(hydroxymethyl) phenoxymethyl-linked polystyrene (Wang) resins were obtained from GL Biochem (Shanghai, China). TIS, EDT, *N*,*N*-diisopropylethylamine (DIEA), TFA, DMB, and piperidine were Merck–Schuchardt (Darmstadt, Germany) products and used without further purification. Dichloromethane, *N*,*N*-dimethylformamide (DMF), distillated over ninhydrin and stored under preactivated molecular sieves 4A, 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 using the standard Fmoc-based strategy. Amino acids were introduced protected as Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Arg(Pbf)-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.

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

HPLC Analysis

The analytical HPLC chromatograms were run on a Waters Millenium (Milford, CT) apparatus with a photodiode array detector 996. The spectra were acquired at 214 and 280 nm. A reverse-phase Discovery C18 column and a flow rate of 1 ml/min were used. Gradient elution was performed with the following solvents: A, H_2O/0.1% TFA and B, CH_3CN/0.1% TFA either from 100 to 60% A for 40 min (elution system I) or from 95 to 50% A for 40 min (elution system II), from 80 to 50% A for 30 min (elution system III), from 90 to 50% A for 40 min (elution system IV), depending on the lipophilicity of the peptide. The crude peptides were purified by semi-preparative HPLC on a Waters PrepLC 4000 system associated with a reversed-phase Discovery C18 column (25 cm ×10 mm) running at a flow rate of 4.7 ml/min. The purity of the final products was checked by analytical HPLC.

Electrospray Mass Spectroscopy (ESI-MS)

Electrospray mass spectra were obtained on a Micromass (Manchester, England) Platform II quadrupole mass spectrometer. Samples were dissolved in the mixture H_2O/CH_3CN/HCOOH (49 : 49 : 2) and injected into the ESI source at a flow rate of 5 µl/min. The source temperature was adjusted at 60 °C, while the cone voltage was set to 60 V.

NMR Spectroscopy

The 1H NMR spectra were recorded on a Bruker AMX-400 spectrometer at 300 K. The NMR samples were prepared by dissolving the solid material in DMSO-d_6 at a concentration of 5 mM.

RESULTS AND DISCUSSION

In various crude peptide amides, synthesized on the Rink amide resin by the Fmoc strategy and independently of their sequence, we observed, during HPLC purification, the presence of a second fraction corresponding to a percentage of up to 35%. This by-product exhibited an absorbance at 280 nm in the UV detector despite the fact that aromatic residues were not present in the peptide sequence (Figure 1A

Table 1 ESI-MS and HPLC data of the studied model peptides

Peptide[a]	Expected MW	Found MW	Retention time (min)	HPLC elution system[b]
H-Gly-Ala-NH_2	145.1	145.1	1.9	I
H-Ser-Trp-Arg-NH_2	446.5	446.6	6.1	II
Ac-Cys- Ser-Trp-Arg-NH_2	591.6	591.6	12.6	II
Ac-Val-Ser-Glu-Ala-Arg-Val-Leu-Glu-Asp-Arg-Pro-Leu-Ser-Asp-Lys-NH_2	1754.9	1755.3	10	III
H-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2	947.0	947.3	5.7	IV
H-Met-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2	1077.1	1077.7	11.5	IV

[a] The peptide amides were synthesized on the Rink amide resin.
[b] See *Materials and Methods* (HPLC analysis).

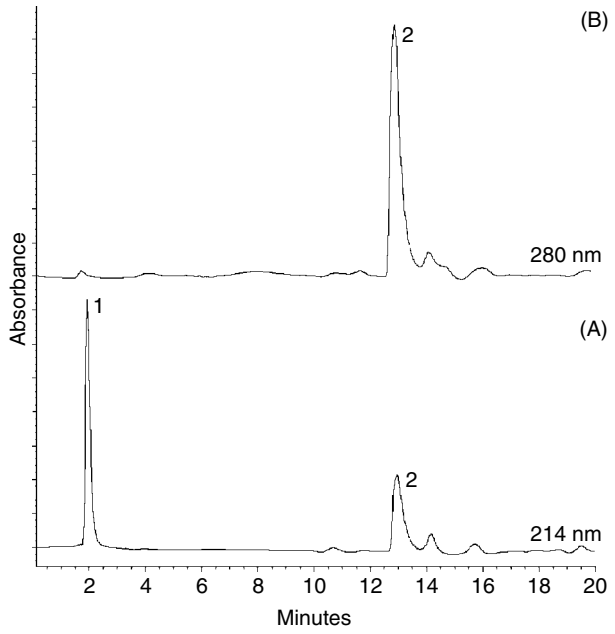

Figure 1 HPLC profiles at 214 nm (A) and 280 nm (B) of the crude H-Gly-Ala-NH_2 synthesized on the Rink amide resin and cleaved with 95% TFA, 2.5% TIS, 2.5% H_2O. The numbers denote the desired peptide (1) and the *C*-terminal alkylated amide by-product (2).

and B, and Table 2). The ESI mass spectra revealed the presence of a molecular ion with an increased molecular weight of 106 a.m.u. compared to that of the desired peptide. Although a similar phenomenon was previously observed only in Trp-containing peptides synthesized on the Wang resin (Figure 2A), the fact that, in the case of the Rink amide resin, the presence of an aromatic residue was not a prerequisite indicates a different nature and mechanism of formation of this by-product. It is worth mentioning here that the by-product with an increased molecular weight of 106 a.m.u. compared to that of the desired peptide could not result from a reaction between the peptide and the carbocations formed from side-chain deprotection. On the basis of these data and the fact that similar by-products were not observed when the Rink amide AM resin was used, we decided to define the origin of the obtained by-product focusing our attention on the resin linker. As is shown in Figure 2B, a *C*-terminal alkylated amide resulting from an inappropriate decomposition of the linker at positions 2 and 3 could explain the experimental results. This hypothesis was confirmed by recording the 1H NMR spectrum of the by-product obtained during the synthesis of H-Gly-Ala-NH_2. The 1H NMR spectrum clearly demonstrates the presence of the *p*-hydroxybenzyl group covalently attached on the *C*-terminal amide nitrogen (Figure 3). All the

Table 2 Yields of the crude peptides synthesized on the Rink amide resin by the Fmoc strategy and cleaved with various cleavage mixtures

Entry number	Peptide	Cleavage mixture[a] (time)	Peptide (%)	Peptide + 106[b] (%)	Other by-products (%)
1	H-Gly-Ala-NH_2	I (2 h)	51	36	13
2		II (2 h)	100	–	–
3	H-Ser-Trp-Arg-NH_2	I (3 h)	58	26	16
4		III (3 h)	90	–	10
5		IV (3 h)	92	–	08
6		V (3 h)	68	–	32
7		II (3 h)	63	–	37
8	Ac-Cys-Ser-Trp-Arg-NH_2	VI (3 h)	61	27	12
9		III (3 h)	78	–	22
10	Ac-Val-Ser-Glu-Ala-Arg-Val-Leu-Glu-Asp-Arg-Pro-Leu-Ser-Asp-Lys-NH_2	I (4 h)	46	16	38
11		III (4 h)	69	–	31
12		II (4 h)	57	–	43
13	H-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2	I (3 h)	73	21	06
14		VII (3 h)	67	25	08
15		VIII (3 h)	59	12	29
16		II (3 h)	87	–	13
17		III (3 h)	93	–	07
18	H-Met-Asn-Asn-Pro-Leu-Tyr-Lys-Glu-Ala-NH_2	II (3 h)	100	–	–

[a] Composition of the cleavage mixtures: 95% TFA, 2.5% TIS, 2.5% H_2O (I), 95% TFA, 5% DMB (II), 92.5% TFA, 5% DMB, 2.5% TIS (III, Reagent I), 92.5% TFA, 7.5% DMB (IV), 92.5% TFA, 5% DMB, 2.5% H_2O (V), 94% TFA, 2.5% EDT, 2.5% H_2O, 1% TIS (VI), 95% TFA, 5% TIS, 2.5% H_2O (VII), 90% TFA, and 1.5% TIS, 8.5% H_2O (VIII).
[b] The yields were estimated from the peak areas of the analytical HPLC chromatograms. The contribution of the *p*-hydroxybenzyl chromophore at 214 nm was not estimated in the given values.

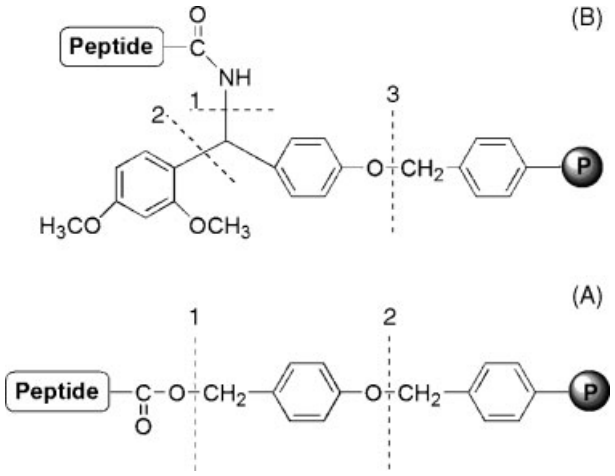

Figure 2 Linker structure of the Wang (A) and the Rink amide (B) resins. Dashed lines show the possible cleavage positions.

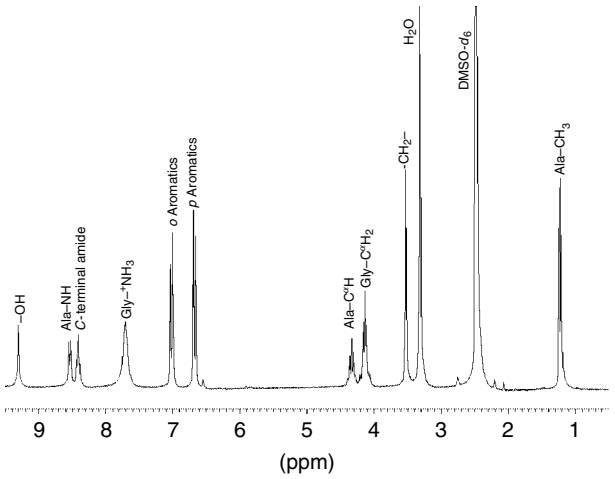

Figure 3 400 MHz 1H NMR spectrum of H-Gly-Ala-$NHCH_2$PhOH *C*-terminal *N*-alkylated amide by-product in DMSO-d_6 at 300 K.

resonances are well resolved. The *C*-terminal amide proton appears as a triplet at 8.47 ppm, the hydroxyl proton as a singlet at 9.36 ppm, the N-terminal Gly ammonium group ($-^+NH_3$) as a broad resonance at 7.77 ppm [20], the Ala amide proton as a doublet at 8.60 ppm, the o- and m-aromatic protons as two separate doublets at 7.07 and 6.74 ppm, and the $-CH_2-$ benzyl group as a singlet at 3.55 ppm, respectively. 1H NMR spectra for other *C*-terminal *N*-alkylated peptides also confirmed the presence of the *p*-hydroxybenzyl group covalently attached on the *C*-terminal amide nitrogen. Since the percentage of this by-product was in some cases very high, we investigated the cleavage conditions that could minimize its formation. In this investigation, we have tried to avoid the use of thiols due to their malodorous properties. The possible cleavage positions shown in Figure 2B prompted us to use DMB as a component of the cleavage mixture. DMB represents the parent molecule of the resulting carbocation if the linker decomposition occurs at position 2. This scavenger has a boiling point of 85–87 °C and therefore can be easily removed from the cleavage mixture under reduced pressure.

To define the optimal composition of the cleavage mixture, we varied the percentage of DMB from 2.5 to 10% while keeping unchanged at 2.5% the percentage of TIS. The cleavage mixtures consisting of 95% TFA: 5% DMB (v/v) and 92.5% TFA: 7.5% DMB (v/v) were also tested. As is shown in Figures 4 and 5, and in Table 2, the cleavage mixture 92.5% TFA: 2.5% TIS: 5% DMB suppresses almost quantitatively the formation of *C*-terminal alkylated amide peptides. Although very good results can be obtained with the cleavage mixture 92.5% TFA: 7.5% DMB (v/v) (Table 2, entry 5), it is not recommended for Cys-containing peptides. If the Cys residue is present in the peptide sequence, then the presence of TIS in the cleavage mixture (Reagent I) is required, since, otherwise, the incomplete deprotection of the sulfydryl group becomes a major problem [9,21]. Incomplete deprotection of the -Asn(Trt)- residue was also observed (<5%), but only in the case when it occupies the N-terminal position in the sequence (Table 2, entry 17). This well-known problem [22] seems to be independent of the cleavage

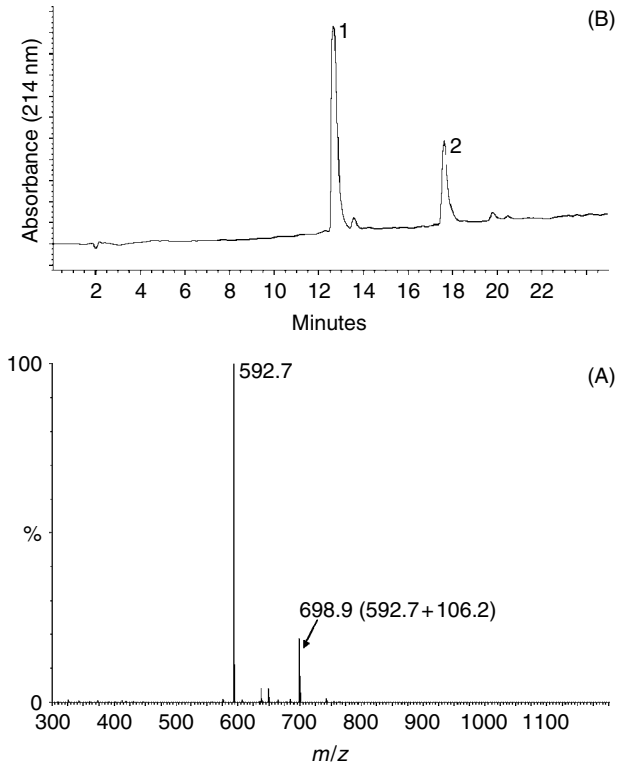

Figure 4 ESI-MS spectrum (A) and HPLC profile (B) of the crude Ac-Cys-Ser-Trp-Arg-NH_2 synthesized on the Rink amide resin and cleaved with 94% TFA, 2.5% EDT, 2.5% H_2O, 1% TIS. The numbers denote the desired peptide (1) and the *C*-terminal alkylated amide by-product (2).

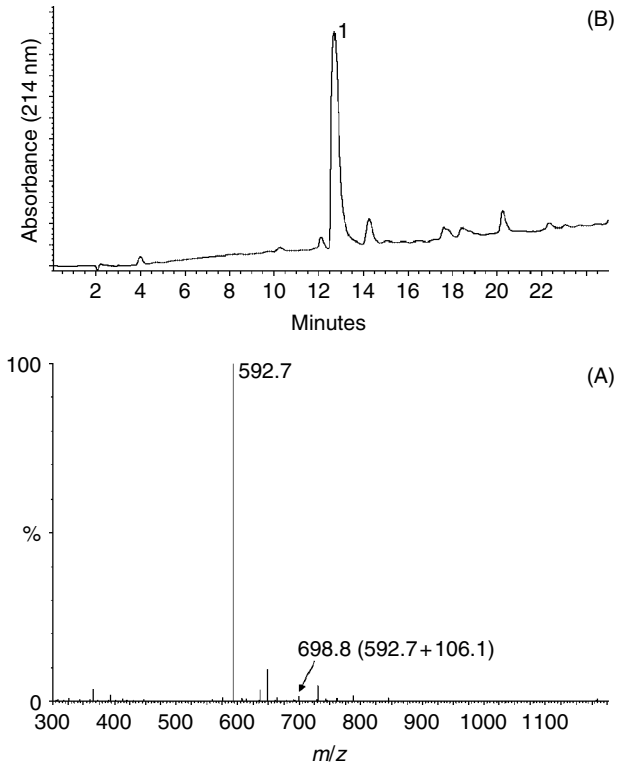

Figure 5 ESI-MS spectrum (A) and HPLC profile (B) of the crude Ac-Cys-Ser-Trp-Arg-NH_2 synthesized on the Rink amide resin and cleaved with 92.5% TFA, 5% DMB, 2.5% TIS (Reagent I). The formation of the *C*-terminal alkylated amide by-product is almost completely suppressed.

mixture used. It is worth mentioning here that, using Reagent I for the cleavage of the peptides from the Rink amide resin, we have not observed oxidation of Met residues or formation of disulfide bridges in Cys-containing peptides.

Table 2 summarizes the results obtained for various cleavage mixtures applied to peptides synthesized on the Rink amide resin. From these data, it is concluded that Reagent I is a suitable cleavage mixture, which prevents formation of *C*-terminal alkylated amides.

The above findings prompted us to apply Reagent I as the cleavage mixture of Trp-containing peptides synthesized on the Wang resin (data not shown). When the Trp residue was incorporated in the peptide sequence as Boc protected in the side chain, the alkylation by the linker decomposition was prevented or occurred at very low percentages either using Reagent I or other known cleavage mixtures [12]. However, as observed for other cleavage mixtures, Reagent I cannot suppress the formation of Trp side-chain alkylated by-products when Trp is used in the side-chain unprotected form. This finding provides indirect evidence regarding the different mechanism of alkylation of the Trp side chain and the *C*-terminal amide group by Wang and Rink amide linkers' decomposition respectively. In the former case, decomposition of the Wang linker results in the formation of the carbocation (Figure 2A), which next reacts with the indole ring of the Trp side chain, while, in the latter case, cleavage of the Rink amide linker at position 2 favors the cleavage of the peptide from the resin at position 3 (Figure 2B) without formation of the intermediate carbocation. Therefore, on the basis of the above-mentioned data, we can hypothesize that DMB does not act as a strong scavenger of the produced carbocations, but it contributes by preventing the inappropriate decomposition of the Rink amide linker at position 2.

CONCLUSION

In this work, we have demonstrated that decomposition of the Rink amide resin linker during the TFA cleavage step leads to the formation of *C*-terminal *N*-alkylated amide by-products. This sequence-independent side reaction can be suppressed by using as cleavage mixture Reagent I. DMB was estimated to contribute to this result by preventing the formation of carbocations that originated from the inappropriate Rink amide linker decomposition. The use of thiols was not required in the cleavage mixture of the Cys- and Met-containing peptides since no oxidation reactions were observed in the tested peptides.

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