Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 989–997
Published online 14 April 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1032

JOURNAL OF PEPTIDE SCIENCE

Short Communication

Mechanistic studies of amide bond scission during acidolytic deprotection of Pip containing peptide

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Received 19 November 2007; Revised 1 February 2008; Accepted 11 February 2008

Abstract: Unusual TFA catalyzed cleavage reaction is reported for peptide containing pipecolic acid residues. Although the use of TFA under standard cleavage conditions is sufficiently mild to prevent degradation of the desired products, the amide bond between consecutive pipecolic acid residues is unexpectedly hydrolyzed by standard TFA treatment. The hydrolysis is proposed to proceed via an oxazolinium ion intermediate. This mechanism is supported by H/D exchange as observed by ESI-MS and NMR experiments. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: pipecolic acid; TFA cleavage; peptide degradation; SH3 domain

The nonproteinogenic amino acid pipecolic acid (so named pipecolinic acid or 2-piperidine carboxylic acid) is a proline homolog which contains a six-membered ring. It is found in several important natural products [1] and it has been extensively used as a proline substitute in several syntheses of peptidomimetics [2]. In order to probe the role of proline ring size in peptide recognition by proline recognition domains (PRDs) and in particular by SH3 domains, we designed and synthesized a compound which contains (Pip) (named peptide **2**) in place of all proline residues into the suitable SH3 ligand peptide of sequence H-Pro-Pro-Pro-Leu-Pro-Pro-Lys-Pro-Lys-Phe-OH (named peptide **1**), corresponding to a Pro-rich peptide from HPK1 kinase [3].

Pipecolic rich peptide was synthesized manually on solid phase (SPPS) using an Fmoc/*t*-Bu-based strategy with stepwise coupling. Coupling reactions were performed using HATU as coupling reagent. Cleavage from the resin and side-chain deprotection were carried out by acidolysis with TFA using H_2O and triisopropylsilane (TIS) as scavengers [4], and the crude peptides were precipitated by addition of cold ethyl ether. Surprisingly, RP-HPLC analysis of the crude compound showed the presence of three components (named **2a, 2b**, and **2c**, respectively) in the crude peptide (Figure 1(A)). ESI-MS analysis of the individual components revealed that the MH^+ of component **2a** was 644.390 mass units, corresponding to the fragment 6–10 of the parent Pip-rich peptide. The other two compounds **2b** and **2c** showed identical MH^+ values of 576.349 mass units as determined with an accuracy of ±0.05 by calibration against a mixture of neurotensin, angiotensin, and bradykinin at a concentration of 1 pmol/µl as external standard, corresponding to the fragment 1–5 of the Pip-rich peptide. The presence of two fragments with identical MH^+ value (compound **2b** and **2c**) indicated that an amino acid residue racemized during the amide bond cleavage reaction. The amino acid involved in epimerization is the Pip5 residue, which is equilibrated to give a 3:2 ratio of L/D as determined from the ratio of HPLC peaks (Figure 1(A)). The identity of the L- and D-isomers was established by the synthesis of authentic H-Pip-Pip-Pip-Leu-(L- or D-)Pip-OH isomers, respectively.

The resin-bound peptide was alternatively treated with TFA and 1,2-ethanedithiol (EDT) as scavengers. Also, in this case the whole Pip-rich peptide was not detected by ESI-MS analysis, while two distinct compounds corresponding to the fragment 6–10 (**2a**) and the *C*-terminal 2-thioethyl thioester of fragment 1–5 (**2d**) (Scheme 1) have been detected and characterized by the ESI-MS analysis (Figure S1).

These evidences indicated that the observed peptide fragmentation is due to an unexpected TFA catalyzed amide bond cleavage reaction under the standard cleavage/deprotection conditions used in SSPS carried out on acid labile Wang resin (Scheme 1).

Since peptides are routinely treated with strong acids such as TFA and anhydrous HF without loss

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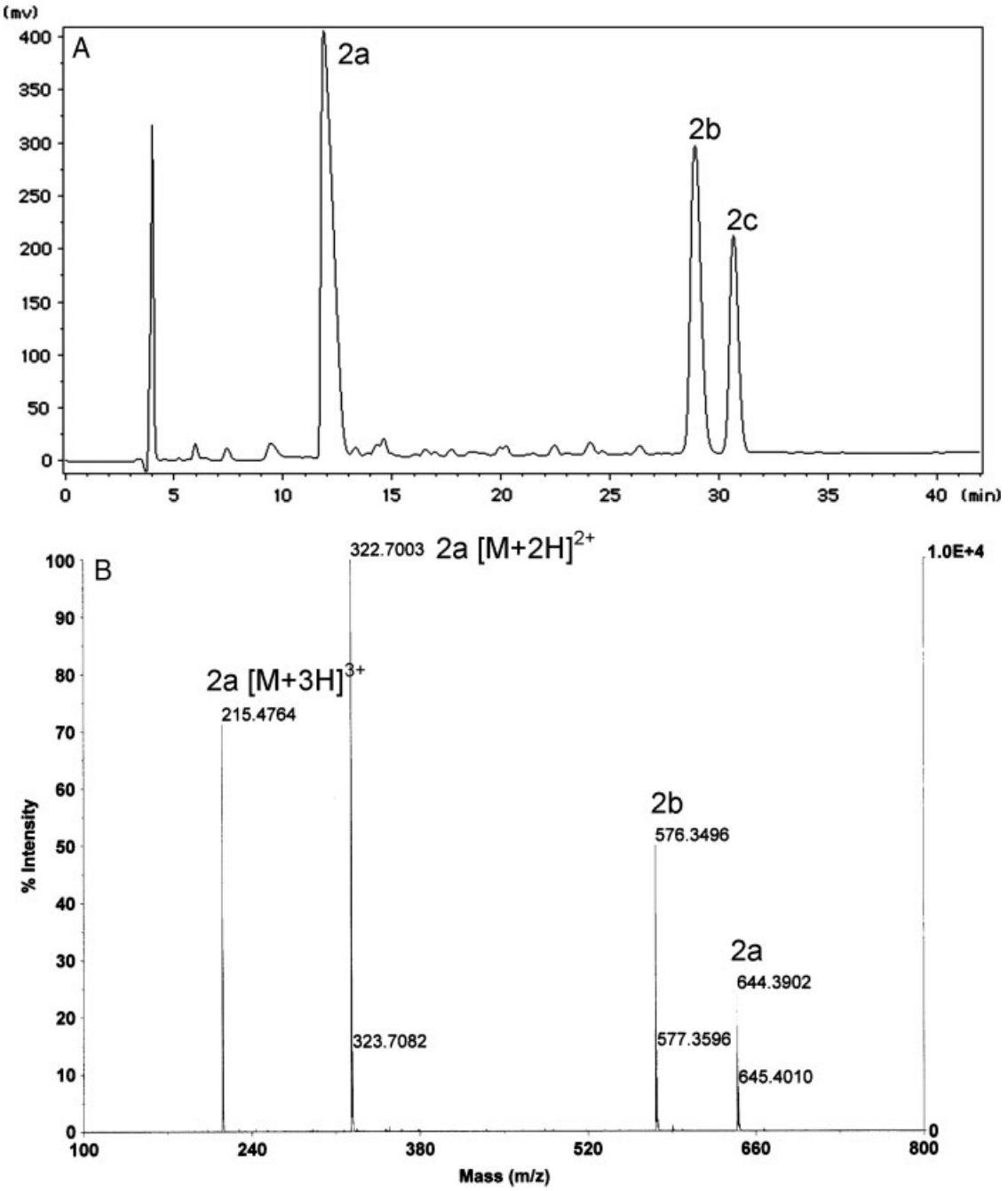

Figure 1 RP-HPLC chromatogram (A), and ESI-MS mass spectrum (B) of crude Pip-rich peptide **2** obtained by SPPS on Wang resin.

of the backbone integrity, this facile amide bond cleavage is highly unusual. Indeed, peptide bonds are commonly cleaved under harsher conditions, whereas the cleavage at room temperature requires other types of catalysis (proteases and lanthanides [5]). However, certain peptide sequences are known to undergo spontaneous rearrangements or cleavage under mild conditions [6,7]. In particular, the cleavage of amide bonds between consecutive *N*-methyl amino acids by acids such as TFA has been reported by Goodman and his coworkers [8]. A massive acid-catalyzed bond cleavage was also observed for the Asp–Pro sequence [9], although the hydrolysis of consecutive Pro residues is a far more unusual event as confirmed by solution and gas-phase fragmentation studies [10].

Despite the importance of pipecolic acid, little is known about side-reactions involving this residue during peptide synthesis and, in particular, during deprotection protocols. Consequently, the knowledge and understanding of this unexpected amide bond degradation will be useful in peptide chemistry. For this purpose, Pip-rich peptide was synthesized minimizing

DOI: 10.1002/psc

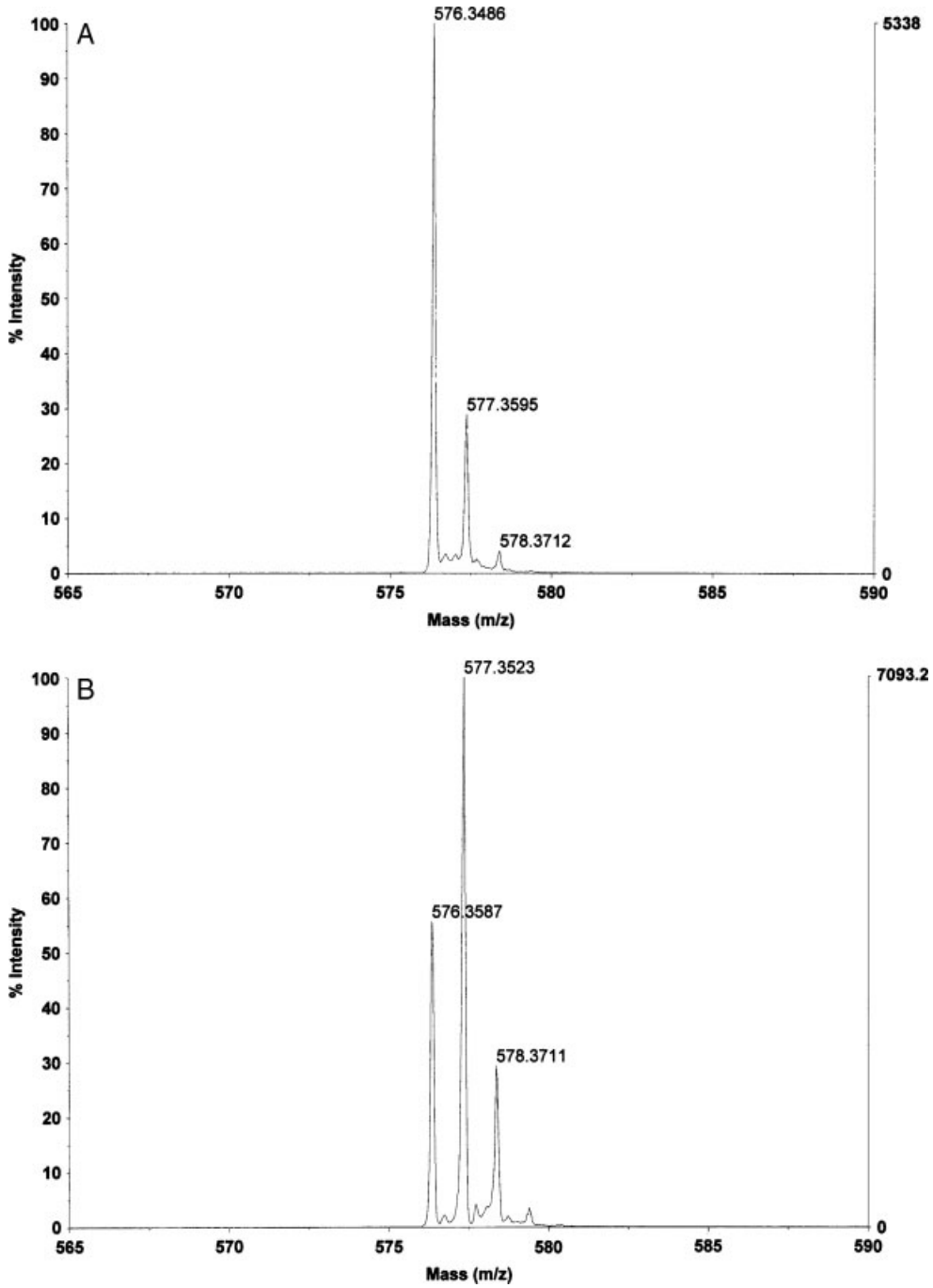

Figure 2 ESI-MS mass spectra of compound **2b** obtained by treatment either with TFA (A), or with deuterated TFA (B).

the use of TFA as far as possible. Peptide was synthesized using the hyper-acid labile 2-Cl-Trt resin [11] and the *iv*Dde moiety [12] as protecting group of the side-chain amino group of Lys residues. After deprotection of Lys residues, peptide was cleaved from the resin using a 1% TFA solution in DCM, and a good yield of the expected peptide was obtained (Table 1).

The relationships between fragmentation during cleavage, composition of the cleavage cocktail, and time of cleavage were studied in detail. Five different conditions were used to examine the stability of peptide to acid conditions. In all cases, *ca* 10–15 μmol of purified peptide was used to study the hydrolysis in solution. The cleavage conditions were the following: **A** – TFA-TIS-H_2O 95 : 4 : 1, r.t., 60 min; **B** – 50% TFA in DCM, r.t., 60 min; **C** – 1% TFA in DCM, r.t., 60 min; **D** – 0.1 M HCl, r.t., 24 h; **E** – 0.1 M HCl, r.t., 10 days. After the indicated time period, each solution was immediately analyzed by RP-HPLC. In addition, methods **A, B**, and **C** were also used for the treatment of the peptide bound (10–20 mg) to both Wang and 2-Cl-Trt resins.

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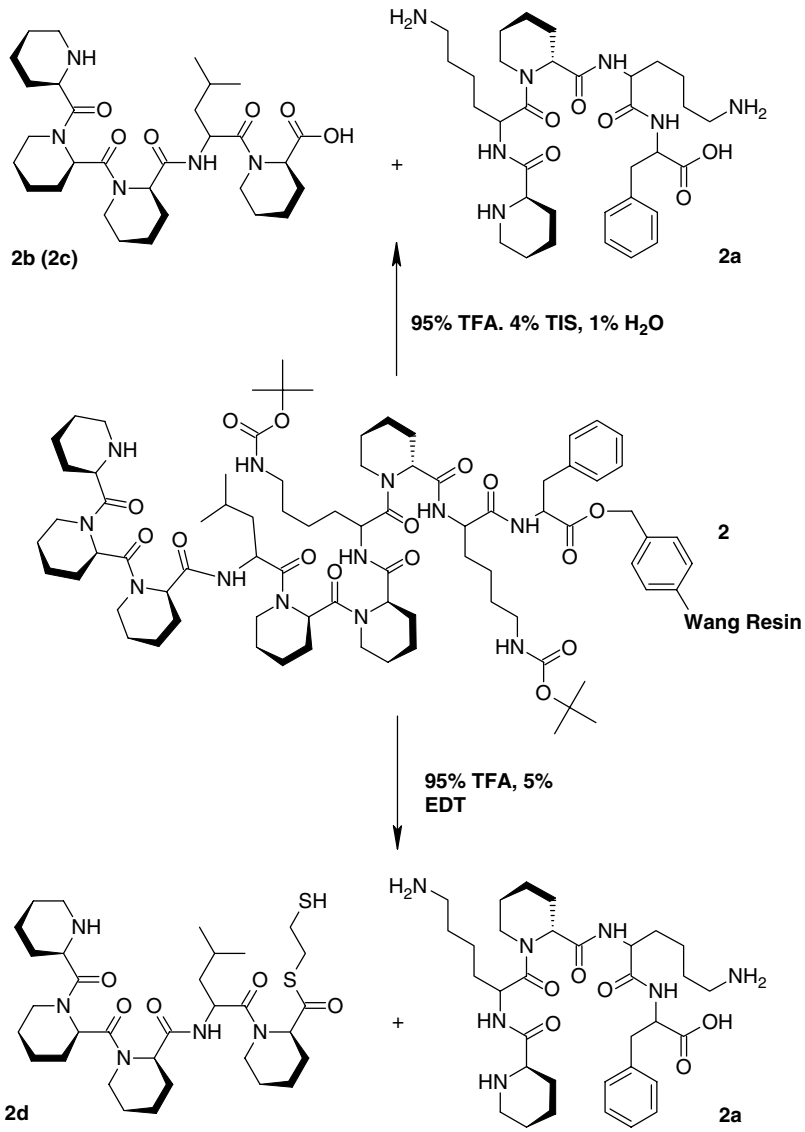

Scheme 1 Acidolysis of Pip-rich peptide **2**.

Table 1 Physicochemical properties of Pip-rich peptide an its **a, b**, and **c** fragments

Peptide	MW		Amino acid ratios in 6 N HCl hydrolyzates				t_r (min)[a]
	Calculated for	Found	Pip	Leu	Phe	Lys	
2	$C_{63}H_{100}N_{12}O_{11}$ 1200.76	1201.758	5.98 (6)	0.98 (1)	1.01 (1)	2.05 (2)	28.0[b]
2a	$C_{33}H_{53}N_7O_6$ 643.41	644.390	1.97 (2)	—	1.05 (1)	2.03 (2)	11.8[c]
2b	$C_{30}H_{49}N_5O_6$ 575.37	576.349	3.98 (4)	0.99 (1)	—	—	28.8[c]
2c	$C_{30}H_{49}N_5O_6$ 575.37	576.349	4.01 (4)	0.99 (1)	—	—	30.6[c]

[a] Elution conditions are: column Jupiter C18 (10 μ, 250 × 4.6 mm); eluents (A) 0.05% TFA in H_2O, (B) 0.05% TFA in 9 : 1 CH_3CN/H_2O; flow rate 1 ml/min; detection at 216 nm.
[b] Isocratic elution at 5% B for 3 min, and then linear gradient from 5 to 50% B in 39 min.
[c] Isocratic elution at 15% B for 3 min, and then linear gradient from 15 to 45% B in 35 min.

Treatment with either 95 or 50% TFA solution (methods **A** or **B**) typically results in a complete or almost complete peptide fragmentation. Partial fragmentation was also observed on leaving the peptide in aqueous acidic solution for a very long period (method **E**). On the contrary, the peptide was stable

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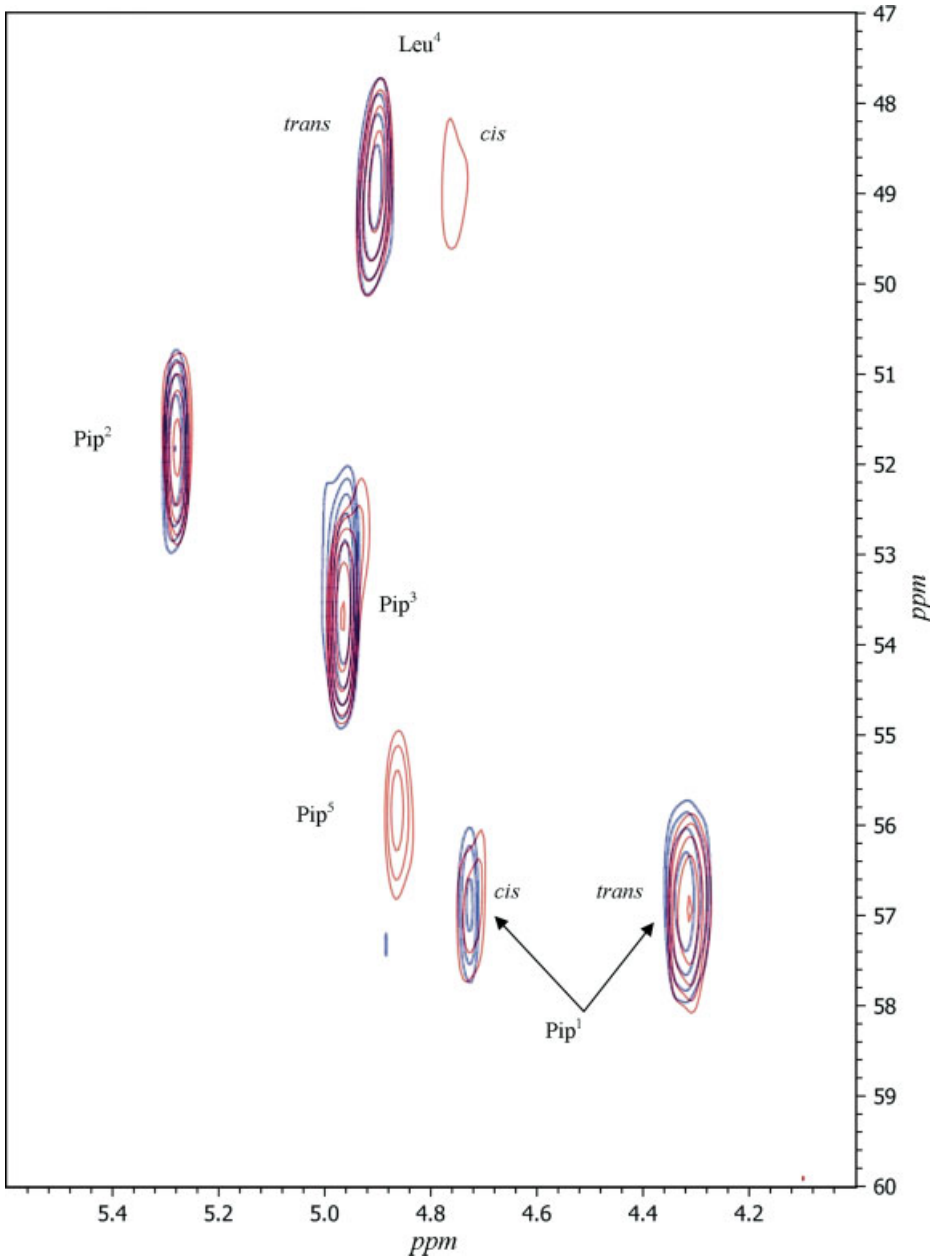


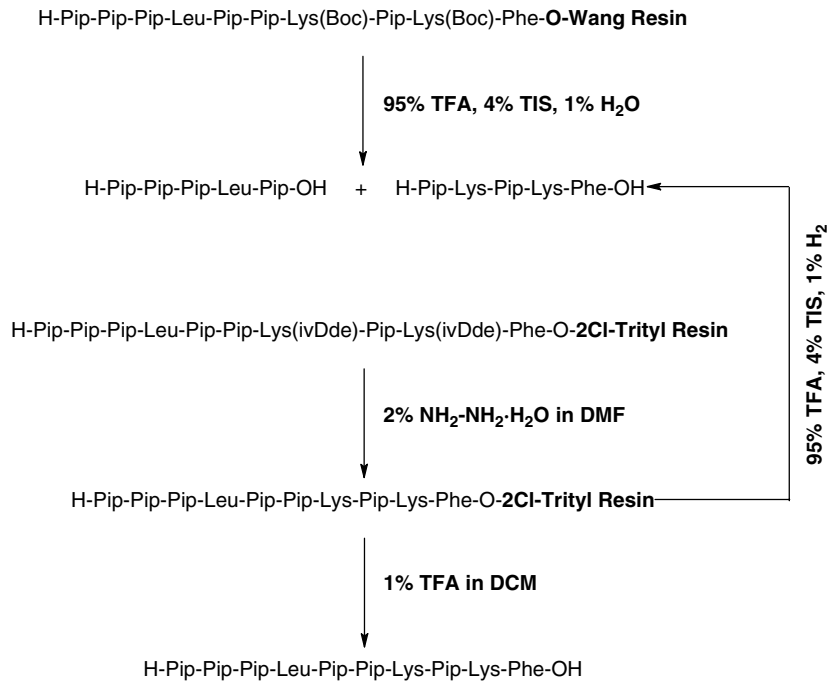
Figure 3 C^{α}-region of the ^{1}H, ^{13}C-HMQC spectra of Pip-rich peptide fragment **2b** obtained by treatment either with TFA (red) or with TFA-*d* (blue).

to treatment with both dilute TFA (method **C**) and 0.1 M HCl for a shorter time (method **D**). In all cases, the number of peptide fragments did not increase when a longer period of cleavage was used. Very similar results were obtained treating the resin-bound peptide using **A, B**, or **C** methods.

To study the mechanism of this amide bond cleavage reaction *ca* 15 μmol of Pip-rich peptide was treated with deuterated TFA-*d*. Hydrolysis catalyzed by TFA-*d* resulted in deuterium incorporation into both the diastereoisomeric *N*-terminal fragments (compounds **2b** and **2c**) (Figure 2), while the *C*-terminal fragment (compound **2a**) did not incorporate any deuterium atom. The comparison of the heteronuclear ^{1}H, ^{13}C-HMQC NMR spectra (Figure 3) of compound **2b** afforded by the treatment with either TFA or TFA-*d*, localized the deuterium atom on the α-carbon of the Pip5 residue supporting an almost complete deuterium exchange. This evidence indicates that the mechanism for TFA catalyzed hydrolysis of the amide bond occurs via an oxazolonium ion intermediate [13] and not from the conventional tetrahedral AAC2 mechanism [14].

As indicated in Scheme 2, we propose that the mechanism for acidolysis proceeds via formation of an intramolecular tetrahedral intermediate. This oxazolone-like intermediate is obtained from a species which is protonated at the Pip5 residue by nucleophilic

DOI: 10.1002/psc



Scheme 2 Products of TFA treatment of Pip-rich peptide **2** anchored either to Wang or 2-Cl-Trt resins.

attack of the adjacent carbonyl group of the Leu4 residue. Once the tetrahedral intermediate is formed, the lone-pair electrons on the nitrogen of Pip6 are no longer in conjugation with the carbonyl π-bond of the preceding residue. As an amine-like structure, the nitrogen of the Pip6 residue becomes a proton acceptor. Thus, the peptide fragment 6–10 is ejected, and the system collapses to an oxazolonium ion intermediate that immediately reacts with water. The direct hydrolysis of the keto-form to give the *N*-terminal fragment is found to be much slower than tautomerization to the enol-form as evidenced by H/D exchange.

This proposed mechanism (Scheme 3) is in sharp contrast with the observed acid-catalyzed hydrolysis of the homologous proline residue, which activates and cleaves preferentially at the *N*-terminal amide bond, while it is similar to that described for the acid-catalyzed hydrolysis of peptides containing consecutive *N*-alkylated residues proposed by Goodman [10] and Urban [15], respectively, and involving the activation and the cleavage of the amide bond on the *C*-terminal side of the *N*-alkylated residue. Mechanistic investigations carried out by these authors led them to postulate the acidolytic formation of an oxazolonium species, which would then undergo hydrolytic ring opening in the presence of trace of water in the reaction solvent.

This facile peptide bond cleavage arises mainly from the relatively favorable formation of a *cis* peptide bond characteristic of the Xaa–Pip peptide sequence, which brings parts of the *cis*-isomer in close proximity leading to tetrahedral cyclic intermediates. Indeed, the Xaa-Pip peptide bond shows a high tendency to adopt a *cis* conformation largely due to the destabilization of the *trans* form by unfavorable steric interactions between the ε position of the pipecolic acid and the α position of the preceding residue [16].

With the aim to examine the factors affecting the position of peptide bond hydrolysis, we synthesized the corresponding per-acetylated Pip-rich peptide analog (peptide **3**). ESI-MS analysis of the product obtained by acidolysis with TFA-H_2O-TIS revealed that the peptide underwent fragmentation not only between Pip5 and Pip6, but also between Pip1 and Pip2, as showed by the presence of both 2–10 (**3a**) and 6–10 (**3b**) fragments (Figure 4). In addition, the presence of the fragment 2–5 (**3c**) in the ESI-MS spectrum (Figure 4) suggested that the acetylated fragment 1–5 underwent an additional hydrolysis at the amide bond between Pip1 and Pip2.

Apparently, the hydrolysis position is mainly affected by the nature of the substituent on the *N*-terminal side of the activated pipecolic acid. The important factor here is the charge density on the oxygen of the carbonyl of the activable Pip residue that is strictly related to the inductive effect of the whole molecule on this carbonyl. Thus peptides in which this residue is preceded by one additional amino acid show different behavior depending on the *N*-terminal acylation. Peptides with free (protonated) *N*-terminus (-I effect) are completely stable, while peptides with acetylated *N*-terminus are readily hydrolyzed. Comparison of the data obtained by TFA treatment of the derivative of Pip-rich peptide indicated that this inductive effect involved amino acid residues until the third position from the *N*-terminus;

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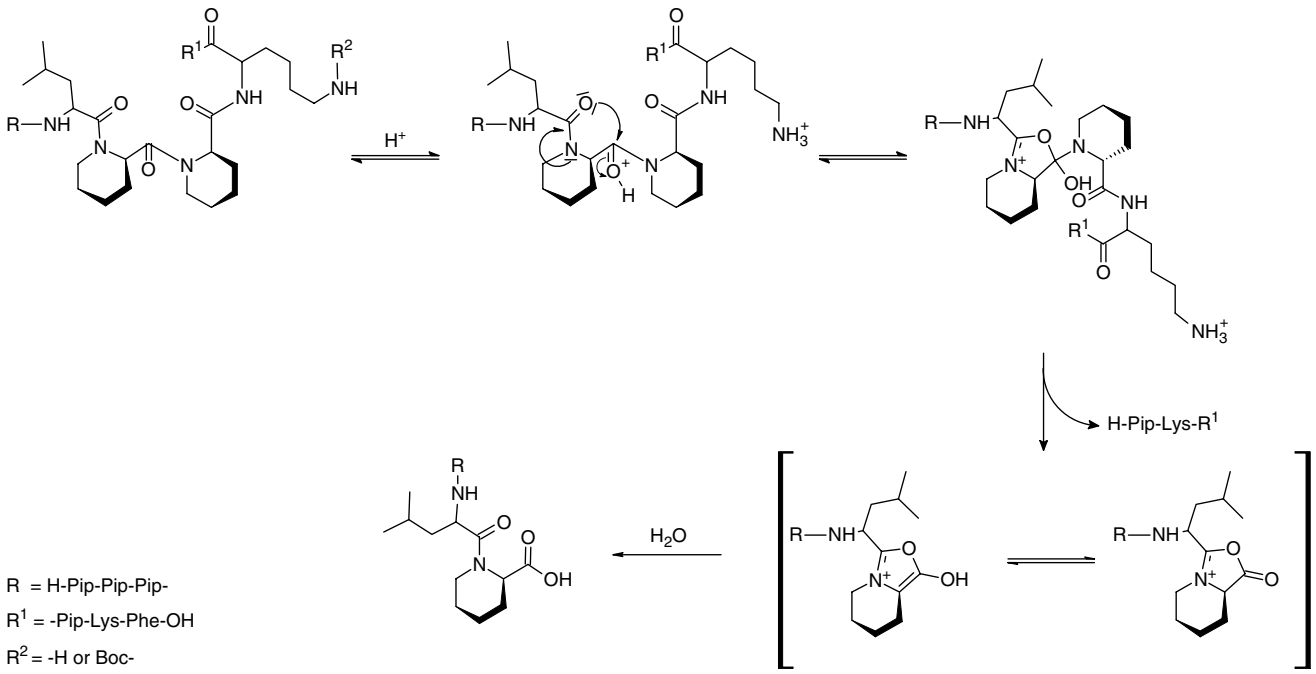

Scheme 3 Proposed mechanism of TFA catalyzed hydrolysis of Pip-rich peptide.

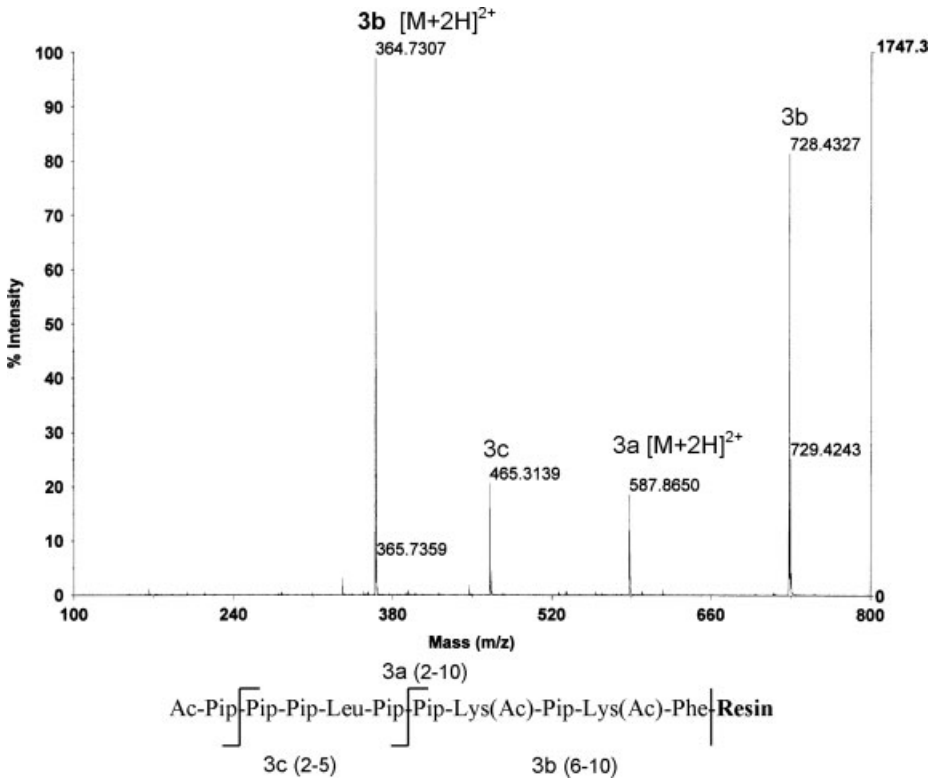

Figure 4 ESI-MS mass spectrum of crude per-acetylated Pip-rich peptide **3** after TFA treatment.

consequently, the first activable residue in the Nα-free peptide is the pipecolic acid in the 5 position.

Our results demonstrated that under standard TFA cleavage/deprotection conditions peptides containing consecutive pipecolic acid residues undergo facile hydrolysis at the amide bond adjacent to the *C*-terminus of the activable pipecolic residue. The mechanism, strongly supported by NMR and ESI-MS experimental data, involved the presence of an oxazolonium ion intermediate. On the other hand, the extremely fast hydrolysis rate of the Pip-rich peptide with respect to the stability of analogs containing either

DOI: 10.1002/psc

NMeAla or Sar residues instead of Pip (data not shown) emphasizes on the steric effect of the side-chain of the pipecolic residue on the peptide bond conformation and consequently on the hydrolysis rate.

EXPERIMENTAL PROCEDURES

General Methods for Peptide Synthesis and Purification

Protected amino acids, coupling reagents, and preloaded resins were obtained by Calbiochem-Novabiochem (Läufelfingen, Switzerland). Deuterated solvents and reagents were obtained from Aldrich (Milwaukee, WI, USA).

Solid phase peptide elongation was carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Synthesis of peptides was carried out with 0.10 mmol of either Fmoc–Phe–Wang resin (0.68 mmol/g) or H–Phe–2-Cl-Trt resin (0.90 mmol/g). HATU activation employed a three-fold molar excess (0.40 mmol) of Fmoc-amino acids in DMF solution for each coupling cycle unless otherwise stated. Coupling yields were monitored on aliquots of peptide–resin either by the Kaiser test for the amino groups or by evaluation of Fmoc displacement.

Cleavage from the resin and deprotection were performed by treatment with TFA-TIS-H_2O (95 : 4 : 1 v/v). After 45 min the resin was removed by filtration, and the crude peptide was precipitated by addition of cold ethyl ether. Alternatively, the peptide–resin was treated with a mixture of TFA-anisole-EDT-H_2O (90 : 4 : 5 : 1 v/v) for 45 min and worked as previously described. Peptide synthesized on 2-Cl-Trt resin was first treated with 5 ml of 2% hydrazine monohydrate in DMF (2×3 min) to remove the *iv*Dde protecting groups. Peptide was then cleaved from the resin by treatment with 1% TFA in DCM (10 ml) at r.t. for 15 min. The solution was filtered into a flask containing 10% pyridine in methanol (2 ml). Treatment with 1% TFA was repeated four times, and the combined filtrates were concentrated under reduced pressure and the peptide was precipitated by addition of ethyl ether.

Per-acetylated peptide was obtained by treatment of Nα, Nε-free peptide–2-Cl-Trt resin (30 mg) with 3 ml of a mixture containing 0.95 ml of Ac_2O and 0.45 ml of DIEA, in 20 ml of dry DMF [17]. The reaction was allowed to proceed for 45 min at room temperature, and then the per-acetylated peptide–resin was washed with DMF and DCM, and treated with TFA-TIS-H_2O as previously reported.

Treatment of Pip-rich peptide with TFA-*d* was performed using a mixture of 98% TFA-*d* in the presence of D_2O-d_2, and then the mixture was worked as reported.

Peptides were purified by preparative RP-HPLC using a Shimadzu model LC-8 system with a Vydac 218TP1022 column. RP-HPLC analyses were performed on a Shimadzu liquid chromatography model LC-10 fitted with a Jupiter C18 column (10 μ, 250 × 4.6 mm).

Molecular weights were determined by ESI-MS on a Mariner (PerSeptive Biosystem) mass spectrometer.

The amino acid composition in the acid hydrolysates was determined by a Carlo Erba 3A30 amino acid analyzer.

NMR experiments. NMR samples were prepared by dissolving appropriate amounts of compound **b** (isolated by preparative HPLC after treatment with either TFA or TFA-*d*, respectively) in D_2O-d_2 (100% isotopic purity, Aldrich) to approximately 2-mM solutions.

All spectra were run on a 600 MHz Bruker DMX spectrometer equipped with a gradient triple resonance probe. Proton and carbon chemical shifts, in parts per million (ppm) are referenced to the DSS internal standard [18]. All NMR experiments have been carried out at a temperature of 298 K, using the BVT2000 temperature control unit.

One-dimensional (1D) NMR spectra were acquired using typically 16–32 scans with 32K data size. For the two-dimensional (2D) experiments, pulse programs of the standard Bruker library were used. All 2D experiments were acquired in the phase-sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increments (TPPI). Typically 512 experiments of 48 scans each were performed: relaxation delay 1 s; size 2K; 6024 Hz spectral width in F2; zero filling to 1K in F1; square cosine or gaussian multiplication was used in both dimensions before the Fourier transformation. Mixing time of 75 ms was used for TOCSY. ROESY experiments were run at mixing times of 350 ms.

Heteronuclear HMQC experiment was used for the assignment of α-carbon resonances as well as for the evaluation of deuterium substitution. Heteronuclear spectra were obtained by recording 240 experiments of 600 transients each. A relaxation delay of 1.0 s was allowed after each acquisition. The spectral widths were 5430 and 16 000 Hz in proton and in carbon dimension, respectively.

Supplementary Material

Supplementary electronic material containing the sequences of the fragments obtained by TFA treatment of peptide **2** and per-acetylated derivative (peptide **3**), NMR data of peptide **2b**, ESI-MS spectrum of TFA degradation of peptide **2** in presence of EDT is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

DOI: 10.1002/psc

Acknowledgement

This work was supported by a grant from the National Research Council (CNR) of Italy.

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