MODIFICATION, PERMETHYLATION AND MASS SPECTROMETRY OF ARGININE-CONTAINING OLIGOPEPTIDES AT THE 100 NANOMOLAR LEVEL

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SUMMARY

The amino acid sequence of arginine-containing oligopeptides, including bradykinin, has been determined by mass spectrometry. The guanidino groups of the arginine residues in 100 nmoles of peptide were reacted with acetylacetone under mild conditions to produce N^{δ}-2-(4,6-dimethyl)pyrimidylornithine residues. N-Acetylation and N,O-permethylation then produced peptide derivatives that were suitable for vaporization. This procedure makes arginine-containing oligopeptides accessible for mass spectrometric sequence analysis.

Sequence analysis of oligopeptides by mass spectrometry has been substantially facilitated by N,O-permethylation (1) utilizing methyl iodide and sodium methylsulfinylmethide. This procedure has been refined recently (2) to be applicable to peptides containing residues of all the usual amino acids, including cysteine (3), methionine (4) and histidine (5), with the single exception of arginine. The mass spectra of acetylated and permethylated arginine-containing peptides contained sequence information from the N-terminus up to, but neither including nor beyond the arginine residue (6).

To circumvent this difficulty, three approaches are applicable: (a) enzymatic hydrolysis with trypsin to produce peptides which have either C-terminal arginine or lysine, (b) conversion of arginine to ornithine by hydrazinolysis, and (c) condensation of the guanidino group with mono- or dicarbonyl compounds.

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For the sequencing of unknown peptides by mass spectrometry, condensation reactions are best suited because a unique derivative of arginine is produced. β-Dicarbonyl compounds such as 1,1,3,3-tetraalkoxypropane (7) and acetylacetone (7-9) have been used. Shemyakin et al. (7), and Bacon et al. (9) employed reaction conditions under which peptide bond cleavage is possible: alcoholic HCl. Vetter-Diechtl et al. (8) utilized milder conditions to convert arginine, 175-600 mg, to Orn (DMP)*.

Thomas et al. (6) stated that "permethylation of (the resulting) basic pyrimidylornithine derivative would form an undesirable quaternized salt," and removed the guanidino group by selective hydrazinolysis, a procedure which also cleaves peptide bonds.

Permethylation of the 1,2-cyclohexanedione condensation product of argininyl peptides (1-3 mg) was accomplished by Lenard and Gallop (10), with methyl iodide and silver oxide as catalyst. They reported sequence information of the four residues of bradykinin from both the N- and the C-termini. This is the only report to date on the successful permethylation of modified arginyl peptides.

We wish to report on the modification and permethylation of arginyl peptides at the 100 nmolar level, using acetylacetone for the arginine modification and methyl iodide/methylsulfinylmethide carbanion for permethylation.

MATERIALS AND METHODS

The commercial sources of the compounds utilized in these experiments are: Arg·HCl·H_2O, Nutritional Biochemicals; Arg-Arg·2HAc (F2050), Fox Chemical Co., Los Angeles; Ac-Ser-Arg-His-Pro·HAc·H_2O (M-3265), Cyclo Chemical Co.,; bradykinin·3HAc·H_2O (50C-0490), Sigma Chemical Co.; 1,1,1,5,5,5-hexafluoroacetylacetone, PCR, Inc., Gainesville, Fla.; sodium hydride (57%

* Abbreviations used are: Ac = acetyl, TFA = trifluoroacetyl, OMe = methoxy, Orn(DMP) = N^{δ}-2-(4,6-dimethyl)pyrimidylornithine, and the standard amino acid abbreviations.

dispersion in mineral oil), Ventron Chemical Co., Beverly, Mass. Other chemicals were reagent grade obtained from a variety of suppliers.

The Formation of Orn(DMP) from Arg.

The procedure reported by Vetter-Diechtl et al. (8) was utilized with the following modifications. To 100 nmoles of each peptide, 10 μl 10% $NaHCO_3$, 20 μl 95% ethanol and 20 μl $CH_3COCH_2COCH_3$ were added and the mixture heated at 100° C for four hours. The reaction appeared complete at this point, judged by paper chromatography. In order to hydrolyze the Schiff bases, 1.0 ml 0.5 M CH_3COOH was added and the reaction mixture heated an additional ten minutes. The self-condensation product of acetylacetone, 3,5-dimethyl-2-acetylphenol, was removed by ether extraction and the aqueous phase was dried in vacuo over P_2O_5.

Acylation and N,O-Permethylation

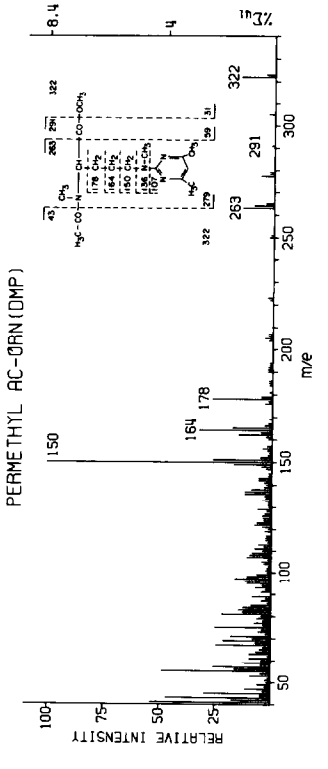
To avoid quaternization during permethylation (6), the free amine groups of the modified peptides were acetylated first (2). To prevent possible acyl migration (11) under the conditions necessary for N^{α}-acetylation of bradykinin, trifluoroacetylation was done in aqueous medium (12). The modified peptide was dissolved in 50 μl 2% Na_2CO_3 and reacted with 5 μl $CF_3COSC_2H_5$ at 45° C for two hours. C_2H_5SH was removed under nitrogen in the hood and the reaction mixture dried as before.

The optimal reaction conditions for permethylation previously described were employed (2).

Low resolution mass spectra were obtained on an LKB 9000 mass spectrometer with sample introduction by direct probe into the ion source. The ionizing electron energy was 70 e V, the ionizing current 60 μA, and the accelerating voltage 3.5 k V. Ion source temperature was 250° C and the probe temperature varied from 85° C to 220° C.

RESULTS

The mass spectra of the derivatized Arg, Arg-Arg, Ser-Arg-His-Pro, and



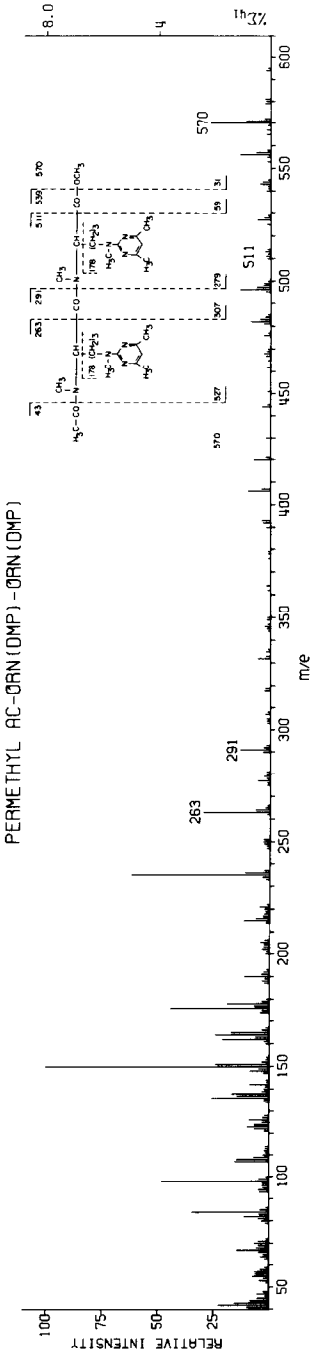


Figure 1 (upper) Mass spectrum of N-permethyl Ac-Orn(DMP)-OMe. (85° C)

Figure 2 (lower) Mass spectrum of N-permethyl Ac-Orn(DMP)-Orn(DMP)-OMe. (185° C)

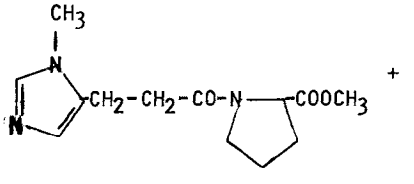
bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) are given in Figures 1-4, respectively.

In all spectra, fragments from the side chain (R) of Orn(DMP) dominate the spectra. These ions are found at m/e 107, 136, 150, 164, and 178 (R). The genesis of these ions is indicated in the fragmentation scheme in Figure 1. Frequently, ions occur at m/e R+12 and R-2.

The spectrum and fragmentation scheme of derivatized arginine are given in Figure 1. The molecular ion (M) is found at m/e 322, M-OMe at m/e 291 and M-COOMe at m/e 263.

Figure 2 contains the spectrum and fragmentation scheme of derivatized Arg-Arg. The sequence is defined by the occurrence of peaks at m/e 263, 291, 511 and 570 (M), corresponding to N-terminal fragments. Fragments from the C-terminus are found at m/e 279 and 527. The molecular ion of the diketopiperazine is found at m/e 496. Ions at m/e 482 and 556 result because of incomplete methylation of the diketopiperazine and the dipeptide, respectively. The ion at m/e 235 corresponds to the unmethylated fragment $[DMP\text{-}NH\text{-}(CH_2)_2\text{-}CH{=}CH\text{-}COOCH_3]^+$, occurring via N - C^{α} cleavage of the C-terminal arginine. The loss of portions of R from the dipeptide are observed at m/e 420 (M-150), 406 (M-164), and 392 (M-178). Corresponding fragments from the diketopiperazine are found at m/e 346, 332 and 318, respectively.

The mass spectrum and fragmentation scheme of derivatized Ser-Arg-His-Pro are given in Figure 3. The sequence is defined by the series of peaks occurring at m/e 130, 158, 378, 406, 543, 571, 668 and 699 (M). All these N-terminal sequence peaks are accompanied by satellite peaks 32 mass units lower due to the loss of methanol from the methylated serine residue. The ions at m/e 293 and 541 correspond to fragments from the C-terminus. The peak at m/e 221 is ascribed to $H_3\overset{+}{C}\text{-}NH{=}CH\text{-}(CH_2)_3\text{-}N(CH_3)\text{-}DMP$ and the ion at m/e 265 to



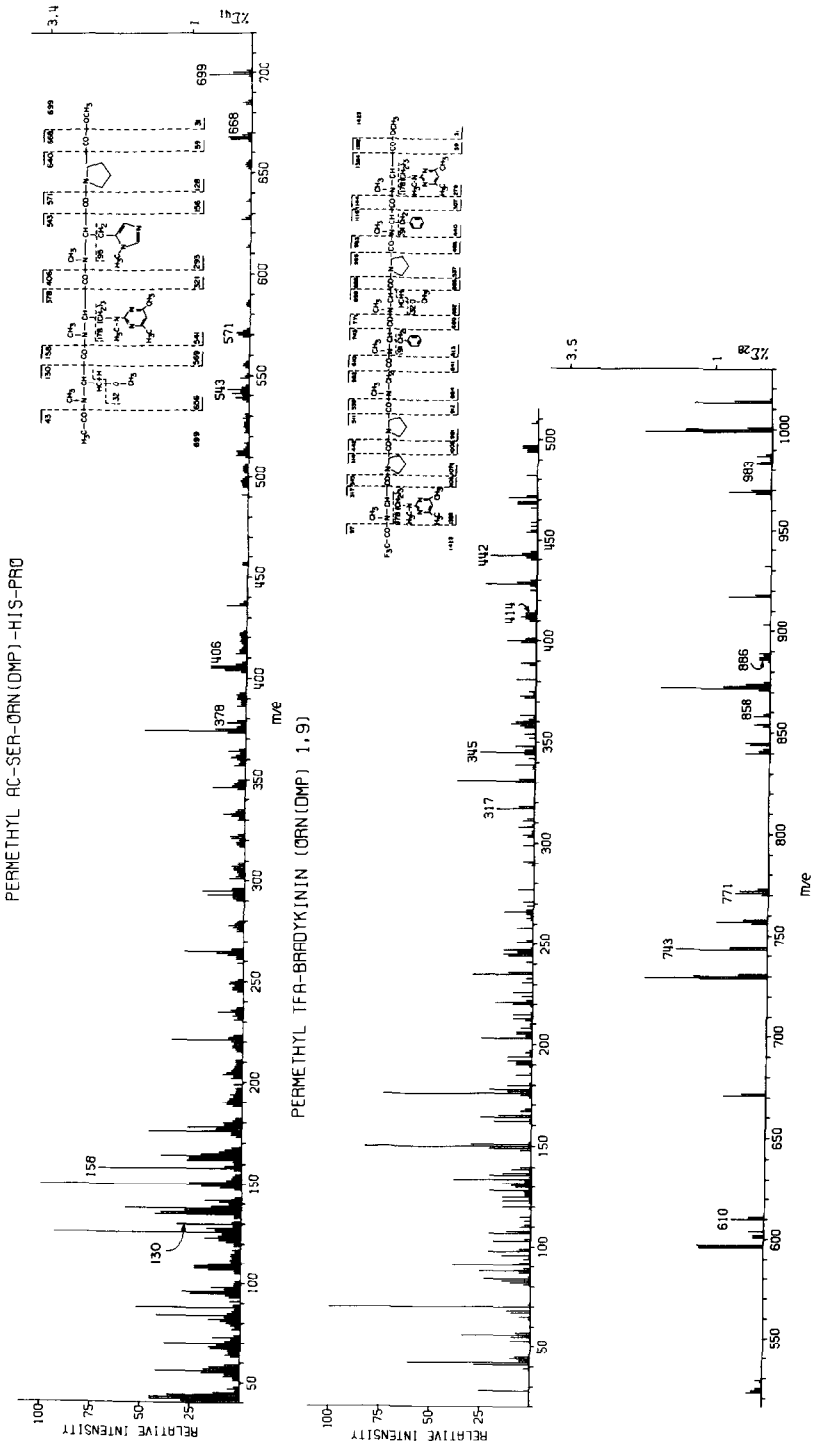


Figure 3 (upper) Mass spectrum of N,O-permethyl Ac-Ser-Orn(DMP)-His-Pro-OMe. (200° C)

Figure 4 (lower) Mass spectrum of N,O-permethyl TFA-Orn(DMP)1,9-bradykinin. (220° C)

The N - C^{α} cleavage of the latter case is common when there is an aromatic group in the side chain (13). The C-terminal fragments at m/e 511 and 627 arise also from N - C^{α} cleavage. Figure 3 also contains the superimposed spectrum of incompletely methylated tetrapeptide.

The mass spectrum and the fragmentation pattern of derivatized bradykinin are given in Figure 4. "Sequence peaks" corresponding to N-terminal fragments up to the eighth residue are found at m/e 317, 345, 414, 442, 610, 743, 771, 858, 886, 983. The peak at m/e 1013 is assigned to the N-terminal fragment resulting from N - C^{α} bond cleavage at the aromatic residue Phe^{8} (13). All these peaks have abundant satellites 14 mass units lower, indicating incomplete methylation in the first residue. No C-terminal "sequence peaks" are found. C-Terminal fragment ions corresponding to N - C^{α} cleavage of the Phe^{8} and $Orn(DMP)^{9}$ bonds occur at m/e 412 and 235, respectively. The genesis of the peaks at m/e 671 and 917 is under investigation. However, the lack of abundant satellite peaks 14 mass units lower indicates that the N-terminus is not present in the ions corresponding to these two peaks.

DISCUSSION

The expected usefulness of the dimethylpyrimidine moiety *in lieu* of the polar guanidino group of arginine in peptides has been demonstrated by the mass spectra presented above. The behavior of the modified arginine residue upon electron impact is very similar to that of aromatic amino acid residues, particularly with respect to N - C^{α} cleavage and the intensity of side chain fragments. Possible quaternization (6) of the pyrimidyl group has been avoided under the controlled conditions of our permethylation procedure (2).

In all cases a superimposed mass spectrum of incompletely methylated material was noted. Apparently the N^{δ}-position of the Orn(DMP) residue is difficult to methylate. In the case of bradykinin, the electron withdrawing power of the TFA group may prevent methylation of the N^{α}-group as well. The TFA-bradykinin was considerably more volatile than the N^{α}-acetyl derivative. In an attempt to increase the volatility further, the guanidino group was

derivatized with $CF_3COCH_2COCF_3$. The mass spectra of these derivatives were inferior to those we have presented.

The dimethylpyrimidine derivative of arginine makes all natural amino acid residues accessible to our general technique for acylation and permethylation of oligopeptides on the 100 nanomolar level.

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