Differences in the Formation and Fragmentation of Sodium Adduct Ions between Tertiarybutoxycarbonyl-Protected Prolylproline Diastereomers in Fast Atom Bombardment Mass Spectrometry

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Received February 24, 1999; accepted April 20, 1999

The effect of Na^+ ions on the fragmentation of tertiarybutoxycarbonyl (Boc) protected prolylproline (Pro-Pro) diastereomers, Boc-Pro-Pro and Boc-D-Pro-Pro, was studied in positive-ion fast atom bombardment (FAB) and tandem mass spectrometry. The formation of the $[M+Na]^+$ ion for Boc-D-Pro-Pro was more predominant than that for Boc-Pro-Pro on the addition of sodium chloride in positive-ion FAB mass spectrometry, suggesting that Boc-D-Pro-Pro has a stronger Na^+ ion affinity than Boc-Pro-Pro. In the collisional-activated decomposition mass spectra of the $[M+Na]^+$ ions, the abundance of the $[M+Na-C(CH_3)_3+H]^+$ ion, which is due to the loss of a tertiarybutyl group from the $[M+Na]^+$ ion for Boc-D-Pro-Pro, was higher than that for Boc-Pro-Pro. These results indicate that the interaction of the Na^+ ion with Boc-Pro-Pro is different from that with Boc-D-Pro-Pro in the FAB condition, and these diastereomers are distinguished by the addition of a Na^+ ion in FAB and tandem mass spectrometry.

Key words prolylproline; diastereomer; peptide; sodium ion; FAB MS/MS

Proline is the only imino acid among the 20 common L-α-amino acids. It is found in many biologically active peptides and proteins, and it plays an important role in their three-dimensional structure.[1,2)]

We have reported significant differences in negative-ion fast atom bombardment (FAB) and collisional-activated decomposition (CAD) mass spectra among the intensities of the fragment ions formed by cleavage of the benzyloxycarbonyl (Z)-group from the Z-protected tri- and tetrapeptides containing Pro, depending on the number and positions of the prolyl residues.[3—6)] This indicates that the conformational difference in the tri- and tetrapeptide derivatives due to the existence of Pro influences the fragmentation of the peptide molecules in FAB and tandem mass spectrometry. It was also reported that the conformation of synthetic cyclic peptides containing D-Pro-Pro was studied by NMR, and this sequence plays a significant role in the secondary structure of the peptides.[7)] These results prompted us to examine the fragmentational differences between the diastereomeric dipeptides, Pro-Pro and D-Pro-Pro in FAB mass spectrometry. Then, we measured the FAB and CAD mass spectra of the $[M+H]^+$ and $[M+Na]^+$ ions for Pro-Pro and D-Pro-Pro. As a result, similar cleavage patterns were obtained for these two diastereomers. It is therefore impossible to distinguish them by FAB and tandem mass spectrometry.

E. Mammoliti *et al*. reported *S,S* and *R,S* isomeric differentiation for Z-protected dipeptides by high-energy FAB mass-analyzed ion kinetic energy studies.[8)] In addition, B. L. Schwartz *et al*. showed the differentiation between LLL and DLL diastereomer pairs of the tripeptides by tandem mass spectrometry on a hybrid tandem mass spectrometer.[9)] When we synthesized the tertiarybutoxycarbonyl (Boc)-protected Pro-Pro and D-Pro-Pro, and confirmed their chemical structures in positive-ion FAB mass spectrometry, we found a significant difference in the formation of the $[M+Na]^+$ ions between each on the addition of sodium chloride. Our finding suggests differences in affinity for Na^+ ions between these diastereomers.

In this paper, we report differences in the formation of the $[M+Na]^+$ ions in positive-ion FAB mass spectrometry and in CAD of the $[M+Na]^+$ ions between Boc-Pro-Pro and Boc-D-Pro-Pro on the addition of sodium chloride, and show that the Na^+ ion is effective for differentiation between a pair of Boc-Pro-Pro diastereomers in FAB mass spectrometry.

Experimental

Materials and Methods Boc-Pro and Boc-D-Pro were obtained from the Protein Research Foundation (Osaka, Japan). The ethyl ester hydrochloride of Pro (Pro-OEt HCl) was prepared using thionyl chloride and absolute ethanol.[10)] All chemicals were of either analytical or reagent grade. The purity of each compound was checked by thin-layer chromatography on Kiesel gel plates 60 PF_{254} (Merck, Darmstadt, Germany) with chloroform–methanol–ethyl acetate (4 : 3 : 1, v/v) and chloroform–ethyl acetate (10 : 1, v/v) as developing systems. All melting points were uncorrected. The chemical structures of the synthesized compounds were confirmed with the aid of FAB mass spectrometry. ^{1}H-NMR spectra were measured at 500 MHz with a Varian UNITY-plus 500 system. The results of elemental analyses obtained for C,H and N were within $\pm 0.3\%$ of the theoretical values.

Synthesis of Boc-Pro-Pro Diastereomers Boc-Pro-Pro-OEt was synthesized by coupling Boc-Pro with Pro-OEt HCl using dicyclohexylcarbodiimide (DCC) as a coupling reagent. Boc-Pro-Pro was prepared by the saponification of Boc-Pro-Pro-OEt. This product was recrystallized from methanol. mp 186—187 °C, *Anal*. Calcd for $C_{15}H_{24}N_2O_5$: C, 57.68; H, 7.75; N, 8.97. Found: C, 57.70; H, 7.66; N, 8.84. Positive-ion FAB mass spectrometry: *m/z* 313 ($[M+H]^+$). In the ^{1}H-NMR, the positions of protons in Boc-Pro-Pro diastereomers were indicated in Chart 1 using the alphabet (a—l). ^{1}H-NMR (DMSO-d_6) δ: 1.29—1.37 (9H, H-a), 1.70—1.87 (4H, m, H-e, f, j, k), 1.90—1.97 (2H, m, H-e, j), 2.06—2.23 (2H, m, H-f, k), 3.27—3.38 (2H, m, H-d, i), 3.46—3.65 (2H, m, H-d, i), 4.24 (1H, dd, J=8.8, 4.2 Hz, H-l), 4.35—4.42 (1H, m, H-g), 12.4 (1H, br s, –COOH).

Boc-D-Pro-Pro was prepared as described above for Boc-Pro-Pro. mp 196—197 °C, *Anal*. Calcd for $C_{15}H_{24}N_2O_5$: C, 57.68; H, 7.75; N, 8.97. Found: C, 57.68; H, 7.69; N, 8.82. Positive-ion FAB mass spectrometry: *m/z* 313 ($[M+H]^+$). ^{1}H-NMR (DMSO-d_6) δ: 1.30—1.38 (9H, H-a), 1.70—1.86 (4H, m, H-e, f, j, k), 1.87—2.00 (2H, m, H-e, j), 2.05—2.22 (2H, m, H-f, k),

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3.24—3.35 (2H, m, H-d, i), 3.38—3.62 (2H, m, H-d, i), 4.16 (1H, dd, J=8.7, 3.8 Hz, H-l), 4.40—4.54 (1H, m, H-g), 12.5 (1H, br s, –COOH).

The diastereomeric purity of Boc-Pro-Pro and Boc-D-Pro-Pro was ascertained by that of Boc-Pro-Pro-dopamine (DA) and Boc-D-Pro-Pro-DA using HPLC methods. Boc-Pro-Pro-DA diastereomers were synthesized by coupling Boc-Pro-Pro diastereomers with DA by using DCC and 1-hydroxybenzotriazole. These compounds were under investigation as prodrug candidates which evade the first-pass metabolism of DA in gastrointestinal absorption. Synthesis of these compounds will be reported in detail elsewhere. The HPLC system (Shimadzu LC-6A) consisted of an ultraviolet (UV) spectrometric detector (SPD-6A), chromatopac (C-R6A) and system controller (SCL-6B). The column used was Shimpack (Shimadzu CLC-ODS(M), 4.6 mm i.d.×25 cm) and the mobile phase was a methanol/phosphate buffer (0.1 M, pH 3.0) containing 1-hexanesulfonic acid sodium salt (5 mM) and EDTA (0.08 mM) (50/50, v/v). The UV detector was set at 280 nm and the flow rate was 0.8 ml min^{-1} at 40 °C. The retention times of Boc-Pro-Pro-DA and Boc-D-Pro-Pro-DA were 10.8 and 12.7 min, respectively. As a result, the diastereomeric purity of Boc-Pro-Pro-DA diastereomers was found to be more than 99%. These results confirmed the diastereomeric purity of Boc-Pro-Pro diastereomers.

Measurement Conditions for FAB and Tandem Mass Spectrometry All mass spectra were acquired with a JEOL SX/SX102A tandem mass spectrometer of BEBE geometry, which was controlled by a JEOL DA-7000 data system (Tokyo, Japan). The positive-ion FAB mass spectra were obtained using only the first spectrometer. The samples were diluted in methanol at a concentration of 1 μg/μl. The solution (1 μl) was then subjected to analysis. *m*-Nitrobenzylalcohol (*m*-NBA) and *m*-NBA saturated with sodium chloride were used as a matrix. The ions were produced by bombardment with a neutral xenon atom at 5 kV. The mass range (*m*/*z* 1—1000) was scanned for 5 s under an ion source accelerating potential of 10 kV, and the averaged peak intensities were recorded in decade scans. The pseudo-molecule ions generated by FAB mass spectrometry were selected as precursor ions, and were then collided with argon molecules in the third field-free region. The argon pressure was sufficient to attenuate the primary ion beam by 50%. The fragment ions were dispersed by the second spectrometer and the spectra were recorded as CAD spectra.

Results and Discussion

FAB and CAD Mass Spectra for Boc-Pro-Pro and Boc-D-Pro-Pro A positive-ion FAB mass spectrum for Boc-Pro-Pro was compared with that for Boc-D-Pro-Pro. Figure 1(a) shows the mass spectrum for Boc-Pro-Pro. The fragment ion at *m*/*z* 213 ($[M+H-Boc+H]^+$) due to the loss of the Boc group from the $[M+H]^+$ ion was the base peak and a protonated molecule ion at *m*/*z* 313 was observed with high abundance. In addition, by the cleavage of the Boc group, an ion at *m*/*z* 257 due to the loss of a tertiary(*tert*)-butyl group and an ion at *m*/*z* 239 due to the loss of *tert*-butoxy group from the $[M+H]^+$ ion were observed with fairly high abundance. The abundance of the ion at *m*/*z* 335 ($[M+Na]^+$) was very low. The other major fragment ions were at *m*/*z* 114 ($[O(CO)NCH_2CH_2CH_2CH+H]^+$), 70 ($[N\text{-}CH_2CH_2CH_2CH+H]^+$) and 57 ($[C(CH_3)_3]^+$).

On the other hand, the cleavage pattern for Boc-D-Pro-Pro was similar to that for Boc-Pro-Pro (Fig. 1(b)). However, the abundance of the $[M+H]^+$ ion for Boc-D-Pro-Pro was almost as high as that for a fragment ion at *m*/*z* 257, though the former for Boc-Pro-Pro is twice as high as that for the latter. These results show that the $[M+H]^+$ ion for Boc-Pro-Pro was more stabilized than that for Boc-D-Pro-Pro under a FAB condition. Figure 2(a) shows the CAD mass spectrum of the $[M+H]^+$ ion for Boc-Pro-Pro. In this spectrum, an ion at *m*/*z* 257 was the base peak and an ion at *m*/*z* 213 was observed with fairly high abundance. These results indicate that these fragment ions were formed from the $[M+H]^+$ ion for this compound. A similar CAD spectrum was also obtained for Boc-D-Pro-Pro, indicating that no significant difference was found in the cleavage of the $[M+H]^+$ ions between these two diastereomers.

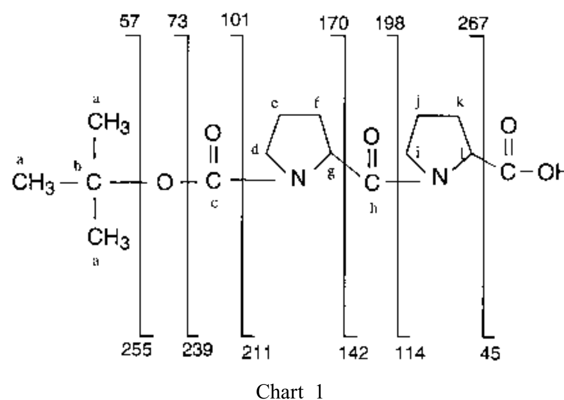

Chart 1

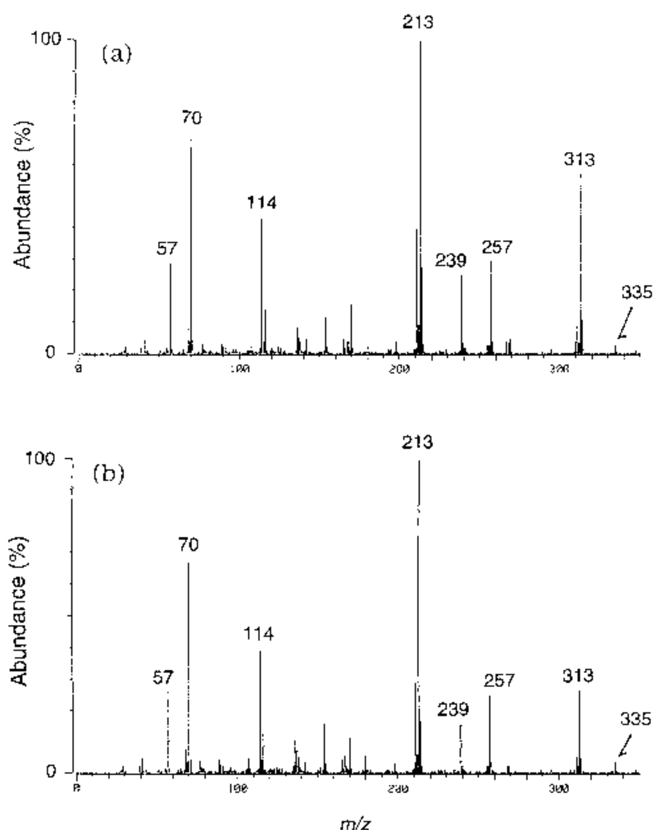

Fig. 1. Positive-Ion FAB Mass Spectra for (a) Boc-Pro-Pro and (b) Boc-D-Pro-Pro

FAB and CAD Mass Spectra for Boc-Pro-Pro and Boc-D-Pro-Pro on the Addition of Sodium Ion The formation of the $[M+Na]^+$ ion for Boc-Pro-Pro was compared with that for Boc-D-Pro-Pro on the addition of sodium chloride. In the positive-ion FAB mass spectrum for Boc-Pro-Pro, the ion at *m*/*z* 213 was the base peak and the $[M+Na]^+$ ion at *m*/*z* 335 was observed with almost the same abundance as that of the $[M+H]^+$ ion (Fig. 3(a)). The ion at *m*/*z* 235, which is assigned as a $[213+Na]^+$ ion, was also observed. On the other hand, in the spectrum for Boc-D-Pro-Pro, the $[M+Na]^+$ ion was the base peak and the abundance of this ion was about four times as high as that of the $[M+H]^+$ ion (Fig. 3(b)). These results imply that the affinity of the Na^+ ion for Boc-D-Pro-Pro is much stronger than that for Boc-Pro-Pro. Furthermore, the ratio of the abundance of the ion at *m*/*z* 235 to

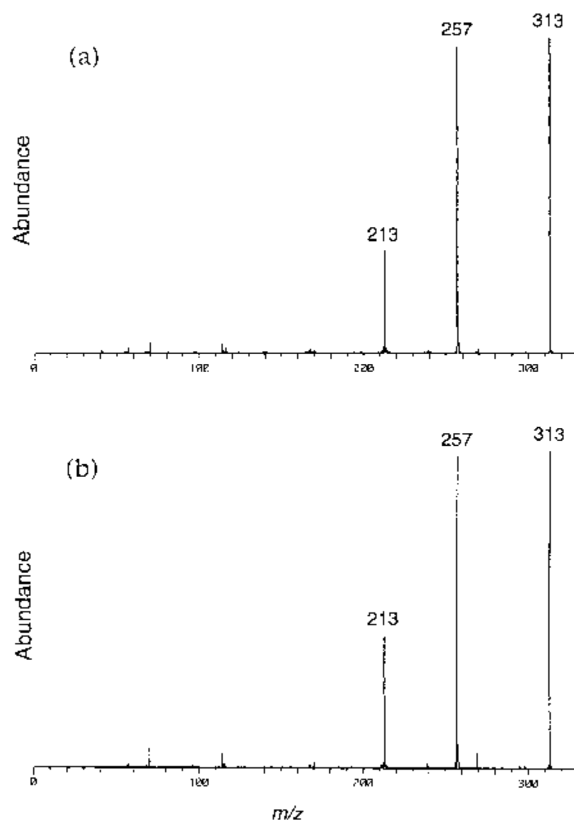

Fig. 2. FAB CAD Tandem Mass Spectra of the $[M+H]^+$ Ions for (a) Boc-Pro-Pro and (b) Boc-D-Pro-Pro

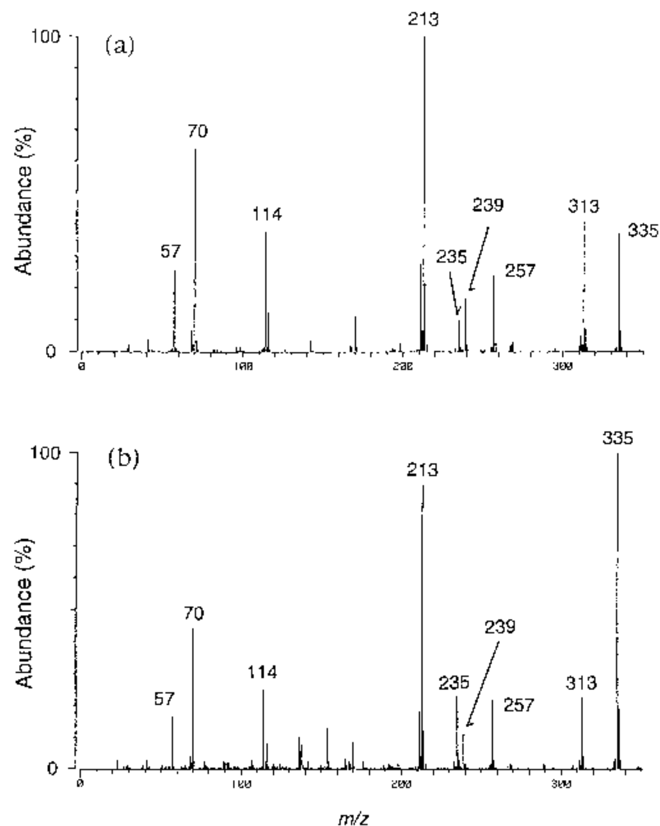

Fig. 3. Positive-Ion FAB Mass Spectra for (a) Boc-Pro-Pro and (b) Boc-D-Pro-Pro on the Addition of Sodium Chloride

the ion at *m*/*z* 213 for Boc-D-Pro-Pro was larger than that for Boc-Pro-Pro. These results indicate that the affinity of the Na^+ ion with the fragment ion for Boc-D-Pro-Pro was stronger than that for Boc-Pro-Pro. This difference may be due mainly to the conformational difference of the Boc-dipeptides induced by L- or D-Pro at the amino terminal of these peptide derivatives. It is therefore thought that Boc-Pro-Pro can be distinguished from Boc-D-Pro-Pro by the addition of a Na^+ ion in positive-ion FAB mass spectrometry.

In addition, in the CAD mass spectrum of the $[M+Na]^+$ ion for Boc-D-Pro-Pro, the ion at *m*/*z* 279 ($[M+Na-C(CH_3)_3+H]^+$), due to the loss of the *tert*-butyl group from the $[M+Na]^+$ ion, was observed in fairly high abundance, while in the spectrum for Boc-Pro-Pro, the abundance of this ion was very low (Fig. 4(a) and (b)). These results indicate that the elimination of the *tert*-butyl group from the $[M+Na]^+$ ion for Boc-D-Pro-Pro, which has a stronger Na^+ ion affinity than Boc-Pro-Pro under a FAB condition, is easier than that from Boc-Pro-Pro in the gas phase. It is therefore thought that the Na^+ ion is closer to the *tert*-butyl group in Boc-Pro-Pro than to that in Boc-D-Pro-Pro, and it prevents protonation to this site. Furthermore, the possibility that decarboxylation from the ion at *m*/*z* 279 for Boc-Pro-Pro may be easier than that for Boc-D-Pro-Pro in the gas phase cannot be ruled out.

It has been reported that the CAD spectra of the [M+(alkali metal)]$^+$ ions for the amino acid and peptide derivatives were distinguishable profiles from those of the $[M+H]^+$ ions, and the former was informative for the structural characterization of the molecules.[11—14] In our results, the $[M+H-C(CH_3)_3+H]^+$ ions were the base peak in the CAD spec-

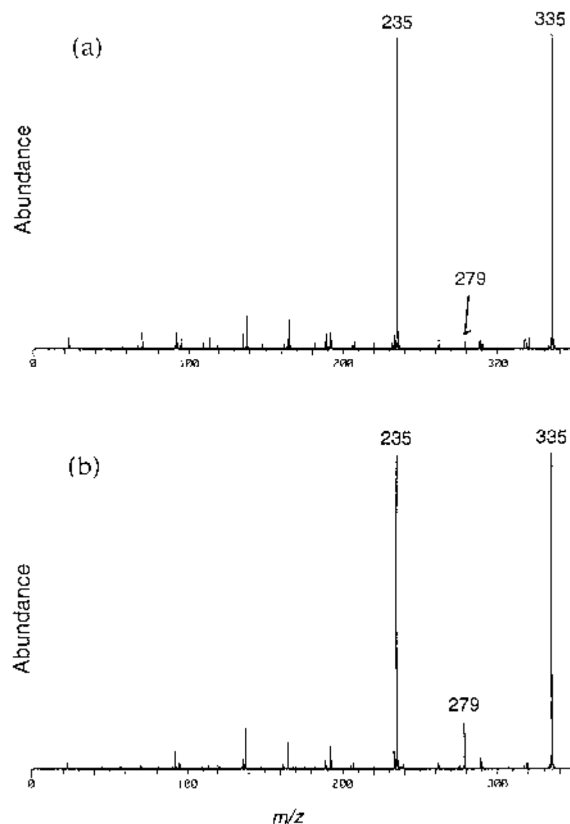

Fig. 4. FAB CAD Tandem Mass Spectra of the $[M+Na]^+$ Ions for (a) Boc-Pro-Pro and (b) Boc-D-Pro-Pro

tra of the $[M+H]^+$ ions for these two diastereomers (Fig. 2 (a) and (b)), while the $[M+Na-Boc+H]^+$ ions were the base peak in the spectra of the $[M+Na]^+$ ions (Fig. 4(a) and (b)). A significant difference was found between the CAD of the $[M+Na]^+$ ion and that of the $[M+H]^+$ ion for the Boc-protected Pro-Pro diastereomers. It is therefore thought that the cleavage of the *tert*-butyl-oxygen bond in Boc-Pro-Pro diastereomers is suppressed in the presence of a Na^+ ion in the gas phase.

The interaction of the Boc-dipeptide diastereomers containing amino acids except for Pro with a Na^+ ion is an interesting problem. At present, work along these lines is in progress in our laboratory.

Conclusion

A significant difference was found in the formation of $[M+Na]^+$ ions between Boc-Pro-Pro and Boc-D-Pro-Pro on the addition of a Na^+ ion in positive-ion FAB mass spectrometry. Furthermore, the CAD mass spectrum of the $[M+Na]^+$ ion for Boc-Pro-Pro is different from that for Boc-D-Pro-Pro. Therefore, Boc-Pro-Pro is discriminated from Boc-D-Pro-Pro by the addition of a Na^+ ion in positive-ion FAB and tandem mass spectrometry.

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