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# Stability of N-Derivatized and α-Methyl Analogues of Aspartame to Hydrolysis by Mammalian Cell-Surface Peptidases†

Nigel M. Hooper,*,‡ Richard J. Hesp,‡ Stephen Tieku,‡ Willy H. J. Boesten,§ Claudio Toniolo,∥ and Johan Kamphuis§

Department of Biochemistry and Molecular Biology, The University of Leeds, Leeds LS2 9JT, United Kingdom, Department of BioOrganic Chemistry, DSM Research, P.O. Box 18, 6160 MD Geleen, The Netherlands, and Biopolymer Research Centre, CNR, Department of Organic Chemistry, University of Padova, Via Marzolo 1, 35131 Padova, Italy

The biological stability of the N-derivatized (*N*-formyl, *N*-formylcarbamoyl, and *N*-carbamoyl) and α-methyl analogues of aspartame (L-α-aspartyl-L-phenylalanine methyl ester; APM) to hydrolysis by human and porcine intestinal and kidney microvillar membranes and by purified preparations of the cell-surface peptidases aminopeptidase A (EC 3.4.11.7) and aminopeptidase W (EC 3.4.11.16) has been examined. Of the N-derivatized analogues of APM, only *N*-formylcarbamoyl-APM was hydrolyzed slightly by the human and porcine intestinal microvillar membrane preparations [1.1 nmol $min^{-1}$ (mg of protein)$^{-1}$ as compared to 80.1 nmol $min^{-1}$ (mg of protein)$^{-1}$ for APM with the human jejunal microvillar membranes]. However, the pattern of inhibition of the hydrolysis of *N*-formylcarbamoyl-APM was distinct from that observed for APM, being inhibited (>80%) by actinonin or 1,10-phenanthroline but not by amastatin, bestatin, or rentiapril. In contrast to APM, *N*-formylcarbamoyl-APM and the other N-derivatized analogues of APM were resistant to hydrolysis by aminopeptidases A and W. All of the α-methyl derivatives of APM were resistant to hydrolysis by both the microvillar membrane preparations and the purified peptidases.

**Keywords:** *Aspartame analogues, N-formylcarbamoylaspartame, intestinal peptidases, metabolism*

## INTRODUCTION

Aspartame (L-α-aspartyl-L-phenylalanine methyl ester; Nutrasweet; APM) is a dipeptide sweetener that is approximately 200 times as potent as sugar (Stegink and Filer, 1984; Walters et al., 1991) (Figure 1, structure 1). APM is widely used as an artificial sweetener in a variety of applications, e.g., in soft drinks and as a table-top sweetener. The major problem with APM is that on exposure to moisture, high pH values, or elevated temperatures, the esterified dipeptide is converted nonenzymically to a variety of decomposition products, none of which are sweet (Homler, 1984; Lipton et al., 1991). The major decomposition product, which is generated by the nonenzymic elimination of methanol, is the cyclic diketopiperazine 3-(carboxymethyl)-6-benzyl-2,5-dioxopiperazine.

Protection of the free amino group in APM with an electron-withdrawing substituent prevents formation of the diketopiperazine. Several N-derivatized analogues of APM have been synthesised, including *N*-formylaspartame (F-APM; Figure 1, structure 2), *N*-formylcarbamoyl aspartame (FC-APM; Figure 1, structure 3), and *N*-carbamoyl aspartame (C-APM; Figure 1, structure 4), of which only FC-APM displays a sweetness potency comparable

* Author to whom correspondence should be addressed (telephone +44 532 333163; fax +44 532 333167).

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‡ The University of Leeds.
§ DSM Research.
∥ University of Padova.

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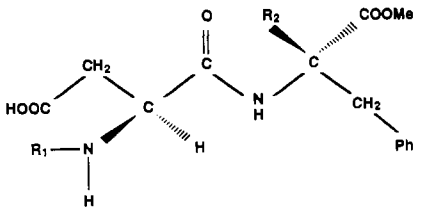

| Structure | $R_1$= | $R_2$= | Compound | Abbreviation | Relative sweetness on mole base (sucrose = 1) |
|---|---|---|---|---|---|
| 1 | -H | H | aspartame | APM | 200[a] |
| 2 | -CH(=O) | H | N-formyl aspartame | F-APM | 0[a] |
| 3 | -C(=O)-NH-C(=O)-$NH_2$ | H | N-formylcarbamoyl aspartame | FC-APM | 200[a] |
| 4 | -C(=O)-$NH_2$ | H | N-carbamoyl aspartame | C-APM | 0[a] |
| 5 | -H | Me | α-methyl aspartame | αMe-APM | 200[b] |
| 6 | -C(=O)-$NH_2$ | Me | N-carbamoyl α-methyl aspartame | CαMe-APM | 0[b] |
| 7 | -C(=O)-NH-C(=O)-$NH_2$ | Me | N-formylcarbamoyl α-methyl aspartame | FCαMe-APM | 200[b] |

**Figure 1.** Structures and sweetness of the N-derivatized and α-methyl analogues of aspartame. [a] Data from Boesten et al. (1991). [b] Data from Polinelli et al. (1992).

to that of APM (Boesten et al., 1991). Another series of APM analogues have been synthesized in which the H atom on the α-carbon of the Phe moiety is replaced with a methyl group, producing α-methylaspartame (αMe-APM; Figure 1, structure 5) (Kamphuis et al., 1991; Polinelli et al., 1992). This compound can also be N-derivatized, giving rise to *N*-carbamoyl α-methyl-aspartame (CαMe-APM; Figure 1, structure 6) and *N*-formylcarbamoyl α-methyl aspartame (FCαMe-APM; Figure 1, structure 7) (Kamphuis et al., 1992; Polinelli et al., 1992). Of these α-methyl analogues, only αMe-APM and FCαMe-APM display a sweetness potency comparable to that of APM (Polinelli et al., 1992).

Recently we have identified two cell-surface peptidases in human and porcine intestinal microvillar membranes that are capable of hydrolyzing APM and the unblocked dipeptide, αAsp-Phe (Hooper et al., 1994), and thus may play a role in the *in vivo* hydrolysis of ingested APM. Selective peptidase inhibitors were used to identify aminopeptidase A (EC 3.4.11.7) as the enzyme responsible for the majority (>80%) of the hydrolysis of APM by the intestinal microvillar membranes. In addition, a purified preparation of aminopeptidase A hydrolyzed APM with a $K_m$ of 0.25 mM and $V_{max}$ of 126 μmol $min^{-1}$ $mg^{-1}$. Another cell-surface peptidase, aminopeptidase W (EC 3.4.11.16), also hydrolyzed APM ($K_m$ 4.96 mM; $V_{max}$ 110 μmol $min^{-1}$ $mg^{-1}$) but was responsible for maximally 20% of the observed hydrolysis of APM by the human and porcine intestinal microvillar membranes. Similar results were also obtained with porcine kidney microvillar membranes (Hooper et al., 1994), which are often used to study the effect of the battery of cell-surface peptidases acting together on the hydrolysis of a biologically active peptide [discussed in Hooper (1993)].

As both of these aminopeptidases require the substrate to possess a free N-terminal α-amino group (Danielsen et al., 1980; Gee and Kenny, 1985), we reasoned that the N-derivatized analogues of APM (Boesten et al., 1991; Kamphuis et al., 1992; Polinelli et al., 1992) would be resistant to hydrolysis by aminopeptidases A and W. Thus, in the present study we have examined the ability of purified preparations of porcine kidney aminopeptidase A and aminopeptidase W to hydrolyze both the N-derivatized and the α-methyl analogues of APM. In addition, we have compared the hydrolysis by human and porcine intestinal and porcine kidney microvillar membrane preparations of APM, αMe-APM, and their N-derivatized analogues.

## MATERIALS AND METHODS

**Materials.** F-APM, C-APM, FC-APM, αMe-APM, CαMe-APM, and FCαMe-APM were synthesized as described previously (Boesten et al., 1991; Toniolo et al., 1991; Polinelli et al., 1992). APM, actinonin, amastatin and bestatin were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 1,10-Phenanthroline was purchased from Aldrich Chemicals, Gillingham, U.K. Rentiapril (SA 446) was a gift from Santen Pharmaceutical Co., Ltd., Osaka, Japan. Cilastatin (MK 0791) was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ. Pig kidneys and intestines were kindly provided by ASDA Farmstores, Lofthousegate, West Yorkshire, U.K. Post-mortem human intestine was obtained from The General Infirmary at Leeds and St. James's University Hospital, Leeds, U.K. All other materials were from sources previously noted.

**Membrane and Enzyme Preparations.** Microvillar membranes were prepared from porcine kidney cortex by the method of Booth and Kenny (1974) and from human and porcine intestine by the method of Kessler et al. (1978). Aminopeptidase A, aminopeptidase N, aminopeptidase W, and membrane dipeptidase were purified from porcine kidney cortex (Hesp, Smith, and Hooper, unpublished results; Bowes and Kenny, 1987; Gee and Kenny, 1985; Littlewood et al., 1989; respectively). All four enzyme preparations were apparently homogeneous as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Aminopeptidase A had a specific activity of 39.1 μmol of *p*-nitroanilide $min^{-1}$ (mg of protein)$^{-1}$ with α-Glu-*p*-nitroaniline as substrate, aminopeptidase N a specific activity of 41.2 μmol of 4-methyl-7-coumarylamide $min^{-1}$ (mg of protein)$^{-1}$ with L-Ala-4-methyl-7-coumarylamide as substrate, aminopeptidase W a specific activity of 15.0 μmol of $PheNH_2$ $min^{-1}$ (mg of protein)$^{-1}$ with αAsp-$PheNH_2$ as substrate, and membrane dipeptidase a specific activity of 56.8 μmol of D-Phe $min^{-1}$ (mg of protein)$^{-1}$ with Gly-D-Phe as substrate.

**Peptide Assays.** Aminopeptidase A activity was routinely assayed with α-Glu-*p*-nitroaniline (2.5 mM) as substrate in 0.1 M Tris-HCl and 1 mM $CaCl_2$, pH 7.4, at 37 °C in the absence or presence of the inhibitor amastatin (0.1 mM final concentration). The released *p*-nitroaniline was quantitated by its absorption at 405 nm. Aminopeptidase N was routinely assayed with L-Ala-4-methyl-7-coumarylamide (0.2 mM) as substrate in 0.1 M Tris-HCl, pH 7.4, at 37 °C in the absence or presence of the inhibitor actinonin (0.1 mM final concentration). The released methyl-7-coumarylamide was detected fluorometrically at an excitation wavelength of 370 nm and an emission wavelength of 442 nm. Aminopeptidase W was routinely assayed with αAsp-$PheNH_2$ (1 mM) or Glu-Trp (0.5 mM) as substrate in 0.1 M Tris-HCl, pH 7.0, at 37 °C in the absence or presence of the inhibitor rentiapril (0.1 mM final concentration) with the products being quantitated by reversed-phase HPLC (Tieku and Hooper, 1992). Membrane dipeptidase was routinely assayed with Gly-D-Phe (1 mM) as substrate in 0.1 M Tris-HCl, pH 8.0, at 37 °C in the absence or presence of the specific inhibitor cilastatin (0.1 mM final concentration) with the products being quantitated by reversed-phase HPLC (Littlewood et al., 1989). Assays with APM and its analogues (all at 1 mM) were carried out in 0.1 M Tris-HCl, pH 7.4. After incubation at 37 °C, 5 μL of 0.5 M sodium acetate, pH 4.0, was added and the samples were heated at 100 °C prior to HPLC analysis. Enzyme samples and inhibitors were preincubated for 15 min at 4 °C. For inhibitor studies maximally 20% substrate breakdown was observed.

**HPLC.** The hydrolysis of APM and its derivatives was monitored by reversed-phase HPLC using a Waters/Millipore Maxima 2 Workstation on a μBondapak $C_{18}$ column with a UV (214 nm) detector at a flow rate of 1.5 mL/min and a 15-min gradient of 4.5–30% (v/v) acetonitrile in 0.08% (v/v) $H_3PO_4$ at pH 2.5, followed by a 5-min elution at the final conditions. The retention times of the peptides were as follows: APM, 12.5 min;

**Table 1. Hydrolysis of the N-Derivatized Analogues of Aspartame by Human and Porcine Microvillar Membrane Preparations[a]**

| microvillar membrane | sp act. [nmol min$^{-1}$ (mg of protein)$^{-1}$] | | | |
|---|---|---|---|---|
| | APM | F-APM | FC-APM | C-APM |
| human jejunal | 80.1[b] | 0.0 | 1.1 | 0.0 |
| porcine duodenal | 58.7[b] | 0.0 | 0.3 | 0.0 |
| porcine kidney | 60.6[b] | 0.2 | 6.7 | 0.1 |

[a] Compounds were incubated with microvillar membrane preparations (human jejunum, 6.0 μg of protein; porcine duodenum, 4.9 μg; porcine kidney, 16.8 μg) in 0.1 M Tris-HCl, pH 7.4, at 37 °C for up to 4 h as described under Materials and Methods. Hydrolysis of the compounds was assessed from the decrease in peak area of the substrate on the HPLC chromatogram, except for APM where the amount of PheMe produced was assessed. Results are the mean of duplicate incubations. [b] Data from Hooper et al. (1994). Under identical conditions no hydrolysis of αMe-APM, CαMe-APM, or FCαMe-APM was observed by any of the membrane preparations.

C-APM, 17.0 min; F-APM 17.8 min; FC-APM, 19.0 min; αMe-APM, 16.1 min; CαMe-APM, 18.9 min; and FCαMe-APM, 21.1 min. Quantification of the substrates and products was by calibration from standard curves where applicable.

## RESULTS

**Hydrolysis of the N-Derivatized and α-Methyl Analogues of Aspartame by Microvillar Membrane Preparations.** The ability of microvillar membrane preparations from human jejunum, porcine duodenum, and porcine kidney to hydrolyze the N-derivatized and α-methyl derivatives of APM was examined (Table 1). FC-APM was hydrolyzed by all three microvillar membrane preparations, whereas C-APM and F-APM were hydrolyzed by only the porcine kidney microvillar membranes, albeit at substantially (>9-fold) lower rates than APM. After prolonged incubation (18 h) at 37 °C, FC-APM was virtually completely degraded by the porcine kidney microvillar membranes, while C-APM and F-APM were only partially hydrolyzed (6% and 12%, respectively). After incubation of FC-APM with the porcine kidney microvillar membranes, two products (retention times of 4.6 and 7.4 min) were detected on the HPLC chromatogram of which the product with retention time of 7.4 min coeluted with a Phe marker. The α-methyl derivatives of APM (αMe-APM, CαMe-APM, and FCαMe-APM) were all resistant to hydrolysis by the human and porcine microvillar membrane preparations even after incubation for 18 h at 37 °C.

**Effect of Inhibitors on the Hydrolysis of *N*-Formylcarbamoylaspartame by the Porcine Kidney Microvillar Membrane Preparation.** The effect of inhibitors on the hydrolysis of FC-APM by the porcine kidney microvillar membrane preparation was examined (Table 2). In contrast to APM, the hydrolysis of FC-APM by the kidney microvillar membranes was not inhibited by the broad-acting aminopeptidase inhibitors amastatin and bestatin or by the converting enzyme inhibitor rentiapril, which has been shown to be selective among the cell-surface aminopeptidases for aminopeptidase W (Tieku and Hooper, 1992). However, as compared to APM the hydrolysis of FC-APM was more susceptible to inhibition by the supposedly selective inhibitor of aminopeptidase N, actinonin (Tieku and Hooper, 1992), and the metal chelator 1,10-phenanthroline. The degree of activation by $CaCl_2$ was similar for both APM and FC-APM.

**Hydrolysis of the N-Derivatized and α-Methyl Analogues of Aspartame by Purified Cell-Surface Peptidases.** The ability of purified preparations of porcine kidney aminopeptidase A and aminopeptidase W to hydrolyze the N-derivatized and α-methyl analogues of APM was examined (Table 3). All of the analogues were resistant to hydrolysis by aminopeptidase A and aminopeptidase W even after incubation for 4 h at 37 °C. Under identical conditions APM is completely hydrolyzed to Asp and PheMe by both aminopeptidase A and aminopeptidase W. Purified preparations of porcine kidney aminopeptidase N (EC 3.4.11.2) and membrane dipeptidase (EC 3.4.13.11) also did not hydrolyze any of the N-derivatized or α-methyl analogues of APM (results not shown). Although neither aminopeptidase A nor aminopeptidase W was capable of hydrolyzing any of the N-derivatized or α-methyl analogues of APM, we considered whether these compounds could be inhibitors of either enzyme (Table 3). However, even at a concentration of 0.1 mM none of the APM analogues inhibited significantly (maximally 12.5%) the activity of either aminopeptidase A or aminopeptidase W.

**Table 2. Effect of Inhibitors on the Hydrolysis of *N*-Formylcarbamoylaspartame by Porcine Kidney Microvillar Membranes[a]**

| inhibitor | concn (mM) | rel act. (%) | |
|---|---|---|---|
| | | APM | FC-APM |
| none | | 100.0 | 100.0 |
| actinonin | 0.1 | 64.9 | 20.6 |
| amastatin | 0.1 | 0.0 | 99.0 |
| bestatin | 0.1 | 42.2 | 91.0 |
| 1,10-phenanthroline | 0.5 | 48.7 | 19.0 |
| rentiapril | 0.1 | 48.1 | 93.7 |
| $CaCl_2$ | 5.0 | 153.1 | 149.4 |

[a] APM and FC-APM were incubated with porcine kidney microvillar membranes (4.2 and 16.8 μg of protein, respectively) in 0.1 M Tris-HCl, pH 7.4, at 37 °C for 90 min as described under Materials and Methods. Hydrolysis of FC-APM was assessed from the increase in peak area of the product with retention time of 7.4 min on the HPLC chromatogram, and for APM the amount of PheMe produced was assessed. Results are the mean of duplicate incubations.

**Table 3. Hydrolysis of Aspartame Analogues by Aminopeptidases A and W and Inhibitory Effect of the Analogues on Aminopeptidases A and W[a]**

| compd | sp act. [μmol min$^{-1}$ (mg of protein)$^{-1}$] | | inhibition (%) | |
|---|---|---|---|---|
| | aminopeptidase A | aminopeptidase W | aminopeptidase A | aminopeptidase W |
| APM | 101.4[b] | 15.4[b] | 0.5 ± 2.1 | nd[c] |
| F-APM | 0.0 | 0.0 | 6.1 ± 2.1 | 2.0 ± 0.2 |
| FC-APM | 0.0 | 0.0 | 10.2 ± 2.1 | 2.0 ± 0.1 |
| C-APM | 0.0 | 0.0 | 12.5 ± 2.7 | 0.0 ± 0.3 |
| αMe-APM | 0.0 | 0.0 | 6.9 ± 2.0 | 0.0 ± 0.2 |
| CαMe-APM | 0.0 | 0.0 | 2.1 ± 2.1 | 0.0 ± 1.0 |
| FCαMe-APM | 0.0 | 0.0 | 0.0 ± 2.7 | 6.3 ± 0.1 |

[a] Compounds were incubated with 100 ng of aminopeptidase A or 100 ng of aminopeptidase W for up to 4 h at 37 °C as described under Materials and Methods. Hydrolysis of the compounds was assessed from the decrease in peak area of the substrate on the HPLC chromatogram, except for APM where the amount of PheMe produced was assessed. Inhibition of aminopeptidases A and W was assessed by preincubating the analogues (0.1 mM final concentration) with the peptidases for 15 min at 37 °C, prior to the addition of either αGlu-*p*-nitroanilide or Glu-Trp as substrate, respectively. Results are the mean (±SEM) of triplicate determinations. [b] Data from Hooper et al. (1994). [c] nd, not determined.

## DISCUSSION

The discovery of the sweet taste of FC-APM (Boesten and Schiepers, 1980) challenged the concept that the zwitterionic structure of the aspartic acid moiety is a prerequisite for a sweet taste (Lelj et al., 1976). The pronounced stability of FC-APM (Figure 1, structure 3)

compared to APM in neutral and weakly alkaline environments (pH 6–9) and at elevated temperatures (25–80 °C) makes FC-APM a potential candidate for special applications. More recently, the α-methyl analogue of APM (Figure 1, structure 5) and its N-derivatized analogue FCαMe-APM (Figure 1, structure 7) have been synthesized (Polinelli et al., 1992). These latter two compounds are also as potent as APM (Figure 1).

A previous study demonstrated that the *in vitro* metabolic clearance of FC-APM in rat tissue fractions occurred at a slower rate than that of APM (Boesten et al., 1991). Recently we observed that the hydrolysis of APM and related peptides in human and porcine intestinal microvillar membranes was due to the actions of two cell-surface aminopeptidases, aminopeptidase A and aminopeptidase W (Hooper et al., 1994). As both of these enzymes have a strict requirement for a free α-amino group on the N terminus of their substrates, we reasoned that the enhanced stability of FC-APM in tissue homogenates may be due to the inability of aminopeptidases A and W to hydrolyze this N-blocked compound. We therefore examined the ability of purified preparations of porcine kidney aminopeptidase A and aminopeptidase W to hydrolyze FC-APM and other N-derivatized and α-methyl derivatives of APM. In addition, we also examined the stability of these compounds to hydrolysis by the battery of cell-surface peptidases, including aminopeptidases A and W, that are present on human and porcine intestinal and kidney microvillar membranes and which present a very effective barrier to the passage of small peptides into the epithelial cells (Hooper, 1993).

Of the N-derivatized analogues of APM, only FC-APM was hydrolyzed to any measurable extent by microvillar membranes prepared from human intestine or porcine intestine or kidney, albeit at a rate substantially slower than that of APM (Table 1). On incubation of FC-APM with porcine kidney microvillar membranes two products were formed, one of which had the identical retention time as Phe. The inhibitor profile of the activity involved in the hydrolysis of FC-APM was distinct from that involved in the hydrolysis of APM (Table 2). In particular, the hydrolysis of FC-APM was not inhibited by amastatin, bestatin, or rentiapril, inhibitors that are effective against aminopeptidases A and W (Tieku and Hooper, 1992; Hooper et al., 1994). Consistent with this observation was the inability of purified preparations of aminopeptidases A and W to hydrolyze FC-APM (Table 3). Although the supposedly selective inhibitor of aminopeptidase N, actinonin, substantially inhibited the hydrolysis of FC-APM by the porcine kidney microvillar membranes, a purified preparation of aminopeptidase N failed to hydrolyze FC-APM. Thus, the inhibitory effects of actinonin and the metal chelator 1,10-phenanthroline may be due to inhibition of an as yet uncharacterized zinc metallopeptidase activity. Such an activity was also noted in the study on the hydrolysis of APM (Hooper et al., 1994). This slight hydrolysis of FC-APM after incubation with the microvillar membranes for several hours may be due either to the action of an esterase and subsequent release by a carboxypeptidase of the unprotected Phe or to the action of an, as yet, uncharacterized hydrolase releasing the PheMe which is then rapidly deesterified. It should be noted, however, that a carboxypeptidase with the specificity for removing C-terminal Phe residues from short peptides has not been identified in intestinal or kidney microvillar membranes (Hooper, 1993).

No information is currently available on the biological stability of αMe-APM and its N-derivatized analogues. However, from the present study it is clear that αMe-APM and FCαMe-APM are completely resistant to hydrolysis by the human and porcine microvillar membranes and the purified preparations of aminopeptidases A and W (Tables 1 and 3). Even without an N-protective group, the presence of the methyl group on the α-carbon atom of the phenylalanine moiety is enough to protect αMe-APM from hydrolysis by the battery of cell-surface peptidases.

To date, no totally selective inhibitor of either aminopeptidase A or aminopeptidase W is available (Tieku and Hooper, 1992). Thus, we investigated whether any of the N-derivatized or α-methyl analogues of APM could act as inhibitors of either peptidase. However, none of the N-derivatized analogues of APM or αMe-APM caused significant inhibition of either aminopeptidase A or aminopeptidase W (Table 3). The failure of αMe-APM to inhibit either enzyme suggests that the presence of the methyl group on the α-carbon atom of the phenylalanine moiety is enough to prevent this peptide with a free N-terminal α-amino group from even binding at the active sites of the peptidases.

In addition to the pronounced physical stability of FC-APM and FCαMe-APM compared to APM at elevated pH and temperature, the results of the present study clearly show that these two compounds are also more stable than APM to hydrolysis by the intestinal microvillar membrane peptidases. This enhanced biological stability of FC-APM and FCαMe-APM may make these compounds attractive as alternatives to APM in certain applications. One such possibility is as an artificial sweetener for phenylketonuriacs, those heterozygous for phenylketonuria and other individuals susceptible to symptoms due to ingestion of APM (Novick, 1985; Pardridge, 1986), in whom the hydrolysis of APM can cause increased plasma levels of Phe which may then have deleterious effects (Caballero et al., 1986; Matalon et al., 1988; Stegink et al., 1988).

## ABBREVIATIONS USED

APM, aspartame; C-APM, *N*-carbamoyl aspartame; F-APM, *N*-formylaspartame; FC-APM, *N*-formylcarbamoylaspartame; αMe-APM, αMe-aspartame; CαMe-APM, *N*-carbamoyl αMe-aspartame; FCαMe-APM, *N*-formylcarbamoyl-αMe-aspartame.

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