N-Formylcarbamoylaspartame, a new aspartame-like sweetener. Synthesis and studies on stability and biological properties


Published in: Journal of Agricultural and Food Chemistry

DOI: 10.1021/jf00001a030

Published: 01/01/1991

Citation for published version (APA):
N-Formylcarbamoylaspartame, a New Aspartame-like Sweetener. Synthesis and Studies on Stability and Biological Properties

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A new aspartame-like sweetener, N-formylcarbamoylaspartame (FC-APM), was efficiently prepared from aspartame (APM). Due to the N-protective group, FC-APM is less prone to diketopiperazine formation and therefore much more stable than aspartame at higher pH values and at higher temperatures, thereby limiting its molecule.

INTRODUCTION

Aspartame (Figure 1, structure 1) or L-aspartyl-L-phenylalanine methyl ester (APM) is a dipeptide sweetener, approximately 150-200 times as sweet as sugar. Aspartame is widely used in a variety of applications, e.g., for table-top use and in soft drinks. A drawback of aspartame, however, is its relatively low stability at higher pH values and at higher temperatures, thereby limiting its use.

A major degradation pathway of aspartame is the decomposition to its diketopiperazine analogue (DKP) via reaction of the amino group of the aspartic acid moiety with the methyl ester of the phenylalanine part of the molecule. This reaction can be suppressed by protection of the amino group with an electron-withdrawing substituent. However, an unsubstituted amino group in combination with a free carboxylic acid group (zwitterionic structure of the aspartic acid moiety) has been generally considered a prerequisite for a sweet taste (cf. Kawai et al. [1980]). Numerous derivatives of aspartame or dipeptide analogues are known that do not taste sweet. Recently, studies on sweeteners and the sweet taste perception mechanism were comprehensively reviewed by van der Weel et al. (1987).

In this paper we report on the synthesis of a new N-derivated aspartame analogue, N-formylcarbamoylaspartame (FC-APM; Figure 1, structure 2), with a sweetness comparable to that of aspartame. FC-APM was discovered in the research laboratories of DSM (Boesten and Schiepers, 1980).

Some preliminary data regarding stability and metabolic and genotoxic characteristics are presented.

MATERIALS AND METHODS

Synthesis of N-Formylcarbamoyl-L-α-aspartyl-L-phenylalanine Methyl Ester. L-α-Aspartyl-L-phenylalanine methyl ester (APM; Figure 1, structure 1) [8.5 g (0.029 mol)] was dissolved in a solution of 4.6 g (0.058 mol) of potassium cyanate in 100 mL of water and subsequently stirred at room temperature for 24 h. After that time, the turbid suspension obtained was filtered and the filtrate

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1 DSM Research.
2 State University Limburg.
the panel on each compound.

was able to achieve reproducible taste intensities involving sucrose

year-old nonsmoking male was incubated for 72 h at 37 OC with

Sweetness Intensity Determinations. APM, C-APM, FC-

Sweetness intensities were determined by a ranking test, with

Genotoxicity Tests. A Salmonella/mammalian microsome

testers to three volunteers from our laboratories. The panel

Effects of FC-APM on SCE frequency of human peripheral

FC-APM was added of 0.2, 2.0, and 20.0 mM concen-

purified, and supernatants being filtered through a

According to the Martin rule the logarithm of the

Preparation of Organ Samples and Incubation Assays

Preparation of Organ Samples and Incubation Assays

Formation of Metabolites. One mL of sample was injected, and the collected eluates were

Prior to incubation with FC-APM or APM, microsomal suspensions were diluted with buffer to protein concentrations of 1 mg/mL. Homogenates were diluted conformably to the protein content ratio between organ homogenates and microsomes, resulting in protein end concentrations of 4–8 mg/mL. To stimulate microsome metabolic activity, a NADPH generating system consisting of 4 mM NADP, 4 mg glucose 6-phosphate, and 1 unit/mL glucose-6-phosphate dehydrogenase was added. FC-APM (1 mM) or APM (1 mM) was incubated with respectively tissue homogenate or microsomal fraction in buffer (final volume 2 mL) at 37 °C during 1 h. All incubations were performed in triplicate. Subsequently, samples were centrifuged at 3000 rpm, supernatants being filtered through a 0.22-μm Millipore filter and stored on ice for immediate HPLC analysis.

HPLC. High-performance liquid chromatographic analysis of FC-APM and APM in homogenate or microsome supernatant was adapted from the method of Verzella and Mangia (1985). A Kratos Analytical HPLC apparatus equipped with a 783 variable UV detector set at λ = 212 nm and AU = 0.2 was employed. The stationary phase consisted of a 150 × 4.6 mm 5 RP 18 column in combination with an appropriate guard column (Chrompack, Middelburg, The Netherlands). The mobile phase contained 0.1 M KH2PO4 (pH 2.5) and acetonitrile (80:20 v/v) and was recycled at a flow rate of 2 mL/min. Filtered supernatants of tissue homogenate or microsome fractions were directly analyzed (injection volume of 20 μL). No internal standard was applied. Calibration concentrations of FC-APM and APM were dissolved in liver microsome supernatant. For preparative chromatography of metabolites a single incubation, in the presence of microsomal fractions, of 10 mL of FC-APM at a concentration of 11.5 mM was made (see Metabolic Studies). Prior to HPLC, the incubate was filtered through a Bakerbond C8 SPE column and eluted with 4 mL of eluent. Nine times 1.5 mL of sample was injected, and the collected eluates were combined, adjusted to pH 5, and evaporated at reduced pressure at 60 °C to a moist residue. A Varian 5000 HPLC (Varian, Walnut Creek, CA) equipped with a Waters 481 variable-wavelength detector (Waters, Milford, MA) was used for preparative purpose (system I); an HP 1090 M DR5 system with built-in diode array detector was used as an analytical liquid chromatograph (Hewlett-Packard, Waldbronn, GFR) to check the purity and the identity of the isolated fractions.

Chromatograms were recorded at 200 nm. Three phase systems were utilized (see Table I). Separation times for the analytes studied in systems II and III are equivalent, but only the isocratic system III allows the application of the Martin rule (Karger et al., 1973), while the overall resolution in system II is larger (see Figure 3). According to the Martin rule the logarithm of the capacity factor of an analyte is the summation of the logarithms of the capacity factor contributions of its constituent groups. All chemicals used were of HPLC grade.

Determination of Amino Acids. Concentrations of amino acids, e.g., phenylalanine, tyrosine, aspartame, and glutamate,
in filtered supernatants of incubated mixtures were analyzed after deproteinization with sulfosalicylic acid (5% w/v) on a LKB 4400 amino acid analyzer running a physiological program with 5 Li+ buffer system according to the method of van Eijck et al. (1988).

NMR. High-resolution 13C NMR spectra were obtained with composite pulse decoupling on a Bruker AM-400 at 100.61 MHz. Samples were measured in DMSO-d6. Chemical shifts are reported relative to the DMSO signal at δ 39.7. Spectra were recorded with 64K data points, and approximately 12 000 scans were collected with a relaxation delay of 3 s. Before Fourier transformation, exponential multiplication was used (line broadening of 2 Hz).

RESULTS

Sweetness and Stability of N-Formylcarbamoylaspartame. Surprisingly, it was found that FC-APM (Figure 1, structure 2), lacking the zwiterionic structure thought to interact with the taste receptor, still elicits a sweet taste qualitatively and quantitatively comparable with that of aspartame (Table II). Moreover, derivatization of analogous dipeptide sweeteners (Figure 1, structures 3 and 4) with a N-formylcarbamoyl substituent also resulted in the formation of products with a sweet taste. In contrast, both the corresponding carboxamid and the corresponding formyl derivatives do not taste sweet.

**Table III. Percentage Decrease of 1 mM Incubating Concentrations of FC-APM and APM (Mean ± SD, in Triplicate Incubation) after 1 h in Vitro Metabolism in Tissue Homogenates or Microsomes of Rat Gastrointestinal Tract**

<table>
<thead>
<tr>
<th>organ</th>
<th>% metabolized APM</th>
<th>% metabolized FC-APM</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>100.0 ± 0.0</td>
<td>42.2 ± 3.7</td>
</tr>
<tr>
<td>stomach</td>
<td>100.0 ± 0.0</td>
<td>10.2 ± 1.3</td>
</tr>
<tr>
<td>ileum</td>
<td>100.0 ± 0.0</td>
<td>71.5 ± 1.3</td>
</tr>
<tr>
<td>colon</td>
<td>100.0 ± 0.0</td>
<td>0.0 ± 1.7</td>
</tr>
</tbody>
</table>

**Table IV. Amino Acid Concentrations after Incubation of 1 mM APM or FC-APM**

<table>
<thead>
<tr>
<th>amino acid level, μM</th>
<th>organ</th>
<th>Asp</th>
<th>Glu</th>
<th>Phe</th>
<th>Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. In Tissue Homogenates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver control</td>
<td>38</td>
<td>78</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>APM</td>
<td>619</td>
<td>76</td>
<td>70</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>FC-APM</td>
<td>34</td>
<td>67</td>
<td>33</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>stomach control</td>
<td>32</td>
<td>67</td>
<td>12</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>APM</td>
<td>780</td>
<td>69</td>
<td>838</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>FC-APM</td>
<td>34</td>
<td>55</td>
<td>36</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>ileum control</td>
<td>38</td>
<td>69</td>
<td>26</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>APM</td>
<td>1330</td>
<td>1440</td>
<td>1290</td>
<td>545</td>
<td></td>
</tr>
<tr>
<td>FC-APM</td>
<td>850</td>
<td>1400</td>
<td>980</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>colon control</td>
<td>38</td>
<td>68</td>
<td>39</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>APM</td>
<td>721</td>
<td>72</td>
<td>856</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>FC-APM</td>
<td>38</td>
<td>68</td>
<td>39</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

**Table V. Capacity Factor Contributions (Log k') Due to Several Groups**

<table>
<thead>
<tr>
<th>compd</th>
<th>log k'</th>
<th>AP methyl</th>
<th>carbamoyl</th>
<th>formyl-carbamoyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>0.20</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>APM</td>
<td>0.78</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>C-APM</td>
<td>1.05</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>F-APM</td>
<td>1.15</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>C-APM metab</td>
<td>0.47</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>F-APM metab</td>
<td>0.57</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>FC-APM metab</td>
<td>0.72</td>
<td>0.20</td>
<td>0.57</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Due to the electron-withdrawing substituent, it can be expected that formylcarbamoylaspartame is less prone to DKP formation and thus will be more stable than aspartame, especially at higher pH values and at higher temperatures. Results of some of the stability studies are graphically represented in Figure 2, from which data it can be concluded that FC-APM is indeed dramatically more stable at room temperature and 80 °C than aspartame at least at neutral and more basic pH values. The difference in stability decreases when the pH is lowered, partly because at lower pH values aspartame is less prone to DKP formation and partly because hydrolysis of the formyl group of FC-APM occurs, resulting in the corresponding nonsweet carbamoyl derivative. Another aspartame decomposition pathway at lower pH values is ester hydrolysis, yielding the corresponding nonsweet dipeptide dicarboxylic acid. A similar decomposition reaction also occurs with FC-APM.

**Metabolic Studies.** Results of in vitro biotransformation of FC-APM and APM in tissue fractions of rat gastrointestinal tract are presented in Table III. It is clearly indicated that APM is completely metabolized by fractionized organs of the gastrointestinal tract as judged from the disappearance of APM from the reaction mixture; FC-APM, however, is only metabolized completely by ileum homogenate, whereas homogenates of other organs are less effective.

Microsomal fractions are not or less capable of in vitro degradation of APM and FC-APM. It is furthermore shown that in vitro metabolism of APM and FC-APM varies with the site of origin of the metabolizing organ fractions, homogenates as well as microsomal fractions of the small intestine being most active.

Table IV shows results of amino acid analysis of incubated mixtures.
Table VII. Effects of FC-APM

Table VI. **13C NMR Data of FC-APM and Metabolite in DMSO-d6 (ppm)**

<table>
<thead>
<tr>
<th>compd</th>
<th>C-a</th>
<th>C-b</th>
<th>C-c</th>
<th>C-d</th>
<th>OMe</th>
<th>C=O</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC-APM</td>
<td>36.9</td>
<td>52.3</td>
<td>54.2</td>
<td>37.0</td>
<td>49.8</td>
<td></td>
</tr>
<tr>
<td>metab</td>
<td>37.9</td>
<td>51.4</td>
<td>56.0</td>
<td></td>
<td>153.0, 163.3, 170.7, 172.0, 172.0</td>
<td></td>
</tr>
</tbody>
</table>

* See for numbering Figure 1. a Obscured by the DMSO-d$_6$ signal.

**Genotoxicity Testing.** FC-APM did not show mutagenic activity (results not shown) within the concentration range 0.5–10 000 μg of FC-APM in the Salmonella/microsome assay with or without metabolic activation (S9 mix from Aroclor induced rats). Results on SCE testing of FC-APM (Table VII) show significant increases at FC-APM concentrations of 0.2 and 20.0 mM in the absence of S9 mix. Linear regression analysis showed no statistically reliable concentration–effect relations, indicating no distinct effect of FC-APM on lymphocyte SCE incidence.

Application of a metabolic system prevented any effects of FC-APM on lymphocyte SCE frequency, suggesting that the demethylated metabolite of FC-APM has no genotoxic potential.

**DISCUSSION**

The discovery of FC-APM (Figure 1, structure 2; Boesten and Schiepers, 1980) was again an example of serendipity. In the search for a new route to APM, the sweet taste of an unknown intermediate was observed. Spectral analysis and independent synthesis established its structure as N-formylcarbamoylaspartame. Subsequent research showed that other dipeptide sweeteners (Figure 1, structures 3 and 4) with the N-formylcarbamoyl substituent also elicit a sweet taste. However, the corresponding N-carbamoyl dipeptides, intermediates in the synthesis of the N-formylcarbamoyl dipeptides, do not taste sweet. Before 1980, the only N-protected aspartame-derivative reported to have a sweet taste was N-trifluoroacetyl aspartame. Tinti et al. (1981) showed that most probably the receptor sites for the NHCOCF$_3$ group and the α-NH$_3^+$ group of the dipeptide ester are different. Tinti and Nofre (1984) described the most potent class of N-protected dipeptide sweeteners discovered up to now, which are para-substituted arylureas having sweetness potencies 10 000–14 000 times that of sucrose.

All these results are seriously challenging the concept that the zwitterionic structure of the aspartic acid moiety is a prerequisite for a sweet taste (cf. Kawai et al. (1980)).

The easy preparation from APM and the pronounced stability compared to APM in weakly neutral and alkaline environments (pH 6–9) at room temperature and higher temperatures (80 °C), make FC-APM a potential candidate for special applications, e.g., bakery.

Our results from the in vitro studies on the metabolic fate of FC-APM in rat tissue fractions demonstrate that the metabolic clearance of FC-APM occurs at a slower rate than of APM, which is metabolized quickly and completely by gastrointestinal tissue homogenates. Contrast, a biologically rather stable metabolite has been found in vitro and characterized as the demethylated formylcarbamoylaspartame phenylalanine ester. The bio-
logical fate of this compound is not known. Further research should focus on kinetics and metabolism of this metabolite in relation with the parent compound FC-APM, to be studied in intact organisms.

The results on the in vitro genotoxicological properties of FC-APM indicate that FC-APM is not mutagenic to Salmonella typhimurium tester strains and has limited capacity to induce chromosome damage in human lymphocytes. Since in vitro metabolism of FC-APM reduces this effect on SCE frequency completely, it is concluded that this slight genotoxic effect of FC-APM probably will not be expressed in vivo.

To complete the genotoxicological studies on FC-APM, possible induction of structural DNA damage, e.g., point mutations, should be evaluated in an in vitro mammalian (preferably human) cell system.

ACKNOWLEDGMENT

H. A. J. Linssen, T. A. Verstappen, and T. G. J. M. van Dooren are gratefully acknowledged for their technical assistance.

LITERATURE CITED

Burke, M. D.; Orrenius, S. Isolation and comparison of endoplasmatic reticulum membranes and their mixed function oxidase activities from mammalian extrahepatic tissues. Pharmacol. Ther. 1979, 7, 549-599.
Tinti, J. M.; Nofre, C.; Durozard, D. Studies on sweeteners requiring the simultaneous presence of both the nitrogen dioxide/cyanide and carboxyl groups. Naturwissenschaften 1981, 68, 143.

Received for review February 12, 1990. Revised manuscript received July 11, 1990. Accepted July 16, 1990.

Registry No. 1, 22839-47-0; 2, 82778-17-4; 2 (demethyl derivative), 129966-82-1; C-APM, 82778-16-3.