European Food Safety Authority

The EFSA Journal (2007) 581, 1-43

Neotame as a sweetener and flavour enhancer [1]

Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food

(Question No EFSA-Q-2003-137)

Adopted on 27 September 2007

PANEL MEMBERS

Fernando Aguilar, Herman Autrup, Sue Barlow, Laurence Castle, Riccardo Crebelli, Wolfgang Dekant, Karl-Heinz Engel, Natalie Gontard, David Gott, Sandro Grilli, Rainer Gürtler, John Chr. Larsen, Catherine Leclercq, Jean-Charles Leblanc, F. Xavier Malcata, Wim Mennes, Maria Rosaria Milana, Iona Pratt, Ivonne Rietjens, Paul Tobback, Fidel Toldrá.

SUMMARY

Following a request from the European Commission, the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) was asked to deliver a scientific opinion on the safety of neotame as a sweetener and flavour enhancer.

Neotame is a dipeptide methyl ester derivate. Its chemical structure is N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester. It is intended for use in food as a sweetener and flavour enhancer. Neotame has a sweetness factor approximately 7000 to 13000 times greater than that of sucrose and approximately 30 to 60 times greater than that of aspartame, depending upon the food application.

Neotame is manufactured by the reaction of aspartame and 3,3-dimethylbutyraldehyde, followed by purification, drying, and milling. Neotame is generally stable under conditions of intended use as a sweetener across a wide range of food and beverage applications. Neotame degrades slowly in aqueous conditions such as those in carbonated soft drinks. The hydrolysis of neotame results in equimolar amounts of N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) and methanol. Three other minor degradation products, N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester (NC-00777) formed by cyclisation, N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester (NC-00764) formed by β-rearrangement of neotame, and N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine (NC-00779) formed by methyl ester hydrolysis of NC-00777,

[1] For citation purposes: Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from European Commission on Neotame as a sweetener and flavour enhancer. *The EFSA Journal* (2007) 581, 1-43

are not detected at the anticipated concentrations and conditions of neotame use in carbonated soft drinks.

At least 30% of neotame ingested is rapidly absorbed in all species. The major metabolic pathway is de-esterification by non-specific esterases to NC-00751, which accounts for approximately 80% of the neotame dose as NC-00751 in all species tested. Peak plasma concentrations of neotame and NC-00751 are observed at approximately 0.5 hours and within 1 hour, respectively. Neotame is completely eliminated from the body with recovery in urine and faeces exceeding 98% in the human and greater than 93% in the rat and the dog within 72 hours. The majority of radioactivity is excreted in faeces in all species. The major component in the faeces is NC-00751.

Studies with radiolabelled neotame given orally to rats indicate no accumulation in tissues. The highest radioactivity is associated with the contents of the gastrointestinal tract and organs of metabolism and excretion (liver, kidney, urinary bladder). In whole body autoradiography studies with pregnant rats no radioactivity has been reported in the fetus.

The safety of neotame has been investigated in *in vitro* studies and in short and long-term studies in mice, rats, rabbits and dogs. The results indicate that neotame is not genotoxic, carcinogenic, teratogenic or associated with any reproductive/developmental toxicity. The consistent findings in animal studies were reduced feed consumption, body weight and body weight gain relative to that of controls, with no clear dose response. These effects are considered not adverse or indicative of toxicity but a consequence of reduced palatability of the neotame-containing diets. Therefore body weight parameters were not considered appropriate endpoints for setting no-observed-adverse-effect levels (NOAELs) in these studies.

A consistent finding was an increased activity of alkaline phosphatase (AP) of hepatic origin in dogs in the 13-week study at doses of 600 and 1200 mg/kg bw and in the 52-week study at 800 mg/kg bw. In the latter study AP activity was also increased compared to controls at 200 mg/kg bw in females in weeks 26 and 52. The Panel noted that the mean baseline value in this dose group was 20% greater than that of controls and AP was not significantly increased at any time point compared to controls by analysis of covariance for repeated measures using predosing AP activity as the covariate. Although the increase in serum AP seen at the two highest dose levels in both dog studies was not accompanied by any other indication of hepatotoxicity, the Panel considered the increase in AP to be the critical endpoint and established a NOAEL of 200 mg/kg neotame/kg bw/day for setting an ADI.

The major degradation product and metabolite NC-00751 will have been present in both the human and animal studies. Its safety has also been established by *in vitro* genotoxicity studies. The three minor degradation products (NC-00764, NC-00777 and NC-00779) have been shown to have a low acute toxicity and are not genotoxic. No treatment-related adverse effects were observed in a 4-week dietary study in rats with a mixture of the three minor degradation products. Based on the above, the dietary exposure to degradation products is not considered to pose any safety concern.

The results of human studies demonstrated that neotame was well tolerated by healthy and diabetic human subjects at dose levels up to 1.5 mg/kg bw/day (the highest dose tested).

The exposure to methanol, which may result from ingestion of neotame-containing foods and beverages is considered negligible compared to that from other dietary sources and as such of no concern from the safety point of view.

The Panel noted that the additional phenylalanine intake expected from ingestion of neotame as a general purpose sweetener represents a relatively small increment in the exposure to phenylalanine of the phenylketonuric homozygous child.

The hypothetical formation of nitrosamines in the gastrointestinal tract from reaction of nitrite with neotame and its major degradation product and/or metabolite NC-00751 has been considered by the Panel. No nitrosated neotame (N-nitroso-(3,3-dimethyl)-L-aspartyl-L-phenylalanine methyl ester, N-nitrosoneotame; NC-00799) and no nitrosated de-esterified neotame (N-nitroso-(3,3-dimethylbuthyl)-L-aspartyl-L-phenylalanine, NC- 00800) could be detected under simulated gastric juice conditions. Furthermore, both compounds were synthesised and shown to be without mutagenic activity in the Ames test. In view of the high sensitivity of the Ames test to genotoxic nitrosocompounds, the Panel considered that nitrosation of neotame, should it occur, is not a matter of safety concern.

After considering all the data on stability, degradation products and toxicology, the Panel concluded that neotame is not of safety concern with respect to the proposed uses as a sweetener and flavour enhancer.

The Panel established an Acceptable Daily Intake (ADI) of 0-2 mg/kg bw/day based on the application of a 100-fold safety factor to the NOAEL of 200 mg/kg bw from a 52-week dog study.

Conservative estimates of dietary exposure both in adults and children suggest that it is very unlikely that the ADI would be exceeded at the proposed use levels.

The Panel recommends that the limit for lead in the specifications should not be higher than 1 mg/kg.

Key words:

Neotame, E 961, CAS N° 165450-17-9, L-phenylalanine, N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-,1-methyl ester artificial sweetener, intense sweetener, food additive.

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Neotame is a dipeptide methyl ester derivative that is chemically related to aspartame, an artificial sweetener, already approved as a food additive (SCF, 1984). Neotame has a sweetness factor that is approximately 7000 to 13000 times greater than that of sucrose and approximately 30 to 60 times greater than that of aspartame, depending upon the food application.

Neotame has been approved for use as a food additive in a number of countries including the following: USA, Australia, New Zealand, Mexico, Costa Rica, China, Guatemala, Russia and Philippines. Neotame has been temporarily approved in Czech Republic and Poland. These national authorisations were valid for two years, in accordance with Article 5 of Directive 89/107/EEC concerning food additives for use in foodstuffs intended for human consumption, and ceased on 30 April 2006.

Neotame was evaluated by the Joint FAO/WHO Expert Committee of Food Additives (JECFA) on its sixty-first meeting in June 2003 and assigned an Acceptable Daily Intake (ADI) 0 –2 mg/kg bw (JECFA, 2003a).

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety of neotame for use in food as a sweetener and flavour enhancer.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Additives Working Group for the preparation of this opinion:

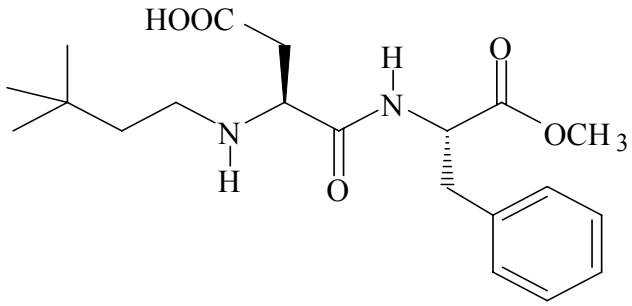
Fernando Aguilar, Dimitrios Boskou, David Gott, Sandro Grilli, Werner Grunow, Karolina Hulshof, John Chr. Larsen, Catherine Leclercq, Jean-Charles Leblanc, Wim Mennes, Alicja Mortensen, Dominique Parent-Massin, Annette Pöting, Iona Pratt, Ivonne Rietjens, Gerrit Speijers, Paul Tobback, Fidel Toldrá.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food wishes to thank RIVM for their technical support.

ASSESSMENT

1. Technical data

The chemical name of neotame is L-phenylalanine, N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-,1-methyl ester. The Chemical Abstract Service (CAS) number is 165450-17-9. The synonyms are N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester or N(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester. The empirical formula of neotame is $C_{20}H_{30}N_2O_5$ and its molecular weight of 378.47. Its structural formula is presented below:



Neotame is a white to off-white powder that has a sweetness factor approximately 7000 to 13000 times greater than that of sucrose and approximately 30 to 60 times greater than that of aspartame. The melting point of neotame ranges between 80.9˚C and 83.4˚C. The solubility of neotame in water is 4.75% (w/w) at 60˚C. It is soluble in ethanol and ethyl acetate. The refractive index and pH of a 0.5% aqueous solution of neotame at 20˚C are 1.3338 and 5.80 respectively.

The raw materials used in the manufacture of neotame are aspartame, 3,3,-dimethylbutyraldehyde, methanol, hydrogen, palladium/carbon catalyst, and diatomaceous earth. Neotame is manufactured by the reaction of aspartame and 3,3-dimethylbutyraldehyde in methanol for several hours at ambient temperature under hydrogen pressure. The catalyst is removed by filtration. The filtration may be aided by the use of diatomaceous earth. The isolation and purification of neotame is carried out by the distillation of the methanol solvent followed by the addition of water. The mixture is cooled for a number of hours and the neotame is then isolated by centrifugation. Additional water is used to wash the product. The neotame is vacuum dried. The product may be milled to obtain the desired particle size. Additional product may be obtained by further processing of filtrate obtained from the initial neotame isolation.

As presented by the petitioner in manufacturing specifications, the purity of neotame is not less than 97.0% and no more than 102.0% calculated on a dry weight basis. The proposed content of N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) is not more than 1.5% and of other structurally related substances, not more than 2.0%. The proposed limit for lead by the petitioner is not more than 2 mg/kg, of water not more than 5%, of residues after ignition not more than 0.2%. The manufacturing specifications are in good agreement with analytical

results obtained from 5 different batches. It is noted that the proposed limit of lead is twice as much as in the JECFA purity criteria (JECFA, 2003b).

Evaluation of chemical stability of neotame in bulk dry form in samples stored at conditions relevant to commercial use (25ºC and 60% relative humidity) and analysed at intervals for up to 78 weeks indicated the inherent stability of neotame. Neotame remained within the range of 99.7% to 100.9% with the levels of NC-00751 ≤ 0.3%. An indication of slight instability was observed only after storage at 40ºC and 75% relative humidity for 52 weeks. Neotame content decreased to between 96.2% to 99.0%. Content of NC-00751 increased to between 1.2% to 3.5%, and N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine (NC-00769) was found in amounts of 0.2% or less. Neotame and NC-00751 combined accounted for a total mass of 99.7% or greater under all conditions evaluated through 78 weeks.

Fluorescent lighting and polyethylene packaging have no effect on the stability of neotame.

The data from stability studies submitted by the petitioner shows that the levels of formation of degradation products depend upon variables of moisture, pH, time and temperature.

The petitioner has indicated that it is very difficult to measure neotame and identify degradation products in chewing gums. Therefore, identification of degradation products was performed only in model beverage matrices instead. Consequently, the petitioner considers the safety assessment of degradation products identified in model beverages as representative of the safety assessment for neotame degradation products in general. Model beverages contained approximately 200 mg/l of neotame, which is approximately 10 times higher than expected use levels in most processed foods and beverages.

Evaluation of neotame stability in various aqueous model systems expected to be encountered in typical uses (i.e. carbonated beverage applications) demonstrated that the major route leading to loss of neotame is hydrolysis of the methyl ester group to generate NC-00751 and methanol in equimolar quantities. Additional degradation products which may be formed include N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester (NC-00777) formed by cyclisation, N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester (NC-00764) formed by beta rearrangement, and N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine (NC-00779) formed by methyl ester hydrolysis of NC-00777. Furthermore, a number of additional degradation products including phenylalanine formed under unrealistic conditions were identified by the petitioner. These would not be expected to be present in beverages under normal conditions of manufacture, storage and use.

The stability of neotame in aqueous solutions was studied under a variety of conditions covering a typical range of pH (2.8, 3.2, 3.8, and 4.5), various temperatures (5, 20, 30 and 35ºC), and times of storage (up to 26 weeks). Model beverages containing 200 mg/l of neotame, a concentration approximately 13 times higher than the anticipated use level were used in order to be able to quantify the potential degradation products. An additional set of model beverage samples, prepared at the anticipated commercial beverage use level of 15 mg/l of neotame at pH 3.2, was stored at each temperature and analysed concurrently with the 200 mg/l model beverage samples for comparison of the kinetics and disappearance of neotame.

The stability of neotame in the 200 mg/l model beverages was pH, temperature, and time dependent. The rate and extent of degradation product formation was increased under acidic conditions (lower pH) and at higher temperatures with the majority of degradation product formation occurring after extended storage.

Storage for 8 weeks at pH 3.2 and at 20ºC was considered by the petitioner as representative conditions for evaluation of the stability of non-nutritive sweeteners in carbonated soft drinks. Under these conditions neotame was quantified to 89.3% of the initial levels and only three degradation products were formed: NC-0751 (7. 35% relative to initial levels of neotame), NC-00764 (0.97%) and NC-00777 (0.82%). After 10 weeks of storage three additional degradation products, NC-00779, NC-00754 and NC-00769 were detected at levels higher than 0.5% but less than approximately 1%. After 26 weeks of storage the concentrations of neotame in samples at pH 2.8, relative to the initial concentration, ranged from 86.2% at 5ºC to 9.7% at 35ºC. At pH 3.2, the concentrations of neotame ranged from 91.3% at 5ºC to 23.2% at 35ºC. At pH 3.8, the concentrations of neotame ranged from 94.4% at 5ºC to 43.1% at 35ºC. Finally, at pH 4.5, the concentrations of neotame ranged from 95.4% at 5ºC to 56.9% at 35ºC.

Samples of the model beverages containing 15 mg/l of neotame yielded neotame values below the range of the standard curve (12.5 mg/l to 250 mg/l). This indicates that the majority of degradation products quantified in the 200 mg/l neotame model beverages would not be detectable in commercial products.

Stability of neotame through thermal processing was tested in model beverages prepared at pH of 3.2 and 6.5 to simulate noncarbonated still beverages and dairy products, respectively and containing approximately 200 mg/l of neotame. Each beverage was exposed to conditions of 88˚C for 30 seconds and was cooled slowly to 4, 21, and 32˚C to simulate different plant cooling procedures. No significant loss of neotame (<0.5%) was found after exposure to these thermal processing conditions. Concentrations of NC-00751 were ≤ 1.0 µg/ml.

A stability study of neotame in chewing gum, at a concentration of approximately 100 mg/kg, demonstrated that encapsulation enhances the stability of the compound. When double-encapsulated neotame was used, the concentration of neotame in the chewing gum samples stored for 52 weeks at 25˚C and 60% relative humidity remained at 78% of its initial concentration (NP00-002).

Neotame is a secondary amine and could hypothetically form a nitrosoneotame (N-nitroso-(3,3-dimethybutyl)-L-aspartyl-L-phenylalanine methyl ester, NC-00799) in the presence of a nitrosating agent. Since neotame might easily undergo de-esterification, yielding the dicarboxylic acid (NC-00751), nitrosated de-esterified neotame (N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine, NC-00800) could hypothetically also be formed. The possible nitrosation of neotame could occur in beverages and in certain dairy products (as other food categories are not conducive to nitrosation reactions either because of the lack of water or higher pH), and in the stomach (low pH) following ingestion of neotame. According to the petitioner exposure to nitrosamine would not be higher than 10.4 pg/kg bw/day from the hypothetical nitrosation of neotame in 0.3 L beverages/day and 37 pg/kg bw/day from the hypothetical nitrosation of neotame in the stomach.

Following initial discussion in the Panel, the petitioner was asked to provide further information on the possible formation of nitrosamines.

The two nitrosation products, NC-00799 and NC-00800, have been synthesised by the petitioner and the stability in gastro-intestinal tract studied.

The stability of NC-00799 and NC-00800 in simulated gastric fluids, with and without enzymes, was tested after incubation at 37°C, within the range of pH 1 to 8. Samples were taken after 1, 5, 15, 30, 60, and 120 minutes and analysed by HPLC. NC-00799 was stable in simulated gastric fluid with or without pepsin and in simulated intestinal fluid without pancreatin. However, NC-00799 showed rapid decomposition in simulated intestinal fluid containing pancreatin, with marked decreases in NC-00799 peaks in the HPLC traces within 5 minutes of the start of the incubation period and could no longer be detected after 120 minutes. Concomitant with the disappearance of NC-00799 a peak corresponding to NC-00800 appeared. The results showed that NC-00799 is unstable in simulated intestinal fluid containing pancreatin, with rapid conversion to the deesterified product, NC-00800. Assuming first order reaction kinetics, the decomposition half-life of NC-00799 was calculated to be 6.3 minutes, with the only decomposition product found being NC-00800. NC-00800 was found to be stable over the incubation time period of 120 minutes in simulated gastric fluid (with or without pepsin) and in simulated intestinal fluid (with or without pancreatin).

Furthermore, mixtures of neotame and nitrite was added to simulated gastric fluid [an aqueous solution of sodium chloride (2g) and hydrochloric acid (0.1 M)]. The reaction was adjusted to pH 3.0 to optimise formation of nitrosated neotame (NC-00799) and allowed to proceed for 2 hours at 37°C, followed by HPLC analysis for detection of NC-00799 and NC-00800. The optimum pH of 3.0 for the formation of nitrosamines in the presence of nitrite has been established by Mirvish (1975). Two experiments were conducted in duplicate, the first using 17.8 µM neotame (6.7 mg/L) and 35.6 mM nitrite (large excess) and the second using 55 µM neotame (20.8 mg/L) and 50 µM nitrite (higher than the 17 - 31 µM considered by the Panel to be worst cases). No nitrosation of neotame, i.e. formation of NC-00799 and NC-00800 could be detected. The detection limit was 340 nM for experiment 1 and was improved to 56 nM for experiment 2.

2. Case of need

According to the petitioner, neotame is a new highly intense sweetener with desirable chemical and sensory characteristics, which will provide industry with greater flexibility in the formulation of foods and beverages. In addition, neotame can modify the flavour of foods and beverages.

3. Dietary exposure

3.1. Intended use and typical use levels

The petitioner proposes neotame for use as a sweetener and flavour enhancer in a range of food applications in accordance with good manufacturing practice.

According to the petitioner, neotame can be used to sweeten foods and beverages. It may be used as a replacement for sucrose or other sweeteners in any product application such as carbonated and non-carbonated soft drinks, beverage concentrates, beverage mixes, dairy beverages, alcoholic drinks, non-dairy desserts, gelatin, ice cream, sorbet and their mixes and

other dessert mixes, toppings, topping mixes, fillings, filling mixes, yoghurt, various types of dairy desserts, confectionery such as hard and soft candies, chocolate confectionery, chewing gum and breath freshener products, fruit spreads, fruit preparations, salad dressings, condiments, breakfast cereals, fine bakery wares and baking mixes, and as a table top sweetener for foods such as hot coffee, tea, fruit and desserts, and cereals. Neotame can be used alone or with other sweeteners.

Typical levels of use mentioned by the petitioner vary from 8 to 17 mg/kg for beverages and from 15 to 35 mg/kg in solid foods. For chewing gums the typical use level of 250 mg/kg is mentioned.

3.2. Estimated dietary exposure to neotame

The estimation of neotame consumption performed by the petitioner is based on the assumption that the compound will be used in the same products as aspartame and that carbonated soft drinks represent about 70% of the consumption of aspartame in the European Community (EU). The petitioner estimated mean and 90th percentile consumption of neotame as 0.01 and 0.05 mg/kg bw/day for Europe, and 0.02 and 0.05 mg/kg bw/day in the United States. The petitioner estimates neotame consumption will be highest in young children as they consume more food or energy on a body weight basis than other members of population. According to the petitioner, children aged 1 to 5 years are expected to have median dietary exposures of 0.029 mg/kg bw/day and of 0.090 mg/kg bw/day for the heavy consumer based on aspartame survey data from the United Kingdom. A similar estimate for young children has been obtained by the petitioner based on Market Research Corporation of America post-marketing surveys on use of aspartame from 1984 to 1992: a mean dietary exposure of 0.03 mg/kg bw/day and of 0.08 mg/kg bw/day for the heavy consumers.

Theoretical dietary exposure estimates were performed by the Agence Française de Sécurité Sanitaire des Aliments (AFSSA) based on the hypothesis that all foods and beverages likely to be sweetened contained neotame at the typical use level provided by the petitioner. Under this scenario, on the basis of United Kingdom and French consumption data, average exposure in the population would be in the range of 0.1 to 0.2 mg/kg bw/day for adults *(AFSSA, 2004)*. For children conservative estimates of exposure to aspartame had been performed previously by the United Kingdom and the Netherlands considering the use of aspartame at Maximum Permitted Level in all products in which it is allowed. Potential exposure would range from 0.4 to 16 mg/kg bw (European Commission, 1999). Assuming that neotame would substitute aspartame in a ratio of 1 to 30 (based on its sweetness potency), this would lead to a conservative estimate of exposure of 0.5 mg neotame/kg bw in a 15 kg child.

3.3. Estimated dietary exposure to neotame degradation products

According to the petitioner, degradation product data from the model beverage study, combined with the known consumption patterns for carbonated soft drinks, can be used to conservatively estimate the human dietary exposure to the individual degradation products of neotame.

The petitioner used a weighted average approach based on estimated concentrations of degradation products in model beverage at specified weekly intervals and estimated intake of carbonated soft drink for any given day post-production.

In using a weighted average approach to calculate the dietary exposure to the major degradation product NC-00751, the mean total exposure (by all age groups within the total population) was estimated by the petitioner to be approximately 0.96 µg/kg bw/day or 2.40 µg/kg bw/day for the 90th percentile consumer. The Panel has calculated the potential exposure to this degradation product in the EU on the basis of the above-mentioned conservative estimates of exposure to neotame in the adult population (0.2 mg/kg bw) and children (0.5 mg/kg bw), leading to a conservative estimate of exposure to NC-00751 of 9.6 µg/kg bw/day in adults and 24 µg/kg bw/day in children.

The mean exposures to each of the three minor degradation products NC-00777, NC-00764 and NC-00779 by all age groups within the total population were estimated by the petitioner to be 0.15 µg/kg bw/day or less with a 90th percentile dietary exposure of 0.38 µg/kg bw/day or less. The Panel has calculated the potential exposure to these degradation products in the EU on the basis of the above mentioned conservative estimates of exposure to neotame in the adult population (0.2 mg/kg bw) and children (0.5 mg/kg bw), leading to a conservative estimate of 1.52 µg/kg bw/day in adults and 3.8 µg/kg bw/day in children for each of these minor degradation products.

Another degradation product to which consumers would be exposed via consumption of neotame-containing products is methanol. Equimolar amounts of methanol are formed following the hydrolysis of neotame to NC-00751. Using the data provided by the petitioner and based on the petitioner's estimated maximum exposure, the estimated exposure to methanol from all food and beverage application of neotame would be approximately 4 µg/kg bw/day for the 90th percentile consumer.

The Panel has estimated the exposure to methanol in the EU calculated on the basis of conservative estimates of exposure to neotame in the adult population (0.2 mg/kg bw) and children (0.5 mg/kg bw). The maximum quantity of methanol consumed is estimated to be approximately 16 µg/kg bw/day for adults and 40 µg/kg bw/day for children. For comparison, the exposure to methanol from the consumption of fruit juice and wine is about 1 mg/kg bw/day and the methanol content in neotame sweetened carbonated beverages is estimated to be 1.37 mg/l while fruit juices may contain from 64 to 326 mg/l of methanol (U.S. FDA, 2002).

If consumers were to be exposed to phenylalanine via metabolism of neotame, one litre of beverage sweetened with neotame would provide 1.5 mg/person of phenylalanine considering that the phenylalanine content in neotame is 44% by weight and assuming a partial metabolism of approximately 20% of neotame to phenylalanine. Under assumption of complete metabolism of neotame to phenylalanine this figure would be approximately 7.5 mg/person.

The Panel has estimated the amount of phenylalanine presented to humans from the ingestion of neotame. Using the conservative approach for neotame dietary exposure in Europe (0.2

mg/kg bw /day for an adult and 0.5 mg/kg bw/day for a child) and assuming total hydrolysis of neotame to phenylalanine, the exposure to phenylalanine would be approximately 88 µg/kg bw for an adult and 220 µg/kg bw for a child. For comparison, the daily dietary exposure to phenylalanine for a healthy human may range from 2.5 to 10 g/person/day (corresponding to from 42 to 167 mg/kg bw for a 60 kg an adult). The daily dietary exposure to phenylalanine by a phenylketonuric homozygous child (20 kg) has been reported to range from 0.2 to 0.4 g/person/day (corresponding to from 10 to 20 mg/kg bw/day) (Trefz, 2002) or from 0.4 to 0.6 g/person/day (corresponding to from 20 to 30 mg/kg bw) (US. FDA, 2002).

4. Biological data

All the information below was provided in the petitioner's dossier. The PCR numbers cited refer to the individual study reference number in the dossier.

4.1. Absorption, distribution, excretion and metabolism

The metabolism and pharmacokinetics of neotame were examined in mice, rats, dogs, rabbits and humans. The data presented by the petitioner demonstrate that neotame is rapidly but incompletely absorbed in all species. Approximately 30% of doses of neotame (15 and 120 mg/kg bw orally, and 15 mg/kg bw iv) labelled with ^{14}C in the C-1 position of the 3,3-dimethylbutylamine moiety was absorbed in rat and dogs, based on the oral/iv ratio of urinary radioactivity excretion (PCR 1028, PCR 1029). At least 34% of a dose of 18.75 mg (approximately 0.25 mg/kg bw) was absorbed in the human based on urinary ^{14}C excretion following oral administration (PCR 1039). The Panel noted that there was extensive excretion of neotame metabolites in faeces, which indicates that absorption could be higher.

Absorbed radioactivity was not concentrated or retained in tissues. Whole body autoradiography and quantitative tissue distribution studies in rats dosed with neotame labelled with ^{14}C in the 3,3,-dimethylbutylamine moiety demonstrated very low amount of radiolabel in tissues such as brain, muscle, or fat. The highest concentrations of radioactivity were in the contents of the gastrointestinal tract with much smaller amounts in the liver and kidney. Radioactivity was not retained in any organ (PCR 0958, PCR 0959). Radioactivity in the fetus was below the detection limit by autoradiography (PCR 1031).

The major metabolic pathway for neotame in humans and animals is de-esterification by non-specific esterases to NC-00751 and formation of methanol. In the human combined neotame and NC-00751 accounted for greater than 90% of both the peak radioactivity and the area under the ^{14}C plasma concentration time curve. Two other metabolites were detected at greater than 1% of the dose. One of them was N-(3,3-dimethylbutyl)-L-aspartic acid (NC-00754). It accounted for a mean of approximately 4.9% of the dose and was found in the faeces in humans (PCR 1039). It was also identified as both urinary and faecal metabolite in the rat and the dog (PCR 1027, PCR 1029). The other metabolite NC-00784 was the carnitine conjugate of the metabolite 3,3-dimethylbutyric acid (NC-00785) (PCR 1215). It accounted for a mean of 3.2% of the dose and it was identified in human urine (PCR 1039, PCR 1215). NC-00785 was also identified as a urinary metabolite in the rat and the dog (PCR 1214, PCR 1029). The rat excreted NC-00785 as both glucuronide and carnitine conjugate while the dog excreted it only as a glucuronide conjugate.

Absorbed neotame was rapidly cleared in animals and humans by de-esterification to NC-00751 followed by faecal and urinary excretion. The half-life of neotame in humans was approximately one-half hour (PCR 1035, PCR 1039). NC-00751 was cleared with an apparent half-life of approximately two hours (PCR 1035, PCR 1039).

Elimination of ^{14}C following oral dosing of neotame labelled with ^{14}C in the 3,3-dimethylbutylamine moiety was rapid and complete with more than 80% of the dose recovered in urine and faeces within 48 hours. Total recoveries within 72 hours were approximately 98% in the human (PCR 1039) and greater than 93% in the rat and dog (PCR 1119, PCR 1029). The majority of total radioactivity was excreted in faeces in all species. After oral doses, approximately 99 – 102%, 80-87% and 63.7% of the dose were excreted in faeces in the rat, dog and human, respectively (PCR 1119, PCR 1029, PCR 1039). NC-00751 was the major metabolite in the faeces (PCR 1027, PCR 1119, PCR 1029). It could result from de-esterification of unabsorbed neotame but could also be the result of biliary or gastrointestinal secretion since radioactivity was detected in rat and dog faeces following intravenous administration (PCR 1027, PCR 1029).

Analysis of plasma samples in the toxicology studies in mice, rats, rabbits and dogs demonstrated that the plasma concentrations of neotame and NC-00751 increased with the dose with no evidence of accumulation (PCR 1044 located in PCR 1014, PCR 1013 located in PCR 1011, PCR 1049 located in PCR 1000, PCR 1023, PCR 0990, PCR 1020 located in PCR 1017).

4.2. Toxicological studies

4.2.1. Animal studies

4.2.1.1. Diet preference studies

Two studies were conducted in rat to determine the palatability/preference of neotame in the diet. In the dietary preference feasibility study test groups (5 animals/sex/group) received 50, 150, 500, 1500, 5000, or 15000 mg/kg neotame in the diets for 14 days. The controls with basal diet and positive control were also included (PCR 1132). Based on the results from this study a definitive 15-day dietary preference study was conducted with neotame at the same dose-levels using 14 male and 14 female rats per group. Basal diet was clearly preferred to diet containing neotame at concentrations as low as 150 to 500 mg/kg, and complete preference for basal diet was observed at concentrations greater than or equal to 5000 mg/kg. Addition of neotame to diets produced an immediate but transient decrease in total feed intake. On the first day of treatment, diets containing neotame at concentrations greater or equal to 5000 mg/kg were refused. Feed intake in these animals returned to near control values by the second or third day of the treatment phase. Rats that had accommodated to neotame continued to prefer basal diet rather than a diet containing neotame at dietary concentrations of 50 to 150 mg/kg or greater. A complete preference for the basal diet was recorded at dietary concentrations of 5000 mg/kg or greater. The results indicate that neotame decreased the palatability of diets even at relatively low concentrations (PCR 1150).

4.2.1.2. Sub-chronic studies

In a 13-week dietary toxicity study in rats followed by a 4-week reversibility period neotame was administered to groups of 20 animals of both sexes at dietary concentrations to provide doses of 0, 100, 300, 1000, and 3000 mg/kg bw/day. Five additional rats/sex per group were included in the control and 2 highest dose groups in order to provide for the random assignment of 5 rats per group to a 4-week reversibility period. Dietary administrations of neotame at 100 and 300 mg/kg bw produced no test article-related effects. Body weight, body weight gain and feed consumption were decreased in the 3000 mg/kg bw group compared to the controls, the difference being statistically significant for males only. Slight but statistically significant increases in alkaline phosphatase (AP), red blood cells counts (3000 mg/kg bw females only), and decreases in cholesterol and mean cell volumes (males only) were observed in the 1000 and 3000 mg/kg bw groups. The individual values for these parameters were generally within respective historical control ranges, the differences were not seen in the reversibility period, nor were they associated with histological or other clinical pathology changes. The absolute organ weights of adrenals, heart, kidneys, liver, prostate, spleen and thymus were decreased in males at 3000 mg/kg bw compared to the controls. The relative weights of spleen were decreased, and the relative weights of the brain and testes were increased in males at 3000 mg/kg. There were no microscopic findings or changes in blood chemistry parameters indicative of damage in these organs. All affected organ weights, with the exception of the prostate, were similar to controls following the 4-week reversibility period. An increased incidence of slight cortico-medullary mineralization in the kidneys of females, a common finding in female rats at the time of sexual maturation, was found by microscopy at 1000 (not statistically significant) and at 3000 mg/kg bw. There was no microscopic evidence of tubular damage or changes in clinical chemistry or urine analysis parameters in females, which would have indicated toxicity or altered renal function. The NOEL was considered by the Panel to be 300 mg/kg bw based on the increase in AP (PCR 0988).

In a 13-week range-finding study in mice neotame was administered to groups of 20 animals of both sexes at dietary concentrations to provide doses of 0, 100, 1000, 4000, and 8000 mg/kg bw/day. A satellite group of 20 animals/sex/group was used for periodic sampling for pharmacokinetic analysis. There were no test article-related effects at dosages up to 1000 mg/kg bw. Decreases in body weight gain were observed in the 4000 and 8000 mg/kg bw male groups (88 and 85% of controls) and in the 8000 mg/kg bw female group (93% of controls). These decreases were consistent with increased feed scattering on day 1 by the 4000 and 8000 mg/kg bw male and female groups and on day 2 at 8000 mg/kg bw group. Small but statistically significant decreases in mean corpuscular volume were observed in females in the 4000 and 8000 mg/kg bw groups, but these were within historical reference range and were not accompanied by any other changes in clinical pathology. Absolute liver weights at 8000 mg/kg bw and relative liver weights at 4000 and 8000 mg/kg bw were statistically significantly increased in both sexes. These increases were not accompanied by microscopical changes. The NOEL was considered by the Panel to be 1000 mg/kg bw based on the effect on relative liver weight (PCR 0989).

In a 13-week dietary toxicity study in dogs followed by a 4-week reversibility period neotame was administered to 5 groups of beagle dogs of both sexes at concentrations to provide doses of 0, 60, 200, 600, or 2000 mg/kg bw. Control and the two highest dose groups consisted of 6

animals/sex/group while the other two groups had 4 animals/sex/group. The additional 2 dogs/sex in the control and 2 highest dose level groups were used for the reversibility phase of the study.

As feed consumption and body weight in 2000 mg/kg bw group were significantly reduced during the first 2 weeks, the dietary concentration of neotame was decreased from 5% to 3.5% corresponding to an upper dose of 1200 mg/kg bw. There were no deaths and no test-article related findings at 60 or 200 mg/kg bw. Discolouration of faeces (white and grey) was recorded in the 2 highest dose groups. Some dogs in the highest dose group had a thin appearance. Neither of these clinical signs were observed after the first week of the reversibility period. Statistically significantly increased serum AP activity was recorded in females at 200 mg/kg bw in week 13 compared with controls, although it was not increased compared with the predose, baseline value for that group. At the 600 and 1200 mg/kg bw/day dose levels, serum AP activities were statistically significantly increased in both sexes after 6 and 13 weeks. The increases in serum AP activity at the high dose were less than 4 –fold above the control values and were approximately 2.5 times the predosing values. Serum AP activity returned to normal levels in both males and females during the 4-week recovery period. The observed effects on haematology parameters were decreased red blood cell counts, haemoglobin concentrations, and haematocrit at 1200 mg/kg during week 13. The only effect on organ weights was a statistically significantly increased relative liver weight at 600 mg/kg bw for females and for both sexes at 1200 mg/kg bw. Microscopically, a higher incidence of minimal to moderate liver glycogen was observed in both sexes at 600 and 1200 mg/kg bw dosages. The NOEL was considered by the Panel to be 200 mg/kg bw based on the effect on AP (PCR 0990).

4.2.1.3. Chronic studies

In a 52-week toxicity study by dietary administration to Sprague Dawley CD rats with exposure *in utero* and followed by a 4-week reversibility period a parent generation (F_0) (25 animals/sex/group) received neotame at dietary concentrations to provide doses of 0, 10, 30, 100, 300, and 1000 mg/kg bw/day for 4 weeks before and throughout pairing. Females from the F_0 generation continued treatment throughout gestation and lactation to weaning at day 21 but the dose in the highest dose group was reduced to 300 mg/kg bw from day 14 to 21 after littering to minimise differences in body weight of pups at the time when they began to consume solid feed. After weaning, 1 male and 1 female from each litter were chosen for a 52-week toxicity study. Groups consisted of 20 animals of each sex and were exposed to the same dose levels as the parental generation. An additional 10 animals/sex/group were selected for a 4-week reversibility study. Satellite groups consisted of 6 animals/sex/group for dose levels of 0, 10, and 30 mg/kg bw and of 24 animals for doses of 100, 300, and 1000 mg/kg bw. Feeding neotame had no effect on mortality, behaviour or clinical appearance with the exception of a brown staining on the muzzle of males in 1000 mg/kg bw group (considered by the petitioner as a result of the brown diet adhering to the muzzle). No effects were seen on physical conditions, clinical signs, haematology, clinical chemistry, urine composition, and organ weights, gross or microscopic findings. There was no indication of organ toxicity, including immunotoxicity or neurotoxicity. The only effects recorded were on body weight and feed intake. The body weight of both sexes was not affected at 10 and 30 mg/kg bw throughout the study and at doses of 100, 300, and 1000 mg/kg up to 13 weeks. Thereafter, a slight reduction in body weight was recorded for males but the reduction was not dose-related and it was not statistically significant at the 2 highest doses. The body weights of males at the 3 highest doses were 90%, 93% and 96% of controls at the end of the study. In females, consistent decreases in

body weight and body weight gain compared to the controls were recorded at 100, 300, and 1000 mg/kg bw from week 24 of the study. The body weights of females in the 3 highest doses were 88%, 87% and 87% of controls at the end of the study. Feed intake of females at 100, 300, and 1000 mg/kg bw was consistently reduced from week 14 and throughout the study. There were no neotame-related effects on feed conversion efficiency during the active growth period (weeks 1-13). The effects on feed consumption and body weight were considered to be linked to the reduced palatability of diet containing neotame and not due to toxicity of the compound (PCR 1011). It was noted that there were no effects on AP in this study.

In a 52-week toxicity study by dietary administration to beagle dogs followed by a 4-week reversibility period neotame was administered as dietary admix in doses of 20, 60, 200, and 800 mg/kg bw. Control animals and all animals in the reversibility period were fed a basal diet. Control and the 2 highest dose groups consisted of 6 animals/sex/group while the other 2 groups had 4 animals/sex/group. The additional 2 dogs/sex in the control and 2 highest dose level groups were used for the reversibility phase of the study. Feeding neotame had no effect on mortality. No effects were observed during physical, neurologic, ophthalmologic, or electrocardiographic examinations. There were no test article-related haematology findings or effects on organ weights, gross or microscopic findings. There was no indication of organ toxicity, including immunotoxicity or neurotoxicity. Feed consumption was reduced for males at 800 mg/kg bw during the first two weeks and it was lower (not statistically significantly) until week 4. Cumulative body weight gains were generally decreased for males at 800 mg/kg bw during the first 8 weeks of the study. The only clinical observation that was considered test article-related was grey faeces at 800 mg/kg bw, which was not observed after the first day of the reversibility period. The only consistent and treatment-related effect was a statistically significantly increased serum AP activity at 800 mg/kg bw in both sexes at all time points tested (weeks 13, 26, 39, 52). This effect was reversible when animals were placed on basal (control) diet following 52 weeks of treatment (4-week reversibility period). AP serum activity of females at 200 mg/kg bw was statistically significantly higher than controls during week 26 and 52 by a standard pair wise comparison. However the Panel noted that the mean baseline value in this dose group was 20% greater than that of the baseline value for controls. There was no statistical difference at any time point at 200 mg/kg when serum AP data from all dose groups were analysed by analysis of covariance with repeated measures using predosing AP activity values as covariate. Isozyme analysis demonstrated that the increase in serum AP was due to elevation of the hepatic form of the enzyme. The elevation of AP was not accompanied by any microscopic evidence of cholestasis or hepatotoxicity, lesions in intestines or bone, or other clinical chemistry evidence of effects on the hepatobiliary system. The Panel considered the NOEL in this study to be 200 mg/kg bw neotame based on the effect on AP activity (PCR 1017).

4.2.1.4. Carcinogenicity studies

In a carcinogenicity study by dietary administration to Sprague Dawley CD rats with exposure *in utero* a parent (F_0) generation (85 animals/sex/group) was administered neotame at dietary concentrations providing doses of 50, 500, and 1000 mg/kg bw/day for 4 weeks before and throughout pairing. Control group (170 animals/sex) received basal diet only. Females from the F_0 generation continued treatment throughout gestation and lactation to weaning at day 21 after littering. The dose for dams from the mid- and high-dose groups was reduced to 300 mg/kg bw from day 14 to 21 after littering to minimise differences in body weight of pups at the time when they began to consume solid feed. This dietary concentration was also given to selected

F_1 offspring from weaning to the beginning of the carcinogenicity phase. Where possible, one male and one female from each litter were selected for the carcinogenicity phase. The F_1 generation (75 animals/sex/group) was exposed to the same dose levels as the parental generation for 104 weeks. Controls (147 animals/sex) received basal diet only. An additional 12 animals/sex/group were selected to form satellite groups at dose levels of 0, 50, 500, and 1000 mg/kg bw.

At termination, the survival was 31%, 45%, 56% and 53% for males and 32%, 39%, 49% and 44% for females in controls and groups receiving neotame at doses of 50, 500, and 1000 mg/kg bw/day, respectively. A brown staining on the muzzle was recorded for males at 500 and 1000 mg/kg bw/day. Body weight, body weight gain and feed consumption were reduced (in no dose-dependent manner) in all treated groups of both sexes compared to controls. Body weights as percentages of controls were 95%, 96%, 97% for males and 97%, 96%, 97% for females in order of ascending dose by week 13. By week 26, body weights in all dose groups in both sexes were 93 to 94% of controls. By week 52, body weights in all dose groups were 90 to 92% of controls for males and 85 to 90% for females. After week 78 the body weights as percentages of controls were 90, 88, and 90 for males and 89, 84, and 86 for females in order of ascending dose. There were no neotame-related effects on feed conversion efficiency during the active growth period (weeks 1-13). Although there were statistically significant decreases in males at 50 mg/kg bw/day and females at 50 and 500 mg/kg bw/day (94-95% of controls) no differences were observed in males and females at higher dosages (98-100% of controls). Several changes in absolute and relative organ weights, secondary to reduction in body weights, were recorded. Non-neoplastic findings, although with sporadically statistically significant incidence, were those commonly observed in aging Sprague-Dawley rats and did not show a dose related response or trend suggestive of a treatment-related effects. The onset and incidence of neoplasms in all treatment groups of both sexes was not statistically significantly different compared to controls except for an increased incidence of adenomas in kidneys of males at 50 mg/kg bw. This finding was regarded as incidental, since no tumours were observed in the high dose group. The results of the study indicated no carcinogenic potential of neotame in doses up to 1000 mg/kg bw, the highest dose level tested. The effects on feed consumption and body weight were regarded as linked to palatability of neotame-containing diet and not due to toxicity of the compound (PCR 1000).

In a carcinogenicity study by dietary administration to CD -1 mice the animals (70/sex/group) were administered neotame at dietary concentrations to provide doses of 50, 400, 2000, and 4000 mg/kg bw/day for 104 weeks. Control group (140 mice/sex) received basal diet. In addition, 35 mice/sex/group were assigned to satellite groups. The survival was not affected by neotame. After 104 weeks the survival ranged from 37% to 54%. Body weight, body weight gain and feed consumption of both sexes were generally lower compared to the controls at 400, 2000, and 4000 mg/kg bw. At week 52 body weights of treated mice ranged from 93 to 95% of controls. At week 78, body weights were 90 to 94% of controls. Feed consumption in both sexes was lower on several occasions at 400, 2000, and 4000 mg/kg bw. The decrease was most apparent from week 15 to 77. There were no treatment-related effects on absolute or relative organ weights except for those secondary to reductions in body weights. The incidences of non-neoplastic lesions were within expected limits of aging CD -1 mice with one exception. In the kidney, an increase in the incidence of chronic inflammation and a decrease in the incidence of chronic progressive nephropathy were observed in males at 2000 and 4000 mg/kg bw when compared to controls. Since chronic inflammation is usually an early component of progressive nephropathy the apparent increase is considered to be related to the

lower incidence of nephropathy at the higher dose levels. The types and incidences of neoplasms in all groups were those expected in this strain and age. The incidence of hepatocellular adenomas in males and of bronchiolar-alveolar carcinomas in females at 4000 mg/kg bw were higher (but not statistically significantly) when compared to controls. The increased incidences of both tumors were considered unrelated to treatment because of the absence of pre-neoplastic changes to support a finding of carcinogenicity. The results of the study demonstrated no carcinogenic effect of neotame in doses up to 4000 mg/kg bw, the highest dose level tested. The effects on feed consumption and body weight were regarded by the Panel as linked to palatability of neotame containing diet and not due to toxicity of the compound (PCR 1014).

4.2.1.5. Reproductive and developmental toxicology

In a two-generation reproductive study by dietary administration to Sprague-Dawley CD rats the F_0 generation (28 animals/sex/group) was administered neotame at dietary concentrations to provide doses of 100, 300, and 1000 mg/kg bw/day (based on the results of 2 reproductive range-finding studies in rats: PCR 0987 and PCR 1007). Control animals received basal diet only. F_0 males were treated for 10 weeks and females for 4 weeks before pairing. Treatment continued throughout pairing, gestation, and lactation to weaning on day 21 after littering and until selection for the next generation. Offspring (F_1) were weaned at day 21 of age, and developmental, behavioural and performance parameters were evaluated. At approximately 4 weeks of age offspring were selected to form the breeding F_1 generation and were treated for a minimum of 10 weeks before pairing to generate F_2 offspring, which were raised to day 21 of age before termination. Growth and reproductive parameters were assessed in the F_0 and F_1 generations. Growth, physical maturation and behavioural parameters were assessed for the offspring. All adult animals were subjected to detailed necropsy and the determination of reproductive organ weights.

The only effects recorded were those on body weight, body weight gain and feed consumption. In F_0 males the decreases in body weight and feed intake were recorded during the whole pre-mating period at 100 and 1000 mg/kg bw. In F_0 females, body weight was decreased at 1000 mg/kg bw in pre-mating period and through gestation. On day 20 of gestation, body weight of high dose females was 95% of controls. In F_1 generation the decreased body weights were recorded on several occasions: at birth for males receiving 300 mg/kg bw, and for males, females, and combined sexes receiving 1000 mg/kg bw, on day 21 for both sexes in groups of 300 and 1000 mg/kg bw, and prior to mating for both sexes at 1000 mg/kg bw. Body weights of F_1 females remained lower compared to controls throughout gestation at 1000 mg/kg. In F_2 generation a lower body weight was recorded on day 21 in both sexes at 1000 mg/kg bw. The recorded changes in body weights were accompanied by lower feed consumption compared to controls. There were no test article–related effects on reproductive parameters (i.e. oestrus cycle, mating performance, fertility, gestation length, parturition and gestation index) throughout 2 successive generations up to the highest dose of 1000 mg/kg. Litter size, sex ratio and offspring viability indices were unchanged by treatment. There were no effects on offspring development in either generation. No test article-related effects were observed on physical development or on auditory or visual performance, activity or learning ability or physical maturation of F_1 offspring. In adult animals there were no test article-related effects on gross findings or organ weights at necropsy. The results indicate that neotame had no effect on the reproduction and fertility of rats exposed to neotame at levels up to 1000 mg/kg bw/day for two generations (PCR 1001).

In a dietary teratology study in the rat 24 female Sprague-Dawley CD rats/group were administered neotame at dietary concentrations to provide doses of 100, 300, and 1000 mg/kg bw for four weeks before pairing (with untreated males), and throughout gestation until necropsy for examination of uterine contents on day 20 after mating. Control animals were fed basal diet. Fetuses were weighed and examined externally at necropsy. Approximately 50% of fetuses were examined internally and processed and examined for skeletal development. The remaining fetuses were examined for visceral abnormalities by sectioning. The only effects observed were decreases in feed consumption and relative body weight of females prior to mating. There was an immediate and transient decrease in feed consumption on day 1 of the premating phase at 300 and 1000 mg/kg bw resulting in decreased body weight gain (57% of controls) for the first week of premating at 1000 mg/kg. Body weight gain at 1000 mg/kg bw improved to 87% of controls by the end of the 4-week premating period and was 98% of controls on day 20 of gestation. There were no clinical signs and no effects on pregnancy rate, corpora lutea count, and pre- and post-implantation loss or litter size. Fetal survival, fetal weights and development (incidences of skeletal or soft tissue malformation) were unaffected in all doses. The results indicate that neotame at doses up to 1000 mg/kg bw had no adverse effects on fertility and the developing fetus (PCR 0999).

In a teratology study in the rabbit, 20 mated New Zealand White females/group were given neotame at doses of 50, 150, or 500 mg/kg bw by gavage (10 ml/kg) between day 6 and 19 (inclusive) after mating. Controls were given vehicle. Approximately 20% of the rabbits in each group, including controls, were not pregnant and 5 animals were added to the control and 500 mg/kg bw dose groups to supplement the number of litters for fetal examination. All dams were subjected to a detailed necropsy on day 29 after mating. Fetuses were weighed and examined externally and internally at necropsy. The heads of one third of the fetuses were fixed and examined following serial sectioning. Fetuses were processed and examined for skeletal development. Additionally satellite groups (5 pregnant females/group) were used for a pharmacokinetic study. Blood samples were collected on dosing days 1, 8, and 14 for measurement of plasma concentration of neotame and NC-00751 concentrations. Mean feed consumption was not affected in the first week of dosing. During the second week, lower feed consumption was recorded in small numbers of animals from all groups, including controls. This effect (although not statistically significant) was most pronounced at 500 mg/kg bw. The mean feed consumption in the high-dose group was 71% in week 2 and 83% in the entire dosing period of that in the controls. Overall feed consumption and body weight gain at 500 mg/kg bw were not statistically significantly different from controls after exclusion of 3 dams (see below). Six litters were not carried to term. Total litter loss was observed in 1 control dam and in 1 dam from 500 mg/kg bw dose group (loss of a single implantation early in pregnancy). One dam at 150 mg/kg bw died due to a gavage error. Furthermore, one dam died on day 20 and 2 others aborted on day 28 or 29 in 500 mg/kg bw group. These 3 rabbits showed markedly lower feed consumption in the second week of dosing and body weight losses. At 150 mg/kg bw one litter from the most affected female (with reference to decreased feed consumption and body weight loss) showed four small and grossly abnormal foetuses. Since the effect was not dose related and rabbits are particularly sensitive to body weight loss, these results were not considered significant by the Panel. While the possibility of some maternal toxicity at 500 mg/kg bw cannot be ruled out, there were no effects on the progress and outcome of pregnancy in rabbits with litters carried to term when neotame was administered orally by gavage in doses up to 500 mg/kg bw (PCR 1023).

4.2.1.6. Genotoxicity

Neotame was not mutagenic in the *Salmonella typhimurium/Escherichia coli* plate incorporation assay both in the absence and the presence of an exogenous source of metabolic activation (Aroclor 1254-induced rat liver S9) at concentrations up to 10000 µg/plate without producing cytotoxicity, when tested in five histidine-requiring *Salmonella typhimurium* strains (TA1535, TA 1537, TA 1538, TA98, TA 100) or with *Escherichia coli* strain WP2 *uvrA* (PCR 0963). In the mouse lymphoma cell gene mutation assay at concentrations up to 1000 µg/ml neotame showed no evidence of mutagenic activity in replicate tests, both with and without metabolic activation at concentrations ranging up to 800 µg/ml. Mutant frequency at a concentration of 1000 µg/ml was not evaluated in this study because of toxicity (PCR 0965). In an *in vitro* chromosome aberration assay in Chinese hamster ovary cells (CHO), neotame at concentrations up to 500 µg/ml without metabolic activation, and up to 1000 µg/ml with metabolic activation demonstrated no genotoxic activity (PCR 0964). In an *in vivo* mouse bone marrow micronucleus assay, neotame in doses of 500, 1000 or 2000 mg/kg bw administered by oral gavage to groups of 5 male and 5 female CD -1 mice did not induce any changes in the ratio of polychromatic erythrocytes to total erythrocytes, or in the frequency of micronucleated polychromatic erythrocytes (PCR 1026).

4.2.2. Human studies

The objectives of the clinical testing program were to evaluate the metabolism and pharmacokinetics, and the safety in healthy subjects and those with Non-Insulin Dependent Diabetes Mellitus (NIDDM).

Neotame in single doses of 0.10, 0.25, or 0.50 mg/kg bw in solution was well tolerated by 19 healthy male subjects (N=6-7 subjects /dose) (PCR 1035) and by 12 healthy male subjects in a three-way cross over study (with washout periods of at least 72 hours) (PCR 1111). In both studies, no observations of clinical significance of any biochemical, haematological, physiological or subjective findings were recorded. The maximum plasma concentration of neotame was reached approximately 0.5 hours after administration. Mean plasma half-lives were short, approximately 0.75 hours for neotame and about 2 hours for NC-00751. The pharmacokinetics of neotame and NC-00751 were approximately linear across the administered dose range as measured by AUC and C_{max} (PCR 1035, PCR 1111).

Neotame in single doses of 20 mg as a solution or in capsules was well tolerated by healthy subjects (12/sex). The relative bioavailability of neotame from capsules was greater than from solution in females but not in males. The relative exposure to NC-00751 was greater from capsules than from solution in all subjects, with no differences between sexes (PCR 1112).

Eight single doses of 0.25 mg/kg bw neotame in solution ingested at hourly intervals (total dose of 2 mg/kg bw) were well tolerated by 12 healthy males. No test article-related changes in any biochemical, haematological, or physiological parameters, or in assessment of subjective findings were recorded. Neotame was rapidly absorbed and eliminated. The mean apparent half-life in plasma for neotame was 0.88 hr. Steady state plasma concentrations were achieved after 2 hours after the first dose. Approximately 3% of the administered dose of neotame was excreted unchanged in the urine, and approximately 23% of the administered dose was recovered in the urine as NC-00751. A biphasic plasma elimination of NC-00751 was observed

in this study: about 93%was eliminated with an apparent half life of approximately 1.3 hours and the remaining 7% with a terminal half life of approximately 14 hours (PCR 1145).

Neotame in capsules administered 3 times a day, equivalent to a daily dose of 0.5 or 1.5 mg/kg bw was well tolerated by healthy subjects (12/sex/group) during 14 days. Reported adverse events were evenly distributed among treatment groups without evidence of an effect of test article administration. For example, the incidence of headache, the most frequently reported adverse event, was 21%, 29% and 15% of subjects in the placebo, 0.5 mg/kg, and 1.5 mg/kg groups. No test article-related changes in biochemical, haematological, or physiological parameters, ECG results, vital signs, or assessment of subjective findings were recorded. Plasma concentrations of neotame were not detectable after overnight fast. Steady state concentrations of NC-00751 were achieved within 72 hours of the first dose of neotame (PCR 1113).

Neotame in capsules administered 3 times a day and equivalent to a daily dose of 0.5 or 1.5 mg/kg bw for 13 weeks was well tolerated by healthy subjects (76 males and 75 females). There were no test article-related changes in any biochemical, haematological, or physiological parameters, ECG results, vital signs, or assessment of subjective findings. Reported adverse events were evenly distributed among all treatment groups without evidence of any effect of test article administration. Headache was the most frequent adverse event reported with incidences of 31%, 29% and 27% in the placebo, 0.5 mg/kg bw, and 1.5 mg/kg bw per day dose groups, respectively. The number of subjects reporting adverse events was not statistically significantly different between the treatment groups (PCR 1114).

The potential influence of neotame on carbohydrate metabolism was studied in 18 male and 19 female Non-Insulin Dependent Diabetes Mellitus (NIDDM) subjects. Neotame in capsules administered 3 times a day and equivalent to a daily dose of 0.5 or 1.5 mg/kg bw during 14 days was well tolerated by this population. There were no test article-related differences in any safety parameters. Ten subjects experienced 13 adverse events, with the most frequently reported events being flu syndrome, headache, and sinusitis. More subjects reported adverse events while on placebo. One serious adverse event (myocardial infarction) occurred in one subject after one week on placebo and was considered unrelated to treatment. Administration of neotame did not affect plasma glucose and insulin concentration in NIDDM subjects (PCR 1115).

4.2.3. Studies with degradation products

In addition to the toxicological studies, the stability of NC-00764 and the extent of de-esterification to NC-00769 were studied *in vitro* in simulated gastric and intestinal fluids. NC-00764 was rapidly and completely de-esterified to NC-00769 in simulated intestinal fluid by enzymes in pancreatin. The results indicated that de-esterification of NC-00764 would be expected *in vivo* (PCR 1229).

4.2.3.1. Studies with NC-00751

The major metabolite and degradation product NC-00751 was tested in the Ames/*Salmonella* assay (strains TA1535, TA1537, TA98, TA100, TA102) both in the absence and the presence

of an exogenous source of metabolic activation (Aroclor 1254-induced rat liver S9) at concentrations up to 5000 µg/plate (PCR 1137) and in the CHO (AS52) mutation assay with and without metabolic activation (Aroclor 1254-induced rat liver S9) at concentrations up to 5000 µg/ml (PCR1138). The compound showed no evidence of genotoxicity in either test.

4.2.3.2. Studies with NC-00764

The acute toxicity of NC-00764 was examined in Sprague-Dawley rats (10/sex/group) given doses of 0.6, 2.0 or 6.0 mg/kg bw by oral gavage and observed for 14 days. There were no effects attributed to administration of NC-00764 in either male or female rats at doses up and including 6.0 mg/kg bw (PCR 1134). NC-00764 in the Ames/*Salmonella* assay (strains TA98, TA100, TA102, TA1535, and TA1537), both in the presence and absence of an Aroclor-induced rat liver metabolic activation system, showed no evidence of mutagenic activity at concentrations up to 5000 µg/plate (PCR 1086). NC-00764 at concentrations up to 5000 µg/ml either with or without metabolic activation demonstrated no mutagenicity in an *in vitro* gene mutation assay in CHO cells with the xanthine-guanidine phosphoribosyl transferase gene locus (AS52/XRT) (PCR1087). In an *in vivo* mouse bone marrow micronucleus assay, NC-00764 in doses of 500, 1000 or 2000 mg/kg bw administered to CD -1 mice (5 mice/sex/time point for each dose group) by oral gavage did not induce chromosome damage, thus demonstrating no genotoxic activity (PCR 1090).

4.2.3.3. Studies with NC-00777

The acute toxicity of NC-00777 was examined in Sprague-Dawley rats (10/sex/group) given doses of 0.6, 2.0 or 6.0 mg/kg bw by oral gavage and observed for 14 days. There were no effects attributed to administration of NC-00777 in either male or female rats at doses up and including 6.0 mg/kg bw (PCR 1189). NC-00777, tested in the Ames/*Salmonella*/microsome assay (strains TA97A, TA98, TA100, TA1535, and TA102), both in the presence and absence of an Aroclor-induced rat liver metabolic activation system, showed no evidence of mutagenic activity at concentrations up to 5000 µg/plate (PCR 1191). NC-00777 at concentrations up to 390 µg/ml either with or without metabolic activation demonstrating no genotoxic activity in an *in vitro* gene mutation assay in CHO cells with the xanthine-guanidine phosphoribosyl transferase gene locus (AS52/XRT) (PCR1192). In an *in vivo* mouse bone marrow micronucleus assay, NC-00777 in doses of 500, 1,000 or 2000 mg/kg bw administered to CD -1 mice (5 mice/sex/time point for each dose group) by oral gavage did not induce chromosome damage, thus demonstrated no genotoxic activity. Yellow staining around the genitalia was observed in some animals at 1000 and 2000 mg/kg bw (PCR 1196).

4.2.3.4. Studies with NC-00779

NC-00779 tested in the Ames/*Salmonella*/microsome assay (strains TA97A, TA98, TA100, TA1535, and TA102) both in the presence and absence of an Aroclor-induced rat liver metabolic activation system, showed no evidence of mutagenic activity at concentrations up to 5000 µg/plate, (PCR 1201). NC-00779 at concentrations up to 5000 µg/ml either with or without metabolic activation demonstrated no genotoxic activity in an *in vitro* gene mutation assay in CHO cells with the xanthine-guanidine phosphoribosyl transferase gene locus (AS52/XRT) (PCR 1202). In an *in vivo* mouse bone marrow micronucleus assay, NC-00779 in doses of 500, 1000 or 2000 mg/kg bw administered to CD -1 mice (5 mice/sex/time point for

each dose group) by oral gavage did not induce chromosome damage, thus demonstrating no genotoxic activity (PCR 1206). The acute toxicity of NC-00779 was examined in Sprague-Dawley rats (10/sex/group) given doses of 0.3, 1.0 or 3.0 mg/kg bw by oral gavage and observed for 14 days. There were no effects attributed to administration of NC-00779 in either male or female rats at doses up to and including 3.0 mg/kg bw (PCR 1199).

4.2.3.5. NC-00764/NC-00777/NC-00779 mixture study

The mixture of NC-00764, NC-00777 and NC-00779 was administered in the diet to Sprague-Dawley rats (15/sex/group) to provide dosages at 0.2/0.2/0.1, 0.6/0.6/0.3, 2.0/2.0/1.0,or 6.0/6.0/3.0 mg/kg during 4 weeks. The relative concentration of the components in the test-mixture was formulated to simulate the ratio of such components in soft drink beverages to which consumers might be exposed. There were no unscheduled deaths and no clinical signs attributed to the treatment during the study. The findings were: (I) reduced feed consumption of females from the first 3 dose groups during the first 4 days, which was attributed to reduced palatability of the diet, (II) slight but statistically significant increase in serum concentration of calcium in females from the two highest dose groups and of phosphorus in the highest dose group, (III) mineralization and dose-dependent focal and multi-focal nephropathy in females, but the values were within the historical control limits. It was concluded that there were no test article-related effects on mean body weights, body weight gain, feed consumption, ophthalmology, haematology, clinical chemistry or urinanalysis parameters, no effects on organ weights, organ weights ratios, or macroscopic or microscopic findings (PCR 1186).

4.2.3.6. Studies with NC-00799 and NC-00800

NC-00799 and NC-00800 were studied for mutagenicity in 4 tester strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in *Escherichia coli* (WP2uvrA). Mutagenicity was tested using a pre-incubation method in the presence and absence of a rat liver metabolizing system (S-9) at 10% and 30%. All positive control compounds were mutagenic indicating the sensitivity of the test method. The carcinogenic nitrosamine, diethylnitrosamine (DEN), also produced dose-related mutagenicity in 3 tester strains of *S. typhimurium* in the presence of 30% S-9 and in WP2uvrA with a greater response at 30% S-9 than at 10%. NC-00799 and NC-00800 tested up to concentrations of 5000 µg/plate, which in some tests was toxic, produced no significant increase in mutants and therefore failed to show any evidence of mutagenicity.

4.2.3.7. Supplementary studies with neotame and /or NC-00751

The effect of neotame on hepatic xenobiotic metabolising enzyme activities was examined in rats. Three groups of 6 male and 6 female Crl:CD BR rats received neotame in the diet at the dose level of 100, 300, or 1000 mg/kg/day for 14 days. An untreated control (6 rats/sex) received a basal diet. Another group (6 rats/sex), which received a basal diet and sodium phentobarbital, a known hepatic enzyme inducer, by oral gavage (75 mg/kg bw), served as a positive control. Neotame did not affect liver weight, liver cytosol nonprotein thiol concentrations, hepatic microsomal or cytosolic protein, microsomal cytochrome P_{450} concentrations, or the activity of other cytochrome P_{450}-related enzyme activities after dietary administration to rats of doses up to 1000 mg/kg bw for 14 days (PCR 1032).

The *in vitro* binding of ^{14}C-neotame to human and dog plasma proteins and of ^{14}C-NC-00751 to human, dog, and rat plasma proteins were evaluated. Both test articles were labelled with ^{14}C in the C-1 position of the 3,3 dimethylbutylamine moiety. Additionally the protein binding of both test substances to human plasma albumin and α_1-acid glycoprotein were evaluated. The binding of neotame to human plasma protein ranged from 94% to 98%, predominantly to albumin. There was no evidence of saturation with increasing concentrations. The binding to α_1-acid glycoprotein was negligible. The binding of NC-00751 to human plasma protein and α_1-acid glycoprotein was less than that of neotame. The binding of neotame and NC-00751 to dog plasma protein and of NC-00751 to rat plasma protein was lower than that in the human (PCR 1208).

4.2.3.8. Other studies

These studies were done in order to examine potential pharmacological effects of neotame and NC-00751 on the gastrointestinal, autonomic nervous, cardiovascular, respiratory and renal systems, and furthermore to evaluate the potential of neotame to interact with hepatic metabolism of hexobarbital or its pharmacodynamic effect on the central nervous system.

Neotame at doses of 5 or 15 mg/kg bw by gavage did not affect gastrointestinal motility in a charcoal propulsion test in rats (PCR 1169). Neotame and NC-00751 did not affect basal tone or the autonomic responses to agonist-induced spasmogenic effects of the isolated guinea pig ileum (PCR 1170). Neotame administered intraduodenally to anaesthetised Beagle dogs in doses of 5 or 15 mg/kg bw did not affect any of the cardiovascular or respiratory parameters recorded. There were no test article-related effects in urinary excretion of protein or electrolytes at both dose levels (PCR 1167).

Neotame in doses of 5 or 15 mg/kg bw administered orally to rats had no effect on hexobarbital-induced sleeping time (PCR 1168). This indicates that neotame did not interfere with the metabolism of hexobarbital or its pharmacodynamic effects on the central nervous system.

4.3. Analysis of data on feed consumption and body weight in sub-chronic, chronic and carcinogenicity studies.

Two studies, submitted by the petitioner, investigated whether the changes in body weight gain in sub-chronic, chronic and carcinogenicity studies with neotame were adverse or secondary to decrements in feed consumption (Flamm *et al.,* 2003, Mahew *et al.,* 2003).

In the first study, feed consumption and body weight data were extracted for weeks 26, 52, and 78 from studies on dietary administration of neotame to Sprague-Dawley rats (PCR 1011, PCR 1000), from published studies with saccharin and sucralose, and from a number of dietary restriction or dietary optimisation studies involving restrained eating in Sprague-Dawley rats. These data were used for evaluation of the relationship between changes in feed consumption and body weight. The percent reduction in feed consumption resulted in a correspondingly greater percent reduction in body weight gain after 26, 52, or 78 weeks of dosing with neotame, saccharin, sucralose, or various degrees of dietary restriction in all studies available for evaluation. Further analysis of 52-week data demonstrated that percent changes in body weight gain were allometrically consistent with the observed percent changes in feed consumption for

these intense sweeteners, including neotame. Furthermore, the authors proposed an approach to delineate body weight changes due to toxicity from those secondary to reduced feed consumption using objective criteria. Accordingly, body weight effects should be considered not adverse, when there is evidence of decreased feed consumption, of palatability effects on feed consumption, of lack of dose response over more than 10-fold range of doses for decrease in body weight/body weight gain and/or feed consumption. Furthermore, unaltered feed assimilation and utilization, as assessed by feed conversion efficiency calculations during the period of active growth, lack of adverse clinical observations and clinical pathology parameters, unaltered water consumption, lack of adverse effects on mortality, moribundity, lack of evidence of identifiable organ toxicity, and of treatment-related macroscopic and microscopic findings, all support the conclusion that changes in body weight and body weight gain are not adverse (Flamm *et al.*, 2003).

The aim of the second study was distinction between toxicity and non-adverse palatability-related effects on feed consumption in sub-chronic (PCR 0988, PCR 0990) and chronic studies in rats and dogs (PCR 1011, PCR 1017), in carcinogenicity studies in rats and mice (PCR 1000, PCR 1014), and in the two generation study in rats (PCR 1001). The authors assessed the effects of the concentrations of neotame required for dietary dosing in these studies in terms of palatability and dietary preference based on the results from the dietary preference study in rats (PCR1150). Accordingly, when offered a choice, rats preferred basal diet to neotame-containing diet at relatively low concentrations. When no choice was available, increasing the concentration of neotame in diets decreased palatability, thus rats ate less feed. In all species tested, changes in neotame concentrations in feed were accompanied by changes in feed consumption. Reductions in feed consumption were due to the concentration of neotame in diet rather than the dose of neotame achieved. At neotame concentrations up to approximately 500 mg/kg the diets were palatable. This concentration was comparable to approximately a dose of 50 mg/kg bw/day at about week 13 in rat studies, when no changes in feed consumption, body weight and body weight gain were recorded. The range of neotame dietary concentrations from approximately 500 to 35000 mg/kg resulted in reduced palatability. These concentrations were comparable to doses of 40-3000 mg/kg bw/day to 350 g rats eating 30 g/day of feed, 75-500 mg/kg bw/day to 40 g mice eating 6g/day, and 20-1600 mg/kg bw/day to 9 kg dog typically eating 400 g/day. Dietary concentrations increased as animals grew but generally remained within this range of reduced palatability throughout dosing in rats for dose groups receiving 50-1000 mg/kg bw/day, in mice for dose groups receiving 100-4000 mg/kg bw/day, and in dogs for dose groups receiving 20-800 mg/kg bw/day. When dietary concentrations were greater than 35000 mg/kg, the palatability of neotame-containing diets was poor, and rats, mice and dogs refused these diets. Marked reductions in feed consumption, body weight and body weight gain occurred in all species tested at doses requiring dietary concentrations of neotame greater than 35000 mg/kg. Consequently, dosages provided by concentrations greater than 35000 mg/kg exceeded the maximum tolerable doses (the highest doses where decrease in body weight gain is not greater than 10%) and also exceeded the maximum palatable doses (the highest doses where animals would eat and drink sufficient to remain healthy) for rats, mice and dogs. Doses requiring concentrations of 35000 mg/kg or higher were used in 13-week studies in rats (3000 mg/kg bw/day) and mice (8000 mg/kg bw/day), and were attempted in 13-week dog study (dose of 2000 mg/kg bw). Thus, the authors concluded that the maximum tolerated doses in the 13-week studies in rats, dogs, and mice were limited by the poor palatability of the feed containing concentrations of neotame greater than 35000 mg/kg, and the maximum tolerated doses for chronic, carcinogenicity, and two generation studies were limited by the reduction in body weight and body weight gain relative to controls rather than toxicity. There were no neotame related changes in feed conversion efficiency during active growth in

chronic and carcinogenicity studies in rats indicating that neotame did not alter nutrient digestion, metabolism, or assimilation. Feed consumption, body weight and body weight gain were not reduced in a dose –dependent manner over the 10-fold range of doses in the rat chronic study, over the 20-fold in the rat carcinogenicity study, or over the 80-fold range of doses in the carcinogenicity study in mice. Reduced body weight and body weight gain were the only consistent finding in neotame safety studies. The authors concluded, that these changes were adequately explained by the small decrements in feed consumption, and that consideration of the objective criteria proposed in the first study (Flamm *et al.,* 2003) supported the conclusion that reduced body weight and body weight gain observed in neotame studies were neither adverse nor a manifestation of toxicity, and that they were not appropriate endpoints for setting NOEL and ADI for neotame (Mahew *et al.,* 2003)

DISCUSSION

The metabolism and pharmacokinetics of neotame have been studied in mice, rats, dogs, rabbits and humans. Approximately 20-30% of orally administered neotame is absorbed in all species. The major metabolic pathway in humans and animals is de-esterification to NC-00751 and methanol. Two other metabolites are detected in humans at levels greater than 1% of the dose: a carnitine conjugate of the metabolite NC-00785 found in urine, and NC-00754 found in faeces. Both metabolites were formed in rats and dogs. Thus their safety is considered established via exposure during dosing with neotame of both animal species and in humans.

Distribution studies in rats given radiolabelled neotame by gavage demonstrate that the absorbed radioactivity does not accumulate in tissues. The radioactivity is primarily associated with the gastrointestinal tract and organs of metabolism and excretion (liver and kidney). No radioactivity has been observed in the fetus. Elimination of radiolabelled neotame is rapid with more than 80 % of the dose recovered in the urine and faeces within 48 hours. Total recoveries are approximately 98% in the human and greater than 93% in the rat and dog.

The safety of neotame has been investigated in *in vitro* studies and in a large number of short and long-term studies in mice, rats, rabbits and dogs. The results indicate that neotame is not genotoxic, or carcinogenic or associated with any reproductive/developmental toxicity, apart from possible maternal toxicity in the rabbit.

In these studies, the consistent findings reported were reduced feed consumption, body weight and body weight gain relative to that of controls with no clear dose response. These findings could be linked to the poor palatability of the diet containing neotame in the increasing concentrations necessary to provide the constant dosages throughout all dietary studies as indicated by several observations. One of them is the high incidence of food scattering recorded in the mouse 13-week dietary study. Another one is a partial reversibility of the body weight changes in the rat and dog 13-week studies when the animals were returned to basal diet during the 4-week period at the end of the study. Furthermore, reduced feed consumption often occurred at the start of treatment at all doses, followed by some degree of adaptation as the animals adjusted to the diet. Body weight changes were not closely correlated to the dose of neotame as would be expected if changes were due to treatment-related toxicity. No changes in body weight gain or feed conversion efficiency were recorded for rapidly growing rats consuming neotame-containing diets in the 1-year rat study and in the carcinogenicity study in

rats. When the effect of neotame on palatability of the diet of rats was specifically examined in a preference study comparing basal diets with and without neotame at concentrations of 50-15000 mg/kg diets, rats showed a clear preference for a diet without neotame when the neotame concentration was 150 mg/kg diet and above. The decreases in feed consumption and body weights were not accompanied by any changes in relative organ weights. Furthermore, the reduced palatability of animal diets, with added high concentrations of an intense sweetener has been previously reported for saccharin and sucralose (Chowaniec and Hicks, 1979; Grice and Goldsmith, 2000). An analysis of a relationship between the feed consumption and body weight changes in the neotame safety studies (Mayhew *et al.,* 2003; Flam et al. 2003) provides additional support to the hypothesis that the effects on feed consumption and body weight gain were the result of poor palatability of neotame-containing diet. In the light of the above considerations, the Panel considered that the decreases in body weight or body weight gain were attributable to palatability problems and were not appropriate endpoints for setting NOAELs for neotame.

Increased levels of AP were recorded in 13-week studies in rats and dogs and in a 52-week study in dogs. Regarding the 13-week study in rats, the statistically significant increase in AP at the two highest dose levels (1000 and 3000 mg/kg bw) compared to the concurrent control in week 13 was not considered an adverse effect for several reasons. Although the level of AP in the highest dose group was statistically significantly higher compared to controls, there was no increase within this dose group compared with baseline. All mean values were within the age- and sex- matched laboratory historical control data. The increase was not seen following the reversibility period and there were no associated microscopical or other clinical pathology changes. Furthermore, the increase in AP was not observed in 1-year and 2-year chronic studies in rats. In the dog, however, the increase in AP has been observed in two studies. In a 13-week study AP was increased at the two highest doses (600 and 1200 mg/kg bw) in both sexes in week 6 and 13. It was also increased at 200 mg/kg bw in females at week 13, but as the AP activity in females at 200 mg/kg was not increased from pre-dosing levels, only the increases at the two higher doses were considered treatment-related. In the one-year dog study elevated serum AP levels were recorded at 200 mg/kg bw in females in weeks 26 and 52, and at 800 mg/kg bw for males and females as early as in week 13 and until the end of the study. However, the difference in AP levels at 200 mg/kg was attributable to a higher pre-dosing level of the enzyme compared with controls. Furthermore, it was not statistically significant by analysis of covariance with repeated measures. Therefore the elevation in serum AP activity was considered limited to the highest dose in this study. In both dog studies the increased AP levels were demonstrated to be of hepatic origin. The elevation of hepatic AP activity was not associated with any microscopic changes in the liver, biliary tract, gut or bone. There were no significant effects of neotame on other liver enzymes (e.g. gamma glutamyl transferase, alanine aminotransferase, and aspartate aminotransferase). Serum bilirubin levels were normal in both sexes at high doses of neotame (an increase would have been seen if cholestasis was occurring). In both studies serum AP activity returned to normal levels at the end of recovery period. Furthermore, the mildly increased liver glycogen at the two highest doses in the 13-week study was not recorded in the 52-week study. Although the increase in serum AP seen at the two highest dose levels in both dog studies was not accompanied by any other indication of hepatotoxicity, the Panel considered increase in AP to be the critical endpoint and established a NOAEL of 200 mg neotame/kg bw/day for setting an ADI.

The results of human studies demonstrated that neotame was well tolerated by healthy and diabetic human subjects at dose levels up to 1.5 mg/kg bw/day. In these studies, a headache

was the most frequently noted adverse experience, but the incidence was comparable for the treated and control groups and therefore it was considered not treatment-related.

Neotame is stable under conditions of intended use as a sweetener across a wide range of food and beverage applications. Neotame degrades slowly in aqueous conditions such as those in carbonated soft drinks. The major pathway is hydrolysis of neotame to NC-00751 and methanol. The three minor degradation products NC-00764, NC-00777 and NC-00779 are not detected at the anticipated concentrations and condition of neotame use in carbonated soft drinks.

The major degradation product and metabolite NC-00751will have been present in both the human and animal studies. Its safety has also been estimated by an *in vitro* genotoxicity study. The three minor degradation products (NC-00764, NC-00777 and NC-00779) have been shown to have a low acute toxicity and are not genotoxic. Furthermore, no treatment-related adverse effects were observed in a 4-week dietary study in rats with a mixture of the three minor degradation products. Based on the above, the dietary exposure to degradation products is considered to pose no safety concerns.

The exposure to methanol, which may result from ingestion of neotame-containing foods and beverages, is considered negligible compared to that from other dietary sources and as such of no concern from the safety point of view.

The Panel calculated that the amount of phenylalanine exposure from neotame, assuming 100% hydrolysis, based on a conservative estimate of exposure to neotame in the adult population (0.2 mg/kg bw) could be 5.28 mg/person/day by a 60 kg adult. This corresponds to 0.05 to 0.21% of what is expected to be ingested from the normal diet (2.5 to 10 g phenylalanine/person/day). This amount of exposure is of no safety concern for a healthy consumer.

For a phenylketonuric homozygous child, the additional phenylalanine intake expected from ingestion of neotame as a general purpose sweetener (0.5 mg/kg bw/day) would be 4.4 mg/person/day for a 20 kg individual. This corresponds to 0.73 to 2.2% of the normal daily dietary exposure of the homozygous child to phenylalanine (0.2 to 0.6 g/person/day). The Panel noted that this represents a relatively small increment in the exposure of the phenylketonuric homozygous child to phenylalanine.

The Panel considered the hypothetical formation of nitrosamines in the gastrointestinal tract from reaction of nitrite with neotame and its major degradation product and/or metabolite NC-00751. Two studies provided by the petitioner demonstrated that neither nitrosoneotame (NC-00799) nor nitrosated de-esterified neotame (NC-00800) could be detected when neotame and nitrite were incubated under simulated gastric juice conditions.

However, the Panel noted that any detection of nitrosoneotame, if formed under the simulated gastric conditions, would be dependent on the sensitivity of the analytical chemical method

used. In the first study, a very high nitrite concentration was used and nitrosoneotame should have been easily detected at the detection limit of 340 nM if it had been formed according to the accepted reaction kinetics for nitrosamine formation from nitrosatable secondary amines. In the second study, a more realistic (low) nitrite concentration was used. However, in this experiment, although the detection limit had been improved, the detection limit of 56 nM would not have been sufficient to detect nitrosoneotame if the formation (if any) had followed the anticipated reaction kinetics. In that case, the nitrosoneotame concentration would have been lower than 1 nM. Taken together these results did not indicate formation of nitrosoneotame from neotame and nitrite under conditions resembling those in the human stomach.

Both nitrosamines were adequately tested for genotoxicity in the Ames test. The Panel noted that the Ames test was the most appropriate and sensitive test to detect genotoxic nitrosocompounds. Neither of the nitrosated compounds was mutagenic in the Ames test using a protocol optimised for the detection of genotoxic nitrosamines. The Panel considered that the information available is sufficient to conclude that any possible nitrosation of neotame, should it occur, is not a matter of concern.

Concerning the specifications, the level for lead proposed by the petitioners is twice as much as in the JECFA purity criteria (JECFA, 2003b). It seems more appropriate to base the specification of neotame as a food additive not on the lead level proposed by the petitioners, but on the JECFA criterion for lead (not more than 1 mg/kg) considering that neotame may be used in a variety of food products.

CONCLUSIONS AND RECOMMENDATIONS

There are extensive *in vitro* and in vivo animal studies and some human tolerance studies on neotame. After considering all the data available, the Panel concluded that neotame is not carcinogenic, genotoxic or associated with any reproductive/developmental toxicity. The only relevant treatment-related effect observed in animal studies was an increase in serum AP activity in 13-week and 52-week studies in the dog. The Panel considered that the NOAEL for this effect was 200 mg neotame/kg bw in the 52-week study in dogs.

Studies in humans demonstrated that daily doses of neotame up to 1.5 mg/kg bw/day were well tolerated.

The Panel considered that the information available is sufficient to conclude that any possible nitrosation of neotame, should it occur, is not a matter of concern.

After considering all the data on stability, degradation products and toxicology, the Panel concluded that neotame is not of safety concern with respect to the proposed uses as a sweetener and flavour enhancer.

The Panel established an ADI of 0-2 mg/kg bw based on the application of a 100-fold safety factor to the NOAEL of 200 mg/kg bw from a 52-week dog study.

Conservative estimates of neotame dietary exposure both in adults and children suggest that it is very unlikely that the ADI would be exceeded at the proposed use levels.

The Panel recommended that the limit for lead in the specifications should not be higher than 1 mg/kg, which is in line with the limit for other permitted sweeteners.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier prepared and submitted by the NutraSweet Company.
2. Additional information: Response to request from AFSSA (and where appropriate, SCF) for additional information concerning the "Request for an opinion on the authorization of neotame as a food additive (1999-SA-0059)".
3. Chemical stability of NC-00723 in chewing gum – 52 week final report, study number NP00-002 in Additional information, Appendix D.
4. Report on investigation into the potential for nitrosation of neotame. August 2006.
5. Report on bacterial mutagenicity testing of nitrosated neotame and nitrosated de-esterified neotame. August 2007.
6. NC-00799 and NC-00800 bacterial mutation test. Final report. August 2007.

REFERENCES

AFSSA, 2004. Avis de l'Agence relatif à l'emploi d'un édulcorant néotame comme additif alimentaire.Available at: <http://www.afssa.fr/Object.asp?IdObj=25589&Pge=0&CCH=041015114702:26:4&cwSID=234011A99895468086870D94CB33E2BA&AID=0>

Chowaniec, J., Hicks, R.M., 1979. Response of the rat to saccharin with particular reference to the urinary bladder. *Br. J. Cancer* 39, 355-375.

European Commission, 1999. Report From The Commission On Dietary Food Additive Intake In The European Union: <http://europa.eu.int/comm/food/food/chemicalsafety/additives/flav15_en.pdf>

Grice, H.C., Goldsmith, L.A., 2000. Sucralose – an overview of the toxicity data. *Food. Chem. Toxicol*. 38 (Suppl. 2), S1-S6.

Flamm, W.G., Blackburn, G.L., Comer, C.P., Mayhew, D.A., Stargel, W.W., 2003. Long-term food consumption and body weight changes in neotame safety studies are consistent with the allometric relationship observed for other sweeteners and during dietary restrictions. *Regul. Toxicol. Pharmacol.* 38, 144-156.

JECFA, 2003a. Summary and conclusions of the sixty-first meeting of the Joint FAO/WHO Expert Committee of Food Additives, Rome, 10-19 June 2003.

JECFA, 2003b. Specifications for neotame. FNP 52, Add. 11. FAO, Rome.

Mahew, D.A., Comer, C.P., Stargel, W.W. 2003. Food consumption and body weight changes with neotame, a new sweetener with intense taste: differentiating effects of palatability from toxicity in dietary safety studies. *Regul. Toxicol. Pharmacol*. 38, 124-143.

Mirvish, S.S. 1975. Formation of N-nitroso compounds: Chemistry, Kinetics, and *in vivo* occurrence. *Toxicol. Appl. Pharmacol*. 31, 325-352.

SCF. 1984. Reports from the Scientific Committee for Food (16th series). Opinion expressed 1984. *Food science and techniques*, 1985.

Trefz, F. 2002. Letter to Stargel W.W. with opinion on the implications of the use of the new sweetener neotame. In: Additional information submitted by the petitioner.

U.S. FDA, Department of Health and Human Services. 2002. Food additives permitted for direct addition to food for human consumption; Neotame. *Federal Register*, 67 (131), pp 45300- 45310.

APPENDICES

APPENDIX A

Identification of key compounds.

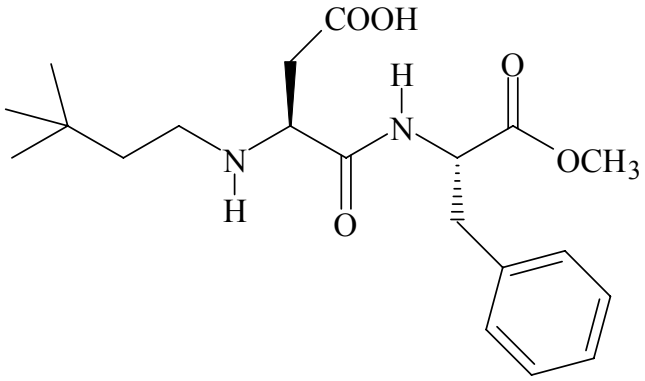


Figure 1. **NC-00723. N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester**

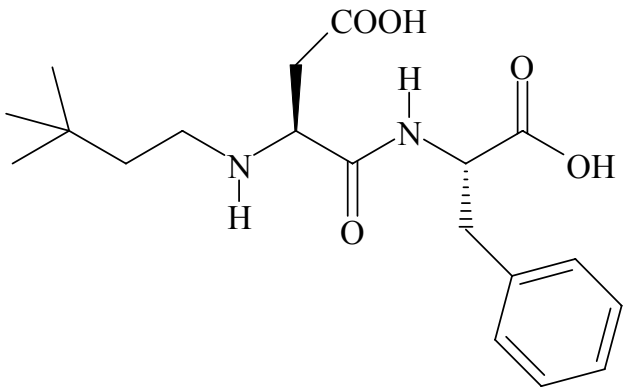


Figure 2. **NC-00751. N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine**

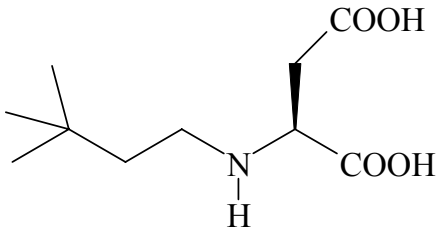


Figure 3. **NC-00754. N-[N-(3,3-dimethylbutyl)-L-aspartic acid**

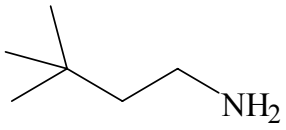


Figure 4. **NC-00759. 3,3-dimethylbutylamine**

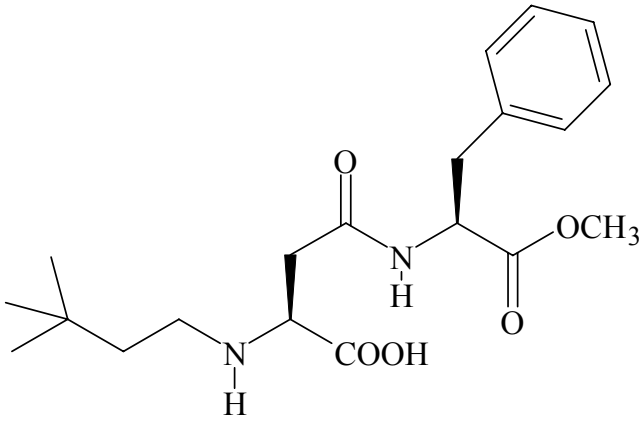


Figure 5. **NC-00764. N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester**

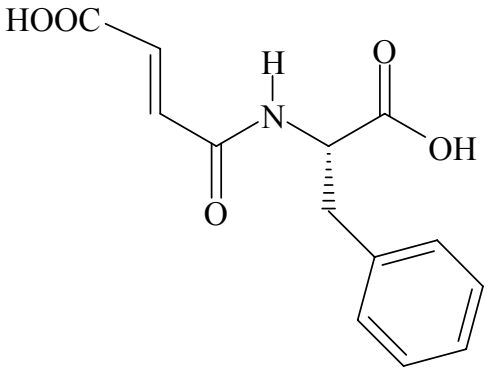


Figure 6. **NC-00767. Fumarylphenylalanine**

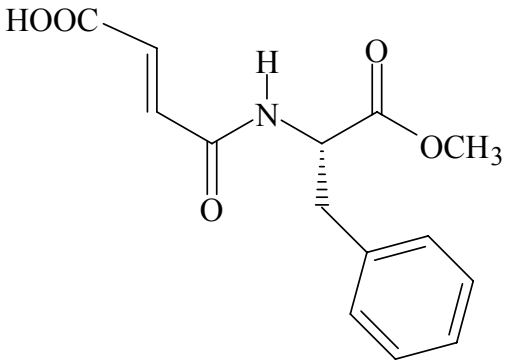


Figure 7. **NC-00768. N-Fumarylphenylalanine 1-methyl ester**

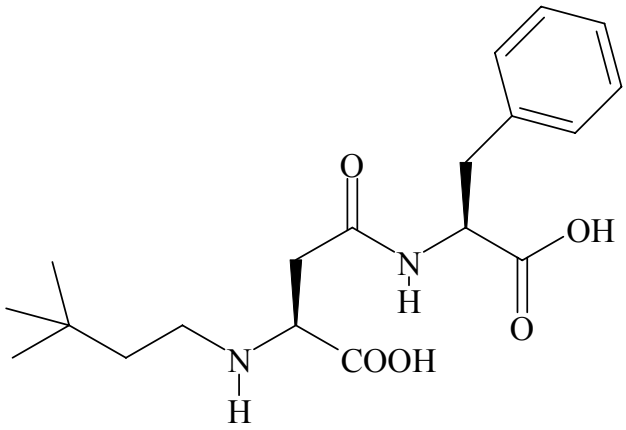


Figure 8. **NC-00769. N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine**

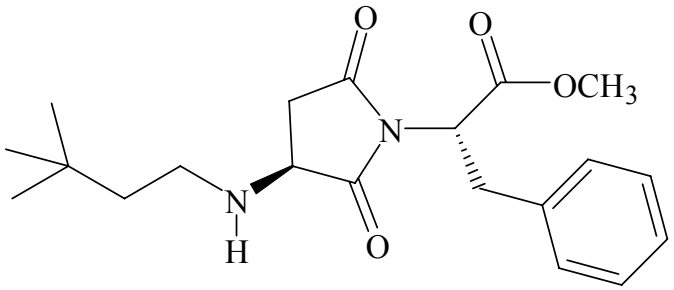


Figure 9. **NC-00777. N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester**

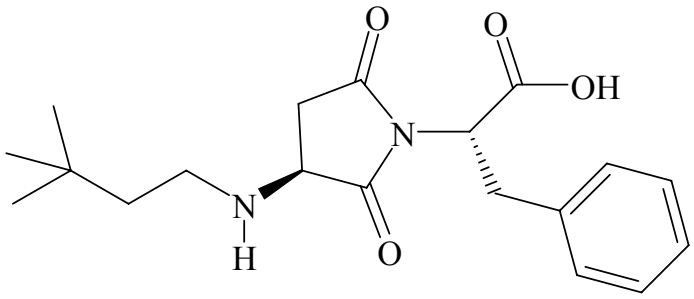


Figure 10. **NC-00779. N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine**

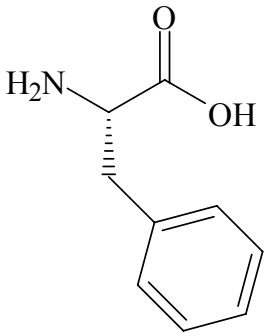


Figure 11. **L-Phenylalanine**

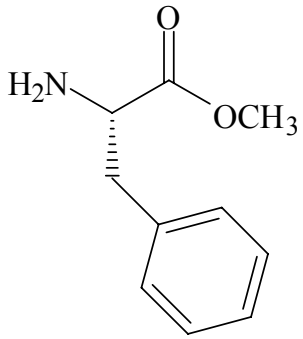
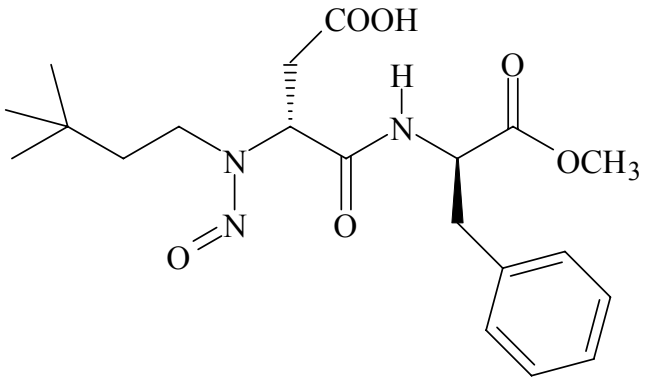
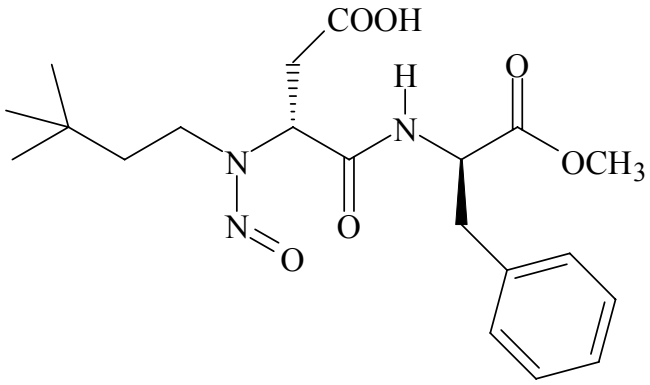


Figure 12. **L-Phenylalanine methyl ester**

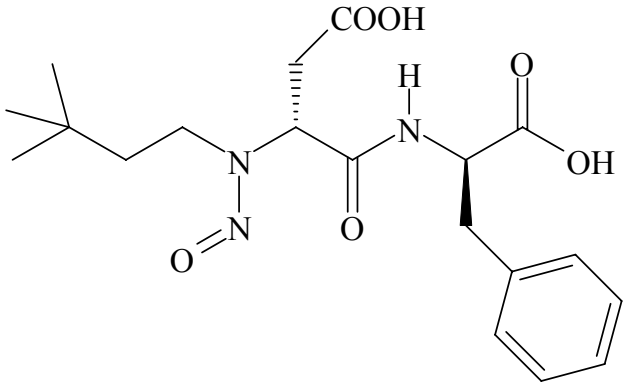


E-Isomer

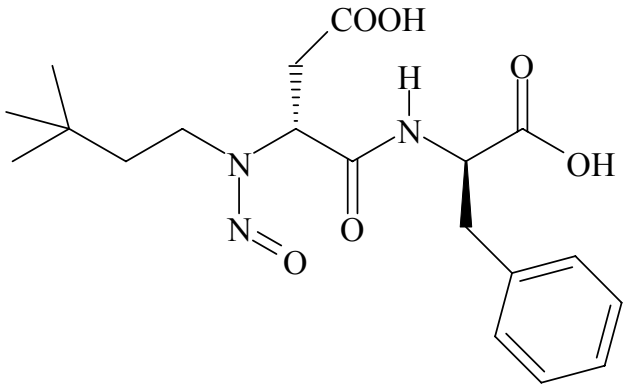


Z-Isomer

Figure 13. NC-00799. N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester



E-Isomer



Z-Isomer

Figure 14. NC-00800. N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine

APPENDIX B

Reports of biological and toxicological studies with neotame and degradation products submitted in the dossier.

PCR 0957. Metabolism of ^{14}C-NC-00723 in the rat pilot investigation.

PCR 0958. Determination of ^{14}C-NC-00723 distribution in rats by whole-body autoradiography.

PCR 0959. Tissue distribution of ^{14}C-NC-00723 in the rat.

PCR 0960. Metabolism of ^{14}C-NC-00723 in the dog pilot investigation.

PCR 1027. Metabolism of ^{14}C-NC-00723 in the rat.

PCR 1028. NC-00723: Pharmacokinetics of single doses in the rat after oral and intravenous administration.

PCR 1029. Metabolism and pharmacokinetics of ^{14}C-NC-00723 in the dog.

PCR 1031. Determination of ^{14}C-NC-00723 distribution in the pregnant and non-pregnant rats by whole-body autoradiography.

PCR 1032. NC-00723 effect on hepatic xenobiotic metabolising enzyme activities in rats by dietary administration for 14 days.

PCR 1117. ^{14}C-NC-00723 and ^{14}C-NC-00751: Storage stability in fortified human faeces.

PCR 1119. Metabolism of ^{14}C-NC-00751 in the rat.

PCR 1141. ^{14}C-NC-00723 and ^{14}C-NC-00751: Storage stability in fortified rat and dog urine.

PCR 1142. ^{14}C-NC-00723 and ^{14}C-NC-00751: Storage stability in rat and dog faeces.

PCR 1172. Synthesis of [1-^{14}C]-butyl-NC00723.

PCR 1208. ^{14}C-NC-00723 and ^{14}C-NC-00751: Studies of plasma protein binding *in vitro* (rat, dog and human).

PCR 1214. ^{14}C-NC-00723: Metabolite isolation from the rat.

PCR 1218. Stability of NC-00723 and NC-00751 in simulated gastric and intestinal fluids.

Rat toxicology

PCR 0949. Two-week dietary toxicity study of NC-00723 in rats.

PCR 0994. Two-week range-finding dietary study of NC-00723 in rats.

PCR 0988. NC-00723: Toxicity study by dietary administration to CD rats for 13-weeks followed by a 4-week reversibility period.

PCR 1000. Carcinogenicity study by dietary administration of NC-00723 to CD rats with exposure *in utero.*

PCR 1011. NC-00723: 52-week toxicity study by dietary administration to CD rats with exposure *in utero* and followed by a 4-week reversibility period.

PCR 1132. NC-00723: Dietary preference feasibility study.

PCR 1150. NC-00723: Dietary preference study.

Mouse toxicology

PCR 0936. Two-week dietary range-finding study of NC-00723 in mice.

PCR 0989. 13-week dietary range-finding study of NC-00723 in mice.

PCR 0992. Two-week dietary range-finding study of NC-00723 in mice.

PCR 1014. 104-week dietary carcinogenicity study with NC-00723 in CD -1 mice.

Dog toxicology

PCR 0952. Two-week dietary range-finding study of NC-00723 in dogs.

PCR 0990. 13-week dietary toxicity study of NC-00723 in dogs followed by a 4-week reversibility period.

PCR 1017. 52-week dietary toxicity study of NC-00723 in dogs followed by a 4-week reversibility period.

Reproductive and developmental toxicology studies

PCR 0987. Reproductive performance range-finding dietary study of NC-00723 in rats.

PCR 0998. NC-00723: Tolerance study in the rabbit.

PCR 0999. NC-00723: Dietary teratology study in the rat.

PCR 1001. NC-00723: Two generation reproductive study by dietary administration to CD rats.

PCR 1007. Reproductive performance range-finding dietary study of NC-00723 in rats.

PCR 1023. NC-00723: Teratology study in the rabbit by gavage.

PCR 1038. NC-00723: Maternal toxicity range-finding study administered by gavage to the rabbit.

Genotoxicity

PCR 0963. *Salmonella-Escherichia Coli*/Microsome plate incorporation assay of NC-00723.

PCR 0964. Measurement of chromosomal damage in Chinese hamster ovary (CHO) cells treated with NC-00723.

PCR 0965. L5178Y mouse lymphoma (MOLY) cell tk+/-→tk-/- gene mutation assay with NC-00723.

PCR 1026. Mouse bone marrow micronucleus assay of NC-00723.

Studies with degradation products

PCR 1086. Ames/*Salmonella* assay of NC-00764.

PCR 1087. AS52/XPRT gene mutation assay with NC-00751.

PCR 1090. Mouse bone marrow micronucleus assay of NC-00764.

PCR 1134. Single gavage dose study in rats with NC-00764.

PCR 1137. Ames/*Salmonella* assay of NC-00751

PCR 1138. AS52/XPRT gene mutation assay with NC-00751.

PCR 1186. Four-week dietary study of NC-00764/NC-00777/NC-00779 mixture in rats.

PCR 1189. Single gavage dose study in rats with NC-00777.

PCR 1191. Evaluation of the mutagenic potential of NC-00777 in the Ames/*Salmonella* microsome assay.

PCR 1192. AS52/XPRT gene mutation assay with NC-00777.

PCR 1196. An evaluation of the potential of NC-00777 to induce micronucleated polychromatic erythrocytes in the bone marrow of mice (micronucleus test).

PCR 1199. Single gavage dose study in rats with NC-00779.

PCR 1201. Evaluation of the mutagenic potential of NC-00779 in the Ames/*Salmonella* microsome assay.

PCR 1202. AS52/XPRT gene mutation assay with NC-00779.

PCR 1206. An evaluation of the potential of NC-00779 to induce micronucleated polychromatic erythrocytes in the bone marrow of mice (micronucleus test).

PCR 1229. ^{14}C-NC-00764 stability in simulated gastric fluid and intestinal fluid.

Studies with nitrosated neotame and nitrosated de-esterified neotame

NC-00799 and NC-00800 bacterial mutation test. Final report. Charles River Laboratories. August 2007.

Pharmacological studies

PCR 1167. NC-00723: Cardiovascular, respiratory and renal evaluation in the anaesthesised dog following intraduodenal administration.

PCR 1168. NC-00723: Assessment of hexobarbital sleeping time in rats (oral administration).

PCR 1169. NC-00723: Charcoal propulsion test in rats (oral administration).

PCR 1170. NC-00723 and NC00751: Effects on isolated guinea-pig ileum.

Human studies

PCR 1035. Single dose tolerance of NC-00723 in healthy male subjects.

PCR 1039. Pharmacokinetic study of ^{14}C-NC-00723 in healthy male subjects.

PCR 1111. Assessment of dose related pharmacokinetic profile of NC-00723 in solution administered to healthy male subjects.

PCR 1112. Comparison of the pharmacokinetic profile of NC-00723 in solution capsules administered to healthy subjects.

PCR 1113. Two-week tolerance study of NC-00723 administered to healthy male and female subjects.

PCR 1114. 13-week tolerance study of NC-00723 administered to healthy male and female subjects.

PCR 1115. Effect of multiple doses of NC-00723 compared to placebo on plasma glucose and insulin concentrations in non-insulin dependent diabetes mellitus (NIDDM) subjects.

PCR 1215. An investigation of a urinary metabolite in healthy male subjects after administration of [^{14}C/^{13}C]-NC-00723.

PCR 1145. Effect of repeated ingestion of NC-00723 in solution administered in healthy male subjects.

PCR 1215. An investigation of urinary metabolite in healthy male subjects after administration of [^{14}C/^{13}C]-NC-00723.

GLOSSARY / ABBREVIATIONS

AFC	Scientific Panel on Food Additive, Flavourings, Processing Aids and Materials in Contact with Food
NOAEL	No Observed Adverse Effect Level
AP	Alkaline Phosphatase
ADI	Acceptable Daily Intake
JECFA	Joint FAO/WHO Expert Committee on Food Additives
CAS	Chemical Abstract Service
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
NOEL	No Observed Effect Level