Applicant: Catalysta Industries Pvt. Ltd

Inventors:

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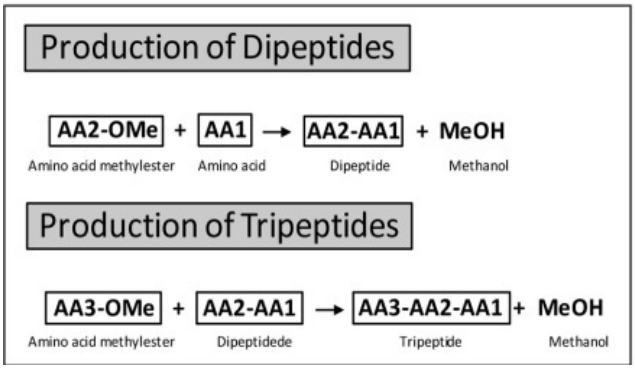
Chemical Formula: CH18N2O5

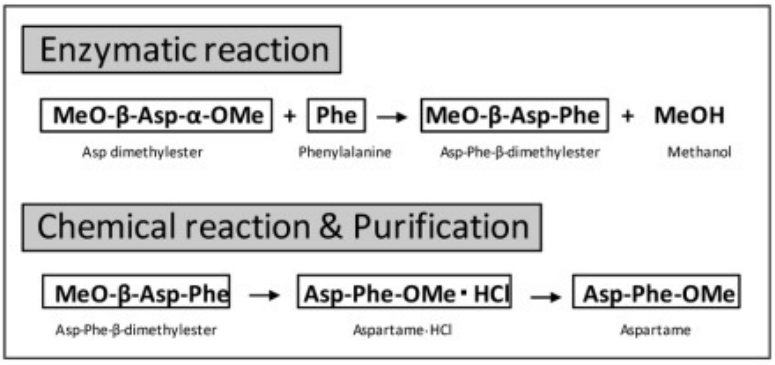
Chemical Name: Aspartame

Chemical synthesis routes:

- **Lab scale reaction:**

  Production of Aspartame through enzymatic and chemical synthesis. The following figure shows the reaction steps.





Raw materials: L-Aspartic Acid, Methanol, L-Phenylalanine, α-Amino Acid Ester Acyltransferase (AET), Hydrochloric Acid (HCl), Borate Buffer.

Chemical reaction steps with reaction conditions:

Methylation of Aspartic Acid (Asp):
Reaction: L-Aspartic Acid + Methanol ⟶ α,β-dimethylester [Asp-(OMe)2] + Water
Catalyst: Typically an acid catalyst such as Hydrochloric Acid (HCl)
Balanced Chemical Equation:
HOOCCH2CH(NH2)COOH + CH3OH -> HOOCCH2CH(NH2)COOCH3 + H2O

Reaction conditions: The concentration of the aspartic acid solution is in the range of 0.1 to 1 M (moles per liter), with reflux temperature in the range of 60-80°C.approximately 8 hours to reach completion under these temperature conditions.
yield:80%

1) Enzymatic production of Asp-(OMe)-Phe from Asp-(OMe)2 and Phe by AET
   Reaction:α,β-dimethylester [Asp-(OMe)2] + L-Phenylalanine ⟶
   L-Aspartyl-L-Phenylalanine Methyl Ester + Water
   Enzyme: α-amino acid ester acyltransferase (AET)
   Buffer: Borate buffer
   Balanced Chemical equation:
   HOOCCH2CH(NH2)COOCH3 + CH2CH(NH2)COOH⟶
   HOOCCH2CH(NH2)COCH2CH(NH2)COOCH3 + H2O

   Reaction conditions: temperature 18°C, pH 9
   Yield: 65%

2) chemical transformation of Asp-(OMe)-Phe to Asp-Phe-OMe · HCl
   The presence of hydrochloric acid helps in protonating the amino and carboxyl groups, facilitating the reaction with methanol. Methanol serves as a nucleophile in this process, leading to the esterification of the carboxyl group of Asp-(OMe)-Phe, resulting in the formation of Asp-Phe-OMe. The hydrochloride salt form of Asp-Phe-OMe is obtained due to the presence of excess HCl in the reaction mixture.
   Yield: 87%
   Additional information:
   4 types of derivatives, that is, Asp-Phe-OMe, Asp-(OMe)-Phe, Asp-Phe, and α-l-aspartyl-l-phenylalanine dimethylester [Asp-(OMe)-Phe-OMe] were found to exist at equilibrium. Among them, only Asp-Phe-OMe (aspartame) was found to be selectively precipitated with high yields as Asp-Phe-OMe · HCl

3) Crystallization of Aspartame Hydrochloride:
   The process of crystallizing aspartame hydrochloride involves adding hydrochloric acid to the reaction mixture containing Asp-Phe-α-OMe, causing the

formation of the salt. Stirring ensures complete salt precipitation. The resulting crystals are filtered using vacuum filtration and washed with a solvent like cold water or ethanol to remove impurities. Lastly, the purified crystals are dried to produce high-quality aspartame hydrochloride.

Reaction conditions: 20°C-40°C.
Yield: 80%

4) Neutralization Step Using Sodium Hydroxide: The process involves neutralizing aspartame hydrochloride to obtain α-Aspartame using sodium carbonate. α-APM hydrochloride is dissolved in water, and the pH of the solution is adjusted to 4.8 using a saturated solution of sodium carbonate ($Na2CO3$).
Crystallization: The neutralized solution is kept at 5°C overnight, leading to the precipitation of α-APM crystals. Crystals are collected by filtration and dried to obtain purified α-APM.
Yield: 65.25%

PROCEDURE:

To carry out the enzymatic synthesis of aspartame (Asp-Phe-α-OMe) in a laboratory setting:

Methylation of Aspartic Acid (Asp):

1. Dissolve aspartic acid in a suitable solvent (e.g., water or methanol). The concentration of the aspartic acid solution in the range of 0.1 to 1 M (moles per liter) is suitable for most reactions.
2. Add methanol and a suitable catalyst (e.g., sulfuric acid). you might start with adding around 0.5 to 5 mol% (molar percentage) of sulfuric acid relative to the amount of aspartic acid.
3. Heat the reaction mixture under reflux conditions for a specified period of 8 hours. Reflux meaning the reaction mixture is heated to boiling and the vapors are condensed and returned to the reaction flask. The reflux temperature can vary, but it's often in the range of 60-80°C.
4. Once the reaction is complete, remove the solvent and excess methanol under reduced pressure by using rotary evaporation.

   Steps for rotary evaporation setup:

   a) Setup: Set up a rotary evaporator apparatus according to manufacturer instructions. This typically involves connecting the rotary evaporator to a vacuum pump and a condenser.
   b) Transfer: Transfer the reaction mixture containing the solvent and excess methanol to a round-bottom flask suitable for use with the rotary evaporator.

   c) Heating Bath: Place the round-bottom flask containing the reaction mixture onto the heating bath of the rotary evaporator. The heating bath is heated to a suitable temperature, usually below the boiling point of the solvent and methanol.

d) Vacuum: Turn on the vacuum pump to create a reduced pressure inside the system. This reduces the boiling point of the solvent and methanol, facilitating their removal.

e) Rotation: Rotate the round-bottom flask at a moderate speed to increase the surface area of the solution exposed to the vacuum. This promotes faster evaporation of the solvent and methanol.

f) Condensation: As the solvent and methanol evaporate, they travel through the system and are condensed back into liquid form in the condenser. The condensed liquid is collected in a separate flask.

g) Monitoring: Monitor the evaporation process closely to prevent overheating or bumping of the solution. Adjust the temperature of the heating bath and the vacuum pressure as needed.

h) Completion: Continue the evaporation process until the desired amount of solvent and excess methanol has been removed, leaving behind the desired product in the round-bottom flask.

i) Isolation: Once the solvent and excess methanol have been removed, the remaining product can be isolated by transferring it to a suitable container for further processing or analysis.

j) Purify the MeO-β-Asp-α-OMe product using techniques such as column chromatography or recrystallization.

Condensation of Aspartic Acid Dimethyl Ester with Phenylalanine (Phe):

1. Dissolve MeO-β-Asp-α-OMe and phenylalanine in a suitable solvent like water, buffer solutions (borate buffer), and organic solvents like methanol or ethanol..
2. Add an enzyme catalyst specific to the condensation reaction (e.g., aminoacylase).
3. Incubate the reaction mixture at the appropriate temperature and pH for the AET to catalyze the condensation reaction. the reaction temperature ranges from 20°C to 40°C, and the pH is maintained within a specific range of 8.5-9.
4. Monitor the reaction progress and optimize reaction conditions if necessary.
5. Once the desired conversion is achieved, stop the reaction by heat inactivation or enzyme removal.

Spontaneous Transfer of Methyl Ester:

1. Allow the reaction mixture containing OMe-β-Asp-Phe to stand at room temperature.
2. Monitor the reaction progress by analytical techniques such as TLC (thin-layer chromatography) or HPLC (high-performance liquid chromatography).

   Thin-Layer Chromatography (TLC):

   a. TLC is a simple and quick technique used to separate and identify compounds in a mixture.

b. Spot a small amount of the reaction mixture containing Asp-Phe-α-OMe onto a TLC plate alongside reference standards.
c. Develop the TLC plate using a suitable solvent system.
d. Visualize the spots under UV light or by using a suitable staining reagent.
e. Compare the Rf (retention factor) value of the spot corresponding to Asp-Phe-α-OMe with that of reference standards to confirm its formation.

High-Performance Liquid Chromatography (HPLC):

a) HPLC is a more precise and quantitative technique for separating and analyzing compounds in a mixture.
b) Inject a small aliquot of the reaction mixture onto an HPLC column equipped with a suitable stationary phase.
c) Elute the compounds using a mobile phase gradient.
d) Detect and quantify the Asp-Phe-α-OMe peak using a UV-visible detector.
e) Compare the retention time and peak area of the Asp-Phe-α-OMe peak with those of reference standards to confirm its formation and quantify its concentration.

Crystallization of Aspartame Hydrochloride:

- Acidify the reaction mixture containing Asp-Phe-α-OMe with hydrochloric acid to form aspartame hydrochloride.
- Stir the mixture to ensure complete precipitation of the salt.
- Filter the precipitated aspartame hydrochloride crystals using vacuum filtration.
- Wash the crystals with a suitable solvent (e.g., cold water or ethanol) to remove impurities.
- Dry the purified aspartame hydrochloride crystals under vacuum or by air drying.

Purification and Isolation:

- Additional purification steps, such as recrystallization or chromatography, should be performed if necessary to obtain highly pure aspartame hydrochloride.
- Characterize the final product using analytical techniques such as NMR (nuclear magnetic resonance) spectroscopy or mass spectrometry to confirm its identity and purity.

Byproducts according to each step of the process:

Methylation of Aspartic Acid (Asp):
Partially methylated aspartic acid derivatives
Hydrolyzed products of methyl ester or peptide bonds in aspartic acid

Enzymatic Condensation of Asp-(OMe)2 with Phenylalanine (Phe):
Partially acylated intermediates containing aspartic acid, phenylalanine, and methyl ester groups
Unreacted starting materials, such as residual aspartic acid or phenylalanine
Hydrolyzed products of ester or peptide bonds

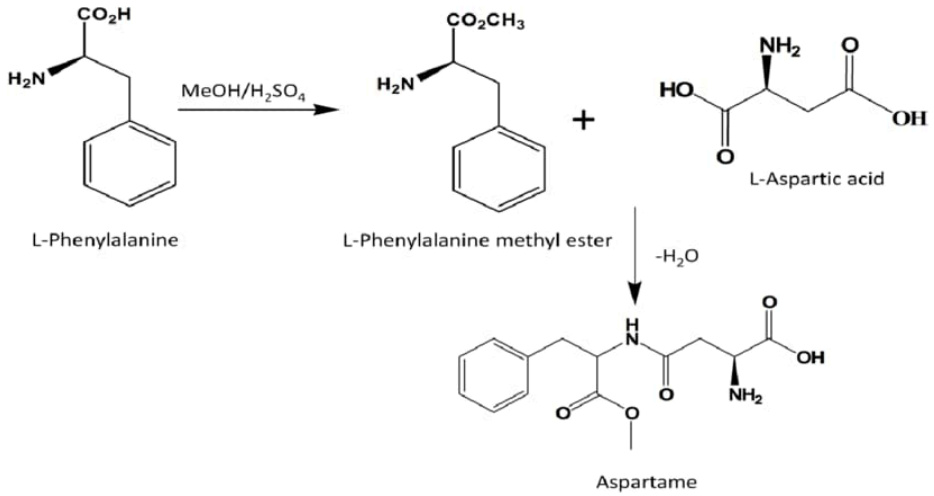
Spontaneous Transfer of Methyl Ester:

While this step may not yield significant byproducts under controlled conditions, there could still be minor hydrolyzed products or side reactions.

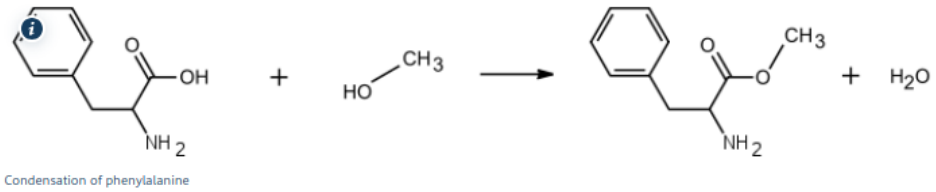
Crystallization of Aspartame Hydrochloride:
Despite efforts to purify, impurities might still be present, including residual starting materials, partially reacted intermediates, or side products from previous steps.

Alternate method:

Raw material**:** L- phenylalanine, Methanol and H2SO4



Reaction:



Condensation of phenylalanine

Fermentation
By using the bacteria Brevibacterium flavum and Corynebacterium glutamicum through direct fermentation, the amino acids L-aspartic acid and L-phenylalanine are formed. The fermentation process starts with a pure culture of bacteria, which will multiply in the presence of the necessary nutrients.
When sufficient bacteria have been formed during the pre-fermentation, the bacteria are transferred to a tank which contains all the nutrients necessary to form more bacteria. In addition, carbon and nitrogen sources are present, which are necessary for forming large quantities of amino acids. This mass is continuously kept in motion with a mixer and with a pump filtered compressed air is added to the mix. The bacteria can produce large

quantities of amino acids in a fermentation tank containing the same nutrients. The desired pH-value is maintained by using ammonia water.

Isolation
When sufficient amino acids have been formed, the desired amino acids have to be separated from those not needed. Using a centrifuge, a large quantity of the amino acids is separated from the bacteria. The required amino acids are further separated and purified in an ion exchanger. These amino acids are then pumped into a crystallization tank, after which the formed crystals are removed using a centrifuge. Subsequently, the amino acids are dried and prepared for synthesis to aspartame.

Modification
Usually, the synthesis of aspartame starts with the modification of aspartic acid and phenylalanine.
The aspartic acid is modified in such a way that certain parts of the molecule are shielded, by for example benzene rings, ensuring that further chemical reactions will only take place with the right acid group of the aspartic acid.

Synthesis
The modified amino acids are then pumped to a reactor, where they are mixed at room temperature during 24 hours. Next, the temperature is increased to 65°C, and this temperature will be maintained for another 24 hours. After cooling the mixture down to minus 18°C, a solvent is added to dilute the mixture, so crystallization can take place. The crystals which are formed during this process are isolated by filtration and dried.

Modification
In a large tank, the crystals that were formed are converted to aspartame through a reaction with acetic acid. In this tank the crystals, acetic acid solution, palladium catalyst and hydrogen are mixed together intensely for about 12 hours, causing them to chemically react with each other.

Purification
After the 12 hours of chemical reactions have passed, the palladium catalyst is removed by filtration and the solvent is removed through distillation. The remaining solid residue is dissolved with ethanol and after that recrystallized. The aspartame crystals that were produced during this process are filtered and dried.

Enzymatic synthesis may offer high selectivity and efficiency, reducing the need for purification steps and minimizing waste. Chemical synthesis routes can be well-established and optimized for large-scale production. Processes may be more easily controlled and reproducible compared to fermentation-based methods.

References:

1) Journal article: https://academic.oup.com/bbb/article/85/2/464/6056131
2) ResearchGate:

   https://www.researchgate.net/figure/Synthesis-of-aspartame_fig2_338073242

3) Patent:

   https://pubchem.ncbi.nlm.nih.gov/compound/Aspartame#section=Mechanism-of-Action

4) Website: https://www.safefoodfactory.com/en/knowledge/35-aspartame/

List the contributions of each author:

- Author 1,2,3 did a significant chunk while finalizing the reaction, and the alternative reaction also that can also be done on a lab scale
- Authors 4,5 found out the efficiency of the process, yield, and other parameters necessary for this report. They also gave a proofreading session focusing on minor problems and details.

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