THE SHIKIMATE PATHWAY

The ShikiFactory100 project aims towards the production of 100 high-added value compounds from the shikimate pathway, a hub in cell metabolism, for application in food, pharma and cosmetics. To fully comprehend this project and the research activities that it entitles, it is necessary to take a step back and explore some of the key terminology. For example, what do we mean by cell metabolism? What is the shikimate pathway? Or what are amino acids, and what role do they play in living organisms?

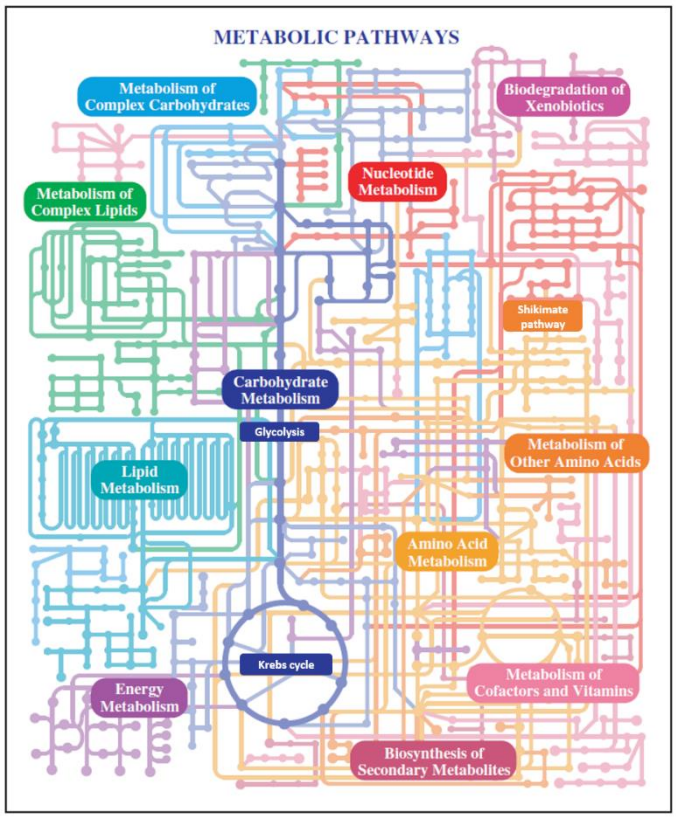

Figure 1 Metabolic roadmap of eukaryotic cells. Image adapted from 'Principles of Biochemistry' 4th edition by D. L. Nelson and M. M. Cox.

Cell metabolism refers to the series of reactions occurring within a cell which includes many metabolic pathways that are crosslinked and interrelated to each other. Each **metabolic pathway** is a chemical transformation that involves several steps. Metabolic pathways can be classified into **catabolic and** anabolic, where the catabolic group includes all those pathways that break down compounds into smaller units, and the anabolic group gathers the metabolic routes that use small molecules to produce larger compounds. In some cases, certain metabolic pathways involve both catabolic and anabolic reactions, and are known as **amphibolic pathways**.

A well-known catabolic pathway is **glycolysis.** Organisms use glycolysis to covert 1 equivalent of glucose into 2 equivalents of a smaller molecule, **pyruvate**, key for the metabolism of cells and organisms. The energy released from this process is conserved as ATP and NADH, but what are those? In living cells, the energy required to power a specific reaction or process can be supplied by different molecules one of them being adenosine triphosphate (ATP); phosphate bonds 'accumulate' energy, which is released when the bond is broken. In biological reactions, electrons are transported from one reaction to another thanks to the co-factor nicotinamide adenine dinucleotide (NAD) which exists in an oxidised (NAD^+) and reduced form (NADH). NADH can pass electrons to O_2, and the energy from these electron transfers can drive the synthesis of more ATP.

The pyruvate formed during glycolysis can be used in different ways depending on the presence or lack of oxygen (O_2). In aerobic conditions (O_2 is present), pyruvate can be fully **oxidised**, producing CO_2, H_20 and energy for the cell. An oxidation reaction is that in which the molecule of interest gives

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up electrons. On the contrary, a reduction is a reaction in which a molecule receives electrons. As mentioned above, the electrons resulting from the oxidation of pyruvate are taken by the NAD^+ co-factor to form NADH as a way to conserve the energy released during the process. Subsequently, NADH transfers those electrons to molecules of O_2, returning to its oxidised form, NAD^+, required for the glycolysis to continue. On the other hand, in anaerobic conditions (absence of O_2), pyruvate is **reduced** to form products such as lactic acid or ethanol in a process known as **fermentation** (it should be noted that some organisms show fermentative behaviour in the presence of oxygen). As mentioned above, in the presence of O_2, NADH is able to give up electrons to O_2 molecules and return to its oxidised form necessary for the glycolysis to continue. However, in the absence of O_2, this is not possible, and the pyruvate molecules are the ones that accept the electrons, being thus, reduced to ethanol or lactic acid, products of the fermentation process.

Fermentation has been used for centuries to prepare alcoholic beverages or bread. Nowadays, fermentation is also exploited industrially for the production of chemical compounds such as ethanol or lactic acid, among others. The ethanol produced by fermentation can be used as a biofuel by blending it with gasoline, and lactic acid is commercially used as a food preservative and to produce plastics. Other examples of fermentation products are succinic and citric acids. Succinic acid is considered a very important intermediate for the production of several consumer products.

As mentioned above, in the presence of O_2, pyruvate can be fully oxidised producing CO_2, H_2O and energy for the cell. This process is known as **cell respiration** and involves three main stages, including the **Krebs cycle**, another well-known metabolic pathway. The Krebs cycle (also known as the citric acid cycle) is a good example of amphibolic pathway as it includes catabolic and anabolic reactions. At the first stage of cell respiration, pyruvate is converted to acetyl-coenzyme A (acetyl-CoA), which enters the Krebs cycle during the second stage of cell respiration, and it is oxidised to form CO_2 and energy conserved in the form of NADH and $FADH_2$. In the final stage, these electron carriers pass electrons to O_2, and the energy released during this process is stored in the form of ATP. It should be noted that cell respiration is not the only metabolic role of the Krebs cycle, as many intermediates of this cycle are precursors of other metabolic pathways. Figure 1, which depicts the metabolic map of a eukaryotic cell, highlights the central role of the Krebs cycle in the metabolism of organisms.

The **shikimate pathway** is less well-known than the above-mentioned glycolysis or Krebs cycle, as it has been less exploited commercially. The shikimate pathway is an anabolic route that organisms use for the synthesis of amino acids and in particular, aromatic amino acids.

Amino acids are essential for all living organisms, as they are the building blocks for proteins, which are formed by hundreds to thousands of amino acid units linked to each other. Proteins are vital to all living organisms as most biological processes rely on proteins for regulation, catalysis of cell reactions, protection against external agents, etc. Although more than 500 amino acids have been observed in nature, only 20 of them are present in the human genetic code and therefore form human proteins. These 20 amino acids are, therefore, the most commonly studied amino acids and are generally categorised into essential amino acids – those that cannot be produced by the human body – **non-essential amino acids** – the human body is able to produce them – and **conditionally non-essential amino acids** – only produced by the human body under certain conditions.

Aromatic amino acids can be found within the group of essential amino acids (phenylalanine, tryptophan and tyrosine – it should be noted that tyrosine has been classified as conditionally non-essential amino acid because it can only be produced by the human body in the presence of

phenylalanine). Aromatic amino acids are those containing one or more aromatic rings in their structure (see Figure 2). Aromatic rings are chemical forms that are particularly stable thanks to its structure: cyclic, planar and with resonant bonds. In a six-atom ring, three resonant bonds are required to make the molecule aromatic. In addition to the formation of proteins, animals also use aromatic amino acids for the production of key molecules for their metabolisms such as serotonin, dopamine or thyroxine. It should be noted that aromatic amino acids are not only crucial to animals and their metabolisms; these compounds are also used industrially in food and pharma applications, and for the production of chemical compounds (both as products and intermediates). As an example, phenylalanine is industrially used as a precursor for the preparation of L-aspartame, an artificial sweetener which production reaches 15000 tons/year worldwide.

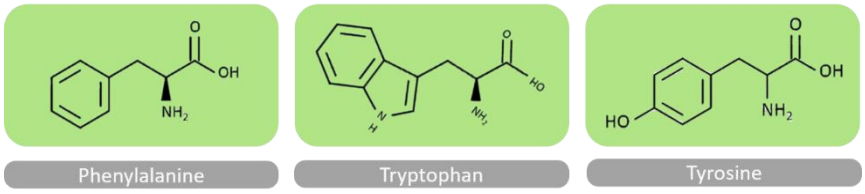

Figure 2 Aromatic amino acids.

For industrial applications, complex molecules like aromatic amino acids can be either chemically synthesised or extracted from plants. However, both methods present clear challenges. On the one hand, the chemical synthesis of aromatic amino acids tends to result in the production of undesired by-products or racemic mixtures due to unclear or not fully understood reaction mechanisms. In addition, food and pharma applications require very high-quality standards; obtaining this high level of purity can significantly increase the cost of the synthesis. On the other hand, extraction from plants is generally very costly due to the low concentration of the target compounds inside the plant. Additionally, extraction from plants requires the use of arable land for the growth of the feedstock which can cause competition with food crops and price fluctuations due to availability issues. As a result of the challenges associated with the chemical synthesis and extraction from biomass, much attention has been put towards the microbial preparation of complex compounds such as aromatic amino acids. Microbial production refers to the production of the target compounds by a host organism (e.g. yeast or bacteria) through a biosynthetic pathway that is well-known and fully characterised. The living organisms used in the production are known as cell factories. The aromatic amino acids phenylalanine, tryptophan and tyrosine can be produced by yeast and bacteria through the shikimate pathway.

The **shikimate pathway** includes seven steps to transform the starting compounds into chorismate, the precursor of the three aromatic amino acids: phenylalanine, tyrosine and tryptophan (Figure 3). The shikimate pathway uses as starting materials phosphoenolpyruvate, formed as an intermediate in the above-mentioned glycolysis pathway, and erythrose-4-phosphate – resulting from the pentose phosphate pathway, a catabolic route through which organisms transform glucose 6-phosphate into pentose phosphates, key molecules for the formation of nucleotides, the basic units that form DNA. In the shikimate pathway, each step is catalysed by an enzyme. A catalyst is a compound that accelerates the reaction rate, but it is not consumed during the reaction, and an enzyme is a biological catalyst that accelerates a specific biological reaction.

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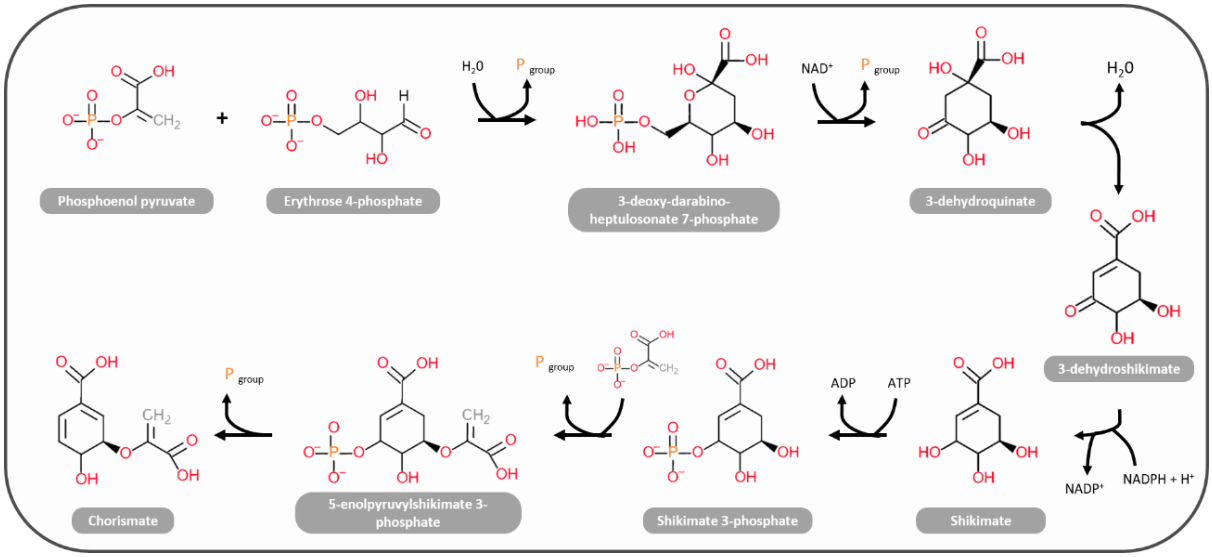

Figure 3 Shikimate pathway. Figure adapted from 'Principles of Biochemistry' 4th edition by D. L. Nelson and M. M. Cox.

The importance of the shikimate pathway derives from the fact that aromatic amino acids can serve as precursors for many other metabolites, and the intermediates formed throughout the shikimate pathway are also branch points for many other compounds. It should be noted that the shikimate pathway occurs in bacteria and other prokaryotic organisms, plants and fungi, but not in animals (remember that aromatic amino acids are categorised as essential amino acids because they cannot be produced by the human body).

So far, we have only mentioned the importance of metabolic pathways and the molecules produced through them. However, regulating the metabolic activity is as important. In bacteria, the shikimate pathway is mainly related to the formation of proteins. On the other hand, this metabolic pathway plays a much more complex role in plants, where the compounds derived from the process are involved in the formation of hormones and **regulatory compounds**. Organisms have mechanisms in place to regulate metabolic pathways, which generally involve the use of molecules that trigger or inhibit those pathways. As an example of regulatory mechanism, glucose 6-phosphate (the first intermediate of the above-mentioned glycolysis process) inhibits the production of the enzyme that enhanced the production of this compound in the first place to avoid a chain reaction. Similarly, significant research has been done to find compounds that could inhibit key metabolic pathways in plants and bacteria for the preparation of key chemical compounds such as antibiotics and herbicides. In the shikimate pathway, the sixth step of the process can be inhibited by the molecule N-[phosphonomethyl]glycine, a well-known herbicide.

In summary, cell metabolism includes hundreds of crosslinked metabolic pathways, such as glycolysis, the Krebs cycle and the shikimate route. The latter allows organisms to synthesise aromatic amino acids, which play key roles in the metabolism of these organisms, including protein formation, regulation or catalysis. These molecules are not only important for metabolic purposes, but also industrially as they play a key role in the commercial preparation of food, pharma and chemical compounds. Nevertheless, current industrial production methods (chemical synthesis and extraction from biomass) present several challenges. This has created an interest in alternative production routes, such as microbial production, where living organisms such as bacteria and yeast are used for the production of the target compounds. The aim of the Shikifactory100 project is to use world-leading synthetic biology tools for the microbial production of 100 molecules derived from the shikimate

pathway, with applications in food, pharma and cosmetics, using bacteria and yeasts as host organisms.

This article was written by Dr Andrea Muñoz Garcia, Senior Consultant at NNFCC.

About the ShikiFactory100 project

The SHIKIFACTORY100 project aims towards the production of a universe of more than 100 high-added value compounds from the shikimate pathway, a hub in cell metabolism, through the development of an optimized shikimate chassis (based in 3 sub-hubs: Phe, Trp and Tyr) and the proposal and implementation of novel biosynthetic routes exploring enzyme promiscuity to introduce new pathways for the production of known and newly designed compounds. Further information about the project and the partners involved are available under www.shikifactory100.eu.

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