STUDY MATERIALS

PHARMACEUTICAL CHEMISTRY
PART-

SEC-I, SEMESTER I

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Vitamins

Vitamin B2

Vitamin B2 is also called riboflavin, which takes its name from its yellow colour (flavus). It is essential for the proper functioning of all the flavoproteins, since riboflavin is the central component of the FAD and FMN cofactors. These are involved in oxidation–reduction reactions, which are key activities in the energy metabolism of carbohydrates, fats, ketone bodies and proteins. Vitamin B_2 is also involved in the metabolism of other vitamins such as B_6, B_3 and A, in glutathione recycling and homocysteine metabolism. The highest amounts of riboflavin in food can be found in *crimini mushrooms* and spinach, but also in asparagus, green beans, yogurt and cow's milk.

Industrial riboflavin production is a paradigm of how biotechnology can turn a chemical synthesis into a bioprocess with significant cost reductions by employing a genetic and metabolic bioengineering approach. Chemically, it is produced from d-glucose by three different processes. More than 9000 t/a of riboflavin were produced in 2010, around 75% being used for feed additive and the rest for human food and pharmaceuticals (Hoffmann-La Roche, BASF, ADM, Takeda). This compound is naturally produced by several microorganisms such as ascomycete fungi (*Ashbya gossypii, Eremothecium ashbyii*), by yeasts such as *Candida flaeri* and *Candida famata*, and also by bacteria such as *B. subtilis* and *Corynebacterium ammoniagenes*. Several metabolic approaches have been developed in *B. subtilis* by overexpression of the gene cluster involved in riboflavin synthesis and including multiple copies of these genes. Other approaches have attempted to express heterologous genes involved in riboflavin accumulation but only modest results have been achieved and some modifications guided by transcriptional analysis have afforded a strain able to accumulate 15 g l^{-1} riboflavin. However, most metabolic bioengineering strategies have been carried out in the main industrial producer *A. gossypii*. All six genes of the riboflavin synthetic pathway have been overexpressed and its use to improve riboflavin production patented. Some other genes have been reported to accumulate the vitamin when they are overexpressed, deregulated, or disrupted. These genetic alterations lead to an accumulation ranging from 1.4- to ten-fold increases relative to the wildtype, affording strains able to produce more than 13 g l^{-1}. In recent

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years, efforts in metabolic bioengineering have also been performed using *C. famata* and strains accumulating 4.1-fold the wild-type amount of the vitamin have been constructed.

Vitamin B_{12}

Vitamin B_{12} is a group of water-soluble compounds that contain the element cobalt, which

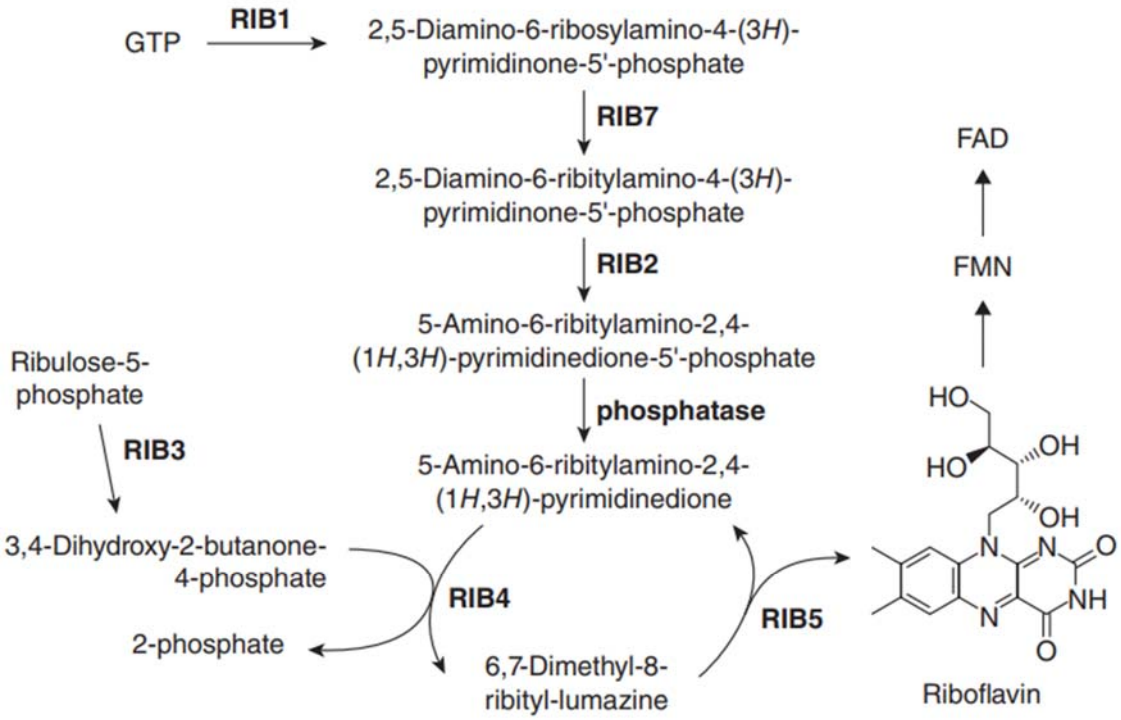

Fig. 21.6 Biosynthetic pathway of riboflavin in *Ashbya gossypii*. GTP = guanosine 5'-triphosphate; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; RIB (1–5 and 7) = riboflavin biosynthesis gene(s).

leads to them being called 'cobalamines.' There are two active forms; methylcobalamine (Fig. 21.12) and 5-deoxyadenosylcobalamine.

This vitamin is involved in DNA synthesis, neurological function and red blood cell formation. It is a cofactor directly involved in the methylation of DNA, RNA, hormones, lipids and proteins, and also in protein and fat metabolism. In the diet it is found bound to proteins and it must be released in the stomach to be absorbed. It is mainly present in animal products, such as meat, fish, poultry, milk and eggs, and because of this, vegetarians may have to obtain this vitamin from fortified foods.

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Vitamin B_{12} is therefore produced industrially for pharmaceutical products, fortified foods and animal feed. It is produced biologically from Pseudomonas and Propionibacterium fermentation (10 t/a, Rhône-Poulenc, Aventis). On the one hand, in the case of *Propionibacterium shermanii*, the vitamin is produced in two steps: (1) bacterial growth and the production of intermediates and (2) the production of vitamin B12 from corn steep liquor, glucose and $CoCl_2$, reaching production levels of 25–40 mg l^{-1}. On the other hand, when made from Pseudomonas denitrificans in a medium with sugar beet molasses and 5,6-dimethylbenzimidazol, a production of 150 mg l^{-1} was obtained.

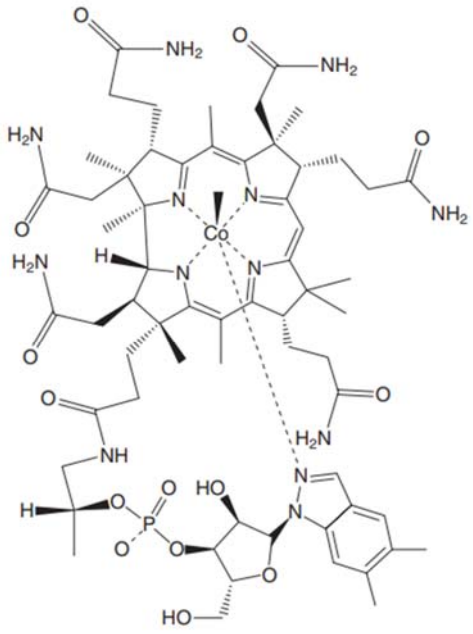

Fig. 21.12 Chemical structure of methylcobalamine.

These naturally overproducing strains have been modified in order to increase the vitamin production rate. Metabolic engineering has been carried out in *P. shermanii* by overexpression of a gene of the biosynthetic pathway, cobA and recently genome shuffling has been carried out, obtaining a 61% improvement in cobalamine production. Extensive metabolic engineering approaches have been developed in the model organism Bacillus megaterium, where biosynthetic and regulatory genes and operons were overexpressed, by-products from second pathway branches were silenced by antisense RNA, and three cobalamine-binding proteins were expressed heterologously to avoid feedback inhibition.

The most important producer, P. denitrifi cans, has also been genetically engineered. The copy-number of one operon (cobF-cobM) encompassing the eight genes involved in the synthesis of the vitamin was increased (30%); two independent genes (cobA and cobE) were also amplified (20% increase) and, in addition, strong inducible promoters, highly efficient ribosomal binding sites and terminator sequences were applied to another important gene (cobB). In order to avoid some regulatory steps, such as substrate inhibition, the heterologous expression of genes from *Methanobacterium ivanovii* and *Rhodobacter capsulatus* has been

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implemented. The combination of both approaches – genetic engineering and random mutagenesis – have led this process to being used to synthesize 80% of the world's production of vitamin B_{12}.

Vitamin C

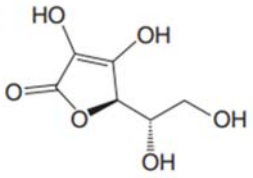

Fig. 21.13 Chemical structure of ascorbic acid.

Vitamin C is an essential dietary component that humans are unable to synthesize. It is also known as l-ascorbic acid (Fig. 21.13). It is an important antioxidant which might prevent or delay certain cancers, cardiovascular and other diseases in which oxidative stress is a crucial factor. It is also involved in the biosynthesis of collagen, *l-carnitine* and certain neurotransmitters, and in protein metabolism. It also plays an important role in immune function and improves the absorption of nonhaem iron. It is absorbed in the intestine via an active transporter and can be found in different food sources, mainly in fruits and vegetables. Citrus fruits, tomato and potatoes are major contributors of vitamin C. It is used as a food and feed antioxidant and in pharmaceuticals (110,000 t/a, Hoffmann-La Roche, Dalry, Belvidere, Takeda, etc). Normally, starch from corn or wheat is converted to glucose, which can be transformed chemically into sorbitol. From this sorbitol, and via a series of biotechnical, chemical processing and purification steps, vitamin C is produced. The so-called Reichstein method used to be the market-dominating process of synthesis but over the past two decades some bioconversions have simplified this method. The Reichstein process consists of seven steps. First, *l-glucose* is transformed into d-sorbitol. Second, *Gluconobacter oxydans* regiospecifically oxidizes *d-sorbitol* into *l-sorbitol*. *l-sorbitol* is then crystallized and condensed with acetone and sorbose-diacetone is formed, which is later oxidized to 2-keto-l-gluconic acid (2KLGA). Then, this is enolized and lactonized to form l-ascorbic acid, with a final yield of 50%. This process is still expensive owing to the high energy consumption of some steps, so an alternative is required. Most microbial methodologies lead to the production of 2-KLGA and some single-strain and mixed cultures have been developed. The single strain process includes the use of *Gluconobacter*, *Acetobacter*, *Ketogulonicigenium*, *Pseudomonas*, Erwinia and Corynebacterium. The mixed culture processes include two fermentation steps: first to produce diacetone-ketogluonic acid and second to produce 2-KLGA. One example of this process is that carried out by Erwinia or Acetobacter (first step) and Corynebacterium

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(second step), although other microorganisms have been used in mixed methods, such as *Pseudomnoas striata*, *G. oxydans* and *B. megaterium*. Several genetic and metabolic bioengineering approaches have been developed to produce 2-KLGA from different microorganisms. *Erwinia herbicola* was bioengineered to express heterologous genes from Corynebacterium, reaching a production of 120 g l^{-1} of 2-KLGA. *G. oxydans* was also genetically engineered to express genes from other strains and some promotors were exchanged to optimize their expression, producing 130 g l^{-1} 2-KLGA. Pseudomonas putida has also been engineered to express genes from *G. oxydans,* reaching 16 g l^{-1} 2-KLGA, much lower than the production values of the earlier microorganisms.

After identification of the enzymes involved in direct conversion to l-ascorbic acid, these were expressed in *G. oxydans*, but only 4.2 g l^{-1} of vitamin C was produced. The yeast *S. cerevisiae* has also been engineered since it is able to produce *d-erythroascorbic acid*. The biosynthetic pathway was modified by overexpression of some genes and heterologous expression of an A. thaliana gene, generating a yeast able to produce 100 mg l^{-1} of *l-ascorbic acid*. Some microalgae are also under study to produce vitamin C directly, such as *Prototheca moriformis* or *Chlorella pyrenoidosa,* which were able to produce 2 g l^{-1} of ascorbic acid. The main drawback of microalgal cultures are their slow rates of growth and metabolic activities, which make them unprofitable.

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