Available online at www.sciencedirect.com
SCIENCE DIRECT®
Metabolic Engineering 5 (2003) 277–283
METABOLIC ENGINEERING
http://www.elsevier.com/locate/ymben

Minireview

Metabolic engineering for microbial production of shikimic acid

Marco Krämer,* Johannes Bongaerts, Roel Bovenberg, Susanne Kremer, Ulrike Müller, Sonja Orf, Marcel Wubbolts, and Leon Raeven

DSM Biotech GmbH, Karl-Heinz-Beckurts-Straße 13, D-52428 Jülich, Germany

Received 21 April 2003; accepted 25 September 2003

Abstract

Shikimic acid is a high valued compound used as a key starting material for the synthesis of the neuramidase inhibitor GS4104, which was developed under the name Tamiflu® for treatment of antiviral infections. An excellent alternative to the isolation of shikimic acid from fruits of the *Illicium* plant is the fermentative production by metabolic engineered microorganisms. Fermentative production of shikimic acid was most successfully carried out by rational designed *Escherichia coli* strains by blocking the aromatic amino acid pathway after the production of shikimic acid. An alternative is to produce shikimic acid as a result of dephosphorylation of shikimate-3-phosphate. Engineering the uptake of carbon, the regulatory circuits, central metabolism and the common aromatic pathway including shikimic acid import that have all been targeted to effect higher productivities and lower by-product formation are discussed.

Keywords: Shikimic acid; Shikimate; Shikmate-3-phosphate; Quinic acid; Aromatic Amino Acid Pathway; PEP; E4P; Dehydroshikimic acid; Dehydroshikimate; DHS

1. Introduction

Due to its highly functionalized, six membered carbocyclic ring and three asymmetric centers shikimic acid can be regarded as attractive enantiomerically pure building block for the synthesis of biologically important compounds. Shikimic acid has emerged as a key chiral starting material for the synthesis of the neuramidase inhibitor GS4104 that was discovered by Gilead Sciences and developed by Roche Pharmaceuticals under the name of Tamiflu® to be used as an orally active antiviral compound for prevention and treatment of influenza infections. Based on the discovery synthesis (Kim et al., 1997, 1998), Rohloff and co-workers (Rohloff et al., 1998) had developed an improved synthesis starting from quinic acid. This synthesis was sufficient for the production of kilogram quantities of Tamiflu® for toxicological and phase I clinical studies. However, to enable large-scale production of Tamiflu® a synthesis route suited for large-scale industrial application with shikimic acid as starting material was developed (Federspiel et al., 2001). Isolation of shikimic acid from the fruits of the *Illicium* plant is cumbersome and costly and precludes its use in commercial-level syntheses. In order to improve shikimic acid's availability, fermentative production processes from renewable resources like glucose present an excellent and even more sustainable alternative to meet the current market volume at a competitive price level. Other applications of shikimic acid—albeit at a much smaller scale—are its use as an additive to food and feed and injectables.

2. Metabolic pathway to shikimic acid

Shikimic acid was one of the first compounds of the common aromatic amino acid pathway to be identified (Davis, 1950). The pathway, which has become known as the shikimic acid pathway, is present in microorganisms and plants leading to L-phenylalanine, L-tyrosine and L-tryptophan (reviewed by Pittard (1996) for *Escherichia coli* and by Herrmann (1995) for plants).

The common aromatic amino acid pathway starts with the condensation of phospho*enol*pyruvate (PEP) and erythrose-4-phosphate (E4P) to 3-deoxy-D-*arabino*-

*Corresponding author.
E-mail address: marco.kraemer@dsm.com (M. Krämer).

1096-7176/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2003.09.001

heptulosonate-7-phosphate (DAHP) (Fig. 1). As a general rule such entrance reactions are tightly regulated. In *E. coli* three different DAHP synthase isoenzymes exist—encoded by the *aroF*, *aroG* and *aroH* genes—that are subject to feedback inhibition by the individual aromatic amino acids L-tyrosine, L-phenylalanine, and L-tryptophan, respectively. The DAHP synthase of *Bacillus subtilis* (encoded by *aroA*(*G*)) is inhibited by the pathway intermediate chorismate (Jensen and Nester, 1966a, b). Dehydroquinate synthase, encoded by *aroB* (*E. coli*), converts DAHP into 3-dehydroquinate (DHQ). DHQ dehydratase, encoded by *aroD* (*E. coli*), catalyzes the elimination of H_2O to obtain 3-dehydroshikimate (DHS). Subsequently, shikimate dehydrogenase, encoded by *aroE* (*E. coli*), reduces DHS to shikimic acid whereby NADPH is consumed. The common aromatic amino acid pathway proceeds via formation of shikimate-3-phosphate (S3P), which is catalyzed in *E. coli* by two shikimate kinase isoenzymes encoded by *aroK* and *aroL*, and 5-*enol*pyruvylshikimate-3-phosphate (EPSP), which is catalyzed by EPSP synthase encoded by *aroA*, to the pathway branch point compound chorismic acid. In *E. coli* the enzymes DHQ synthase, DHQ dehydratase and shikimate dehydrogenase are expressed constitutively in contrast to transcriptional regulation of the DAHP synthases (*aroG*, *aroF* and *aroH*) and shikimate kinase II. Regulation by feedback inhibition only occurs at the level of shikimic acid inhibiting shikimate dehydrogenase (Dell and Frost, 1993).

The rate-limiting enzymes of the aromatic amino acid pathway of *E. coli* were identified as DHQ synthase (encoded by *aroB*), shikimate kinase (encoded by *aroL* or *aroK*) by analysis of accumulated intermediate metabolites (Dell and Frost, 1993).

3. Metabolic engineering of shikimic acid production

There are two different approaches for the fermentative production of shikimic acid. The best-elucidated system was achieved by blocking the aromatic amino acid pathway after the biosynthesis of shikimic acid by elimination of the metabolic step to S3P catalyzed by shikimate kinase. This was performed with either genetic engineered *E. coli* strains, which were deficient in both shikimate kinase genes (Draths et al., 1999) or with *Bacillus* or *Citrobacter* strains, which as a result of random mutagenesis have lost their shikimate kinase activity (Iomantas et al., 2002; Shirai et al., 2001).

An alternative approach for shikimic acid production was carried out by EPSP synthase deficient strains (Iomantas et al., 2002), blocking the aromatic amino acid pathway after the production of S3P. Presumably by activity of bacterial phosphatases S3P was converted to shikimic acid (Iomantas et al., 2002). This system has been applied with *B. subtilis* (Iomantas et al., 2002) and *E. coli* (Krämer et al., unpublished).

4. Shikimic acid production by shikimate kinase deficient strains

Microbial production of shikimic acid by metabolic engineering is most advanced in rationally designed *E. coli* strains (Draths et al., 1999). In these strains the aromatic amino acid pathway has been blocked after the stage of shikimic acid, which was performed by transduction of disrupted *aroK* and *aroL* genes encoding shikimate kinase I and II (Draths et al., 1999).

In order to increase the carbon flux from the central metabolism into the aromatic amino acid pathway the feedback resistant DAHP synthase gene $aroF^{fbr}$ was introduced. This gene was combined with the *aroB* gene encoding DHQ synthase in order to circumvent polar effects caused by *aroK* disruption and to overcome the rate-limiting DHQ synthase step. Furthermore, an additional gene coding for shikimic acid dehydrogenase (*aroE*), as compensation for the enzymes feedback inhibition by shikimic acid, was introduced (Draths et al., 1999).

A complication of shikimic acid dehydrogenase overexpression is the ability of the enzyme to reduce dehydroshikimic acid to quinic acid as well. Compensation of the feedback inhibition of shikimic acid dehydrogenase therefore interferes with a decreased production of shikimic acid due to the formation of quinic acid (Draths et al., 1999). This reaction is similar to a quinic acid dehydrogenase of *Klebsiella pneumoniae* (Draths et al., 1992), which had been used to establish a fermentative route from glucose to quinic acid in *E. coli* (Draths et al., 1992; Ran et al., 2001).

5. Reduction of by-product formation

The production of high amounts of the by-products dehydroshikimic acid (to 4.4 g/L) and quinic acid (12.6 g/L) is a complication for the fermentative production of shikimic acid (27 g/L) (Draths et al., 1999). The formation of these by-products significantly reduces the shikimic acid yield and more importantly the presence of quinic acid impairs the down stream processing. The efficiency of crystallization of shikimic acid and thus the purity and quality is reduced (Knop et al., 2001).

To investigate the equilibrium between shikimic acid and by-products a shikimic acid producing strain was cultivated in shikimic acid containing medium, which led to the formation of quinic acid and dehydroshikimic acid (Draths et al., 1999). This observation suggested that a shikimic acid producing strain can make quinic

acid and dehydroshikimic acid from initially synthesized shikimic acid. This was explained by the reversibility of the normal shikimic acid producing pathway caused by the equilibrium between shikimic acid and quinic acid and dehydroshikimic acid (Draths et al., 1999). In order to verify that the dehydroshikimic acid and quinic acid production is indeed based on equilibrium, the de novo synthesis of shikimic acid was prevented. This was carried out by disruption of the *aroF*^{fbr} gene of a shikimic acid producing *E. coli* strain (Knop et al., 2001) and inhibiting the three DAHP synthases (AroF, AroG and AroH) to prevent the production of shikimic acid from glucose. The observed ratio of shikimic acid to quinic acid and dehydroshikimic acid thus provided evidence for the uptake of shikimic acid from the culture medium and the operation of the pathway in the reverse direction (Knop et al., 2001).

The reduction of shikimic acid uptake activity was investigated as a strategy for minimizing quinic acid contamination (Draths et al., 1999). The transport systems for re-uptake of shikimic acid were targeted by either increasing glucose availability, by disrupting the shikimic acid transporter encoded by gene *shiA*, by using a glucose analogue and by using substrate channeling (Knop et al., 2001).

5.1. Increasing glucose availability

Shikimic acid transport (Pittard and Wallace, 1966; Brown and Doy, 1976; Whipp et al., 1998) in *E. coli* may be an evolutionary relict of a previous ability to catabolize shikimic and quinic acids as a carbon source for growth and metabolism (Draths et al., 1999). Since utilization of non-glucose carbon sources is often subject to catabolite repression, it was envisioned that increasing D-glucose availability might repress shikimic acid transport, thereby minimizing the formation of quinic acid. Therefore, the availability of glucose was increased, which drastically reduced the formation of quinic acid by 90%. However, this method also led to a reduction of the shikimic acid titer of about 25% and no improvements in the reduction of dehydroshikimic acid formation was observed (Draths et al., 1999).

5.2. Use of glucose mimics

Since the shikimic acid transport systems were expected to be controlled by catabolite repression, the glucose-mimic methyl-α-D-glucopyranoside was added to the culture medium of a shikimic acid synthesizing *E. coli* in order to reduce shikimic acid uptake (Knop et al., 2001). When methyl-α-D-glucopyranoside was added to glucose limited cultivations, the formation of quinic acid was strongly reduced from 19 to 2.8 g/L. Addition of methyl-α-D-glucopyranoside thus increased the titer of shikimic acid from 28 to 35 g/L and the yield from 0.14 to 0.19 mol/mol based on glucose (Knop et al., 2001).

5.3. Disruption of shikimate transporter gene shiA

Shikimic acid can be used to supply the aromatic amino acid and aromatic vitamin requirements of *E. coli* strains blocked in any of the first three steps of the aromatic amino acid pathway (Pittard and Wallace, 1966). This ability is related to the presence of the shikimic acid transport system encoded by the gene *shiA* (Whipp et al., 1998).

Inactivation of the shikimic acid transport gene (*shiA*) was therefore another approach to prevent re-uptake of shikimic acid. In isogenic systems, in which the *shiA* gene was either active or disrupted, the production of by-products from externally added shikimic acid was studied (Knop et al., 2001). Although the formation of the equilibration was slower in the strain having an intact *shiA* gene as compared to the *shiA* mutant, the experimental results still pointed out that transport and equilibration of shikimic acid was occurring in the *shiA* disrupted strain (Knop et al., 2001). This observation indicates that additional transport systems for shikimic acid are present, which are not based on the presence of an intact *shiA* locus (Knop et al., 2001).

5.4. Substrate channeling

The plant derived *aroD·E* genes, which encode a single bifunctional DHQ dehydratase and shikimate dehydrogenase, were studied to investigate the effect of substrate channeling on 3-dehydroshikimic acid side product formation (Knop et al., 2001). Substrate channeling is defined as the transfer of the reaction product from one enzyme to the next in a metabolic sequence (Geck and Kirsch, 1999). Therefore, the genes *aroD·E* of *Nicotiana tabacum* were introduced into a shikimic acid producing *E. coli* strain, which lacked wild-type *aroD* and *aroE* activities (Knop et al., 2001). The channeling of enzyme-bound 3-dehydroquinic acid directly into shikimic acid formation would possibly omit the accumulation of DHS in the cytoplasma.

It was, however, observed that substantial quantities of dehydroshikimic acid remained, possibly as a consequence of the inhibition by shikimic acid of the shikimate dehydrogenase domain of *N. tabacum* *aroD·E* (Knop et al., 2001). In contrast, quinic acid formation was reduced in this strain, which was most probably caused by the selectivity of AroD·E activity of *N. tabacum* that favors the reduction of DHS to shikimic acid over reduction of 3-dehydroquinic acid to shikimic acid (Knop et al., 2001).

6. Engineering of central metabolism

The fermentative production of shikimic acid has been improved by increased availability of the intermediates of central metabolism E4P and PEP, which condensate at the beginning of the aromatic amino acid pathway. Under glucose-rich culture conditions, which reduced the production of the by-product quinic acid, (Knop et al., 2001) overexpression of the transketolase gene (*tktA*) meant to increase availability of E4P increased the total yield of the produced hydroaromatic compounds, i.e. the sum of shikimic acid, quinic acid and dehydroshikimic acid from 0.15 to 0.24 mol/mol based on glucose (theoretical maximum is 0.43 mol/mol) (Knop et al., 2001). The shikimic acid yield was increased from 0.12 to 0.18 mol/mol (Knop et al., 2001) with a theoretical maximum of 0.43 mol/mol (Patnaik and Liao, 1994; Patnaik et al., 1995), and the shikimic acid titer was increased from 38 to 52 g/L.

For improving shikimic acid production two different strategies were carried out to increase availability of PEP, both increasing the theoretical stoichiometric yield of shikimic acid from 0.43 to 0.86 mol/mol based on glucose (Chandran et al., 2003).

One approach to increase PEP supply was carried out by recycling pyruvate by PEP synthase (encoded by *ppsA*) to PEP along with consumption of ATP to AMP and one molecule of inorganic phosphate (Chandran et al., 2003). The shikimic acid titer (66 g/L) and yield (0.23 mol/mol based on glucose) were significantly increased.

Glucose uptake in *E. coli* is maintained by the PEP consuming phospho*enol*pyruvate-phosphotransferase system (PTS). In order to increase the availability of PEP, the PTS was inactivated and replaced by a PEP independent, but ATP dependent uptake and phosphorylation system consisting of the glucose facilitator (*glf*) and the glucokinase (*glk*) from *Zymomonas mobilis* (Gibson et al., 2001; Chandran et al., 2003). In combination with an overexpressed *tktA* gene this resulted in a high shikimic acid titer (71 g/L) and yield (0.27 mol/mol based on glucose). The high titer and yield was even improved when the mineral fermentation medium was supplemented with yeast extract in a 10-L up-scale process. A shikimic acid titer to 84 g/L and a yield of 0.33 mol/mol based on glucose was reached compared to 62 g/L shikimic acid and a yield of 0.26 mol/mol of the unsupplemented process in the same scale (Chandran et al., 2003).

The impact of the Glf-mediated glucose transport in strains carrying an intact PTS depended on whether the *glk* gene of *Z. mobilis* was introduced. When cultivating the isogenic strain, but with an intact PTS, production of shikimic acid was reduced to 46 g/L with a yield of 0.21 mol/mol based on glucose. Acetate titer was increased presumably as a consequence of high glucose-6-phosphate formation and therefore overabundance of PEP by sequential action of pyruvate kinase, pyruvate dehydrogenase, phosphotransacetylase and acetate kinase (Chandran et al., 2003). Omitting the introduction of the *glk* encoded glucokinase of *Z. mobilis* left only the genomic *E. coli* glucokinase for phosphorylation leading to an increased titer of shikimic acid (70 g/L) combined with a high yield (0.24 mol/mol based on glucose) compared to the latter strain. Acetate accumulation was absent presumably as a consequence of lower rates of glucose-6-phosphate formation and PEP generation (Chandran et al., 2003).

7. Metabolic engineering of *Bacillus subtilis* and *Citrobacter freundii*

Similar to *E. coli* fermentative production of shikimic acid from glucose was also carried out by using *B. subtilis*, which as opposed to *E. coli*, needs creation of only one defect allele of shikimate kinase (*aroI*). An *aroI* deficient strain produced shikimic acid (8.5 g/L) but mostly the by-product DHS in relatively high amounts (9.5 g/L) with a ratio of DHS to shikimic acid to about 1.1 (Iomantas et al., 2002). The *aroA*(*G*) gene encoding the DAHP synthase was introduced on a plasmid into the *aroI* deficient *B. subtilis* strain, which resulted in an even higher ratio of DHS to shikimic acid (2.7). Presumably, this is the result of inhibition of shikimate dehydrogenase by shikimic acid, although the shikimic acid titer was not affected (Iomantas et al., 2002). Overproduction of shikimate dehydrogenase by introducing the plasmid encoded gene from *B. amyloliquefaciens* successfully improved the shikimic acid production to a titer of 14 g/L and DHS of 6.8 g/L and lowered the ratio of DHS to shikimic acid to 0.48. Because of the low plasmid stability *aroD* was chromosomally integrated and *aroA*(*G*) was introduced on a plasmid. This resulted in an increased shikimic acid titer to 19.7 g/L with 9.8 g/L DHS (Iomantas et al., 2002).

Strain improvements to obtain shikimic acid producing strains have been described using *Citrobacter freundii*, resulting in a titer up to 10 g/L shikimic acid (Shirai et al., 2001).

8. Shikimic acid production by EPSP synthase deficient strains

The basis of this strategy was pointed out when Davis and Mignioli observed that an *aroA* deficient *E. coli* strain secreted S3P into the culture medium. Heating or acidification of the supernatant converted S3P to shikimic acid (Davis and Mignioli, 1953). Therefore, shikimic acid production can also be accomplished by blocking the pathway at the level of EPSP synthase. However, there are only a few reports describing the

production of shikimic acid via EPSP synthase deficient strains.

A *B. subtilis* strain, which was devoid of EPSP synthase activity accumulated 1.1 g/L shikimic acid and 0.2 g/L of DHS and no S3P was detected presumably as a result of dephosphorylation of S3P to shikimic acid (Iomantas et al., 2002). For overcoming a putative feedback inhibition step in the pathway to shikimic acid the gene encoding a shikimate dehydrogenase of *B. amyloliquefaciens* was introduced and overexpressed resulting in an increased shikimic acid titer of 2.8 g/L shikimic acid and 1.1 g/L DHS (Iomantas et al., 2002).

As already pointed out, a disadvantage of shikimic acid production by shikimate kinase deficient *E. coli* strains is the production of high amounts of DHS (Draths et al., 1999) caused by the equilibrium reaction of DHS and shikimic acid. The production of S3P, however, pulls this equilibrium between DHS and shikimic acid into the direction of S3P.

For the fermentative production of shikimic acid via S3P an *E. coli* strain was constructed that was devoid of the EPSP synthase gene *aroA*. Deregulation of the aromatic amino acid pathway to S3P was accomplished by overexpression of a feedback resistant DAHP synthase ($aroF^{fbr}$), DHQ synthase (*aroB*) and shikimate kinase II (*aroL*) (Krämer et al., unpublished).

After 48 h of cultivation a shikimic acid plus S3P titer of up to 20 g/L was reached. This route to shikimic acid reduced the ratio of DHS to shikimic acid plus S3P plus DHS to about 40% (0.15 ± 0.02 mol/mol based on carbon) compared to the isogenic shikimate kinase deficient strain (0.25 ± 0.02 mol/mol based on carbon) (Krämer et al., unpublished).

For further decrease of the DHS pool the gene *shiA* encoding the shikimate importer was disrupted, creating a strain, which is unable to import shikimic acid. Therefore, by preventing uptake of shikimic acid by ShiA a futile flux from external to internal shikimic acid is eliminated and this should result in an increase of the external shikimic acid pool. However, experimental data pointed out that disruption of *shiA* did not lead to any significant improvements concerning the ratio of DHS to the products shikimic acid plus S3P (Krämer et al., unpublished). Therefore, it can be assumed that in accordance with other experimental approaches (Knop et al., 2001) more than one shikimic acid uptake system is present in *E. coli*.

9. Conclusions and outlook

Shikimic acid production is a good example of a successful approach of rational strain design by metabolic pathway engineering for the sustainable production of a high valued product. Metabolic engineering for the fermentative production of shikimic acid is almost restricted to *E. coli*, only minor attempts have been made with *B. subtilis and C. freundii*.

Although the production of shikimic acid can also be carried out by EPSP synthase deficient strains, almost all production systems use shikimate kinase deficient strains. The best strain resulted in a shikimic acid titer up to 84 g/L with a 0.33 mol/mol yield of shikimic acid on glucose (Chandran et al., 2003).

The production strategy for shikimic acid as an intermediate of the aromatic amino acid pathway is different from the production of the aromatic amino acids of this pathway. This is based on the fact that one has to deal more intensively with the reduction of unexpected by-products than with the reduction of acetate (Gerigk et al., 2002). As pointed out these by-products are based on equilibrium reactions or on the occurrence of unexpected side activities of pathway enzymes than on the reduction of compounds involved in overflow metabolism.

Besides producing shikimic acid as an intermediate of the aromatic amino acid pathway metabolic engineering has already been used for the sustainable production of quinic acid (Draths et al., 1992; Ran et al., 2001), protocatechuate (Draths and Frost, 1994a), catechol (Draths and Frost, 1994a), gallic acid (Kambourakis et al., 2000) and pyrogallol (Kambourakis et al., 2000) (Fig. 1).

A pathway to quinic acid was established by introducing or overexpression of genes encoding dehydroquinate dehydrogenase, which has either been encoded by *qad* from *K. pneumoniae* or by overexpression of *aroE* encoding the shikimic acid dehydrogenase from *E. coli* (Draths et al., 1992; Ran et al., 2001) (Fig. 1).

From dehydroshikimic acid the aromatic amino acid pathway was broadened by DHS dehydratase activity encoded by *aroZ* from *K. pneumoniae*, which resulted in the formation of protocatechuate (Draths and Frost, 1994a) and subsequently decarboxylated by protocatechuate decarboxylase encoded by *aroY* from *K. pneumoniae* to catechol (Draths and Frost, 1991) (Draths and Frost, 1994a, b) (Fig. 1).

Protocatechuate was converted to gallic acid by activity of the product of $pobA^*$ encoding a mutant *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa* (Kambourakis et al., 2000) (Fig. 1). A pathway from gallic acid was further engineered to pyrogallol by activity of protocatechuate decarboxylase (Kambourakis et al., 2000). These examples indicate how far the pathway to shikimic acid has been exploited yet and what future potential there is.

Acknowledgments

Financial support from the BioRegio program of the Bundesministerium für Bildung und Forschung (BMBF, Grant 0311644) is gratefully acknowledged.

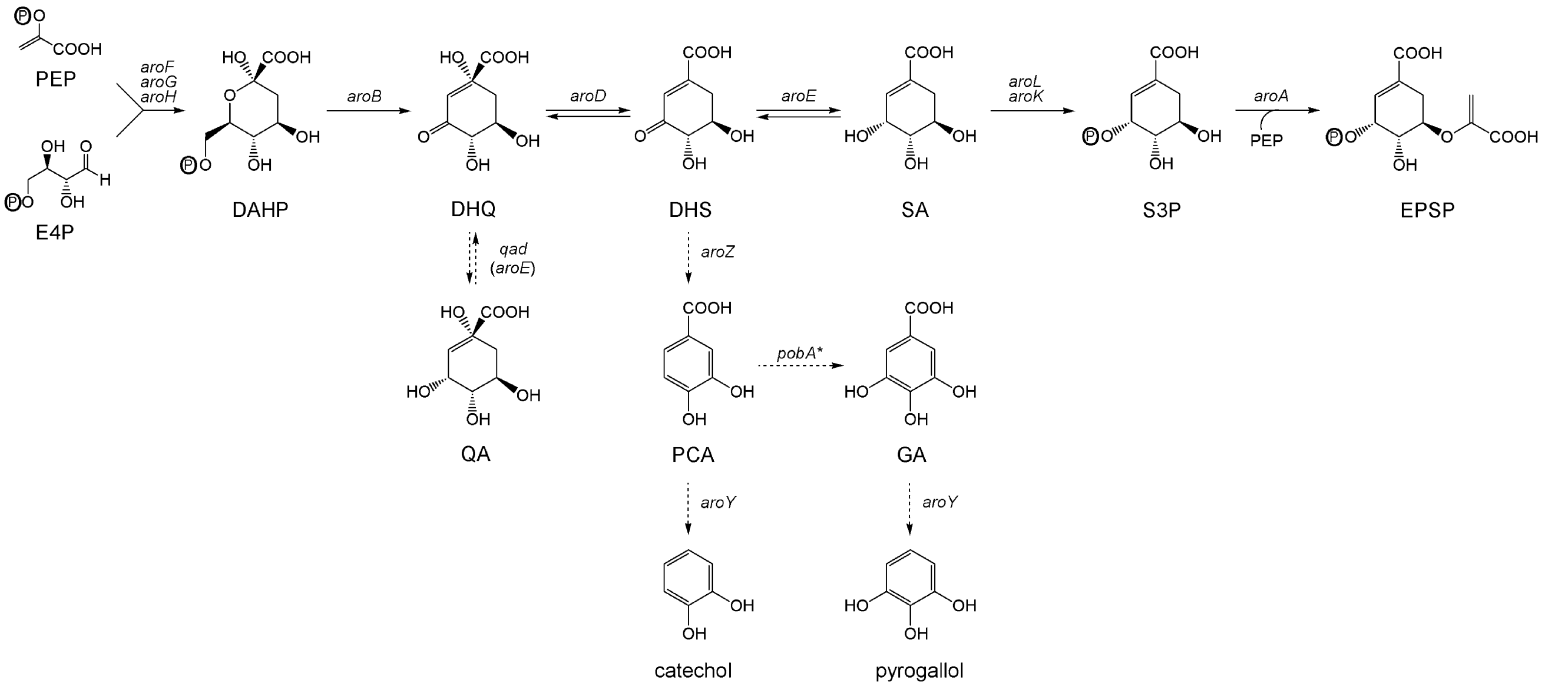

Fig. 1. Pathway of aromatic amino acid biosynthesis to 5-*enol*pyruvylshikimate-3-phosphate in *E. coli* and heterologous pathways to hydroaromatic and aromatic compounds derived thereof (indicated as dashed arrows). *Abbreviations*: E4P, erythrose-4-phosphate; PEP, phospho*enol*pyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; SA, shikimic acid; S3P, shikmate-3-phosphate; EPSP, 5-*enol*pyruvylshikimate-3-phosphate; QA, quinic acid; PCA, protocatechuate; GA, gallic acid. *Genes*: *aroF*, DAHP synthase (L-tyr); *aroG*, DAHP synthase (L-phe); *aroH*, DAHP synthase (L-trp); *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroL*, shikimate kinase II; *aroK*, shikimate kinase I; *aroA*, EPSP synthase; *qad*, dehydroquinate dehydrogenase; *aroZ*, dehdroshikimate dehydratase; *aroY*, protocatechuate decarboxylase; *pobA**, mutant *p*-hydroxybenzoate hydroxylase.

References

Brown, K.D., Doy, C.H., 1976. Transport and utilization of the biosynthetic intermediate shikimic acid in *Escheichia coli*. Biochim. Biophys. Acta 428, 550–562.

Chandran, S.S., Yi, J., Draths, K.M., Von Daeniken, R., Weber, W., Frost, J.W., 2003. Phosphoenolpyruvate availability and the biosynthesis of shikimic acid. Biotechnol. Prog. 19, 808–814.

Davis, B.D., 1950. Aromatic biosynthesis I. The role of shikimic acid. J. Biol. Chem. 191, 315–325.

Davis, B.D., Mignioli, E.S., 1953. Aromatic biosynthesis I. Accumulation of two derivatives of shikimic acid by bacterial mutants. J. Bacteriol. 66, 129–136.

Dell, K.A., Frost, J.W., 1993. Identification and removal of impediments to biocatalytic synthesis of aromatics from D-glucose: rate-limiting enzymes in the common pathway of aromatic amino acid biosynthesis. J. Am. Chem. Soc. 115, 11581–11589.

Draths, K.M., Frost, J.W., 1991. Conversion of D-glucose into catechol: the not-so-common pathway of aromatic biosynthesis. J. Am. Chem. Soc. 113, 9361–9363.

Draths, K.M., Frost, J.W., 1994a. Environmentally compatible synthesis of adipic acid from D-glucose. J. Am. Chem. Soc. 116, 399–400.

Draths, K.M., Frost, J.W., 1994b. Microbial biocatalysis synthesis of adipic acid from d-glucose. In: Anastas, P.T., Farris, C.S. (Eds.), Benign by Design. ACS Symp. Ser. No. 577. American Chemical Society, Washington, DC, pp. 32–45.

Draths, K.M., Ward, T.L., Frost, J.W., 1992. Biocatalysis and nineteenth century organic chemistry: conversion of D-glucose into quinoid organics. J. Am. Chem. Soc. 114, 9725–9726.

Draths, K.M., Knop, D.R., Frost, J.W., 1999. Shikimic acid and quinic acid: replacing isolation from plant sources with recombinant microbial biocatalysis. J. Am. Chem. Soc. 121, 1603–1604.

Federspiel, M., Fischer, R., Hennig, M., Mair, H.J., Oberhauser, T., Rimmler, G., Albiez, T., Bruhin, J., Estermann, H., Gandert, C., Göckel, V., Hoffmann, U., Huber, G., Janatsch, G., Lauper, S., Röckel-Stäbler, O., Trussardi, R., Zwahlen, A., 2001. Industrial synthesis of the key precursor in the synthesis of the anti-influenza drug oseltamivir phosphate (Ro 64-0796/002, GS-4104-02): ethyl (3R,4S,5S)-4,5-epoxy-3-(1-ethyl-propoxy)-cyclohex-1-ene-1-carboxylate. Org. Process Res. Dev. 3, 266–274.

Geck, M.K., Kirsch, J.F., 1999. A novel, definitive test for substrate channeling illustrated with the aspartate aminotransferase/malate dehydrogenase system. Biochemistry 38, 8032–8037.

Gerigk, M., Bujnicki, R., Ganpo-Nkwenkwa, E., Bongaerts, J., Sprenger, G., Takors, R., 2002. Process control for enhanced L-phenylalanine production using different recombinant *Escherichia coli* strains. Biotechnol. Bioeng. 80, 746–754.

Gibson, J.M., Thomas, P.S., Thomas, J.D., Barker, J.L., Chandran, S.S., Harrup, M.K., Draths, K.M., Frost, J.W., 2001. Benzene-free synthesis of phenol. Angew. Chem. Int. Ed. 40, 1945–1948.

Herrmann, K.M., 1995. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 7, 907–919.

Iomantas, Y., Abalakina, E.G., Polanuer, B., Yampolskaya, T.A., Bachina, T.A., Kozlov, J.I., 2002. Method for producing shikimic acid. US Patent 6,436,664.

Jensen, R.A., Nester, E.W., 1966a. Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. J. Biol. Chem. 241, 3365–3372.

Jensen, R.A., Nester, E.W., 1966b. Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. II. The enzymology of feedback inhibition of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase. J. Biol. Chem. 241, 3373–3380.

Kambourakis, S., Draths, K.M., Frost, J.W., 2000. Synthesis of gallic acid and pyrogallol from glucose: replacing natural product isolation with microbial catalysis. J. Am. Chem. Soc. 122, 9042–9043.

Kim, C.U., Lew, W., Williams, M.A., Liu, H., Zhang, L., Swaminathan, S., Bischhofberger, N., Chen, M.S., Mendel, D.B., Tai, C.Y., Laver, W.G., Stevens, R.C., 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J. Am. Chem. Soc. 119, 681–690.

Kim, C.U., Lew, W., Williams, M.A., Wu, H., Zhang, L., Chen, X., Escarpe, P.A., Mendel, D.B., Laver, W.G., Stevens, R.C., 1998. Structure–activity relationship studies of novel carbocyclic influenza neuraminidase inhibitors. J. Med. Chem. 41, 2451–2460.

Knop, D.R., Draths, K.M., Chandran, S.S., Barker, J.L., Frost, J.W., 2001. Hydroaromatic equilibrium during biosynthesis of shikimic acid. J. Am. Chem. Soc. 123, 10173–10182.

Patnaik, R., Liao, J.C., 1994. Engineering of *Escherichia coli* central metabolism for aromatic metabolite production with near theoretical yield. Appl. Environ. Microbiol. 60, 3903–3908.

Patnaik, R., Spitzer, R., Liao, J.C., 1995. Pathway engineering for production of aromatics in *Escherichia coli*: confirmation of stoichiometric analysis by independent modulation of AroG, TktA, and Pps activities. Biotechnol. Bioeng. 46, 361–370.

Pittard, A.J., 1996. Biosynthesis of aromatic amino acids. In: Neidhardt, F.C., Curtiss III, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., Umbarger, H.E. (Eds.), *Escherichia coli* and *Salmonella*, Cellular and Molecular Biology. American Society of Microbiology, Washington, DC, pp. 458–484.

Pittard, A.J., Wallace, B.J., 1966. Gene controlling the uptake of shikimic acid by *Escherichia coli*. J. Bacteriol. 92, 1070–1075.

Ran, N., Knop, D.R., Draths, K.M., Frost, J.W., 2001. Benzene-free synthesis of hydroquinone. J. Am. Chem. Soc. 123, 10927–10934.

Rohloff, J.C., Kenneth, M.K., Postich, M.J., Becker, M.W., Chapman, H.H., Kelly, D.E., Lew, W., Louie, M.S., McGee, L.R., Prisbe, E.J., Schultze, L.M., Yu, R.H., Zhang, L., 1998. Practical total synthesis of the anti-influenza drug GS-4104. J. Org. Chem. 63, 4545–4550.

Shirai, M., Miyata, R., Sasaki, S., Sakamoto, K., Yahanda, S., Shibayama, K., Yonehara, T., Ogawa, K., 2001. Microorganism belonging to the genus *Citrobacter* and process for producing shikimic acid. European Patent 1,092,766.

Whipp, M.J., Camakaris, H., Pittard, A.J., 1998. Cloning and analysis of the *shiA* gene, which encodes the shikimate transport system of *Escherichia coli* K-12. Gene 209, 185–192.