Synthesis of Vanillin from Glucose

Kai Li and J. W. Frost*

Department of Chemistry
Michigan State University
East Lansing, Michigan 48824

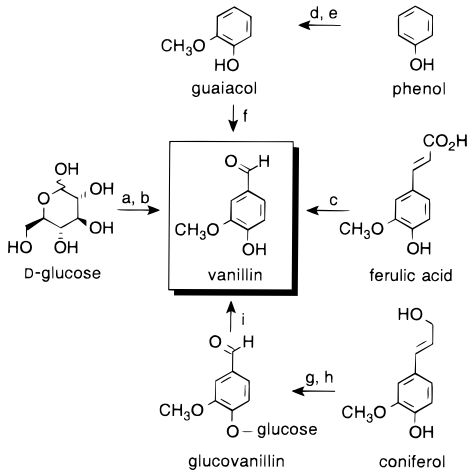
Received May 21, 1998

Condensation of glyoxylic acid with benzene-derived guaiacol (Scheme 1) is currently the dominant route for vanillin manufacture.[1] Natural vanillin is produced from glucovanillin (Scheme 1) when the beans of the orchid *Vanilla planifolia* are submitted to a multistep curing process.[1a] Because of the extreme care that must be exercised during vine cultivation, bean harvesting, and hand pollination of flowers, natural vanillin can supply only 2×10^4 kg/yr of the world's 1.2×10^7 kg/yr demand for vanillin.[1b] This has resulted in the substitution of synthetic vanillin for natural vanilla in most flavoring applications. Limited vanilla bean supplies have also led to extensive research into the use of plant tissue culture and microbes to convert ferulic acid (Scheme 1) into vanillin suitable for labeling as a natural or nature-equivalent flavoring.[2] A synthesis of vanillin from glucose (Scheme 1) has now been elaborated. Glucose is converted into vanillic acid by a recombinant *Escherichia coli* biocatalyst under fed-batch fermentor conditions. Reduction of vanillic acid to vanillin is catalyzed by aryl aldehyde dehydrogenase isolated from *Neurospora crassa*. This synthesis qualifies both as a route to natural vanillin and as a first step toward large-scale, environmentally benign manufacture of vanillin using biocatalysis.

Vanillate-synthesizing *E. coli* KL7 biocatalysts carried a mutated *aroE* locus and an *aroBaroZ* cassette inserted into the *serA* locus. The lack of *aroE*-encoded shikimate dehydrogenase resulted in the synthesis of 3-dehydroshikimic acid which was converted into protocatechuic acid by genome-localized, *aroZ*-encoded 3-dehydroshikimate dehydratase. Plasmid-localized P_{tac}*COMT* encoded catechol-*O*-methyltransferase for conversion of protocatechuic acid into vanillic acid (Scheme 2). Plasmid pKL5.97A carried two P_{tac}*COMT* loci, while only a single P_{tac}*COMT* locus was present in pKL5.26A. These plasmids also carried an *aroF*^{FBR} and a *serA* insert. The *aroF*^{FBR} insert encodes a 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase isozyme insensitive to feedback inhibition which increased carbon flow into the common pathway. Due to a mutation in the genomic *serA* locus required for L-serine biosynthesis, growth in minimal salts medium and plasmid maintenance followed from expression of plasmid-localized *serA*. The two genomic copies of *aroB* increased 3-dehydroquinate synthase (Scheme 2) activity to the point where this enzyme no longer impeded carbon flow.[3]

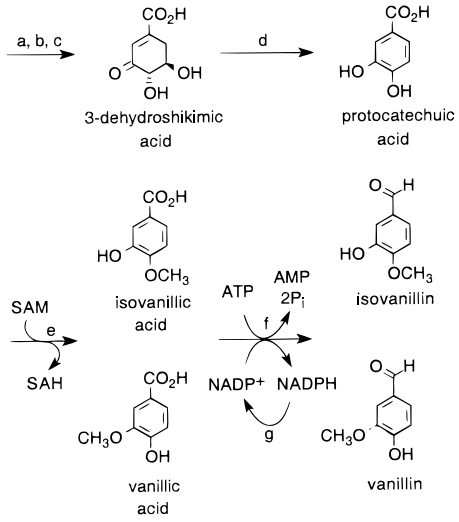
KL7/pKL5.26A and KL7/pKL5.97A were cultured for 48 h under fed-batch fermentor conditions at 37 °C, pH 7.0, and dissolved oxygen at 20% of saturation. Extracellular accumulation (Figure 1) of vanillic, isovanillic, protocatechuic, and 3-dehydroshikimic acids began in mid log phase of microbial growth. 3-Dehydroshikimic acid usually constituted 5−10 mol % of the total product mixture, indicating that the rates for its biosynthesis and dehydration were nearly equal. However, the molar dominance of protocatechuic acid (Figure 1, Table 1) relative to vanillic acid pointed to inadequate catechol-*O*-methyltransferase activity. Although increasing the specific activity (Table 1) of catechol-*O*-methyltransferase in KL7/pKL5.97A relative to KL7/pKL5.26A had little impact on the concentrations (Table 1) of synthesized vanillic acid, supplementation with L-methionine nearly doubled the amount of vanillic acid synthesized by both biocatalysts (Table 1). The 4-fold to 6-fold molar excess of vanillic acid synthesized relative to

Scheme 1[a]



[a] Key: (a) KL7/pKL5.26A or KL7/pKL5.97A. (b) *N. crassa* aryl aldehyde dehydrogenase. (c) Microbial catabolism. (d) HCO_3H, HCO_2H. (e) Me_2SO_4. (f) (i) $HCOCO_2H$, (ii) O_2, (iii) H^+. (g) UDP-glucose:coniferyl alcohol glucosyltransferase. (h) Unidentified enzymes. (i) β-glucosidase.

Scheme 2[a]



[a] Key: (a) 3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase (*aroF*^{FBR}). (b) 3-Dehydroquinate synthase (*aroB*). (c) 3-Dehydroquinate dehydratase (*aroD*). (d) 3-Dehydroshikimate dehydratase (*aroZ*). (e) Catechol-*O*-methyltransferase (*COMT*). (f) Aryl aldehyde dehydrogenase. (g) D-glucose 6-phosphate dehydrogenase; D-glucose 6-phosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

* Author to whom correspondence should be addressed. Phone: 517-355-9715, ext 115. FAX: 517-432-3873. E-mail: frostjw@argus.cem.msu.edu.

(1) (a) Ranadive, A. S. In *Spices, Herbs, and Edible Fungi*; Charalambous, G., Ed.; Elsevier: Amsterdam, 1994; p 517. (b) Clark, G. S. *Perfum. Flavor.* **1990**, *15*, 45. (c) Esposito, L.; Formanek, K.; Kientz, G.; Mauger, F.; Maureaux, V.; Robert, G.; Truchet, F. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed.; Kroschwitz, J. I., Howe-Grant, M., Eds.; Wiley: New York, 1997; Vol. 24, p 812.

(2) (a) Falconnier, B.; Lapierre, C.; Lesage-Meessen, L.; Yonnet, G.; Brunerie, P.; Colonna-Ceccaldi, B.; Corrieu, G.; Asther, M. *J. Biotechnol.* **1994**, *37*, 123. (b) Lesage-Meessen, L.; Delattre, M.; Haon, M.; Thibault, J.-F.; Ceccaldi, B. C.; Brunerie, P.; Asther, M. *J. Biotechnol.* **1996**, *50*, 107. (c) Lesage-Meessen, L.; Haon, M.; Delattre, M.; Thibault, J.-F.; Ceccaldi, B. C.; Asther, M. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 393. (d) Labuda, I. M.; Goers, S. K.; Keon, K. A. U.S. Patent 5,279,950, 1994. (e) Westcott, R. J.; Cheetham, P. S. J.; Barraclough, A. J. *Phytochemistry* **1994**, *35*, 135.

(3) Snell, K. D.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 5605.

S0002-7863(98)01774-0 CCC: $15.00
Published on Web 09/25/1998

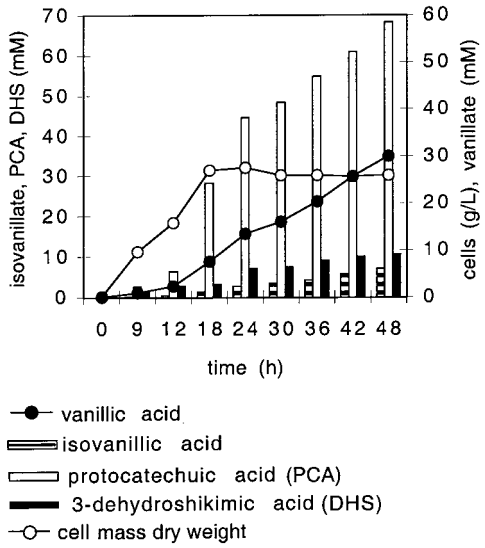


Figure 1. Fermentor culture of KL7/pKL5.97A supplemented with L-methionine.

Table 1. Products Formed after 48 h under Fed-Batch Fermentor Conditions as a Function of Catechol-*O*-methyltransferase Activity and L-Methionine Supplementation

	KL7/pKL5.26A[a]		KL7/pKL5.97A[b]	
L-methionine[c]	−	+	−	+
COMT[d]	0.0060	0.0055	0.012	0.010
vanillic acid[e]	2.5	4.9	3.0	5.0
isovanillic acid[e]	0.4	1.3	0.6	1.2
protocatechuic acid[e]	9.7	7.1	12.9	10.5
3-dehydroshikimic acid[e]	0.9	1.0	1.0	1.8

[a] *aroF*^{FBR}*P*$_{tac}$*COMTserA*. [b] *aroF*^{FBR}*P*$_{tac}$*COMTP*$_{tac}$*COMTserA*. [c] 0.4 g/L added every 6 h beginning at 12 h. [d] Specific activity, μmol/min/mg. [e] g/L.

isovanillic acid (Table 1) conforms to the reported selectivity of catechol-*O*-methyltransferase toward *meta*-hydroxyl group methylation.[4]

Aryl aldehyde dehydrogenase[5] in *N. crassa* mycelial extract was purified from an unwanted dehydrogenase which reduced vanillin to vanillyl alcohol. Vanillic, protocatechuic, and isovanillic acids were extracted into EtOAc after acidification of the fermentor broth. A subsequent reprecipitation step increased the vanillic acid/protocatechuic acid ratio from 1:2 to 2.5:1 (mol/mol). The resulting aromatic mixture was incubated with glucose 6-phosphate dehydrogenase (to recycle $NADP^+$) and aryl aldehyde dehydrogenase at 30 °C and pH 8.0 using 0.07 equiv of $NADP^+$ and 2 equiv of ATP relative to vanillic acid. Reduction of vanillic acid to vanillin (Scheme 2) proceeded in 92% yield in 7 h. Reduction of protocatechuic acid was slower with a 33% yield of protocatechualdehyde obtained after 7 h. Vanillin was extracted from the enzymatic reduction with CH_2Cl_2, leaving protocatechualdehyde and protocatechuic acid in the aqueous phase. Isovanillin at 10 mol % remained as the only contaminant. Extraction of the fermentor broth, selective precipitation to remove excess protocatechuic acid, aryl aldehyde dehydrogenase reduction, and the final CH_2Cl_2 extraction led to a 66% overall yield (mol/mol) for the conversion of vanillic acid into vanillin.

Use of an intact microbe to reduce vanillic acid will be essential for future large-scale vanillin synthesis. Vanillic acid synthesized by one microbe from glucose could be reduced to vanillin by a second, different microbe. Conversion of glucose into vanillin using a single vanillate-synthesizing microbe expressing aryl aldehyde dehydrogenase may also be possible. Irrespective of the strategy employed, improved protocatechuic acid methylation will be essential. The lack of significantly improved protocatechuic acid methylation with increased catechol-*O*-methyltransferase activity and the improvement in methylation observed with L-methionine supplementation suggest that cosubstrate *S*-adenosylmethionine availability and/or feedback inhibition[6] may be limiting in vivo methyltransferase activity. Improving regioselectivity for protocatechuic acid *meta*-oxygen methylation using a different isozyme of widely distributed[4] catechol-*O*-methyltransferase would also be advantageous.

Biocatalytic synthesis of vanillin from glucose has a number of advantages relative to other biocatalytic vanillin syntheses (Scheme 1). Coniferol, formed during phenylpropanoid biosynthesis, is converted into coniferin by a glucosyltransferase in *V. planifolia*.[1a] Coniferin is then transformed into glucovanillin which is finally hydrolyzed by a β-glucosidase.[1a] Synthesis of vanillin via 3-dehydroshikimic, protocatechuic, and vanillic acids circumvents phenylpropanoid biosynthesis and glucosylation/deglucosylation reactions. This substantially reduces the number of enzymes required to synthesize vanillin. Ferulic acid (Scheme 1) can be microbially converted into vanillin. However, these routes produce vanillin titers below 1 g/L.[2] Cultured plant tissue in medium supplemented with ferulic acid has also been used to produce vanillin although this conversion is slow and scale-up is relatively difficult.[2d,e] A problem with all vanillin syntheses from ferulic acid is the absence of low-cost, commercial production of this phenylpropanoid.

Biocatalytic synthesis of vanillin from glucose, although a longer-term commercial prospect, also has advantages relative to synthetic vanillin manufacture[1c] (Scheme 1). Phenol and guaiacol are toxic and are derived from carcinogenic benzene.[7] Nontoxic 3-dehydroshikimic, protocatechuic, and vanillic acids are derived from innocuous glucose. Corrosive H_2O_2 used for the oxidation of phenol into catechol requires special handling precautions[7] while biocatalytically synthesized vanillin derives its oxygen atoms from the oxygen atoms of glucose. Dimethyl sulfate, a carcinogen,[7] has historically been used to methylate catechol. Protocatechuic acid methylation employs *S*-adenosylmethionine generated and consumed intracellularly. Finally, synthetic vanillin manufacture is based on the use of nonrenewable petroleum, whereas glucose is derived from abundant, renewable starch. This difference in feedstock utilization is important, given projected fierce international competition as global petroleum production diminishes.[8]

Acknowledgment. Research was supported by the United States Department of Agriculture and the National Science Foundation. Orion Corporation provided the *COMT* gene.

Supporting Information Available: Enzyme isolation, fermentor synthesis, and enzymatic reduction (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA9817747

(4) Guldberg, H. C.; Marsden, C. A. *Pharmacol. Rev.* **1975**, *27*, 135.
(5) (a) Gross, G. G.; Bolkart, K. H.; Zenk, M. H. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 173. (b) Gross, G. G.; Zenk, M. H. *Eur. J. Biochem.* **1969**, *8*, 413. (c) Gross, G. G. *Eur. J. Biochem.* **1972**, *31*, 585. (d) Zenk, M. H.; Gross, G. G. *Recent Adv. Phytochem.* **1972**, *4*, 87.
(6) Coward, J. K.; Slisz, E. P.; Wu, F. Y.-H. *Biochemistry* **1973**, *12*, 2291.
(7) Lewis, R. J., Sr. *Hazardous Chemicals Desk Reference*, 3rd ed.; Van Nostrand Reinhold: New York, 1993.
(8) Campbell, C. J.; Laherrère, J. H. *Sci. Am.* **1998**, *278* (3), 78.