the negative value of $\Delta S^{\circ}_{PT}$.[7] This was explained through electrostatic interaction between the solute ion pair and the solvent dipole. For solvent molecules with negligible or zero dipole moment, the ion pair engages in little or no reordering of solvent molecules; however, the ion pair does engage in a large reordering of solvents with large dipoles. As a corollary, the larger the dipole moment of the solvent, the greater the electrostatic field strength of the rearranged solvent, and the larger the interaction, $\Delta H^{\circ}_{PT}$, between the ion pair and solvent cage. When applying this to proteins, it is recalled that the two acid-base side chains are already arranged in hydrogen bond formation. Furthermore, the amino acid side chains immediately adjacent to the ion pair are also fixed in space through protein folding. In this particular case, side chains are spatially arranged so their dipoles engage in supportive electrostatic interaction. Since there is little reordering, $\Delta S^{\circ}_{PT}$ for the process is negligible or a small negative value. However, these highly ordered side chains now engage in a strong electrostatic interaction with the ion pair, contributing a substantial negative value to $\Delta H^{\circ}_{PT}$. The small negative $\Delta S^{\circ}_{PT}$ coupled with the large negative $\Delta H^{\circ}_{PT}$, when fitted into eq 7, yields a large negative $\Delta G^{\circ}_{PT}$. Equilibrium is displaced to the right, driving the proton transfer process to completion.

**Acknowledgment.** This work was supported in part by the National Institutes of Health through a grant from the National Eye Institute, EY07587 to P.E.B.

**Registry No. 1**, 62480-45-9; phenol, 108-95-2; methylcyclohexane, 108-87-2; 3-methylpentane, 96-14-0; retinal, 116-31-4.

# Biocatalytic Synthesis of Aromatics from D-Glucose: The Role of Transketolase

**K. M. Draths,[†] D. L. Pompliano,[†] D. L. Conley,[†] J. W. Frost,*[,†] A. Berry,*[,‡] G. L. Disbrow,[‡] R. J. Staversky,[‡] and J. C. Lievense[‡]**

*Contribution from the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, and Genencor International, Inc., 1700 Lexington Avenue, Rochester, New York 14652.* *Received June 26, 1991*

**Abstract:** The percentage of D-glucose that microbes such as *Escherichia coli* convert into aromatics is one of the key considerations that will determine the long-term utility of such biocatalytic syntheses. Considerable research has focused on improving percent conversions by increasing the in vivo catalytic activity of the first enzyme involved in aromatic biosynthesis, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase. This report details a different strategy based on increasing the in vivo catalytic activity of the enzyme transketolase when DAHP synthase catalytic activity is amplified. A genetic locus (*tkt*) encoding transketolase was used to vary the catalytic activity of transketolase in *E. coli*. The catalytic activity of DAHP synthase was manipulated with an *aroG* gene encoding an isozyme of DAHP synthase that was insensitive to feedback inhibition. Increases in DAHP synthase catalytic activity ultimately reached a point where no further improvements in aromatic biosynthesis were observed. At this limiting level of aromatic biosynthesis, amplification of transketolase catalytic levels achieved an additional 2-fold increase in the percentage of D-glucose siphoned into aromatic biosynthesis.

Chemists have long been aware of the potential advantages of using simple carbohydrates such as D-glucose as synthetic starting materials.[1] Carbohydrates are relatively inexpensive and provide an almost boundless pool of chiral building blocks. Unfortunately, conversion of carbohydrates into noncarbohydrate products often requires long syntheses. Only a small amount of product is typically produced at the end of these synthetic efforts. An alternate approach to small-molecule synthesis entails genetic alteration of microbes to create a new organism capable of converting carbohydrates such as D-glucose into a noncarbohydrate product. Genetic modification of an organism requires as many tedious manipulations to create the new organism as chemical synthesis does to convert carbohydrate starting material into the desired product. The key advantage of the genetic approach is that a catalytic entity is created.

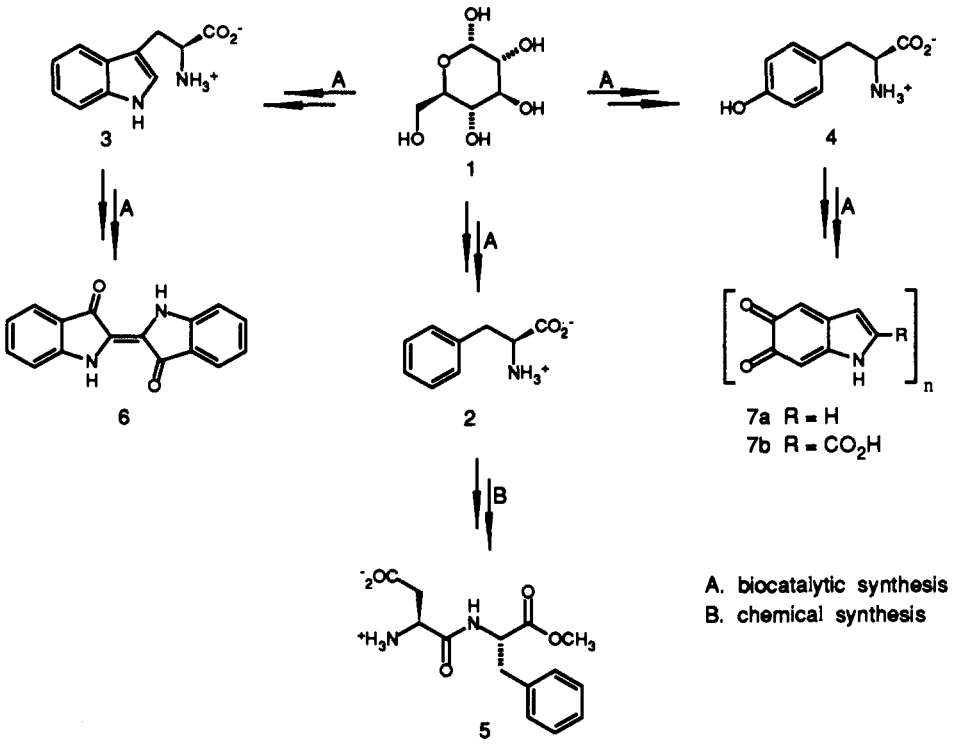
Perhaps the most spectacular example of the use of simple carbohydrates as starting materials in the synthesis of natural products is the conversion of D-glucose (**1**) by plants and microbes into aromatic amino acids and related metabolites (Scheme I).[2] Tremendous strides have been taken in the biocatalytic synthesis of L-phenylalanine (**2**) and L-tryptophan (**3**) from D-glucose.[3] These aromatic amino acids along with L-tyrosine (**4**) are used as human and animal dietary supplements. Of equal importance are the molecules that can be chemically or biocatalytically derived from the aromatic amino acids. L-Phenylalanine is transformed into the artificial sweetener aspartame (**5**) by traditional chemical methodology[4] while L-tryptophan and L-tyrosine can be converted to indigo (**6**) and eumelanin (**7**), respectively, by suitably constructed *Escherichia coli*.[5,6] Indigo imparts denim jeans with their distinctive, faded-blue coloration. Melanins provide the basic color pigmentation in mammals and birds while classes of melanin such as eumelanin possess unique UV-absorbing characteristics.[7]

[†] Purdue University.
[‡] Genencor International, Inc.

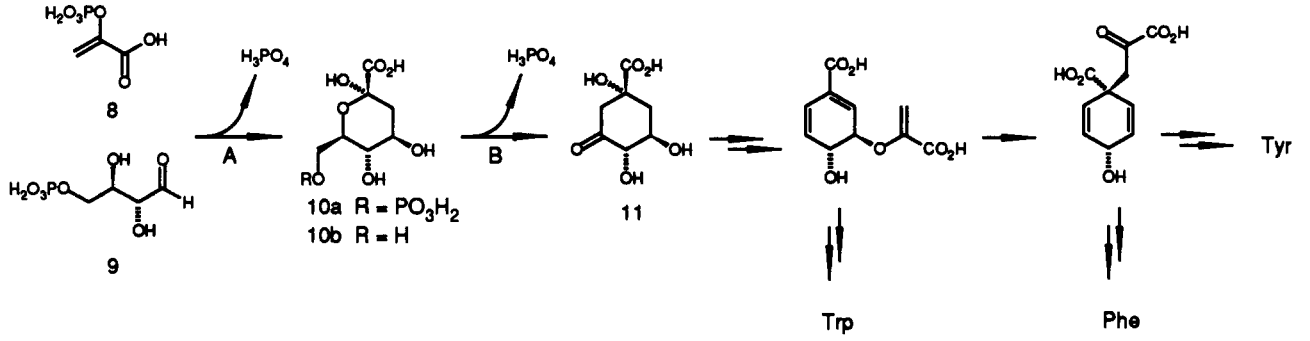
(1) Hanessian, S. *Total Synthesis of Natural Products: The "Chiron" Approach*; Pergamon: New York, 1983.
(2) (a) Haslam, E. *The Shikimate Pathway*; Wiley: New York, 1984. (b) Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley: New York, 1980. (c) Ganem, B. *Tetrahedron* **1978**, *34*, 3353. (d) Dewick, P. M. *Nat. Prod. Rep.* **1989**, *6*, 263. (e) Dewick, P. M. *Nat. Prod. Rep.* **1990**, *7*, 165.
(3) (a) Nakayama, K. In *Comprehensive Biotechnology*; Moo-Young, M., Ed.; Pergamon: New York, 1985; Vol. 3, Chapter 29. (b) Enei, H.; Hirose, Y. In *Comprehensive Biotechnology*; Moo-Young, M., Ed.; Pergamon: New York, 1985; Vol. 3, Chapter 27.
(4) (a) Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. *J. Am. Chem. Soc.* **1969**, *91*, 2684. (b) Davey, J. M.; Laird, A. H.; Morley, J. S. *J. Chem. Soc. C* **1966**, 555. (c) Pietsch, H. *Tetrahedron Lett.* **1976**, 4053. (d) Vinick, F. J.; Jung, S. *Tetrahedron Lett.* **1982**, *23*, 1315.
(5) Ensley, B. D.; Ratzkin, B. J.; Osslund, T. D.; Simon, M. J.; Wackett, L. P.; Gibson, D. T. *Science* **1983**, *222*, 167.
(6) della-Cioppa, G.; Garger, S. J.; Sverlow, G. G.; Turpen, T. H.; Grill, L. K. *Biotechnology* **1990**, *8*, 634.

0002-7863/92/1514-3956$03.00/0 

Scheme I




Scheme II



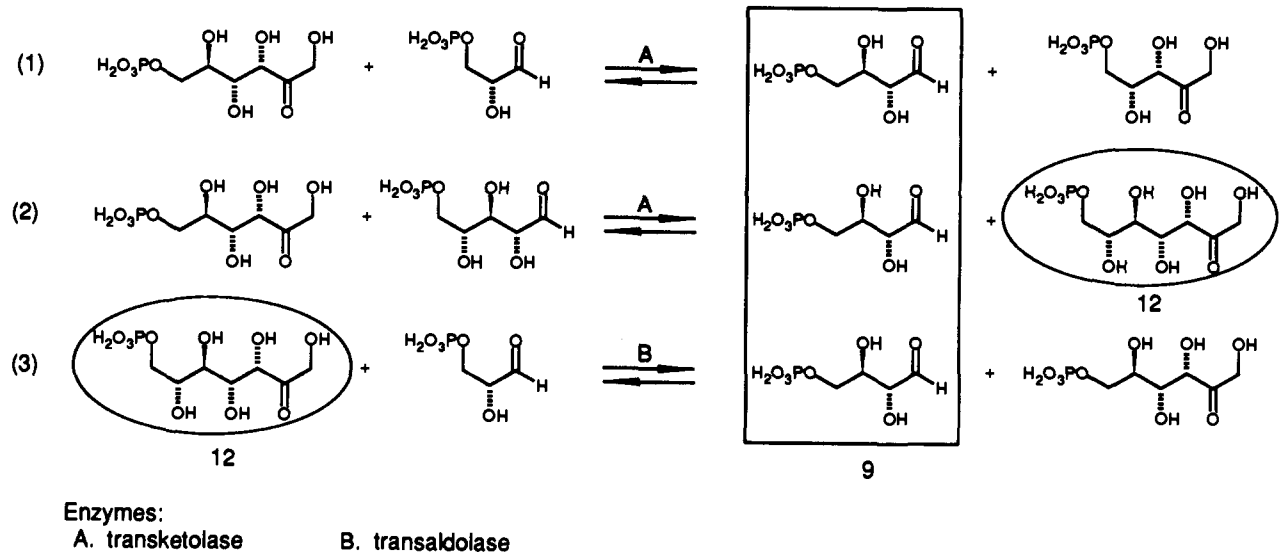

Whether biocatalytic synthesis of indigo will replace traditional chemical syntheses or whether biocatalytic synthesis will produce sufficiently inexpensive eumelanin for widespread industrial uses significantly depends on the microbial culture's percent conversion of the feedstock D-glucose into aromatic amino acid precursors. Therefore, just as improvements in percent yield of product from starting material are of preeminent concern in chemical synthesis, the percent conversion of D-glucose into desired small molecule product is also important in microbial biosynthesis. Traditionally, increasing the percent conversion of carbohydrates such as D-glucose into aromatics has focused on the catalytic activity of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase.[8] DAHP synthase is the first biosynthetic enzyme unique to the common pathway of aromatic amino acid biosynthesis.[2] This manuscript examines altered catalytic activity of the enzyme transketolase[9] as a new approach for increasing the percentage of D-glucose siphoned into aromatic biosynthesis.[10]

## Results and Discussion

**The Predicted Impact of Transketolase Catalysis on the Biosynthesis of Aromatics.** DAHP synthase catalyzes (Scheme II) the irreversible condensation of phosphoenolpyruvate (**8**) with D-erythrose 4-phosphate (**9**) resulting in the formation of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP, **10a**) and inorganic phosphate.[8] DAHP synthase (tyr), DAHP synthase (phe), and DAHP synthase (trp) are three different isozymes found in *E. coli* which are feedback inhibited by tyrosine, phenylalanine, and tryptophan. The loci *aroF*, *aroG*, and *aroH* encode the tyrosine-, phenylalanine-, and tryptophan-sensitive isozymes,

(7) Bell, A. A.; Wheeler, M. H. *Annu. Rev. Phytopathol.* **1986**, *24*, 411.
(8) (a) Herrmann, K. M. In *Amino Acids: Biosynthesis and Genetic Regulation*; Herrmann, K. M., Somerville, R. L., Eds.; Addison-Wesley: Reading, MA, 1983; Chapter 17. (b) Pittard, A. J. In *Escherichia coli and Salmonella typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1987; Vol. 1, Chapter 24.
(9) (a) Racker, E. In *The Enzymes*; Academic: New York, 1961; Vol. V, Chapter 24A. (b) Horecker, B. L. In *Comprehensive Biochemistry*; Florkin, M., Stotz, E. H., Eds.; Elsevier: New York, 1964; Vol. 15, Chapter 2. (c) Demuynck, C.; Fisson, F.; Bennani-Baiti, I.; Samaki, H.; Mani, J.-C. *Agric. Biol. Chem.* **1990**, *54*, 3073.
(10) (a) Frost, J. W.; Reimer, L. M.; Draths, K. M.; Pompliano, D. L.; Conley, D. L. *Biocatalysis*; Abramowicz, D. A., Ed.; Van Nostrand Reinhold: New York, 1990; Chapter 4. (b) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 1657. (c) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 9630.

Scheme III




respectively. A variety of approaches to elevate DAHP synthase catalytic activity have been shown to increase D-glucose equivalents channeled into aromatic biosynthesis. For example, increased expression of the gene encoding a particular DAHP synthase isozyme can increase the in vivo concentrations of DAHP synthase.[11,8b] Alternatively, mutations can be introduced into the genetic locus encoding a DAHP synthase isozyme that render the protein resistant to feedback inhibition by aromatic amino acids in vivo.[12]

In contrast to the catalytic activity of DAHP synthase, comparatively little attention has been given to small-molecule limitations on the flow of carbon into the common pathway of aromatic amino acid biosynthesis. A prime candidate for such a limitation is D-erythrose 4-phosphate. There currently exists no convincing evidence for detection of D-erythrose 4-phosphate in a living system.[13] This may reflect a phenomenon where the rate of D-erythrose 4-phosphate formation is closely matched by the rate of utilization of the aldose phosphate,[13b,c] allowing biological systems to circumvent the unwieldy chemical characteristics of D-erythrose 4-phosphate.[14] An example where such a strategy has been successfully employed is the cell-free multistep enzymatic synthesis of DAHP involving transketolase-catalyzed conversion of D-fructose 6-phosphate to D-erythrose 4-phosphate followed by condensation with PEP mediated by DAHP synthase.[15] Under optimized conditions, the C-4 aldose phosphate was not detected during the enzymatic synthesis.

Consideration of which enzymes influence the in vivo concentration of D-erythrose 4-phosphate leads to the pentose phosphate pathway. Uniquely situated between glycolysis and a variety of biosynthetic cascades, the pathway can be divided into an oxidative and nonoxidative pathway. The oxidative pentose phosphate pathway converts D-fructose 6-phosphate into D-ribose 5-phosphate and carbon dioxide. Concomitant formation of NADPH provides the reducing equivalents needed for reductive biosynthesis. The nonoxidative pentose phosphate pathway converts D-fructose 6-phosphate into varying equivalents of D-ribose 5-phosphate, D-sedoheptulose 7-phosphate, and D-erythrose 4-phosphate, which are required respectively for the biosynthesis of nucleotides, gram-negative bacterial lipopolysaccharide, and aromatic amino acids. Nonoxidative pentose phosphate pathway interconversion between D-fructose 6-phosphate and pentoses allows organisms such as *E. coli* to use the pentoses D-ribose, D-xylose, and D-arabinose as exclusive sources of carbon.

The siphoning of D-glucose equivalents from glycolysis into the pentose phosphate pathway involves the catalytic interplay between the enzymes transketolase and transaldolase. Reactions that lead to D-erythrose 4-phosphate (**9**) formation are summarized in Scheme III. Overall, two D-glucose equivalents in the form of two molecules of D-fructose 6-phosphate can be converted into three molecules of D-erythrose 4-phosphate by the nonoxidative pentose phosphate pathway. Notably, two of the three reactions (Scheme III) involving D-erythrose 4-phosphate formation are catalyzed by transketolase, while the third reaction involving transaldolase catalysis is dependent on transketolase-catalyzed generation of D-sedoheptulose 7-phosphate (**12**, Scheme III).

Additional clues relevant to the role of transketolase in aromatic biosynthesis can be gleaned from microbial mutants lacking transketolase activity. Though mutants of *Bacillus* that completely lack transketolase activity are characterized by a buildup of D-ribose in the organisms' culture supernatant,[16] *Salmonella typhimurium* and *E. coli* mutants[17] lacking transketolase do not accumulate D-ribose. This seems to be due to retention of some level of transketolase activity in the gram-negative mutants. The levels of residual transketolase activity are insufficient to enable the mutants to exploit pentoses such as D-arabinose, D-ribose, or D-xylose as exclusive sources of carbon during growth. Normal growth of the mutants on minimal salts and D-glucose requires supplementation with shikimic or aromatic amino acids.[17b] This implied reduction in the availability of aromatic amino acids due to diminished transketolase activity along with the analysis of the reactions of Scheme III leads to a prediction that transketolase is the pivotal enzyme in determining D-erythrose 4-phosphate concentrations.

**Isolation and Characterization of a Transketolase-Encoding Gene.** Complementation of an *E. coli* strain possessing a mutation in the gene (*tkt*) encoding transketolase was the first step toward defining the role of this enzyme in aromatic biosynthesis. Unsuccessful attempts to use transposon Tn5 mutagenesis to isolate an *E. coli* mutant lacking transketolase necessitated use of a strain, *E. coli* BJ502, which carried a "leaky" mutant phenotype.[17b,c] In

(11) (a) Cobbett, C. S.; Morrison, S.; Pittard, J. *J. Bacteriol.* **1984**, *157*, 303. (b) Garner, C. C.; Herrmann, K. M. *J. Biol. Chem.* **1985**, *260*, 3820. (c) Cobbett, C. S.; Delbridge, M. L. *J. Bacteriol.* **1987**, *169*, 2500.

(12) (a) Ogino, T.; Garner, C.; Markley, J. L.; Herrmann, K. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5828. (b) Weaver, L. M.; Herrmann, K. M. *J. Bacteriol.* **1990**, *172*, 6581.

(13) (a) Paoletti, F.; Williams, J. F.; Horecker, B. L. *Anal. Biochem.* **1979**, *95*, 250. (b) Williams, J. F.; Arora, K. K.; Longenecker, J. P. *Int. J. Biochem.* **1987**, *19*, 749. (c) Williams, J. F.; Blackmore, P. F.; Duke, C. C.; MacLeod, J. F. *Int. J. Biochem.* **1980**, *12*, 339.

(14) (a) Blackmore, P. F.; Williams, J. F.; MacLeod, J. F. *FEBS Lett.* **1976**, *64*, 222. (b) Duke, C. C.; MacLeod, J. K.; Williams, J. F. *Carbohydr. Res.* **1981**, *95*, 1.

(15) Reimer, L. M.; Conley, D. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 8010.

(16) (a) Sasajima, K.; Yoneda, M. *Agric. Biol. Chem.* **1971**, *35*, 509. (b) Sasajima, K.; Yoneda, M. *Biotech. Bioeng. Rev.* **1984**, *2*, 175.

(17) (a) Eidels, L.; Osborn, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1673. (b) Josephson, B. L.; Fraenkel, D. G. *J. Bacteriol.* **1969**, *100*, 1289. (c) Josephson, B. L.; Fraenkel, D. G. *J. Bacteriol.* **1974**, *118*, 1082.

**Table I.** Purification of Transketolase from Wild-Type *E. coli* K-12

| step | total units[a] | specific activity, units/mg | *X*-fold purification | yield, % |
|---|---|---|---|---|
| crude | | | | |
| DEAE open column (linear phosphate gradient) | 1.2 | 0.016 | 1 | 100 |
| HPLC DEAE column (linear KCl gradient) | 0.70 | 0.39 | 24 | 58 |
| HPLC DEAE column (linear phosphate gradient) | 0.38 | 1.4 | 88 | 32 |

[a] Unit = 1 μmol of NADPH produced per minute at 25 °C.

our hands, *E. coli* BJ502 was determined to possess 15% of the units of transketolase activity found in unmutagenized wild-type *E. coli* K-12. A genomic library was constructed in phage lambda by ligation of *Bam*HI partially digested genomic DNA into cosmid pLAFR3[18] and subsequent packaging in lambda phage heads. Transformation into BJ502 followed by screening for colonies with both a restored ability to grow on pentoses as the exclusive source of carbon and normal growth rates on D-glucose lacking shikimate supplementation led to isolation of cosmid p10 which contained a 24-kb insert. Subcloning of p10 yielded plasmid pKD44B which carried a 5-kb *Bam*HI insert capable of complementing the transketolase mutation in BJ502.

Support that pKD44B encoded transketolase was obtained by enzymological evaluation of transketolase purified to homogeneity from wild-type *E. coli* K-12 and from *E. coli* BJ502/pKD4413. Two continuous assays used to gauge transketolase activity relied on measured absorbance changes at $OD_{340}$ due to either NADPH formation or NADH consumption.[13a,19] Neither of these assays could give a reliable measure of transketolase activity in crude lysates. Since linear changes at $OD_{340}$ were observed only after purification by (diethylaminoethyl)cellulose (DE52) anion exchange chromatography, fold purification and percent yield of transketolase were based on material purified through the initial DEAE column. Subsequent steps used to purify transketolase from wild-type *E. coli* K-12 are outlined in Table I.

Gel electrophoresis (Figure 1) indicated that the purified *E. coli* transketolase was homogeneous. Although partial purification of transketolase from *E. coli* has been reported,[20] this is the first purification of *E. coli* transketolase to homogeneity. Cross-linking of the purified transketolase with dimethyl suberimidate[21] followed by gel electrophoresis under denaturing, reducing conditions with Coomassie Brilliant Blue visualization revealed that the enzyme was a homodimer having a subunit molecular weight of 72 500. Gel electrophoresis (Figure 1) also indicated that genome-encoded transketolase purified from *E. coli* K-12 comigrated with plasmid-encoded transketolase purified from *E. coli* BJ502/pKD44B. This comparison established that transketolase derived from the plasmid pKD44B has the same molecular weight and subunit composition as genome-encoded transketolase.

**The Impact of Transketolase Catalysis on Biosynthesis of Aromatics.** Expression of plasmid localized *tkt*, genomic *tkt*, and the mutated *tkt* locus of *E. coli* BJ502 leads to transketolase specific activities of 1.5, 0.17, and 0.03 units/mg, respectively. However, manipulation of the *tkt* locus is only the first step necessary to establish the impact of transketolase catalysis on aromatic biosynthesis. Transketolase-catalyzed reactions have an equilibrium constant of approximately unity,[10a] while DAHP synthase catalyzes an essentially irreversible reaction. In order for increased transketolase catalytic activity to translate into increased aromatic biosynthesis, D-erythrose 4-phosphate must be condensed with PEP at a rate in excess to those rates associated with transaldolase- and transketolase-mediated consumption of

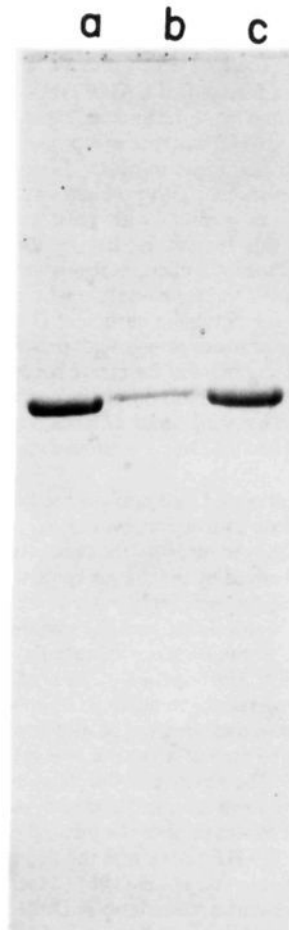



**Figure 1.** Comparison of transketolase purified from (lane a) *E. coli* BJ502/pKD44B, (lane b) *E. coli* K-12, and (lane c) *E. coli* BJ502/p10.

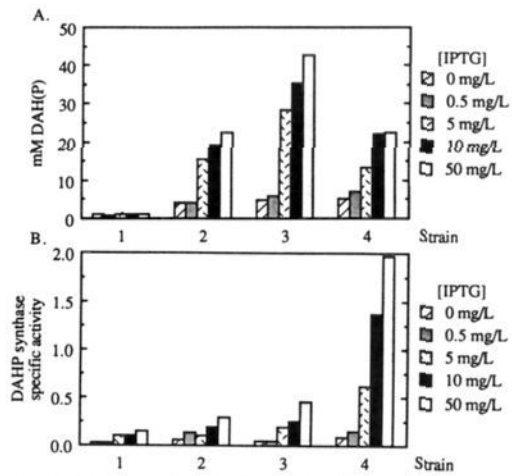



**Figure 2.** (A) Concentration (mM) of DAH(P) that accumulated in the culture supernatants of *E. coli aroB* strains after 21 h of growth. (B) Specific activity of DAHP synthase (μmol $min^{-1}$ $mg^{-1}$) after 21 h of growth. DAH(P) concentrations and DAHP synthase specific activities were determined for each construct at IPTG concentrations of 0, 0.5, 5, 10, and 50 mg/L. Constructs examined included BJ502/pRW5 (strain 1), AB2847/pRW5 (strain 2), AB2847/pRW5tkt (strain 3), and AB2847/pRW5/pRW300 (strain 4).

D-erythrose 4-phosphate. To identify the required levels of DAHP synthase activity, the *aroG* locus encoding the phenylalanine-sensitive isozyme of DAHP synthase was placed under *lac* promoter control. The plasmid (pRW5) carrying *aroG* also contained the *lacI* gene encoding *lac* repressor protein. With this plasmid,

(18) Friedman, A. M.; Long, S. R.; Brown, S. E.; Buikema, W. J.; Ausebel, F. M. *Gene* **1982**, *18*, 289.
(19) Sasajima, K.; Yoneda, M. *Agric. Biol. Chem.* **1974**, *38*, 1297.
(20) Yaphe, W.; Christensen, D. D.; Biaglow, J. E.; Jackson, M. A.; Sable, H. Z. *Can. J. Biochem.* **1966**, *44*, 91.
(21) (a) Davies, G. E.; Stark, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 651. (b) Stach, R. W.; Shooter, E. M. *J. Biol. Chem.* **1974**, *249*, 6668.

the amount of DAHP synthase specific activity expressed by the host *E. coli* strain is dictated by the concentration of isopropyl β-D-thiogalactopyranoside (IPTG) present in the culture medium.

One concern with amplified DAHP synthase activity was that an unacceptable amount of the microbe's metabolic currency may be funneled into DAHP synthase overproduction. The resulting metabolic burden might lead to widely different growth rates for the microbial constructs. This problem was avoided by supplementing the culture medium with yeast extract. A potential complication of this remedy is that feedback inhibition may quantitatively be the most important regulation of DAHP synthase activity in *E. coli*.[12] Supplementation with yeast extract might thus ameliorate the metabolic burden of DAHP synthase overexpression, but the aromatic amino acids present in the supplement would negate improvements in the in vivo concentrations of DAHP synthase via feedback inhibition of the enzyme. This problem was circumvented by using an *aroG* locus possessing a mutation that renders the encoded DAHP synthase insensitive to feedback inhibition.

Another critical consideration is the methodology required to gauge the D-glucose equivalents committed to aromatic biosynthesis. One option is to measure the concentrations of aromatic amino acids synthesized by the various constructs. Unfortunately, such measurements would likely not give an accurate measure of the D-glucose equivalents actually channeled into aromatic biosynthesis due to the presence of rate-limiting enzymes and branch pathways.[9c] Introduction of an *aroB* mutation into the host strains' genomes circumvents these complications by allowing the flow of D-glucose equivalents to be measured immediately after the first committed step of aromatic biosynthesis catalyzed by DAHP synthase. The absence of the enzyme 3-dehydroquinate synthase in *E. coli aroB* is characterized by accumulation in the microbe's culture supernatant of the missing enzyme's phosphorylated substrate, DAHP (**10a**), and the dephosphorylated form of the enzyme's substrate, DAH (**10b**) (Scheme II).[30a] Most of the heptulosonate that accumulates is DAH. Measurement of DAH(P) concentrations thus allows quantitation of the number of D-glucose equivalents funneled into aromatic biosynthesis.

Use of *aroB* strains and DAH(P) quantitation provides a clear indication of the level of aromatic biosynthesis in BJ502*aroB*/pRW5. This construct contains diminished levels of transketolase due to a mutated genomic *tkt* locus. Construct BJ502*aroB*/pRW5 also carries a plasmid (pRW5) encoding feedback resistant, *aroG*-encoded DAHP synthase. Very little DAH(P) is synthesized (Figure 2A) by BJ502*aroB*/pRW5 even with elevated DAHP synthase catalytic activity (Figure 2B) resulting from increased IPTG concentration in the culture supernatant. Similar incremental increases in DAHP synthase (Figure 2B) in *aroB* mutant AB2847/pRW5 resulted in significantly different levels of DAH(P) synthesis (Figure 2A). The 10- to 20-fold improvement in DAH(P) synthesized by *E. coli aroB* strain AB2847/pRW5 which expresses normal, wild-type levels of transketolase (0.17 units/mg) relative to BJ502*aroB*/pRW5 expressing reduced levels of transketolase (0.03 units/mg) points directly to the importance of transketolase catalysis in aromatic biosynthesis.

While the impact of diminished transketolase catalysis on aromatic amino acid biosynthesis has now been well established, the consequences of amplifying transketolase catalysis over wild-type levels have not clearly been delineated. Specifically, will amplified levels of transketolase catalysis result in an increase in aromatic amino acid biosynthesis? Resolution of this query focused on the DAH(P) concentrations synthesized by AB2847/pRW5 and AB2847/pRW5tkt. Both strains carry a genomic copy of *tkt*. At identical concentrations of IPTG, both strains express comparable levels of DAHP synthase (Figure 2B) due to the high copy plasmid-localized *aroG* that is encoded by pRW5 and pRW5tkt. The key difference between these two constructs is that pRW5 lacks the plasmid-encoded *tkt*, which is present in pRW5tkt. Higher levels of transketolase catalytic activity are thus expressed by AB2847/pRW5tkt relative to the transketolase activity expressed in AB2847/pRW5. When cultured under identical conditions, AB2847/pRW5tkt synthesizes DAH(P) concentrations that are twice the concentrations of the DAH(P) synthesized by AB2847/pRW5.

Another view of the importance of transketolase in directing additional D-glucose equivalents into aromatic biosynthesis arises from comparison of the concentrations of DAH(P) synthesized by AB2847/pRW5 and AB2847/pRW5/pRW300. Both constructs possess the same genomic *tkt* and plasmid *aroG*. However, AB2847/pRW5/pRW300 carries an extra, high-copy plasmid (pRW300) encoding *aroG*. This results in AB2847/pRW5/pRW300 expressing DAHP synthase levels (Figure 2B) that are about 6-fold higher than those measured in AB2847/pRW5 for identical concentrations of IPTG. Nonetheless, DAHP synthase overproducing AB2847/pRW5/pRW300 does not synthesize higher concentrations of DAH(P) than AB2847/pRW5. This result stands in marked contrast to the elevated concentrations of DAH(P) that are synthesized when the levels of transketolase catalysis are increased relative to transketolase levels in AB2847/pRW5 upon insertion of *tkt* into pRW5 to generate AB2847pRW5tkt.

There can be little question that the catalytic activity of DAHP synthase is an essential factor determining the level of D-glucose equivalents siphoned into aromatic biosynthesis. However, this study demonstrates that increases in DAHP synthase catalytic activity reach a level where further improvements in aromatic biosynthesis are not achieved (i.e., AB2847/pRW5 relative to AB2847/pRW5/pRW300). At this limiting level of aromatic biosynthesis, amplification of transketolase above wild-type levels (i.e., AB2847/pRW5 relative to AB2847/pRW5tkt) results in a substantial increase in the D-glucose equivalents committed to aromatic biosynthesis.

Ultimate translation of increased levels of transketolase catalysis into improved conversions of D-glucose into aromatic amino acids and other aromatic molecules will depend on removal of rate-limiting enzymes and branch pathways that are situated between DAHP synthase and synthesis of the desired aromatic product.[9c] The in vivo availability of small molecules other than D-erythrose 4-phosphate may also limit aromatic biosynthesis.[22] Nonetheless, transketolase catalysis has now been firmly established as a variable that can be manipulated to improve the percent conversion of simple carbohydrates into aromatic molecules. Such a discovery will likely have a significant impact upon future strategies for biocatalytic conversion (Scheme I) of D-glucose into L-phenylalanine (**2**), L-tyrosine (**4**), L-tryptophan (**3**), and potentially metabolites such as indigo (**6**) and eumelanin (**7**).

## Experimental Section

**Assays for Protein (A), Transketolase (B), and DAHP Synthase (C).** (A) Protein was assayed by using the Bradford dye-binding procedure[23] with protein assay solution purchased from Bio-Rad. A standard curve was prepared by using BSA.

(B) Transketolase activity was assayed in either of two ways. In the first method[13a] the assay solution (1 mL) contained triethanolamine buffer (150 mM), pH 7.6, $MgCl_2$ (5 mM), thiamine pyrophosphate (0.1 mM), NADP (0.4 mM), β-hydroxypyruvate (0.4 mM), D-erythrose 4-phosphate (0.1 mM), glucose 6-phosphate dehydrogenase (3 units), and phosphoglucose isomerase (10 units). The D-erythrose 4-phosphate was prepared from D-glucose 6-phosphate by the method of Sieben.[24] After incubation for 5 min to allow any glucose 6-phosphate to react, enzyme solution was added and the reaction monitored at 340 nm for 20–30 min. One unit of activity is defined as the formation of 1 μmol of NADPH per minute.

A modification of the method of Sasajima[19] was the second assay method used for transketolase activity. The assay solution (1 mL) containing (final concentration) potassium 3-(*N*-morpholino)propanesulfonate buffer (200 mM), pH 7.5, D-ribose 5-phosphate (2 mM), D-ribulose 5-phosphate 3-epimerase (1 unit), and phosphoriboisomerase (3 units) was incubated at 25 °C for 15 min. NADH (0.5 mM), thiamine pyrophosphate (0.1 mM), $MgCl_2$ (0.1 mM), $AsO_4$ (25 mM), and gly-

(22) Miller, J. E.; Backman, K. C.; O'Conner, M. J.; Hatch, R. T. *J. Ind. Microbiol.* **1987**, *2*, 143.
(23) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
(24) Sieben, A. S.; Perlin, A. S.; Simpson, F. J. *Can. J. Chem.* **1966**, *44*, 663.

ceraldehyde 3-phosphate dehydrogenase (5 units) were then added followed by the transketolase-containing solution. Absorbance at 340 nm was monitored continuously with 1 unit of transketolase activity defined as the loss of 1 μmol of NADH per minute.

(C) Phosphoenolpyruvate[25] and D-erythrose 4-phosphate[24] required for the assay of DAHP synthase activity were synthesized according to literature procedures. DAHP synthase was assayed by the procedure of Schoner.[26] Two different solutions were prepared and incubated separately at 37 °C for 10 min. The first solution (1 mL) contained potassium phosphate buffer (250 mM), pH 7, phosphoenolpyruvate (12.5 mM), D-erythrose 4-phosphate (6.5 mM), and ovalbumin (1 mg). The second solution (0.5 mL) consisted of the DAHP synthase-containing sample diluted in potassium phosphate (50 mM), pH 7, phosphoenolpyruvate (0.5 mM), and 1,3-propanediol (250 mM). After the two solutions were mixed, 0.15-mL aliquots were withdrawn at timed intervals and quenched by addition of 0.10 mL of 10% trichloroacetic acid. Precipitated protein was separated by centrifugation and the DAHP in the supernatant measured by thiobarbiturate assay.[27] One unit of DAHP synthase activity is defined by the catalyzed formation of 1 μmol of DAHP per minute.

**Purification of *E. coli* Transketolase.** Bio-Rad hydroxylapatite, Whatman (diethylaminoethyl)cellulose (DE52), and a 7.5 mm × 7.5 cm DEAE-5PW TSK analytical (purchased from Beckman) HPLC column were used during the purification. Buffers included buffer A, potassium phosphate (50 mM), $MgCl_2$ (1 mM), and dithiothreitol (0.2 mM), pH 7.35; buffer B, potassium phosphate (250 mM), $MgCl_2$ (1 mM), and dithiothreitol (0.2 mM), pH 7.35; buffer C, potassium phosphate (10 mM) and $MgCl_2$ (1 mM), pH 7.35; buffer D, potassium phosphate (10 mM), $MgCl_2$ (1 mM), and KCl (500 mM), pH 7.35; and buffer E, potassium phosphate (250 mM) and $MgCl_2$ (1 mM), pH 7.35. All protein manipulations were carried out at 4 °C. Protein solutions were concentrated by ultrafiltration (PM-10 Diaflo membranes from Amicon).

Frozen cell paste (18 g) of *E. coli* K-12 was thawed in 45 mL of buffer A. The cells were lysed by two passes through a French press at 16 000 psi. Cell debris was removed by centrifugation at 18000*g* for 20 min. A slurry of hydroxylapatite in 30 mL of buffer A was added, and the mixture was centrifuged at 18000*g* for 20 min. The pellet was resuspended in 30 mL of buffer A and centrifuged at 18000*g* for 7 min. The two resulting supernatants were combined and applied to a column of DEAE-cellulose (110 mL) equilibrated with buffer A. Elution with buffer A (110 mL) was followed by elution with a linear gradient (500 mL + 500 mL, buffer A–buffer B). Fractions containing transketolase were concentrated and applied to the HPLC DEAE column equilibrated with 77:23 buffer C/buffer D. Transketolase was eluted with a linear gradient (77:23 buffer C/buffer D–68:32 buffer C/buffer D). Fractions containing transketolase were concentrated and desalted by using buffer C. The protein was reapplied to the HPLC DEAE column equilibrated with 70:30 buffer C/buffer E. Transketolase was eluted with a linear gradient (70:30 buffer C/buffer E–50:50 buffer C/buffer E). Active fractions were concentrated, quick frozen in liquid nitrogen, and stored at −70 °C.

Polyacrylamide gel electrophoresis of purified transketolase in the presence of sodium dodecyl sulfate was performed according to Laemmli.[28] The stacking gel was buffered with Tris-HCl at pH 6.8, and the separating gel (12.5% acrylamide) was buffered with Tris-HCl at pH 8.8. Typically, 20 μg of protein was loaded per lane. Gels were fixed with acetic acid solution and visualized with Coomassie Brilliant Blue. Standards used for molecular weight determination included lysozyme (14 300), β-lactoglobulin (18 400), ovalbumin (43 000), bovine serum albumin (68 000), phosphorylase B (97 000), and myosin-H chain (200 000). Cross-linking of purified transketolase followed the protocol of Stach[21b] and entailed reaction (in a total volume of 0.05 mL) of transketolase (0.5 mg/mL) with dimethyl suberimidate (0–10 mg/mL) in 0.2 M borate, pH 9.5, for 3 h at 25 °C. Addition of 10% sodium dodecyl sulfate (0.005 mL) and β-mercaptoethanol (0.0005 mL) was followed by incubation at 37 °C for 2 h. Subsequent separation by polyacrylamide gel electrophoresis followed the procedure of Davies.[21a] The separating gel (5% acrylamide) was buffered with 0.1 M sodium borate and 0.1 M sodium acetate, pH 8.5, and contained sodium dodecyl sulfate (0.1%).

**Culture Medium.** All growth solutions were prepared in distilled, deionized water. LB medium[29] contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and sodium chloride (10 g) in 1 L of water. M9 medium[29] contained $Na_2HPO_4$ (6 g), $KH_2PO_4$ (3 g), NaCl (0.5 g), $NH_4Cl$ (1 g), $MgSO_4$ (0.12 g), thiamine (1 mg), and shikimic acid (50 mg) where indicated. M9/glucose, M9/arabinose, M9/ribose, and M9/xylose consisted of M9 medium containing respectively D-glucose (4 g/L), D-arabinose (4 g/L), D-ribose (4 g/L), and D-xylose (4 g/L). YE medium contained $K_2HPO_4$ (14 g), $KH_2PO_4$ (16 g), $(NH_4)_2SO_4$ (5 g), $MgSO_4$ (1 g), yeast extract (15 g), D-glucose (15 g), and two drops of P-2000 antifoam. Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed. Antibiotics, thiamine, amino acids, and shikimic acid were introduced by syringe through sterilizing 0.2-μm membranes (Nalgene). Agar (1.5% w/v) plates were prepared with either Difco agar for LB solid medium or from agarose (Type II, medium EEO, Sigma) for M9/glucose, M9/arabinose, M9/ribose, and M9/xylose solid medium.

**Genetic Manipulations.** Transductions with P1 were performed according to Miller.[29a] Other recombinant DNA techniques followed the procedures detailed in Maniatis.[29b] Alteration of *E. coli* BJ502[17b,c] to an *aroB* phenotype followed from P1 transduction of the *aroB* locus from JB5 (*aroB malT*::Tn5).[30b] Selection of transductants for resistance to kanamycin (50 mg/L) followed by screening for lack of growth on M9/glucose and growth on M9/glucose supplemented with shikimate yielded a number of BJ502 *aroB* candidates. These candidates were then transformed with pKD44B and assayed for accumulation of DAH(P) in the growth supernatant in order to isolate BJ502 *aroB* strains.

**Isolation of a Transketolase-Encoding *tkt*.** Chromosomal DNA purified from[31] *E. coli* RB791 was partially digested with *Bam*HI. The resulting 20–30-kb fragments were ligated into cosmid[18] pLAFR3 that had previously been digested with *Bam*HI and then treated with alkaline phosphatase. Ligated product was added to freshly prepared lambda extract[29b] for incorporation into phage heads. Following transformation into BJ502, transformants were selected for the ability to grow on M9/glucose, M9/arabinose, M9/ribose, and M9/xylose solid medium lacking aromatic supplementation. The *tkt* mutation of BJ502 was complemented by cosmid p10. Digestion of p10 with *Bam*HI followed by ligation and transformation into BJ502 resulted in isolation of cosmid pKD44B, which similarly complemented the *tkt* mutation of BJ502. All subsequent plasmids encoding transketolase were constructed from the 5-kb *Bam*HI insert initially isolated in cosmid pKD44B. Plasmid pRW5 was constructed by cloning a promoterless *aroG* gene encoding a feedback resistant DAHP synthase (phe) isozyme under control of tandem *lac* promoters in a pACYC184-based[32] plasmid that also contained a *lacI* gene encoding the *lac* repressor. Plasmid pRW5tkt was constructed by inserting the 5-kb *tkt*-containing fragment from pKD44B into the unique *Bam*HT site of pRW5. Plasmids pRW300 and pRW5 contain the same *aroG* gene under *lac* promoter control and the same *lacI* gene. These plasmids differ in that pRW300 is a pBR322-based[33] plasmid. Plasmids pRW300 and pRW5 can thus be stably maintained in the same strain. When plasmids pRW5 and pRW300 were simultaneously present in the same strain, chloramphenicol (for pRW5) and tetracycline (for pRW300) were both present at 50 mg/L.

**Biocatalytic Synthesis of Aromatics As Measured by DAH(P) Accumulation.** Cultures used for determination of DAH(P) accumulation were grown at 35 °C with agitation at 250 rpm in YE medium. Accumulation cultures (30 mL of YE medium in a 250-mL baffled Erlenmeyer flask) were inoculated with washed cells recovered from overnight growth in YE medium. The initial cell density as measured by optical density at 660 nm ($OD_{660}$) of the cultures was 0.2. Growth was monitored at $OD_{660}$, and IPTG was added when the cultures reached an $OD_{660}$ of 2.0. Following induction with IPTG, the pH of the cultures was maintained at 6.5–6.7 by addition of a 45% (w/v) KOH solution. At the indicated timed intervals, 1-mL samples of the cultures were removed for the assay of DAH(P). The cells were removed from the samples by centrifugation and the supernatants stored at 4 °C until all samples had been collected. The concentration of DAH(P) in the samples was determined by the thiobarbiturate assay.[27] After growth for 21 h, the cells

(25) (a) Clark, V. M.; Kirby, A. J. *Biochem. Prep.* **1966**, *11*, 101. (b) Hirshbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765.

(26) Schoner, R.; Herrmann, K. M. *J. Biol. Chem.* **1976**, *251*, 5440.

(27) Gollub, E.; Zalkin, H.; Sprinson, D. B. *Methods Enzymol.* **1971**, *17A*, 349.

(28) Laemmli, U. K. *Nature* **1970**, *227*, 680.

(29) (a) Miller, J. M. *Experiments in Molecular Genetics*; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1972. (b) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1982.

(30) (a) Frost, J. W.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4465. (b) Frost, J. W.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470.

(31) Silhavy, T. J.; Berman, M. L.; Enquist, L. W. *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1980.

(32) (a) Chang, A. C. Y.; Cohen, S. N. *J. Bacteriol.* **1978**, *134*, 114. (b) Rose, R. E. *Nucleic Acids Res.* **1988**, *16*, 355.

(33) Bolivar, F.; Rodriguez, R. L.; Greene, P. J.; Betlach, M. C.; Heynecker, H. L.; Boyer, H. W. *Gene* **1977**, *2*, 95.

from each culture were also saved. Extracts prepared from these cells were assayed for DAHP synthase activity as described above.

**Acknowledgment.** Research (J.W.F., K.M.D., D.L.P., D.L.C.) was partially supported by the Searle Scholars Program, a Camille and Henry Dreyfus Teacher-Scholar Grant, and the Sloan Foundation.

**Registry No.** DAHP synthase, 9026-94-2; D-glucose, 50-99-7; transketolase, 9014-48-6.

# Synthesis and Polymerization of *N*-Z-L-Serine-β-lactone and Serine Hydroxybenzotriazole Active Esters

**Michael E. Gelbin and Joachim Kohn***

*Contribution from the Department of Chemistry, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903.* *Received June 27, 1991*

**Abstract:** The reaction of Z-L-serine **1a** with 1-hydroxybenzotriazole (HOBt) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) yielded oligomeric poly(*N*-Z-serine ester) **4a** and not Z-L-serine-β-lactone (**2**, R = Z), as described in a widely cited report by König and Geiger. After modifying the reaction conditions, the elusive HOBt active ester of Z-L-serine (**3**, R = Z) was obtained in 90% yield. Bulk polymerization of **3** gave poly(Z-L-serine ester) **4a** with a weight average molecular weight ($M_w$) of about 22000 da. Reaction of N-protected serine derivatives **1b–d** with HOBt and DCC afforded poly(serine esters) **4b–d** directly, typically with $M_w$ in excess of 20000 da. In contrast, *N*-Z-threonine **5** failed to yield poly(*N*-Z-threonine ester). The optical purity of L-serine was preserved during the synthesis of the HOBt active ester and the subsequent polymerization. The approach described here represents the first, convenient multigram synthesis of serine derived polyesters, new polymers with potential biomedical applications.

## Introduction

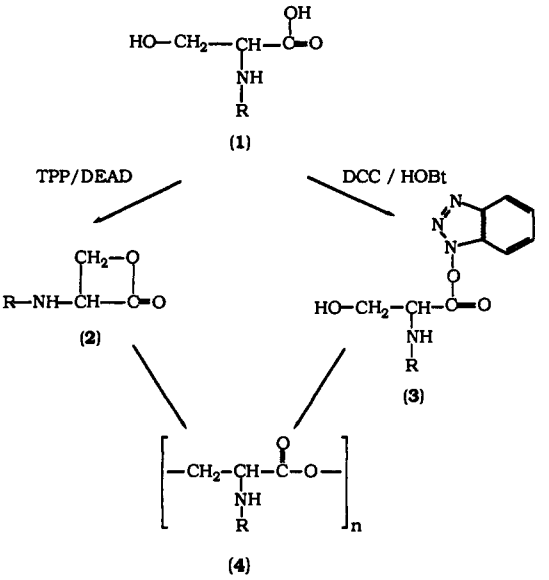
Serine-β-lactones **2** (Scheme I) are important intermediates in amino acid synthesis and polymer chemistry: Among other transformations, Vederas et al.[1,2] recently reported the synthesis of N-protected β-substituted alanines via **2**, while Zhou and Kohn[3] as well as Spassky et al.[4] described the ring-opening polymerization of **2** leading to poly(N-protected serine esters) **4**. Polyesters **4** belong to a new group of polymers defined as "pseudo" poly(amino acids)[5] which are currently being investigated as biodegradable implant materials in a variety of medical applications.[6]

Lactones **2**, however, are not readily available. Several methods giving **2** have been reported but are laborious, give low yields, or are limited to specific protecting groups. For example, Sheehan et al.[7] described the cyclization of *N*-trityl-L-serine (**1**, R = trityl) using *N,N'*-diisopropylcarbodiimide. The corresponding lactone was obtained with 15% yield. This method was later modified by employing (dimethylamino)pyridine as a catalyst and *N,N'*-diisopropylcarbodiimide[8] or *N,N'*-dicyclohexylcarbodiimide[4] as the carboxyl activation agents. The reported yields, however, were not significantly higher than the yields obtained by Sheehan's approach. An alternate method via Hofmann rearrangement of *N*-(benzenesulfonyl)asparagine[9,10] gave higher yields (45%) but appears to be restricted to the use of the benzenesulfonyl protecting group.

So far, only two procedures have been reported that provide N-protected serine-β-lactones with yields in excess of 50%. Most noteworthy is the synthesis published in 1970 by König and Geiger,[11] furnishing **2** (R = Z) in 91% yield in a simple, one-step reaction of **1a** with *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). Unfortunately, König and Geiger provided only melting point and elemental analysis as the sole identifying properties for their reaction product. This synthesis was disclosed in their pioneering paper on the use of HOBt in peptide synthesis. Today this paper is frequently cited and has been included in major textbooks on peptide synthesis.[12,13]

More recently, Vederas et al.[1,2] reported the successful synthesis of **2** (R = Z, Boc) by means of a modified Mitsunobu[14] reaction in yields of up to 81%. Vederas et al. published a detailed structural analysis of their products. Furthermore, in a footnote

**Scheme I**




| 1, 4 | R |
|---|---|
| a | benzyloxycarbonyl (Z) |
| b | p-methoxybenzylcarbonyl (Moz) |
| c | p-nitrobenzyloxycarbonyl |
| d | tert-butoxycarbonyl (Boc) |

* To whom correspondence should be addressed at Department of Chemistry, Rutgers, The State University of New Jersey, P.O. Box 939, Piscataway, NJ 08855.

(1) Arnold, L. D.; Kalantar, T. H.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7105–7109.
(2) Arnold, L. D.; Drover, J. C. G.; Vederas, J. C. *J. Am. Chem. Soc.* **1987**, *109*, 4649–4659.
(3) Zhou, Q.; Kohn, J. *Macromolecules* **1990**, *23*, 3399–3406.
(4) Fietier, I.; LeBorgne, A.; Spassky, N. *Polym. Bull.* **1990**, *24*, 349–353.
(5) Kohn, J.; Langer, R. *J. Am. Chem. Soc.* **1987**, *109*, 817–820.
(6) Kohn, J. *Pseudopoly(amino acids)*; Marcel Dekker, Inc.: New York-Basel-Hong Kong, 1990; pp 195–229.

0002-7863/92/1514-3962$03.00/0 