P3OT films was 0.2 ± 0.1 with **3a** and 0.3 ± 0.1 ng/mm^2 with **3b**.[20]

Surface modification using PFPAs was combined with photolithography to generate micrometer-sized patterns. A PS film was spin-coated with a nitromethane solution of **1a**, baked, and irradiated[23] through a high-resolution photomask with a minimum feature size of 0.5 μm. The film was then dipped in nitromethane for 20 s, air-dried, and allowed to react with a solution of 5-(aminoacetamido)fluorescein in ethanol (4 mg/mL) at 25 °C for 1 h followed by thorough rinsing with ethanol. Figure 1 shows the resulting micrometer-sized patterns observed under a fluorescence microscope, further demonstrating this new surface modification strategy.[24] The smallest features (0.5 μm) are resolved though slightly broadened, probably owing to diffraction effects.

This same methodology was also used for the surface modification of preformed polymer microstructures. A micrometer-scale pattern of PS, which had previously been fabricated on a silicon wafer using deep-UV lithography, was dipped in a nitromethane solution of **1a** for 10 s, baked, and photolyzed as before. The sample was then immersed in a solution of *N*-(5-aminopentyl)biotinamide in DMF (1 mg/0.2 mL) for 4 h and washed with DMF followed by ethanol. Taking advantage of the strong affinity of avidin for biotin,[25] fluorescein–avidin was attached to the surface by incubating the wafer in a solution of the fluorescent protein in pH 8.2 buffer (3.2 mg/0.5 mL) for 4 h. The micrometer-sized patterns and control shown in Figure 2 indicate the covalent attachment of the biotin–avidin–fluorescein assembly on the preformed PS microstructure.

This new surface modification strategy should find application in microelectronics and in the construction of novel micrometer-scale biosensors.

Acknowledgment. This work was supported by grants from the Office of Naval Research, the Oregon Resource Technology Development Corporation, and the National Institute of General Medical Sciences (GM 27137). We thank Dr. Gary Goncher of Tektronix Inc. for his help in carrying out the deep-UV exposure.

(21) An HRP molecule has a molecular weight of around 40 000 and a radius of 2.67 nm in the hydrated state. (Steiner, H.; Dunford, H. B. *Eur. J. Biochem.* **1978**, *82*, 543–549.) Assuming a flat polymer surface, the surface coverage of a monolayer of HRP is 2.7 ng of HRP/mm^2.

(22) The nitrene insertion reaction probably involves CH bonds in the octyl side chain and retention of polymer conductivity. See: Cai, S. X.; Nabity, J. C.; Wybourne, M. N.; Keana, J. F. W. *J. Mol. Electron.* **1991**, *7*, 63–68.

(23) Photolysis was carried out in a KSM Karl Suss deep-UV contact aligner.

(24) As a control, a PS film without a spin-coating of NHS active ester **1a** was photolyzed, developed, and treated with 5-(aminoacetamido)-fluorescein. No fluorescent patterns were observed under the fluorescence microscope.

(25) Heitzmann, H.; Richards, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3537–3541.

Isolation and Structure Determination of 2-Amino-2-deoxyisochorismate: An Intermediate in the Biosynthesis of Anthranilate†

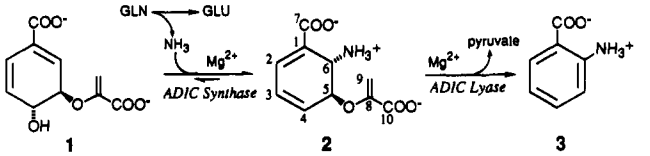
Anthony A. Morollo,*,‡ M. G. Finn,§ and Ronald Bauerle‡

Department of Biology and the Molecular Biology Institute University of Virginia, Charlottesville, Virginia 22903 Department of Chemistry, University of Virginia Charlottesville, Virginia 22903

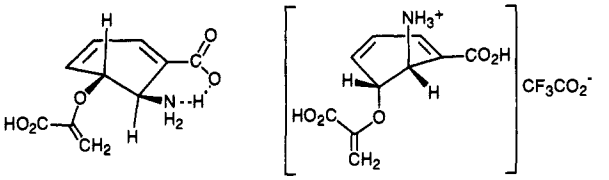
Received September 24, 1992

Anthranilate synthase (AS) catalyzes the initial reaction in the biosynthesis of tryptophan from chorismate[1] (Scheme I). The formation of anthranilate (*o*-aminobenzoate) **3** from chorismate **1** and glutamine has long been suggested to occur through an intermediate, *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid, **2**,[2] commonly called 2-amino-2-deoxyisochorismate (ADIC), although a cis rather than trans stereochemistry has also been proposed.[3] Attempts to detect the accumulation of **2** during enzymatic turnover have heretofore been unsuccessful;[4] however, synthetic **2**·TFA has been shown to be a competent substrate for anthranilate formation by AS.[5] Additionally, the accumulation of a C5-*R*-lactyl aminocyclohexadiene has been demonstrated in enzymatic incubations using a C5-*R*-lactyl analog of chorismate as substrate, although the stereochemistry of the lactylaminocyclohexadiene was not defined.[6] Here we report the isolation of an aminated intermediate as the product of an enzymatic activity of the *Salmonella typhimurium* AS and present the formal structural identification of this intermediate as **2**.

Scheme I



Scheme II



The results of ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-COSY 2D NMR analysis of **2** are shown in Figure 1. The assignments are supported by ^{1}H,^{1}H correlations establishing the H2–H3–H4–H5–H6 and H9–H9′ spin systems, expected chemical shift values, and ^{1}H,^{13}C correlation to the carbon resonances. The coupling constant of 12.3 Hz measured for H5 and H6 is indicative of trans configuration at C5 and C6. The N-substituted carbon was identified from the proton-decoupled natural abundance ^{13}C NMR spectrum of a sample of **2** enriched in ^{15}N, which demonstrated splitting of the C6 resonance at 52.9 ppm into a doublet with J_{CN} = 6 Hz (Figure 1, inset). All data are consistent with the structure of **2** with stereochemistry as shown.

The ^{1}H NMR data obtained for **2** differ significantly from those reported for synthetic **2**·TFA,[5a] particularly the H6 coupling constant,[9] suggesting that either **2** and **2**·TFA exist in different

† Abbreviations used: AS, anthranilate synthase; TrpE, catalytic subunit of AS; ADIC, *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid, commonly known as 2-amino-2-deoxyisochorismate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

‡ Department of Biology and the Molecular Biology Institute, University of Virginia.

§ Department of Chemistry, University of Virginia.

* Author to whom correspondence should be addressed. Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22903.

(1) Bentley, R. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 307–384.

(2) (a) Levin, J. G.; Sprinson, D. B. *J. Biol. Chem.* **1963**, *239*, 1142–1150. (b) Srinivason, P. R. *Biochemistry* **1965**, *4*, 2860–2865.

(3) Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley: New York, 1980; pp 238–246.

(4) Walsh, C. T.; Liu, J.; Rusnak, F.; Sakaitani, M. *Chem. Rev.* **1990**, *90*, 1105–1129.

(5) (a) Policastro, P. P.; Au, K. G.; Walsh, C. T.; Berchtold, G. A. *J. Am. Chem. Soc.* **1984**, *106*, 2443–2444. (b) Teng, C. P.; Ganem, B. *J. Am. Chem. Soc.* **1984**, *106*, 2463–2464.

(6) Walsh, C. T.; Erion, M. D.; Walts, A. E.; Delany, J. J., III; Berchtold, G. A. *Biochemistry* **1987**, *26*, 4734–4745.

(7) The mutant AS complex, with a single amino acid substitution at HIS398 of the TrpE subunit (His → Met), was purified as described previously in the following: Caligiuri, M. G.; Bauerle, R. *J. Biol. Chem.* **1991**, *266*, 8328–8335. The preparation was homogeneous as determined by SDS-PAGE. The AS specific activity of the enzyme was ≈1% of the wild-type enzyme, assayed as described in the following: Bauerle, R.; Hess, J.; French, S. *Methods Enzymol.* **1987**, *142*, 366–386. The properties and kinetic behavior of this enzyme will be reported in more detail elsewhere (manuscript in preparation).

0002-7863/93/1515-816$04.00/0

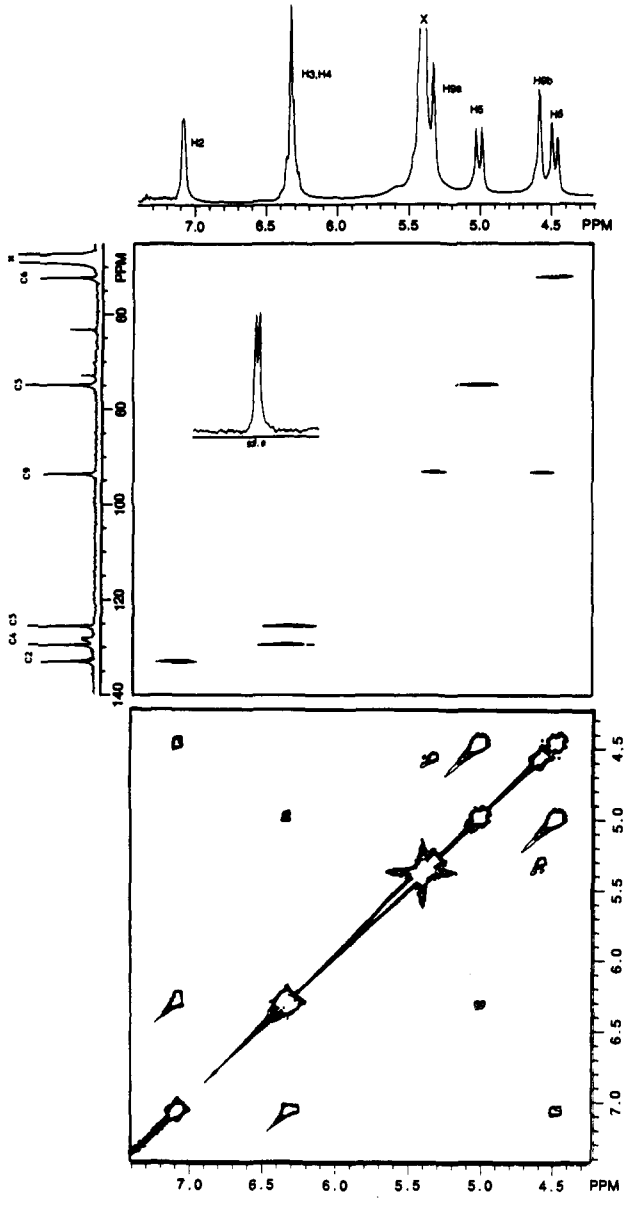


Figure 1. NMR spectra of 2-amino-2-deoxyisochorismate. Assignments are with respect to the numbering shown in Scheme I. Bottom: $^1H,^1H$-correlated spectrum. Top: $^1H,^{13}C$-correlated spectrum, with 1D spectra appended ("x" marks residual resonance of solvent). Chemical shifts are in parts per million relative to residual CHD_2OD at 3.30 ppm. ^1H NMR (CD_3OD 300.1 MHz): δ 7.05 (1 H, br s), 6.28 (2 H, m), 5.34 (1 H, s), 4.99 (1 H, d, J = 12.3 Hz), 4.59 (1 H, s), 4.45 (1 H, d, J = 12.3 Hz). ^{13}C NMR (CD_3OD 75.1 MHz): δ 171.1, 169.6, 154.1, 134.0, 130.4, 129.2, 126.5, 94.3, 75.6, 52.9. Spectra were acquired at −30 °C on a GE GN-300 spectrometer. Inset: Proton-decoupled natural abundance ^{13}C spectrum of ^{15}N-labeled **2** showing the C6 resonance. The spectrum was obtained at −10 °C on a GE Omega-500 spectrometer operating at 125.5 MHz. Methods: A reaction mixture consisting of 100 mM NH_4HCO_3, pH 8.0, 5 mM $MgCl_2$, 35 mM diammonium chorismate, and 13 μM mutant $TrpE^{H398M}$ AS complex[7] was incubated at 20 °C for 3.25 h, extracted with an equal volume of $CHCl_3$, and lyophilized.[8] The residue was dissolved in 5% acetic acid, filtered, and fractionated by RP-HPLC using isocratic elution with 5% acetic acid. Column fractions containing **2** were pooled, lyophilized, reconstituted with D_2O, lyophilized, and dissolved in 0.5 mL of CD_3OD for NMR analysis. [^{15}N]-**2** was purified from an enzymatic reaction mixture consisting of 50 mM Tricine, pH 8.0, 50 mM $(^{15}NH_4)_2SO_4$, 5 mM $MgCl_2$, 15 mM chorismate, and 13 μM mutant $TrpE^{H398M}$ AS complex.

conformations or the enzymatic and synthetic compounds are different stereoisomers. To distinguish these possibilities, a ^1H NMR spectrum of **2** in CD_3OD was acquired at −30 °C and the critical coupling constant was measured as 14 Hz. After addition of 1 equiv of TFA to the sample, a coupling constant of 4.8 Hz was measured between the same protons (assignments confirmed by $^1H,^1H$-COSY NMR shown in supplementary material),[10] consistent with that reported[5a] for synthetic **2**·TFA. Subsequent addition of $NaOCD_3$ was accompanied by a return to the initial 14-Hz coupling constant. These results establish the existence of alternative conformations for **2**; the free acid exists as the diequatorial conformer, and the TFA salt assumes the diaxial conformer (Scheme II).[11] A similar, though less dramatic, change in conformation has been characterized for chorismic acid and its dianionic salt.[12]

We have also noted a marked difference in the relative stabilities of the two forms of **2**. RP-HPLC and ^1H NMR analyses of **2**·TFA revealed substantial decomposition of the material during preparation, with breakdown occurring predominantly by the same route previously described for synthetic **2**·TFA, i.e., conversion to 3-carboxyphenylpyruvic acid by Claisen rearrangement and elimination of ammonia.[5b] In contrast, the free acid form of **2** is much more stable; degradation is negligible after 2 months of storage as an aqueous solution at −80 °C. We conclude that the diaxial conformer adopted by **2**·TFA is more favorable for spontaneous Claisen rearrangement than the diequatorial conformer assumed by the free acid form.

Isomeric hydroxy- and aminochorismate compounds have been isolated as products of enzymes, which are similar to AS at the primary structural level,[13] that catalyze the first steps in enterobactin[14] and *p*-aminobenzoate[15] biosynthesis, respectively. The results described here establish that a similar aminochorismate compound, **2**, is an intermediate in the anthranilate synthase reaction, supporting suggestions that all three enzymes share features of a common mechanism[4] as well.

Acknowledgment. We thank Laurie Kelsh and Jeff Ellena for collecting NMR spectra and Bruce Ganem for communicating low-field spectra. This work was supported by a NIH research grant (GM35889) to R.B. and an American Cancer Society Junior Faculty Research Award to M.G.F.

Supplementary Material Available: ^1H and $^1H,^1H$-COSY NMR spectra of **2**·TFA, with peak assignments (2 pages). Ordering information is given on any current masthead page.

(8) In this experiment, **2** had accumulated to approximately 20 mol % when the reaction was quenched, a level over 100-fold greater than that detected for the wild-type enzyme.

(9) Although ^1H NMR resonances of synthetic **2**·TFA have been reported but not assigned (see ref 5a), signals at 5.63 and 5.07 ppm, with J = 3 Hz, appear to be olefinic resonances, leaving a doublet at 4.53 ppm with J = 6 Hz that must be assigned to H6. In contrast, we find J = 12.3 Hz between H5 and H6 of the free amino acid form of **2**.

(10) **2**·TFA: ^1H NMR (CD_3OD 300.1 MHz) δ 7.37 (1 H, d, J = 5.1 Hz), 6.50 (2 H, m), 5.60 (1 H, d, J = 3.0 Hz), 5.08 (1 H, d, J = 3.0 Hz), 4.90 (1 H, t), 4.50 (1 H, d, J = 4.8 Hz).

(11) The analogous coupling constants for the diequatorial and diaxial conformations of chorismic acid have been predicted to be approximately 13 and 3.2 Hz, respectively (see ref 12). The change in conformation upon acidification can be understood by invoking an interaction between adjacent amino and carboxyl groups to stabilize the diequatorial conformation for **2** (H5–H6 dihedral angle ≈90°), and release to the favored diaxial conformation (H5–H6 dihedral angle ≈170°) for **2**·TFA (see Scheme II). In the absence of an attractive interaction between the substituents at C1 and C6, a steric interaction between them is expected to render the diequatorial conformation less stable than the diaxial one. For a related example in which conformation changes with solvent due to a disruption of a stabilizing H-bond, see: Rabideau, P. W., Ed. *The Conformational Analysis of Cyclohexenes, Cyclohexadienes, and Related Hydroaromatic Compounds*; VCH Publishers: New York, 1989; pp 72–73.

(12) Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5008–5013.

(13) (a) Elkins, M. F.; Earhart, C. F. *FEMS Microbiol. Lett.* **1988**, *56*, 35–40. (b) Ozenberger, B. A.; Brickman, T. J.; McIntosh, M. A. *J. Bacteriol.* **1989**, *171*, 775–783. (c) Goncharoff, P.; Nichols, B. P. *J. Bacteriol.* **1984**, *159*, 57–62.

(14) Young, I. G.; Batterham, J. J.; Gibson, F. *Biochim. Biophys. Acta* **1969**, *177*, 389–400.

(15) Anderson, K. S.; Kati, W. M.; Ye, Q.; Liu, J.; Walsh, C. T.; Benesi, A. J.; Johnson, K. A. *J. Am. Chem. Soc.* **1991**, *113*, 3198–3200.