(19) Epstein, L. M.; Straub, D. K.; Maricondi, C. *Inorg. Chem.* **1967**, *6*, 1720–1724.
(20) Brunori, M.; Giacometti, G. M.; Antonini, E.; Wyman, J. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3141–3144.
(21) See, Saffran, W. A.; Gibson, Q. H. *J. Biol. Chem.* **1977**, *252*, 7955–7958.
(22) (a) Geibel, J.; Cannon, J.; Campbell, D.; Traylor, T. G. *J. Am. Chem. Soc.* **1978**, *100*, 3575–3585. (b) White, D. K.; Cannon, J. B.; Traylor, T. G. *Ibid.* **1979**, *101*, 2443–2454. (c) Mincey, T.; Traylor, T. G. *Ibid.* **1979**, *101*, 765–766. (d) Traylor, T. G.; Chang, C. K.; Geibel, J.; Berzinis, A.; Mincey, T.; Cannon, J. *Ibid.* **1979**, *101*, 6716–6731.
(23) Smith, M. H. *Biochem. J.* **1959**, *73*, 90–101.
(24) (a) K(Im): Brault, D.; Rougee, M. *Biochem. Biophys. Res. Commun.* **1974**, *57*(3), 654–659. (b) K(Py): Brault, D.; Rougee, M. *Biochemistry* **1974**, *13*(22), 4591–4597.
(25) Balch, A. L.; Watkins, J. J.; Doonan, D. J. *Inorg. Chem.* **1979**, *18*, 1228–1231.
(26) The optical spectra show that although K_2 is small but finite, for purposes of the analysis of kinetic data we may ignore the binding of the second base. The linear dependence of k_{obsd} on [CO] and its simultaneous independence of [Im$^-$] show that loss of base and subsequent CO binding contribute minimally compared with direct CO addition to the five-coordinate species.
(27) Sundberg, R. J.; Martin, R. B. *Chem. Rev.* **1974**, 471–517.
(28) (a) Viscosities from: "Handbook of Chemistry and Physics", 54th ed.; CRC Press: Cleveland, 1973. (b) Dielectric constants from: "Lange's Handbook of Chemistry", 10th ed.; McGraw-Hill: New York, 1967.
(29) Fulton, G. P.; LaMar, G. N. *J. Am. Chem. Soc.* **1976**, *98*, 2119–2124.
(30) Foster, R. "Organic Charge-Transfer Complexes", Academic Press: New York, 1969.
(31) Barry, C. D.; Hill, H. A. O.; Mann, B. E.; Sadler, P. J.; Williams, R. J. P. *J. Am. Chem. Soc.* **1973**, *95*, 4545, and references therein.

Structure of Arogenate (Pretyrosine), an Amino Acid Intermediate of Aromatic Biosynthesis

Lolita O. Zamir,*[1a,b] Roy A. Jensen,[1b] B. H. Arison,[1c] A. W. Douglas,[1c] G. Albers-Schönberg,[1c] and John R. Bowen[1b]

Contribution from the Departments of Chemistry and Biology, State University of New York at Binghamton, Binghamton, New York 13901, and Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065. *Received October 29, 1979*

Abstract: L-(8*S*)-Arogenate (previously named pretyrosine), a newly recognized precursor of L-phenylalanine and L-tyrosine biosynthesis, is widely distributed in nature. Proof of structure for arogenate, β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine, was established through the application of spectroscopic techniques (ultraviolet, ^{1}H NMR, ^{13}C NMR, and mass spectrometry) following the application of an improved procedure for isolation of L-arogenate from the culture supernatant of a mutant strain of *Neurospora crassa*. The (*S*) configuration of the chiral amino acid carbon at C-8 of L-arogenate was established by circular dichroism.

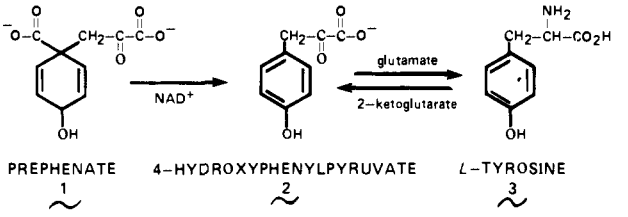
Introduction

Until 1974, the only biochemical route known for the biosynthesis of L-tyrosine in nature was as illustrated in Scheme I. Prephenate is the last nonaromatic intermediate in this 4-hydroxyphenylpyruvate sequence. A second sequence, the arogenate branchlet of L-tyrosine biosynthesis (Scheme II), has been identified as the sole route of L-tyrosine biosynthesis in species of cyanobacteria,[2,3] coryneform bacteria,[4-6] and in at least one yeast organism.[7] In the latter cases arogenate is the last nonaromatic intermediate. Pseudomonad bacteria[8-10] and plants[11] possess both enzymatic sequences simultaneously.

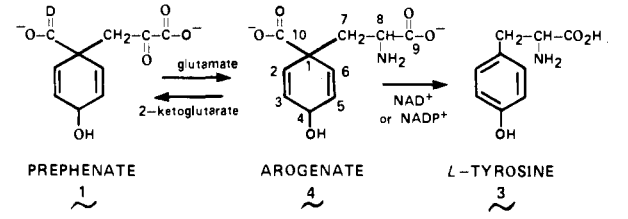
The structure depicted in Scheme II for arogenate was deduced[2] on the basis of enzymological results. Transamination of prephenate via a partially purified aminotransferase from *Agmenellum quadruplicatum* in the presence of L-leucine as the amino donor produced a ninhydrin-positive product that was easily distinguished from L-leucine by thin-layer chromatography. A dehydrogenase was partially purified which was able to oxidize the unknown compound to tyrosine in the presence of either NAD^+ or $NADP^+$. Synonymy of tyrosine and the product formed by the dehydrogenase was established by thin-layer chromatography, fluorescence profile, and amino acid analysis. Furthermore, acid treatment of arogenate yielded phenylalanine in a reaction analogous with the nonenzymatic conversion[12] of prephenate to phenylpyruvate at acidic pH. Since in some organisms[13] arogenate is a substrate for arogenate dehydratase, an enzyme which forms phenylalanine, the previous name (pretyrosine) is now abandoned in favor of the more appropriate designation.[14]

Rigorous chemical proof of structure for arogenate has not been offered prior to this study. In particular, the existence of the carboxyl group at C_1, the positioning of the double bonds in the cyclohexadiene moiety, and the stereochemistry of the C_8 chiral carbon (*R* or *S*) were not established. The ubiquity in nature of this newly found amino acid intermediate highlights the importance of structural data. The purpose of this work is (i) to establish the structure of arogenate and (ii) to assign the configuration of the asymmetric carbon at C-8.

Scheme I. 4-Hydroxyphenylpyruvate Pathway to L-Tyrosine



Scheme II. Arogenate Pathway to L-Tyrosine



Results and Discussion

Isolation of Arogenate. Small amounts of arogenate were originally made by using prephenate as substrate for a partially

0002-7863/80/1502-4499$01.00/0

Table I. Purification of Arogenate (Agn) from *N. crassa* Accumulation Medium

procedural step[a]	volume	m*M* Agn	mg free acid[b]	yield
original supernatant	12.6 L.	0.1	302	(100)%
eluate from Dowex 21K	775 ml	1.6	295	98%
supernatant from $BaCl_2$ ppt. of AG1–X8 (pH 8.1) eluate	378 ml	3.2	290	96%
eluate from Dowex AG1–X8 (pH 11.0)	315 ml	3.1	232	77%
eluate from Sephadex G–10[c]	99 ml	5.5	130	43%

[a] See narrative for full explanation of procedural steps. [b] Based upon a molecular weight of 238 for arogenate. [c] 70 mL of the 315 mL available from the previous step was used. Hence, the values given are calculated to anticipate the values expected had the entire 315 mL been used.

purified preparation of prephenate aminotransferase obtained from *A. quadruplicatum.*[2] Later we reported a method for the isolation and preparation of arogenate from a multiple auxotroph of *Neurospora crassa.*[16] An improved modification of this procedure is presented (Table I) in this paper.[17] Briefly, our current procedure eliminates an initial time-consuming evaporation step and no longer employs the inherently variable steps of barium salt precipitation. A series of anion exchange chromatography steps at several pH conditions is followed by a final desalting purification step via gel filtration (G-10), which yields the sodium salt of arogenate. Since acidification of arogenate promotes quantitative conversion to phenylalanine, arogenic acid must be regarded as intrinsically unstable. However, the disodium salt of arogenate at basic pH (7.5) is quite stable in the lyophilized state. For example, no detectable decomposition of the latter preparation occurred, even after storage at room temperature for over a month.

Purity of Arogenate. The purity of arogenate obtained by this procedure was 95% based on ^1H NMR spectra obtained at 300 MHz. An assay was devised in order to detect arogenate in various biological sources in minute amounts. This assay is based upon the conversion of arogenate to a dansyl derivative which is highly fluorescent. An even more sensitive test uses [G-^3H]dansyl-chloride, since the resultant [^3H]dansyl-arogenate is then detected in tracer amounts. At this level of sensitivity a minor compound was detected which was optically active and which yields a fluorescent dansyl derivative. This compound, probably an unknown amino acid, exists at greater levels in unfractionated preparations of culture supernatant used as the source of arogenate. The separate investigation of this unknown structure is currently underway. The procedural steps in isolation and purification of arogenate did not alter its biochemical properties as probed through the use of enzymes requiring arogenate as substrate. Thus, a crude enzyme preparation of dehydrogenase from *Pseudomonas syringae* (ATCC 19872)[10] was extremely active with a sample of arogenate and NAD^+ (specific activity equals 13 nmol of tyrosine obtained per mg of protein per min). This activity was totally abolished by acid treatment and reneutralization of the arogenate substrate preparation.

Detection of Arogenate in Minute Quantities. At acidic pH, arogenate is quantitatively converted to phenylalanine, which can be measured with a sensitive fluorometric assay.[18] This assay is not always reliable with crude preparations such as culture supernatants which contain a variety of molecules that may interfere with the assay. The fluorometric assay is also not satisfactory for analytical determinations to be made on samples that contain L-phenylalanine in addition to arogenate. We have developed sensitive methods[19] for detection and

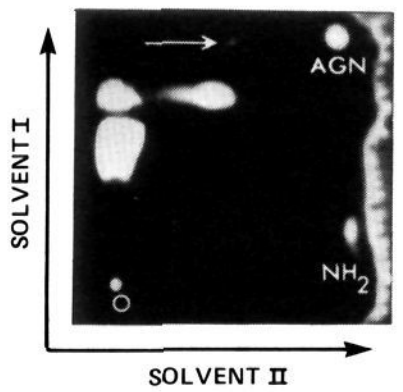


Figure 1. Photograph of dansyl-arogenate after two-dimensional TLC as described in the text. The migration positions of dansyl-arogenate (AGN) (actually dansyl-phenylalanine following acidification subsequent to migration in solvent I, see text), dansyl-amide (NH_2), a dansyl impurity (arrow), and other reaction byproducts were photographed under UV illumination.

quantitation of arogenate without interference of other amino acids, keto acids, or proteins. At basic pH the fluorescent dansyl-chloride is covalently attached, probably on the amino group. Dansyl-arogenate is then separated from the byproducts of the reaction by thin-layer chromatography on polyamide plates (5 cm × 5 cm) coated on both sides. A standard mixture of known dansyl-amino acids is applied on one side. The TLC plate is developed in two directions in two different solvents, the second development following the in situ conversion of dansyl-arogenate to dansyl-phenylalanine. Fluorescent dansyl compounds are detected under UV light. Dansyl-arogenate migrates to a different R_f position than known dansylated amino acids.

Figure 1 illustrates the migration position of acid-treated dansyl-arogenate following two-dimensional TLC. The separation of a small amount of an organic impurity (less than 5%) is also shown in Figure 1. Altnernatively, we use high-performance liquid chromatography (LC) for the analytical detection and quantitation of arogenate. The high-performance LC equipped with a fluorometric detector is used to quantitate dansyl-arogenate through measurement of peak area. Individual steps of arogenate purification from *N. crassa* supernatants are monitored by dansylation followed either by thin-layer chromatography on polyamide plates or by high-performance LC analysis.

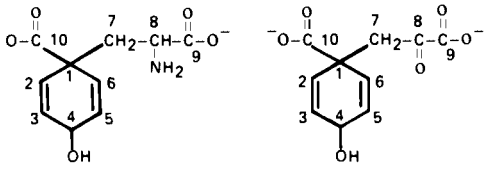
UV Profile and Circular Dichroism Spectrum of Arogenate. The ammonium salt of arogenate was derived from sodium arogenate via ion exchange chromatography. The UV profile of both salts of arogenate indicated clearly that the double bonds in the cyclohexadienyl moiety were not conjugated, the only absorption being an end absorption at λ 222 nm (ϵ = 10 000) at pH 13.55 in aqueous solution.

In contrast to prephenate, arogenate lacks a plane of symmetry and therefore is expected to exhibit optical activity. Indeed, arogenate shows an intense positive Cotton effect in its circular dichroism spectrum below 230 nm, $[\theta] = +4.6 \times 10^4$.

^{13}C NMR of Arogenate and Prephenate. The ^{13}C NMR data of arogenate and prephenate rigorously establish the structure of arogenate as that shown in Scheme II. Assignments of the constituent carbons are shown in Table II. In arogenate one can clearly distinguish two carbonyls at low field: 182.3 and 183.7 ppm. We cannot differentiate between C_9 and C_{10}. In prephenate three carbonyls are evident. The lowest field signal is attributed to the carbonyl group at C_8, which is α to a carboxylic acid (204.6 ppm). A carbonyl group is expected to shift an adjacent carboxylic carbon upfield, and, indeed, one of the carboxylates in prephenate appeared at 170.5 ppm, while the other appeared at 180.7 ppm. The signal at 170.5 is therefore attributed to C_9.

The asymmetry introduced at the C_8 moiety of arogenate accounts for the nonequivalence of the four carbons in the

Table II. ^{13}C NMR Data for Sodium Arogenate and Sodium Prephenate[a]



Carbons	Chemical shifts in ppm (off-resonance multiplicity)	
C-9	182.3/183.7 (singlet)	170.5 (singlet)
C-10	182.3/183.7 (singlet)	180.7 (singlet)
C-3	132.5/131.9 (doublet)	132.1 (doublet)
C-5	132.5/131.9 (doublet)	132.1 (doublet)
C-2	129.6/129.9 (doublet)	128.5 (doublet)
C-6	129.6/129.9 (doublet)	128.5 (doublet)
C-4	62.1 (doublet)	62.2 (doublet)
C-8	54.8 (doublet)	204.6 (singlet)
C-1	51.1 (singlet)	49.4 (singlet)
C-7	45.0 (triplet)	48.7 (triplet)

[a] The chemical shifts of the decoupled spectrum are described as well as the off-resonance spectrum multiplicity. Where assignments are uncertain between a pair of carbon atoms, both are listed.

olefinic region. Prephenate, on the other hand, having a plane of symmetry (perpendicular to the plane of the paper) exhibits equivalence of carbons C_2 and C_6, which differ from C_3 and C_5 which are also equivalent. We observed only two signals corresponding to the four olefinic carbons at 132.1 and 128.5 ppm. The C_8 carbonyl is expected to influence the positions of C_2 and C_6 more than C_3 and C_5 relative to arogenate. Comparison of the values of the olefinic carbons in arogenate with prephenate showed the largest shift to be from 129.6/129.9 to 128.5 ppm. We therefore assign C_2 and C_6 in prephenate at 128.5 ppm, while C_3 and C_5 correspond to 132.1 ppm. In arogenate, C_3 and C_5 appeared at 132.5 and 131.9 ppm, while C_2 and C_6 were located at 129.6 and 129.9 ppm, respectively. Although we could not discern C_3 from C_5 or C_2 from C_6, this information is not critical to the structure determination. The C_4 carbon ought to be influenced least by C_8, an expectation consistent with the finding that in both arogenate and in prephenate C_4 was observed at an almost identical chemical shift (62.1 and 62.2 ppm).

The off-resonance decoupled spectra of arogenate and prephenate fit the assignments deduced from evaluation of the fully decoupled ^{13}C NMR spectra. The quaternary carbon at C_1 was found at 51.1 ppm in arogenate and at 49.4 ppm in prephenate. The off-resonance spectra confirm the absence of attached hydrogens, since both signals are singlets. The observation of this quaternary carbon confirms with certainty the synonymy of arogenate with the structure assigned in Scheme II. The upfield shift of C_1 as well as C_7 in prephenate is attributable to the carbonyl at C_8. The ^{13}C NMR data of arogenate and prephenate provided the basis for unambiguous structural assignments that were confirmed decisively by the remaining spectroscopic data.

^1H NMR of Arogenate and Prephenate. Our ^1H NMR studies were carried out with the sodium salts of prephenate and arogenate at 300 MHz in D_2O using deuterated trimethylsilyl sodium propionate (TSP) as the internal standard. Our data obtained with prephenate isolated as the natural product were very similar to that previously reported by Danishefsky and Hirama,[20] who obtained ^1H NMR data at 250 MHz with disodium prephenate in D_2O using 3-trimethylsilyl-1-propanesulfonic acid as a standard. The vinylic protons in prephenate appear as a typical AB portion of an ABX pattern of two doublets of doublets (Figure 2). The absence of observed exchange of the C_7-methylene protons in D_2O noted in our ^1H NMR analysis was confirmed by inspection of ^{13}C NMR spectra of prephenate and arogenate, where no significant deuterium exchange occurred in the C_7-methylene group. The assignments made for $H_{3,5}$ and $H_{2,6}$ in prephenate are based upon the likelihood that the larger coupling with H_4 (J = 3.1 Hz) reflects a vicinal relationship. Therefore, the signal at δ 5.94 is assigned to $H_{3,5}$ and the one at δ 6.03 (J = 1.5-2.0 Hz) is linked to $H_{2,6}$. The vinylic region is very characteristic of the arogenate structure and is shown expanded in Figure 3. The presence of four vinylic hydrogens was established by integration in both disodium arogenate and diammonium arogenate. The chiral center introduced at C_8 in arogenate alters the pattern of the vinylic hydrogens seen in prephenate. The spectral pattern obtained is not first order; we tentatively interpret it as two sets of partially overlapping AB systems (Figure 3).

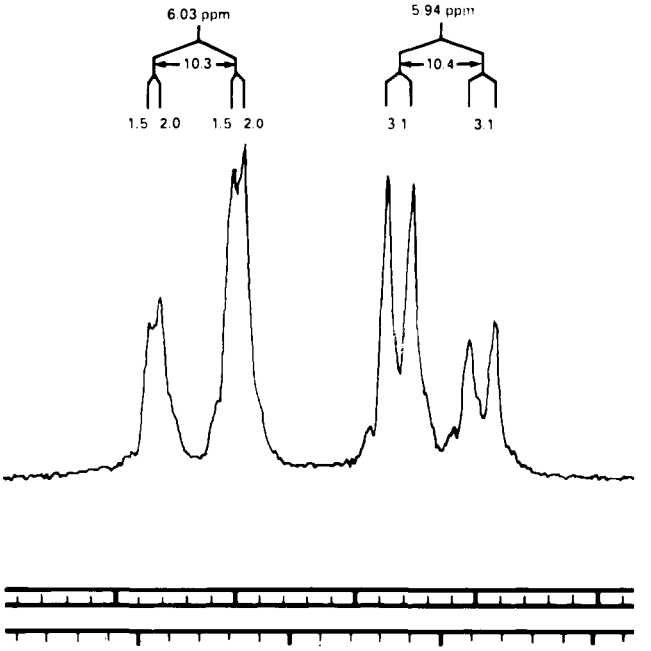
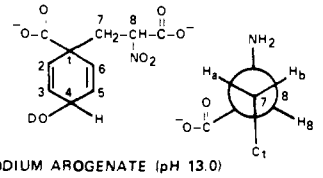


Figure 2. ^1H NMR spectrum of the vinylic protons in disodium prephenate.

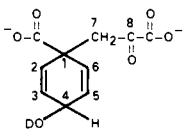
The ^1H NMR of arogenate was pH dependent (Table III). All of the signals were shifted in concert upfield with increasing pH. While the C_7-methylene hydrogens of prephenate (relative area: 2 H) form a sharp singlet, the C_7-methylene protons of arogenate are nonequivalent (J_{gem} = 13.8-13.9 Hz) and further split by the adjacent methine hydrogen at C_8. Space-filling models show that the vicinal coupling of 4.8 Hz corresponds to the coupling between H_b of the C_7-methylene and H_8, since C_8 is of the *S* configuration (as shown in the following section). On the other hand, the second vicinal coupling of 7.2 is best assigned to the coupling between H_a and H_8. As expected, H_7 is observed as two doublets of doublets and H_8 as a doublet of doublets. Since the spectra are highly pH dependent, H_4 in the diammonium salt of arogenate was barely detected under the H_2O peak, and its multiplicity was not discerned. In the basic disodium salt of arogenate, H_4 appeared as a broad singlet of half-width (6 Hz).

Mass Spectroscopy of Arogenate. The disodium salt of arogenate is thermally unstable (decomposes to phenylalanine) and is nonvolatile. Various ingenious methods have been devised recently for the mass spectroscopy of organic salts and thermally labile species with little or no prior thermal decomposition.[21-30] However, since these techniques were not available to us, we decided to derivatize arogenate in order to increase its volatility. To improve the solubility we converted the sodium salt to the ammonium salt on an ion-exchange

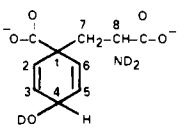
Table III. ^{1}H NMR Data for Disodium Arogenate (pH 13.0), Diammonium Arogenate (pH 6.9), and Disodium Prephenate (pH 13.0)



DISODIUM AROGENATE (pH 13.0)



DISODIUM PREPHENATE (pH 13.0)



DIAMMONIUM AROGENATE (pH 6.9)

Signal Observed	δppm	Signal Pattern	J,Hz
$H_{5,6}$ $H_{2,3}$	5.54	Doublet	10
	5.66	Doublet	10
$H_{5,6}$ $H_{2,3}$	~5.63	Doublet	~10
	~5.69	Doublet	~10
H_4	4.22	Broad singlet	$\omega_{1/2}$ ~ 6 Hz
H_7	1.81	Doublet of doublets	Jgem(a,b) 13.9 Jvic(b,8) 4.8
	1.55	Doublet of doublets	Jgem(b,a) 13.8 Jvic(a,8) 7.2
H_8	2.77	Doublet of doublets	Jvic(b,8) 4.8 Jvic(a,8) 7.2

Signal Observed	δppm	Signal Pattern	J,Hz
$H_{2,6}$	6.03	Doublet of doublets	$J_{(2,4)(6,4)}$ 1.5–2.0 $J_{(2,3)(6,5)}$ 10.3
$H_{3,5}$	5.94	Doublet of doublets	$J_{(3,4)(5,4)}$ 3.1 $J_{(3,2)(5,6)}$ 10.4
H_4	4.52	Multiplet	—
H_7	3.14	Singlet	—

Signal Observed	δppm	Signal Pattern	J,Hz
$H_{5,6}/H_{2,3}$	5.99	Doublet	10
	6.06	Doublet	10
$H_{5,6}/H_{2,3}$	6.9	Broad singlet (2H)	—
H_4	4.59	Singlet	—
H_7	2.33	Doublet of doublets	Jgem 15 Jvic 3.0–3.5
	2.23	Doublet of doublets	Jgem 15 Jvic 8.5
H_8	3.53	Doublet of doublets	Jvic 8.5 Jvic 3.0–3.5

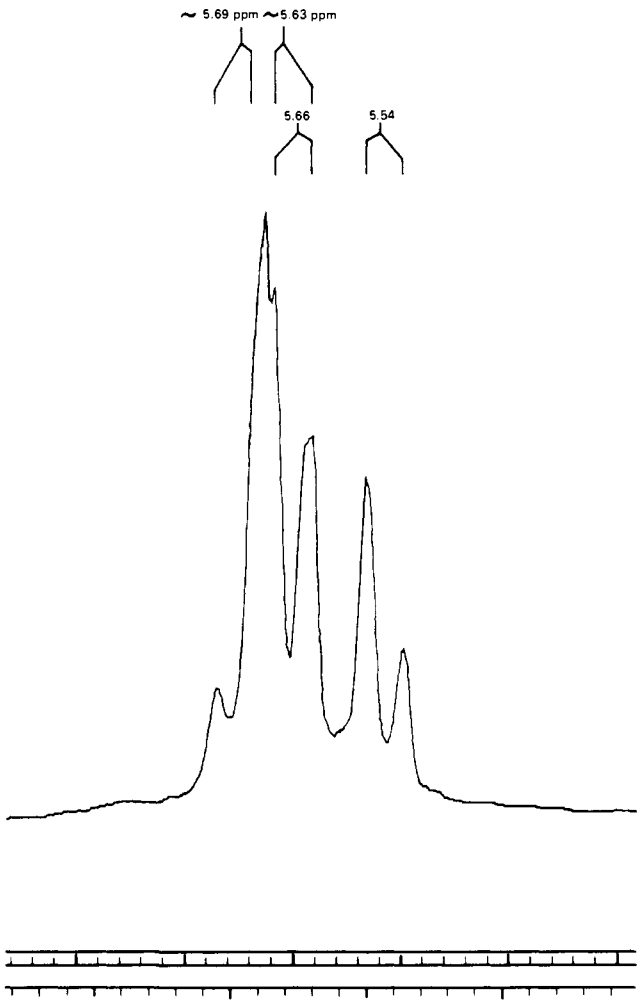


Figure 3. ^{1}H NMR spectrum of the vinylic protons in disodium arogenate.

column. The diammonium arogenate was dissolved in dimethylformamide and then treated at room temperature with bis(trimethylsilyl)trifluoroacetamide. The derivative was not isolated but inserted directly through the inlet source of the mass spectrometer. We obtained a very pure spectrum of arogenate to which four trimethylsilyl groups had been attached. The high-resolution mass spectrum proved the structure unambiguously. Indeed, the calculated molecular weight for four trimethylsilyl groups attached to the OH, NH_2, and the two carboxylates of arogenate is 515.2356 ($C_{10}H_{13}NO_5 \cdot 4Me_3Si$) compared to the value obtained of 515.2353. The mass spectrum of tetra(trimethylsilyl) arogenate is shown in Figure 4. The fragment ion at *m/e* 398 corresponds to loss of CO_2 and trimethylsilyl, and one can discern a metastable ion that could be assigned to this fragmentation at *m/e* 308.5. Further loss of a hydrogen radical and ·O-Si(Me_3)$_3$ yields the ion at *m/e* 308. A metastable peak is observed for the cleavage of the ion at mass *m/e* 398 to the ion at mass *m/e* 308. The fragment 398 can also lose CO_2 and HSi(Me_3)$_3$ to give a fragment at *m/e* 280. The other fragmentation peaks are the same ones observed in bis(trimethylsilyl)phenylalanine as expected, since the quaternary carboxyl group and the ·O-Si(Me_3)$_3$ are very easily cleaved. The fragments 398, 410, and 500 are specific to the arogenate moiety.

Configuration of Disodium Arogenate at C_8. (*R*)-D- or (*S*)-L-phenylalanine racemizes extremely slowly in acid. In order to establish the configuration at the C_8 position, we acidified disodium arogenate at room temperature and recorded the UV as well as the circular dichroism (CD) of the resulting phenylalanine (Figure 5). Only (*S*)-L-phenylalanine was obtained. The CD analysis revealed two positive Cotton effects, one of low intensity exhibiting fine structures centered at λ 260 nm and one of high intensity in the vicinity of 218 nm. The CD of authentic (*S*)-phenylalanine[31,32] was superimposable with the CD of the phenylalanine obtained from arogenate (Figure 5). The arogenate molecule contains C_8 as its only chiral center, the carbons at C_1 and C_4 being meso prochiral centers. Prephenate, which has identical substituents at C_1 and C_4, has been shown to have the carboxyl group at C_1 and the hydroxyl group at C_4 in a cis spatial relationship.[33] Since arogenate was derived biosynthetically from prephenate, it is reasonable to assume that the C_1-carboxyl group is probably cis to the C_4-hydroxyl group.

Experimental Section

Instrumentation. ^{13}C NMR spectra were obtained by Fourier transform at 25.15 MHz on a Varian Associates XL-100 spectrometer. Observations were made at ambient probe temperature which was about 25 °C for the XL-100. The ^{13}C NMR runs were made with D_2O as solvent and dioxane as internal reference at 67.4 ppm. Pulse nutation angle was between 35 and 40° with 1.0-s repetition rate. Free induction decays were weighted by exp($-t$/0.5 s) before Fourier transformation. ^{1}H NMR spectra were obtained in D_2O on a Varian SC 300-MHz spectrometer equipped with a Fourier transform accessory. Flip angles between 60 and 80° and acquisition times of 1 or 2 s were used for the accumulations. All spectra were obtained at

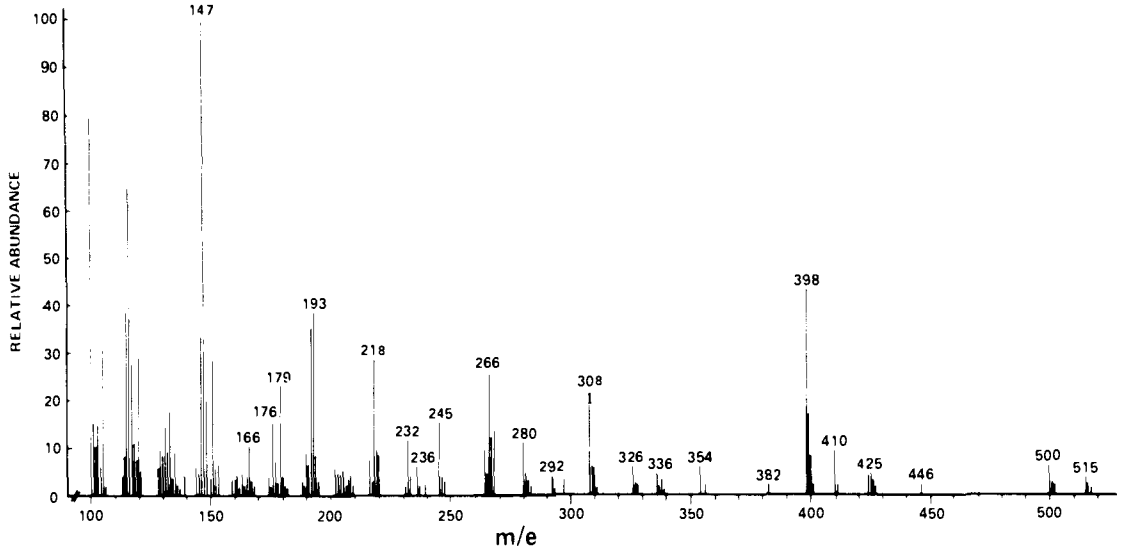


Figure 4. Mass spectrum of tetra(trimethylsilyl) arogenate.

ambient temperature (ca. 22 °C) on approximately 0.02 M solutions. The mass spectrum of the tetra(trimethylsilyl) arogenate was recorded by Mr. Jack Smith by direct insertion into the inlet source on an electron impact mass spectrometer LKB 9000. The high-resolution mass spectrum was taken on a Varian MAT 731 mass spectrometer. The ultraviolet and circular dichroism measurements were carried out using a JASCO Model ORD/UV-CD-5 instrument. Amino acid analyses were performed on a Glenco MM-70 amino acid analyzer using a single column methodology with the Pico Buffer (R) System supplied by Pierce Chemical Company, Rockford, Ill.[34]

Accumulation of Arogenate. Cultures of a multiply blocked aromatic auxotroph of *N. crassa* (75001/5212/C-167) were maintained at room temperature by conidial transfer on the solidified media of Westergaard and Mitchell[15] containing 250 mg per L each of L-phenylalanine, L-tyrosine, and L-tryptophan (25 mL per 250-mL Erlenmeyer flask). The accumulation medium for arogenate[15] contained 1% (w/v) sucrose, 25 mg per L each of L-tyrosine and L-tryptophan as well as 5 mg per L of L-phenylalanine. The medium was adjusted to pH 7.5 with 10 N NaOH before autoclaving. Sucrose and $CaCl_2$ solutions were autoclaved separately. A carboy containing 15 L of accumulation medium was inoculated with an aqueous suspension of conidia obtained from six starter cultures, and incubation was carried out at room temperature. The pH of the culture was continuously maintained within the range of 7.0–7.5 by addition of concentrated NH_4OH. These conditions are essentially those of Jensen et al.[16]

Purification of Arogenate. After 6 days, the culture was filtered sequentially through a single layer of miracloth (Chicopee Mills) and a 5.0-μm filter (GA-1, Gelman). The pH of the filtrate was adjusted to pH 10.5 with 10 N NaOH, and the solution was applied to a column of the anion-exchanger Dowex 21K (5 cm × 33 cm, Cl^-) at 17 mL per min. The column was subsequently washed at 3.7 mL per min with 3 L of 0.1% (v/v) *N*-ethylmorpholine-HCl buffer (pH 8.6) and a linear gradient prepared from 2.75 L each of 0.1% (v/v) *N*-ethylmorpholine-HCl (pH 8.6) and 1 M NaCl in the same buffer.

Fractions of 12.7 mL were collected. Fractions containing arogenate (fractions 178–240) were combined and diluted 1:10 with H_2O, and the solution was adjusted to pH 10.5 with 10 N NaOH. This solution was applied to a column of AG1-X8 (200–400 mesh, 5 × 23 cm Cl^-, Bio-Rad) at a rate of 19 mL per min. The column was subsequently washed at 3.9 mL per min with 1.5 L of 0.1% (v/v) *N*-ethylmorpholine-HCl (pH 8.1), and a linear gradient was prepared from 2.2 L each of 0.1% *N*-ethylmorpholine-HCl (pH 8.1) and 1.0 M NaCl in the same buffer. Fractions of 14.3 mL were collected.

Fractions containing arogenate (141–165) were combined, 32 mL of 1.5 M $BaCl_2$ was added, and the resulting suspension was centrifuged. To the supernatant was added 44 mL of 0.5 M Na_2CO_3. The resulting suspension was centrifuged, and the supernatant was filtered through a 1.2-μm Millipore filter (Ga-3, Gelman). The filtrate was diluted to 3.4 L with H_2O and applied to a column of Dowex AG1-X8 as above. The column was subsequently washed at 3.9 mL per min with 1 L of 0.5% (v/v) 2-amino-2-methyl-1-propanol-HCl (pH 11.0), and a gradient was prepared from 2.2 L each of that buffer and 1.0 M NaCl dissolved in that buffer. The absorbance of the effluent at 240 nm was monitored with a Gilson spectrophotometer and 14.3-mL fractions were collected. Fractions (108–130) containing arogenate

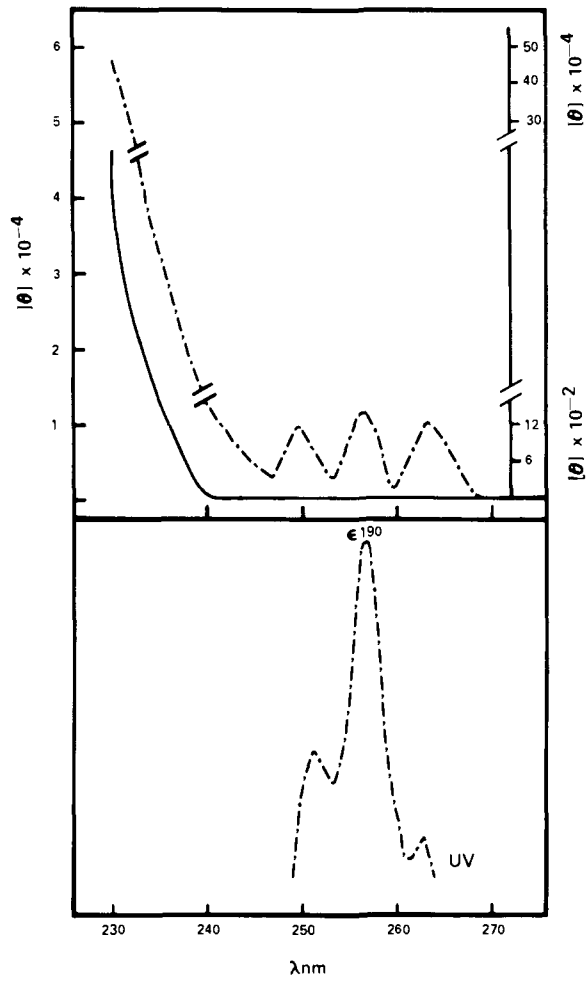


Figure 5. (Top) Circular dichroism of disodium arogenate (—). Acid treatment of the sample (pH 1.5) yielded phenylalanine. The circular dichroism of the resulting phenylalanine (---) shows it to be of the *S* configuration at the C_8 chiral carbon. (Bottom) UV profile of the phenylalanine product arising from the same acid-treated arogenate sample (---)

and comprising a symmetrical and smooth peak of A_{240} were combined and lyophilized.

The residue was suspended in 30 mL of H_2O, filtered through a 0.45-μm filter (Nuclepore), and chromatographed in 5-mL aliquots over Sephadex G-10 (Pharmacia, 2.5 × 67 cm). The column was equilibrated with 0.1 N NaOH. The A_{240} of the effluent was monitored as above, and 5.3-mL fractions were collected. Fractions (33-36) comprising the center of the arogenate peak were combined and lyophilized, and the sodium salt of arogenate was stored at 4 °C. Under these conditions, disodium arogenate is stable indefinitely. The purification is summarized in Table I.

Preparation of Ammonium Arogenate. All operations were performed in closed vessels. Forty milligrams of sodium arogenate was dissolved in 5 mL of H_2O. The solution was applied to a column of Dowex AG1-X4 (50-100 mesh, Cl^-, 1.3 × 2 cm Bio-Rad). The column was subsequently washed with 10 mL of H_2O, and arogenate was eluted with 10 mL of 1 M $(NH_4)_2CO_3$ in H_2O. The solution was lyophilized to give 22 mg of ammonium arogenate.

Preparation of Sodium Prephenate. The barium salt of prephenate was prepared as described by Metzenberg and Mitchell.[12] Seventy milligrams of barium prephenate (85% pure) was dissolved in 5 mL of 0.1 mM NaOH, chromatographed on Sephadex G-10 as above. The prephenate concentration was determined from the molar absorption of phenylpyruvate (in 1 N NaOH) formed by treatment of an aliquot of prephenate with 0.1 N HCl at 37 °C for 15 min.

Dansylation. To 100 μL of sample in a 6 × 50 mm tube were added 10 μL of 3.4 M $KHCO_3$ (pH 9.8) and 100 μL of 0.5% (w/v) dansyl-chloride (Pierce Chemical Co.) in acetone. Tubes were sealed with Parafilm, heated to 37 °C for 30 min, and cooled on ice.

Thin-Layer Chromatography. A 100-μL sample was dansylated as described above, mixed with 10 μL of perchloric acid, and chilled. A 1-μL aliquot was spotted near one corner of a polyamide TLC plate (5 × 5 cm, Schleicher and Schuell A1700). The plate was developed in the ascending direction in 3.4 M NH_4OH, dried in an air stream, exposed to the vapors of 88% formic acid for 15 min, and developed ascendingly in the second dimension in benzene-acetic acid-pyridine (50:5:1). The plate was observed under long-wave UV illumination.

Estimation of the Impurity in Arogenate. Dansylation of the arogenate samples consistently demonstrated the presence of a small amount of impurity. In order to quantify it, we used radioactive [G-^{3}H]dansyl-chloride (0.01 mCi of [G-^{3}H]dansyl-chloride/10.5 × 10^{-3} μM) to dansylate 10 μL of a 2.9 mM solution of arogenate in 85 μL of acetone. Unlabeled dansyl-chloride [3 μL of a 5% (w/v) dansyl-chloride in acetone] was also added to the arogenate sample. Five microliters of 3.4 M $KHCO_3$ was added and the tube was incubated at 37 °C for 30 min and then cooled on ice. Five microliters of $HClO_4$ was added to quench the reaction. The reaction mixture was spotted on a polyamide thin-layer chromatographic plate and developed in two directions as described previously. The fluorescence of dansyl-arogenate and dansyl impurity were readily detected. A 6-mm square of polyamide containing the dansyl impurity was counted in 15 mL of aquasol (New England Nuclear) in a scintillation counter. A second polyamide square containing dansyl-arogenate was likewise counted in 15 mL of aquasol. Background values were taken from a plate of the same size where no radioactivity was expected. The ratio of dansyl-arogenate to the dansyl impurity yielded an estimate of 3% for the impurity.

High-Performance Liquid Chromatography. To the solution of dansylated amino acid was added 200 μL of carbon tetrachloride. The phases were mixed and separated by centrifugation. To 100 μL of the aqueous phase was added 7 μL of 5.1 M $KClO_4$. The suspension was chilled on ice, and, after $KClO_4$ had settled, a 20-μL aliquot of the supernatant was injected onto a column of LiChrosorb RP-18 (3.2 × 250 mm), which was subsequently developed with a logarithmic gradient (exponent = 2) prepared from (A) 20 mM sodium phosphate (pH 6.0) and (B) 32% (v/v) 10 mM sodium acetate (pH 5.0) in acetonitrile. The initial volume ratio of A to B was 3:7, and the final composition was 100% B. The solvent velocity was 18.6 cm per min and the detector was a Gilson Filter-Glo fluorimeter (excitation filter 7-51x and emission filter 3-70M). Dansylated amino acids were identified by retention time and quantitated from peak height.

Acknowledgments. This investigation was supported by Public Health Service Research Grant GM-26880 (L.Z.) and by Biomedical Research Support Grant Funds (RR07149) from the National Institutes of Health. We gratetully acknowledge the help of Dr. Larry Verbit with the circular dichroism studies, Dr. Karl Wilson for amino acid analyses, Dr. Graham Byng for the prephenate preparations, and Ms. Elyse Jung for competent technical help.

References and Notes

(1) (a) Department of Chemistry; (b) Department of Biology; (c) Merck, Sharp and Dohme Research Laboratories.
(2) Stenmark, S. L.; Pierson, D. L.; Glover, G. I.; Jensen, R. A. *Nature (London)* **1974,** *247,* 290-292.
(3) Jensen, R. A.; Pierson, D. L. *Nature (London)* **1975,** *254,* 667-671.
(4) Fazel, A.M.; Jensen, R. A. *J. Bacteriol.* **1979,** *138,* 805-815.
(5) Fazel, A. M.; Jensen, R. A. *J. Bacteriol.* **1979,** *140,* 580-587.
(6) A tyrosine auxotroph isolated from wild-type *Brevibacterium flavum* has been shown to lack arogenate dehydrogenase and to accumulate arogenate behind the genetic block: Fazel, A. M.; Bowen, J.; Jensen, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1980,** *77,* 1270-1273.
(7) Bode, R.; Birnbaum, D. *Biochem. Physiol. Pflanzen.* **1978,** *173,* 44-49.
(8) Patel, N.; Pierson, D. L.; Jensen, R. A. *J. Biol. Chem.* **1977,** *252,* 5839-5846.
(9) Patel, N.; Stenmark-Cox, S.; Jensen, R. A. *J. Biol. Chem.* **1978,** *253,* 2972-2978.
(10) In a collaborative study with American Type Culture Collection, over 100 species representing all subgroup taxa of the *Pseudomonas* genus resemble *P. aeruginosa*[8,9] in the presence of the dual pathways to L-tyrosine biosynthesis (Byng, G. S.; Whitaker, R.; Jensen, R. A., submitted for publication).
(11) Rubin, J. L.; Jensen, R. A. *Plant Physiol.* **1979,** *64,* 727-734.
(12) Metzenberg, R. L.; Mitchell, H. K. *Arch. Biochem. Biophys.* **1956,** *64,* 51-56.
(13) The previously named pretyrosine dehydratase,[8,9] arogenate dehydratase, has been identified in *P. aeruginosa*,[8,9] in dozens of pseudomonad species (Byng, G. S., Whitaker, R., Jensen, R. A., submitted for publication), and in a species of *Klebsiella pneumoniae* (Jensen, R. A., in preparation).
(14) The Greek roots of aro-genate—gen, giving rise to; aro, aromatic compound—imply the potential of arogenate to serve as a precursor of phenylalanine, tyrosine, and perhaps other aromatic derivatives.
(15) Westergaard, M.; Mitchell, H. K. *Am. J. Bot.* **1947,** *34,* 573-577.
(16) Jensen, R. A.; Zamir, L. O.; St. Pierre, M.; Patel, N.; Pierson, D. L. *J. Bacteriol.* **1977,** *132,* 896-903.
(17) We expect to obtain better starting accumulations in culture supernatants through the use of a tyrosine auxotroph of *B. Flavum* (see ref 6).
(18) (a) McCamon, M. W.; Robins. E. J. *Lab. Clin. Med.* **1962,** *59.* 885-888. (b) Shapiro, C.; Jensen, R. A.; Wilson, K. A.; Bowen, J., submitted for publication.
(19) Detailed descriptions of methodological procedures now in use are in preparation for publication (Zamir, L. O.; Bowen, J.; Brown, M.; Jensen, R. A.).
(20) Danishefsky, S.; Hirama, M. *J. Am. Chem. Soc.* **1977,** *99,* 7741-7743.
(21) (a) Beckey, H. D. In "Biochemical Applications of Mass Spectrometry"; Walter, G. R., Ed.; Wiley-Interscience: New York, 1972; Chapter 30. (b) Schulten, H. R.; Rollgen, F. W. *Org. Mass. Spectrom.* **1972,** *10,* 649-659.
(22) Schulten, H. R.; Beckey, H. D. *Org. Mass. Spectrom.* **1972,** *6,* 885-895.
(23) Large, R.; Knof, H. *J. Chem. Soc., Chem. Commun.* **1974,** 935-936.
(24) Schulten, H. R.; Beckey, H. D. *Adv. Mass. Spectrum.* **1974,** *6,* 499-507.
(25) Games, D. E.; Games, M. P.; Jackson, A. H.; Olavssen, A. H.; Rossiter, M.; Winterburn, P. J. *Tetrahedron Lett.* **1975,** 2377-2380.
(26) Macfarlane, R. D.; Torgerson, D. F. *Science* **1975,** *191,* 920-925.
(27) Beuhler, R. J.; Flanigan, E.; Greene, L. J.; Friedman, L. *Biochem. Biophys. Res. Commun.* **1972,** *46,* 1082-1088.
(28) Hunt, D. F.; Schabanowtlz, J.; Botz, F. V.; Brent, D. A. *Anal. Chem.* **1977,** *49,* 1160-1163.
(29) Soltmann, B.; Sweeley, C. C.; Holland, J. F. *Anal. Chem.* **1977,** *49,* 1164-1166.
(30) Frick, W.; Barofsky, E.; Daves, G. D.; Barofsky, D. F.; Chang, D.; Folkers, K. *J. Am. Chem. Soc.* **1978,** *100,* 6221-6225.
(31) Legrand, M.; Viennet, R. *Bull. Soc. Chim. Fr.* **1965,** 679-681.
(32) Verbit, L.; Heffron, P. J. *Tetrahedron Lett.* **1967,** *23,* 3865-3873.
(33) Danishefsky, S.; Hirama, M.; Fritsch, N.; Clardy, J. *J. Am. Chem. Soc.* **1979,** *101,* 7013-7018.
(34) Benson, J. R. 1972, Durrum Resin Report No. 4.