Tetrahedron Letters,Vol.24,No.28,pp 2815-2818,1983 0040-4039/83 $3.00 + .00
Printed in Great Britain

DIFFERENTIAL ACID-CATALYZED AROMATIZATION OF PREPHENATE, AROGENATE, AND SPIRO-AROGENATE

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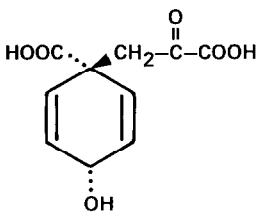
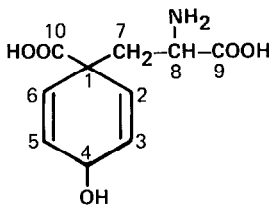
Summary: The family of closely related 2,5-cyclohexadiene, 4-hydroxy-carboxylic acid molecules are differentially labile to aromatization at mildly acidic pH in the order: prephenate > L-arogenate > spiro-L-arogenate.

For many years[1] prephenate (1) was assumed to be the universal molecule utilized by microbes and higher plants as the last non-aromatic precursor of L-phenylalanine and L-tyrosine. The initial exception was upturned in species of cyanobacteria[2] wherein L-arogenate (2) was shown to be the last non-aromatic precursor of L-tyrosine, and this was soon followed by a series of reports of other exceptions. The considerable biochemical diversity of aromatic biosynthesis subsequently described in the contemporary literature has been reviewed recently, and it seems clear that L-arogenate (see ref. 4 for proof-of-structure and characterization) is commonly employed in nature as a precursor of L-tyrosine and/or L-phenylalanine. Recently, a novel spiro-γ-lactam derivative (3) of L-arogenate (denoted spiro-arogenate) was isolated as a natural product from Neurospora crassa[5] and was chemically characterized[6]. The significance of spiro-arogenate in vivo has not yet been established. Each of these compounds, sharing in common a 2,5-cyclohexadiene, 4-hydroxy-carboxylic acid structure, is aromatized at acid pH. The stereochemical configurations of 2 and 3 have not been proven but undoubtedly are like 1.



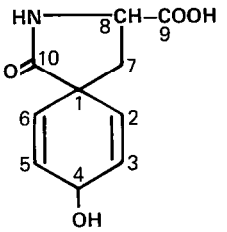
Prephenate

1



8-[*S*]-Arogenate[a]

2



8-[*S*]-Spiro-Arogenate

3

Aromatization of compounds 1, 2 and 3, yields phenylpyruvate, L-phenylalanine and L-phenylalanine, respectively. We suspect that the hydrate of spiro-arogenate is a transient intermediate between 2 and 3. If so, then it is likely that spiro-arogenate hydrate may be a fleeting intermediate in the aromatizations of both 2 and 3 to form L-phenylalanine. Since the early description of 1 (initially named prephenylalanine) by Davis[7] and Katagiri and Sato[8], only qualitative descriptions of the acid-catalyzed aromatization of 1 have been offered. The most quantitative information[9] indicated half-life values at 25°C of 130 hr at pH 7.0, 13 hr at pH 6.0 and 1.0 min in 1 N HCl. Quantitative data have not been published to define the acid labilities of 2 and 3 before now. The differential proneness of these three natural products to non-enzymatic conversion to aromatic compounds are important in analytical methodologies to detect them, to separate them in mixtures, and to devise enzyme assays. The order-of-magnitude differences in stability found for structures 1, 2 and 3 may prove to be significant for interpretations of biological strategies employed by different organisms during evolution of aromatic biosynthesis[10]. In this report a quantitative comparison of the relative labilities of cyclohexadiene molecules 1, 2 and 3 to acid-catalyzed aromatization is given.

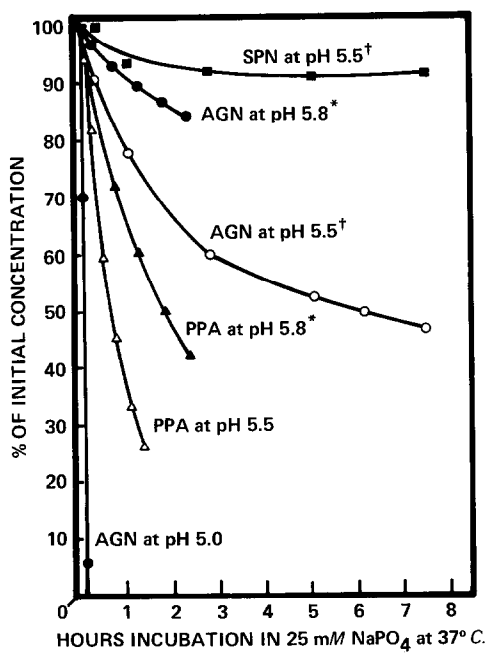
The aromatization rates of 1, 2 and 3 were followed in solution within the pH range of 5.0-6.0. In order to provide an ideal internal control for this type of experiment, mixtures of 1 and 2 or 2 and 3 were monitored for degradation. With the mixture of 1 and 2, a 1-ml solution of 0.26 mM sodium arogenate and 0.38 mM sodium spiro-arogenate was prepared from fresh stock solutions buffered with 25 mM sodium phosphate, pH 7.6. In similar fashion, the mixture prepared of 2 and 3 contained 0.55 mM sodium arogenate and 0.34 mM sodium prephenate. At ice temperature the mixture of 1 and 2 was adjusted to pH 5.8 by slow addition of 1 N HCl. The mixture of 2 and 3 was adjusted to pH 5.5 in the same manner. In separate experiments 1-ml mixtures were incubated in a water bath at 37°C, and 50-µl samples were taken periodically for HPLC analysis.

In the mixture of 2 and 3, L-arogenate, spiro-L-arogenate, and L-phenylalanine solutes were separated on an Altex Ultrasphere ODS HPLC column (5 µ, 4.5 x 150 mm). Retention times were about 1.1 min (L-arogenate), 3.1 min (spiro-L-arogenate), and 8.6 min (L-phenylalanine). The mobile phase was 25 mM sodium phosphate, pH 7.2. In the mixture of 1 and 2, a Whatman Partisil 1025 SAX anion exchange column (4.6 x 250 mm) was operated in series with the afore-

mentioned ODS column to separate solutes eluting with the following order of retention times: L-arogenate (∿6.0 min), prephenate (∿8.0 min), L-phenylalanine (∿11.4 min), and phenylpyruvate (∿18.1 min). The mobile phase was 20 mM sodium phosphate, pH 7.3. In these HPLC separations an Altex Model 110A pump was used to maintain flow rates of 1.0 ml per min., and solutes were detected by their UV absorbance at 215 nm using a Gilson Model HM UV-VIS spectrophotometer.

The initial concentrations of 1, 2 and 3 were determined by acidifying an aliquot of each stock solution and quantitatively converting 1 to phenylpyruvate, 2 to L-phenylalanine, and 3 to L-phenylalanine. Absorbance of phenylpyruvate was read at 320 nm in 2.5 M NaOH (ε=17,500). L-Phenylalanine concentrations were determined by HPLC using the ODS column. Peak profiles of L-phenylalanine obtained from samples were compared to a standard curve prepared from authentic L-phenylalanine (Sigma Chemical Co.).

Progressive decreases in the relative peak heights of the cyclohexadienyl compounds during incubation was a valid measurement of the relative labilities of the solutes because peak height was proportional to concentration at a given retention time. Because peak height reproducibility for replicate samples was reasonably good (within 10%), changes in absolute peak heights as a function of time could be used to generate degradation curves suitable for estimation of half-life times as shown below.



* = AGN + PPA Contained in the Same Solution
† = AGN + SPN Contained in the Same Solution
PPA: Prephenate
AGN: *L*—Arogenate
SPN: Spiro—Arogenate

The results show that order-of-magnitude lability differences at mildly acidic pH distinguish between 1, 2 and 3 in a range which could prove to be biologically significant. At pH 5.5 and 37°C $t_{\frac{1}{2}}$ values obtained were 40 min (1), 4-6 hrs (2), and > 24 hrs (3). At pH 5.8 and 37°C $t_{\frac{1}{2}}$ values were 2 hrs (1), 10-20 hrs (2), and days (3). The more labile compound in mixture 1 and 2 or 2 and 3 was added to a concentration lower than that of the second solute, a precaution ensuring that any deviation from first-order kinetics of aromatization would not produce an apparent order of labilities that was opposite of the true order. Semi-log plots of the data showed generally good adherance to pseudo first-order kinetics. The $t_{\frac{1}{2}}$ ranges given for 2 reflect deviations that most likely derive from the limited buffering capacity of phosphate at pH 5.5 - 5.8. In these cases the lower limit of the $t_{\frac{1}{2}}$ ranges given were obtained by extrapolation from the pseudo first-order rates that were maintained for about 2.5 hrs. Other experiments in which the three compounds were incubated separately at various pH values (e.g., see data for PPA at pH 5.5 and AGN at pH 5.0) indicated that a general proportionality of the relative half-life values shown above were maintained through an extended pH range.

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Acknowledgments These studies were supported by grants from the National Institutes of Health (L.O.Z) and from the Department of Energy (R.A.J.)

(Received in USA 22 February 1983)