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Evidence for the Generation of α-Carboxy-α-hydroxy-*p*-xylylene from *p*-(Bromomethyl)mandelate by Mandelate Racemase

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Mandelate racemase [EC 5.1.2.2] from *Pseudomonas putida* catalyzes the interconversion of (*R*)- and (*S*)-mandelates and requires only a divalent cation (e.g., Mg^{2+}) for this reaction that presumably proceeds via a carbanionic intermediate.[1] Concern over the intermediacy of such a high-energy carbanion[2] and our recent interest in the generation of quino dimethane derivatives[3] have prompted us to synthesize *p*-(bromomethyl)mandelate (**1**) and evaluate its reaction with mandelate racemase. We report here that reaction of **1** with the racemase uniquely affords *p*-methylbenzoylformate (**4**) and bromide elimination. These findings, consonant with our previous work on a related system,[3] provide a strong argument for the intermediacy of the carbanion **2** and of the unusual *p*-xylylene derivative **3** (Scheme I).

p-(Bromomethyl)mandelate[4] (**1**) is subject to a facile buffer-dependent solvolysis to *p*-(hydroxymethyl)mandelate (**5**). At pH 6.0 (0.1 M MES buffer, 1 mM $MgCl_2$) the $t_{1/2}$ is approximately 65 min, and UV analysis (Figure 1a) reveals the smooth first-order decay of **1** (1 mM; λ_{max} 235 nm) and the formation of **5** (λ_{max} 222 nm) with a sharp isosbestic point at 224 nm.[5] Addition of mandelate racemase (2.6 units)[6] to an identical mixture afforded a more complex spectral change (Figure 1b), the most striking feature being the formation of a new product (λ_{max} 264 nm) in

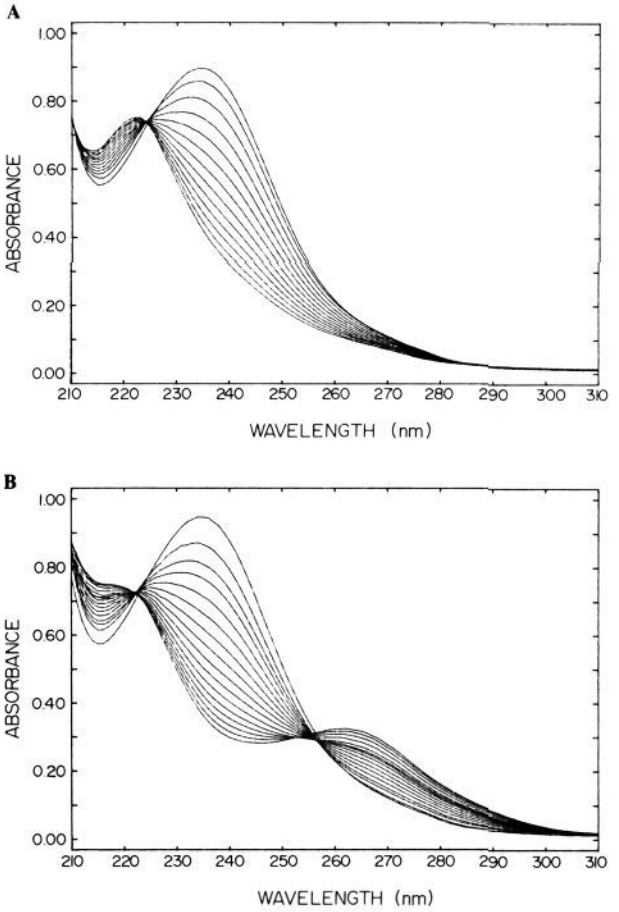
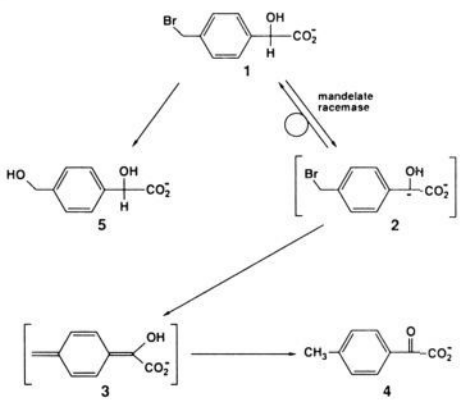


Figure 1. UV spectra (1-mm pathlength) of the chemical and enzymatic reactions of *p*-(bromomethyl)mandelate (**1**; 1 mM; λ_{max} 235 nm) in 0.1 M MES (pH 6.0) and 1 mM $MgCl_2$: (a) solvolysis (5-min intervals) and (b) reaction with mandelate racemase (2.6 units, 4-min intervals).

Scheme I



(5) The identification of **5** was established by ^1H NMR and HPLC comparison with an authentic sample.[4] The conversion of **1** to **5** was also monitored by ^1H NMR (D_2O). No exchange of the methine proton was detected.

(6) Mandelate racemase (510 units/mg) was isolated according to Hegeman (Hegeman, G. D. *Methods Enzymol.* **1970**, *17A*, 670). Activity was measured by circular dichroism: Sharp., T. R.; Hegeman, G. D.; Kenyon, G. L. *Anal. Biochem.* **1979**, *94*, 329.

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(4) Ethyl-*p*-(bromomethyl)benzoylformate (1 g) (Barnish, I. T.; Cross, P. E.; Danielewicz, J. C.; Dickinson, R. P.; Stopher, D. A. *J. Med. Chem.* **1981**, *24*, 399) was treated with sodium borohydride (3 equiv) in ethanol (90 mL total volume) under N_2 for 90 min at 0 °C. Workup afforded ethyl *p*-(bromomethyl)mandelate (0.85 g, 84% yield): ^1H NMR ($CDCl_3$) δ 7.5 (4 H, s), 5.3 (1 H, s), 4.8 (2 H, s), 4.4 (2 H, q), 1.6 (3 H, t). Hydrolysis in refluxing 10% HBr gave **1** as light orange solid (100% yield); mp 140–42 °C; UV (H_2O) λ_{max} = 235 nm, ϵ = 7050 M^{-1} cm^{-1}; ^1H NMR (acetone-d_6) δ 7.5 (4 H, q), 5.3 (1 H, s), 4.7 (2 H, s); EI mass spectrum *m/e* (rel intensity) 246, 244 (M^+, 1.39, 0.92), 201, 199 (M^+ – COOH, 22.61, 23.02), 165 (M^+ – Br, 14.8) 120 (M^+ – Br – COOH, 30.77). A satisfactory microanalysis was obtained (±0.4 of calculated values). Stock solutions of **1** were freshly prepared in ethanol and were quite stable at 0 °C.

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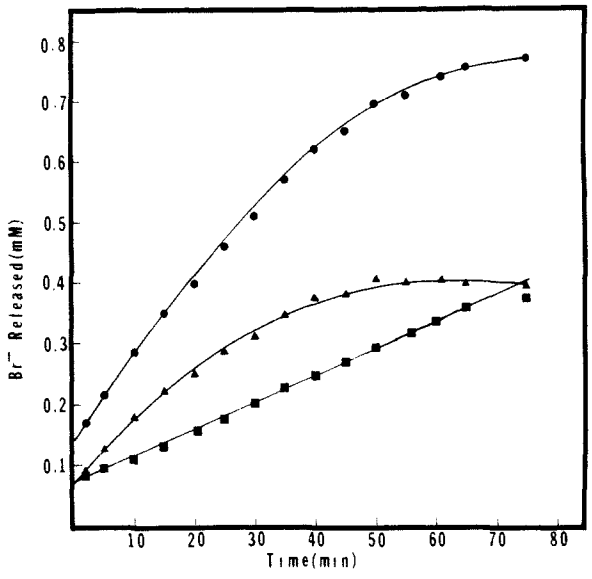

Figure 2. Analysis of bromide ion release from *p*-(bromomethyl)-mandelate (**1**; 1 mM) in 0.1 M MES (pH 6.0) by mandelate racemase:[8] no racemase (■); racemase added (255 units; ●); difference curve (▲).

addition to **5**. The new product was determined to be *p*-methylbenzoylformate (**4**) based on λ_{max}, HPLC analysis (comparison of retention time with that of authentic **4**[4]), and its reaction with benzoylformate decarboxylase [EC 4.1.1.7].[3,7]

(7) Our preliminary results suggest that k_{cat} for the formation of **4** from **1** is less than 1% of the estimated k_{cat} for racemization of **1**.

Bromide elimination was confirmed by analysis with a bromide ion electrode (Figure 2).[8] In the absence of enzyme, the release of bromide ion (closed squares) parallels the solvolysis of **1**; however, addition of the racemase results in an acceleration of bromide ion formation (closed circles). The difference curve (closed triangles) is consistent with the enzyme-catalyzed elimination of bromide to afford the *p*-xylylene derivative **3** which undergoes tautomerization to **4** (Scheme I).

The detection and chemical properties of xylylene derivatives have received considerable attention in recent years.[9] Their instability has largely precluded isolation. Our previous work with benzoylformate decarboxylase provided direct evidence for a thiamine-stabilized *p*-xylylene derivative generated from *p*-(halomethyl)benzoylformates.[3] The results reported here cleanly demonstrate a similar phenomenon for an enzyme which does not proceed through a covalent substrate–cofactor intermediate. The unique conversion of **1** to **4** by mandelate racemase provides compelling evidence for a carbanionic mechanism for this enzyme. Moreover, the indisputable formation of **3** as an obligatory intermediate suggests that enzyme-catalyzed halide elimination through an intervening aromatic ring may be a general method for the formation and analysis of a variety of unstable xylylene derivatives.

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(8) Bromide ion release was measured as described previously.[3]

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Computer Software Reviews

TrueSTAT. Version 1.0. TrueBASIC, Inc.: 39 South Main St., Hanover, NH. List price: $49.95. Educational discounted price: $19.95 (with a total of $500 purchase by institution).

TrueSTAT is an interactive, statistical simulation and data analysis system designed primarily as an educational tool. It incorporates a menu driven mode, a command mode, and a programming mode which allow several modes of graphical output (XY plots, Histograms, Box plots, Residual plots), the ability to generate test data from several types of distributions (normal, binomial, poisson, etc.), and some simple statistical routines.

Features: Versions of this program are available for the IBM-PC, XT, or AT, Macintosh, and Amiga. A version is in development for the Atari ST. The copy tested was for the IBM machines, and some of the following comments are specific to that version. The stated requirements for running this program include 256 Kb of RAM (the main program occupies 165 Kb), a graphics adapter, and DOS 2.0 or higher. This package is not copy protected and can be backed up with standard DOS commands.

The program was examined on a Heath-Zenith Data System Model 248 with an Enhanced Graphics Adapter and an IBM-XT with a Color Graphics Adapter. The latter was also equipped with a math coprocessor. No problems were encountered in operating this program in either of these environments. This included concurrent running of a memory resident program. The program automatically checks for the presence of the math coprocessor and utilizes it if available.

Overall, the program is easy to use and easy to learn. The manual is well-written and organized. A modest help facility is provided which provides information on commands and syntax. It fulfills nicely the stated goal of an instructional aid for anyone concerned with fundamental statistical analysis (i.e., courses in quantitative analysis, chemometrics).

The program features three methods of command entry: menu driven, direct command, and a programming language. With respect to the latter, a line editor is provided for generating code. While this is functional for short programs, it is more convenient to use a full blown text editor/word processor if long procedures are to be written. In the menu mode, selections are made with the soft function keys. One awkward feature is that the various menu screens are not labeled and in one case the same command performs different functions within different screens ("PRINT" command). Data can be entered directly by the modes discussed above or by creating a suitable ASCII file. The latter is particularly useful for large data sets. While data can be saved to disk there are two annoying aspects. Contrary to stated claims files can only be saved on the default drive. In addition significant figures are lost in numbers with more than 6 significant figures when they are saved and then retrieved from disk. The only way to preserve these figures is to create data files external to the program.

In addition to providing many fundamental statistical computations (i.e., mean, standard deviation, t-tests, confidence intervals, and linear regression) this program includes several interesting features particularly useful to an educator. Sets of random numbers can be generated from normal, binomial, Poisson, exponential, and uniform distributions. One can also generate random y values about a straight line for a corresponding set of x values. Datasets can be manipulated by arithmetic functions and operations (+, −, ×, ÷, square root, log, etc.).

One of the attractive features of this package is its plotting routines. These routines generate XY plots of datasets, histograms, a plot of the best fitting line for sets of ordered pairs, box plots, residual plots, confidence intervals, and a fit of a normal distribution to a dataset. It is a simple matter to simultaneously display several plots even of different type.

Overall, this program is a bargain at its modest price. The documentation is clear and concise and the program is easy to use. Despite some minor flaws, it would be a useful addition to the software collection of any educator or scientist concerned with the quantitative manipulation of data.

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