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New substrates for β-methylaspartase

The enzyme β-methylaspartase (3-methyl-L-aspartic acid ammonia-lyase, EC 4.3.1.2) was shown by BARKER *et al.*[1] to utilize as substrates not only *threo-* and *erythro*-β-methyl-L-aspartate, but also L-aspartate. Of these three, the first was deaminated at the fastest rate. BARKER *et al.*[2] reported that β-ethylaspartate was also a substrate for the enzyme, with a deamination rate approx. 27% that of the methyl homolog. Higher homologs have not previously been synthesized or tested in the enzyme system. The question arose as to how limiting a parameter was constituted by the size of the β-alkyl group, and it appeared that useful information about the size of the enzyme active site might result from a study of higher homologs.

Accordingly, the following β-alkyl-DL-aspartic acids were synthesized and examined as substrates for β-methylaspartase: β-ethyl-, β-propyl-, β-isopropyl-, and β-butylaspartic acid. The last three of these are new compounds. Although the enzyme is specific for the L-configuration, no inhibition is displayed by the D isomer, and therefore the racemic mixtures were not resolved. Substrate concentrations were based on 50% L isomer. The *erythro* or *threo* configuration of these homologs is not known, but it is most probably *threo*. The known methods for synthesizing the β-alkylaspartic acids all yield over 90% *threo* isomer of β-methylaspartate. The reaction catalyzed by the enzyme is:

$$\underset{\text{(L- configuration)}}{{}^{-}OOC\text{–}CHR\text{–}CHNH_3^{+}\text{–}COO^{-}} \rightleftarrows \underset{\textit{(trans)}}{{}^{-}OOC\text{–}RC{=}CH\text{–}COO^{-}} + NH_4^{+}$$

The assay system was that of WILLIAMS AND SELBIN[3]. The alkylaspartates were judged to be substrates on the basis of the rate at which they were deaminated to the corresponding olefinic acids. The formation of product was followed in the Beckman spectrophotometer, Model DB, at 240 mμ for no longer than 10 min at several different enzyme concentrations. The lower limit of sensitivity was of the order of 10^{-9} mole·ml^{-1}·min^{-1}.

The β-alkylaspartic acids were synthesized by the method of GREENSTEIN AND WINITZ[4]. Homologous α-bromoesters were purchased from Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y. Ion-exchange resins were obtained from Bio-Rad Laboratories, Richmond, Calif. Carbon/hydrogen analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Nitrogen was based on the quantitative ninhydrin method of MOORE AND STEIN[5]. All melting points were determined on a Fisher–Johns melting point apparatus. A reference sample of ethylfumaric acid was kindly supplied by Dr. W. R. VAUGHAN.

The method of synthesis consists generally of condensing together equimolar amounts of the appropriate α-bromoester and ethyl acetamidocyanoacetate, dissolved in absolute ethanol containing an equivalent amount of sodium ethoxide. The condensation product is hydrolyzed in 6 M HCl, and the HCl removed under vacuum. The amino acid is separated from other components of the solution by column chromatography on Dowex 1(acetate). The full experimental procedure has been published in detail[4].

*β-Ethyl-*DL*-aspartic acid.* From 0.5 mole each of ethyl α-bromobutyrate, ethyl

acetamidocyanoacetate, and sodium ethoxide, we obtained 35 g of slightly discolored crude product (49% yield). Recrystallization from ethanol–water yielded a white solid which melted at 235–237°. (Found: C, 43.22, 43.04; H, 6.73, 6.81; N, 8.96. $C_6H_{11}O_4N$ requires C, 44.72; H, 6.88; N, 8.69%.)

*β-Propyl-*DL*-aspartic acid.* From 0.12 mole each of ethyl α-bromovalerate, ethyl acetamidocyanoacetate, and sodium ethoxide, we obtained 548 mg of recrystallized white solid, melting at 224–227°. (Found: C, 47.73, 47.82; H, 7.46, 7.39; N, 8.36. $C_7H_{13}O_4N$ requires C, 47.99; H, 7.48; N, 8.00%.)

*β-Isopropyl-*DL*-aspartic acid.* From 0.2 mole each of ethyl α-bromo-β-methylbutyrate, ethyl acetamidocyanoacetate, and sodium ethoxide, we obtained 355 mg of recrystallized white solid, melting at 235–237°. (Found: C, 47.76, 47.78; H, 7.45, 7.31; N, 8.70. $C_7H_{13}O_4N$ requires C, 47.99; H, 7.48; N, 8.00%.)

*β-Butyl-*DL*-aspartic acid.* From 0.2 mole each of ethyl α-bromohexanoate, ethyl acetamidocyanoacetate, and sodium ethoxide, we obtained 910 mg of recrystallized white solid, melting at 230–233°. (Found: C, 50.68, 50.54; H, 7.90, 8.08; N, 7.80. $C_8H_{15}O_4N$ requires C, 50.78; H, 7.99; N, 7.40%.)

R_F values. The β-alkylaspartic acids were chromatographed on Whatman No. 1 filter paper at room temperature in a solvent system consisting of *n*-butanol–acetic acid–water (4:1:1, v/v/v). The R_F values for the series were (by alkyl group): methyl, 0.238; ethyl, 0.308; propyl, 0.462; isopropyl, 0.418; and butyl, 0.550.

Reaction rates were determined for a series of substrate concentrations and Lineweaver–Burk plots were prepared, from which the constants v_{max} and K_m were calculated. Relative velocities and K_m values for the series are shown in Table I.

Interpretation of the K_m data is based on kinetic studies of the β-methylaspartase system with *threo*-β-methylaspartate as substrate[6]. In this case BRIGHT[6] has shown that K_m is the dissociation constant of the enzyme–substrate complex. If the enzyme mechanism is the same for the homologous substrates, K_m is a valid inverse index of the affinity of the substrate for the enzyme active sites. The relative v_{max} values should be a measure of the catalytic efficiency of the enzyme in the rate-determining step.

From these considerations it is clear on inspection of Table I that β-methylaspartate is by far the best substrate from the standpoint of both binding and catalysis, and that β-butylaspartate is not a substrate at all under the conditions of assay.

Omitting for the moment a consideration of aspartate, we see that, as the size of the β-alkyl substituent increases, the corresponding K_m increases and v_{max} decreases. The only exception to this regular progression is β-propylaspartate, which gives a slightly larger K_m and smaller v_{max} than the isopropyl isomer. If these differences are significant, the discrepancy could mean that the length of the propyl side chain interferes with binding, whereas the bulky isopropyl group decreases the rate of elimination.

The elimination of ammonia from β-methylaspartate has been shown to occur by a carbanion mechanism, the slow step of which involves formation of the double bond[6]. The alkyl substituents, therefore, might be expected to exert a certain influence on the developing double bond in the transition state, not unlike the influence of alkyl groups in the base-catalyzed elimination of HX from alkyl halides[7]. Although the mechanism in the latter case is concerted, the events occurring in the transition state are similar to those in the β-methylaspartase reaction. For the HX eliminations

TABLE I

APPARENT MICHAELIS CONSTANTS AND RELATIVE LIMITING VELOCITIES FOR HOMOLOGOUS L-ASPARTIC ACIDS

L-*Substrate*	K_m *(M)*	v_{max} *based on β-methyl-aspartate (%)*
Aspartate*	$2.3 \cdot 10^{-3}$	1.1
β-Methylaspartate *(threo)*	$6.5 \cdot 10^{-4}$	100
β-Ethylaspartate	$2.5 \cdot 10^{-3}$	33**
β-*n*-Propylaspartate	$1.9 \cdot 10^{-2}$	2.5
β-Isopropylaspartate	$0.9 \cdot 10^{-2}$	0.4
β-*n*-Butylaspartate	—	0

* Based on data of BARKER[1].

** Data for ethylaspartate and higher members are based on $\varepsilon_{240\ m\mu}$ for ethylfumarate equal to $6.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at pH 7.0 and 25°, as determined by us.

cited, the rate constants for the substituted ethyl bromides decreased in the following order: methyl → ethyl → propyl → H. This is the same order as the v_{max} values shown in Table I.

A second factor to consider is the increasing steric hindrance in the elimination reaction, resulting from increasing size of the alkyl group. However, the abrupt decrease in v_{max} resulting from changing the alkyl group from ethyl to propyl cannot be explained adequately by electromeric effects or internal steric hindrance of elimination alone. Probably further steric interactions with the enzyme surface begin to occur at this point, and the rate falls off accordingly.

Studies on the active sulfhydryl group of β-methylaspartase have indicated that the active site occurs within a crevice or fold in the enzyme surface[8]. The results reported here suggest that this region may have the topology of an oblate spheroid and a size somewhere between that of β-isopropylaspartate and β-butylaspartate.

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