Enzymic Synthesis of 3-Halogenoaspartic Acids using β-Methylaspartase: Inhibition by 3-Bromoaspartic acid

Mahmoud Akhtar, Mark A. Cohen, and David Gani*

The Department of Chemistry, The University, Southampton SO9 5NH, U.K.

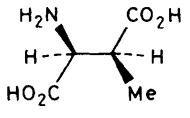
In the presence of ammonia, β-methylaspartase catalyses the conversion of halogenofumaric acids into the corresponding β-halogenoaspartic acids; one of the products, bromoaspartic acid, is an irreversible inhibitor.

Amino acids substituted at C-3 with potential leaving groups have attracted much interest recently owing to their ability to irreversibly inactivate pyridoxal 5′-phosphate-dependent enzymes.[1—3] We decided to investigate the possibility of using enzymes to synthesize these compounds.

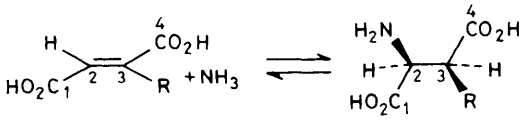
β-Methylaspartase (L-*threo*-3-methylaspartate ammonia-lyase EC 4.3.1.2) from *Clostridium tetanomorphum* catalyses the reversible α,β-elimination of ammonia from (2*S*,3*S*)-3-methylaspartic[4] acid (**1**) which is formed from L-glutamate *in vivo* through the action of coenzyme B_{12}-dependent glutamate mutase. It has been shown previously that β-methylaspartase also catalyses the deamination of L-aspartic acid and some 3-alkylaspartic acids at low to moderate rates.[5] It was also known that in the reverse direction the addition of ammonia to the double bond of both mesaconic (**2**, R = Me) and fumaric acid (**2**, R = H) occurs stereospecifically *via* N-attack at the *si*-face of C-2, with protonation occurring from the *re*-face of C-3, Scheme 1.[6] We reasoned that a similar reaction should occur with halogenofumaric acids (**2**, R = F, Cl, Br, and I) provided that the C-3 substitution did not reduce the susceptibility of conjugate addition of ammonia at C-2 through a positive mesomeric effect, or prevent active-site binding through a steric effect.

Successful reactions would then yield new highly functionalized amino acids containing two chiral centres.

The potential substrates for the enzyme, fluoro-,[7] chloro-,[8] bromo-,[9] and iodo-fumaric[10] acid were prepared using litera-

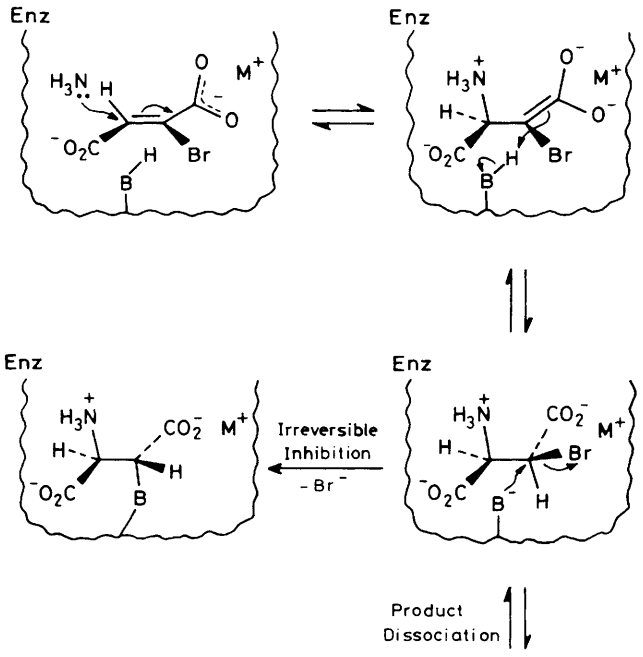


(**1**)



(**2**) (**3**)

Scheme 1



Scheme 2

ture procedures with minor modification. Analytical and spectral data were consistent with expected structures. In the first instance each of the halogenofumaric acids, as well as acetylenedicarboxylic acid, fumaric acid, and mesaconic acid were separately incubated with partially purified β-methylaspartase (20 units mg^{-1} protein) in the presence of an excess of ammonia and the necessary metal ions at pH 9. The optical density (o.d.) at 240 nm was monitored using dilution techniques.† Each of the incubations containing mesaconate, fumarate, chlorofumarate, or bromofumarate showed an enzyme-dependent decrease in o.d. The other substrates showed no decrease.

In order to identify the products of the reaction, each of the experiments was repeated on a larger scale in a 10 mm n.m.r. tube. Typically incubations contained 1—2 mmol of the diammonium salts of the substrate, K^+, Mg^{2+}, 50 μl MeOH (for a ^{13}C n.m.r. reference), 100—200 μl D_2O (for deuterium signal locking), and enzyme in a total volume of 2.2 ml protium oxide at pH 9. The reactions were started by the addition of enzyme and were followed using $\{^1H\}^{13}C$ n.m.r. spectroscopy. The decoupler power was optimally set to maximize Overhauser enhancements and to minimize sample heating and spectra were recorded for each sample at intervals over a period of two days. From these spectra it became apparent that iodofumarate and acetylenedicarboxylate did not react. Chlorofumarate was converted smoothly into chloroaspartate [δ 176 and 172 (2 × CO_2H), 62.4 (C-3), 56.5 (C-2)] and bromofumarate was converted into bromoaspartate [δ 176 and 172 (2 × CO_2H), 56.4 (C-2), 55 (C-3)].

During the bromofumarate conversion the enzyme became inactive and the aminated product rapidly cyclized to give an aziridine [δ 173.4 (2 × CO_2H), 36.3 (C-2 and C-3)]. When a second aliquot of enzyme was added to the incubation mixture (now containing aziridine) the fresh enzyme was not inactivated more rapidly suggesting that the aziridine is not a $k_{cat.}$ inhibitor. Also, pre-incubation of bromofumarate with the enzyme in the absence of ammonia did not lead to irreversible inactivation. The inactivation of the enzyme by both 3-bromoaspartate (formed *in situ*) and bromoacetate was substantially reduced in the presence of alternative substrates. This results suggests that alkylation of a specific active-site base/nucleophile occurs during the inactivation process. A possible mechanism is outlined in Scheme 2.

The incubation containing fluorofumarate initially appeared to show no reaction. However, when the quantity of enzyme was increased 10 to 20 fold a small amount (*ca.* 5%) of fluoroaspartic acid could be detected (after prolonged incubation) by ^1H n.m.r. spectroscopy[11] [δ 5.17 (1H, dd, *J* 46.8 and 1.7 Hz, 3-H), 4.04 (1H, dd, *J* 32 and 1.7 Hz, 2-H)]. This sample contained many other products.

The initial rates of amination for chlorofumarate, bromofumarate, fumarate, and mesaconate at 0.5—1 M concentration are similar and about as fast as the deamination of (2*S*,3*S*)-3-methylaspartate, the best substrate. This observation is interesting because the rate of deamination of (2*S*)-aspartate is slow (*ca.* 1%) compared to (2*S*,3*S*)-3-methylaspartate. We have used the enzyme to prepare gramme quantities of L-aspartic acid [100% conversion, 90% isolated yield], 3-chloroaspartic acid {*ca.* 75% conversion, 60% isolated yield, m.p. 168—171 °C, $[\alpha]_D$ −38.8° (*c* 0.5, 0.1 M HCl)} and also on a smaller scale 2,3-aziridinedicarboxylic acid. On the basis of the known stereochemical course of the enzymic amination of fumaric and mesaconic acid we propose that each of the 3-halogenoaspartic acids described in this communication possesses (2*R*,3*S*)-stereochemistry, but this has not yet been confirmed.

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† The K_M for substituted fumaric acids is very high (*ca.* 10^{-1} M) thus activity cannot be measured directly spectrophotometrically.

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