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Kinetic Characterisation of Ene-Diol-Based Inhibitors of α-Amylase

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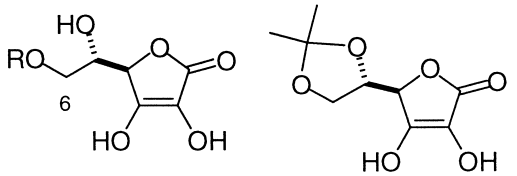
Abstract—A kinetic analysis of the inhibition of malt α-amylase by compounds based on ascorbic acid has shown the mode of inhibition to be competitive for the parent compound, but more complex for its derivatives. We have further simplified the ascorbic acid ene-diol pharmacophore by demonstrating that dihydroxyfumaric acid is also a good inhibitor of malt α-amylase. © 2000 Elsevier Science Ltd. All rights reserved.

α-Amylases play a key role in the breakdown of starch, and have attracted considerable attention in recent years, in the light of the wealth of structural information that has become available.[1] Inhibition of α-amylases has been reported to induce carbohydrate tolerance, satiety and weight loss, and to prolong gastric emptying.[2] α-Amylase inhibitors thus have potential as therapeutics for the treatment of obesity and non-insulin dependent diabetes mellitus.[3] Perhaps the best-characterised small molecule inhibitor of α-amylase is acarbose, a compound thought to be a transition state analogue of the natural substrate.[4] Acarbose has been prescribed for the treatment of diabetes,[5] but side effects of this compound have piqued medical interest in the development of alternative inhibitors. We have previously reported[6] a novel class of inhibitors of α-amylase, based on ascorbic acid derivatives, following an obscure report by Palla and Verrier.[7] A structure–activity relationship study revealed that the ene-diol moiety was the essential pharmacophore for inhibition.[6] In this paper we further simplify the pharmacophore and determine the mode of inhibition of the parent ascorbic acid-based inhibitors.

A kinetic analysis was carried out on selected ascorbic acid-based inhibitors of malt α-amylase to examine the mode of inhibition. This evaluation was carried out, as previously described, using the Megazyme assay.[6,8] In the first instance, IC_{50} measurements were made for ascorbic acid **1**, a simple derivative **2** and an elongated and more complex derivative **3**. The data for ascorbic acid are shown in Figure 1. The IC_{50} for ascorbic acid was determined to be 2.65×10^{-5} M. For compound **2**, the $IC_{50}=2.8\times10^{-5}$ M, and for **3**, $IC_{50}=6.25\times10^{-5}$ M. Inhibition is thus largely maintained upon substitution at the 6-hydroxy position.



A detailed kinetic analysis was undertaken on ascorbic acid in order to establish the mode of inhibition of this new and simple class of inhibitor.[9] Initial rates were measured at five different substrate concentrations, and the data analysed using Enzfitter (Biosoft, Cambridge, UK). The data fitted the Michaelis–Menten model at concentrations less than 10^{-4} M ($r^2=0.92$) and the pattern of inhibition was consistent with competitive inhibition. The K_I was calculated to be 4.34×10^{-5} M. Results were less definitive for compounds **2** and **3**, which fitted the Michaelis–Menten model only at very low inhibitor concentrations ($<4\times10^{-5}$ M).[10]

In an attempt to simplify the pharmacophore further, we tested a commercial sample (Aldrich®) of dihydroxyfumaric acid in our assay system. At 5 mM, this compound inhibited 92% of the α-amylase activity, a comparable figure to the 95% displayed by ascorbic acid.[6] This suggests that an intact ring structure is not a requirement for inhibition. Work is therefore underway to determine structure activity relationship studies on simple ene-diols based on dihydroxyfumaric acid.

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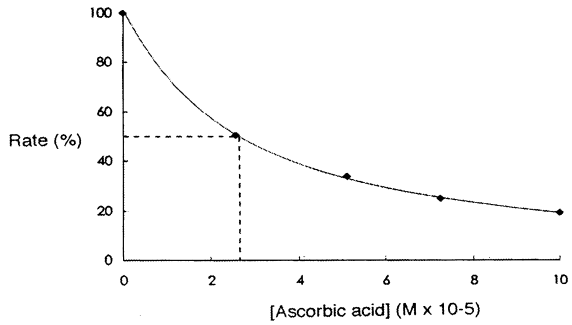

Figure 1. A typical IC_{50} graph of rate (%) versus inhibitor concentration ($M \times 10^{-5}$).

The binding site of α-amylase has been shown by X-ray crystallographic studies to comprise a cleft that can accommodate five or more sugar residues.[11] We suggest that ene-diol inhibitors bind in the active site cleft, at any one of the sugar binding subsites, explaining the competitive inhibition pattern. For more complex derivatives, the mode of inhibition is more complex, suggesting a dual functionality in which the inhibitor is bound to the active site cleft and a neighbouring site. Current work is exploring substitution of ascorbic acid with an acarbose-like substrate mimic, in an attempt to produce inhibitors that are more potent than either class of inhibitor alone.

Acknowledgements

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9. Assay method: α-Amylase was Type VIII-A Barley Malt (2.1 U/mg solid), obtained from Sigma The assay was used in accordance with the manufacturer's instructions, varying the substrate concentration as required. Rigorous testing ensured the linearity of the assay in the presence of inhibitors over the timeframe of the incubation. Barley malt (2.3 mg) was dissolved in enzyme extract buffer (20 mL) and stored on ice until required. New solutions were prepared every 3 h. Standard conditions for initial screening of inhibitory activity were carried out with a 5 mM solution of inhibitor and the values are expressed as a % relative to a control without inhibitor. Each inhibitor tested was prepared as a 0.1 mM solution in deoxygenated water. The water was degassed by bubbling N_2 through for 5 min. Solutions were made just prior to use. Stringent controls ensured that the inhibitors were stable in the assay conditions. The concentrations of the inhibitor solutions were prepared such that the highest and lowest values would give absorbance readings of between 0.0 and 1.0 over all substrate concentrations at 410 nm. For each inhibitor, four solutions were prepared within the following concentration. Ascorbic acid $= 5.12 \times 10^{-4}$ -2.00×10^{-3} M; compound **2** $= 3.27 \times 10^{-4}$ -1.31×10^{-3} M. All measurements were made in triplicate.
10. Enzfitter returned ambiguous results for the mode of inhibition by **2**, suggesting that derivatisation at the 5 and 6 positions may interfere with pure competitive binding.
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