Inhibitors of angiotensin-converting enzyme containing a tetrahedral arsenic atom

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A series of tetrahedral oxo acids of Group VA and VIA elements and of silicon and boron were examined as inhibitors of angiotensin-converting enzyme. Arsenate is a competitive inhibitor with a K_i of 27 ± 1 mM, at least 10-fold more potent than phosphate. Dimethylarsinate is a competitive inhibitor with a K_i of 70 ± 9 mM, 2-fold more potent than dimethylphosphinate. Oxo acids of boron, silicon, antimony, sulphur and selenium are not inhibitors. On the basis of these results and the strong inhibition of this zinc metallopeptidase by substrate analogues containing a tetrahedral phosphorus atom, two substrate analogues containing a tetrahedral arsenic atom were prepared. 2-Arsonoacetyl-L-proline is a competitive inhibitor with a K_i of 18 ± 7 mM, more than 2000-fold weaker than that of its phosphorus analogue 2-phosphonoacetyl-L-proline. 4-Arsono-2-benzylbutanoic acid is a mixed inhibitor with a K_i of 0.5 ± 0.2 mM, indistinguishable in potency from its phosphorus analogue 2-benzyl-4-phosphonobutanoic acid.

Substrate analogues containing a tetrahedral phosphorus atom have been shown to be powerful inhibitors of angiotensin-converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (Galardy, 1980, 1982; Galardy *et al.*, 1983; Thorsett *et al.*, 1982; Petrillo & Ondetti, 1982). These compounds are all oxo acids of phosphorus or derivatives thereof and include phosphonic acids, phosphinic acids, phosphoramidates and phosphonamidates. In these inhibitors the tetrahedral phosphorus atom is thought to resemble a reaction intermediate containing a tetrahedrally hybridized carbon at the position of the trigonal planar carbonyl carbon of the scissile amide bond in the ground state. Therefore these tightly bound inhibitors are probably analogues of an intermediate resembling the transition state for amide hydrolysis.

The binding affinity of a transition-state analogue is proportional to its ability to duplicate the unknown interatomic distances and bond angles found in the actual transition state for the enzyme-

catalysed reaction (Wolfenden, 1972). The success of oxo acids containing a tetrahedral phosphorus atom in mimicking the transition state for amide hydrolysis does not exclude the possibility that tetrahedral oxo acids of other elements may equally or more accurately duplicate the geometry and interatomic distances of the transition state. Fig. 1 shows a schematic model of the active site of angiotensin-converting enzyme and the proposed binding of substrate and substrate analogues containing tetrahedral atoms in place of the trigonal planar carbonyl carbon atom.

Since phosphate itself is a weak inhibitor of angiotensin-converting enzyme (K_i approx. 300 mM; Galardy, 1982) and triphosphate a moderately potent competitive inhibitor (K_i 18 μ M; Galardy, 1982), simple tetrahedral oxo acids of six elements of Groups IIIA, IVA, VA and VIA were tested as inhibitors of this zinc metallopeptidase. The simple oxo acids borate, sulphate, and selenate had K_i values greater than 300 mM and thus were not inhibitors. Arsenate and dimethylarsinate were competitive inhibitors with K_i values of 27 ± 1 and 70 ± 8 mM respectively, more than 10-fold and 2-fold more potent than their corresponding phosphorus analogues. On the basis of these promising results, two substrate analogues containing a tetra-

Abbreviations used: IC_{50} , concentration of inhibitor giving 50% inhibition of the enzyme at a specified substrate concentration; m.s.(e.i.), electron-impact mass spectrometry; m.s.(c.i.), chemical-ionization mass spectrometry.

Fig. 1. Model for the active site of angiotensin-converting enzyme, adapted from Cushman *et al.* (1977)

Proposed binding modes for: (a) natural or synthetic substrates; (b) a tetrahedral reaction intermediate occurring during substrate hydrolysis; (c) tetrahedral oxo acids of the element X which might mimic the tetrahedral intermediate of (b), where X is phosphorus, arsenic, antimony, boron, silicon, sulphur or selenium.

hedral arsenic atom were synthesized and their inhibitory potency was compared with that of their respective phosphorus analogues.

Figs. 2(c) and 2(d) show the four substrate-analogue inhibitors, two containing tetrahedral phosphorus and two containing tetrahedral arsenic. 2-Arsonoacetyl-L-proline was bound over 2000-fold more weakly than was its phosphorus analogue 2-phosphonoacetyl-L-proline, both of which were designed to resemble the substrate analogue glycyl-L-proline ($IC_{50} = 450 \mu M$; Cheung *et al.*, 1980). DL-4-Arsono-2-benzylbutanoic acid was bound as well as its phosphorus analogue 2-benzyl-4-phosphonobutanoic acid, both of which were designed to resemble glycyl-L-phenylalanine ($IC_{50} = 630 \mu M$; Cheung *et al.*, 1980). Adams *et al.* (1983) have described 4-arsono-2-hydroxybutanoic acid, the arsonomethyl analogue of 3-phosphoglycerate, which has a K_m for phosphoglycerate kinase about 5-fold higher than that of 3-phosphoglycerate and a catalytic constant about 1300-fold smaller. The present work and this previous report provide the only examples of the interaction of arsonic acid substrate analogues and inhibitors with enzymes.

Fig. 2. Proposed binding of substrate (a) and a tetrahedral reaction intermediate (b) in the model of Fig. 1

(c) and (d) The proposed binding of two types of inhibitors where X is phosphorus or arsenic; (e) the proposed binding of the competitive inhibitor triphosphate.

Experimental

Chemicals and synthesis

H_3BO_3 , dimethylarsinic acid $[(CH_3)_2As(O)OH]$, i.e. cacodylic acid and Na_2SO_4 were from the Fisher Scientific Co., Fairlawn, NJ, U.S.A. Na_2AsO_4 was from Allied Chemical, Norristown, NJ, U.S.A. Na_2SeO_4 and dimethylphosphinic acid $[(CH_3)_2P(O)OH]$ were from Alfa Products, Danver, MA, U.S.A.

All the starting materials and solvents used in this work were reagent grade in generally 98% or higher purity, and were used without further purification or prepared by standard literature procedures. Since most of the starting organoantimony compounds mentioned in this paper are sensitive to O_2 or moisture, all manipulations of these compounds were performed in an atmosphere of dry O_2 -free N_2 , unless indicated otherwise. T.l.c. was on silica gel 60F-254 (EM Reagents, Elmsford, NY, U.S.A.). Compounds were located by using the phosphomolybdate spray (Bandurski & Axelrod, 1951), u.v. light and I_2 vapour. Purification by column chromatography was performed on silica gel (100–200 mesh; EM Reagents). Proton-n.m.r. spectra were recorded on a Varian EM-390 spec-

trometer for dilute solutions (10%), with tetramethylsilane as internal standard. ^{13}C -n.m.r. spectra were recorded on a Varian XL-200 instrument at 49.5 MHz in $^2\text{H}_2\text{O}$, with $[\text{H}_6]$ dioxan as internal standard. The m.s.(e.i.) spectra were measured with a Hitachi 90E spectrometer, and m.s.(c.i.) spectra on a Finnigan 3300 GC/MS at 150 eV with a solid probe. Melting points were taken on a hot stage and are corrected. Boiling points, which are given for short-path evaporative distillation, are uncorrected. The yields given were based on isolated products after purification.

Trimethoxydimethylantimony (Meinema & Noltes, 1972) was obtained from the reaction of freshly prepared trichlorodimethylantimony (Morgan & Davies, 1926), in 29% yield, m.p. 81–87°C (literature m.p. 80–86°C), n.m.r. δ (p.p.m.) ($[\text{H}_6]$ -dimethyl sulphoxide) 1.40 (6H, s, $2 \times \text{CH}_3$), 3.62 (9H, s, $3 \times \text{OCH}_3$). Ethyl 2-benzyl-4-bromobutanoate was prepared by passing a stream of dry HBr through an ethanolic solution of α -benzyl- γ -butyrolactone at 0°C for 6 h (Galaray & Kortylewicz, 1984). The yield of pure product was 78%, b.p. 108–110°C/0.05 mmHg, n.m.r. δ (p.p.m.) (C_2HCl_3) 1.15 (3H, t, CH_3), 2.07 (2H, N, CH_2), 2.78 (3H, m, CH_2CHCO), 3.31 (2H, m, CH_2Br), 4.05 (2H, q, OCH_2) and 7.15 (5H, m, C_6H_5).

Dimethyldihydroxysilane was prepared by the method of Takiguchi (1959), m.p. 98–100°C (literature m.p. 99–100°C). The average yield from two preparations was 77%. The product could be stored without decomposition in purified n-hexane at 0°C for several weeks; n.m.r. δ (p.p.m.) ($[\text{H}_6]$ -dimethyl sulphoxide) 0.32 (6H, s, $2 \times \text{CH}_3$) and 5.35 (2H, s, $2 \times \text{OH}$).

Hydroxy(oxo)dimethylantimony (dimethyl antimonite) was prepared as described by Meinema & Noltes (1972) as a colourless solid on hydrolysis of trimethoxydimethylantimony (1) in water followed by freeze-drying; n.m.r. δ (p.p.m.) ($[\text{H}_6]$ -dimethyl sulphoxide) at 60°C (8% solution) 0.93 (6H, s, $2 \times \text{CH}_3$) and 1.82 (1H, s, OH, exchangeable with $^2\text{H}_2\text{O}$). This compound does not melt. [Analysis (atomic absorption): Found: Sb, 65.65. Calc. for $\text{C}_2\text{H}_7\text{SbO}_2$: Sb, 65.89%.]

N-(Arsonoacetyl)-L-proline (1). The following modification of the method of Palmer (1923) was used. As_2O_3 (2.86 g, 15 mmol) and 4.35 g (109 mmol) of NaOH were dissolved in water (40 ml), and the solution was cooled at 25°C. Bromoacetyl-L-proline (5.12 g, 22 mmol) was added in one portion, and the mixture was stirred for 98 h. After this time more than 65% of the As_2O_3 had reacted, by titration of Na_3AsO_3 with I_2 . The solution was then carefully neutralized with acetic acid. The mixture was set aside for 12 h at 20°C, filtered, and added to aqueous BaCl_2 solution (19 g in 55 ml of water). The barium salt was washed, dried

at 50°C under high vacuum (yield 3.16 g, 62%), and decomposed at room temperature by addition of 1 M- H_2SO_4 . The mixture was filtered and extracted with ethyl acetate (3×50 ml) to remove unchanged bromoacetyl-L-proline, identified by t.l.c. The arsonic acid was isolated from the aqueous solution by freeze-drying, as solutions of the free acid were partially converted into another product, as shown by t.l.c. This mixture had an n.m.r. spectrum whose exchangeable protons integrated to about two protons, instead of three, and an m.s. identical with that of pure (1). Rapid recrystallization of solid, by warming then cooling to 4°C, was necessary (ethanol/water, 1:1, v/v), otherwise the white crystals melted to brown oil. We presume that the mixture and the oil contain an anhydride-like structure. The yield of crystalline *N*-(arsonoacetyl)-L-proline, m.p. 94–97°C, was 1.17 g (19%); n.m.r. (p.p.m.) ($[\text{H}_6]$ -dimethyl sulphoxide) 2.08 (4H, m, Pro-CH_2), 3.98 (2H, s, CH_2CO), 4.35 (1H, t, Pro-CH), 9.10 [2H, s, $\text{As}(\text{OH})_2$, exchangeable with $^2\text{H}_2\text{O}$], 10.85 (1H, s, CO_2H , exchangeable with $^2\text{H}_2\text{O}$), m.s.(c.i.) m/z 282 ($M^+ + 1$), 263 ($M^+ - \text{H}_2\text{O}$), 250, 206, 160, 129, 91, 71. [Analysis (atomic absorption): Found: As, 26.52. Calc. for $\text{C}_7\text{H}_{12}\text{NO}_6\text{As}$: As, 26.67%.]

DL-4-Arsono-2-benzylbutanoic acid (2). This compound was prepared by the route described by Quick & Adams (1922) for the preparation of aliphatic arsonic acids, but a much longer time was required to complete the reaction. As_2O_3 (2.63 g, 13.3 mmol) and NaOH (3.16 g, 79 mmol) were dissolved in water (50 ml) to produce Na_3AsO_3 . The solution was cooled at 15°C, and 6.0 g (20 mmol) of ethyl 2-benzyl-4-bromobutyrate (3) was added in one portion. After 1 h the mixture became homogeneous, and the clear solution was stirred at 50°C until titration of 1 ml of the reaction mixture with standard I_2 showed that most of the Na_3AsO_3 (65–70%) had reacted (about 58 h). Neither prolonged reaction time nor higher temperatures increased the degree of transformation. The reaction mixture was concentrated to one-half of its original volume and filtered to remove the NaBr. The filtrate was then neutralized with HCl to pH 8.0, again concentrated to one-half its volume under reduced pressure and filtered from sodium halide. On standing at 5°C overnight an additional small amount of As_2O_3 and NaBr were separated. The solution was acidified to pH 4.0 with HCl. An oily mixture of product (9) and α -benzyl- γ -butyrolactone (4) containing a small quantity of NaBr separated from the aqueous solution, and the lactone was purified from this layer on a short silica-gel column with dichloromethane/n-hexane (1:3, v/v) as eluent. The crude arsonic acid (1.1 g, 18%) was washed out from the silica gel by extraction with warm diethyl ether. Recrystallization from ethanol/water (1:1,

v/v) gave 0.76g (16.5%) of pure arsonic acid (2), m.p. 77–79°C, n.m.r. δ (p.p.m.) [$^2\text{H}_6$]dimethyl sulphoxide) 1.64–2.30 (2H, m, CH_2As), 2.65–3.30 (5H, m, CH_2CHCH_2), 7.23 (5H, m, C_6H_5) and 11.40 [3H, s broad, CO_2H and (O)As(OH) $_2$, exchangeable with $^2\text{H}_2\text{O}$], ^{13}C -n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$) 179.3, 138.8, 129.0, 126.6, 126.5, 47.8, 37.2, 26.0 and 22.9 ($\text{CH}_2\text{AsO}_3\text{H}_2$) and ^{13}C -n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$) 18.2 [(CH_3) $_2\text{As(O)OH}$]. [Analysis (atomic absorption): Found: As, 24.37%. Calc. for $\text{C}_{11}\text{H}_{15}\text{AsO}_5$: As 24.82%.]

DL-2-Benzyl-4-phosphonobutanoic acid (3). This compound was prepared by alkylation of the sodium salt of dibenzyl phosphite with ethyl 4-bromo-2-benzylbutyrate (method A) or by condensation of diethyl benzylmalonate with vinyl diethylphosphonate via Michael conjugated addition (Pudovik & Grishina, 1972) followed by hydrolysis and decarboxylation of the tetraester (method B).

Method A. Dibenzyl phosphite (4.60g, 17.5 mmol) in dimethylformamide (40 ml) was converted into the sodium salt with NaH (1 equiv.). To this solution 5.10g (17.55 mmol) of ethyl 2-benzyl-4-bromobutyrate was added. The mixture was stirred at room temperature overnight, concentrated and partitioned between diethyl ether and water. The ether layer was chromatographed on a silica-gel column in n-hexane/ethyl acetate (1:1, v/v). Two products were obtained. One was ethyl 2-benzyl-4-dibenzylphosphonobutyrate (4) (1.73g, 21% yield), t.l.c. R_f 0.45 (silica, ethyl acetate/n-hexane, 1:1, v/v), n.m.r. δ (p.p.m.) (C^2HCl_3) 1.10 (3H, t, CH_3), 1.45–1.84 (4H, m, CH_2CH_2), 2.40–2.76 (3H, m, CH_2CH), 3.76 (2H, q, OCH_2), 4.78 (4H, dd, POCH_2) and 7.12 (15H, m, $3 \times \text{C}_6\text{H}_5$). The other was ethyl 2-benzyl-4-benzylethylphosphonobutyrate (5) (0.96g, 11% yield), t.l.c. R_f 0.30 (silica, ethyl acetate/n-hexane, 1:1, v/v), n.m.r. δ (p.p.m.) (C^2HCl_3) 1.10 (3H, t, CH_3), 1.17 (3H, t, CH_3), 1.35–2.10 (4H, m, CH_2CH_2), 2.44–3.07 (3H, m, CH_2CH), 3.86–4.18 (4H, m, $2 \times \text{OCH}_2$), 4.93 (2H, d, POCH_2), 7.06 (5H, m, C_6H_5), 7.27 (5H, m, C_6H_5).

The hydrolysis of both esters in 10M-KOH under reflux for 6h, followed by acidification with conc. HCl to pH1, extraction with diethyl ether and evaporation of the solvent, yielded one common product, 2-benzyl-4-(O-benzylphosphonobutanoic acid (6) (1.12g, 75% yield), n.m.r. δ (p.p.m.) [$^2\text{H}_6$]dimethyl sulphoxide) 1.47–2.14 (4H, m, CH_2CH_2), 2.43–2.76 (3H, m, CH_2CH), 5.02 (2H, d, POCH_2), 7.26 (10H, m, $2 \times \text{C}_6\text{H}_5$) and 9.73–10.12 [2H, s, CO_2H and (O)POH, exchangeable with $^2\text{H}_2\text{O}$]. The crude monobenzyl ester (6) (1.0g, 3.88 mmol) was suspended in 20ml of water, and 1 ml of 1M-NaOH was added (3 equiv.). The mixture was stirred until the solution became homogeneous,

then was hydrogenolysed (120mg of 5% Pd on carbon) until the reaction was completed. The catalyst was removed by filtration and the filtrate acidified with conc. HCl to pH1.0. The oily product was extracted with diethyl ether, and the extract was dried (over Na_2SO_4) and evaporated under high vacuum. The crude product was recrystallized from boiling water, giving 0.64g (67% yield) of pure triacid (3), m.p. 72–74°C after two recrystallizations. The overall yield in this method was 23%; n.m.r. δ (p.p.m.) [$^2\text{H}_6$]dimethyl sulphoxide) 1.35–2.00 (4H, m, CH_2CH_2), 2.37–2.85 (3H, m, CH_2CH), 7.26 (5H, m, C_6H_5), 10.2 [3H, s very broad, CO_2H and (O)P(OH) $_2$, exchangeable with $^2\text{H}_2\text{O}$], m.s.(e.i.) m/z 212 ($M^+ - \text{CO}_2$), 180, 126, 108 and 91. On drying under vacuum at 56°C, cyclization occurred, giving the cyclic anhydride of compound (9), m.p. 123–125°C, n.m.r. δ (p.p.m.) [$^2\text{H}_6$]dimethyl sulphoxide) 1.57–1.65 [2H, m, $\text{CH}_2\text{CH}_2\text{P(O)OH}$], 1.84–2.76 [5H, CH_2CH and $\text{CH}_2\text{P(O)OH}$], 5.60 [1H, s, P(O)OH, exchangeable with $^2\text{H}_2\text{O}$], 7.19 (5H, m, C_6H_5), m.s.(c.i.) m/z 241 ($M^+ + 1$), 221 ($M^+ - \text{H}_2\text{O}$), 203, 201 and 91.

Method B. A saturated solution of sodium ethoxide (1.40g of Na dissolved in 27 ml of ethanol) was added very slowly to the reaction mixture which consisted of 10.0g (61 mmol) of diethyl vinylphosphonate and 15.24g (61 mmol) of diethyl benzylmalonate to give an exothermic reaction (the mixture reached 70°C). After about 20 min the reaction subsided, and addition of sodium ethoxide was stopped (65% of the theoretical quantity of sodium ethoxide was added). The mixture was stirred for 3h at room temperature, and 20g of ice was added. After acidification with cold conc. HCl and dilution with water to 100 ml, the oily layer was extracted into diethyl ether, and the extracts were washed with 5% NaHCO_3 and water, and dried over Na_2SO_4 . Distillation gave 21.5g (85% yield) of pure tetraester (7) diethyl 2-benzyl-2-(2'-diethylphosphonoethyl)malonate, b.p. 168–171°C/0.05 mmHg, t.l.c. R_f 0.45 (silica, dichloromethane/n-hexane, 1:1 v/v), n.m.r. δ (p.p.m.) (C^2HCl_3) 1.05 (12H, m, $4 \times \text{CH}_3$), 3.14 (2H, s, $\text{C}_6\text{H}_5\text{CH}_2$), 3.74–4.36 (8H, m, $4 \times \text{CH}_2$) and 7.06 (5H, m, C_6H_5).

A suspension of 8.0g (19 mmol) of the tetraester (7) and 10g (178 mmol) of KOH in 50 ml of water was refluxed for 10h, 10 ml of water was evaporated, an additional 2g of KOH was added and the mixture was refluxed for 6h. The mixture was cooled, acidified with conc. HCl to pH1.0, and 20 ml of water was added to dissolve a precipitate of KCl. On standing at 5°C the oily product separated, and it was extracted into diethyl ether. After drying over (Na_2SO_4) the ether extract was evaporated and 4.20g (77%) of crude 2-benzyl-4-(O-ethylphosphono)butanoic acid (8) was obtained

as an oily residue, n.m.r. δ (p.p.m.) ($[\text{}^2\text{H}_6]$ dimethyl sulphoxide) 1.23 (3H, t, CH_3), 1.34–1.84 (4H, m, CH_2CH_2), 2.30–2.91 (3H, m, CH_2CH), 3.56–4.00 (2H, m, OCH_2), 7.20 (5H, m, C_6H_5), 8.20–9.15 [2H, s very broad, CO_2H and $(\text{O})\text{POH}$, exchangeable with $^2\text{H}_2\text{O}$]. Further hydrolysis of 3.0 g of this monester in 3 M-HCl (20 ml) under reflux for 3 h gave 1.05 g (66% yield) of pure DL-2-benzyl-4-phosphonobutanoic acid (3). The overall yield in this method was 41% of pure product, which had m.p. and m.s. and n.m.r. spectra identical with those of the compound obtained by method A and gave the identical anhydride on being dried at 56°C.

Angiotensin-converting enzyme

Angiotensin-converting enzyme was partially purified (Galaray, 1982) from frozen rabbit lungs by hydroxyapatite, ion-exchange and gel-filtration chromatography (Das & Soffer, 1975), with omission of the last step of lectin affinity chromatography. The specific activity of angiotensin-converting enzyme averaged 19 units/mg of protein. One unit of converting enzyme hydrolyses 1 μmol of hippurylhistidyl-leucine/min at 37°C in 100 mM-potassium phosphate buffer, pH 8.3, containing 300 mM-NaCl.

Kinetic studies

All inhibitors were assayed with hippurylhistidyl-leucine as substrate in 50 mM-Tris hydrochloride (adjusted to pH 7.5 with NaOH) containing 300 mM-NaCl (Galaray, 1980) by the fluorometric reaction of histidyl-leucine with *o*-phthalaldehyde (Cheung & Cushman, 1973) with a single 30 min time point. K_m was 0.5 mM. The reaction was initiated by the addition of enzyme to a final concentration of 0.05 nM. Hydrolysis was linear with time to well beyond 30 min, with less than 5% total substrate hydrolysed for all substrate concentrations. Mean K_i values were determined by averaging the K_i values found from a Lineweaver-Burk plot and a Dixon plot of each of two separate

experiments. When these K_i values are compared with K_i and IC_{50} values from the literature determined under different conditions, the buffer composition and pH are given for the literature references. K_i and IC_{50} values determined under different conditions are not strictly comparable.

Results

The K_i values of simple oxo acids of phosphorus and arsenic and of substrate analogues containing these elements are given in Table 1. An IC_{50} from the literature, not a K_i , is reported for 2-phosphonoacetyl-L-proline. This IC_{50} was determined under assay conditions different from those used here. This difference in assay conditions does not affect the great disparity in potency between the phosphorus-containing inhibitor and its arsenic analogue. Fig. 3 shows a Lineweaver-Burk plot and a Dixon plot for the inhibition of angiotensin-converting enzyme by Na_2HAsO_4 . The intercept on the ordinate of the former indicated competitive inhibition. The K_i values of the other oxo acids examined were: borate > 300 mM, dimethyldihydroxysilane > 300 mM, dimethylantimonite > 300 mM, sulphate > 300 mM and selenate > 300 mM. The K_i of borate was unchanged when assayed in 50 mM-Tris/HCl containing 300 mM-NaCl adjusted to pH 8.0 instead of pH 7.5.

Discussion

Comparison of the K_i values of phosphate, borate, arsenate, sulphate and selenate suggested that only a tetrahedral arsenic atom could successfully substitute for a tetrahedral phosphorus atom in a substrate-analogue inhibitor of angiotensin-converting enzyme. The ability of arsenate to inhibit angiotensin-converting enzyme more strongly than phosphate may result from the similarity of its $\text{p}K_a$ values (2.2, 7.0 and 13.0) to those of phosphate (2.1, 7.2 and 12.3; Fasman, 1976) and its in-

Table 1. Phosphorus-containing and arsenic-containing inhibitors of angiotensin-converting enzyme

	K_i (mM), mode
H_3PO_4	$\geq 300^*$
H_3AsO_4	27 ± 1 , competitive
$\text{CH}_3\text{PO}_2\text{HCH}_3$	170 ± 7 , mixed
$\text{CH}_3\text{AsO}_2\text{HCH}_3$	70 ± 8 , competitive
$\text{H}_2\text{PO}_3\text{CH}_2\text{CO-Pro}$	0.008†
$\text{H}_2\text{AsO}_3\text{CH}_2\text{CO-Pro}$ (1)	18 ± 7 , competitive
$\text{H}_2\text{PO}_3\text{CH}_2\text{CH}_2\text{CH(Bzl)CO}_2^-$ (3)	0.2 ± 0.1 , competitive
$\text{H}_2\text{AsO}_3\text{CH}_2\text{CH}_2\text{CH(Bzl)CO}_2^-$ (2)	0.5 ± 0.2 , competitive

* Galaray (1982).

† IC_{50} , 100 mM-phosphate buffer, pH 8.3, containing 300 mM-NaCl (Petrillo & Ondetti, 1982).

Fig. 3. Lineweaver-Burk plot (a) and Dixon plot (b) of the inhibition of angiotensin-converting enzyme by Na_2HAsO_4

creased size (As-O bond distance of 0.175 nm in the crystal compared with 0.156 nm for P-O; Durrant & Durrant, 1962). The S-O and Se-O bond distances in sulphate and selenate (0.156 nm and 0.161 nm respectively; Durrant & Durrant, 1962) are close to the P-O bond distance in phosphate. Lack of detectable inhibition by these oxo acids could therefore be due to their different charge distribution in the dianion, i.e. sulphate and selenate are harder bases ($\text{p}K_a$ -3, 2 and -3, -3 respectively; Albert & Serjeant, 1962) compared with phosphate.

The lack of even weak inhibition of angiotensin-converting enzyme by boric acid is surprising, since boronic acids have been shown to be strong inhibitors of chymotrypsin (Matteson *et al.*, 1981; Phillip & Bender, 1971; Koehler & Lienhard, 1971), subtilisin (Matthews *et al.*, 1975; Lindquist & Terry, 1974), acetylcholinesterase (Koehler & Hess, 1974) and aminopeptidase (Baker *et al.*, 1983), and since boric acid itself is an inhibitor of *Streptomyces griseus* proteinase 3 (Bauer & Pettersson, 1974). The studies with the serine hydrolases (chymotrypsin, subtilisin, acetylcholinesterase and proteinase 3) suggest that the boronic acid and boric acid can form a tetrahedral adduct with the active-site-serine hydroxy group. The boronic acid inhibitor of the zinc metallopeptidase amino-

peptidase is also thought to form a tetrahedral adduct with an unknown nucleophile at the active site of the enzyme (Baker *et al.*, 1983). Boric acid (H_3BO_3) ionizes at pH 9.2 by forming a tetrahedral adduct $\text{B}(\text{OH})_4^-$ with OH^- ion (Cotton & Wilkinson, 1980). However, at pH 8, where several-fold more of this adduct is present compared with pH 7.5, boric acid still did not inhibit angiotensin-converting enzyme.

Of the dimethyl oxo acids examined, only the dimethyl oxo acid of arsenic mimicked the inhibition of angiotensin-converting enzyme by dimethylphosphinate. The tetrahedral dimethyl oxo acids of silicon and antimony were prepared and compared with dimethylphosphinate owing to the low solubility in water of the simple silicates and antimonates. These two compounds did not inhibit the enzyme at concentrations of 300 mM.

The more powerful inhibition of angiotensin-converting enzyme by arsenate and dimethylarsinate compared with phosphate and dimethylphosphinate suggested that substitution of an arsenic atom for the phosphorus atom in a substrate analogue would lead to a more potent inhibitor. However, 2-arsonoacetyl-L-proline was found to be over 2000-fold weaker an inhibitor than 2-phosphonoacetyl-L-proline. The As-C and As-O bond

distances in the former are significantly longer than the P-C and P-O distances in the latter (compare DL-2-amino-4-arsonobutanoic acid and DL-2-amino-4-phosphonobutanoic acid; Kamiya *et al.*, 1983). These greater bond distances may place an arsenic oxygen atom too far to the left of the active-site zinc atom in angiotensin-converting enzyme (see Fig. 2) for the arsenic analogue to be as effectively bound as the shorter phosphorus analogue. For example, 2-phosphonopropanoyl-L-proline is strongly bound 6-fold less tightly than is the shorter inhibitor 2-phosphonoacetyl-L-proline (Petrillo & Ondetti, 1982). In order to test whether decreasing the distance from the carboxy terminus of the inhibitor to the tetrahedral arsenic atom would decrease its K_i , DL-4-arsono-2-benzylbutanoic acid was prepared, along with DL-2-benzyl-4-phosphonobutanoic acid for comparison. These compounds can be thought of as partial analogues of the inhibitor glycyl-L-phenylalanine, $IC_{50} = 630 \mu M$ (Cheung *et al.*, 1980), with the peptide bond replaced by an ethylene bridge. Replacement of the peptide bond by an ethylene bridge usually causes a large decrease in inhibitory potency (Condon *et al.*, 1982).

DL-4-Arsono-2-benzylbutanoic acid was over 25-fold more potent an inhibitor than was 2-arsonoacetyl-L-proline, and nearly equipotent to its phosphorus analogue DL-2-benzyl-4-phosphonobutanoic acid. However, both compounds were rather weak inhibitors, with K_i of 0.5 mM and 0.2 mM respectively. It is not possible to rule out the possibility that these two inhibitors bind in reverse order in the angiotensin converting-enzyme model shown in Fig. 2, i.e. with the carboxylate in the position of the zinc atom, the benzyl side chain in the S_1' site, and the phosphonic acid or arsonic acid group at the cationic site to the right of the S_2' position. The acceptability of phosphate at the cationic site to the right of S_2' was demonstrated by the low K_i of triphosphate for angiotensin-converting enzyme (18 μM ; see Fig. 3) and the fact that the K_i of phosphoglycyl-aminomethylphosphonic acid is indistinguishable from that of its carboxylic acid analogue phosphoglycylglycine (Galary, 1982). However, the weak binding of L-phenylalanyl-glycine ($IC_{50} = 3.7 mM$) compared with its reversed sequence glycyl-L-phenylalanine ($IC_{50} = 630 \mu M$; Cheung *et al.*, 1980) suggests that phosphonobutanoic acid and arsonobutanoic acid probably bind as shown in Fig. 3, with the benzyl group located at S_2' and the tetrahedral heteroatom located at the zinc atom rather than in reverse order.

In summary, in the simple tetrahedral oxo acids examined, only arsenic mimics phosphorus in producing inhibitors of angiotensin-converting enzyme. In inhibitors that resemble substrate ana-

logues, DL-4-arsono-2-benzylbutanoic acid is about as potent an inhibitor as is DL-2-benzyl-4-phosphonobutanoic acid. 2-Arsonoacetyl-L-proline is more than 2000-fold weaker an inhibitor than is 2-phosphonoacetyl-L-proline, possibly because of the increased As-C and As-O bond distances of the former compared with the P-C and P-O distances of the latter.

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