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Catalytic irreversible inhibition of bacterial and plant arginine decarboxylase activities by novel substrate and product analogues

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Arginine decarboxylase (ADC) activity from *Escherichia coli* and two plant species (oats and barley) was inhibited by five new substrate (arginine) and product (agmatine) analogues. The five compounds, (*E*)-α-monofluoromethyldehydroarginine (Δ-MFMA), α-monofluoromethylarginine (MFMA), α-monofluoromethylagmatine (FMA), α-ethynylagmatine (EA) and α-allenylagmatine (AA), were all more potent inhibitors of ADC activity than was α-difluoromethylarginine (DFMA), the only irreversible inhibitor of this enzyme described previously. The inhibition caused by the five compounds was apparently enzyme-activated and irreversible, since the loss of enzyme activity followed pseudo-first-order kinetics, was time-dependent, the natural substrate of ADC (arginine) blocked the effects of the inhibitors, and the inhibition remained after chromatography of inhibited ADC on Sephadex G-25 or on overnight dialysis of the enzyme. DFMA, FMA, Δ-MFMA and MFMA were effective at very low concentrations (10 nM–10 μM) at inhibiting ADC activity in growing *E. coli*. FMA was also shown to deplete putrescine effectively in *E. coli*, particularly when combined with an inhibitor of ornithine decarboxylase, α-monofluoromethyl-putrescine. The potential uses of the compounds for the study of the role of polyamine biosynthesis in bacteria and plants is discussed.

INTRODUCTION

Putrescine, spermidine and spermine are found in virtually all cells and are important regulators of cell division, growth and differentiation (Pegg & McCann, 1982). Although most of the work in the polyamine field has centred on the enzyme ornithine decarboxylase (ODC), which produces putrescine directly from ornithine, in recent years increasing attention has been focused on the alternate biosynthetic pathway to the polyamines which occurs in some cell types, namely the arginine decarboxylase (ADC) pathway.

ADC catalyses the decarboxylation of arginine to agmatine (Morris & Pardee, 1966; Pegg & Williams-Ashman, 1981). Agmatine can then be hydrolysed to putrescine by agmatine amidinohydrolase (agmatinase; EC 1.5.3.11) or agmatine iminohydrolase (agmatine deiminase; EC 3.5.3.12), and putrescine can be further converted into spermidine and spermine (Pegg & Williams-Ashman, 1981). Although ADC was originally described by Gale (1940) as a biodegradative enzyme which is induced by growth of bacteria in low-pH medium, it has now been recognized that the biosynthetic form of the enzyme, which is responsible for the biosynthesis of putrescine and the polyamines, has a distinct role in the normal physiology of bacteria and higher plants (Tabor & Tabor, 1985; Galston, 1983).

Elucidation of the role of ADC in normal bacterial and plant cell physiology was aided by the development of a specific enzyme-activated irreversible inhibitor of ADC, DL-α-difluoromethylarginine (DFMA) (Kallio *et al.*, 1981). Studies utilizing DFMA alone and in conjunction with specific enzyme-activated irreversible inhibitors of ODC such as DL-α-difluoromethylornithine (eflornithine), and other similar compounds, clearly demonstrated that ADC activity in bacteria is sufficient to maintain intracellular putrescine concentrations at normal values (Kallio *et al.*, 1982). Complete inhibition of either ODC or ADC activity in *Escherichia coli* produces no effect on intracellular putrescine concentration. In fact, when only one of the decarboxylases is inhibited, there seems to be compensatory increases in the second decarboxylase, and putrescine concentration is maintained (Kallio & McCann, 1981). However, if ADC and ODC are inhibited simultaneously with DFMA and DL-α-monofluoromethylputrescine, respectively, there is a fall in putrescine concentration (Kallio *et al.*, 1982). Furthermore, a combination of monofluoromethylornithine (inhibitor of ODC), DFMA (inhibitor of ADC) and cyclohexylamine (inhibitor of spermidine synthase) significantly lowered polyamine concentrations in *Pseudomonas aeruginosa* and *E. coli* and slowed the growth of both species, demonstrating the utility of specific inhibitors for studying the role(s) of polyamines in bacteria (Bitonti *et al.*, 1982, 1983, 1984).

Plant physiologists have used DFMA to demonstrate the contribution of ADC and putrescine produced by this route to normal plant physiology. A previous report showed that embryogenesis in carrots could be completely blocked by addition of DFMA to the culture medium (Feirer *et al.*, 1984). Flores & Galston (1981) demonstrated that osmotic stress increased putrescine concentrations in oat leaves, and this increase was apparently due to an increased ADC activity since the increase in putrescine was completely blocked by DFMA and not by difluoromethylornithine. Others have also shown that plants respond to stimulatory hormones by induction of ADC activity and increased putrescine,

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); ADC, arginine decarboxylase (EC 4.1.1.19); DFMA, DL-α-difluoromethylarginine; Δ-MFMA, (*E*)-α-monofluoromethyldehydroarginine, MFMA, α-monofluoromethylarginine; FMA, α-monofluoromethylagmatine; EA, α-ethynylagmatine; AA, α-allenylagmatine.

Table 1. Kinetic data for irreversible inhibition of *E. coli* ADC by analogues of arginine and agmatine

E. coli ADC was prepared and assayed as described in the Experimental section. It was incubated with various concentrations of arginine and agmatine analogues, and at selected times residual enzyme activity was determined on samples of the enzyme/inhibitor mixture. From graphs of $t_{\frac{1}{2}}$ versus 1/[inhibitor], apparent K_i values and $t_{\frac{1}{2}}$ at infinite inhibitor concentration were calculated as in Kitz & Wilson (1962), except for the K_i for α-EA, which was calculated from graphs of $1/v$ versus 1/[arginine] determined at different inhibitor concentrations.

	Structure	K_i (μM)	$t_{\frac{1}{2}}$ (min) At infinite concentration	$t_{\frac{1}{2}}$ (min) At 10 μM
Arginine analogues				
α-Difluoromethylarginine (DFMA)	$HOOC{-}C(CHF_2)(NH_2){-}(CH_2)_3{-}NH{-}C(=NH){-}NH_2$	850	0.7	54
α-Monofluoromethylarginine (MFMA)	$HOOC{-}C(CH_2F)(NH_2){-}(CH_2)_3{-}NH{-}C(=NH){-}NH_2$	65	1.0	7.3
(*E*)-α-Monofluoromethyldehydroarginine (Δ-MFMA)	$HOOC{-}C(CH_2F)(NH_2){-}CH{=}CH{-}CH_2{-}NH{-}C(=NH){-}NH_2$	34	1.1	4.8
Agmatine analogues				
α-Monofluoromethylagmatine (FMA)	$H_2N{-}CH(CH_2F){-}(CH_2)_3{-}NH{-}C(=NH){-}NH_2$	9	0.6	1.0
α-Ethynylagmatine (EA)	$HC{\equiv}C{-}CH(NH_2){-}(CH_2)_3{-}NH{-}C(=NH){-}NH_2$	11	12.6	55
α-Allenylagmatine (AA)	$H_2C{=}C{=}CH{-}CH(NH_2){-}(CH_2)_3{-}NH{-}C(=NH){-}NH_2$	7	3	5

since the promotion of the elongation of dwarf pea internodes produced by gibberellin was inhibited by DFMA (Dai *et al.*, 1982; Slocum *et al.*, 1984). It is clear from these studies and many others [reviewed by Slocum *et al.* (1984) and Galston (1983)] that potent specific inhibitors of ADC will be important for the further elucidation of the roles of ADC and polyamines in plant growth and development.

Recently we have synthesized a number of arginine (substrate) and agmatine (product) analogues as potential irreversible inhibitors of ADC. We detail in this paper the kinetic characteristics of these new compounds against *E. coli* ADC, the effects of the compounds on ADC activity and polyamine concentrations in growing *E. coli*, and the inhibition of plant-derived ADC by the compounds.

EXPERIMENTAL

Preparation of *E. coli* ADC

Freeze-dried *Escherichia coli W* (A.T.C.C. 9637) were suspended in 10 mM-Tris/HCl (pH 7.5) containing 1 mM-dithiothreitol and 0.1 mM-EDTA (Disrupting Buffer), disrupted by sonication (5 × 30 s; setting 3, Branson 350 sonicator) and then centrifuged at 100000 *g* for 30 min. The supernatant, which contained 7–15 mg of protein/ml, was used as the source for ADC activity and was stored frozen at −20 °C for extended periods without appreciable loss of enzyme activity. Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Plant materials and preparation of plant ADC

Oats (*Hordeum vulgare*) and barley (*Avena sativa*) seeds were germinated in flats in a mixture of peat moss and vermiculite (1:1) as described by Birecka *et al.* (1985*b*). First leaves were sampled on day 4 after germination and homogenized in 5 vol. of a solution of 200 mM-Tris/HCl (pH 8.0) containing 10 mM-dithiothreitol, 0.1 mM-EDTA and 0.1 mM-pyridoxal phosphate, with a Polytron at the highest setting. The homogenates were centrifuged at 20000 *g* for 20 min, and the supernatant was used to assay ADC activity.

Assay for ADC

Biosynthetic ADC activity of *E. coli* was measured by a modification of the previously described method of Wu & Morris (1973). The incubation mixture contained 100 mM-Tris/HCl (pH 8.25), 4 mM-$MgSO_4$, 20 μM-pyridoxal phosphate, 2 mM-L-arginine, 0.5 μCi of DL-[1-^{14}C]arginine, 0.5 mM-dithiothreitol and 150–250 μg of protein in a total volume of 1 ml. The incubations were carried out at 37 °C for 60 min and terminated by the addition of 1 ml of 40% trichloroacetic acid. The

reaction mixtures were then incubated for an additional 30 min at 37 °C while $^{14}CO_2$ was trapped on a filter paper saturated with 50 μl of methylbenzethonium hydroxide. The K_m for arginine determined under these assay conditions was 350 μM.

ADC activity from oats and barley was assayed as described by Birecka *et al.* (1985*a*). Portions of the 20000 ***g*** supernatant containing 4.3 mg of protein/ml of barley extract and 8.1 mg of protein/ml of oat extract were assayed in 80 mM-Tris/HCl (pH 8.0) containing 1 mM-L-arginine, 0.5 μCi of DL-[1-^{14}C]arginine, 1.6 mM-dithiothreitol, 0.04 mM-pyridoxal phosphate and 0.04 mM-EDTA in a total volume of 0.2 ml. The assays were initiated and terminated just as for the assays of *E. coli* ADC activity.

Time-dependent inhibition of *E. coli* ADC

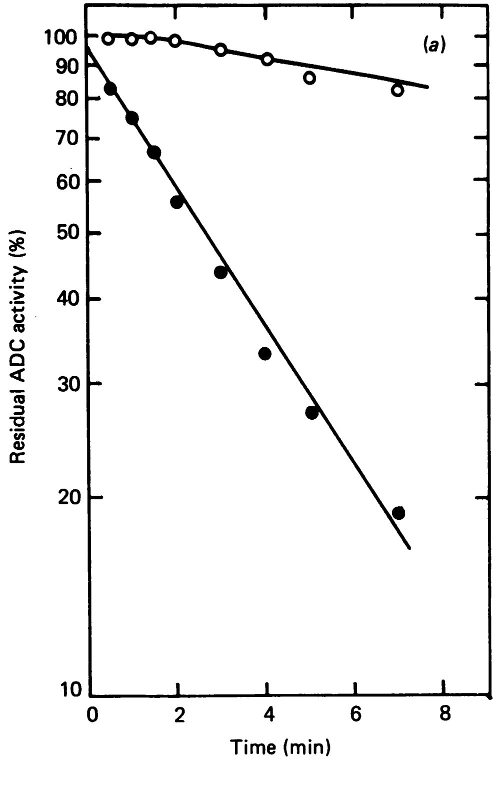
This was determined by modification of the method previously described by Kallio *et al.* (1981). Typically, 200 μl of a solution containing 300 mM-Tris/HCl (pH 8.25), 12 mM-$MgSO_4$, 3 mM-dithiothreitol and 120 μM-pyridoxal phosphate was mixed with 200 μl of *E. coli* ADC. After incubation of this mixture for 2 min at 37 °C, a 50 μl sample was removed for measurement of uninhibited enzyme activity and inhibitors were added immediately to the mixture at various concentrations in 150 μl of water. At selected times 50 μl samples were removed from the enzyme/inhibitor mixture and added to reaction vessels on ice in which ADC activity was to be measured. These reaction vessels contained all the components for the ADC reaction, except [1-^{14}C]arginine, in a total volume of 850 μl. The dilution of the enzyme/inhibitor mixture into the reaction vessels on ice effectively terminated time-dependent inhibition. Kinetic analysis of the enzyme-inhibition data was carried out as described by Kitz & Wilson (1962).

Test for irreversibility of inhibition of *E. coli* ADC by analogues of arginine and agmatine

ADC (200 μl) was incubated with 200 μl of inhibitor solution for 30 min at 37 °C. A 50 μl sample of the enzyme/inhibitor mixture was then taken for ADC assay, and the remaining 350 μl of the mixture was chromatographed on a 10 ml Sephadex G-25 column (PD-10; Pharmacia) which had been equilibrated with 25 ml of the disrupting buffer described above. After addition of enzyme to the column, the column was washed with 2.5 ml of disrupting buffer and this wash was discarded. ADC activity was then eluted with an additional 1.5 ml of the buffer, and the eluent was assayed for enzyme activity. Alternatively, after incubation of the enzyme with the inhibitors, a 50 μl sample of the enzyme preparation was taken for enzyme assay and the remaining sample was dialysed overnight (approx. 18 h) against 500 ml of disrupting buffer. ADC activity was then determined on the dialysed enzyme preparations as described above.

Growth of *E. coli* and effects of ADC inhibitors on ADC and polyamines *in vivo*

E. coli (MRC 59) were grown aerobically in minimal medium (Davis & Mingioli, 1950) at 37 °C in a shaking incubator. Bacterial growth was monitored as A_{550}. Bacteria were grown until they were in late-exponential phase (A_{550} = 0.9–1; $14\times10^8-16\times10^8$ cells/ml), at which time the cultures were divided into smaller flasks containing the test compounds at various concentrations. After incubation for an additional 30 min, the bacteria were sedimented by centrifugation, washed twice with 0.125 M-NaCl containing 10 mM-Na_2HPO_4 and 3 mM-KH_2PO_4 (pH 7.2) and then suspended in the disrupting buffer described above, sonicated, and centri-



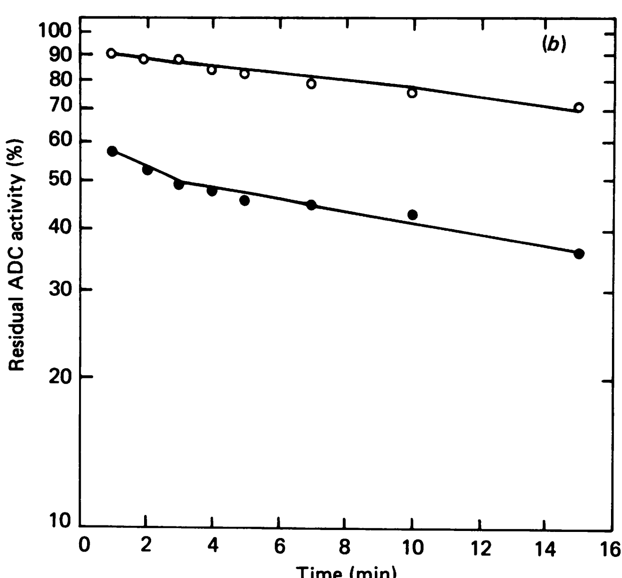


Fig. 1. Protection of *E. coli* ADC activity from inhibition by agmatine analogues by arginine

(*a*) Time-dependent inhibition of ADC by FMA (5 μM) in the absence (●) or presence (○) of 2 mM-L-arginine. (*b*) Time-dependent inhibition of ADC by EA (200 μM) in the absence (●) or presence (○) of 40 mM-L-arginine. *E. coli* ADC activity and time-dependent inhibition were determined as described in the Experimental section.

Table 2. Effects of Sephadex G-25 chromatography on *E. coli* ADC after incubation with analogues of arginine and agmatine

E. coli ADC activity was prepared, treated with inhibitors, chromatographed on Sephadex G-25 and then assayed as described in the Experimental section. Values in parentheses represent percentages of uninhibited activity. Expts. 1 and 2 were carried out separately with two different preparations of *E. coli* ADC.

		ADC (nmol of CO_2/h per mg of protein)	
Inhibitor	Concn. (μM)	Before Sephadex G-25	After Sephadex G-25
Expt. 1			
None	0	251 (100)	195 (100)
DFMA	250	36 (14)	42 (22)
Δ-MFMA	20	20 (8)	24 (12)
FMA	10	27 (11)	27 (14)
EA	500	55 (22)	100 (51)
Expt. 2			
None	0	121 (100)	157 (100)
MFMA	20	15 (12)	34 (22)
AA	10	14 (11)	19 (12)

fuged at 45000 *g* for 20 min. The resulting supernatant was used for the determination of residual ADC activity.

When polyamines were to be measured, *E. coli* in late-exponential phase were sedimented, washed twice with the NaCl solution described above and extracted in a small volume of 0.4 M-$HClO_4$. Proteins were removed from the extracts by centrifugation, and polyamines were determined as described by Wagner *et al.* (1982).

Chemicals

DL-[1-^{14}C]Arginine (47 mCi/mmol) was purchased from Research Products International, and freeze-dried *E. coli* W from Sigma. Oat (*Hordeum vulgare*) and barley (*Avena sativa*) seeds were purchased from Carolina Biological Supply Co. Arginine and agmatine analogues were synthesized in our laboratories.

RESULTS

(*E*)-α-Monofluoromethyl-3,4-dehydroarginine (Δ-MFMA), α-monofluoromethylarginine (MFMA), α-monofluoromethylagmatine (FMA), α-ethynylagmatine (EA) and α-allenylagmatine (AA) were found to be potent time-dependent inhibitors of *E. coli* ADC (Table 1). From plots of half-life ($t_{\frac{1}{2}}$) of enzyme activity versus the inverse of the inhibitor concentration, it was possible to determine an apparent dissociation constant (K_i) for each inhibitor and half-life of the enzyme activity at an infinite concentration of inhibitor. Since there was a large component of competitive inhibition with EA (see Fig. 1*b*), it was necessary to determine a K_i for this compound by graphs of $1/v$ versus 1/[arginine] at various concentrations of the inhibitor. On the basis of these values, it was apparent that the new substrate and product analogues are markedly more potent than DFMA, the only other time-dependent irreversible ADC inhibitor previously described. The most potent compounds were the agmatine analogues (FMA, AA, EA),

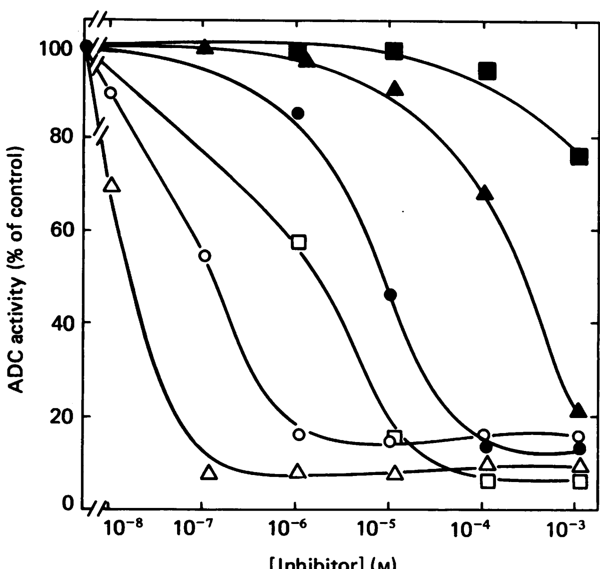


Fig. 2. Effects of analogues of arginine and agmatine on ADC activity in intact *E. coli*

Growing *E. coli* were incubated for 30 min in the absence or presence of the indicated concentrations of Δ-MFMA (○), DFMA (□), FMA (●), EA (■), MFMA (△) or AA (▲), after which the cells were sedimented by centrifugation, washed twice and sonicated in preparation for the assay of residual ADC activity. ADC was assayed as described in the Experimental section.

which had K_i values approx. 1/100 of that for DFMA (K_i = 850 μM).

The inhibition produced by Δ-MFMA, MFMA, AA and FMA was apparently irreversible in nature. Chromatography of ADC, which had been incubated with these inhibitors, on Sephadex G-25 failed to restore enzyme activity (Table 2), as did extensive dialysis of the inhibited enzyme (results not shown). The inhibition produced by EA, on the other hand, was not completely irreversible, since chromatography of inhibited ADC on Sephadex G-25 resulted in a marked restoration of enzyme activity (Table 2), as did overnight dialysis (results not shown). The apparent slight recovery of ADC activity in the samples incubated with Δ-MFMA, MFMA, AA or FMA and then chromatographed on Sephadex G-25 was not reproducible from one experiment to another, and no increases in the inhibited ADC activity larger than those in the representative experiments shown in Table 2 were seen. However, the recovery of ADC activity in samples incubated with EA was consistently as large as the increase shown in Table 2. It seems clear that Δ-MFMA, MFMA, AA and FMA are active-site-directed inhibitors, since low concentrations (2–5 mM) of L-arginine almost completely abolished the rapid time-dependent inhibition caused by these compounds [the inhibition caused by FMA is shown in Fig. 1(*a*) as an example of the inhibition produced by these four compounds]. The inhibition by EA appeared to be somewhat more complex: although 40 mM-L-arginine did provide ADC activity some protection from EA, the protective effect was not as marked as with Δ-MFMA and FMA (Fig. 1*b*).

In order to study the effects of the ADC inhibitors on ADC activity in whole cells, test compounds were added

Table 3. Effects of FMA and α-monofluoromethylputrescine on *E. coli* polyamines

E. coli were grown and extracted for polyamines as described in the Experimental section. Putrescine and spermidine were determined by h.p.l.c. Values in parentheses represent percentages of the control values found in the absence of inhibitors. FMA and α-monofluoromethylputrescine were added to the culture medium at a concentration of 1 mM of each drug before inoculation of the medium with *E. coli* and were present throughout the duration of the cultures.

Additions	Content (nmol/10^8 cells)	
	Putrescine	Spermidine
None	1.54 (100)	0.22 (100)
FMA	0.87 (57)	0.30 (136)
α-Monofluoromethyl-putrescine	1.22 (79)	0.25 (114)
FMA + α-monofluoromethyl-putrescine	0.1 (6)	0.31 (141)

at various concentrations to *E. coli* in late-exponential phase of growth. After 30 min of continuous exposure to the compounds, residual ADC activity was measured in cell-free extracts prepared from washed cells. The results of these experiments are presented in Fig. 2. ADC activity was inhibited by 90% in the whole cells by 0.1 μM-MFMA, 10 μM-DFMA, 1 μM-Δ-MFMA and 100 μM-FMA. EA and AA were much less effective against ADC activity in whole cells, with 1 mM inhibitor being needed to produce significant inhibition.

FMA was examined for its effects on bacterial growth and polyamine concentrations alone and in combination with an ODC inhibitor, α-monofluoromethylputrescine. FMA alone had no effect on *E. coli* growth rate (results not shown), but did cause a decrease in intracellular putrescine (Table 3). When FMA was combined with α-monofluoromethylputrescine, there was a 94% decline in intracellular putrescine but no change in growth rate, probably because of the 40% increase in spermidine caused by the compounds.

ADC activity from oats and barley was examined for inhibition by DFMA, Δ-MFMA, FMA and EA. The relative potencies of the inhibitors seemed to be the same for the plant ADC as for the bacterial ADC (Table 4).

Table 4. Inhibition of barley and oat ADC activity by substrate and product analogues

Barley and oat ADC activity was prepared and assayed as described in the Experimental section. The values in parentheses represent percentages of the ADC activity present in the absence of inhibitors.

Inhibitor	Concn. (μM)	ADC activity (nmol of CO_2/h per mg of protein)	
		Barley	Oat
None		10.6 (100)	40.8 (100)
FMA	1	8.7 (82)	30.2 (74)
	10	2.3 (22)	8.5 (21)
	100	0.6 (6)	1.2 (3)
DFMA	1	10.4 (98)	39.1 (96)
	10	9.7 (92)	30.6 (75)
	100	3.0 (28)	10.1 (25)
Δ-MFMA	1	5.1 (48)	8.3 (20)
	10	0.7 (7)	1.6 (4)
	100	0.1 (1)	0.4 (1)
EA	1	9.3 (88)	35.4 (87)
	10	6.1 (57)	23.4 (57)
	100	2.7 (26)	13.3 (33)

DISCUSSION

ADC activities from *E. coli* and two plant species (oats and barley) are inhibited by FMA, Δ-MFMA, MFMA, AA and EA. All five compounds are more potent inhibitors than DFMA, the only other known irreversible inhibitor of ADC, against ADC from both bacterial and plant sources. The most potent new inhibitors (FMA, EA and AA) are approximately 100 times as potent as DFMA against ADC activity in *E. coli* extracts. All five compounds caused irreversible inhibition of ADC. However, the inhibition by EA is apparently partially reversible, since enzyme activity was restored by chromatography of the inhibited enzyme on Sephadex G-25 or by overnight dialysis. The compounds are most likely active-site-directed and therefore catalytically activated, since addition of L-arginine to incubations of ADC plus the inhibitors protects the enzyme against inactivation.

Three of the new compounds, FMA, MFMA and Δ-MFMA, should be useful for the inhibition of ADC activity in bacteria. Δ-MFMA was about 10 times as potent as DFMA at inhibiting ADC in growing *E. coli*, whereas FMA was slightly less potent than DFMA in this regard but still active at concentrations of approx. 10–100 μM. It was noteworthy that the combination of α-monofluoromethylputrescine and FMA caused a decrease in intracellular putrescine concentration more dramatic than that previously achieved with combinations of ODC inhibitors and DFMA (Bitonti *et al.*, 1982; Kallio *et al.*, 1982). Even though the depletion of putrescine is enhanced in the presence of the two inhibitors, spermidine increased, similar to the result found previously in our laboratory (Kallio *et al.*, 1982; Bitonti *et al.*, 1982). Nonetheless, FMA or one of the other new inhibitors might be more useful for the depletion of polyamines in *E. coli* and other bacteria than DFMA, particularly if cyclohexylamine, an inhibitor of *E. coli* spermidine synthase (Bitonti *et al.*, 1982), is added to the drug combination. This second generation of ADC inhibitors should also be of great value for the study of the role of polyamines in plant physiology, since numerous studies with DFMA have already given credence to the notion that ADC activity and the polyamines produced by this route are important regulators of plant growth and development (Slocum *et al.*, 1984; Galston, 1983).

Undoubtedly another significant utility for the new, more potent, inhibitors of ADC activity could be in the field of parasitology. A preliminary report (Kierszenbaum *et al.*, 1986) suggests the presence of ADC activity in the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease (South American trypanosomiasis). The existence of ADC in this organism would be exciting, since this polyamine-biosynthetic

enzyme has always been regarded as one that was limited to bacteria and plants.

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