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Fungicidal Activity of the Synthetic Putrescine Analogue, (*E*)-1,4-Diaminobut-2-ene, and Derivatives

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Abstract: The putrescine analogue, (*E*)-1,4-diaminobut-2-ene (E-BED), synthesized as the dihydrochloride salt, controlled five economically important crop pathogens, *Erysiphe graminis* DC f.sp. *hordei* Marchal, *Uromyces viciae-fabae* (Pers.) Schroet, *Botrytis fabae* Sardina, *Podosphaera leucotricha* (Ell. & Ev.) Salm. and *Phytophthora infestans* (Mont) De Bary. The *Z*-isomer, Z-BED, was also fungicidal, although less so than E-BED. Post-inoculation treatment with E-BED gave greater control of powdery mildew infection on barley and rust and chocolate spot on broad bean than did pre-inoculation application. It was also effective *in vitro* against *Botrytis cinerea* Pers. ex Fr., *Pyricularia oxyzae* Br. & Cav. and *Pyrenophora avenae* Ito & Karibay. When *P. avenae* was grown in the presence of E-BED dihydrochloride at 81·5 mg litre^{-1}, growth was reduced by 58% and there were significant reductions in soluble ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) activity. These changes were accompanied by a sevenfold increase in putrescine concentration, a 60% increase in spermine concentration and a 32% reduction in spermidine concentration within the fungal tissue.

1 INTRODUCTION

It has been shown that inhibitors of polyamine biosynthesis can effectively control biotrophic fungal pathogens, e.g. rusts and powdery mildews.[1–3] Much of this early work centred on the use of enzyme-activated irreversible inhibitors, e.g. α-difluoromethylornithine (DFMO), which is an inhibitor of the putrescine biosynthetic enzyme ornithine decarboxylase (ODC).[4] DFMO has been shown to deplete intracellular concentrations of putrescine and spermidine in plant pathogenic fungi.[5] However, an alternative method of polyamine perturbation has been demonstrated involving the use of polyamine analogues. Thus, a variety of analogues was shown to alter polyamine metabolism in tumour cells leading to powerful antiproliferative effects.[6] Indeed, three agmatine analogues were found to be more potent inhibitors of arginine decarboxylase than the enzyme-activated, irreversible inhibitor α-difluoromethylarginine.[7] In recent work, keto-putrescine, a commercially available putrescine analogue, was shown to possess fungicidal properties.[8] This paper describes the synthesis and fungicidal evaluation of two putrescine analogues, (*E*)-1,4-diaminobut-2-ene (E-BED) and (*Z*)-1,4-diaminobut-2-ene (Z-BED), as their dihydrochloride salts (Fig. 1). In addition, the fungicidal properties of other salts and of derivatives of these two compounds were also evaluated.

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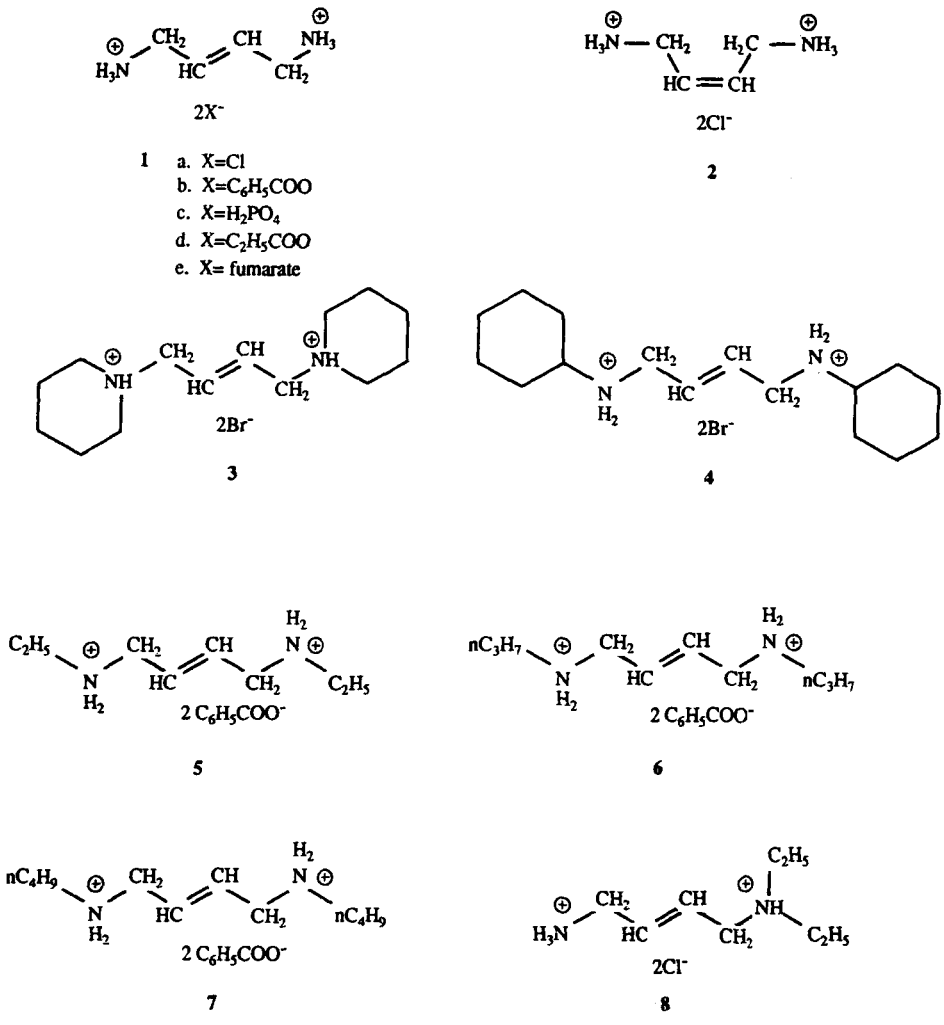

Fig. 1. Structures of compounds studied.

2 EXPERIMENTAL METHODS

2.1 Chemical synthesis

2.1.1 Synthesis of (E)-*1,4-diaminobut-2-ene dihydrochloride* (*E-BED; 1a*)

This synthesis was carried out by adapting the procedure of Macholan.[9] Potassium phthalimide (20 g, 108 mmol) was added in portions over 2 h to a stirred solution of (*E*)-1,4-dibromobut-2-ene (10·7 g, 50 mmol) in *N,N*-dimethylformamide (DMF; 100 ml) at room temperature. The mixture was stirred for a further 3 days at this temperature, then poured into water (100 ml), and the mixture was extracted with dichloromethane (5 × 100 ml). The organic extracts were dried, filtered and concentrated under vacuum to leave DMF (*c.* 30 ml), and a white solid, which was filtered off and washed with ether (3 × 10 ml) to give (*E*)-1,4-diphthalimidobut-2-ene (15·75 g, 91%). [^{1}H]NMR (90 MHz, deuterochloroform) 4·28 (4H, d), 5·92 (2H, m), and 7·79 (4H, m) ppm. Anal: calc. for $C_{20}H_{14}N_2O_4$: C 69·34%, H 4·07%, N 8·09%; found: C 69·22%, H 4·11%, N 8·01%.

(*E*)-1,4-diphthalimidobut-2-ene (15·72 g, 45 mmol) was suspended in glacial acetic acid (160 ml), and concentrated hydrochloric acid (160 ml) was added. The mixture was heated at reflux until all the (*E*)-1,4-diphthalimidobut-2-ene had dissolved, then for a further 24 h. The solution was cooled, filtered and the solvents were concentrated under vacuum to *c.* 10 ml, then 20 ml diethyl ether was added to give a precipitate which was collected and washed with diethyl ether to afford (*E*)-1,4-diaminobut-2-ene dihydrochloride (6·48 g, 90%). [1H]NMR (200 MHz, deuterium oxide) 3·58 (4H, d) and 5·89 (2H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 128·8 (CH), 41·2 (CH_2) ppm. Anal: calc. for $C_4H_{12}N_2Cl_2$: C 30·21%, H 7·60%, N 17·61%; found: C 30·41%; H 7·40%, N 17·78%.

2.1.2 Synthesis of (*E*)-*1,4-diaminobut-2-ene and salts*

(*E*)-1,4-diaminobut-2-ene dihydrochloride (1·21 g, 7·6 mmol) was dissolved in the minimum amount of water (10 ml), added to diethyl ether (100 ml) and stirred vigorously. Potassium carbonate (40 g) was added and the stirring was continued for 0·5 h. The solid was filtered

off and the filtrate was concentrated under vacuum to give (*E*)-1,4-diaminobut-2-ene as an oil (0·46 g, 70%). [^{1}H]NMR (90 MHz, deuterochloroform) 1·80 (4H, s), 3·3 (4H, m) and 5·7 (2H, m) ppm.

2.1.2.1 *E-BED benzoate* (**1b**) (*E*)-1,4-diaminobut-2-ene (1 eq.) was stirred with benzoic acid (2 eq.) in benzene for 1 h. The precipitate was filtered off and washed with ether to afford a white solid (68%). [^{1}H]NMR (200 MHz, deuterium oxide) 3·46 (4H, m), 5·78 (2H, m), and 7·38 (10H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 41·1, 106·3, 129·3, 129·8, 132·8 ppm. IR: 3061, 2032, 1695, 1624, 1547, 1523, 1398 cm^{-1}. MS (*m/z*) 122, 105, 77, 51, 28, 18. Other compounds were prepared by this method.
2.1.2.2 *E-BED phosphate* (**1c**) (2·81 g, 85%). [^{1}H]NMR (200 MHz, deuterium oxide) 3·42 (4H, m) and 5·73 (2H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 41·33, 128·99 ppm. IR: 3069, 3007, 2926, 2872, 1635, 1601, 1466, 1450, 1332, 983, 924 cm^{-1}. MS (*m/z*) 98, 81, 28, 18.

2.1.2.3 *E-BED propionate* (**1d**) (0·41 g, 83%). [^{1}H]NMR (200 MHz, deuterium oxide) 0·84 (6H, t), 1·99 (4H, q), 3·41 (4H, m), and 5·72 (2H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 10·2, 29·9, 41·0, 128·7, 183·5 ppm. IR: 2970, 2934, 1647, 1549, 1406, 976 cm^{-1}. MS (*m/z*) 74, 56, 45, 28, 18.

2.1.2.4 *E-BED fumarate* (**1e**) (0·75 g, 70%). [^{1}H]NMR (200 MHz, deuterium oxide) 3·48 (4H, m) and 5·73 (2H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 41·08, 128·75, 135·49, 172·19 ppm. IR: 3430, 3071, 3009, 1680, 1560, 1450, 1275, 983 cm^{-1}. MS (*m/z*) 116, 98, 88, 45, 27, 18.

2.1.3 (Z)-*1,4-diaminobut-2-ene dihydrochloride (Z-BED; 2)*
A solution of hydrazoic acid (1·0 M in benzene, 30 ml) was added to (*Z*)-2-butene-1,4-diol (1·32 g, 15 mmol) in dry tetrahydrofuran (THF; 20 ml) under nitrogen. A solution of diisopropylazodicarboxylate (6·78 g, 33·9 mmol) in dry THF (10 ml) was then added with stirring. To this mixture was added triphenylphosphine (17·8 g, 67·8 mmol) in dry THF (60 ml), maintaining the reaction temperature at 40°C by cooling. The reaction mixture was stirred for 1 h at room temperature, then heated to 50°C for a further 3 h. Water was added and the heating continued for 3 h. The solvents were removed under vacuum and the residue partitioned between hydrochloric acid (1 M, 80 ml) and dichloromethane (80 ml). The aqueous layer was further extracted with dichloromethane (2 × 80 ml), then evaporated to dryness under vacuum to give a light brown solid. Recrystallization from aqueous ethanol/acetone gave the title compound in 63% yield. [^{1}H]NMR (200 MHz, deuterium oxide) 5·92 (m, 2H), 3·81 (m, 4H) ppm [^{13}C]NMR (50 MHz, deuterium oxide) 127·8 (CH), 37·0 (CH_2) ppm. MS (*m/z*) 69, 56, 43, 36, 30.

2.1.4 (E)-*1,4-bis(cyclohexylamino)-2-butene dihydrobromide (Dichex-E-BED; 4)*
To a stirred solution of cyclohexylamine (2·97 g, 30 mmol) in chloroform (25 ml) was added (*E*)-1,4-dibromobut-2-ene (2·14 g, 10 mmol) in chloroform (75 ml) dropwise at room temperature, and the solution was stirred for 24 h. The solvents were removed under vacuum to give a white solid, which was recrystallized from methanol to yield the title compound, 2·31 g (56%). [^{1}H]NMR (200 MHz, deuterium oxide) 5·90 (m, 2H), 3·62 (m, 4H), 3·00 (m, 2H), 0·70–2·11 (m, 20H) ppm. [^{13}C]NMR (5·0 MHz, deuterium oxide) 129·3 (CH), 57·6 (CH), 45·9 (CH_2), 29·7 (CH_2), 25·4 (CH_2), 24·8 (CH_2) ppm. Anal: calc. for $C_{16}H_{32}N_2Br_2$: C 46·62%, H 7·82%, N 6·80%; found C 46·90%, H 7·66%, N 6·72%.

2.1.5 (E)-*1,4-bis-*(N-*piperidyl*)-2-*butene dihydrobromide (Dipip-E-BED; 3)*
To a stirred solution of piperidine (2·55 g, 30 mmol) in chloroform (30 ml) was added (*E*)-1,4-dibromobut-2-ene (2·14 g, 10 mmol) in chloroform (70 ml) dropwise at room temperature, and the solution was stirred for 24 h. The solvents were removed under vacuum to give a white solid, which was recrystallized from methanol to yield the title compound, 2·30 g (60%). [^{1}H]NMR (200 MHz, deuterium oxide) 5·99 (m, 2H), 3·69 (m, 4H), 2·70 (s, 12H) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 130·2 (CH), 58·9 (CH_2), 43·2 (CH_3) ppm. Anal: calc. for $C_{14}H_{28}N_2Br_2$: C 43·77%, H 7·35%, N 7·29%; found: C 43·49%, H 7·39%, N 7·33%.

2.1.6 (E)-N,N′-*diethyl-1,4-diaminobut-2-ene dihydrobenzoate (E-DED; 5)*
Free base 0·2 g, 28%; salt 1·8 g, 33%. [^{1}H]NMR (200 MHz, deuterium oxide) 1·06 (6H, t), 2·83 (4H, q), 3·48 (4H, m), 5·79 (2H, m) and 7·58 (10H, m) ppm. [^{1}H]NMR (50 MHz, deuterium oxide) 11·3, 43·1, 48·2, 129·3, 130·0, 133·1, 174·4 ppm. IR: 2945, 2799, 1686, 1655, 1581, 1448, 1380 cm^{-1}. MS (*m/z*) 122, 105, 97, 77, 51, 44, 28, 18.

2.1.7 (E)-N,N′-*dipropyl-1,4-diaminobut-2-ene dihydrobenzoate (E-DPD; 6)*
Free base 0·73 g, 85%; salt 1·25 g, 68%. [^{1}H]NMR (200 MHz, deuterium oxide) 0·71 (6H, t), 1·41 (4H, m), 2·66 (4H, t), 3·42 (4H, m) 5·77 (2H, m) and 7·57 (10H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 11·2, 20·0, 48·7, 49·3, 129·3, 130·1, 135·6, 174·2 ppm. IR: 2964, 2941, 1709, 1583, 1448, 1381 cm^{-1}. MS (*m/z*) 122, 112, 105, 82, 77, 51, 39, 28, 18.

2.1.8 (E)-N,N′-*dibutyl-1,4-diaminobut-2-ene dihydrobenzoate (E-DBD; 7)*
Free base 0·45 g, 45%; salt 2·03 g, 91%. [^{1}H]NMR (200 MHz, deuterium oxide) 0·68 (6H, t), 1·12 (4H, m), 1·40 (4H, m), 2·78 (4H, t), 3·47 (4H, m), 5·78 (2H, m) and 7·42 (10H, m) ppm. [^{13}C]NMR (50 MHz, deuterium

oxide) 13·5, 19·9, 28·3, 47·6, 48·7, 113·2, 129·0, 129·1, 129·5, 132·0 ppm. IR: 2955, 2930, 1628, 1599, 1560, 1375 cm^{-1}. MS (m/z) 126, 122, 105, 82, 77, 72, 55, 51, 28, 18. Anal: calc. for $C_{26}H_{38}N_2O_4$: C 70·58%, H 8·59%, N 6·33%; found: C 70·54%, H 8·86%, N 6·21%.

2.1.9 (E)-N,N'-*diethyl-1,4-diaminobut-2-ene dihydrochloride* (*E-UDED; 8*)

This procedure was based on the work of Robins.[10] Potassium phthalimide (18·5 g, 0·1 mol) was added in portions over 5 h to a stirred solution of (*E*)-1,4-dibromobut-2-ene (21·4 g, 0·1 mol) in acetone (200 ml) at 85°C. The suspension was stirred for 24 h at 85°C, then cooled and filtered. The filtrate was concentrated under vacuum to afford a white solid, which was recrystallized three times from acetone to yield (*E*)-1-phthalimido-4-bromobut-2-ene (15·75 g, 56·2%). [^{1}H]NMR (90 MHz, deuterochloroform) 3·88 (2H, d), 4·28 (2H, d), 5·92 (2H, m) and (4H, m) ppm. Anal: calc. for $C_{12}H_{10}NO_2$ Br: C 51·45%, H 3·60%, N 5·00%; found: C 51·66%, H 3·64%, N 5·01%.

The second stage of this synthesis was adapted from the procedure of Samejima *et al.*[11] (*E*)-1-phthalimido-4-bromobut-2-ene (4·2 g, 15 mmol), diethylamine (1·1 g, 15 mmol) and potassium fluoride supported on Celite (7·5 g) were stirred together in acetonitrile (75 ml) at 40°C for 18 h. The solution was filtered, and the filtrate was concentrated under vacuum to afford an oil, which was heated at reflux for 30 h. The solution was cooled, filtered, and the solvents were removed under vacuum to afford (*E*)-*N,N*-diethyl-1,4-diaminobut-3-ene dihydrochloride as a solid (1·32 g, 41%). [^{1}H]NMR (200 MHz, deuterium oxide) 1·17 (6H, t) 3·14 (4H, q) 3·65 (4H, m) and 5·95 (2H, m) ppm. [^{13}C]NMR (50 MHz, deuterium oxide) 9·4 (CH_3) 42·3 (CH_2) 49·5 (CH_2) 129·0 (CH) and 129·7 (CH) ppm. Anal: calc. for $C_8H_{20}N_2Cl_2$: C 44·66%, H 9·37%, N 13·02%; found: C 44·79%, H 9·12%, N 12·88%.

2.2 Determination of the fungicidal effects of the putrescine analogues

(*E*)-1,4-diaminobut-2-ene, (*Z*)-1,4-diaminobut-2-ene (as their dihydrochlorides) and other inhibitors were applied to plants in aqueous solution containing 0·1 ml $litre^{-1}$ 'Tween' 20. Barley and bean seedlings were sprayed to run-off with these solutions (usually 1 mM, unless stated otherwise) using a Shandon spray unit, 3 h before or 3 days after inoculation with pathogens. Apple seedings were sprayed 3 days after inoculation. The only exception to this procedure was in tests using the *Phytophthora infestans* (Mont) De Bary/potato system, where potato leaf discs were floated on an aqueous solution of the compounds.

2.2.1 Determination of protectant and curative action against powdery mildew (Erysiphe graminis *DC f.sp* hordei *Marchal*) *on barley*

Barley seedlings (*Hordeum vulgare* L. cv. Golden Promise) were grown in Fison's Levington compost in 36-cm seed trays. Plants were grown in a glasshouse under natural daylight supplemented for 16 h daily by 400-W mercury vapour lamps. The maximum temperature was 24°C during the day and 9°C at night. Plants at Zadoks growth stage 12 (second leaf unfolded) were sprayed before or after inoculation with the powdery mildew fungus. To study the effects of the time of application on infection, E-BED was applied to barley seedlings 1, 2 and 5 days pre- or post-inoculation with mildew. E-BED was also compared to four commercial fungicides: Propiconazole (250 mg a.i. $litre^{-1}$; 216 g kg^{-1} emulsifiable concentrate); tridemorph (360 mg a.i. $litre^{-1}$; 998 g kg^{-1} emulsifiable concentrate), flutriafol (125 mg a.i. $litre^{-1}$; 120 g kg^{-1} suspension concentrate) and fenpropidin (750 mg a.i. $litre^{-1}$; 824 g kg^{-1} emulsifiable concentrate). In these experiments, all treatments were applied post-inoculation, and E-BED was applied in 0·1 ml $litre^{-1}$ 'Agral' 90. Plants were inoculated simply by dusting them with conidia of *E. graminis* f.sp *hordei*. Infection intensity was assessed 6–10 days after inoculation by estimating the percentage leaf area with pustules using a standard area diagram. Sporulation usually occurred about 6–7 days after inoculation.

2.2.2 Determination of protectant and curative action against rust (Uromyces viciae-fabae *(Pers.) Schroet.*)

Seeds of broad bean (*Vicia faba* L. cv. Express Long Pod) were sown in Fison's Levington compost in 15-cm plastic pots. Plants were grown in a ventilated glasshouse as described above and were used in experiments when they were 20 days old. Bean seedlings were inoculated by painting a spore suspension (25 mg per 100 ml distilled water) onto the leaves with a camel-hair brush. Plants were then covered with clear plastic bags for 48 h in order to maintain the high relative humidity necessary for spore germination. Infection intensity was assessed 17 days after inoculation by estimating the percentage leaf area infected with rust pustules using a standard area diagram. Sporulation usually occurred about 6–7 days after inoculation.

2.2.3 Determination of protectant and curative action against chocolate spot (Botrytis fabae *Sardina*) *on broad bean*

Broad beans were grown as described above. Plants were inoculated by painting leaves with a spore suspension (approx. 4×10^5 conidia ml^{-1}), after which they were loosely covered with clear plastic bags for 48 h in order to provide the high relative humidity required for spore germination. Infection intensity was assessed 7 days after inoculation by estimating the percentage leaf area infected using a standard area diagram.

2.2.4 Determination of the curative action against powdery mildew (Podosphaera leucotricha *(Ell. & Ev.) Salm.) on apple*
Seeds of apple (*Malus bitenfelder*) were stratified by placing them in cold storage (−10°C) for 14 weeks in trays of Fison's Levington compost. After this time the seeds were removed from cold storage and, after 10 days in a heated, ventilated glasshouse, seeds which had germinated were potted individually into 4-cm pots. After a further 12 days, the seedlings were inoculated by gently brushing mildew conidia onto the leaves. Infection intensity was assessed 17 days after inoculation using an infection key. Sporulation occurred about 12–14 days after inoculation.

2.2.5 Determination of the curative action against late blight (Phytophthora infestans) *on potato*
Potato leaf discs (15 mm diameter) were floated on solutions of the inhibitors in plastic Petri dishes. The leaf discs were inoculated with *P. infestans* by pipetting a sporangial suspension (0·1 ml) onto the surface of each leaf disc. Infection intensity was assessed 6 days later using an infection key.

2.3 Measurement of the in-vitro antifungal effects of the putrescine analogues

The effects of E-BED and Z-BED on the mycelial growth of *Botrytis cinerea* Pers. ex Fr., *Pyricularia oryzae* Br. & Cav. and *Pyrenophora avenae* Ito & Kuribay were examined. Filter-sterilized aqueous solutions (10 ml) containing the compounds were added to 140 ml of sterile potato dextrose agar (PDA) at 45–47°C to obtain final concentrations of E-BED and Z-BED dihydrochlorides of 163 and 160 mg litre^{-1} respectively. Control plates contained culture medium only.

Sterile medium (20 ml) containing the analogue was added aseptically to each 90-mm single-vent sterile plastic Petri dish. To obtain inoculum, a sterile 10 mm-diameter cork borer was used to remove plugs of mycelium from the edges of stock cultures. The mycelial plugs were inverted and one placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24°C. Colony growth was measured radially, beyond the 10-mm plug of inoculum, 3, 6 and 8 days after inoculation. Three measurements were made from each Petri dish. All results are the means of six replicates and each experiment was repeated with similar results.

2.4 Measurement of the effects of E-BED and Z-BED on growth, enzyme activities and polyamine concentrations in *Pyrenophora avenae*

Filter-sterilized solutions (10 ml) containing E-BED and Z-BED as their dihydrochloride salts were added to sterile liquid potato dextrose agar (140 ml) in 250-ml flasks, to obtain final concentrations of 81·5 and 80 mg litre^{-1} (0·5 mM each) respectively. These concentrations were used since there was insufficient growth at 1 mM for biochemical analyses. Each flask was then inoculated with a 10-mm disc of mycelium and placed in a Gallenkamp orbital shaker (140 rev min^{-1}) at 24°C. After 4 days, the fungus was washed with distilled water through a fine-mesh sieve and centrifuged at 16 000 g for 10 min. The pellet obtained was weighed and then used for enzyme and polyamine analysis.

The activities of ornithine decarboxylase (ODC; EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) were assayed, and polyamine concentrations determined, as described previously.[5]

3 RESULTS

3.1 Fungicidal activity of E-BED and Z-BED

E-BED and Z-BED applied as their dihydrochlorides, either as pre-inoculation or post-inoculation sprays, gave substantial control of several plant–pathogen interactions (Table 1). However, although E-BED gave greater control of barley powdery mildew and broad bean rust than Z-BED, both compounds provided similar control of chocolate spot on broad bean. E-BED was most effective against infection of broad bean by the chocolate spot pathogen, *B. fabae*, where post-inoculation treatments reduced infection by 91% (Table 1). Although application of E-BED 5 days before inoculation gave substantial control of mildew infection on barley seedlings (65%), best control was achieved when E-BED was applied 2 or 5 days after inoculation (75%; Table 2).

In a glasshouse evaluation where all compounds were applied as post-inoculation treatments, E-BED reduced mildew infection on barley by 70% compared to an 86% reduction obtained using the commercial fungicide, fenpropidin (Table 3). Various salts of E-BED also reduced powdery mildew infection on barley seedlings when used as post-inoculation sprays. Greatest control was obtained using 236 mg litre^{-1} of the propionate or 204 mg litre^{-1} of the furmarate salts of E-BED (69% and 65%, respectively; Table 4). Derivatives of E-BED and Z-BED also provided substantial control of powdery mildew on barley, when applied as post-inoculation sprays. Best control was achieved using a 384 mg litre^{-1} spray of compound **4** (41%; Table 5).

3.2 Effects of E-BED and Z-BED on growth, enzyme activities and polyamine concentrations in *Pyrenophera avenae*

Growth of *P. avenae* was significantly reduced in the presence of E-BED, with 81·5 mg litre^{-1} giving 58% reduction (Table 6). Z-BED had little effect on growth but slightly decreased soluble ODC (31%) and increased soluble AdoMetDC activity (83%; Table 7). In contrast,

TABLE 1
Fungicidal Activity of E-BED[a]

A *Pathogen–plant interaction*	*Leaf area infected (%)*			*Disease control (%)*	
	Control	*E-BED*	*Z-BED*	*E-BED*	*Z-BED*
Erysiphe graminis/barley					
Pre-inoculation	18·9 (±2·75)	5·8 (±0·63)	8·9 (±0·99)	70	53
Post-inoculation	18·9 (±2·75)	3·8 (±0·41)	11·1 (±1·12)	80	42
Uromyces viciae-fabae/broad bean					
Pre-inoculation	22·5 (±2·60)	10·0 (±2·00)	12·9 (±3·10)	56	43
Post-inoculation	22·5 (±2·60)	6·3 (±2·60)	10·0 (±1·11)	72	56
Botrytis fabae/broad bean					
Pre-inoculation	3·2 (±0·68)	0·5 (±0·14)	0·6 (±0·11)	85	82
Post-inoculation	3·2 (±0·68)	0·3 (±0·12)	0·7 (±0·39)	91	79

B *Pathogen–plant interaction*	*Mean disease score*[b]		
	Control	*E-BED*	*Z-BED*
Phytophthora infestans/potato[c]	3	1	1
Podosphaera leucotricha/apple[c]	3	1	1

All treatments differed significantly from the control at $P \leqslant 0{\cdot}001$.
[a] E-BED dihydrochloride was applied at 163 mg litre^{-1} (1 mM).
[b] Late blight on potato leaf discs was assessed using the following key: 1 = A few isolated sporophores, 2 = <50% infection, 3 = >50% infection. Powdery mildew on apple was assessed using the following key: 0 = No infection, 1 = A few isolated spores, 2 = <50% infection, 3 = >50% infection.
[c] Post-inoculation treatments only.

TABLE 2
Effect of Timing of E-BED Dihydrochloride Application, at 163 mg litre^{-1} (1 mM), on Infection of Barley with the Powdery Mildew Fungus, *Erysiphe graminis*

		Leaf area infected (%) Pre-inoculation treatment		
A	*Control*	*5 days*	*2 days*	*1 day*
	15·9 (±1·71)	5·5 (±0·49)[b]	7·4 (±1·78)[a]	5·7 (±1·38)[b]
		Post-inoculation treatment		
B	*Control*	*5 days*	*2 days*	*1 day*
	15·9 (±1·71)	4·0 (±0·44)[b]	4·1 (±0·85)[b]	5·3 (±0·43)[b]

[a,b] Significantly different from the control at $P \leqslant 0{\cdot}01$ and $P \leqslant 0{\cdot}001$, respectively.

E-BED reduced both soluble ODC and soluble AdoMet-DC activity (87 and 82% respectively; Table 7). Exposure to E-BED increased putrescine concentration in fungal tissues (760%), increased spermine concentration (60%) and decreased spermidine concentration (32%; Table 7). Z-BED gave no significant change in putrescine or spermine concentration but reduced spermidine concentration by 41% (Table 7).

4 DISCUSSION

E-BED and Z-BED, (*E*)- and (*Z*)-isomers, respectively, of a putrescine analogue, have been shown here to possess substantial fungicidal activity, controlling powdery mildew on barley and apple, rust and chocolate spot on broad bean and late blight on potato. However, E-BED was generally more active than Z-BED. E-BED, applied

TABLE 3
Comparison of the Effects of E-BED and Commercial Fungicides on Infection of Barley with Powdery Mildew

Treatment	*Leaf area infected (%)*	*Disease control (%)*
Control	4·4 (±0·37)	
E-BED[a]	1·3 (±0·13)	70
Propiconazole[b]	0·9 (±0·09)	80
Tridemorph[b]	0·8 (±0·10)	82
Flutriafol[b]	2·4 (±0·20)	45
Fenpropidin[b]	0·6 (±0·10)	86

All treatments differed significantly from the control at $P \leqslant 0{\cdot}001$.
[a] E-BED dihydrochloride was used at 163 mg litre^{-1} (1 mM).
[b] Propiconazole, tridemorph, flutriafol and fenpropidin were used at 250, 360, 125 and 750 mg litre^{-1} respectively (see Section 2.2.1).

TABLE 4
Comparison of the Effects of Different Salts of E-BED on Infection of Barley with Powdery Mildew

Treatment[a]	*Leaf area infected (%)*	*Disease control (%)*
Control	19·0 (±1·94)	
E-BED dihydrobromide	12·2 (±1·03)[b]	36
Control	15·7 (±0·82)	
E-BED dihydrobenzoate	6·8 (±0·46)[c]	57
E-BED phosphate	9·1 (±0·52)[c]	42
E-BED fumarate	5·5 (±0·45)[c]	65
E-BED dihydropropionate	4·9 (±0·55)[c]	69

[a] Salts were used at 1 mM, i.e. 316, 288, 222, 204 and 236 mg litre^{-1} respectively, and were applied as post-inoculation treatments.
[b,c] Significantly different from the control at $P \leqslant 0{\cdot}01$ and $P \leqslant 0{\cdot}001$, respectively.

TABLE 5
Effects of Derivatives of E-BED on Powdery Mildew of Barley

Treatment[a]	*Leaf area infected (%)*	*Disease control (%)*
Control	22·0 (±2·21)	
Compound **3**	17·0 (±1·37)	23
Compound **4**	12·9 (±1·00)[c]	41
Control	16·0 (±1·14)	
Compound **5**	12·9 (±0·71)[b]	20
Compound **6**	12·9 (±0·75)[b]	20
Compound **7**	13·7 (±0·77)	14
Compound **8**	15·9 (±1·38)	1

[a] Derivatives were used at 1 mM i.e. 412, 384, 384, 408, 432 and 215 mg litre^{-1} respectively and were applied as post-inoculation treatments. All compounds were used as salts (see Fig. 1).
[b,c,d] Significantly different from the control at $P \leqslant 0{\cdot}05$, $P \leqslant 0{\cdot}01$ and $P \leqslant 0{\cdot}001$, respectively.

TABLE 6
Effect of E-BED and Z-BED on in-vitro Growth of *Botrytis cinerea*, *Pyricularia oryzae* and *Pyrenophora avenae*

	Mycelial growth (mm)		*Mycelial weight (g)*
Treatment	*B. cinerea*	*P. oryzae*	*P. avenae*
Control	17·0 (±1·20)	27·9 (±0·20)	3·8 (±0·22)
E-BED[a]	2·2 (±0·30)[b]	25·6 (±0·20)[b]	1·6 (±0·03)[b]
Z-BED[a]	7·4 (±0·90)[b]	24·9 (±0·30)[b]	3·4 (±0·26)

[a] E-BED and Z-BED as their dihydrochlorides were used at 163 and 160 mg litre^{-1} (1 mM) against *B. cinerea* and *P. oryzae* and at 81·5 and 80 mg litre^{-1} (0·5 mM) against *P. avenae*.
[b] Significantly different from the control at $P \leqslant 0{\cdot}001$.

as a post-inoculation treatment against barley powdery mildew, produced an EC_{50} value of 10·58 mg litre^{-1} (data not shown), which compares very favourably with an EC_{50} value of 61 mg litre^{-1} obtained for the commercially available putrescine analogue, keto-putrescine, against the same pathogen.[8]

In a time-course experiment against *E. graminis*, greatest control was achieved when E-BED was applied 5 days after inoculation. This control may well be related to perturbation of polyamine biosynthesis in the germinating conidia on the leaf surface. It has been shown previously that the ODC inhibitor DFMO can inhibit the germination of rust uredospores.[12] Moreover, germination of conidia of *Aspergillus nidulans* (Eidam) Winter was delayed in the presence of keto-putrescine.[13]

In the glasshouse, E-BED compared favourably to four commercially available fungicides and outperformed flutriafol. Although E-BED gave only 70% disease control, it was used at a lower concentration than the other compounds, with the exception of flutriafol. The greatest disease control was achieved using a 750 mg litre^{-1} spray of fenpropidin (86%). This difference may reflect the superior formulation of the fenpropidin and the other active ingredients, since E-BED was applied at 163 mg litre^{-1} in 0·1 ml litre^{-1} 'Agral' 90.

E-BED was originally synthesized as the dihydrochloride salt. Five other salts were tested and gave reasonable control of powdery mildew on barley (ranging from 36% to 69%), although none was superior to the dihydrochloride salt. Various derivatives of E-BED and Z-BED were tested; the activity of these compounds was variable against *E. graminis* on barley seedlings, best

TABLE 7
Effects of E-BED and Z-BED on ODC and AdoMetDC Activity and on Polyamine Concentrations in *Pyrenophora avenae*

A Treatment[a]	*Enzyme activity (pmol CO_2 mg protein h^{-1})*	
	ODC	*AdoMetDC*
Control	7·7 (±0·62)	7·4 (±1·50)
E-BED (81·5 mg litre^{-1})	1·0 (±0·08)[b]	1·3 (±0·66)[b]
Z-BED (80 mg litre^{-1})	5·3 (±0·43)[c]	13·5 (±2·09)[b]

B Treatment[a]	*Polyamine concentration (μmol g^{-1} f.wt)*		
	Putrescine	*Spermidine*	*Spermine*
Control	62 (±11·1)	199 (±11·5)	47 (±3·4)
E-BED (81·5 mg litre^{-1})	538 (±25·2)[b]	135 (±6·3)[b]	76 (±6·9)[b]
Z-BED (80 mg litre^{-1})	62 (±19·6)	116 (±4·9)[b]	57 (±3·8)

[a] E-BED and Z-BED used as dihydrochlorides.
[b,c] Significantly different from the control at $P \leqslant 0{\cdot}001$ and $P \leqslant 0{\cdot}01$, respectively.

control being achieved by 384 mg litre^{-1} of Dichex-E-BED dihydrobromide (Compound **4**; 41%).

E-BED and Z-BED both gave significant reductions in mycelial growth of *B. cinerea*, *P. oryzae* and *P. avenae*. In particular, E-BED inhibited growth of *B. cinerea* by 87% and *P. avenae* by 58%. Again, these results compare favourably with those obtained for keto-putrescine, where growth of *B. cinerea* was reduced by only 27% and *P. avenae* by only 25% (although in the case of *P. avenae* growth was on solid media and may not be directly comparable).[8,14]

E-BED was shown to alter polyamine biosynthesis in *P. avenae*. Thus, soluble ODC and AdoMetDC activities were reduced by 87% and 82% respectively. Interestingly, these reductions were accompanied by a very significant accumulation of putrescine within the cells, and a 33% reduction in spermidine. The latter could be accounted for by the reduced AdoMetDC activity. It is possible that E-BED reduces putrescine catabolism and/or reduces putrescine efflux from fungal cells. Preliminary experiments have indicated that treatment with E-BED leads to a substantial reduction in DAO activity in *P. avenae* (Havis & Walters, unpublished results). Nevertheless, the inhibition of fungal growth produced by E-BED may be due to the high putrescine levels which accumulated in the fungus. Davis and Ristow[15] have shown that in a mutant of *Neurospora crassa* Shear & Dodge which concentrates polyamines, addition of 5 mM putrescine to the medium led to an intracellular concentration of greater than 200 nmol putrescine mg^{-1} dry weight (nominally 80 mmol). Under these circumstances, *N. crassa* grew at half the normal rate. In *P. avenae* treated with E-BED, the putrescine pool increased from 62 μmol g^{-1} fresh weight to 540 μmol g^{-1} fresh weight (*c.* 65 mmol). This elevated putrescine concentration may be responsible, in part, for the antifungal effects of E-BED. Putrescine could be acted upon by diamine oxidase to yield free radicals and hydrogen peroxide, both of which could damage membranes. Such a mechanism has been shown to be responsible for putrescine-induced wounding in maize roots.[16] High intracellular putrescine levels have also been shown to be toxic in the cyanobacterium *Anabaena*, where the lethal effect of putrescine was believed to be the result of conjugation with cellular ribosomes.[17]

Z-BED produced a small decrease in soluble ODC activity and an increase in soluble AdoMetDC activity (83%) in *P. avenae*. However, fungal tissue from inhibitor-amended media contained 41% less spermidine than the control, while spermine levels were increased slightly, although this was not significant. Moreover, growth of *P. avenae* was not significantly altered by the presence of Z-BED in the medium. Since spermidine is known to be important for fungal growth, in some fungi being an absolute requirement, e.g. in *N. crassa*,[16] it may be that the spermidine pool present in the fungus (117 μmol g^{-1} fresh weight) was sufficient to support fungal growth. Putrescine is an obligatory activator of AdoMetDC in both mammals and fungi.[18] It is therefore interesting that the isomers E-BED and Z-BED, both putrescine analogues, produce such different effects on AdoMetDC activity in *P. avenae*. Thus, E-BED decreases AdoMetDC activity, while Z-BED stimulates activity.

Since powdery mildew cannot be grown axenically and rusts can be cultured *in vitro* only with difficulty, the mode of action of these two compounds against these pathogens cannot be fully determined.

In conclusion, E-BED, and to a substantially lesser extent Z-BED, possess fungicidal activity, which may be related to a perturbation of polyamine biosynthesis.[19] These results, together with those for keto-putrescine,[8] show that perturbation of polyamine biosynthesis using polyamine analogues provides a useful alternative mode of action for the development of new fungicides.

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