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An antibacterial pyrazole derivative from *Burkholderia glumae*, a bacterial pathogen of rice

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ABSTRACT

Burkholderia glumae, a bacterial pathogen on rice, produced compounds in liquid culture that, in agar diffusion assays, gave strong inhibitory action against *Erwinia amylovora*, the bacterium responsible for fire blight disease of apple and pear trees. Products were isolated from culture medium by cation exchange and then purified by bioassay-guided chromatographic methods. Two major products were obtained, one of which was not active when fully purified. Each product showed a single ninhydrin-staining spot on TLC and a single HPLC peak. The non-active product was deduced from NMR, MS, and chemical data, to be the tripeptide L-alanyl-L-*homo*serinyl-L-aspartate. The NMR data for the active product demonstrated that it contained the same tripeptide, but functionalised at the β-carboxyl of the C-terminal aspartate, by a moiety that provided an additional 98 mass units to the parent tripeptide. Various data led to the interpretation that this moiety was a highly unusual oxygenated pyrazole structure, and thus the bioactive product was deduced to be 3-[L-alanyl-L-*homo*serinyl-L-aspartyl-β-carboxy]-4-hydroxy-5-oxopyrazole. This compound was found to inhibit the growth of a number of different bacterial species.

1. Introduction

Fire blight disease, caused by the bacterium *Erwinia amylovora*, is a serious and increasing problem for apple and pear orchardists in many parts of the world (Hale and Mitchell, 2002). Severe outbreaks of this disease can cause significant economic loss to growers and also necessitate removal of infected trees. Various options for the control of fire blight are based on biological, chemical and orchard management methods. In the past the preference for chemical control has been spray applications of streptomycin, but agricultural use of streptomycin is now becoming limited. Because of the paucity of chemical control options we have searched for new candidates that might be suitable for such an application, either in their own right or by way of chemical modification. A target source for new chemical products has been plant pathogenic species of *Pseudomonas* and *Burkholderia*, because many potently bioactive products have been previously found from these organisms, some with antibacterial activity (Bender et al., 1999; Mitchell, 1991). In a survey of 121 strains, 34 were identified as candidates for closer investigation for anti fire blight products (Mitchell, 1993). Previously we have reported on some new iminopyrrolidine products from *Burkholderia plantarii*, with strong *in vitro* inhibitory activity against *E. amylovora* (Mitchell and Teh, 2005). We report here the chemical structure of a new compound isolated from liquid cultures of the rice pathogen *Burkholderia glumae* (syn *Pseudomonas glumae*). The compound consists of a substituted pyrazole, linked to the aspartate-β-carboxyl of the tripeptide L-alanyl-L-*homo*serinyl-L-aspartate. The compound has high *in vitro* inhibitory activity in an agar minimal medium against the growth of *E. amylovora*.

2. Results and discussion

Liquid cultures of *B. glumae* were centrifuged to remove bacterial cells and then passed through a cation exchange resin. The inhibitory activity was recovered from the resin by elution with 5% ammonia solution, and then subjected to various purification sequences (chromatography on LH20 Sephadex, and ion-exchange Sephadex). The course of isolation and purification was monitored by the inhibition of *E. amylovora* by agar diffusion assay. Concurrent TLC and TLE analyses of fractions with inhibition activity, indicated that two ninhydrin-staining components (compounds **1** and **2**) in particular were prominent. However, one of these, compound **1**, had no biological activity when it was fully purified, while compound **2** retained strong inhibitory activity. Because ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** had striking similarities, we chose first to establish the nature of compound **1**. FAB-MS showed MH^+ at 306 and accurate mass data established a MF $C_{11}H_{19}N_3O_7$. Hydrolysis with 6 M HCl and 2D TLE/TLC analysis indicated the

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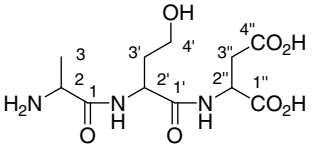
amino acid components to be alanine, aspartic acid and *homo*serine, all with the L-configuration by GC analysis. ^{1}H and ^{13}C NMR data were in full accord with these three amino acids (Table 1), and a linear tripeptide of them has the MF as deduced by MS. When the tripeptide was derivatised with 2,4-dinitrofluorobenzene and then hydrolysed, 2,4-dinitroalanine was obtained (TLC analysis) and thus alanine is the N-terminal amino acid. The electrospray MS had a strong fragment ion at *m*/*z* 173, assigned to an ala-homoserinyl ion arising from amide cleavage, and thus the tripeptide sequence is established, with Asp being C-terminal. Daughter-ion MS analysis was consistent with a C-terminal aspartic acid. Compound **1** is therefore L-alanyl-L-*homo*serinyl-L-aspartic acid (structure **1**).

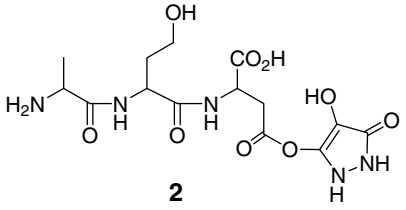
The structure of compound **2** was clearly closely related to **1**. ^{1}H and ^{13}C NMR, and 2D experiments (COSY, HSQC) showed the presence of the same three amino acids as in **1**, and therefore it would be reasonable to expect the same amino acid sequence. In fact, the only significant difference in the ^{1}H NMR spectrum of compound **2** was the chemical shift of the two aspartyl C-3 protons (δ 3.36 and 3.27 ppm), downfield shifts of 0.67 and 0.68 ppm from δ 2.69 and 2.59 in **1** (Table 1). Although the coupling pattern of these was the same (dd), there were substantial changes in the coupling constants (e.g. the geminal coupling in compound **2** 18.6 Hz up from 15.9 Hz in **1**). These data indicate an ester moiety linked to the aspartyl C-4 carboxyl, and the electronic and spatial effects of this moiety on the protons at C-3 must account for the altered NMR characteristics from the "parent" carboxylic acid. Consistent with the presence of an ester-linked moiety in compound **2**, the ^{13}C NMR had only **1** carboxylic acid signal, compared with two such signals in **1** (Table 1). Overall, the ^{13}C NMR of compound **2** was similar to that of **1**, but in addition, there were three weaker signals present in the carbonyl region.

Mass spectral data (FAB and ES) demonstrated MH^+ at *m*/*z* 404, with an accurate mass indicative of a MF $C_{14}H_{21}N_5O_9$. Furthermore, diagnostic (ES) fragment ions (from amide cleavages) appeared at *m*/*z* 333 [M −71], suggestive of loss of a terminal alanyl unit, and at *m*/*z* 173, indicative of an Ala-*homo*serinyl fragment. These data, supported by accurate mass measurements, are consistent with the presence of the tripeptide Ala-*homo*ser-asp as a major component of the compound **2** structure.

The calculated MW and MF of compound **2**, 403 and $C_{14}H_{21}N_5O_9$, differ from those for Ala-*homo*serinyl-asp (**1**) by 98 mass units and $C_3H_2N_2O_2$, respectively, and these values can therefore be assigned to the chemical constitution of the C-4 aspartyl ester unit. Accordingly, this must be a heterocyclic entity to accommodate the observed data satisfactorily, and the most likely candidates appear to be either imidazole or pyrazole structures. We envisage 4 possible structures (**2–5**) based on these two parent heterocycles that would satisfactorily fit these MS data. In the ES spectral analysis of compound **2**, a fragment ion at *m*/*z* 343 that derives from *m*/*z* 386 (loss of H_2O from MH^+) was shown by accurate mass measurements to be the result of a loss of O=C–NH (43 amu), which may be a fragment from the pyrazole ring structure as depicted in **2**. Another fragment at *m*/*z* 325 (368–O=C–NH) was similarly derived from the ion *m*/*z* 368 (MH^+-2H_2O).



1



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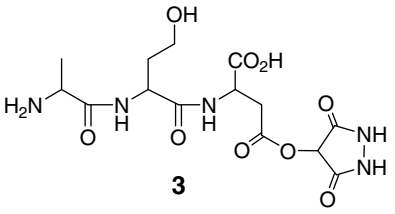

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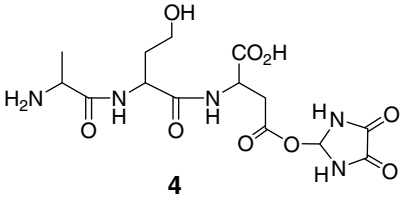

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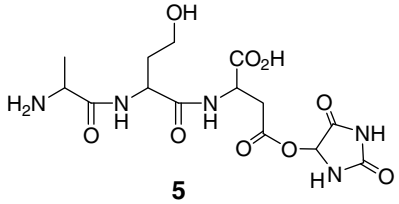

5

NMR data for structures **3–5** would be expected to show a ^{13}C NMR chemical shift for the –O–C–H ring carbon between 60 to 100 ppm, and indeed we found for 5-hydroxyhydantoin (ester moiety corresponding to structure **5**) that this was 77.9 ppm (in CD_3CN) or 74.8 ppm (in H_2O). Because the ^{13}C spectrum of compound **2** had no such signal – all the carbon signals below 100 ppm were fully accounted for by the amino acid components – structures **3–5** are untenable. On this basis, structure **2** is the sole tenable structure for compound **2**, where the ring carbons would all be expected to have ^{13}C chemical shifts above 150 ppm. In support of this proposal, we have sought exhaustively to gain unambiguous evidence for the ^{13}C carbon signals at lower field (carbonyl region), with various preparations of the compound. The results obtained, summarised in Table 1 as a composite from several sets of data, show the presence of seven carbon signals of this type, and are therefore consistent with our overall interpretations. Our work with compound **2** has frequently been hampered by a small degree of undefined instability, and we believe the chemistry of the pyrazole ester moiety of structure **2** could easily account for these events.

Table 1
^{1}H and ^{13}C assignments for compounds **1** and **2** in D_2O (ext Me_4Si)

Position		Compound **1**		Compound **2**	
		^{1}H δppm	^{13}C δppm	^{1}H δppm	^{13}C δppm
1		–	173.45 or 175.24	–	Unassigned[a]
2	1H	4.10 q *J* = 7.1 Hz	51.64	4.10 q *J* = 7.2 Hz	51.64
3	3H	1.54 d *J* = 7.1 Hz	19.19	1.55 d *J* = 7.2 Hz	19.20
1′		–	173.45 or 175.24	–	Unassigned[a]
2′	1H	4.47 dd *J* = 5.2, 9.3	53.90	4.4 dd *J* = 5.4, 9.1	53.92
3′	1H	1.92 mult	35.78	1.91 mult	35.78
	1H	2.08 mult		2.07 mult	
4′	2H	3.67 mult	60.42	3.67 mult	60.41
1″		–	178.71 or 179.06	–	178.93
2″	1H	4.38 dd *J* = 4.5, 8.2	53.94	4.52 dd *J* = 4.5, 6.3	52.97
3″	1H	2.69 dd *J* = 4.4, 15.9	40.04	3.36 dd *J* = 4.8, 18.6	42.26
	1H	2.59 dd *J* = 8.2, 15.9		3.27 dd *J* = 6.4, 18.7	
4″		–	178.71 or 179.06	–	Unassigned[a]

[a] These carbonyls were unassigned – a total of 7 carbons were present at 178.95, 175.2, 175.05, 174.95, 173.6, 173.5, and 161.0.

We conclude that the bioactive compound **2** is 3-[L-alanyl-L-*homo*serinyl-L-aspartyl-β-carboxy]-4-hydroxy-5-oxopyrazole, corresponding to structure **2**.

2.1. Nature of the antibacterial activity

The basis of the antibacterial activity of **2** was investigated by testing all standard protein amino acids for their effectiveness in blocking the antibiotic activity against *E. amylovora*. We found that only L-glutamine, at a concentration ⩾5 mM, prevented the activity of **2**, and in contrast, D-glutamine had no effect. This result may implicate the glutamine synthesis pathway as a target of **2**.

The specificity of **2** was examined by testing a number of plant pathogenic bacterial species for their susceptibility to inhibition by **2**. We found that as well as inhibition of *E. amylovora*, there was a much broader antibacterial activity: susceptible species were *Erwinia carotovora*, *Erwinia carotovora* pv atroseptica, *Erwinia carotovora* pv carotovora, *Pseudomonas syringae* pv syringae, *Pseudomonas syringae* pv tomato, and *Xanthmonas campestris* pv pruni. Two species tested were not affected, *Pseudomonas corrugata* and *Pseudomonas marginalis*. These results suggest that there may be a useful practical application from the chemistry contained in the pyrazole structure **2**.

2.2. Concluding comments

The substitution pattern of the pyrazole ring of compound **2** appears to be new and novel. This compound is another example of the wide range of unusual bioactive chemicals produced by plant pathogenic bacteria, and it undoubtedly plays a role in the bacterial–plant interaction. The bioactivity of **2** clearly is closely linked to the pyrazole moiety, and from this point of view compound **2** is a novel acylated pyrazole, where the tripeptide substituent carries some (undefined) alternative function. Pyrazole structures, such as pyrazofurin isolated from a *Streptomyces sp.*, are widely known to have strong bioactivity.

3. Experimental

3.1. Bacterial strains and culture

The cultures of *B. glumae* used were #3729 and 8657 from the International Collection of Microorganisms from Plants (Landcare Research, Private Bag 92170, Auckland, New Zealand). These were recovered from the lyophilised state by plating onto King's medium B (King et al., 1954), and maintained both on slants of phosphate-salts agar and by storage at −80 °C in 15% glycerol. Liquid shake cultures were 600 ml volumes of HS medium (Hoitink and Sinden, 1970) in 2 l Erlenmeyer flasks, for 3 days at 21 °C, inoculated with 2 ml of a 24 h yeast inoculum culture that was initially inoculated with cells from a 24 h culture on a King's B slant. *E. amylovora* was grown on EA medium (per litre: K_2HPO_4 11.5 g, $KHPO_4$ 4.5 g, $MgSO_4$ 0.12 g, and organics L-asparagine 0.3 g, nicotinic acid 0.05 g, glucose 20 g autoclaved separately then mixed with the inorganic solution).

3.2. Bioassay

Inhibition activity was determined on plates of HS medium with 2% agar added. Plates were overlaid with a mid log-phase culture of *E. amylovora* mixed with an equal volume of warm (c. 50 °C) 2% agar, then test samples were added to 5 mm diameter wells cut into the agar. Inhibition zones appeared after overnight incubation at 26 °C. A number of other plant pathogenic bacterial species were tested for inhibition by the same procedure (see Section 2). Experiments to test the effect of added amino acids on the inhibition of *E. amylovora* used 1 μg of **2** added to each well along with test amino acid solutions at each of the four concentrations 1, 5, 25, 50 mM.

3.3. Column chromatography and analytical

Thin layer chromatography and electrophoresis (TLC and TLE) used cellulose layers and procedures previously described (Mitchell, 1976). Separation methods utilising DEAE and LH20 Sephadex are described elsewhere (Mitchell, 1976; Mitchell and Durbin, 1981); the standard LH20 column applicable to data given below was 105 g packed in 1:1 methanol–water with bed dimensions 3.5 × 43.5 cm. Reverse phase column chromatography used 10 g 55–105 μ C_{18} silica from waters, with a bed volume of 8 × 1.9 cm, and degassed mixtures of H_2O–MeCN. HPLC was with a Hewlett Packard 1050 instrument on a Phenomenex 150 × 0.46 mm 5 μm AQUA column, using isocratic elution with H_2O–MeCN–MeOH (97:2:1) + 0.05% TFA as solvent at a flow rate of 0.4 ml min^{-1}. GC of amino acids used their methyl ester trifluoroacetyl derivatives as previously described (Mitchell, 1985; Nakaparksin et al., 1970). NMR spectra were recorded at 400 MHz or 600 MHz (^{1}H) and 100 MHz or 150 MHz (^{13}C) on a Bruker instrument (University of Auckland), in D_2O, referenced to the HOD signal as 4.70 ppm. 5-Hydroxyhydantoin was purchased from Toronto Research Chemicals Inc, for reference spectral data.

3.4. Isolation and purification of products

All processes were monitored by bioassay. At harvest, bacterial cells were removed by centrifugation. The culture supernatant was then passed through a column of ion exchange resin (Amberlite CG-120, H^+ form) and the column eluted with 5% ammonia; biological activity was confined to 15 ml collected at the start of the pH change in the eluent. This fraction was rotary evaporated and the residue purified by chromatography on LH20 Sephadex and QAE Sephadex, as used for rhizobitoxine (Mitchell et al., 1986). The content of fractions was monitored by TLE with ninhydrin visualisation, and by bioassay. Correlation between ninhydrin-staining TLE components and *E. amylovora* inhibition was by preparative TLE (on a thin layer) and bioassay.

L-alanyl-L-*homo*serinyl-L-aspartic acid, compound **1**. Non-crystalline product. NMR data in Table 1. *m*/*z* (FAB) 306.1350 (MH^+), $C_{11}H_{20}N_3O_7$ requires 306.1301. *m*/*z* (ES) 306 (MH^+), 173. HPLC peak at 7.50 min with solvent H_2O:MeCN:MeOH 97:2:1, UV detector mAU ratio for λ 208:218 = 3.0:1. No inhibition of *E. amylovora*.

3-[L-alanyl-L-*homo*serinyl-L-aspartyl-β-carboxy]-4-hydroxy-5-oxopyrazole, compound **2**. Non-crystalline product. NMR data in Table 1. *m*/*z* (FAB) 404.1412 (MH^+), $C_{14}H_{22}N_5O_9$ requires 404.1412. *m*/*z* (ES) 426.1229 (MNa^+), $C_{14}H_{21}N_5O_9Na$ requires 426.1232, 404.1409 (MH^+), $C_{14}H_{22}N_5O_9$ requires 404.1412, 386.1309 (MH^+-H_2O), $C_{14}H_{20}N_5O_8$ requires 386.1306, 343 (386 – CHNO), 333.1039 (MH^+-71), $C_{11}H_{17}N_4O_8$ requires 333.1041, 232.0564 (MH^+-172), $C_7H_{10}N_3O_6$ requires 232.0564, 173.0921 (ala-*homo*ser), $C_7H_{13}N_2O_3$ requires 173.0921. HPLC peak at 4.60 min with solvent H_2O:MeCN:MeOH 97:2:1, UV detector mAU ratio for λ 208:218 = 2.3:1. Inhibition of *E. amylovora* ca. 18 mm clearance zone from 1 μg.

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