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Antibacterial iminopyrrolidines from *Burkholderia plantarii*, a bacterial pathogen of rice

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Burkholderia plantarii, a bacterial pathogen on rice, produced compounds in liquid culture that gave strong inhibitory action against *Erwinia amylovora*, the bacterium responsible for fire blight disease of apple and pear trees. The active component was hydrophilic and ionic, and was fractionated extensively by passage through SP, DEAE, and LH20 Sephadexes. Final purification was achieved by reverse phase chromatography on C_{18}-bonded silica. Biological activity was associated with two compounds detectable by HPLC (UV λ 218 nm). A third related compound was non-active. The chemical structures of the pure compounds were deduced by NMR and MS, and corroborated by chemical synthesis which also verified the stereochemical assignments. Thus, the two active compounds are reported herewith as 2-imino-3-methylene-5-L(carboxy-L-valyl)-pyrrolidine, and 2-imino-3-methylene-5-L(carboxy-L-threoninyl)-pyrrolidine. The non-active component was deduced to be 2-imino-5-L(carboxy-L-threoninyl)-pyrrolidine.

Introduction

Fire blight disease, caused by the bacterium *Erwinia amylovora*, is an often serious problem for apple and pear orchardists in many parts of the world.[1] Severe outbreaks of this disease can cause significant economic loss to growers through the necessary removal of infected trees. There are various options for the control of fire blight, these being biological, chemical, and orchard management methods. In the past the preference for chemical control has been spray applications of streptomycin. However, agricultural use of streptomycin is now limited. Because of the paucity in control options, particularly chemical, 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. The target source for new chemical products has been plant pathogenic species of *Pseudomonas* and *Burkholderia*, because many potently bioactive products have been previously found, some with antibacterial activity.[2,3] In a survey of 121 strains, 34 were identified as candidates for closer investigation for anti fire blight products.[4] We report here the chemical structures of two related new compounds isolated from liquid cultures of the rice pathogen *Burkholderia plantarii* (syn *Pseudomonas plantarii*). The compounds are amino acid conjugates to a new iminopyrrolidine carboxylic acid structure. These compounds have a high *in vitro* inhibitory activity against the growth of *Erwinia amylovora.*

Results and discussion

The bioactivity in cell-free liquid culture supernatants was not removed by liquid phase extraction into organic solvents. A gentle method of isolation was adopted, initially by sequential precipitation of inorganic media components from the concentrated supernatant during a slow addition of methanol. Filtration, then evaporation of the methanol–water filtrate to dryness, yielded a residue that contained the bioactivity. This bioactivity was extracted from the residue into large volumes of methanol. The activity was weakly retained on both DEAE and SP Sephadexes, and large columns of each of these were used to isolate the products from remaining media components, using dilute ammonium bicarbonate buffer. However, without adequate precaution, the removal of the ammonium bicarbonate buffer caused loss of activity, ascribed to a higher pH level generated in the more concentrated decomposing ammonium bicarbonate solutions after evaporation. Final purity was achieved by column chromatography first on LH20 Sephadex, and then by reverse phase on C_{18}-bonded silica. As the bioassay-guided purification progressed, two sources of bioactivity were found (compounds **1** and **2**). During the course of the purification, analytical and preparative HPLC investigations established a single HPLC peak for each bioactive source as being responsible for the bioactivity, and accordingly final purifications were monitored by HPLC. Neither of these two components gave a ninhydrin colour response after TLC on cellulose. A third product (compound **3**) proved to be non-active when fully purified, but nevertheless had related chemical characteristics to the two active products, and therefore was investigated further. Compounds **1** and **2** applied in 10 µl to 5mm diameter wells cut into agar plates overlaid with *Erwinia amylovora* gave clear zones of growth inhibition with sharp boundaries, after 15 h. Zone diameters were 18–20 mm from 1.0 µg applications of each compound.

The retention of the compounds on DEAE Sephadex was indicative of acidic functionality. Electrophoresis experiments on cellulose thin layers established that the bioactive compounds migrated at pH 2, indicating that **1** and **2** each had basic character, in accordance with their retention on SP Sephadex. Since neither compound gave a colour reaction with ninhydrin on the cellulose thin layers, even at loadings that normally give a strong colour reaction with amino acids and small peptides, the basic property of **1** and **2** is required to be from a moiety other than a primary or secondary amine.

Mass spectral (FAB) results from the 3 compounds indicated compound **1** differed from **2** by a hydroxyl function (in **1**) replaced with a methyl group (in **2**). This was reflected in the HPLC elution times (68 : 25 : 7 H_2O–MeCN–MeOH + 0.05% TFA), 4.0 min for **1**, the more polar, and 6.3 min for **2**.

NMR data (Table 1) verified the relatedness of **1** and **2**. In summary, each compound consisted of two units. One structural unit was common to both compounds, and is referred to as the *Core Structure* (**CS**). 2D NMR data (COSY and HSQC) indicated that **1** contained a threonine unit, and **2** a valine unit. These two amino acids account for the elemental composition difference deduced from the MS data—that is, an –OH in **1** replaced by a $-CH_3$ in **2**. The valine component of **2** was cleaved

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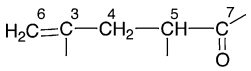
Table 1 ^{1}H and ^{13}C NMR data for compounds **1** and **2** in D_2O (ext Me_4Si)

Position/mult.		Compound **1**		Compound **2**	
		^{1}H (δ ppm, J Hz)	^{13}C (δ ppm)	^{1}H (δ ppm, J Hz)	^{13}C (δ ppm)
1	NH	—	—	—	—
2	C=NH	—	167.43	—	167.34
3		—	138.03	—	137.99
4	1H	3.36 (ddt) (J = 9.1, 2.8, 17.4)	34.67	3.30 (ddt) (J = 9.1, 2.8, 17.4)	34.62
	1H	2.97 (dm) (J = 17.4)		2.89 (dm) (J = 17.4)	
5	1H	4.79 (dd) (J = 9.1, 3.7)	61.09	4.74 (dd) (J = 9.2, 3.5)	60.79
6	1H	5.95 (dd) (J = 4.3, 2.25)	123.81	5.92 (mult) (J = 2.0)	123.70
	1H	6.21 (dd) (J = 4.6, 2.7)	—	6.18 (mult) (J = 2.4)	
7	CONH	—	175.23/176.91	—	175.10/177.84
1′		—	176.91/175.23	—	177.84/175.10
2′	1H	4.35–4.42 (mult)	61.78	4.26 (d) (J = 5.8)	61.63
3′	1H	4.35–4.42 (mult)	69.86	2.21 (oct) ($J \approx$ 6.7)	32.41
4′	3H	1.22 (J = 6.3)	21.67	0.95 (d) (J = 6.65)	19.91/20.98
5′	3H	—	—	0.965 (d) (J = 6.5)	20.98/19.91

by carboxypeptidase A, demonstrating a C-terminal L-valine. This result can be extrapolated to **1**, *i.e.* threonine is C-terminal and -L- in **1**, since **1** and **2** are parallel bioproducts. The presence of a free carboxylic acid was further confirmed when both compounds formed methyl esters with methanol–HCl, giving MH^+ (FAB-MS) 14 mass units higher than the respective parent compound.

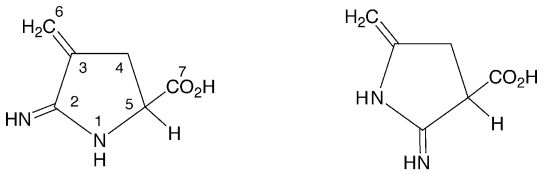
In addition to data for the amino acid moieties, the 2D NMR revealed an extended coupling system common to **1** and **2**, and thus the following part structure for **CS** was assigned, where all but one of the carbon content of each compound is accounted for:



CS part structure

Compounds **1** and **2** were hydrolysed by 6 M HCl liberating the single respective amino acid, and **CS** which gave no colour reaction with ninhydrin, but which was observed at λ 218 nm on HPLC as the sole product. Thus the amino moieties of Thre and Val are the linkage points to **CS** in **1** and **2**, by an amide bond to the carbonyl of the part structure. For each compound the carbonyl of this amide linkage, together with the carboxylic acid of the Val (**2**), or the carboxylic acid and hydroxyl of Thre (**1**), accounts for the total oxygen complement. Clearly then, by arithmetic difference, **CS** contains 2 carbon-bound nitrogens, bonded in a manner that imparts basic properties, but not as a primary or secondary amine. These structural entities are linked to or through C^3 and C^5 of the part structure depicted. The ^{13}C NMR shows 3 carbon signals in the carbonyl region. Two of these (δ 175.2 and 176.9 ppm for **1**, 175.1 and 177.8 for **2**) are accounted for in the free carboxylic acid and amide moieties, while the third (δ 167.4, 167.3 respectively for **1** and **2**), is assigned to a –C=N-moiety. Both **1** and **2** were sufficiently soluble in CD_3CN to acquire complete proton NMR data inclusive of the exchangeable protons. The amide proton appeared as a doublet at δ 7.2 and 7.1 ppm for **1** and **2** respectively; as well there were NH signals at δ 7.45 and 7.55 ppm (**1** and **2**), and another assigned as C=NH at 10.0 and 10.4 ppm for **1** and **2** respectively. Each of these proton signals integrated for 1H.

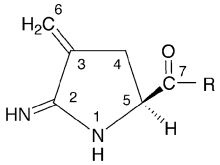
Consideration of the MS data allows completion of the structural detail of **CS**. In the case of **2**, if a valine is subtracted from the molecular formula of $C_{11}H_{17}N_3O_3$ the resultant product as the free carboxylic acid would have the composition $C_6H_8N_2O_2$. This has 4 units of unsaturation: carboxylic acid, methylene $=CH_2$, C=NH, and therefore one ring structure. Thus the two structural options for **CS** are:



Option I Option II

Structures for Options I and II

HMBC NMR data for **1** and **2** were obtained in CD_3CN solutions, and gave 2J and 3J correlations. For each compound, the C^6 exocyclic methylene protons gave strong correlation (3J couplings) to the ring methylene carbon (C^4 in **I**), and the imino ring carbon (C^2 in **I**), a result untenable for option **II**. Therefore **CS** has the structure **I**, and compounds **1** and **2** the corresponding amide structures. The full complement of HMBC correlations for each compound were in accord with these structural assignments.



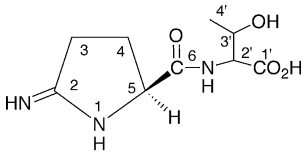
1 R = -NH-$C^{2'}H(CO_2H)C^{3'}HOHC^{4'}H_3$ (threonine)

2 R = -NH-$C^{2'}H(CO_2H)C^{3'}H(CH_3)_2$ (valine)

CS R = OH

Structures for compounds **1**, **2** and **CS**

The structure of compound **3** was deduced on the basis of MS data, proton NMR data, and the correlation and variance of this data with the data and assignments for **1** and **2**. The proton NMR of **3** allowed for the assignment of a threonine unit, relating it to **1**. The most prominent feature in the proton spectrum of **3** when compared with that of **1** was an absence of exocyclic methylene protons, or a methyl group that would have been the result of reduction of the exocyclic methylene. Instead, an additional two methylene protons appeared as a multiplet (ddd?) centered at δ 2.97 ppm and the adjacent (verified by COSY) C^4 ring protons as 1H multiplets at δ 2.63 and 2.24 ppm. This, in conjunction with the MS data which showed a molecular mass 12 units less, defines **3** as 2-imino-5-(carboxy-L-threoninyl)-pyrrolidine.



Compound **3**

In terms of structure–activity it is noteworthy that an absence of the exocyclic methylene at C^3 has abolished the antibacterial activity, suggesting that the chemical reactivity of a conjugated imino alkene part structure is intimately associated with bioactive properties.

Stereochemistry

As described above, the valine unit of **2** was deduced to have the L-configuration on the basis that it was a substrate for CPA. It is reasonable to expect that parallel natural products from the one strain of the same organism will have the same configuration, and therefore the threonine in **1** (and **3**) can be inferred to have the L-configuration in each case. The remaining asymmetric centre, at C^5 of the imino-pyrrolidine ring, is likely to be derived from an L-amino acid, such as L-pyroglutamic acid, or 4-methylene-L-pyroglutamic acid. In search of support for this hypothesis we have synthesised the two possible diastereomers of the structure represented for compound **2** (containing an L-valine moiety). 4-Methylene pyroglutamic acid was synthesised from L-pyroglutamate,[5] and coupled *N*,*N*′-dicyclohexylcarbodiimide to L-valine methyl ester. The ring carbonyl was then converted in a 2-step process to the iminopyrrolidine structure,[6] and the resulting methyl ester product base-hydrolysed to the final iminopyrrolidine structure. The synthesis was duplicated starting with D-pyroglutamic acid. The two synthetic products were compared to naturally occurring compound **2**, with the result that the synthetic L,L-product was identical and biologically active while the synthetic D,L-product was different and not biologically active. These data will be reported separately along with syntheses of additional analogous structures.

The iminopyrrolidine structures reported herewith represent new structural substituent arrangements for iminopyrrolidines. The exocyclic methylene conjugated with the ring imino moiety provides a considerable potential for reactivity and may predictably impart bio-reactivities that are as yet undiscovered. Other alkyl-substituted iminopyrrolidines have been reported recently as nitric oxide synthase inhibitors.[6,7] The amino acid conjugation is another noteworthy feature of the compounds we report. This occurs relatively frequently in bioactive natural products, for example the jasmonates, and the coronatine family of compounds.

Experimental

Bacterial strains and culture

The culture of *Burkholderia plantarii* used in this study was #9424 from the International Collection of Microorganisms from Plants (Landcare Research, Auckland, New Zealand). It was recovered from the lyophilised state by plating onto King's medium B,[8] 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 in 2 l erlenmeyer flasks, for 5 days at 18 °C, inoculated with 2 ml of a 24 h yeast inoculum culture which was initially inoculated with cells from a 24 h culture on a King's B slant. Media used were as described by Hoitink and Sinden.[9] *Erwinia 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).

Bioassay

Inhibition activity was determined on plates of HS medium with 2% agar added. Plates were overlaid with a mid log-phase culture (*ca.* 10^7 cells per ml) of *Erwinia amylovora* mixed with an equal volume of 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.

Column chromatography and analytical

Thin layer chromatography and electrophoresis (TLC and TLE) procedures are described previously.[10] Separation methods utilising DEAE and LH20 Sephadexes are described elsewhere;[10,11] 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 using an ODS Hypersil 250 × 0.4 mm column, and isocratic elution with H_2O–MeCN–MeOH + 0.05% TFA as solvent at a flow rate of 0.8 ml min^{-1}. NMR spectra were recorded at 400 MHz (^1H) and 100 MHz (^{13}C) on a Bruker instrument (University of Auckland).

Isolation and purification of products

All processes were monitored by bioassay. At harvest, bacterial cells were removed by centrifugation and the supernatant (2.4 l) concentrated to *ca.* 50 ml, and refrigerated overnight. It was centrifuged, then the decanted supernatant was stirred slowly during addition of 200 ml of MeOH, and refrigerated overnight. The MeOH–H_2O solution was decanted and evaporated (finished on hi-vac), for 19.15 g. This product in *ca.* 350 ml of 0.02 M NH_4HCO_3 was fractionated on a 50 g column of DEAE Sephadex using 0.02 M NH_4HCO_3 as solvent, and the active fractions from this subjected to further repetitive fractionations (same conditions). Appropriate combinations of fractions were further purified on LH20 Sephadex, and HPLC analysis of LH20 fractions together with bioassay results identified the presence of 3 distinct products, designated as compounds **1**, **2**, and **3**. These were purified to homogeneity by reverse phase chromatography on C_{18}-bonded silica and LH20 Sephadex.

2-Imino-3-methylene-5-(carboxy-L-threoninyl)-pyrrolidine, compound 1. Obtained as a crystalline residue following purification, yield *ca.* 4 mg l^{-1}. *m/z* (FAB) 242.1138 (MH^+). $C_{10}H_{16}N_3O_4$ requires 242.1141. δ_H(400 MHz; CD_3CN; ext Me_4Si) 1.15 (3 H, d, *J* 6.4, C*H*$_3$CHOH), 2.95 (1 H, 2 × mult, J_{gem} 17.4, H_2C=CCH*H*CH), 3.16 (1 H, ddt, *J* 2.8, 8.7 and 17.4, H_2C=CC*H*HCH), 4.27 (1 H, dq, *J* 2.8 and 6.4, CH_3CHCH), 4.37 (1 H, dd, *J* 2.8 and 8.6, $CH_3CHCHNH$), 4.67 (1 H, dd, *J* 3.5 and 8.7, CCH_2CHCO), 5.85 (1 H, dd, *J* 2.4 and 4.1, H*H*C=CCH_2), 6.09 (1 H, dd+, *J* 2.8 and 4.5, *H*HC=CCH_2), 7.21 (1 H, d, *J* 8.4, CON*H*CH), 7.45 (1 H, N*H*), 10.04 (1 H, C=N*H*); δ_C(100.6 MHz; CD_3CN; ext Me_4Si) 20.3 (CH_3), 32.15 (CH_2), 58.75 (CH), 59.63 (CH), 68.02 (CH), 120.59 (CH_2), 137.93, 166.27, 170.65, 172.03.

2-Imino-3-methylene-5-(carboxy-L-valyl)-pyrrolidine, compound 2. Obtained as a crystalline residue following purification, yield *ca.* 2 mg l^{-1}. *m/z* (FAB) 240.1343 (MH^+). $C_{11}H_{18}N_3O_3$ requires 240.1348. δ_H(400 MHz; CD_3CN; ext Me_4Si) 0.93 (3 H, d, *J* 6.0, C*H*$_3$CH), 0.95 (3 H, d, *J* 6.8, C*H*$_3$CH), 2.16 (1 H, oct, *J* 6.6, $(CH_3)_2CHCH$), 2.88 (1 H, 2 × mult, J_{gem} 17.3, H_2C=CCH*H*CH), 3.16 (1 H, ddt, *J* 2.8, 8.8 and 17.4,

$H_2C{=}CCHHCH$), 4.27 (1 H, dd, *J* 5.4 and 8.3, $CHCHNH$), 4.62 (1 H, dd, *J* 3.5 and 8.9, CCH_2CHCO), 5.83 (1 H, dd, *J* 2.35 and 4.0, $HHC{=}CCH_2$), 6.10 (1 H, dd+, *J* 1.8, 2.7 and 4.35, $HHC{=}CCH_2$), 7.14 (1 H, d, *J* 8.05, $CONHCH$), 7.55 (1 H, NH), 10.38 (1 H, $C{=}NH$); δ_C(100.6 MHz; D_2O; ext Me_4Si) 19.91 (CH_3), 20.98 (CH_3), 32.41 (CH), 34.62 (CH_2), 60.79 (CH), 61.63 (CH), 123.70 (CH_2), 137.99, 167.34, 175.10, 177.84.

2-Imino-5-(carboxy-L-threoninyl)-pyrrolidine, compound 3. Obtained non-crystalline, yield *ca.* 0.4 mg l^{-1}. *m/z* (FAB) 230 (MH^+). δ_H(400 MHz; D_2O; ext Me_4Si) 1.18 (3 H, d, *J* 6.4, CH_3CHOH), 2.20–2.29 (1 H, m, CH_2CHHCH), 2.58–2.68 (1 H, m, CH_2CHHCH), 3.81–3.905 (2 H, ddd, *J* 3.9, 6.3 and 11.6, CCH_2CH_2CH), 4.17 (1 H, d, *J* 4.2, $CHCHCO$), 4.25 (1 H, dq, *J* 4.2 and 6.4, CH_3CHCH), 4.69 (1 H, dd, *J* 4.9 and 8.9, CH_2CHCO).

Acknowledgements

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