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# Synthesis of amino acid conjugates to 2-imino-3-methylene-5-carboxypyrrolidine and 2-imino-3-methylene-6-carboxypiperidine

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ABSTRACT

The four stereomers of 2-imino-3-methylene-5-L(carboxy-L-valyl)pyrrolidine, a bacterial metabolite that is inhibitory to the fire blight bacterium *Erwinia amylovora*, were synthesised and compared for antibacterial activity. Several alternative amino acid conjugates with L,L-stereochemistry were also prepared, and the synthesis was extended to 3-methylenepiperidine-6-L-carboxylic acid and a selection of 2-imino-3-methylenepiperidine-6-L-carboxy-L-amino acid conjugates. All synthetic amino acid conjugates (L,L-stereomers) were inhibitory to the growth of *E. amylovora*. The likely participation of the conjugated iminomethylene moiety as a Michael acceptor is implicated.



The structures of two new compounds from liquid cultures of *Burkholderia plantarii*, a bacterial pathogen on rice, were recently elucidated and reported as 2-imino-3-methylene-5-L(carboxy-L-valyl)pyrrolidine (**1**) and 2-imino-3-methylene-5-L(carboxy-L-threoninyl)pyrrolidine (**2**) (Fig. 1).[1] Both compounds inhibited the growth of *Erwinia amylovora* in an agar diffusion assay, the bacterium responsible for fire blight disease in Rosaceae, in particular, apple and pear trees. The newly reported 2-imino-3-methylenepyrrolidine structural component of these compounds has considerable potential significance in that past studies[2] have shown that a series of substituted iminopyrrolidines are potent and selective inhibitors of human inducible nitric oxide synthases, suggesting possible adaptation to therapeutics. More recently, a product from a marine sponge was found to be the related compound 2-imino-1-methylpyrrolidine-5-carboxylic acid and was reported to have allelopathic activity against corals.[3] To confirm the structural assignment for **1** and **2**, and to further define the nature of the antibacterial activity, the synthesis and properties of all four stereomers of the valine conjugate to the 2-imino-3-methylenepyrrolidine product was undertaken, and extended to a number of L,L-analogues with different amino acids. The basis of the structural specificity for the activity against *E. amylovora* was further evaluated by the synthesis of the corresponding six-membered ring homologs of a selection of the products. This required first the synthesis of 2-oxo-3-methylenepiperidine-6-carboxylic acid and then adaptation of parallel synthetic procedures used for the iminopyrrolidine compounds.

The synthesis of 2-imino-3-methylenepyrrolidine-5-carboxyvaline stereomers is summarised in Schemes 1A and 2**A**. The first stage (Scheme 1A) inserts the exocyclic 3-methylene group[4,5] into each of L- and D-pyroglutamic acid, resulting in the required product 2-oxo-3-methylene-pyrrolidine-5-carboxylic acid (**3A**-L and **3A**-D).

These were then coupled (Scheme 2) to L- or D-valine methyl ester (as the free base), resulting in the four lactam analogues (**4A**) of the targeted products, confirmed by FAB-MS ($MH^+$ 255) and NMR (Supplementary data) following purification (HPLC data, Table 1). Conversion of the lactam carbonyl of these four products to the imino functionality was accomplished in a two-step reaction (Scheme 2A). Purified products were base hydrolysed to the final iminopyrrolidine compounds **5A**. Each imino acid product displayed an $MH^+$ at 240 (FAB-MS), and had $^1H$ and $^{13}C$ spectra consistent for the required products (Supplementary data).

The four synthetic stereomers were compared for their antibacterial activity against *E. amylovora*, by agar diffusion assay[1] (Table 2). The L,L-stereomer (natural product configuration) inhibited growth at 0.1 μg per application site (10 mm zone), and gave

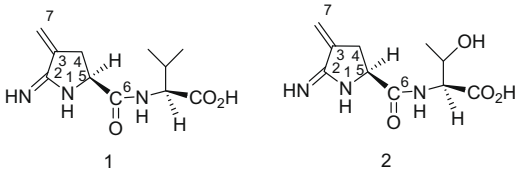

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**Figure 1.** Natural product structures.

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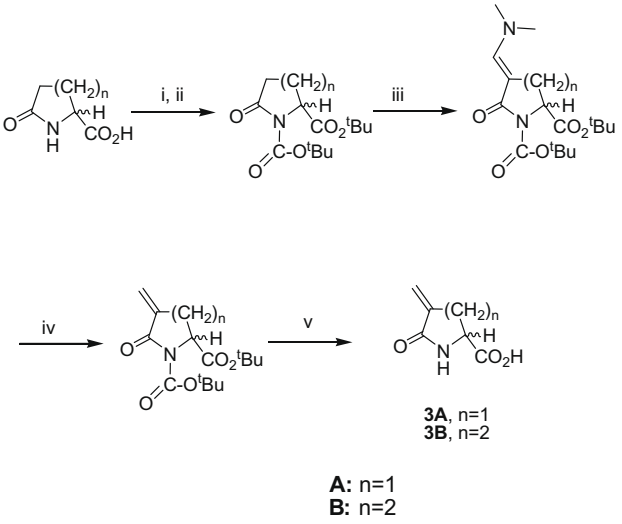



**Scheme 1.** Reagents and conditions: (i) $^{t}$BuOAc, $HClO_4$; (ii) $(^{t}BuO)_2CO$, DMAP, MeCN, 0 °C; (iii) $HC[N(CH_3)_2]_3$, $CH_3OCH_2CH_2OCH_3$, 72 °C, 88%; (iv) $[(CH_3)_2CHCH_2]_2AlH$, THF, −78 °C, 61%; (v) TFA/$CH_2Cl_2$, 10 M, 72%.

**Table 1**
HPLC characteristics of stereomers[a]

| Stereomer | Elution times (min) | |
|---|---|---|
| | Lactam **4** | Iminopyrrolidine **5** |
| L,L- and D,D- | 4.97 | 4.29 |
| D,L- and L,D- | 5.23 | 4.54 |

[a] HPLC used an ODS Hypersil $C_{18}$ column, 250 × 0.4 mm, flow rate 0.8 ml $min^{-1}$, and isocratic elution with solvent compositions of $CH_3CN/H_2O$/MeOH + 0.05% TFA, 70:25:5 for **4**, 50:45:5 for **5**.

**Table 2**
Antibacterial activity[a] of iminopyrrolidine stereomers

| Stereomer | Zone size (mm) at loading of | | | |
|---|---|---|---|---|
| | 0.1 μg | 1.0 μg | 10 μg | 100 μg |
| L,L- | 10 | 19 | | |
| L,D- | – | 6 | 10 | 19 |
| D,L- | | | – | 10 |
| D,D- | | | – | – |

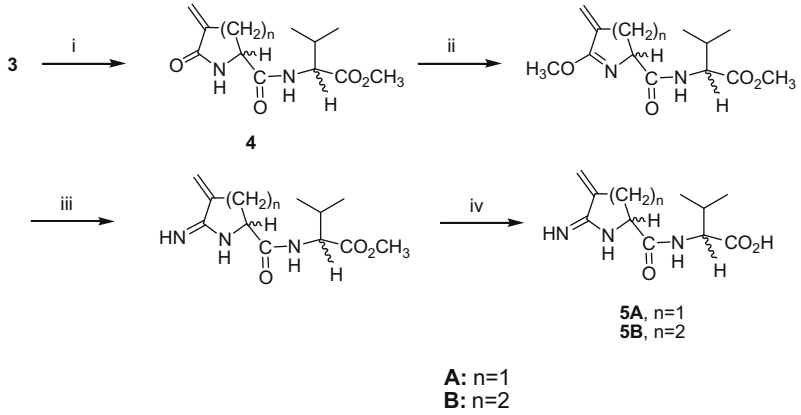
[a] Agar diffusion assay, samples applied in 10 μL water to 4 mm diameter wells.

strong inhibition at 1 μg (19 mm zone), comparable to that of the natural product. In contrast, the D,D-stereomer gave no inhibition at loadings up to 100 μg (i.e., >$10^4$ less active). The L,D- and D,L-stereomers showed intermediate degrees of inhibition: L,D- at a 10 μg loading was comparable to L,L- at 0.1 μg, and D,L- at 100 μg was comparable to L,L- at 0.1 μg. The estimated differentials in concentrations for L,L-: L,D-: D,L-: D,D- to give comparable inhibitions therefore are 1: $10^2$: $10^3$: >$10^4$. The activity observed for the L,D-stereomer was concluded to be real rather than from the presence of a low concentration of L,L-stereomer. HPLC analysis of L,D-stereomer spiked with ca. 1% of the L,L-product clearly showed this as a resolved 'pimple' not present in the parent, indicating that the L,D-stereomer did not contain the L,L-stereomer. If there were a presence of L,L-stereomer at a concentration below HPLC detectability this would be significantly below the concentration to account for the activity observed for the L,D-stereomer.

The natural product 2-imino-3-methylene-5-L(carboxy-L-valyl)pyrrolidine was found to contain an L-valine and was proposed to have the L-stereochemistry at the 5-position of the iminopyrrolidine ring.[1] In the present study, the HPLC characteristics of synthetic L,L-stereomer were the same as those of the natural product, and differed from the D,L-stereomer, and therefore the natural product is confirmed to be the L,L-stereomer.[1]

The same methodology outlined was also used to prepare several additional 2-imino-3-methylene-5-L-carboxy→L-amino acid conjugates for comparative antibacterial testing. Each was purified to homogeneity, as evaluated by HPLC, and their identity confirmed by MS. The amino acid component of these included glycine, alanine, β-alanine, 2-methyl alanine, leucine, phenylalanine, proline, and the dipeptide valylleucine. These products generally displayed an antibacterial activity similar to that of the threonine- and valine-containing natural products, although some (β-alanine, glycine, val-leu) were active to a lesser degree (2.5-, 5-, 20-fold, respectively).

Synthesis of the six-membered ring homologs started with 2-oxopiperidine-6-L-carboxylic acid (Scheme 1B), which was obtained from the cyclisation of 2-L-aminoadipic acid.[6] Introduction of the exocyclic methylene lead to **3B**, 3-methylenepiperidine-6-carboxylic acid (Scheme 1B). This was then coupled with various amino acids (as their methyl ester) followed by conversion of the lactam carbonyl to the 2-imino functionality, and base hydrolysis to the free carboxylic acid (Scheme 2B). Three amino acid-coupled products were prepared (L-Val, L-Ile, L-Thr); in all cases only low overall yields were achieved, but sufficient material was obtained for characterisation (HPLC data and FAB-MS, and for the Val conjugate, full NMR analysis) and antibacterial testing. Each of the com-




**Scheme 2.** Reagents and conditions: (i) DCCD, MeCN, then ValOMe(base), 75%; (ii) $Me_3O^+BF_4^-$, $CH_2Cl_2$ 2 h, product used without purification; (iii) $NH_4Cl$, MeOH, 70 °C 16 h, 19% from i; (iv) aq 0.1 M NaOH, 62%.

pounds displayed strong inhibition of *E. amylovora* in the agar diffusion assay, comparable to their iminopyrrolidine analogues.

The five- and six-membered 2-imino-3-methylenelactam ring structures, when coupled to various amino acids through the carboxyl group, provide a diverse family of compounds with antibacterial activity. New and unusual chemistry is represented in the iminomethylene lactams, and their bioactivity may well be attributable to the conjugated iminomethylene moiety acting as a Michael acceptor. The conjugation to amino acids is a phenomenon not uncommon in biology, and probably represents some biophysical requirement for functionality. It is feasible that various further chemical modifications may provide scope for the development of new antibacterial products. Given the known nitric oxide synthase activity of related compounds, there would appear to be considerable potential for discovery of additional bioactivities in these compounds.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.128.

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