The Journal of Antibiotics (2018) 71:438–446
https://doi.org/10.1038/s41429-017-0014-y

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

ARTICLE

Agrocin 108 is a 5'-cytidine nucleotide bacteriocin containing a carbocyclic phosphoryl-ascorbate group

Christopher M. Elvin[1,2] · Robert E. Asenstorfer[1] · Maarten H. Ryder[1] · Scott C. Donner[1,3] · Graham P. Jones[1] · Max E. Tate[1]

Received: 9 August 2017 / Revised: 26 November 2017 / Accepted: 1 December 2017 / Published online: 18 January 2018

Abstract

Agrocin 108 is a 3′-*O*-β-D-xylopyranosyl-cytidine-5′-*O*-phosphodiester of an ascorbate-carbocyclic cyclopentenone analogue, with bacteriocin-like properties. This bacteriocin exhibits orders of magnitude greater than the inhibition zone diameter towards the indicator strain than either ampicillin or streptomycin. It has been isolated from cultures of *Rhizobium rhizogenes* strain K108. The structure of the agrocin 108 without detail, has been previously published. We now report a detailed structure elucidation, including the hitherto undetermined residual 5′-phospho-diester fragment by a combination of 1D and 2D NMR studies at various pH values in H_2O/D_2O, high resolution MS, pKa determination, and chemical degradation.

Introduction

Agrocins are highly selective bacteriocins. Agrocin 84 (a fraudulent, *N*-6, *O*-5′-disubstituted adenine arabinofuranosyl nucleotide) together with agrocin 434 (an *N*-4-substituted cytidine nucleoside, with 3′-*O*-enol ether blocking group) are two representatives of a class of non-peptide bacteriocins that have been successfully used in combination for four decades [1], to effect biological control of the dicotyledonous plant cancer known as crown gall. This disease is induced by pathogenic strains of *Agrobacterium tumefaciens* and *A. rhizogenes* (now known as *Rhizobium rhizogenes*) which can carry a tumour-inducing (Ti) plasmid. An interesting feature of agrocins is that they have a toxic moiety and an uptake moiety, and certain bacterial permeases designed for specific nutrients allows the entry of the bacteriocin. Agrocin 84 produced by *R. rhizogenes* strain K84 is a fraudulent adenosine and only bacteria with a specific permease are sensitive to agrocin 84 [2]. This uptake system has been described as a Trojan Horse [3]. To protect against crown gall, both agrocin 84 and agrocin 434 are delivered via application of a live non-pathogenic *R. rhizogenes* (strain K1026) producer of these agrocins to the roots, cuttings or seeds of susceptible plants, prior to planting. We suggest that a thorough understanding of the mode of action [4] of these long-standing and remarkably effective nucleoside and nucleotide bacteriocin-like antibiotics will provide clues as how to avoid the build-up of resistant pathogens which is very much the characteristic of broad spectrum antibiotics.

We now report the structure of yet another low-molecular weight nucleotide bacteriocin: agrocin 108. A proposed structure of agrocin 108 by ME Tate with neither stereo-chemistry nor structural information was first published in a review in 1995 [5]. We wish to provide further details regarding the structure of this compound.

Experimental procedures

Bacterial strains

Rhizobium rhizogenes strain K108 and *R. rhizogenes* strain K46 (syn. TR101) were obtained from Professor Allen Kerr of the University of Adelaide, Waite Campus. Both the

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-017-0014-y) contains supplementary material, which is available to authorised users.

✉ Robert E. Asenstorfer
robert.asenstorfer@adelaide.edu.au

[1] School of Agriculture, Food and Wine, Waite Campus, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia

[2] CWAN Technologies Pty Ltd, 28 Dexter Street, Tennyson, QLD 4105, Australia

[3] Department of Environment, Water and Natural Resource, Level 4, 81 Waymouth St, Adelaide, SA 5000, Australia

producer strain (K108) and the sensitive strain (K46) were maintained at 4 °C on yeast mannitol agar and long-term as freeze-dried cultures. Routine subcultures of strain K108 were grown on yeast mannitol agar and incubated for 2 days at 26 °C. The *R. rhizogenes* indicator strain K46 is available as TR101 from the International Collection of Phytopathogenic Bacteria housed by USDA in Maryland. The producer strain *R. rhizogenes* K108 has been deposited with The International Collection of Micro-organisms from Plants, Landcare Research, Private Bag 92170, Auckland Mail Centre, Auckland 1142, New Zealand.

Production of agrocin 108 and bioassay of activity

Strain K108 was grown in 0.5 l modified Ghent's medium in 1 l Erlenmeyer flasks. The medium contained, per litre: glucose 2.0 g, NH_4SO_4 1.0 g, sodium glutamate monohydrate 1.1 g, trisodium citrate dihydrate 0.56 g, $K_2HPO_4.3H_2O$ 2.28 g, KH_2PO_4 5.45 g, $MgSO_4.7H_2O$ 0.2 g, $FeCl_3.6H_2O$ 5 mg, thiamine 10 mg, biotin 0.2 mg (added after autoclaving), pH adjusted to 7.0. The 1 l flasks were each inoculated with 10 ml starter cultures that had been grown in the same liquid medium for 2 days at 26 °C and incubated for a further 60 h at 26 °C on a rotary shaker at 150 r.p.m. Biological activity of agrocin 108 was assessed on Petri dishes containing Stonier's agar [6] plus biotin (0.2 mg/l). The producer strain was patched to the centre of the Petri dish and allowed to grow for 2 days. Filter sterilised bacterial culture medium and purified compounds (including antibiotics ampicillin and streptomycin) were either added to wells (5 mm diameter) in the agar or applied to filter paper circles (5 mm diameter). Glass Petri dishes together with agar and producer strains or test samples, were sterilised with chloroform vapour, from a $CHCl_3$ cotton wool patch taped to its lid for approximately five minutes and then opened in a laminar flow cabinet to remove $CHCl_3$ and finally overlaid with soft buffered agar containing the indicator strain *R. rhizogenes* K46 (syn. TR101). The tests were incubated for 2 to 4 days at 26 °C.

Isolation of agrocin 108

BDH activated decolourising charcoal (ash 1.2%) was added at 2 g per liter of liquid culture broth and stirred for 24 h at room temperature. The slurry was filtered using a large Büchner funnel lined with Whatman 451 filter paper. The carbon cake residue, containing the nucleotides, was stirred for 12 h with 25 ml/g 50% aqueous isopropanol (*v*/*v*) containing 0.25% ammonia solution at room temperature. The slurry was centrifuged at 16,250×*g* for 30 min. The straw coloured supernatant was taken to dryness by rotary evaporation at 40 °C. The crude charcoal desorbed bacteriocin, followed by adsorption on Dowex $50H^+$, was eluted with 1 M NH_4OH and evaporated to remove excess NH_3. Crude agrocin 108 in water (60,000 273 nm units), was applied to a Sephadex DEAE-A25 anion exchange column (620 × 38 mm diameter) and eluted with 0.1 M triethylammonium acetate pH 5.0 (4 l). Fractions (17.8 ml) were collected at 1 ml/minute. UV absorbing material was monitored by electrophoresis at pH 1.7 (0.1 M formic/acetic buffer) and separated into three major fractions. The first fraction contained unresolved components. Agrocin 108 (m. p. 229 °C followed immediately by decomposition; molar absorption coefficient ε 34,663, 273 nm, pH 7; specific rotation coefficient $[\alpha]$ 11.3 ± 0.3, 589 nm, pH 5.5) was found in the second fraction whilst a small amount of a substance, referred to as agrocin 108B, was found in the third fraction.

Enzymatic degradation of agrocin 108

(a) Snake venom phosphodiesterase I (E.C.3.1.4.1) type VII from *Crotalus atrox* (0.39 units/mg; Sigma-Aldrich). Incubations performed in 0.1 M Tris-HCL buffer at pH 8.0 at 37 °C released phosphorylated "snake venom cytidine nucleotide" and a UV absorbing fragment. This experiment was repeated in the same buffer for 2 h at 28 °C. The degradation products were purified by HVPE (pH 1.7, formic acetic buffer), ultraviolet absorbing bands were eluted and the UV spectrum measured. (b) Bacterial alkaline phosphatase (E.C.3.1.3.1) type III from *E. coli* in 2.5 M $(NH_4)_2SO_4$ solution (264 units/ml; Sigma-Aldrich), incubations in 0.1 M NH_4OH buffer at pH 10.0 at 37 °C, showed no effect on snake venom nucleotide. (c) Acid phosphatase (E.C.3.1.3.2) from wheat germ (0.3 units/mg; Worthington Biochemicals), incubation in 0.1 M sodium acetate buffer at pH 4.6 at 25 °C, using either agrocin 108 or snake venom nucleotide, released cytidine. Its identity to a reference sample was established by UV-vis spectrometry, HVPE, TLC, paper chromatography, column chromatography and optical rotation. In addition to release of cytidine, this enzyme also released xylose (detected by paper chromatography, with *p*-anisidine as the detection reagent) indicating the presence of glycosidase activity. Further sealed tube, acid hydrolysis (3 N acetic acid for 18 h at 110 °C) of the cytidine product yielded ribose as identified by HVPE, paper chromatography and GLC, as well as uracil (identified by HVPE) a deamination product of cytosine. (d) Cellulase (E.C.3.2.1.4) (Activity unknown; Onozuka R-10, Yakult Biochemicals Co. Ltd., Nishinomiya, Japan). Incubations were performed in 0.1 M sodium acetate buffer at pH 4.6 at 25 °C. Xylose was released as measured by paper chromatography. (e) β-glucosidase (E.C.3.2.1.21) from almonds (7.2 units/mg; Sigma-Aldrich). Incubations were performed in 0.1 M sodium acetate buffer at pH 4.6 at 37 °C. This enzyme released xylose as measured by HVPE,

paper chromatography and GLC. Specificity of this enzyme helped to establish the likely presence of a β-D-xylopyranoside. (f) Snail-gut chitinase from *Helix pomatia* (activity unknown; Industries Biologique, Francais, France). Incubations were performed in 0.1 M sodium acetate buffer at pH 4.6 at 25 °C. This enzyme also released xylose as measured by paper chromatography.

Acid hydrolysis

(a) Hydrolysis of agrocin 108 in 2.5 M acetic acid at 110 °C for 2 h yielded 5′-CMP as identified by HVPE and compared with the standard (formic/acetic buffer pH 1.7, Rm_{OG} −0.05; 0.05 M $Na_2B_4O_7$, pH 9.2, Rm_{OG} 1.13). Crystals gave needles, m.p. 203–204 °C. (b) Mild acid hydrolysis was performed in a sealed tube, using 3 M formic acid at 100 °C for 1 h to release a substituted nucleotide and the phosphorylated substituent. (c) Cold (0 °C) nitrous acid deamination of agrocin 108 (50 µl, 1% Na agrocin 108) with 2.5% HNO_2 in 0.5 M HCl (50 µl) for 3 h at room temperature yielded a (Rm_{OG} + 0.02) UV positive, $AgNO_3$ positive, $FeCl_3$ positive, *p*-anisidine positive (yellow) product, which was no longer cationic at pH 1.7.

Ammonolysis

Agrocin 108 heated in a sealed tube with 2.5 M ammonia for 16 h at 100 °C unexpectedly and fortuitously yielded a quickly recognised yellow spot detected by HVPE with similar mobility and UV spectrum to that of authentic croconic acid observed in other studies [7].

Mass spectrometry

Initially, low resolution mass spectrometry field desorption MS Fast atom bombardment (MH^+) was performed using a V.G. Analytical ZAB mass spectrometer. Subsequently, high resolution mass spectral analysis was carried out using an electrospray mass spectrometer (MicrOTOF-Q, Bruker Daltonics Corporation, USA) operated in negative ion mode. The capillary voltage was set at 3500 V with an end plate offset potential of −500 V. Data were collected from 50 to 1500 *m/z* with an acquisition rate of 1 spectrum per second. Dry gas was set to 6 l min^{-1} at 200 °C with a nebulization gas pressure of 2.0 bar. Samples were introduced by direct infusion.

NMR spectroscopy

Initial 1D ^{13}C and ^{1}H NMR spectra were recorded in D_2O on a JEOL FX-90Q spectrometer operating at 90 MHz. Subsequent 1D and 2D data were obtained using a Varian Inova-600 spectrometer operating at 600 MHz. Samples were dissolved in D_2O/H_2O (1:9) using water suppression techniques for ^{1}H spectra and TSP as an external reference. MestReNova [8] prediction software was used to help assign the proton and carbon shifts. (NMR spectra, Supplemental Part 3).

Electrophoresis

High voltage paper electrophoresis (HVPE) was carried out according to the method of Tate [9] using Orange G, fructose, 2-deoxyadenosine and inorganic phosphate as markers. Relative mobility (Rm_{OG}) values were estimated using the mobility of either Orange G (Rm_{OG}) or *p*-nitrobenzene sulphonate (Rm_{NBS}) with fructose as the non-migrating marker (Rm 0.0). In the pH range used, Orange G has formal charge −2 while *p*-nitrobenzene sulphonate has formal charge −1. UV absorbing reaction intermediates and end products were detected using a UV lamp (254 nm, 366 nm). Non UV absorbing products were detected by developing chromatograms in silver nitrate reagent [10] for vicinal hydroxyl groups, phosphomolybdate reagent [11] for phosphates and *p*-anisidine [12] for hexoses (yellow) and pentoses (red). Relative mobilities (Rm_{OG}) for a range of non-UV absorbing pH buffers for both agrocin 108 and snake venom nucleotide were measured. The pKa values and the corresponding mobilities of the various ionic species, relative to the standard Orange G, were determined for the pH range 1.7–10.7 (1.68–8.92, 0.1 M oxalate/citrate; 9.00–10.11, 0.1 M oxalate/potassium carbonate) [9, 13] and data were fitted using Wincurvefit software [14].

Formaldehyde assay

Oxidation of compounds possessing either –CH.OH–CH_2OH or –CH.NH_2.CH_2OH terminal groups by iodine tetraoxide (IO_4^-) yields formaldehyde. Formaldehyde was quantified by the method of Hanahan and Olley [15] using erythritol which yields 2 mol of formaldehyde per mole. Agrocin 108 (0.37×10^{-6} mol) yielded 3.0×10^{-7} mol formaldehyde, or approximately 0.81 moles per mole implying the presence of one of the above terminal groups. The absence of formaldehyde in the periodate oxidation of the *N*-acetylated agrocin 108 is only compatible with presence of a terminal –CH.NH_2.CH_2OH group.

Determination of mole ratio of cytidine to xylose

Snake venom nucleotide (200 µl of A_{280} 20.4, pH 1.0; 1.35×10^{-6} mol) was digested with 0.6 mg β-glucosidase, 0.417×10^{-6} moles erythritol (internal standard) and 200 µl 0.1 M sodium acetate buffer (pH 4.6) for 18 h at 37 °C. The digest was fractionated by HVPE and the three fractions

representing the zero mobility neutral sugar fraction (containing some unreacted snake venom nucleotide), cytidine and xylosyl-cytidine were eluted from the paper. Absorbance values at 280 nm (pH 1.0) for the three bands were 5.2, 2.21, and 10.63 for neutral sugar (and unreacted starting material) fraction, cytidine and xylopyranosyl-cytidine, respectively. The estimated mass of cytidine released was 0.167×10^{-6} mol. The neutral sugar fraction was acetylated and its carbohydrate content were analysed by gas chromatography. Based on the initial concentration of erythritol and standard curves for the acetates of xylose and erythritol, the ratio of the areas of xylose and erythritol acetates allowed the estimation of the mass of xylose released: 0.150×10^{-6} mol which equates to a xylopyranoside to cytidine mole ratio of 0.9 and indicative of an equimolar ratio of xylopyranoside to cytidine.

Results

Agrocin 108 inhibition zones (Fig. 1a) show, by comparison with the broad spectrum antibiotics ampicillin or streptomycin, that it achieves, respectively, a 100-fold or 1000-fold increase in its relative potency towards the target, a hairy root disease-inducing pathogen (*R. rhizogenes* K46). Addition of xylose and methylxylose decreased the effectiveness of agrocin 108 (Fig. 1b). Agrocin 108 required 1.5 and 0.08 µg to produce inhibition zones of 3 cm and 1 cm radius, respectively. The toxicity of agrocin 108 to *R. rhizogenes* K46 was compared to the toxicity of agrocin 84 to *A. tumefaciens* strain K57, using data derived from Tate et al. [3] (Supplementary Figure S1). On a molar basis, agrocin 108 was 2.5× to 18× less toxic than the highly potent agrocin 84 in the range of agrocin required to produce inhibition zones of 3 cm and 1 cm radius, respectively. However, it should be noted that agrocin 84 is not toxic to strain K46 and agrocin 108 is not toxic to strain K57. As part of the cancer screening programme a sample of agrocin 108 was submitted to the National Cancer Institute Bethesda Maryland in 1977, but showed no activity against the cancer cell screens at that time.

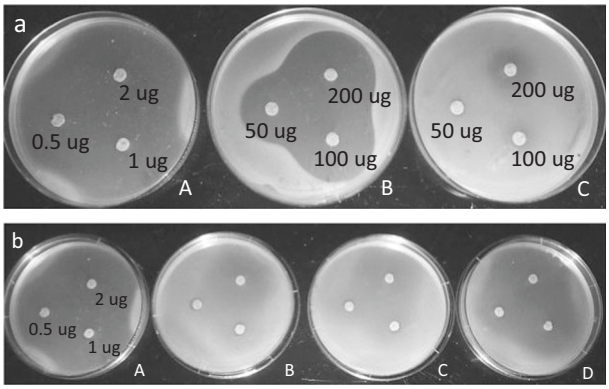

Fig. 1 a Comparative biological activity of (A) agrocin 108, (B) ampicillin, and (C) streptomycin loaded onto sterilised paper disks against *R. rhizogenes* strain K46 (syn. TR101) on Stonier's agar. **b** The effect of addition of (A) no sugar, (B) xylose, (C) 2-O-methyl-d-xylose and, (D) D-arabinose at 3 mM final concentration in Stonier's agar, on the inhibition of *R. rhizogenes* strain K46 (syn. TR101) by agrocin 108 (0.5 to 2 µg) loaded onto sterilised paper disks

The structure of agrocin 108 was determined by a mixture of classical degradation studies and modern spectroscopic techniques. Low Resolution Fast atom Bombardment disodium salt of agrocin 108 (MNa_2^+) gave mass 657.2 *m/z* (calculated $C_{20}H_{28}N_4Na_2O_{16}P$, requires 657.1028), xylopyranosyl-cytidine nucleoside had mass 375 *m/z* ($C_{14}H_{21}N_3O_9$ requires 375.1278), agrocin 108 (MH^+) gave mass 613 *m/z* ($C_{20}H_{30}N_4O_{16}P$ requires 613.1394 *m/z*) while agrocin 108B (MH^+) had mass 481 *m/z*, showing a loss of 132 *m/z*, which is consistent with loss of an anhydropentose (xylopyranose) group from agrocin 108. As the mass of agrocin 108 was 612.13 ($C_{20}H_{29}N_4O_{16}P$) and the mass of 3′-*O*-beta-D-xylopyranosyl-5′CMP was 455.09 ($C_{14}H_{22}N_3O_{12}P$), the calculated mass of the snake venom phosphodiesterase fragment was 175.04 ($C_6O_5NH_9$ requires 175.0481). The high resolution mass spectrum ($M–H^+$) yielded three ions with m/z (relative intensity) 611.1271 (base peak, $C_{20}H_{28}N_4OP^-$ requires 611.1243), 454.0886 (0.02), and 305.0601 (0.05). A neutral loss of 157.0385 ($C_6H_7NO_4$ requires 157.0375) gave a daughter ion 454.0886 *m/z* the same as the mass of snake venom phosphodiesterase fragment (the ascorbate moiety) less H_2O. A mass of 305.0601 *m/z* represents the doubly charged molecular ion ($M/2–H^+$).

The pH mobility profile of agrocin 108 (Fig. 2) showed three observable deprotonations consistent with a vinylogous-carboxyl adjacent to an amino group, (pKa_1, 2.35 ± 0.09) to yield a zero charge species at pH 3.56, a protonated 4-amino group of the cytidine base (pKa_2, 4.77 ± 0.18) to give a mono-anion near pH 5.8 and a quaternary ammonium ion associated with the 5'-phosphodiester fragment (pKa_3, 8.25 ± 0.10) which yields a di-anion at pH > 10. To account for the cationic behaviour below pH 2 in the pH mobility profile, in addition to the three nitrogens of the cytosine moiety, there must be a fourth basic-nitrogen in the fragment moiety. There was no significant change in UV/Vis spectra (data not shown) between pH 7.4 and pH 13.0 although there is a major deprotonation (pKa 8.25) observable in the pH mobility profile in that region. This can only be interpreted as the deprotonation of a quaternary nitrogen which is not part of the 5′-*O*-phosphodiester fragment chromophore.

Acid phosphatase, β-glucosidase and snail-gut chitinase enzymes all released xylose from agrocin 108. Release of D-xylose by β-glucosidase was indicative of the presence of

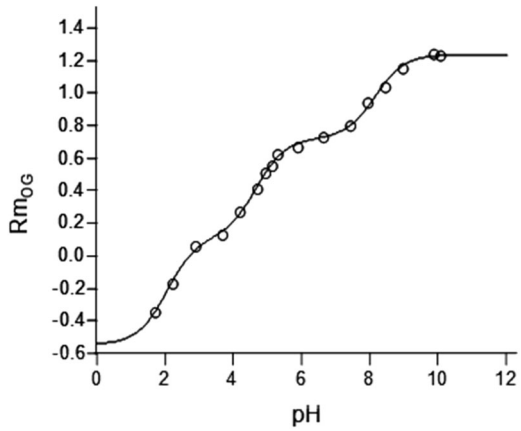

Fig. 2 Proton dissociations of agrocin 108 indicating a strong carboxyl deprotonation (pKa = 2.05 ± 0.16) to yield a zero charge species at pH 3.38, a weak proton dissociation from the cytosine base (4.67 ± 0.07) to yield a mono-anion of net charge of −1 (Rm_{OG} = 0.72) and a third proton dissociation pKa = 8.15 ± 0.07 to yield a di-anion (Rm_{OG} = 1.24)

a β-D-xylopyranosyl moiety. The xylose free product treated with snake-venom phosphodiesterase released a cytidine mono-phosphate nucleotide (Rm_{OG} −0.05 at pH 1.7) with UV spectrum maxima at pH 1.0 (270 nm) and pH 10 (0.1 M NH_4OH) (280 nm) which were superimposable on that for 5′ CMP at the same pH values. The corresponding electrophoretic mobility (Rm_{OG}) of 5′CMP was also −0.05. The xylose to cytidine ratio was 1. The unusual non-phosphorylated fragment cleaved from the biologically active agrocin 108 by snake-venom phosphodiesterase had a relative mobility (Rm_{OG}) at pH 1.7 of −0.53 and UV maxima of 273 nm at pH 1.0 (0.1 M HCl), 294 nm at pH 7.0 (0.1 M phosphate buffer) and 264 nm, 341 nm, 379 nm at pH 10 (0.1 M NH_4OH) (Fig. 3). This latter increment in wavelength is consistent with an ene-diol/enolate ionisation. The intact agrocin 108 does not show these enol-enolate changes at this pH range suggesting the phosphodiester linkage from the cytidine 5′-phosphate is via an enol group of the fragment. The free fragment has zero charge at pH 5.0 and is cationic at pH 1.0 which is consistent with the presence of a vinylogous-carboxylic acid adjacent to a protonated amino group.

Agrocin 108 hydrolysed in 2.5 M ammonia yielded a yellow spot with similar electrophoretic mobility to that of a croconic acid reference. The origin of the five carbon croconic acid could only be from the six carbon snake venom phosphodiesterase cleavage fragment which immediately suggested that the fragment had a five membered-carbon ring with a single hydroxyl methylene substituent adjacent to an amino group on the cyclopent-enediol-one as depicted in Fig. 4.

The acetylation of agrocin 108 under the alkaline conditions employed occurred, whereas 5′CMP was not

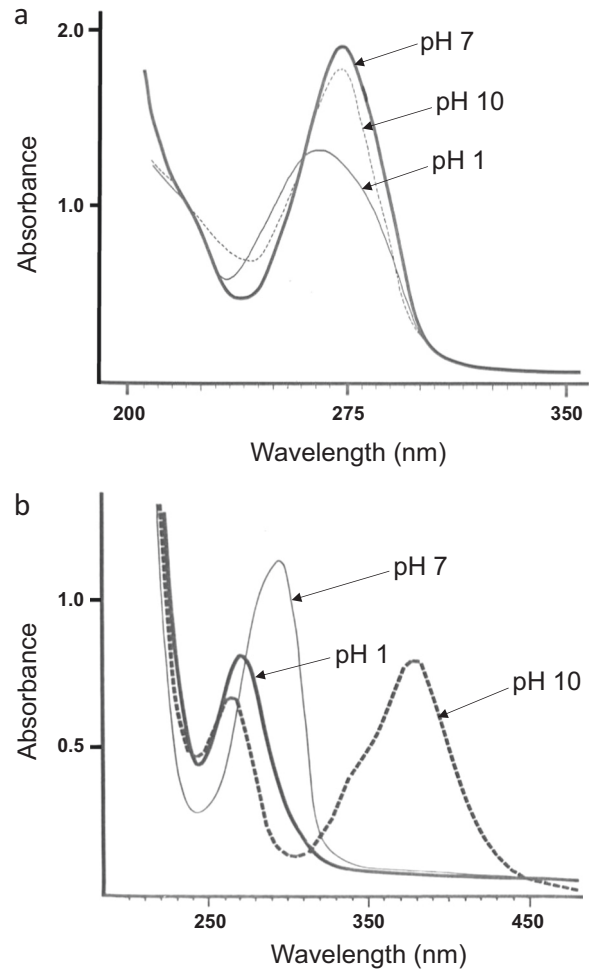

Fig. 3 Absorbance spectra of (**a**) agrocin 108 and (**b**) snake venom phosphodiesterase fragment at pH 1, pH 7, and pH 10

affected, suggests a strongly basic nitrogen pKa (8.15) located on the fragment. This was in accord with the expected charge changes at pH 1.7 from +1 (Rm_{OG} −0.5) to neutral (Rm_{OG} 0.01) and at pH 6.2 from +1 (Rm_{OG} 0.52) to +2 (Rm_{OG} 1.26) determined by electrophoresis (data not shown). Further evidence of the primary nature of this nitrogen was obtained from electrophoretic mobility data where in addition to the 3 nitrogens of the cytosine moiety (pKa 4.58), the presence of a fourth highly basic nitrogen (pKa 8.15) was indicated in order to account for the cationic behaviour below pH 2 in the pH mobility profile of the molecule.

The ^{1}H NMR spectra at pH 6.1 displayed three protons in the aromatic region (δ_H 6.0–8.0 p.p.m.) an anomeric proton (δ_H 4.7 p.p.m.) three methylene protons (δ_H 3.3–4.4 p.p.m.), six oxygenated methine protons (δ_H 3.4–5.5 p.p.m.) and two non-oxygenated methine protons (δ_H 4.4 p.p.m. and δ_H 6.6 p.p.m.). The largest differences in proton shifts between pH 6.1 and pH 10.7 shifts were two oxygenated methine protons (δ_H 4.4 p.p.m. to δ_H 4.2 p.p.m.; δ_H 3.4 p.p.

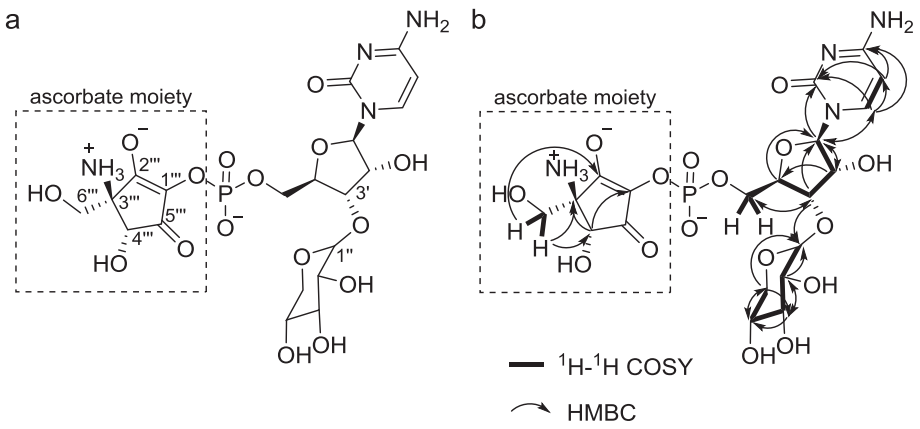

Fig. 4 **a** Structure proposed for agrocin 108 and (**b**) with COSY and HMBC NMR interactions indicated

m. to δ_H 3.6 p.p.m.) and a methylene proton from two doublets (δ_H 4.0 p.p.m., $J = 7.2$ Hz; δ_H 3.9 p.p.m., $J = 12.0$ Hz) to a multuplet (δ_H 3.6 p.p.m.).

The ^{13}C and HMQC spectra showed 20 spectral lines with four bands in the carbonyl region (δ_C 142–188 p.p.m.), three associated with carbonyls and the other with an unsaturated carbon bonded to a NH_2 group (δ_C 167 p.p.m.). There were four carbons in the aromatic region (δ_C 97–126 p.p.m.) with one being an anomeric carbon at δ_C 103 p.p.m. The carbon at 126 p.p.m. showed splitting indicating long-range coupling to a phosphorus. There were 12 aliphatic carbons (δ_C 63–90 p.p.m.) with six of them oxygenated (δ_C 78–70 p.p.m.). Carbons at δ_C 65 p.p.m. and δ_C 82 p.p.m. demonstrated long-range coupling to a phosphorus atom. Two of the carbonyl carbons at δ_C 158 p.p.m. and δ_C 167 p.p.m. were associated with the cytidine and an anomeric carbon at δ_C 103 p.p.m. was associated with xylose. The cytidine moiety had two aromatic carbons (δ_C 97 p.p.m. and δ_C 142 p.p.m.). Two oxygenated and two non-oxygenated aliphatic carbons were associated with the cytidine moiety while xylose accounted for 4 oxygenated carbons and a methylene carbon (δ_C 66 p.p.m.). The remaining moiety had six carbons; two carbonyl carbons (δ_C 188 p.p.m. and δ_C 184 p.p.m.), an aromatic carbon (δ_C 126 p.p.m.), an oxygenated aliphatic carbon (δ_C 74 p.p.m.), a methylene carbon (δ_C 63 p.p.m.) and a non-protonated carbon (δ_C 66 p.p.m.). Seven carbons bands exhibited substantial differences in shift due to pH change with two of these associated with structural change in xylose (C2" δ_C 72 p.p.m. and C3" δ_C 76 p.p.m.) while differences shown by other five carbons, which were associated the remaining moiety, were consistent with de-protonation of a quaternary ammonium ion. The ^{1}H shifts due to pH differences agreed with these data.

HMBC and COSY spectra in aqueous solutions at pH 6.1 clearly indicated the 5′-cytidine mono-phosphate (Fig. 4a, b) and the 3′-*O*-β-D-xylopyranosyl constituents were in accord with spectra of the authentic references (5′ CMP and 1-*O*-β-methyl-xylopyranoside; Supplemental Part 4). The H6 proton (δ_H 7.98 p.p.m.) was correlated with the cytosine carbons, C2 (δ_C 158 p.p.m.) and C4 (δ_C 167 p.p.m.), and C1′ (δ_C 90 p.p.m.) of ribofuranose while the H1' proton (δ_H 6.03 p.p.m.) was coupled with the C2 and C6 carbons confirming a bond between the cytosine ring and the ribose C1' through the cytosine nitrogen. Other interactions between H5 (δ_H 6.13 p.p.m.) and H6 (δ_H 7.98 p.p.m.) and the carbons of the cytosine ring were in agreement with the 5′-cytidine monophosphate standard. The H3' proton (4.50 p.p.m.) of the ribofuranose was coupled with the C1" carbon (δ_C 103 p.p.m.) of the xylose moiety confirming the linkage of the xylopyranose to the C3' carbon (δ_C 78 p.p.m.). HMBC couplings between xylopyranose protons and carbons were consistent with the 1-*O*-β-methyl-xylopyranoside model. As expected, there were no HMBC interactions between the xylosyl-cytidine moiety and the ascorbate moiety. Within the ascorbate moiety, couplings were observed between the H6‴ protons (δ_H 3.97 and 3.85 p.p.m.) and C2‴ (δ_C 184 p.p.m.), C3‴ (δ_C 62 p.p.m.) and C4‴ (δ_C 74 p.p.m.) and were consistent with a hydroxyl methyl group attached with C3‴. The H4‴ proton (δ_H 4.40 p.p.m.) was correlated with C3‴ (δ_C 62 p.p.m.) and C1‴ (δ_C 126 p.p.m.) carbons. While a geminal COSY coupling was observed between the two H6‴ protons (δ_H 3.97 and 3.85 p.p.m.), there was no coupling between the H6‴ and the H4‴ protons.

NMR assignments of the remaining moiety consisting of $C_6H_8NO_4$ are consistent with a phosphorylated-ascorbate-related structure (ascorbate moiety), with an observed P…C coupling of 6.9 Hz for C1‴ (2-bond coupling), but not for C2‴ or C5‴ (3-bond coupling), which from model building, stems from $+90^o$ and -90° torsion angles for the phosphorus atom to the respective carbon atoms. At pH 6.1 all carbon atoms in the terminal phosphodiester moiety could be assigned (Table 1) to peaks in the NMR spectra along with the H4‴ and two H6‴ protons. The HVPE data indicated the presence of a quaternary nitrogen which is not part

Table 1 Agrocin 108 proton (600 MHz), carbon (150 MHz) and 2D NMR, pH 6.1

	^{13}C Assign.	δ^{13}C (p.p.m.)	*j* (Hz)	No. ^{1}H	^{1}H Assign.	δ^{1}H (p.p.m.)		*J* (Hz)	HMBC	COSY
1	C5‴	188.36		0						
2	C2‴	183.81		0						
3	C4	166.77		0						
4	C2	158.19		0						
5	C6	141.99		1	H6	7.982	d	7.8	C1′, C2, C4, C5	H5
6	C1‴	126.26	6.9	0						
7	C1”	103.33		1	H1”	4.586	d	7.2		H2”
8	C5	97.19		1	H5	6.133	d	7.2	C4, C6	H6
9	C1′	89.82		1	H1′	6.033	d	4.8	C2′,C2, C6	H2′
10	C4′	81.92	9.1	1	H4′	4.446	m		C1′	H3′, H5′
11	C3′	77.58		1	H3′	4.501	t	4.8	C1’, C1”, C5'	H2′, H4′, H5′
12	C3”	76.26		1	H3”	3.503	t	9.3	C2”, C4”	H2”, H4”
13	C2′	74.18		1	H2′	4.434	t	5.4	C1′, C4′	H3′
14	C4‴	73.58		1	H4‴	4.400	s		C1‴, C3‴	
15	C2”	73.42		1	H2”	3.397	t	8.4	C1”, C3”	H1”, H3”
16	C4”	69.90		1	H4”	3.678	m		C5”	H3”, H5”
17	C5”	65.78		2	H5”	3.358	t	11.1	C1”, C3”, C4”	H4”, H5”
					H5”	3.991	t	6.3	C1”, C3”	H4”, H5”
18	C5′	65.40	5.7	2	H5′	4.309	m		C3′	H4′, H5′
					H5′	4.389	m			H4′, H5′
19	C3‴	64.02		0						
20	C6‴	62.82		2	H6‴	3.966	d	7.2	C2‴, C4‴	H6‴
					H6‴	3.851	d	12.0	C2‴, C4‴, C3‴	H6‴

Phosphorus δ^{31}P (p.p.m.) −2.794

of the chromophore. The proton, carbon, and HMBC NMR data indicated that the C6‴ carbon is a hydroxyl methyl attached to the C3‴ carbon. Therefore this moiety has a 5-membered ring with carbonyls at the C2‴ and C5‴ positions and the phosphate attached to the C1‴. HMBC couplings between the H4‴ proton and C3‴ and the H6‴ protons with C4‴ establishes the position of this carbon. At pH 10.7 (Table 2), large shifts are noted for C6‴ and its attached protons consistent with the de-protonation of the nitrogen (pKa 8.25) attached to C3‴ of the ascorbate moiety. The ^{31}P NMR absorbances at pH 6.1 (^{31}P −2.79 p.p.m.) and pH 10.7 (^{31}P −2.59 p.p.m.) are as might be expected for a phosphodiester. The cis orientation of the C3‴ hydroxyl-methyl group and the C4‴ hydroxyl group was established from NOESY data. No NOE correlations were observed between H4‴ and either protons attach to C6‴. Both physical model building and MM2 computed distances [16] between H4‴ and C6‴ protons (cis-, 3.461 Å and 3.841 Å; trans-, 2.730 Å and 2.417 Å) indicate this could only occur with the proposed cis configuration.

The structure of agrocin 108 was therefore characterised as an amino-substituted cyclopenten-one-diol analogue of ascorbate-2-phosphate, present as the 5′-phospho-diester of 3′*O*-β-D-xylopyranoside-5′-cytidine as shown in Fig. 4. The absolute (R or S) stereochemistry of carbons 3‴ and 4‴ of the cyclopenten-one-analogue ascorbate entity must await an X-ray crystal structure determination.

Discussion

Agrocin 108 is a 3′-*O*-β-D-xylopyranosyl-cytidine, 5′ phospho-diester of an enediol-methylene, amino-methylene, hydroxyl-methylene-substituted cyclopentenone analogue of ascorbic acid 2-phosphate. Inhibition of *R. rhizogenes* strain K84 by agrocin 108 established that the cyclopentenone is the toxic portion of the molecule and requires the phosphate ester for toxicity [17]. Agrocin 108 appears to be one of the most unusual natural-products with bacteriocin-like properties investigated to date, and appears to exhibit a close

Table 2 Agrocin 108 proton (600 MHz), carbon (150 MHz), and 2D NMR, pH 10.7

	^{13}C Assign.	δ^{13}C (p.p.m.)	*j* (Hz)	No. ^{1}H	^{1}H Assign.	δ^{1}H (p.p.m.)		*J* (Hz)	HMBC	COSY
1	C5‴	190.87		0						
2	C2‴	189.33		0						
3	C4	166.92		0						
4	C2	158.35		0						
5	C6	142.15		1	H6	8.095	d	7.8	C2, C4	H5
6	C1‴	126.22	6.9	0						
7	C1”	103.42		1	H1”	4.672	d	7.8	C3”, C2”, C4”	H3”
8	C5	97.27		1	H5	6.226	d	7.8	C6, C4	H6
9	C1′	89.74		1	H1′	6.129	d	4.8	C6, C2	H2′
10	C4′	82.16	7.9	1	H4′	4.524	s		C3′	
11	C3′	77.78		1	H3′	4.587	t	4.8	C5′	H4′, H2′
15	C4‴	77.01		1	H4‴	4.208	s		C3‴, C6‴	
14	C2”	76.37		1	H2”	3.583	t	9.0	C3”, C4”, C5”	H3”, H4”
13	C2′	74.20		1	H2′	4.516	s		C1’, C4’, C5’	H1′
12	C3”	73.53		1	H3”	3.466	t	8.7	C2”, C1”	H1”, H5”
16	C4”	69.98		1	H4”	3.746	m		C2”, C5”	H5”, H2”
17	C5”	65.90		2	H5”	4.065	dd	5.1, 11.7	C4”, C2”, C1”	H3”, H4”, H5”
					H5”	3.435	d	11.4	C4”	
18	C5′	65.46		2	H5′	4.430	dd	11.7, 40.5	C3′	H5′
20	C6‴	65.50	5.7	2	H6‴	3.780	m			
19	C3‴	62.61		0						

Phosphorus δ^{31}P (p.p.m.) −2.594

structural relationship to ascorbate 2-phosphate, a relatively stable form of Vitamin C, at the terminal 5′ phosphodiester position. In view of this structural relationship between ascorbate 2-phosphate and the 5′ phosphodiester-linked fragment of agrocin 108, the question arises, is there a cytidylic phosphodiester of ascorbic acid in nature, and if so what does it do? Large numbers of diverse bacteria are capable of metabolising ascorbic acid [18]. It is tempting to speculate that the ascorbate-phosphate analogue of 108 either competes with native ascorbate-6-phosphate for effector sites [19] or forms a non-metabolisable phosphorylated intermediate, effectively blocking subsequent metabolic pathways resulting in cell death.

A reduction of inhibition of agrocin 108/*R. rhizogenes* strain K46 with the addition of sugars such as xylose and 2-O-methyl-d-xylose but less so arabinose suggests uptake via a transporter where the agrocin 108 and xylose compete. Alternatively, a reduction of inhibition of agrocin 108/*R. rhizogenes* strain K46 by xylose and 2-O-methyl-d-xylose may indicate agrocin 108 interferes with sugar metabolism. Donner [17] found xylose, cytidine, and cytidine-monophosphate had no effect on the inhibition zone of agrocin 108 acting on *R. rhizogenes* strain K84 and only 3′-O-xylopyranosyl cytidine decreased the inhibition zone indicating that the 3′-O-xylopyranosyl cytidine nucleotide moiety is required for active transport into the cell. This is analogous to the uptake of agrocin 84 by sensitive strains of crown gall pathogens [20, 21]. However, unlike agrocin 84, agrocin 108 inhibition zones do not contain resistant colonies. Agrocin 108 activity against *R. rhizogenes* strain K84 decreased 100-fold with the loss of the xylopyranoside [17] (forming agrocin 108B). Agrocin 108 is an extraordinarily potent inhibitor, ($10^2 \times$ ampicillin and $10^3 \times$ streptomycin) of *R. rhizogenes* strain K46 and its toxicity is similar to, though slightly less than, that of the highly potent agrocin 84.

The spectrum of activity of agrocin 108 differs from that of agrocin 84 (Supplemental Part 1). In vitro tests using either strain K108 or purified agrocin 108 to test toxicity of the agrocin to pathogenic (tumour forming and hairy root-inducing) and non-pathogenic *A. tumefaciens* and *R. rhizogenes* strains, indicated all *A. tumefaciens* strains were insensitive and all *R. rhizogenes* hairy root-inducing strains were sensitive, except 3 tumour-forming *R. rhizogenes* and

3 non-pathogenic *R. rhizogenes*. Interestingly, 5 of the 6 insensitive *R. rhizogenes* strains produced an agrocin 108-like antibiotic. Agrocin 108 appears to be generally toxic to *R. rhizogenes*, irrespective of pathogenicity or opine type.

The structure of agrocin 108, produced by *R. rhizogenes* K108, bears some similarity to the agrocin 84 di-nucleotide [4, 20, 21] and its accompanying agrocin 434 cytidine nucleoside [17, 21] both of which are produced by *R. rhizogenes* K84. The similarity resides in the fact that all three entities have substituents blocking the 3′ locus of the furanosyl loci. Agrocins 84, 434 and now agrocin 108, thereby provide models for revealing a common mode of action that must include an active transport process via individual nutritional permeases with which they have co-evolved. With structural analysis now complete, the opportunity exists to dissect the mode of action of these highly selective and toxic molecular "Trojan horses".

Acknowledgements The authors thank Professor Allen Kerr for the bacterial strains and extensive discussions. Philip Clements is thanked for providing the 600 MHz NMR data and Mark Solomon for the high resolution electrospray mass spectrometry data. The former support of Australian Research Council and latter the University of Adelaide Alumni Association is gratefully acknowledged. This paper is dedicated to the memory of Max E. Tate (deceased 2016).

Author contributions CE and the late MET performed the original 1979 investigation, intermittent studies by SD and the late MET culminated in 2016, when sufficient spectroscopic data were accumulated with the help of RA, for a rational complete structure. MR provided recent microbiological activity information and GPJ assisted in the interpretation of the NMR data. The late MET, RA, and GPJ prepared the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Kerr A. Biological control of crown gall. Australas Plant Pathol. 2016;45:15–8.
2. Kerr A, Htay K. Biological control of crown gall through bacteriocin production. Physiol Plant Path. 1974;4:37–44.
3. Tate ME, Murphy PJ, Roberts WP, Kerr A. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature. 1979;280:697–9.
4. El Sahili A, et al. A pyranose-2-phosphate motif is responsible for both antibiotic import and quorum-sensing regulation in Agrobacterium tumefaciens. PLoS Pathog. 2015;11:e1005071.
5. Clare BG. Agrocins. In: Vining LC, Stuttard C, editors. Genetics and Biochemistry of Antibiotic Production. Reading, Mass., USA: Butterworth-Heinemann, Biotechnology; 1995. Vol. 28, pp. 619–32
6. Stonier T. Agrobacterium tumefaciens Conn. II. Production of an antibiotic substance. J Bacteriol. 1960;79:889–98.
7. Liljegren DR, Tate ME. Spontaneous oscillations in the oxidative degradation of 2,4,6/3,5-pentahydroxycyclohexanone (*scyllo*-inosose). Carbohydr Res. 1980;82:378–84.
8. MestReNova, version 10.0.2-15465, Mestrelab Research, Santiago, Spain. 2015.
9. Tate ME. Determination of ionization constants by paper electrophoresis. Biochem J. 1981;195:419–26.
10. Trevelyan WE, Procter DP, Harrison JS. Detection of sugars on paper chromatograms. Nature. 1950;166:444–5.
11. Harrap FEG. The detection of phosphate esters on paper chromatograms. Analyst. 1960;85:452.
12. Veiga LA, Chandelier EL. Detection and differentiation of sugars on paper by the *p*-anisidine/periodate reaction. Anal Biochem. 1967;20:419–22.
13. Asenstorfer RE, Iland PG, Tate ME, Jones GP. Charge equilibria and pKa of malvidin-3-glucoside by electrophoresis. Anal Biochem. 2003;318:291–9.
14. WinCurveFit, version 1. 1.6, Kevin Raner Software, Mt Waverley, Victoria, Australia. 2001.
15. Hanahan DJ, Olley JN. Chemical nature of monophosphoinositides. J Biol Chem. 1958;231:813–28.
16. ChemDraw3D, version 14.0.0.117, CambridgeSoft Corporation, Perkin Elmer Inc. Waltham, Massachusetts, United States. 2014.
17. Donner, SC Agrocins from Agrobacteria. PhD thesis, The University of Adelaide, Australia. 1997. http://hdl.handle.net/2440/18932.
18. Luo P, et al. Crystal structure of a phosphorylation-coupled vitamin C transporter. Nat Struct Mol Biol. 2015;22:238–41.
19. Campos E, et al. The yiaKLX1X2PQRS and ulaABCDEFG gene systems are required for the aerobic utilization of L-ascorbate in Klebsiella pneumoniae strain 13882 with L-ascorbate-6-phosphate as the inducer. J Bacteriol. 2008;190:6615–24.
20. Murphy PJ, Tate ME, Kerr A. Substituents at N^6 and C-5′ control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in agrobacteria. FEBS J. 1981;115:539–43.
21. Kerr A, Tate ME. Agrocins and the biological control of crown gall. Microbiol Sci. 1984;1:1–4.