

L-Tenuazonic acid, a new inhibitor of *Paenibacillus Larvae*[☆]

Gabriela L. Gallardo¹, Nora I. Peña², Pablo Chacana³, Horacio R. Terzolo³ and Gabriela M. Cabrera^{1,*}

¹Depto de Química Orgánica, FCEyN, UBA, Ciudad Universitaria, Pab. II (1428) Buenos Aires, Argentina

²Depto de Biología, FCEyN, UNMDP, Mar del Plata, Argentina

³Lab. Bacteriología, Depto Prod. Animal, INTA EEA Balcarce, Argentina

*Author for correspondence: Tel./fax: 5411-4576-3346, E-mail: gabyc@qo.fcen.uba.ar

Received 28 August 2003; accepted 27 January 2004

Keywords: *Alternaria brassicicola*, *Alternaria raphani*, American foulbrood, L-Tenuazonic acid, *Paenibacillus larvae*, tetramic acids

Summary

A search for bioactive compounds, inhibitors of *Paenibacillus larvae*, the causal agent of American foulbrood, a honeybees' disease, was carried on. Extracts of two fungal strains, *Alternaria brassicicola* and *Alternaria raphani*, isolated from pollen collected from beehives, exhibited a specific inhibitory activity against this bacterium. From these extracts and by means of chromatographic steps and bioassay-guided fractionation, three tetramic acids were isolated. The compounds were identified by spectroscopic methods and the absolute stereochemistry was chemically determined. L-Tenuazonic acid was shown to be responsible for the antibiotic activity. This compound showed a MIC of 32 µg/ml, comparable with that of oxytetracycline, an antibiotic currently used for the prevention of American foulbrood.

Introduction

American foulbrood (AFB) is a disease very difficult to control, affecting the larvae and pupae of honeybees, and caused by the bacterium *Paenibacillus larvae*. Due to the lack of effective treatments to prevent the disease, beekeepers use the antibiotic oxytetracycline in a routine way in sublethal concentrations. As a consequence of this, the bacterium is capable of acquiring resistance mechanisms to that drug in a very short time. Many new resistant strains are reported everyday. Besides the failure to control disease, this procedure produces contamination of beehives and residual antibiotics are transferred to the honey and other products for human use, generating resistant clinical bacteria with the consequent risk for public health. For the above reasons we began a search for specific antibiotics against *Paenibacillus larvae* produced by fungal cultures. Herewith, we report the isolation and identification of L-Tenuazonic acid from *Alternaria raphani* and *A. brassicicola* cultures, as inhibitor of *P. larvae*. D-allo-Tenuazonic acid, not previously reported as a natural product, and the corresponding tetramic acid of valine were also isolated, but showed no significant activity.

Materials and methods

General

FTIR spectra were recorded on a Nicolet Magna-IR 550 instrument. The u.v. spectra were taken on a Hewlett-Packard 8451A diode-array spectrophotometer. Optical rotations were measured on a Perkin Elmer 343 polarimeter. NMR spectra were recorded on a Bruker AC-200 instrument at 200.1 MHz for ¹H and on a Bruker AM-500 instrument at 125.13 MHz for ¹³C NMR. EIMS were obtained on a Trio-2 quadrupole mass spectrometer (VG Biotech) and the FABMS spectrum was recorded at the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry.

Fungal strains

Alternaria brassicicola (Schw.) Wiltshire and *A. raphani* Groves & Skolko were isolated from pollen and classified, according to the taxonomic criteria by Ellis (Ellis 1971), by Dr Nora Peña, Universidad de Mar del Plata, Argentina, and deposited at the BAFC culture collection (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires). The pollen was collected from beehives of the apiary of Unidad Integrada INTA (Balcarce, Buenos Aires) – Facultad de Ciencias Agrarias, (Universidad de Mar del Plata), Argentina, and

[☆]Dedicated to the memory of Dra Alicia M. Seldes.

diluted with a sterile physiological solution. Dilutions were seeded on agar malt Petri dishes, from which individual colonies were isolated. A Meopta microscope was used for culture observations. The main morphological characteristics were as follows:

Alternaria brassicicola: conidiophores arising singly or branched, isolated or in groups, septate, pale to mid olivaceous, smooth walls, up to 70 μm long and 5–8 μm thick. Conidia mostly in chains of up to 20, some times branched, straight, obclavate, basal cell rounded, apical cell being more or less rectangular with 6–9 transverse septa and up to 6 longitudinal septa, slightly constricted at the septa, pale to dark olivaceous brown, smooth walls, 25–90 μm long, 10–20 μm thick.

Alternaria raphani: conidiophores simple or branched, septate, olivaceous brown, up to 150 μm long and 3–7 μm thick. Conidia generally in chains of 2 or 3, straight or slightly curved, obclavate or ellipsoidal, generally with a short apical beak with 3–7 transverse septa and up to 6 longitudinal or oblique septa, constricted at the septa, golden brown, smooth walls minutely verruculose, 50–70 μm long and 12–20 μm thick.

Fermentation

A well grown agar slant of each fungus was used to inoculate 250 ml Erlenmeyer flasks containing 75 ml of malt extract medium containing malt extract 30 g and peptone 5 g per liter. After a week, each of the above-mentioned media was employed to inoculate 1 l of the same media in 4 \times 4 l Erlenmeyer flasks. The fermentation was carried out at 25 °C for 15 days (stationary phase) under static conditions. The optimal fermentation and harvest conditions were determined in previous small scale experiments and agreed with reported knowledge about secondary metabolite production (Calvo *et al.* 2002).

Extraction and isolation

The cultures of *A. brassicicola* and *A. raphani* were filtered and the culture media were extracted with ethyl acetate (3 \times 500 ml l⁻¹ of culture). The extracts were independently subjected to fractionation on a Sephadex LH-20 column (MeOH, phase: 80 cm h \times 3.5 cm \varnothing), collecting fractions of 200 ml each, and the active fractions (only one per extract) were evaporated and chromatographed on HPLC (Column: YMC C 18, 5 μm , 22.5 \times 2.5 cm; Eluent: MeOH–H₂O 65:35, 5 ml min⁻¹), yielding compounds **1** (2 mg l⁻¹), **2** (65 mg l⁻¹) and **3** (60 mg l⁻¹) from *A. brassicicola* and **1** (1 mg l⁻¹) and **3** (49 mg l⁻¹) from *A. raphani*. Compound **3**, and **1** and **2** in much lesser extent, were the only active components. No activity was lost during the isolation steps.

Antibiotic activity

The antibiotic activity against *P. larvae* was determined by the agar diffusion method (Vanden & Vlietinck

1991), using MPYGP-thiamine 0.01% medium, in the bioassay-guided fractionation. The antibiotic activity of the pure compounds was determined by the agar diffusion method as described above and by the agar dilution method (Vanden & Vlietinck 1991) employing Columbia blood agar. The incubations were carried out at 37 °C in 10% CO₂ and a concentration of 10⁸ bacteria/ml was used in all cases. Three replications were made in each case.

All the strains of *Paenibacillus larvae* used in this work, strains A, C and I–IV, were isolated from different beehives at the province of Buenos Aires, Argentina, and classified and maintained at the Laboratorio de bacteriología, Departamento de Producción Animal, INTA, EEA Balcarce, Argentina. Their morphological and biochemical descriptions will be published in a separate paper.

The antibiotic activity of the crude extracts were also determined against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 18804, by the agar diffusion method using 100 μg of sample/disk. Both extracts were inactive in these assays.

Absolute configuration determination of compounds 1–3

One milligram of each compound was dissolved in acetone and oxidized with Jones reagent (Bowers *et al.* 1953). All the crude product was transferred to a solid phase extraction cartridge, eluted with Cl₂CH₂–MeOH 8:2 and evaporated in vacuum; 0.3 ml of 6M HCl was added to the residue, heated at 120 °C for 8 h and evaporated to dryness. Identification of the amino acids was accomplished by gas chromatography after derivatization (Bodo *et al.* 1985). Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standards.

The GC analyses of *N*-trifluoroacetyl isopropyl ester derivatives were carried out on a Hewlett Packard 5890 gas chromatograph on a Chirasil-Val capillary column (Alltech, 25 m length, 0.25 mm i.d.) with N₂ as carrier gas and a temperature programme: 90–180 °C, 4 °C/min. Retention times (min): L-Val (5.55), D-Val (6.24), D-*allo*-Ile (7.75), D-Ile (8.11), L-*allo*-Ile (8.19), L-Ile (8.44), **1** (5.55), **2** (7.75) and **3** (8.44). Co-injections were realized to confirm the identities.

Compound **1**. Oil. α_{D} = –100 (MeOH, c = 0.13). ¹H NMR (CD₃OD): 3.77 d (3.3), H-5; 2.41 s, H-7; 2.13 m, H-1'; 1.02 d (7.3), H-2'; 0.82 d (6.6), H-3'.

Compound **2**. Oil. α_{D} = +93 (MeOH, c = 0.65). UV (CH₂Cl₂) λ_{max} (log ϵ): 275 nm (4.30). FTIR (KBr) ν_{max} 3300 (OH), 2966 (CH), 2930 (CH), 2880 (CH), 1654 (CO), 1626 (CO), 1469, 1384, 1227 cm⁻¹. EIMS 70 eV *m/z* (rel. int.): 197 [M]⁺• (1), 182 (1), 168 (3), 141 (100), 123 (19), 43 (92). EIMS (Cl, CH₄) *m/z* (rel. int.): 198 [M + H]⁺ (52), 41 (100). FABMS (NaI/Gly): 242 (M + 2 Na-1)⁺. ¹H NMR (CD₃OD): 3.91 d (2.9), H-5; 2.41 s, H-7; 1.92 m, H-1'; 1.28–1.57 m, H-2'; 0.96 t (7.3), H-3'; 0.76 d (6.6), H-4'. ¹³C NMR (CD₃OD): 199.9, C-4;

187.8, C-6; 176.1, C-2; 104.0, C-3; 66.3, C-5; 38.0, C-1'; 27.6, C-2'; 20.4, C-7; 13.3, C-4'; 12.1, C-3'. ^1H NMR (CDCl_3): 6.34 bs, NH_M ; 6.17 bs, NH_m ; 4.03 d (3.2), H-5_m; 3.86 d (3.0), H-5_M; 2.51 s, H-7_m; 2.46 s, H-7_M; 2.03 m, H-1'; 1.42 m, H-2'a; 1.33 m, H-2'b; 0.97 t (7.3), H-3'_m; 0.96 t (7.5), H-3'_M; 0.82 d (6.9), H-4'_m; 0.79 d (6.9) H-4'_M. ^{13}C NMR (CDCl_3): 195.7, C-4; 188.9, C-6_m; 184.6, C-6_M; 175.6, C-2; 107.6, C-3_m; 102.4, C-3_M; 65.8, C-5_M; 62.6, C-5_m; 36.4, C-1'; 26.9, C-2'_M; 26.6, C-2'_m; 20.6, C-7_m; 19.5, C-7_M; 13.1, C-4'_m; 12.9, C-4'_M; 11.7, C-3'.

Compound **3**. Oil. $\alpha_D = -99$ (MeOH, $c = 0.44$). UV (CH_2Cl_2) λ_{max} (log ϵ): 276 nm (3.96). FTIR (KBr) ν_{max} 3322 (OH), 2966 (CH), 2930 (CH), 2880 (CH), 1661 (CO), 1619 (CO), 1455, 1234 cm^{-1} . EIMS 70 eV m/z (rel. int.): 197 [$\text{M}]^+$ (1), 182 (2), 168 (3), 141 (100), 123 (25), 43 (73). ^1H NMR (CD_3OD): 3.84 d (3.3), H-5; 2.42 s, H-7; 1.90 m, H-1'; 1.19–1.43 m, H-2'; 0.99 d (7.3), H-3'; 0.90 t (7.3), H-4'. ^{13}C NMR (CD_3OD): 198.7, C-4; 187.8, C-6; 175.8, C-2; 104.0, C-3; 67.4, C-5; 38.4, C-1'; 24.8, C-2'; 20.7, C-7; 15.9, C-4'; 12.2, C-3'.

Results and discussion

A search for specific antibacterial fungal extracts against the bee-pathogenic bacterium *Paenibacillus larvae* was carried on. Fungal strains were isolated from pollen collected from beehives, under the assumption that most of this natural population would be non-toxic for bees. If so, the isolated strains could be used as fungal biocontrol agents. The extracts of two *Alternaria* fungal strains, *A. brassicicola* and *A. raphani*, inhibited the growth of *P. larvae* and were inactive against the bacterial strains *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. Bioassay-guided fractionation of larger scale extracts by several chromatographic techniques yielded the tetramic acids **1–3**, compound **3** being the responsible for the activity (Figure 1). Compounds **1** and **2** were only slightly active, with *in vitro* MICs larger than 100 μg per disk.

The analysis of the NMR spectra of compounds **1** and **3** allowed their identification as 5-isopropyl-3-(1-hydroxyethylidene)-pyrrolidine-2,4-dione and tenuazonic acid respectively, by comparison with literature data (Nolte *et al.* 1980; Poncet *et al.* 1990).

In order to determine their absolute configuration, both compounds were oxidized with Jones reagent and hydrolysed with 6M HCl to obtain the corresponding amino acids, which were analyzed by GC as a *N*-trifluoroacetyl isopropyl ester derivative in a Chiral-sil-val column. These results determined the L-isoleucine configuration for the amino acid moiety of compound **3** and L-valine for that of compound **1**.

The ^1H NMR spectrum in CD_3OD of compound **2** was very similar to that of compound **3**, the main differences being the signals of H-5, H-3' and H-4'. A doublet at δ 3.91, a triplet at δ 0.96 and a doublet at δ 0.76 were observed respectively for these protons. The

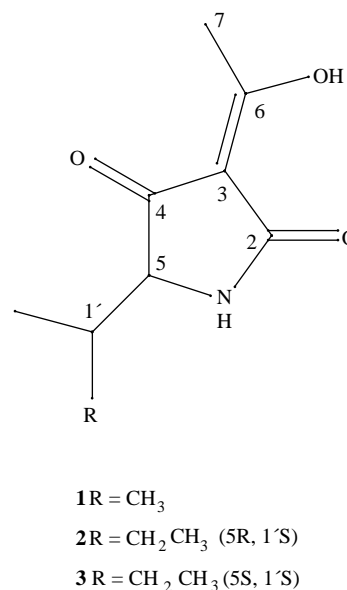


Figure 1. Compounds isolated from *Alternaria* strains from pollen.

mass spectrum revealed a molecular weight of 197, similar to that of compound **3**, confirmed by chemical ionization and fast atom bombardment. These results would indicate that both compounds were diastereomeric. The ^{13}C NMR and DEPT spectra, and a COLOC experiment allowed us to confirm, with the aid of literature (Laatsch 2000), the skeleton of compound **2** to be same as **3**, 5-*sec*-butyl-3-(1-hydroxyethylidene)-pyrrolidine-2,4-dione. The *D-allo*-isoleucine configuration for the amino acid moiety of compound **2** was determined in the same way as above, resulting this compound being *D-allo*-tenuazonic acid. The antibiotic activity was evaluated by the agar diffusion and agar dilution methods, comparing the MIC values for L-tenuazonic acid (**3**) with those of oxytetracycline and gentamicine against six different strains of *P. larvae*. The results are shown in Table 1. The inhibition halos by agar diffusion were slightly smaller for L-tenuazonic acid (**3**) and oxytetracycline, probably due to the lesser diffusion of these compounds in the aqueous media.

L-Tenuazonic acid was previously isolated from *Alternaria tenuis* (Rosset *et al.* 1957; Royles 1995), *Pyricularia oryzae* (Nukina & Saito 1992) and an *Aspergillus* sp. (Miller *et al.* 1963). This compound has been reported to have antiviral activity (Miller *et al.* 1963) and an inhibitory effect on human adenocarcinoma growth (Kazcka *et al.* 1964; Gitterman 1965). L and synthetic *D-allo*-tenuazonic acids exhibited low antibacterial activity against *Bacillus megaterium* (Gitterman 1965), and were essentially inactive against 31 bacterial strains, 48 yeast strains and showed moderate LD₅₀ toxicities, when administered to male mice (Miller *et al.* 1963).

Compound **2** has been synthesized (Poncet *et al.* 1990) in a study of racemization during the synthesis of tetramic acids but has not previously been reported as a natural product.

Table 1. MIC values for L-tenuazonic (3) and commercial antibiotics against six *Paenibacillus larvae* strains.

Compound	Strain					
	A	C	I	II	III	IV
L-Tenuazonic (3) ^a	1.0	1.0	1.0	1.0	5.0	1.0
Oxytetracycline ^a	0.5	0.5	0.5	0.5	0.5	0.5
Gentamicine ^a	0.5	0.5	0.5	0.5	0.5	0.5
L-Tenuazonic (3) ^b	32	32	32	32	32	32
Oxytetracycline ^b	64	32	32	32	16	16
Gentamicine ^b	2	16	4	4	8	8

^a MIC in $\mu\text{g disk}^{-1}$ determined by the agar diffusion method.

^b MIC in $\mu\text{g ml}^{-1}$ determined by the agar dilution method.

L-Tenuazonic acid (3) has proved to be a specific antibiotic against *P. larvae*, as active as oxytetracycline *in vitro*. The possibility of developing *A. raphani* as a fungal biocontrol agent was discarded since it also produced small amounts of altertoxins, which are known mycotoxins (Stack *et al.* 1986). However, it is noteworthy that since the fungal strains were isolated from pollen collected from beehives, all the isolated compounds may be naturally present in honey or other apiculture products. Further studies on the *in vivo* activity and formulation of L-tenuazonic acid are now under way.

Acknowledgements

We thank Dr Jorge A. Palermo and UMYMFOR (CONICET-FCEN, UBA) for the NMR spectra, UMYMFOR (CONICET-FCEN, UBA) and Washington University Resource for Biomedical and Bio-organic Mass Spectrometry for the mass spectra CONICET, ANPCYT and Universidad de Buenos Aires for partial financial support.

References

- Bodo, B., Rebuffat, S., El Hajji, M. & Davoust, D. 1985 Structure of trichorzianine A IIIc, an antifungal peptide from *Trichoderma harzianum*. *Journal of the American Chemical Society* **107**, 6011–6017.
- Bowers, A., Halsall, T.G., Jones, E.R.H. & Lemin, A.J. 1953 The chemistry of the triterpenes and related compounds. Part XVIII. Elucidation of the structure of polyporenic acid C. *Journal of the Chemical Society* 2548–2553.
- Calvo, A.M., Wilson, R.A., Bok, J.W. & Keller, N.P. 2002 Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**, 447–459.
- Ellis, M.B. 1971 *Dematiaceae hyphomycetes*. The Eastern Press, Ltd. London pp. 467–468, 474.
- Gitterman, C.O. 1965 Antitumor, cytotoxic and antibacterial activities of tenuazonic acid and congeneric tetramic acids. *Journal of Medicinal Chemistry* **8**, 483–486.
- Kazcka, E.A., Gitterman, C.O., Dulaney, E.L., Smith, M.C., Hendlin, D., Woodruff, H.B. & Folkers, K. 1964 Discovery of inhibitory activity of tenuazonic acid for growth of adenocarcinoma-1. *Biochemical and Biophysical Research Communications* **14**, 54–57.
- Laatsch, H. 2000 *Antibase-A database for rapid identification of microbial natural products*, Chemical Concepts, Weinheim, Germany.
- Miller, F.A., Rightsel, W.A., Sloan, B.J., Ehrlich, J., French, J.C. & Bartz, Q.R. 1963 Antiviral activity of tenuazonic acid. *Nature* **200**, 1338–1339.
- Nolte, M.J., Steyn, P.S. & Wessels, P.L. 1980 Structural investigations of 3-acylpyrrolidine-2,4-diones by nuclear magnetic resonance spectroscopy and X-ray crystallography. *Journal of the Chemical Society Perkin Transactions I*, 1057–1065.
- Nukina, M. & Saito, T. 1992 Microbial production of a norvaline-analog of tenuazonic acid by *Pyricularia oryzae*. *Bioscience, Biotechnology and Biochemistry* **56**, 1314–1315.
- Poncet, J., Jouin, P., Castro, B., Nicolas, L., Boutar, M. & Gaudemer 1990 Tetramic acid chemistry. Part I. Reinvestigation of racemisation during the synthesis of tetramic acids via Dieckmann cyclisation. *Journal of the Chemical Society Perkin Transaction I*, 611–616.
- Rosset, T., Sankhala, R.H., Stickings, C.E., Taylor, M.E.U. & Thomas, R. 1957 Studies on the biochemistry of microorganisms. 103. Metabolites of *Alternaria tenuis* auct.: culture filtrate products. *Biochemical Journal* **67**, 390–394.
- Royles, B.J.L. 1995 Naturally occurring tetramic acids: Structure, isolation and synthesis. *Chemical Reviews* **95**, 1981–2001.
- Stack, M.E., Mazzola, E.P., Page, S.W., Pohland, A.E., Highet, R.J., Tempesta, M.S. & Corley, D.G. 1986 Mutagenic perylenequinone metabolites of *Alternaria alternata*: Altertoxins I, II, and III. *Journal of Natural Products* **49**, 866–871.
- Vanden, D.A. & Vlietinck, A.J. 1991 Screening methods for antibacterial and antiviral agents from higher plants. In *Assays for bioactivity*, ed. Hostettmann, K. from *Methods in Plant Biochemistry*, Vol. 6, eds. Dey, P.M. & Harborne, J.B. pp. 47–69. Academic Press. ISBN 0-12-461016-1.