Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 1495–1497
Bioorganic & Medicinal Chemistry Letters

Lysianadioic acid, a carboxypeptidase B inhibitor from *Lysiana subfalcata*

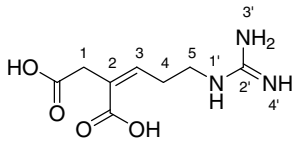
Malcolm S. Buchanan,[a] Anthony R. Carroll,[a] Annette Edser,[a] Melissa Sykes,[a] Gregory A. Fechner,[a] Paul I. Forster,[b] Gordon P. Guymer[b] and Ronald J. Quinn[a,*]

[a]*Eskitis Institute, Griffith University, Brisbane, Qld 4111, Australia*
[b]*Queensland Herbarium, Brisbane Botanic Gardens, Toowong, Qld 4066, Australia*

Received 18 November 2007; revised 21 December 2007; accepted 21 December 2007
Available online 25 December 2007

Abstract—A new natural product, lysianadioic acid, was isolated from the plant *Lysiana subfalcata* as a carboxypeptidase B (CPB) inhibitor. It is a potent inhibitor of CPB with an IC_{50} of 0.36 μM. This is the first known example of a small molecule CPB inhibitor isolated from plant origin. Its structure was determined by NMR spectroscopy.

In a continuation of our investigations to find novel carboxypeptidase inhibitors[1,2] a carboxypeptidase B (CPB) high-throughput screening campaign was conducted. CPB is a pancreatic digestive metalloenzyme, which releases basic amino acids from the carboxyl-terminus of proteins or peptides. The search for lead compounds from natural sources which inhibit CPB resulted in a bioactive MeOH extract of the plant *Lysiana subfalcata* (Hook.) Barlow (Loranthaceae) being studied further.[3] Bioassay-guided purification resulted in the alkaloid lysianadioic acid **(1)** (Fig. 1) being isolated as the active constituent. To date the only CPB inhibitors reported from plants have been large polypeptides from potato[4,5] and tomato.[6,7] Compound **1** is the first known example of a small molecule carboxypeptidase B inhibitor isolated from plant origin. Actinophyllic acid from *Alstonia actinophylla* was previously suggested to be a carboxypeptidase inhibitor, but the activity observed in a coupled enzyme assay was not confirmed in a direct carboxypeptidase assay.[1] This paper reports the isolation, structure elucidation and biological activity of **1**. It is the first known chemical investigation of the plant *L. subfalcata*.



1

Figure 1. Lysianadioic acid **(1)**.

The plant *Lysiana subfalcata* (100 g) was ground and extracted with MeOH. This MeOH extract showed inhibitory activity against the CPB enzyme assay.[8] A series of partitions were then performed on the MeOH extract (22.3 g). Firstly a *n*-hexane/MeOH partition, the MeOH layer was then dried, followed by a H_2O/MeOH(4:1)/CH_2Cl_2 partition, the H_2O/MeOH (4:1) layer was then dried, then finally a butanol/H_2O partition. Testing of the layers directed further purification of the H_2O layer (14.7 g). The H_2O layer was filtered through strongly basic ion-exchange resin (DOWEX 1X8-400A) and washed sequentially with MeOH/H_2O and 2% TFA solution. The MeOH/H_2O wash contained bioactivity and was subsequently filtered through strongly acidic ion-exchange resin (DOWEX 50WX8-400A) and washed sequentially with MeOH/H_2O followed by a 5% aqueous NH_3 solution. CPB inhibitory activity was found only in the NH_3 fraction. Half of the NH_3 fraction (3.98 g) was pre-adsorbed on C_{18} (04K-4348 Sepra C_{18} End-Capped Silica) and loaded into a guard column in line with a semi-preparative

Keywords: Carboxypeptidase B (CPB); Small molecule inhibitor; Natural products; *Lysiana subfalcata*; Structure elucidation; NMR spectroscopy.
* Corresponding author. Tel.: +61 7 3735 6006; fax: +61 7 3735 6001; e-mail: r.quinn@griffith.edu.au

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.12.060

Table 1. ^{1}H (600 MHz), ^{13}C (125 MHz), HMBC and COSY NMR data for lysianadioic acid **(1)** in D_2O[a]

Position	δ_C	δ_H (multi, J Hz)	$^{2,3}J_{CH}$ HMBC (C no.)	COSY (H no.)
1	42.7 CH_2	3.25 (s, 2H)	1-COOH, 2-COOH, 2, 3	
2	132.6 qC			
3	139.1 CH	5.72 (t, 7.2)	2-COOH, 1	4
4	29.9 CH_2	2.62 (q, 7.2)	2, 3, 5	3, 5
5	41.8 CH_2	3.34 (t, 7.2)	3, 4, 2′	4
1′				
2′	158.4 qC			
3′				
4′				
1-COOH	178.8 qC			
2-COOH	174.6 qC			

[a] NMR spectra were recorded at 30 °C on Varian Inova 500 and 600 MHz NMR spectrometers. Samples were dissolved in D_2O (^{1}H) or D_2O + 2% DMSO (^{13}C), chemical shifts calculated relative to the solvent peak (D_2O ^{1}H δ 4.80 and DMSO ^{13}C 39.5 ppm). Multiplicity determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). Standard parameters were used for the 2D experiments, which included gradient gCOSY, gHSQC ($^{1}J_{CH}$ = 140 Hz) and gHMBC ($^{n}J_{CH}$ = 8.3 Hz).

Betasil C_{18}HPLC column (Betasil C_{18} 5 μm 21.2 mm × 150 mm i.d.). Isocratic conditions of H_2O/1%TFA for 40 min, then a gradient to H_2O/1%TFA:-MeOH/1%TFA (9:1) in 20 min (flow 10 mL/min), were used and 120 fractions were collected. A bioactive band eluted early resulting in combining fractions 16–20 (520 mg). This was then chromatographed by gel permeation chromatography using Superdex 30, eluting with water (0.5 mL/min) and 60 fractions were collected. There was a spread of activity in fractions 36–47, which were then combined (288 mg). The combined fraction then underwent two consecutive Synergi Hydro-RP 80Å HPLC (4 μm, 10.0 mm × 250 mm i.d.) purification steps under gradient conditions over 40 min. Firstly the 288 mg fraction (10 separate injections) using a gradient from H_2O/1%TFA to H_2O/1%TFA:MeOH/1%TFA (9:1) in 30 min followed by a 10 min gradient to MeOH/1%TFA (flow 4 mL/min). The desired fractions were combined (4.5 mg), then secondly a gradient from H_2O/1%TFA to H_2O/1%TFA:MeOH/1%TFA (8:2) in 30 min followed by a 10 min gradient to MeOH/1%TFA (flow 4 mL/min) was used. Lysianadioic acid **(1)** (1.30 mg, 0.0026% dry wt) eluted with retention time 32.0 min.

Lysianadioic acid **(1)** was isolated as an optically inactive amorphous solid.[9] A pseudomolecular ion in the positive HRESIMS at *m/z* 216.0983 (Δ 1.9 ppm) allowed a molecular formula of $C_8H_{13}N_3O_4$ to be assigned to **1**. The natural product was analysed using a series of one and two-dimensional NMR experiments in order to determine its structure. The ^{1}H NMR spectrum (Table 1) was very simple showing an olefinic proton {δ_H 5.72 (t, 7.2 Hz)} and six methylene protons {δ_H 2.62 (q, 7.2 Hz, 2H); 3.25 (s, 2H); 3.34 (t, 7.2 Hz, 2H)}, while the ^{13}C NMR spectrum (Table 1) revealed the presence of eight carbons. The isolated methylene singlet at δ_H 3.25 in the ^{1}H NMR spectrum had gHMBC correlations to two carboxylic acid carbonyls {δ_C 178.8, 174.6} and two olefinic carbons {δ_C 132.6 (s), 139.1 (d)}. The proton (δ_H 5.72) attached to the olefinic carbon at δ_C 139.1 had a gCOSY correlation to the methylene protons at δ_H 2.62 and these methylene protons (δ_H 2.62) had a further gCOSY correlation to the methylene protons at δ_H 3.34. This information disclosed a 2-pentene-1,2-dicarboxylic acid moiety. The remaining signal to be accounted for was a downfield quaternary carbon at δ_C 158.4 in the ^{13}C NMR spectrum, which had a chemical shift characteristic for a guanidine carbon. In the gHMBC spectrum there was a correlation between the methylene protons at δ_H 3.34 and the carbon at δ_C 158.4. A guanidine moiety attached to the methylene group at δ_C 41.8 in the 2-pentene-1,2-dicarboxylic acid moiety completed the molecular formula revealed by HRESIMS. Now all that remained was to establish whether the olefinic bond was *Z* or *E*. A ROESY correlation between H_2-1 and H-3 confirmed a *Z* configuration. Therefore, lysianadioic acid was assigned structure **1**, (2*Z*)-2-(3-carbamimidamidopropylidene)butanedioic acid.

Lysianadioic acid **(1)** inhibited CPB with an IC_{50} of 0.36 μM. Based on published data for X-ray structures of small molecule inhibitors in CPB the following can be predicted about **1** in the active site of CPB.[10] The carboxylic acid, 1-COOH, chelates the Zinc, and the carboxylic acid, 2-COOH, and guanidine group form hydrogen bonds (salt bridges) to Arg145 and Asp255, respectively. Compound **1** is a close mimic to the natural substrate arginine and probably binds in a similar manner.

In conclusion, lysianadioic acid **(1)**, a potent small molecule inhibitor of CPB, was isolated from *L. subfalcata.* It is a new arginine analogue containing an unusual dicarboxylic acid moiety and is the first known example of a small molecule CPB inhibitor isolated from plant origin.

Acknowledgments

We thank AstraZeneca for financial support. We are indebted to Dr. Jennifer Mitchell, Griffith University, Brisbane, for HRESIMS analyses.

References and notes

1. Carroll, A. R.; Hyde, E.; Smith, J.; Quinn, R. J.; Guymer, G.; Forster, P. I. *J. Org. Chem.* **2005**, *70*, 1096.
2. Bjoerquist, P.; Buchanan, M.; Campitelli, M.; Carroll, A., Hyde, E.; Neve, J.; Polla, M., Quinn, R. PCT Int. Appl., WO 200503961, 2005; *Chem. Abstr.* **2005**, 142, 423904.
3. The plant sample *Lysiana subfalcata* (Hook.) Barlow (Loranthaceae) was collected at The Pinnacle, Glenlyon Dam-Pinnacle Rd, Darling Downs, Queensland, Australia in February 1994. A voucher sample, AQ600699, was lodged at the Queensland Herbarium, Brisbane, Qld, Australia.
4. Leary, T. R.; Grahn, D. T.; Neurath, H.; Hass, G. M. *Biochemistry* **1979**, *18*, 2252.
5. Rancour, J. M.; Ryan, C. A. *Arch. Biochem. Biophys.* **1968**, *125*, 380.
6. Hass, G. M.; Hermodson, M. A. *Biochemistry* **1981**, *20*, 2256.
7. Hass, G. M.; Ryan, C. A. *Phytochemistry* **1980**, *19*, 1329.
8. *CPB bioassay*: Compound in aqueous solution was added to microplates (5 μL per well). Then to each well was added 50 μL of CPB enzyme (0.063 U/mL) in assay buffer (50 mM HEPES, 0.25% BSA, pH 7.4). The reaction was initiated with addition of 35 μL of the chromogenic substrate, anisylazoformyl-L-lysin (190 μM), in assay buffer. The plate was incubated at ambient temperature (~22 °C) for 90 min and the absorbance at 355 nm was read on a VictorII™ multimode plate reader (Wallac, Turku, Finland). Percent activity for each compound was determined by the following equation:

$$\%\ \text{Activity} = \left[\frac{\text{Abs}_{\text{Cmpd}} - \text{Abs}_{0\%\ \text{Inhibition}}}{\text{Abs}_{100\%\ \text{Inhibition}} - \text{Abs}_{0\%\ \text{Inhibition}}}\right] \times 100\%$$

where 0% inhibition is the absorbance of the full reaction and 100% inhibition is the absorbance following addition of MGTPA (1 μM final concentration), a carboxypeptidase inhibitor.
9. Lysianadioic acid **(1)**, (2*Z*)-2-(3-carbamimidamidopropylidene)butanedioic acid: isolated as an amorphous solid; UV (MeOH) λ_{max} (log ε) 198 (3.65) nm; IR ν_{max} (film) 3452, 1678, 1206, 1140 cm^{-1}; ^{1}H and ^{13}C NMR: see Table 1; positive-HRESIMS *m/z* 216.0983 $[C_8H_{13}N_3O_4 + H]^+$ (calcd 216.0979).
10. Islam, I.; Bryant, J.; May, K.; Mohan, R.; Yuan, S.; Kent, L.; Morser, J.; Zhao, L.; Vergona, R.; White, K.; Adler, M.; Whitlow, M.; Buckman, B. O. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1349.