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L-755,807, a New Non-peptide Bradykinin Binding Inhibitor from an Endophytic *Microsphaeropsis* sp.

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Abstract: A new metabolite, L-755,807, **1**, was isolated from an endophytic *Microsphaeropsis* sp. in the course of searching for a bradykinin binding inhibitor. The structure of **1**, including relative stereochemistry, was determined. **1** showed an IC_{50} of 71 μM in ^{3}H-bradykinin binding to a cloned human B_2 receptor.

The kinins, bradykinin (BK) and kallidin, are endogenous peptides which act on specific cell surface receptors to produce a myriad of physiological responses. The major subtypes of receptors are B_1 and B_2. B_1 receptors are rare. B_2 receptors are expressed by most nonmyeloid cells and mediate the majority of kinin effects, including hyperalgesia, pain, secretion, contraction and relaxation. A B_2 receptor antagonist can afford a potential therapy for inflammatory edema, pain, rhinitis, shock (septic, pancreatitis) and asthma[1,2]. In our search for novel non-peptide BK binding inhibitors, using ^{3}H-BK and intact Chinese hamster ovary (CHO) cells expressing the human B_2 receptor, we found a new metabolite, **1**, from the culture of a *Microsphaeropsis* sp. (Deuteromycotina, Coelomycetes), MF6057. This report describes the isolation and determination of the structure of **1** (Fig. 1).

Results and Discussion

Isolation of **1** from fermentation broth was accomplished by extraction with methyl ethyl ketone and purification by the following sequence of chromatographic steps: a) E. Merck silica gel 60 flash column using stepwise gradients of 0 - 50 % Me_2CO/hexane for elution; b) Whatman Partisil 10 ODS-3 column using 55 % MeCN/H_2O for isocratic elution; and c) DuPont Zorbax Sil column using 15 % Me_2CO/hexane for elution. This process afforded 40 mg of homogeneous **1**.

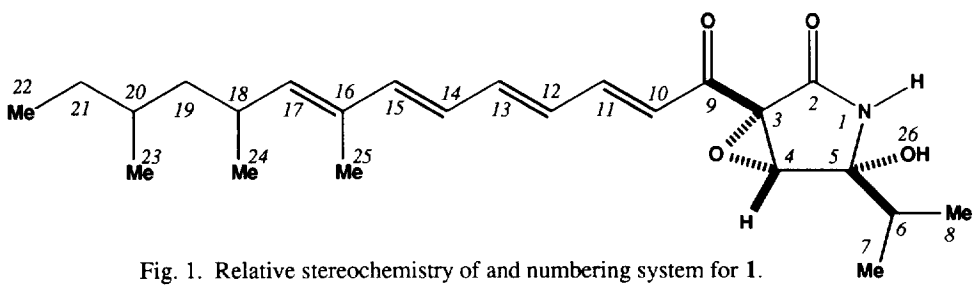

Fig. 1. Relative stereochemistry of and numbering system for **1**.

Table 1. Chemical shifts assignments and ^{1}H-^{13}C long range connectivities for **1** (CD_2Cl_2, 25 °C).

Position	δ_C[a] (100 MHz)	δ_H (400 MHz)	Long-range coupled protons[b]
1		8.03 (br s)	
2	169.9 (s)		4-H
3	60.9 (s)		1-H
4	65.8 (d)	4.28 (d, J = 2.4)	1-H
5	87.6 (s)		1-H, 4-H, 7-H, 8-H
6	33.6 (d)	2.06 (m)	7-H, 8-H
7	17.8 (q)	1.16 (d, J = 6.8)	8-H
8	16.2 (q)	1.11 (d, J = 6.8)	7-H
9	189.8 (s)		10-H, 11-H
10	129.0 (d)	6.30 (d, J =15.2)	12-H
11	146.7 (d)	7.30 (dd, J = 11.6, 15.2)	13-H
12	120.7 (d)	6.32 (dd, J = 10.9, 15)	
13	146.5 (d)	6.68 (dd, J = 10.9, 14.9)	11-H
14	125.9 (d)	6.23 (dd, J = 11, 15.1)	12-H, 15-H
15	144.9 (d)	6.46 (d, J = 15.2)	13-H, 14-H, 17-H, 25-H
16	133.0 (s)		14-H, 15-H, 25-H
17	145.3 (d)	5.43 (br d, J = 9.7)	15-H, 24-H, 25-H
18	31.1 (d)	2.65 (m)	24-H
19	45.0 (t)	1.10 (m)	23-H, 24-H
20	32.7 (d)	1.15 (m)	22-H, 23-H
21	30.4 (t)	0.83 (m)	22-H, 23-H
22	11.4 (q)	0.85 (t, J = 7.0)	
23	19.2 (q)	0.83 (d, J = 6.4)	
24	21.4 (q)	0.97 (d, J = 6.6)	17-H
25	12.6 (q)	1.78 (br s)	15-H
26		4.55 (br s)	

[a] multiplicities deduced from DEPT; [b] correlations deduced from HMBC; abbreviations, s: singlet, d: doublet, t: triplet, q: quartet

Assignments of the ^{13}C and ^{1}H NMR chemical shifts derived from ^{13}C NMR, ^{1}H NMR, DEPT, COSY, HMQC, HMBC and NOESY experiments in CD_2Cl_2 are depicted in Table 1.

The structure of **1** (Fig.1) was derived based on extensive spectroscopic analysis. HREIMS suggested a molecular formula of $C_{24}H_{35}NO_4$ (M^+, 100%, *m/z* found: 401.2577, calculated: 401.2566) for **1**, which required eight double bond equivalents (dbe's). This formula was supported by the presence of 24 lines in its broad band decoupled ^{13}C-NMR spectrum. The carbon types, revealed by a DEPT experiment, included 6 methyls, 2 methylenes, 11 methines and 5 non-protonated carbons, two of which were carbonyls: δ169.9 *(lactam)* and δ189.9 ppm *(ketone)*. This required 33 of the 35 protons as carbon-attached and suggested the remaining two to be exchangeable. Single-frequency decoupling and COSY experiments afforded four partial structures: **A**, **B**, **C** and **D** (Fig. 2). In addition, HMBC and LR-COSY experiments revealed connectivities among these partial structures and the remainder of the molecule (Table 1). Of significance, placements of C-25 and C-16 were established by the key ^1H-^{13}C long range correlations observed between H-25 and C-15/C-16/C-17, H-17 and C-15, and H-15 and C-16/C-25/C-17. A long range ^1H-^1H correlation between H-17 and H-25 (allylic and W-couplings) was also observed in a LR-COSY spectrum in support of this placement. Locating the carbonyl (C-9) adjacent to C-10 was suggested by UV (λ_{max} 361, log ε 4.44) and IR (1710 cm^{-1}) spectra, and confirmed by two HMBC correlations: H-10/C-9 and H-11/C-9. The geometry of the tetraene portion was established as *E,E,E,E*, largely by consideration of the multiplicities and coupling constants (11~15 Hz) observed among H-10~H15. The stereochemistry at the C-16/C-17 double bond was supported by NOESY correlations (0.7 and 0.3 sec mixing time) observed between H-17/H-15. In a ROESY experiment with mixing times of 0.1 and 0.15 sec, correlations were also observed for H-13/H-11 and H_3-25/H-14. This completed the structure of the acyl side chain in **1** and accounted for five of the eight total dbe's.

The remainder of **1** must account for three dbe's: the remaining carbonyl (C-2, δ169.9 ppm, *lactam*) and two rings, including partial structures **C** and **D** together with the quaternary carbons C-5 (δ87.6 ppm) and C-3 (δ60.9 ppm), one exchangeable proton (OH), and an oxygen atom. The presence of a γ-lactam in **1** was suggested by an absorption at 1733 cm^{-1} in its IR spectrum. The HMBC experiment revealed connectivities of the partial structure **C** and **D** at C-5. In addition, correlations between H-4 and C-2, and H-1 and quaternary C-3 were observed. This information together with chemical shift, valence, elemental and dbe considerations suggested the construction of a γ-lactam α,β-fused with an epoxide, the placement of an hydroxyl at C-5 to

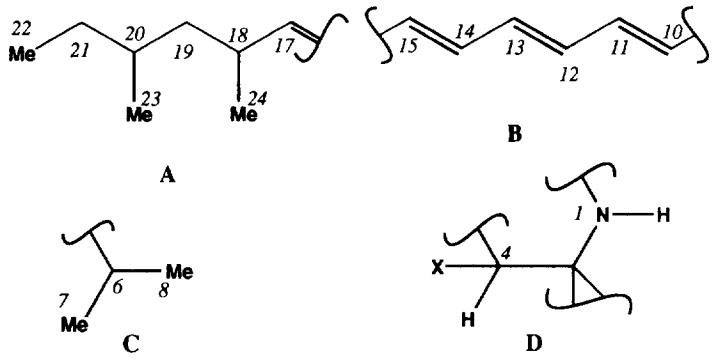

Fig. 2. Partial structures of **1** derived from a COSY experiment.

account for an exchangeable proton, and leaving C-3 as the only point of attachment to the tetraenone sidechain. The results of a deutero-isotope shift experiment (CD_3OD/CD_3OH), summarized in Fig. 3, supported such an arrangement. The order of effects on the ^{13}C resonances of **1** was found to be C-5 > C-2 > C-4 >C-3, in qualitative agreement with their proximity to deutero-exchangeable protons (β and γ shifts). The large deuterium β-shift of 0.18 ppm for C-5 (β and γ shift) confirmed that C-5 was hydroxy-bearing and adjacent to the lactam NHCO group. The planar structure of **1** was thus established. Furthermore in the NOESY experiment, correlations observed between H-4 and H_3-7/H_3-8, established H-4 and the isopropyl group to be *cis*; and together with a fused epoxide constraint allowed the relative stereochemistry for **1** to be assigned as shown.

Only the lactone antibiotic, S39163/F-1, and the diketopiperazine antibiotics, TAN-1496 A~E[3,4], were previously identified from *Microsphaeropsis* spp. A literature survey did not reveal any compound structurally related to **1**. The structural type for **1** is unprecedented. **1** however is similar to a proposed intermediate tetramic acid derivative in the biosynthetic pathway towards the relatively rare class of tenellin-type compounds[5]. Extensive and detailed biosynthetic studies of tenellin have shown that it is derived from a polyketide precursor that incorporates phenylalanine. By analogy then, **1** may be biosynthesized through a pathway starting from polyketide and valine precursors. Carbon alkylation at C-16, C-18 and C-20 by S-adenosylmethionine may introduce the C-25, C-24 and C-23 methyl substituents.

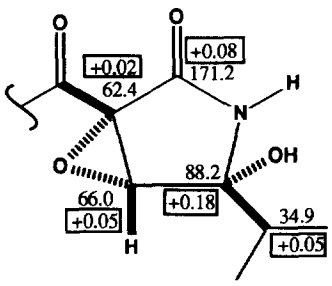

Fig. 3. ^2H-Isotope effects (▭) on ^{13}C chemical shifts (ppm), CD_3OD vs CD_3OH, of γ-lactam portion of **1**.

L-755,807 only showed an IC_{50} of 71 μM in ^3H-BK binding to a cloned human B_2 receptor expressed in CHO cells. For comparison, WIN 64338[6], a known synthetic antagonist, demonstrated an IC_{50} of 61 nM. The potency of **1** is relatively weak in this regard.

Experimental

UV spectra were recorded with a Beckman model DU-70 spectrophotometer. IR spectra were recorded as a neat deposit on a Zn-Se crystal with a Perkin-Elmer model 1750 FT-IR spectrophotometer. MS data were recorded on either Finnigan-MAT 90 (FAB) or TSQ70B (FAB, EI) mass spectrometers. Exact mass measurements were performed on the MAT 90 at high resolution using CsI as the internal standard. Optical rotation was measured on a Perkin-Elmer model 241 polarimeter.

^1H-NMR and ^{13}C-NMR spectra were recorded in 0.75 ml, 40 mM solutions in CD_2Cl_2 at 25 °C, on a Varian Unity 400 NMR spectrometer, using either a standard 5 mm direct or indirect probe. Chemical shifts are given in ppm relative to TMS at zero ppm, using the solvent peaks at δ5.32 ppm (for ^1H spectra) and δ53.8 or (for ^{13}C spectra) as internal standard. Proton-proton chemical shift correlation spectra (COSY) were recorded using the standard pulse sequence[7]. Proton-carbon chemical shift correlations were obtained using the indirect detection methods, HMQC[8] (with $^1J_{XH}$ = 140 Hz) and HMBC[9]. The HMBC spectra were optimized for a $^nJ_{CH}$

of 7 Hz. NOESY experiments[10] were performed with mixing times of 0.7, 0.5 and 0.3 sec. ROESY experiments[11] were performed with mixing times of 0.1 and 0.15 sec. Deuteroisotope shift effects on ^{13}C resonances were observed by recording ^{13}C-NMR spectra in both CD_3OD and CD_3OH in the same instrument, using the solvent peak of CD_3OD at δ49.0 ppm as internal standard, the peak for CD_3OH at 49.12 ppm. In this deuterium exchange experiment with a spectral width of 25000 Hz, 59968 data points were recorded and zero-filling was not used for data processing.

Analytical HPLC assay for evaluating fraction purity was performed on a Zorbax Sil column (4.6 x 250 mm). The column was eluted at 1 ml/minute flow rate with 20 % Me_2CO/C_6H_{14} at room temperature. The effluent was monitored at 400 nm.

Fermentation

The producing fungus, isolated from surface-sterilized living twigs of *Prosopsis glandulosa* (honey mesquite) collected in Chaparral Wildlife Management Area, Dimmit Co., Texas, U.S.A., was identified as a *Microsphaeropsis* sp. (Deuteromycotina, Coelomycetes) and given the accession number, MF6057 (Merck Microbial Resources Culture Collection).

Vegetative mycelia of the fungus was prepared by inoculating 54-ml portion of aq nutrient medium[12] in a 250-ml non-baffled Erlenmeyer flask with 2-ml of mycelia in 10% glycerol that had been stored at -80 °C. This seed culture was incubated for 3 days at 25 °C with 50% relative humidity on a rotary shaker at 220 rpm with a 5-cm throw under constant fluorescent light. A 2-ml portion of the 3-day seed culture was aseptically transferred to 50-ml of production medium in a 250-ml non-baffled Erlenmeyer flask. The production medium contained (g/liter): sucrose, 75; tomato paste, 10; malt extract, 5; $(NH_4)_2SO_4$, 1; soy flour, 1; and KH_2PO_4, 9. The pH of the production medium was adjusted to 7.0 with NaOH before sterilization. Production cultures were incubated at 25 °C at 220 rpm with a 5 cm throw and 50% relative humidity for up to 22 days. All culture media were steam sterilized at 121 °C and 15 psi for 20 minutes.

Isolation of ***1***

The contents of 28 production flasks described above were pooled and extracted with methyl ethyl ketone (1960 ml). The aq layer was discarded. The upper organic layer, after flash evaporation to dryness (2.1 g) was mixed with CH_2Cl_2 (10 ml) and purified on a flash column of 100 g silica gel 60 (E. Merck, 40-63 μm) in C_6H_{14} using a stepwise gradient (0, 5, 10, 15, 20, 25, 30, 35, 40, 50 and 100 % Me_2CO/C_6H_{14}, 250 ml per step). The compound of interest was located in the 25~30 % Me_2CO/C_6H_{14} fraction.

The enriched fraction above was concentrated to dryness (433 mg), dissolved in 1 ml of MeOH and chromatographed as one injection on a 2.21 x 50 cm Whatman partisil 10 ODS-3 column using an isocratic 55 % MeCN (aq) for elution at 15 ml/min and room temperature. The desired fraction, located at elution volumes of 1020~1500 ml, were concentrated to dryness (269 mg) and dissolved in 0.8 ml of C_6H_{14}. A 0.21 ml portion was chromatographed on a 2.12 x 25 cm Zorbax Sil column using an isocratic 15 % Me_2CO/C_6H_{14} for elution at 10

ml/min and room temperature. After pooling a total of three such runs, 40 mg of homogeneous **1** was obtained between 440~480 ml elution volumes.

Physical data for 1

1 . yellow gum, $C_{24}H_{35}NO_4$; $[\alpha]_D^{21}$ = -87.3° (*c* 0.65, MeOH); UV λ_{max}^{MeOH} nm (log ε): 381 (sh, 4.33), 361 (4.44), 256 (3.86); FT-IR (ZnSe) ν_{max} cm^{-1}: 3452 (NH), 3235 (OH), 2962, 2924, 1733 (C=O of γ-lactam), 1710 (sh, C=O of ketone), 1659, 1579 (C=C, *s*-trans), 1407; ^1H and ^{13}C NMR (400/100 MHz, CD_2Cl_2, 25 °C) are detailed in Table 1; EI-MS (M^+, 100%, *m/z* found: 401.2577, calculated: 401.2566), with following fragmentations, *m/z* (%): 385 (4.1), 357 (29.3), 330 (11.9), 313 (15.5), 302 (2.5), 285 (1.3), 259 (3.4), 243 (17.5), 236 (3.4), 215 (9.3), 173 (14.1), 159 (8.2), 145 (14), 131 (14.8), 119 (16), 107 (18.3), 91 (15.3); t_R = 8.3 min.

^3H-Bradykinin binding assay

^3H-Bradykinin binding assay on human B_2 receptors expressed in Chinese hamster ovary cells was performed according to the methods of Hess *et al.*[13]. A human B_2 receptor was cloned from the lung fibroblast cell line CCD-16Lu. Transfection of the receptor cDNA into Chinese hamster ovary cells resulted in the expression of high levels of specific BK binding sites.

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