BENZOMALVINS, NEW SUBSTANCE P INHIBITORS FROM A *Penicillium* sp.

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In the course of screening microbial broths for neurokinin receptor antagonists, a series of new benzodiazepines, benzomalvins A (**1**), B (**2**) and C (**3**), has been isolated from the culture broth of a fungus identified as a *Penicillium* sp. Benzomalvin A (**1**) showed inhibitory activity against substance P with *Ki* values of 12, 42 and 43 μM at the guinea pig, rat and human neurokinin NK1 receptors, respectively. Benzomalvins B (**2**) and C (**3**) were only weakly active. The structures of these compounds were determined by spectroscopic methods including MS measurements and NMR analysis.

Substance P, a neurokinin peptide, and its NK1 receptor are involved in a variety of physiological actions, especially in pain transmission and activation of neurogenic inflammation[1]. Several peptidal[2~7] and nonpeptidal[8~14] NK1 receptor antagonists have recently been reported. Our search for substance P antagonists from microbial broths has resulted in the isolation of a number of new compounds including anthrotainin[15], fiscalins[16], diketopiperazine dimers[17] and cyclic peptides[18]. We now wish to report on a series of new benzodiazepines from a fungal culture *Penicillium* sp. The isolation, structure determination and biological activities of these inhibitors are described herein.

Materials and Methods

Taxonomy

A fungal culture, deposited in Sterling Winthrop's Culture Collection, Collegeville, PA, USA and designated as SC67, was isolated from a soil sample collected at a short grass prairie near Jamestown, North Dakota. The culture was preserved initially on LCSB agar (lactose 1.5%, corn steep liquor 0.5%, peptone 0.5%, NaCl 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.06%, $FeCl_3 \cdot 6H_2O$ 0.0005%, $CuSO_4 \cdot 5H_2O$ 0.0002% and agar 3.0%) where it exhibited isolated colonies with dull green spores and irregular black spots. The mycelial color was white and the culture produced no soluble pigment. The culture was then transferred onto CZAPEK's (sucrose 3.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and agar 1.5%) and malt extract agar (malt extract 2.0%, glucose 2.0%, peptone 0.1%, and agar 2.0%) to form white to pale gray colonies with cottony aerial hyphae. Conidia formed were hyaline, globose, and appeared smooth in dry basipetal chains. The conidiophores were hyaline, ornamented and either biverticillate or singly branched in loose, penicilliate like clusters. Characteristic hard black sclerotia formed abundantly within days on CZAPEK's and malt extract agar. Sclerotia showed no evidence of asci formation after as long as six weeks on any media tested. No reverse of diffusible pigments were observed on any media. Based on an assessment of these taxonomic characteristics we identified this culture as a *Penicillium* species.

Fermentation of Culture SC67 and Preparation of Extracts

Fresh culture plates were washed with a solution of 10% glycerol - 5% lactose, and the spore suspension was stored at -70°C. One ml of this frozen stock was added 30 ml of the VM-FP-1 medium (glucose 2.0%, Pharmamedia 1.5%, yeast extract 0.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003% and $CaCO_3$ 0.4%) in baffled 250-ml flask and incubated at 27°C for 2 days at 220 rpm in a New Brunswick Psychrotherm

shaker. The resulting seed culture (1 ml) was transferred into a 250-ml nonbaffled flask containing 30 ml of production medium (mannitol 4.0%, arginine 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.2%, yeast extract 0.05%, 0.1% of a stock solution of trace elements containing $FeSO_4 \cdot 7H_2O$ 0.1%, $MnCl_2 \cdot 4H_2O$ 0.1%, $ZnSO_4 \cdot 7H_2O$ 0.1%, $CuSO_4 \cdot 5H_2O$ 0.1% and $CoCl_2 \cdot 2H_2O$ 0.1%) and incubated for 2 days using the same conditions as described above. This generated a second-stage seed culture. Production of the bioactive components was scaled up by inoculated the entire 30 ml of the second-stage seed culture into to a 500 ml volume of production medium in a 2.8-liter nonbaffled Fernbach flask. The fermentation was carried out at 27°C in B. Braun BS4 shakers at 220 rpm for 4~5 days.

Neurokinin Receptor Binding Assay

Samples were assayed for SP binding inhibition assay using radiolabeled SP and NK1 receptor preparations obtained from guinea pig submaxillary tissue, rat submaxillary tissue, or human astrocytoma cell membranes. The details of these procedures are described elsewhere[19].

LC/MS Analysis of the Extract of Culture SC67

Thermospray LC/MS was conducted using a Nova Pak C18 15 cm column with at a flow rate of 1.5 ml/minute plus 0.5 ml/minute of 0.5 M ammonium acetate in water. The solvent gradient system: 100% A~100% B in 20 minutes, A=30% MeOH in H_2O, B=MeOH, was used. Column effluents were monitored by a UV detector at 220 nm and detected by a HP5988A thermospray mass spectrometer with source temperature at 350°C scanning from 145 to 800 daltons in 1 second.

Particle beam LC/MS was carried out using the same column as described above with conditions listed as follows: 100% H_2O~50% H_2O-MeOH in 10 minutes and 50% H_2O-MeOH~100% MeOH in 20 minutes; flow rate: 1.5 ml/minute from 0 to 10 minutes and 0.5 ml/minute from 10~40 minutes; and UV detection at 220 nm.

Isolation of Benzomalvins A (**1**), B (**2**) and C (**3**)

The harvested culture (500 ml × 3) was centrifuged at 25°C for 30 minutes at 3,500 rpm. The inactive supernatant fluid was discarded, the cells were resuspended in 125 ml distilled water and extracted with an equal volume of EtOAc for 15 minutes at 220 rpm in a New Brunswick Psychrotherm shaker. The process was repeated, extracts combined and evaporated to dryness.

The extract (520 mg) was fractionated by using high speed centrifugal countercurrent chromatography with a solvent system containing 5:5:4:3 hexanes-$CHCl_3$-MeOH-H_2O (lower layer as stationary phase). The resulting active fractions were further separated using HPLC on a reverse-phase C-18 column with 30% H_2O-MeOH to yield three novel compounds, designated as benzomalvins A (**1**, 10 mg), B (**2**, 5.9 mg) and C (**3**, 4.6 mg).

Physical and Spectroscopic Data of Benzomalvins A (**1**), B (**2**) and C (**3**)

Benzomalvin A (**1**): white solids; $[\alpha]_D$ −106° (*c* 1.0, MeOH); HRFAB-MS MH^+ 382.1525 (calcd for $C_{24}H_{20}N_3O_2$, Δ mmu 3.1); UV (MeOH) λ_{max} 234 (ε 19,600), 269 (ε 7,400), 280 (ε 6,000), 312 (ε 3,100), 323 (ε 2,300); IR (KBr) ν_{max} 3470, 3065, 3030, 2925, 2855, 1680, 1650, 1615, 1590, 1468, 1440, 1380, 1300, 1252, 775 and 698 cm^{-1}; mp 105~115°C; ^1H and ^{13}C NMR, Table 1.

Benzomalvin B (**2**): white solids; $[\alpha]_D$ +158° (*c* 0.59, MeOH); HRFAB-MS MH^+ 380.1403 (calcd for $C_{24}H_{18}N_3O_2$, Δ mmu −0.4); UV (MeOH) λ_{max} 229 (ε 30,000), 234 (ε 30,100), 265 (ε 13,800), 284 (ε 12,700), 312 (ε 6,500), 326 (ε 4,700); IR (KBr) ν_{max} 3440, 3080, 2040, 3015, 2930, 2850, 1695, 1655, 1610, 1590, 1465, 1455, 1370, 1370, 1255, 775, 760, 720 and 690 cm^{-1}; mp >260°C; ^1H and ^{13}C NMR, Table 2.

Benzomalvin C (**3**): white needles crystallized from MeOH-H_2O, $[\alpha]_D$ +69.7° (*c* 0.38, MeOH); HR-FAB-MS MH^+ 396.1348 (calcd for $C_{24}H_{18}N_3O_3$, Δ mmu 0); UV (MeOH) λ_{max} 229 (ε 27,600), 273 (ε 6,700), 283 (ε 5,900), 312 (ε 2,800) and 323 (ε 2,200); IR (KBr) ν_{max} 3470, 3060, 3030, 3010, 2910, 2850, 1700, 1670, 1620, 1590, 1455, 1365, 1250, 775, 760, 710, 690 and 665 cm^{-1}; mp 214°C; ^1H and ^{13}C NMR, Table 3.

Conversion of Benzomalvin B (**2**) to Benzomalvin C (**3**)

Benzomalvin B (**2**, 10 mg) was stirred with 50 mg of *m*-chloroperbenzoic acid in 2 ml CH_2Cl_2 for 3 weeks. After filtering off the solids, the solvent was evaporated under a stream of N_2. The residue was

separated using a C18 reversed phase HPLC column with 30% H_2O-MeOH to yield an epoxide (1 mg) and the starting material (5 mg). The spectroscopic data of the epoxide is identical to those of **3**.

Results and Discussion

It was found that the majority of the activity resided with harvested cell mass. The activity of the extract was retained after heat treatment (90°C) and under acidic conditions, but was lost at alkaline pH. Examination of the extract using photodiode array HPLC indicated the presence of 3 major bioactive components having a similar UV absorbance profile at 270, 280, 312, and 323 nm. LC/MS analyses of this extract using thermal spray and particle beam (Fig. 1) indicated these components have molecular weights of 381, 379 and 395 daltons and differed from compounds previously isolated during our substance P screen[15~18]. Centrifugal countercurrent chromatography and reversed-phase HPLC of the cell extract

Fig. 1. Particle beam LC/MS analysis of the extract from *Penicillium* sp. SC67.

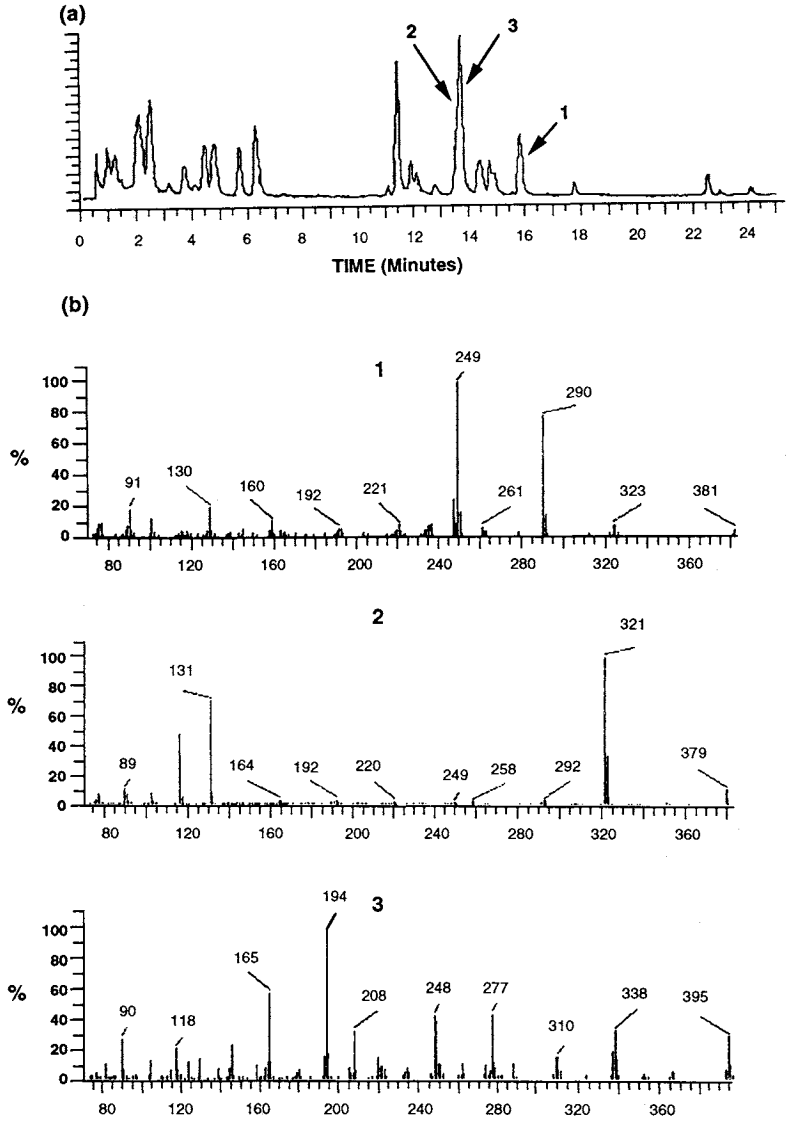
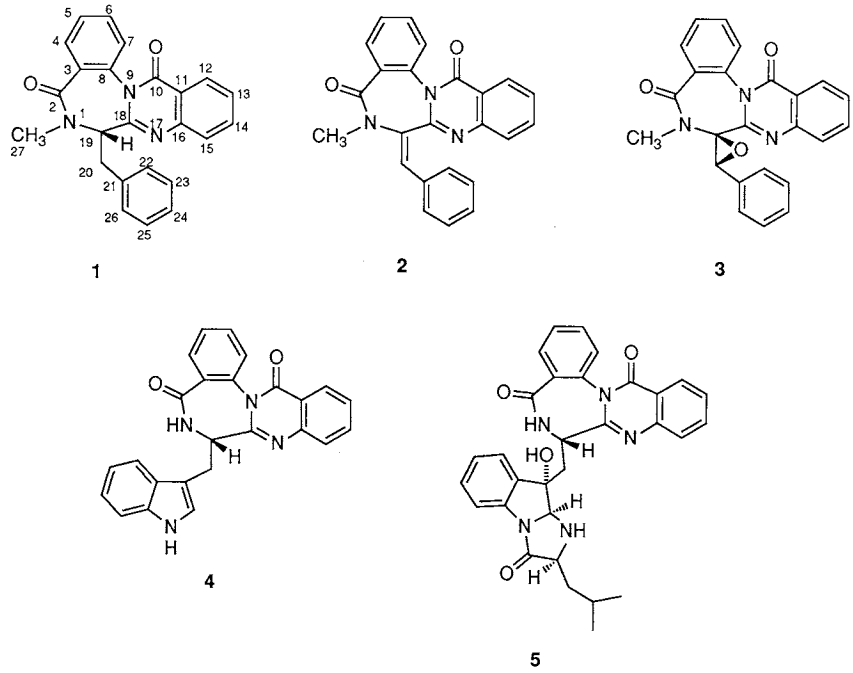


Fig. 2. Structures of benzomalvins A (**1**), B (**2**), C (**3**), asperlicin C (**4**) and asperlicin (**5**).



yielded three active novel compounds (Fig. 2), designated as benzomalvins A (**1**), B (**2**) and C (**3**).

A molecular formula $C_{24}H_{19}N_3O_2$ was established for benzomalvin A (**1**) from HRFAB-MS. The ^{13}C NMR spectrum, and a DEPT experiment, showed the molecule contained 1 methyl, 1 methylene, 1 methine, 13 protonated sp^2, 6 non-protonated sp^2 and 2 carbonyl carbons. Both carbonyls must be involved in amide bonds, as required by their chemical shifts and the molecular formula. The presence of two 1,2-di-substituted and one mono-substituted benzene moieties was evident from analysis of the coupling pattern of the aromatic ^1H signals, 2D COSY, HMQC and HMBC spectra (Table 1). The HMBC spectrum showed that aromatic protons at δ 7.86 (H-4) and 8.26 (H-12) were long-range coupled to carbonyls at δ 167.6 (C-2) and 161.8 (C-10), respectively, indicating the presence of two independent benzoic amide moieties. The ^1H and 2D COSY NMR spectra showed that a methine proton (H-19) at δ 5.07 was coupled with two geminal-coupled methylene protons at δ 3.76 and 3.53 (H-20). Each of these methine and methylene protons was found to have cross peaks with an aromatic carbon (C-21) at δ 138.4 and an amidine carbon (C-18) at δ 153.7, in the HMBC spectrum. The methylene protons were also coupled to two chemically equivalent phenyl carbons at δ 130.0 (C-22 and C-26), thereby establishing connectivity from C-18 to C-26. Furthermore, cross peaks were observed from H-19 to both C-2 and C-27 (N–Me), indicating the presence of a quinazolinobenzodiazepine ring structure with a benzyl group attached at C-19, as depicted in **1**. The chirality of C-19 was assigned as *S* by comparison of optical rotation ($[\alpha]_D$ −106°, *c* 1.0, MeOH) with that of a known related compound, asperlicin C[20] (**4**, $[\alpha]_D$ −76.8°, *c* 0.345, MeOH). Confirmation of structure by total synthesis of **1** was carried out and will be reported elsewhere. It should be noted that in solution we observed that 20% of **1** converts into a second related compound which has also been detected in the extract. Both compounds were separable using HPLC chromatography. However, each compound once purified, reforms into a mixture containing 80% of **1** and 20% of the converted compound

Table 1. ^{1}H and ^{13}C NMR spectral data of benzomalvin A (**1**)[a].

Atom No.	^{13}C δ (m)	^{1}H δ (m, J Hz)	HMBC coupled ^{1}H
2	167.6 (s)	—	4-H, 19-H
3	133.0 (s)	—	5-H, 7-H
4	130.3 (d)	7.86 (ddd, 8.0, 1.5, 0.6)	5-H, 6-H
5	129.5 (d)	7.61 (ddd, 8.0, 7.0, 1.4)	7-H
6	131.3 (d)	7.67 (ddd, 8.1, 7.0, 1.5)	4-H
7	129.0 (d)	7.71 (ddd, 8.1, 1.4, 0.6)	4-H, 5-H
8	134.2 (s)	—	4-H, 6-H
10	161.8 (s)	—	12-H
11	122.8 (s)	—	15-H, 13-H
12	127.9 (d)	8.26 (ddd, 7.6, 1.7, 0.5)	4-H
13	128.2 (d)	7.62 (ddd, 7.6, 7.1, 1.5)	15-H
14	135.6 (d)	7.93 (ddd, 8.1, 7.1, 1.7)	12-H, 13-H
15	128.5 (d)	7.88 (ddd, 8.1, 1.5, 0.5)	13-H
16	147.1 (s)	—	12-H, 14-H, 15-H
18	153.7 (s)	—	20-H, 19-H
19	59.3 (d)	4.87 (dd, 7.5, 7.5)	20-H
20	33.7 (t)	3.79 (dd, 14.6, 7.5), 3.42 (dd, 14.6, 7.5)	19-H, 22/26-H
21	138.4 (s)	—	19-H, 20-H, 23/25-H
22/26	130.0 (d, 2C)	7.39 (br d, 7.3, 2H)	20-H, 24-H
23/25	129.3 (d, 2C)	7.27 (br t, 7.3, 2H)	
24	127.5 (d)	7.20 (br t, 7.3)	22/26-H
27	27.9 (q)	3.03 (s, 3H)	19-H

[a] Recorded in $CDCl_3$.

within 24 hours at room temperature regardless of solvent conditions. This equilibrium phenomenon, not readily apparent from the structure of **1**, is currently under investigation.

Benzomalvin B (**2**) has a molecular formula of $C_{24}H_{17}N_3O_2$ by HRFAB-MS. The existence of the same carbon skeleton as in benzomalvin A (**1**) was evident from the NMR spectral features of ^{1}H and ^{13}C NMR spectra. The structural differences between **2** and **1** were readily recognized from their respective ^{1}H and ^{13}C NMR chemical shifts (Table 2). In the ^{1}H NMR spectrum of **2** the C-19 methine (δ 4.87) and C-20 methylene (δ 3.79 and 3.42), which were present in **1**, were now absent, and an olefinic singlet at δ 6.72 was now present. The corresponding ^{13}C absorptions at δ 132.8 (d, C-20) and 128.9 (s, C-19) in the ^{13}C spectrum of **2** suggested the existence of a trisubstituted double bond, whose position was further confirmed by the long-range CH correlations deduced from HMBC, as listed in Table 2. The *E*-geometry of the exocyclic double bond was determined from the observation of a 10.9% NOE enhancement of the olefinic proton (H-20) upon irradiation of the N–Me (C-27) resonance signal. Unexpectedly we found that benzomalvin B (**2**) possesses optical activity ($[\alpha]_D$ +158°) but without having asymmetric carbons. Examination of the space-filling model of **2** showed that the bulky phenyl group which is *cis* to the quinazoline group may prevent the inversion of benzodiazepine ring thus adopting only one conformation and forming an atropisomer. Further discussion of this phenomenon together with the equilibrium phenomenon of **1** will be described elsewhere.

The HRFAB-MS measurement and the ^{13}C NMR spectrum established the formula $C_{24}H_{17}N_3O_3$ for benzomalvin C (**3**). NMR spectral features were similar to those observed for **1** and **2**, but a ^{1}H signal at δ 3.86 (s) and two ^{13}C resonances at δ 67.8 (d) and 72.1 (s) indicated the presence of an epoxide functionality in **3** (Table 3). Further 2D NMR experiments including COSY, HMQC and HMBC led us

Table 2. ^{1}H and ^{13}C NMR spectral data of benzomalvin B (2)[a].

Atom No.	^{13}C δ (m)	^{1}H δ (m, J Hz)	HMBC coupled ^{1}H
2	165.8 (s)	—	4-H
3	133.2 (s)	—	4-H, 5-H, 7-H
4	130.2 (d)	7.88 (dd, 8.0, 1.5)	
5	130.9 (d)	7.51 (ddd, 8.0, 7.0, 1.5)	4-H
6	128.8 (d)	7.45 (ddd, 8.0, 7.0, 1.5)	
7	127.4 (d)	7.64 (br d, 8.0)	6-H
8	131.7 (s)	—	6-H
10	161.0 (s)	—	12-H
11	121.9 (s)	—	13-H, 15-H
12	127.5 (d)	8.36 (dd, 8.0, 1.5)	14-H
13	128.0 (d)	7.53 (ddd, 8.0, 7.0, 1.5)	
14	135.0 (d)	7.76 (ddd, 8.0, 7.0, 1.5)	12-H
15	127.9 (d)	7.64 (br d, 8.0)	13-H
16	146.8 (s)	—	12-H
18	151.1 (s)	—	
19	128.9 (s)	—	27-H
20	132.8 (d)	6.72 (s)	27-H
21	132.1 (s)	—	
22/26	128.7 (d, 2C)[b]	7.2 (m, 5H)	
23/25	128.9 (d, 2C)[b]		
24	129.4 (d)		22/26-H
27	36.1 (q)	3.45 (s, 3H)	

[a] Recorded in $CDCl_3$.
[b] Interchangeable.

Table 3. ^{1}H and ^{13}C NMR spectral data of benzomalvin C (3)[a].

Atom No.	^{13}C δ (m)	^{1}H δ (m, J Hz)	HMBC coupled ^{1}H
2	165.8 (s)	—	4-H, 27-H
3	131.7 (s)	—	5-H, 7-H
4	129.7 (d)	7.95 (dd, 7.8, 1.5)	6-H
5	129.1 (d)	7.56 (br dd, 7.8, 7.8)	7-H
6	131.1 (d)	7.41 (ddd, 7.8, 7.8, 1.5)	
7	128.6 (d)	6.87 (br d, 7.8, 1.5)	
8	132.0 (s)	—	4-H, 6-H
10	160.5 (s)	—	12-H
11	121.9 (s)	—	13-H, 15-H
12	127.6 (d)	8.24 (dd, 7.7, 1.5)	14-H
13	128.3 (d)	7.53 (br dd, 7.7, 7.0)	
14	135.4 (d)	7.82 (ddd, 8.0, 7.0, 1.5)	12-H
15	128.2 (d)	7.92 (br d, 8.0)	13-H
16	146.1 (s)	—	12-H, 14-H
18	148.1 (s)	—	
19	72.1 (s)	—	20-H
20	67.8 (d)	3.86 (s)	22/26-H
21	130.3 (s)	—	20-H, 23/25-H
22/26	125.9 (d, 2C)	6.60 (br d, 7.5, 2H)	20-H
23/25	128.4 (d, 2C)	7.05 (br dd, 7.5, 2H)	
24	129.5 (d)	7.16 (br dd, 7.5)	22/26-H
27	28.7 (q)	3.21 (s, 3H)	

[a] Recorded in $CDCl_3$.

to conclude that benzomalvin C (**3**) differed from **1** and **2** only by the presence of an epoxide at C-19 and C-20.

We have attempted to establish the relative stereochemistry of the epoxide in **3** by performing a series of 1D difference NOE experiments. Irradiation of CH_3-27, H-20 and H-22/26 gave no detectable NOEs on H-20, CH_3-27 and CH_3-27, respectively. A two dimensional NOESY experiment did not yield cross peaks between CH_3-27 and H-20, nor CH_3-27 and H-22/26. Thus, a chemical conversion of **2** to **3** was undertaken through the epoxidation of **2** using *m*-chloroperbenzoic acid to yield **3**. As well as establishing the relative stereochemistry of **3**, this also confirmed the structure of **3**, as related to **2**.

Benzomalvin A (**1**) showed inhibitory activity against neuropeptide substance P with *Ki* values of 12, 42 and 43 μM at the guinea pig, rat and human neurokinin NK1 receptors, respectively. Benzomalvins B (**2**) and C (**3**) were found to be weakly active against substance P with inhibitions of 24% and 46%, respectively, at a concentration of 100 μg/ml.

Conclusion

It was determined that benzomalvin A (**1**), which belongs to the benzodiazepine class, was mainly responsible for the inhibition of substance P binding detected in the extract of the *Penicillium* sp. The compound inhibits substance P binding to rat, guinea pig and human neurokinin NK1 receptors. Two less active analogs, benzomalvins B (**2**) and C (**3**), have also been found in the same culture extract. All three compounds are new microbial metabolites, appear to be derived biogenetically from phenylalanine and anthranilic acid, and resemble in structure the known cholecystokinin antagonist, asperlicin (**5**)[20~24].

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