ANTHROTAINORIN, AN INHIBITOR OF SUBSTANCE P BINDING PRODUCED BY *Gliocladium catenulatum*

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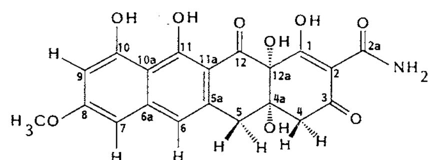
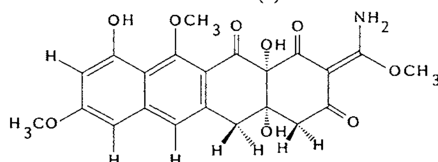
Substance P (SP) is an undecapeptide belonging to a family of chemically related neurotransmitters and neuromodulators known as neurokinins. In our search for SP antagonists, we screened microbial broth extracts for the ability to inhibit radiolabeled SP binding to membranes prepared from rat forebrain. Anthrotainin was isolated from a fungal culture and determined to be a novel tetracyclic compound, with an IC_{50} of 3 μM against [^{125}I]SP. The structure, spectroscopic, chemical, and pharmacological properties of anthrotainin are presented.

Neurokinins are a family of chemically related peptides which act as neurotransmitters and neuromodulators. Three neurokinin receptor subtypes have been proposed, based on the relative potencies of agonists[1~3]. The undecapeptide substance P (SP), the putative endogenous agonist for the NK-1 receptor subtype, has a wide spectrum of pharmacological activities, and is thought to be involved in pain transmission and inflammation[4]. Therefore, an antagonist of SP might prove to be a novel analgesic or anti-inflammatory agent. However, the lack of selective and potent non-peptide compounds has been a limiting factor in exploring this possibility.

In our search for SP antagonists, we screened microbial broth extracts for the ability to inhibit radiolabeled SP binding to membranes prepared from rat forebrain, which contain predominantly neurokinin receptors of the NK-1 subtype. Anthrotainin (1) was isolated from one fungal culture and determined to be a novel tetracyclic compound, with an IC_{50} of 3.0 μM against [^{125}I]SP. While elucidating the structure, some unexpected chemical and spectroscopic properties of the compound were observed. Reacting anthrotainin with diazomethane led to a more stable but unusual methylation product. Spectroscopic and X-ray



Anthrotainin (1)



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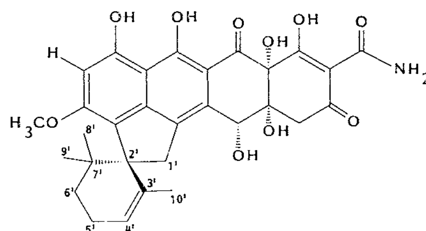


Viridicatumtoxin (3)

diffraction studies were performed on the more stable compound **2**. The structures of these two novel compounds were elucidated by means of NMR and single crystal X-ray diffraction experiments and by chemical synthesis of a partial structure of **2**.

Taxonomy and Fermentation

The producing organism, a fungus, was isolated from a soil sample near a mountain region of Taipei, Taiwan. The culture has been deposited in the Sterling Winthrop Biologicals Culture Collection under the accession number SC1. It has been classified as *Gliocladium catenulatum* based on the following morphological characteristics (observed on malt extract agar, grown after 7, 14 and 21 days). Colonies; pure white, spreading, floccose: Spore color; olive green to bright green in center, developing entirely to dark green in older culture: Conidiospores; branched heads composed of conidial chains in long columns up to 150 μm long, with conidia 4~7.5 × 3~4 μm (personal communication from Dr. JUDITH FRANKE, Applied Sciences, American Type Culture Collection, Rockville, MD). Culture broth produced by *Gliocladium catenulatum* was incubated for four days, the pH adjusted to 7 and the whole broth extracted twice with equal volumes of ethyl acetate.

Isolation

Anthrotainin (**1**) was initially only isolated in small quantities after solvent partitioning and semi-preparative HPLC (see scheme 1). Since anthrotainin is extremely unstable to heat, light and solvent, it was decided to derivatize and isolate a more stable derivative directly from crude extract. Thus ethyl acetate extract of the whole broth was defatted with hexane and the residue containing crude **1** was treated with diazomethane in MeOH - ether. A methylated derivative of anthrotainin was formed and was purified over silica gel eluting with $CHCl_3$ - MeOH to give **2**, as yellow needles.

Spectroscopic Properties and Structures

Spectrophysical characteristics of anthrotainin (**1**) indicated a highly substituted tetracyclic system. The accurate mass analysis by FAB-MS and DCI (CH_4) of **1** *quasi*-molecular ion was $(M+H)^+$, m/z 416.0958 (calc., 416.0982) corresponding to a molecular formula $C_{20}H_{17}NO_9$. A naphtha-anthrone chromophore was suggested from the UV spectrum[5], and IR absorption bands indicated the following; conjugated C=O, 1680 cm^{-1} and aromatic CH functional groups, 3100 and 1100~750 cm^{-1}. The molecular ion of **2** m/z 443.1213 obtained by high resolution FAB mass measurements indicated a molecular formula of $C_{22}H_{21}NO_9$ (calc., 443.1217). From ^1H and ^{13}C NMR of **2** (DEPT, HMQC and HETCOR), seven fully substituted sp^2 carbons (δ 161.3, 160.3, 157.5, 140.0, 137.5, 117.7 and 112.5) were observed, together with three OCH_3 (δ 55.5, 55.8, 63.6 and δ 3.78, 3.83 and 3.90). Two isolated CH_2 groups (δ 47.6

Scheme 1.

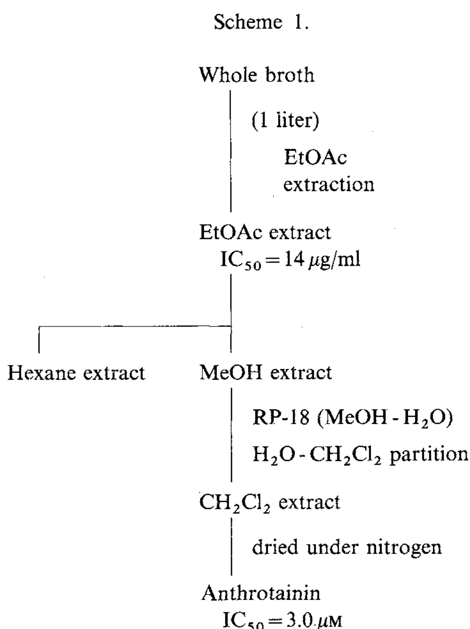


Fig. 1. NOESY, ROESY, N-H, and ^{1}H and ^{13}C long range correlations of **2**.

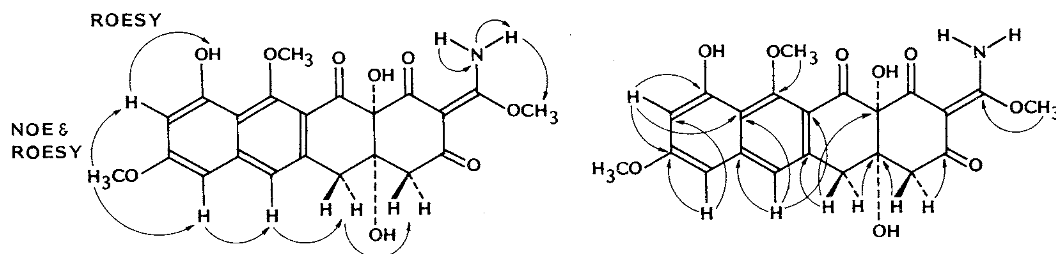


Table 1. ^{13}C NMR assignments for **1**~**3**.

Carbon	**1**	**2**	**3**[a]	Carbon	**1**	**2**	**3**[a]
1	192.1	191.9	191.3	7	99.5	98.2	123.2
2	98.0	97.3	99.9	8	163.2	161.3	161.4
2a	173.5	171.8	173.5	8-OCH_3	55.6	55.5	55.8
2-OCH_3		55.8		9	101.1	102.0	100.3
3	194.3	193.7	193.7	10	159.3	157.5	158.6
4	42.1	47.6	40.6	10a	108.2	112.5	105.9
4a	72.3	72.1	71.9	11	166.7	160.3	166.7
5	37.9	38.7	71.9	11a	106.8	117.7	105.5
5a	135.4	137.5	124.4	11-OCH_3		63.6	
6	117.5	122.3	137.6	12	196.6	195.6	196.0
6a	141.4	140.0	147.8	12a	82.1	83.5	80.6

[a] C-1′ 41.4; C-2′ 60.4; C-3′ 137.1; C-4′ 121.9; C-5′ 23.0; C-6′ 34.1; C-7′ 38.8; C-8′, C-9′ 21.2, 25.7; C-10′ 24.1.

and 38.7) were observed, one set showing strong geminal coupling at δ 2.35 and 2.77, $J=16$ Hz with the other set resonating at δ 3.13. Three sp^2 CH carbons (δ 122.3, 102.0 and 98.2) were accounted for. One set of protons exhibited *meta* coupling at δ 6.70 and δ 6.40, $J=2$ Hz, and the other proton resonating at δ 7.25. There were two quaternary sp^3 carbons linked to heteroatoms (δ 83.5 and 72.1). In addition, 5 D_2O exchangeable protons were observed (δ 5.29, 5.80, 9.06, 9.94 and 11.64).

Comparison of spectral data between **1** and **2** indicated that reaction of CH_2N_2 with **1** led to the addition of two methoxy groups, however their placement was not immediately predicted. Methylation usually occurs at any of the aromatic OH groups, although the 11-OH position is considered less likely due to steric hindrance, and hydrogen binding to the C-12 carbonyl group. From the ROESY and NOESY experiments, the carbon and proton signals at δ 55.8 and δ 3.83, respectively, were assigned to the CH_3 group in the side chain (ring D) and these were differentiated from the aromatic methoxy group assigned to 8-OCH_3 (δ 55.5 and 3.78) by NOESY, ROESY and long range correlation experiments as shown in Fig. 1. In an NOE experiment, irradiating the methoxy proton signal resulted in *ca.* 25% NOE enhancement of the two *meta* coupled protons at δ 6.40 and 6.70 in ring A.

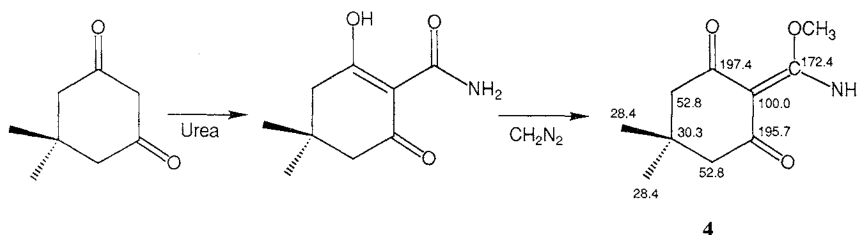
The NOE experiment gave essentially the same result for **1**, thus establishing the OCH_3 at position C-8 (δ 55.6) in the parent compound. The locations of the 8-OCH_3 and 11-OCH_3 groups in **2** were further confirmed through long range HETCOR and HMBC experiments which established their C-H long range correlations as depicted in Fig. 1.

Anthrotainin has a similar oxygenation pattern to viridicatumtoxin (**3**) first isolated from *Penicillium viradicatum*[6]. This structure was determined based on X-ray analysis. We completed extensive proton and carbon experiments (COSY, DEPT, HETCOR), which have now led to the complete assignment of carbon

Table 2. ^{1}H NMR assignments for **1**~**3**.

Proton No.	δ (multiplicity, J=Hz)		
	1	**2**	**3**[a]
2-OCH_3		3.83	
4-H	2.84 (s) (2)	2.35 (d, J=16), 2.77 (d, J=16)	2.74 (s)
5-H	3.41 (br s) (2)	3.13 (br s) (2)	4.55 (s) (1)
6-H	6.90 (s)	7.25 (s)	
7-H	6.49 (d, J=2)	6.70 (d, J=2)	
8-OCH_3	3.87 (s)	3.78 (s)	3.86 (s)
9-H	6.55 (d, J=2)	6.40 (d, J=2)	6.66 (s)
11-OCH_3		3.90 (s)	
2-NH_2	9.01 (s)	9.06 (s), 11.64 (s)	
10-OH		9.94 (s)	

[a] 1'-H (2) 2.86, 3.02 (d, J=16 Hz); 4'-H, 5'-H, 6'-H 7.0~7.2 m; 8'-CH_3 0.48 (s); 10'-CH_3 0.92 (q, J=7 Hz); 9'-CH_3 1.52 (s).

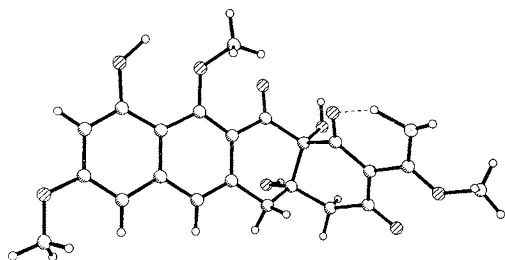


and proton signals for **3** (Tables 1, 2). Comparison of spectral data for **1**, **2** with those of **3**, led to the assignment of chemical shifts at δ 191.9 and 195.6 to the two carbonyl groups at C-1 and C-12, respectively, in **2**.

Although it is an unusual reaction, the methylation of the amide group in **2** was confirmed by chemical synthesis of compound **4**, discussed below. Although 2-amido-1,3-diketone functionality has been found in a number of tetracyclines, there is no report of the functional group at the 2 position of a 1,3-diketone moiety. During derivatization of anthrotainin with diazomethane, an unprecedented methylation reaction occurred at the amide group. Through *keto-enol* tautomerization, the carbonyl group of the amide was enolized and then reacted with diazomethane to generate the methoxy group. This reaction was further confirmed by comparison of formation of compound **4** through methylation of the synthesized 2-carbamoyl-5,5-dimethyl-1,3-cyclohexanedione. Reaction of 5,5-dimethyl-1,3-cyclohexanedione with urea gave the corresponding carbamoyl derivative. Treatment of the carbamoyl derivative with diazomethane led to a mixture of products from which a methylated derivative **4** was isolated. Low resolution MS (DCI - CH_4) gave a MH^+, m/z 198 indicating a molecular formula $C_{10}H_{15}NO_3$ for compound **4**, and ion fragmentation m/z 166 and 141, consistent with the structure. The proton signal at δ 4.0 (OCH_3) together with slight chemical shift changes of the olefinic carbon signals from δ 102.1 to 100.0 and the carbonyl carbon chemical shift from δ 174.2 to 172.4 suggested the occurence of *O*-methylation on the amide. This was further supported by the high element of symmetry in compound **4** as reflected by the same ^{1}H and ^{13}C NMR chemical shifts of two methylenes (δ 2.3, 52.8) and two methyls (δ 1.0, 28.4).

Among two possible tautomeric structures for **2**, the =C(OMe)NH_2 side chain in ring A, appeared

Fig. 2. Single crystal X-ray structure of **2**.



to be the predominant tautomer at room temperature. This was supported by a nitrogen inverse detection experiment in which the nitrogen was shown to link directly to two D_2O exchangeable protons at δ 9.0 and 11.6 through N-H correlation.

Colorless needles were obtained from a solution of **2** in chloroform by vapor diffusion of methanol. A single needle of 0.02 × 0.02 × 0.48 mm was obtained for X-ray diffraction. Twenty five reflections were selected from a rotation photograph and centered using standard software procedures, thus: $a=9.260(1)$ Å; $b=6.919(2)$ Å; $c=16.28(2)$ Å; $\alpha=90.00$; $\beta=105.33(3)$; $\gamma=90.00$. Axial photographs showed symmetry for the b-axis and absence of symmetry for the a and the c-axes, thus confirming the monoclinic crystal class. The cell volume is 1,006.3(3) Å^3. The empirical formula is $C_{22}H_{21}NO_9$ with a formula weight of 443.4 daltons. For two molecules in the unit cell (Z=2) the density calculates to 1.463 g/cm^3. The absorption coefficient for Cu-radiation is 0.928 mm^{-1}. The systematic absence of $P2_1$(No. 4) reflections indicated uniquely the space group.

The single crystal X-ray structure is shown in Fig. 2. The results of the single crystal X-ray diffraction study further showed that the two hydroxyl groups at the ring junction between rings A and B of compound **2** are *cis* to each other and match the 1,2 *cis* diol system in viridicatumtoxin.

Fig. 3. [^{125}I]Substance P binding to rat forebrain: Competition with anthrotainin (**1**) and **2**.

● Anthrotainin (**1**) (IC_{50}=3.0 μM), ■ **2** (IC_{50}>100 μM).

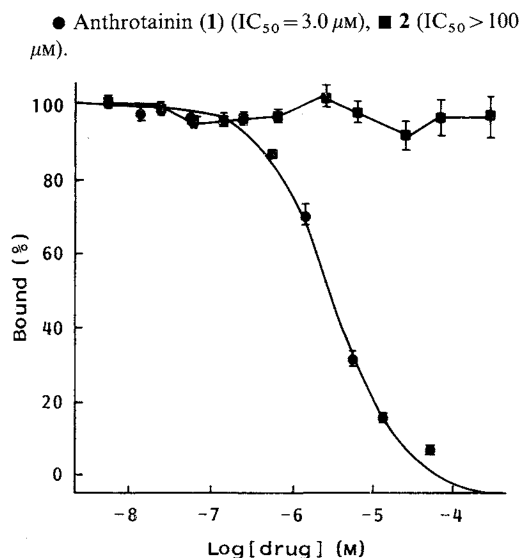
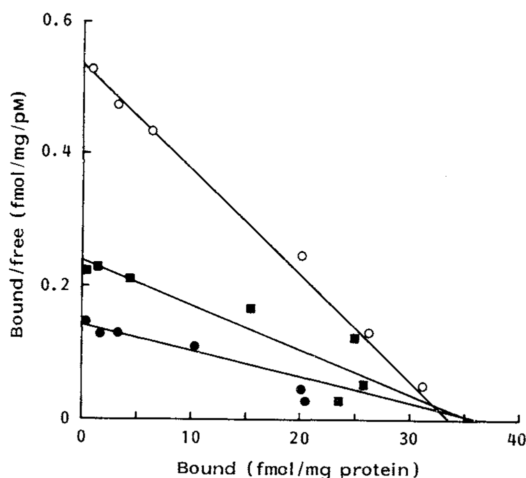


Fig. 4. [^{125}I]Substance P binding: Scatchard analysis of anthrotainin (**1**).

○ Control (no anthrotainin) (*Kd*=0.07 nM; Bmax=35 fmol/mg), ■ 1.2 μM anthrotainin (*Kd*=0.16 nM; Bmax=38 fmol/mg), ● 2.4 μM anthrotainin (*Kd*=0.27 nM; Bmax=38 fmol/mg).



Biological Properties

Anthrotainin produced a concentration-dependent inhibition of [^{125}I]SP binding to membranes prepared from rat forebrain, which is rich in the NK-1 receptor subtype with an IC_{50} value of 3.0 μM (Fig. 3). The more stable methylated product, however, was found to be inactive in the receptor binding

assay at concentrations up to 100 μM. This finding suggests that the site of methylation is not only important for chemical stability and biological activity, but also for interaction at the NK-1 receptor. We also found that viridicatumtoxin (**3**) was inactive up to 2.5 μM under the same conditions described above. The reversibility of the anthrotainin-receptor interaction was established by Scatchard analyses. The results indicated that anthrotainin produced a concentration-dependent increase in the apparent *Kd* of the radioligand and with no change in Bmax (Fig. 4). This suggests that there is a fully reversible interaction of anthrotainin with the NK-1 receptor. To further investigate the nature of this interaction, the dissociation rate of $[^{125}I]$SP was investigated in the presence and absence of the test compound, anthrotainin. The rate of dissociation was significantly decreased from 0.0166 minute^{-1} in the absence of test compound to 0.0123 minute^{-1} in the presence of 50 μM anthrotainin. This suggests either the interaction of anthrotainin with the receptor is not strictly competitive with SP binding, or perhaps that some secondary site or non-specific interaction is occurring at these relatively high concentrations of test compound that influence SP binding properties.

Experimental

Fermentation of Fungus SC1

A frozen stock vial of SC1 was used to inoculate 30 ml of seed medium (VM-1) in a 250-ml Erlenmeyer flask. The flask was incubated at 25°C on a rotary shaker at 220 rpm (New Brusnwick Scientific) for 48 hours. The VM-1 medium consisted of (w/v); glucose 2.0%, Pharmamedia 1.5%, yeast extract 0.5%, ammonium sulfate 0.3%, zinc sulfate 0.003% and calcium carbonate 0.4%. The pH was adjusted to 7.0 prior to autoclaving. A 5.0% (v/v) inoculum of the 48-hour seed culture was used to inoculate a 2.8-liter Fernbach flask containing 500 ml of the fermentation production medium, consisting of (w/v); corn starch 4.0%, Pharmamedia 4.0%, yeast extract 0.5%, potassium diphosphate 0.05%, magnesium sulfate 0.05%, potassium chloride 0.03%, calcium carbonate 0.3% and soybean oil 2.0%. The pH was adjusted to 7.0 before autoclaving. The contents of the flasks were incubated as described above and were harvested after 4 days. Whole broth was extracted twice with equal volumes of ethyl acetate.

NK-1 Receptor Binding

$[^{125}I]$-Bolton Hunter-Substance P ($[^{125}I]$-BH-SP, 2,200 Ci/mmol) was purchased from New England Nuclear. Receptor binding was performed according to the procedure reported by PARK *et al.*[7], using rat forebrain membranes (whole brain minus cerebellum) from male Sprague-Dawley rats.

The binding assay mixture, 0.5 ml, contained 100~150 μg membrane protein, 0.1 mM $[^{125}I]$-BH-SP, and test compound in 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.4 at 25°C, 0.02% bovine serum albumin, 2 μg/ml chymostatin, 4 μg/ml leupeptin, 40 μg/ml bacitracin, and 1.2 mM $MnCl_2$. Nonspecific binding was defined in the presence of 1 μM SP. All assays were run in duplicate, and the reaction mixtures were incubated 20 minutes at 25°C. The assay was terminated by dilution with 4 ml of ice cold Tris-HCl, pH 7.4 at 4°C, 5 mM KCl and 120 mM NaCl (wash buffer) and filtered through GF/C glass fiber filters presoaked in 0.01% polyethyleneimine. The filters were washed two times more with 4 ml ice cold wash buffer. The radioactivity trapped on the filters was counted in a Packard Cobra Gamma Counter. The competition curve data were analyzed by computer nonlinear least squares best fit of the data to the Hill equation which determined the IC_{50} values from at least seven concentrations of the test compound. For dissociation experiments, $[^{125}I]$SP binding was allowed to come to equilibrium for 20 minutes and then 1 μM SP $\pm$ 50 μM anthrotainin was added to the assay mixture at 5-minute intervals up to 50 minutes. The binding reaction was terminated by filtration as outlined above.

Isolation of 1 and 2

Whole broth was extracted with 2 × equal volume EtOAc. The organic layer was concentrated *in vacuo* and partitioned between MeOH and hexane. The MeOH extract was concentrated to dryness *in vacuo*

and the residue chromatographed by HPLC using the following conditions: Column; Merck RP-18, 5μm with a solvent gradient 20% to 100% MeOH in H_2O at a flow rate 1 ml/minute. Anthrotainin elutes approximately after 35 minutes. The fractions were partitioned into H_2O - CH_2Cl_2 and the CH_2Cl_2 extract concentrated and dried under N_2.

Methylation of Ethyl Acetate Extract from SC1 to Produce **2**

Ethyl acetate extract of the whole broth was defatted with hexane. The residue was dissolved in 50 ml methanol and then treated for 25 minutes, with diazomethane, generated from Diazaid under recess light and nitrogen atmosphere. Methylation was monitored by silica gel TLC using chloroform - methanol (10 : 1) as the eluant. The reaction mixture was protected from light and stored under nitrogen atmosphere at room temperature for *ca.* 1 hour. After drying under a stream of nitrogen, the reaction mixture was chromatographed over a silica gel column firstly with dichloromethane - methanol (9 : 1) and then with chloroform - methanol (10 : 1) to afford **2** (4 mg) which crystallized as pale yellow needles in a mixture of methanol and chloroform, mp 232~234°C.

Preparation of **4**

5,5-Dimethyl-1,3-cyclohexanedione (0.28 g) and urea (0.16 g) in 6.5 ml of water were refluxed for six hours. Cooling gave 37.9 mg of 2-carbamoyl-5,5-dimethyl-1,3-cyclohexanedione, mp 142~144°C (uncorrected); ^{1}H NMR ($CDCl_3$,): δ 1.08 (s, 6H, $2CH_3$), 2.34 (s, 2H, CH_2), 2.48 (s, 2H, CH_2), 5.78 (1H, br s, D_2O exchange), 9.46 (1H, br s, D_2O exchange); ^{13}C NMR ($CDCl_3$): δ 28.4 ($2 \times CH_3$), 31.2 (C), 45.9 (CH_2), 51.5 (CH_2), 102.1 (=C), 174.2 (C=O), 197.6 (C=O).

2-Carbamoyl-5,5-dimethyl-1,3-cyclohexanedione (39.8 mg) was dissolved in 10 ml diazomethane - ether solution. The reaction mixture was left overnight and then dried under a stream of nitrogen. Chromatography of the reaction mixture over silica gel with 5% methanol gave **4** (3.5 mg), DCI (CH_4) *m/z*: 198 $(M+H)^+$, 180, 166, 141, 124; ^{1}H NMR ($CDCl_3$): δ 1.0 (s, $2 \times CH_3$), 2.3 (s, $2 \times CH_2$), 4.0 (OCH_3), 6.2 (1H, D_2O exchange), 12.6 (1H, D_2O exchange).

NMR Experiments

^{1}H NMR experiments (COSY, decoupling experiments, quantitative proton), NOE difference experiment, and ^{13}C NMR experiments (DEPT, HETCOR) were performed with a Varian Gemini 300. Instrument $CDCl_3$, DMSO-d_6, CD_3OD and acetone-d_6 were used as solvents.

Inverse detection experiments (HMQC and HMBC), nitrogen and N-H correlation experiments, ROESY and NOESY were performed on a Varian VXR 500 NMR instrument.

MS Analysis

FAB-MS analysis was performed with Finnigan MAT TSQ 70 triple quadruple mass spectrometer using 8 keV xenon atoms, glycerol/thioglycerol, and 3-nitro benzyl alcohol substrates. Accurate FAB-MS was performed on a VG Analytical ZAB-2SE high field mass spectrometer provided with Cs ion gun and using peak matching against reference ions of CsI. Accurate mass DCI measurements were made with the same instrument using methane as chemical ionization gas, ion source temperature 150°C, and perfluorokerosene as mass reference standard. The instrument resolution was set at 5,000 for FAB and 10,000 for DCI analysis.

Single Crystal X-Ray Diffraction

A RIGAKU AFC5 diffractometer was used for the data collection. The CuK doublet ($\gamma = 1.54178$ Å) of an X-ray tube with a rotating anode was employed as radiation source, using a "graphite monochromator". The reflections were measured in an ω-scan mode at a scan-speed of 3.0°/minute. The ω-range was 2.20°. The background was measured with stationary crystal and stationary counter at the beginning and at the end of each scan, each for 50% of the total scan time. Each scan was repeated up to five times until a F/σ (F) of at least ten was obtained. Six reflections were measured as standards after every 100 reflections. The 2θ-range was 3.0 to 110.0° ($-14 \leq h \leq 9$; $0 \leq k \leq 8$; $0 \leq l \leq 18$). 1,430 reflections were collected at room temperature (21°C) of which 1,247 were independent reflections. 988 reflections were observed [$F > 4.0\ \sigma(F)$]. No absorption correction was applied.

SIEMENS' programs SHELXTL PLUS (Release 4.11/V) were used for phase determination and structure refinement. The refinement converged with $R=11.59\%$; $wR=11.81\%$. The largest feature in the Fourier difference map was $0.65\,eA^{-3}$.

Acknowledgments

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