HETEROCYCLES, Vol. 68, No. 10, 2006, pp. 2097 - 2106. © The Japan Institute of Heterocyclic Chemistry
Received, 28th June, 2006, Accepted, 21st August, 2006, Published online, 22nd August, 2006. COM-06-10829

MUCORALACTONE A: AN UNUSUAL STEROID FROM THE LIQUID CULTURE OF *MUCOR PLUMBEUS*

Athar Ata,*[,a] Leigha J. Conci,[a] and Ilkay Orhan[a,b]

[a]Department of Chemistry, The University of Winnipeg, 515 Portage Avenue, Winnipeg, Manitoba, Canada R3B 2E9
[b]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 6330 Ankara, Turkey.
E-mail: a.ata@uwinnipeg.ca

Abstract – Mucoralactone A (**1**), a novel steroid, was isolated from the liquid culture of *Mucor plumbeus*. Its structure was elucidated with the aid of extensive spectroscopic studies. Mucoralactone A (**1**) has a lactone moiety incorporated in its structure, and exhibited acetylcholinesterase enzyme inhibition activity with an IC_{50} value of 19 μM and modest antifungal activity against *Candida albicans*.

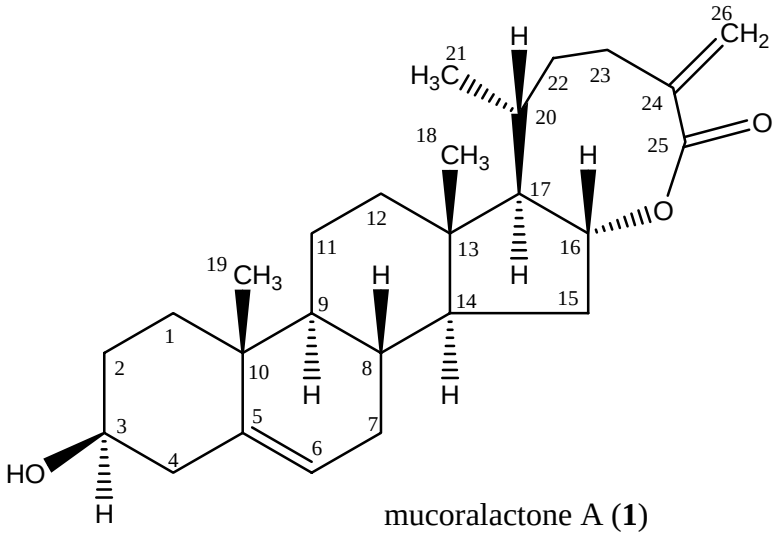
INTRODUCTION

Microbial natural product chemistry has generated a number of bioactive natural products. For instance cyclosporine A FK506 and rapamycin are used as immunosuppressants.[1] Other examples of microbial metabolites, having potential biomedical application include antihyperlipidemics, lovastatin and guggulsterone.[2,3] We are involved in discovering natural products exhibiting antimicrobial, acetylcholinesterase enzyme inhibition activities from marine organisms and other natural sources.[4-6] In our continuing effort to discover lead compound, we discovered that the crude extracts of *Mucor plumbeus* has exhibited acetylcholinesterase (AchE) enzyme inhibition activity. Our detailed chromatographic work on this crude extract resulted in the isolation of mucoralactone A (**1**), a novel steroid containing a lactone moeity incorporated in its structure. Spectroscopic methods were used to

elucidate the structure of this new metabolite. Mucoralactone A (**1**) was found to be responsible for AchE inhibitory activity also showed modest antifungal activity against *Candida albicans*. In this paper, we wish to report the isolation and structure elucidation of mucoralactone A (**1**) as well as its bioactivity data.

RESULTS AND DISCUSSION

The crude ethyl acetate extract of *M. plumbeus* was chromatographed over silica gel column to afford mucoralacton A (**1**) as a yellow colored gummy material. Its UV spectrum displayed maximum absorption at 241 nm indicating the presence of an α, β-unsaturated carbonyl group in **1**.[7] The IR spectrum displayed intense absorptions at 3456 (OH), 2900 (CH), 1780 C=O) and 1596 (C=C) cm^{-1}. Its high-resolution electron-impact mass spectrum (HREIMS) showed molecular ion peak at *m/z* 398.2798, which corresponded to the molecular formula $C_{26}H_{38}O_3$ (calcd 398.2821).



mucoralactone A (**1**)

The ^{1}H-NMR spectrum (acetone-d_6) of **1** displayed two singlets, integrating for three protons each at δ 0.73 and 1.07 due to the C-18 and C-19 methyl protons, respectively. A three-proton doublet at δ 0.95 ($J_{21,20}$ = 6.5 Hz) was due to the C-21 methyl protons. The C-3 and C-16 methine protons resonated at δ 3.82 and 4.99, respectively. Their downfield chemical shift values were indicative of the presence of geminal oxygen functionalities. An olefinic signal at δ 5.36 was due to the C-6 methine proton. The sp^2

hybridized C-28 methylene protons appeared as two singlets at δ 5.56 and 6.06, respectively. The COSY-45^{o} spectrum of **1** showed homonuclear couplings of the C-6 methine proton (δ 5.36) with the C-7 methylene protons (δ 2.39 and 2.01). Allylic couplings of the C-6 methine proton (δ 5.36) with the C-4 methylene protons (δ 2.82 and 3.20) were observed in the TOSCY spectrum which in turn showed vicinal couplings with the C-3 methine proton (δ 3.82). The C-16 methine proton (δ 4.99) exhibited COSY-45^{o} interactions with the C-15 methylene (δ 1.88 and 1.56) and C-17 methine (δ 2.05) protons. The latter in turn exhibited cross-peaks with the C-20 methine proton (δ 1.75). The C-20 methine proton showed vicinal couplings with the C-21 methyl (δ 0.95) and C-22 methylene (δ 2.01 and 1.55) protons. The C-22 methylene protons displayed ^{1}H-^{1}H spin correlations with the C-23 methylene protons (δ 2.80 and 2.43). Allylic couplings of the C-23 methylene protons with the olefinic C-28 methylene protons (δ 6.06 and 5.56) were also observed in the TOCSY spectrum. This detailed interpretation of COSY-45^{o} and TOCSY spectra helped to complete the ^{1}H-NMR chemical shift assignments of **1**.

The ^{13}C-NMR spectrum of **1** showed the resonance of all twenty six carbon atoms. The DEPT experiment was also performed to determine the multiplicity of each carbon signal, present in the broadband ^{13}C-NMR spectrum. This indicated the presence of three methyl, ten methylene and eight methine carbon atoms in **1**. Substraction of the DEPT spectra from the broadband ^{13}C-NMR spectrum suggested the presence of five quaternary carbon atoms. Two downfield aliphatic signals at δ 78.4 and 71.2 were due to the C-16 and C-3 carbon atoms, respectively. Their downfield chemical shift values suggested the substitution of oxygen atom on these carbon atoms. The olefinic C-5, C-6, C-24 and C-28 resonated at δ 139.8, 126.5, 141.0 and 112.5, respectively. Another downfield signal at δ 173.6 was ascribed to the C-25 ester carbonyl carbon. The HSQC spectrum of compound (**1**) was very helpful in determining the ^{1}H/^{13}C one-bond shift correlations of all protonated carbon atoms. Complete ^{13}C-NMR chemical shift assignments and ^{1}H/^{13}C one-bond connectivity of all hydreogen-bearing carbon atoms of **1**, as determined from HSQC, are shown in Table 1.

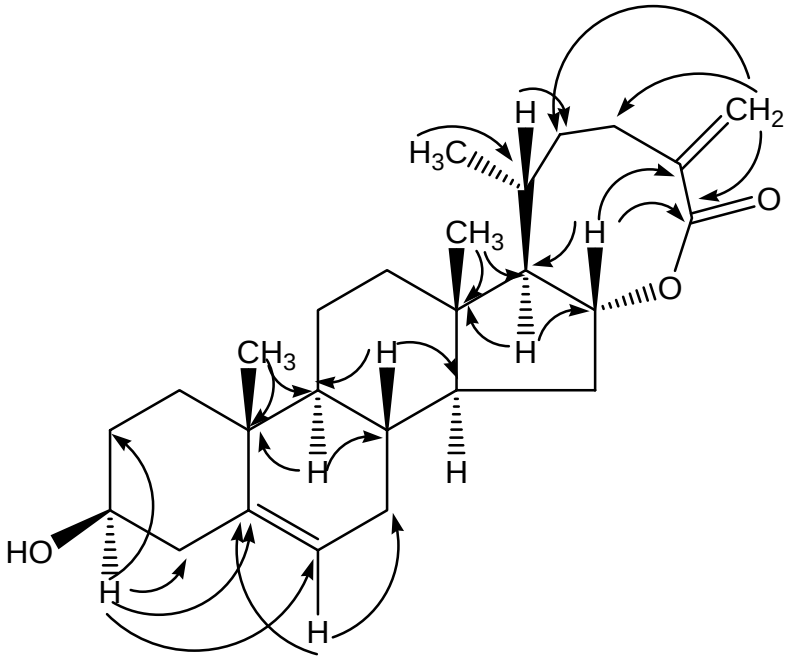


Figure 1: Important HMBC interactions of **1**

The information derived from a combination of the data obtained from ^{1}H-NMR, ^{13}C-NMR, COSY-45^{o}, HSQC and HMBC spectra suggested to us that **1** has a 3-hydroxycholestane type sterol like structure. Most of the ^{1}H- and ^{13}C-NMR signals had the same chemical shift values as reported for this class of steroids in literature.[8-12] A positive Liebermann-Burchard test further suggested that **1** belongs to the steroid family.[10] The presence of a lactone moiety in **1** was inferred from the mass, IR, ^{1}H-NMR, ^{13}C-NMR and HMBC spectra. The molecular formula of **1** ($C_{26}H_{38}O_3$) corresponded to eight degrees of unsaturation. Five of them were accounted for by the steroidal skeleton having a double bond. Two of them were due to another carbon-carbon double bond and a carbonyl group as indicated by the ^{13}C-NMR spectrum and remaining one double bond equivalent could only be satisfied by the presence of a lactone ring E in **1**. The molecular formula also suggested the presence of three oxygen atoms and these oxygen atoms were present as a hydroxyl and an ester group. The IR spectrum also displayed intense absorption bands at 1780 cm^{-1} due to a lactone moiety. The ^{1}H-NMR spectrum of **1** also showed the downfield resonance for C-28 methylene protons, which may be due to the presence of a carbonyl group at C-25. The ^{13}C-NMR spectrum exhibited signal at δ 173.6 due to the C-25 carbonyl. The HMBC spectrum

showed the long-range couplings of the C-20 (δ 1.75) methine proton with the C-21 (δ 18.4), C-22 (δ 39.7), and C-23 (δ 44.2) carbons. The C-16 methine proton (δ 4.99) exhibited cross-peaks with the C-24 (δ 141.0) and C-25 (δ 173.6) carbons. The long-range couplings of the C-28 methylene protons with the C-24 (δ 141.0) and C-25 (δ 173.6) carbons were also observed in the HMBC spectrum. These HMBC spectral observations can only be satisfied by placing an ester moiety between the C-16 and C-24 carbon atoms. These spectral data led us to propose the formation of ring E in **1**. Important HMBC interactions of **1** are presented in Figure 1.

After the structure determination, stereochemistry at various chiral centers was established with the aid of a NOESY spectrum, biogenetic considerations and by the comparison of ^{1}H-NMR chemical shift values of H-3, CH_3-18 and CH_3-19 with those of reported for physanolide A.[11] The C-18 and C-19 methyl groups, and C-8 methine proton have been reported to have β-orientation in physanolide A.[11] This was also observed in the NOESY spectrum, which exhibited cross peaks between the C-18 methyl (δ 0.73)/C-19 methyl (δ 1.07) and C-8 methine (δ 1.86) protons. This *cis* relationship indicated that CH_3-18, CH_3-19 and H-8 have similar orientation in **1**. The chemical shift values of CH_3-18, CH_3-19 and H-8 were also found to be nearly identical to those reported for 3β-hydroxycholestane type sterols in literature.[8-12] Based on these observations we assigned β-stereochemistry for CH_3-18, CH_3-19 and H-8. Once we are sure about the β-orientation of these groups, then NOESY spectrum helped us to determine the stereochemistry at the remaining chiral centers. The C-16 methine proton (δ 4.99) showed an NOE with the C-18 methyl protons (δ 0.73), suggesting a β-orientaion for H-16. The C-9 methine proton (δ 1.95) showed a cross-peak with the C-14 methine proton (δ 1.77), which further exhibited an NOE with the C-17 methine proton (δ 2.05). The ^{1}H- and ^{13}C-NMR chemical shift values of H-9 and H-14 were also found to be nearly identical to those of steroids reported in the literature.[8-12] Based on the ^{1}H-, ^{13}C-NMR and NOE data H-9, H-13 and H-17 were assigned α-orientations in **1**. The C-3 methine (δ 3.82) showed an NOE with the C-9 methine proton (δ 1.95) suggesting α-stereochemistry for H-3 and β-

stereochemistry for C-3/OH. Important NOE interactions of **1** are shown in Figure 2. These spectroscopic studies helped us to establish structure (**1**) for this novel compound.

Antifungal Activity: Mucoralactone A (**1**) was evaluated for antifungal activity against *Candida albicans* using the progressive double dilution method.[13] It exhibited modest antifungal activity against aforementioned fungus with a minimum inhibitory concentration (MIC) value of 23 μg/mL.

Table 1: ^{13}C NMR chemical shift assignments and $^{1}H/^{13}C$ one-bond shift correlations of the hydrogen-bearing carbon atoms of **1**, as determined from HSQC spectrum.

Carbon No.	^{1}H (δ)	Multiplicity	$^{13}C^{\dagger}$ (δ)
1.	2.03 and 1.69 (m)	CH_2	22.1
2.	2.48 and 2.03 (m)	CH_2	24.3
3.	3.82 (m)	CH	71.2
4.	2.82 and 3.20 (m)	CH_2	34.6
5.	---	-C-	139.8
6.	5.36 (br s)	CH	126.5
7.	2.39 and 2.01 (m)	CH_2	33.8
8.	1.86 (m)	CH	31.9
9.	1.95 (m)	CH	35.1
10.	---	-C-	36.9
11	2.01 and 1.65 (m)	CH_2	33.7
12.	2.12 and 1.55 (m)	CH_2	33.9
13	---	-C-	37.1
14.	1.77 (ddd, J = 10.0, 7.5, 6.8 Hz)	CH	38.0
15.	1.88 and 1.56 (m)	CH_2	37.2
16.	4.99 (ddd, J = 7.5, 6.0, 4.5 Hz)	CH	78.4
17.	2.05 (dd, J 5.8, 9.6 Hz)	CH	42.3
18.	0.73 (s)	CH_3	11.9
19.	1.07 (s)	CH_3	15.4
20.	1.75 (m)	CH	49.8
21.	0.95 (d, J = 6.5 Hz)	CH_3	18.4
22.	2.01 and 1.55 (m)	CH_2	39.7
23.	2.80 and 2.43 (m)	CH_2	44.2
24.	---	-C-	141.0
25	---	-C-	173.6
28	5.56 and 6.06 (br s)	CH_2	112.5

†Multiplicities were determined by the DEPT spectra

Acetylcholinesterase Eznyme Inhibition Activity: Acetylcholinesterase (AchE) stops the function of acetylcholine by its hydrolytic destruction.[14] Alzheimer's disease is a progressive degenerative neurologic disorder that results from the deficit of cholinergic function in brain.[14] Enhancement of acetylcholine level in the brain is considered one of the most promising approach for treating Alzheimer's disease.[15] The use of potent inhibitors to inhibit AChE activity is one of the methods to enhance acetylcholine level in brain to cure Alzheimer's disease. Compound (**1**) showed AchE inhibitory activity with an IC_{50} value (inhibition of enzyme activity by 50%) of 19 μM.

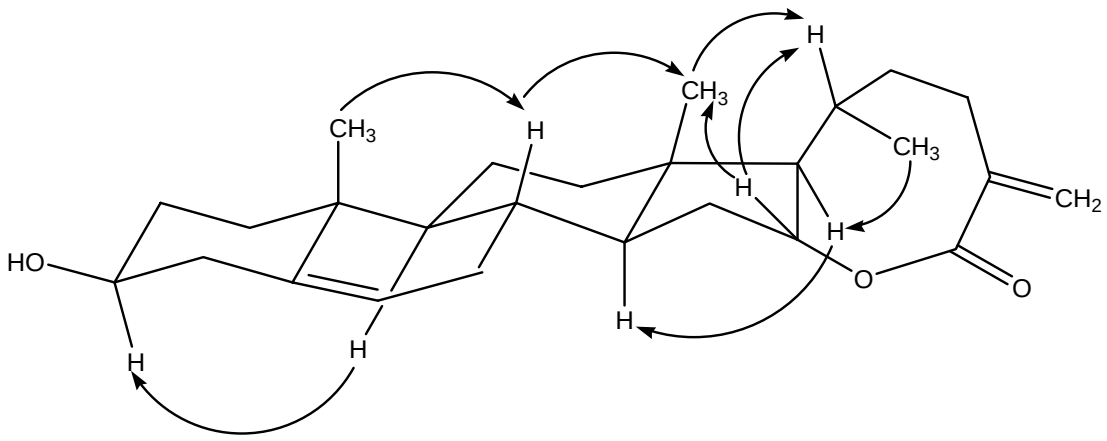


Figure 2: A probable conformations of rings A, B, C, D and E, as obtained from NOESY spectrum, of **1**

EXPERIMENTAL

General experimental procedures: Mass spectrometric measurements were conducted on a Varian MAT 312 double focussing mass spectrometer connected to a DEC PDP 11/34 computer system. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Inova and an AM 300 Bruker NMR spectrometers at 200 and 300 MHz, while the ^{13}C NMR spectra were recorded on an AM 300 Bruker NMR spectrometer at 75 MHz with TMS as an internal standard. The IR spectra were recorded on a Jasco-IRA1 IR spectrophotometer. Hewlett Packard GC-MS was used to monitor the microbial reactions. The UV spectra were recorded on a Shimadzu UV 240 instrument. The optical rotations were measured on a

Polatronic D polarimeter (Hitachi) and the purities of the samples were checked on TLC (silica gel, GF 254 precoated plates purchased from Merck).

***Fungus*:** *Mucor plumbeus* (ATCC 4740), was purchased from ATCC and maintained on Potato Dextrose Agar and stored in a refrigerator at 4 ^{o}C.

Preparation of media: The medium for *M. plumbeus* (ATCC 4740) was prepared by mixing the following ingredients in 1 L of distilled water: Dextrose (20 g), yeast (5 g), sodium chloride (5 g), potassium hydrogen phosphate (5 g) and soy flour (5 g). The pH was adjusted to 7.0 before autoclaving.

Fungal cultivation and isolation of mucoralactone A (1)*:* For the production of **1**, *M. plumbeus* was grown in 30 replicates 500 mL Erlenmeyer flasks having 200 mL of above-mentioned medium. The flasks were placed on a shaker for 10 days, after all the cultures were combined and extracted thrice with AcOEt. The crude extract (12 g) was subjected to silica gel column chromatography using gradient elution with hexane/AcOEt (0 - 100%) and AcOEt/MeOH (0 - 100%) to purify compound (**1**) (10.8 mg) as a yellow colored gum.

Mucoralactone A (**1**): $[\alpha]^{25}_{D}$ = +21^{o} (*c* 7.2, $CHCl_3$). UV(MeOH): λ_{max} = 241 nm. IR ($CHCl_3$): ν_{max} = 3456 (OH), 2900 (CH), 1780 (lactone), and 1596 cm^{-1} (C=C). ^{1}H NMR (300 MHz, $CDCl_3$): δ = see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$): δ = see Table 1; HR-EI-MS *m/z* = 398.2798 ($C_{28}H_{38}O_3$, calcd 398.2821). EIMS *m/z* (rel. int. %) = 398 (11), 383 (25), 138 (30), 241 (100).

Acetylcholinesterase (AChE) inhibitory activity: For this bioassay, AChE (0.04 U/mL) and acetylthiocholine iodide (ATC) (75 mM) were prepared by dissolving in 0.1 M phosphate buffer (pH 8). 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) was made up in 10 mL of 0.1 M phosphate buffer (pH 7.0) containing 15 mg of $NaHCO_3$. Compound (**1**) was dissolved in isopropanol and the concentration of sample solution was 5% (v/v). A control of similar concentration was also prepared.

The colorimetric method was used to determine the inhibition of AChE.[16] A solution of test compound (**1**) (50μL) and AChE (0.5 mL) were mixed in a test tube, and the tube was set on the incubator at 25^{o}C. DTNB (100 μL) and buffer (2.4 mL) were added to the test tube. The tube was incubated at 25^{o}C for 5

minutes as pre-incubation. The addition of ATC (40 μL) initiated the reaction and the mixture was then again incubated at 25°C for 20 min. The absorbance at 412 nm was measured spectrophotometerically and all test and control (without sample) assays were corrected by blanks for nonenzymic hydrolysis. This assay was performed in triplicate.

ACKNOWLEDGMENTS

The Financial support for this project provided by Natural Sceince and Egnieering Research Council (NSERC), Canada and Research Corporation, Tucson, Arizona, USA is gratefully acknowledged by authors. We are also thankful to Dr. Krk Marat, Department of Chemistry, The University of Manitoba, for his help in running two-dimensional NMR experiments.

REFERENCES

1. F. Van Middlesworth and R. J. P. Cannell, 'Dereplication and Partial Identification of Natural Products: Methods in Biotechnology,' Vol. 4, ed by R. J. P Cannell Humana, Totowa, New Jersey, 1998, pp. 279-327.
2. V. Knight, J. –J. Sanflier, D. Ditullio, S. Braccili, P. Bonner, J. Waters, D. Hughes, and L. Zhang, *Appl. Microbiol. Biotechnol*., 2003, **62**, 446.
3. N. L. Urizar, A. B. Liverman, D. T. Dodds, F. V. Silva, P. Ordentlich, Y. Yan, F. J. Gonzalez, R. A. Heyman, D. J. Mangelsdorf, and D. D. Moore, *Science*, 2002, **296**, 1703.
4. A. Ata and J. A. Nachtigall, *Z. Naturforsch.*, 2004, **59c**, 209.
5. A. Ata, H. Y. Win, D. Holt, P. Holloway, E. P. Segstro, and J. S. Gamini, *Helv. Chim. Acta*, 2004, **87**, 1090.
6. A. Ata, J. Ackerman, A. Bayoud, and P. Radhika, *Helv. Chim. Acta*, 2004, **87**, 592.
7. A. Ata and R. G. Kerr, *Heterocycles*, 2000, **53**, 717.
8. M. Denance, M. Guyot, and M. Samadi., *Steroids*, 2006, **71**, 599.
9. E. Ma, H. Kim, and E. Kim, *Steroids*, 2005, **70**, 245.

10 D. L. Coleman, W. W. Wells, and C. A. Baumann, *Arch. of Biochem. and Biophy.*, 1956, **60**, 412.

11. P. -C. Kuo, T. -H. Kuo, A. G. Damu, C. -R. Su, E. -J. Lee, T. -S. Wu, R. Shu, C. -M. Chen, K. F. Bastow, T. -H. Chen, and K. -H. Lee, *Org. Lett*. 2006, **8**, 2953.

12. F. Ronchetti, G. Russo, R. Longhi, and G. Sportoletti, *J. Lab. Comp. and Radiopharm.*, 1978, **14**, 687.

13. P. Vicini, F. Zani, P. Cozzini, and I. Doytchinove, *Eur. J. Med. Chem*., 2002, **37**, 553.

14. T. L. Rosenberry, *Adv. Enzymol. Relat. Areas Mol. Biol*., 1975, **43**, 103.

15. A. Enz, R. Amstutz, H. Boddeke, G. Gmelin, and J. Malonowski, *Prog. in Brain Res.*, 1993, **98**, 431.

16. G. L. Ellman, K. D. Courtney, J. V. Andres, and R. M. Featherstone, *Biochem. Pharmacol*., 1961, **7**, 88.