KIMIKA 13(1): 11-16 (1997)
Printed in the Philippines

Antimutagenic diterpenes from *Andrographis paniculata*

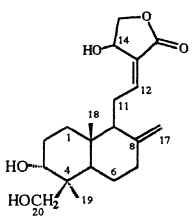
ZENAIDA D. NACPIL AND CONSOLACION Y. RAGASA
Chemistry Department, De La Salle University, 2401 Taft Avenue, Manila, 1004, Philippines

JOHN C. COLL AND JOHN A. RIDEOUT
Central Queensland University, Rockhampton Queensland, 4702, Australia

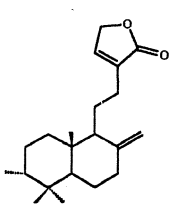
Andrographis paniculata afforded mixtures of diterpenes containing andrographolide (**1**) and deoxyanhydroandrographolide (**2**). The mixtures were acetylated to obtain pure compounds whose structures were elucidated by nmr spectroscopy and mass spectrometry. The structures of the natural products were deduced from the acetylated compounds. Micronucleus test of the crude extract indicated an 84.7% reduction in micronucleated polychromatic erythrocytes (MPCE); the mixture of natural products containing **1**, mixture of natural products containing **2**, and acetylated compounds (**3**, **4**, and **5**) likewise exhibited 89.8%, 86.7%, 93.2%, 94.6% and 91.1% reduction in MPCE, respectively. Therefore, all samples tested possess high antimutagenic activity.

Keywords. *Andrographis paniculata, Acanthaceae*, diterpenes, andrographolide, diacetylanhydroandrographolide, monoacetylanhydroandrographolide, deoxyanhydroandrographolide, diacetyldeoxyanhydroandrographolide, antimutagen, micronucleus test

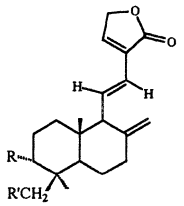
Andrographis paniculata, commonly known as "serpentina" is extensively used as a household medicine in West Indies and India [1]. In the Philippines, it is known to cure a number of diseases, such as rheumatism, arthritis, hypertension, asthma, diarrhea, diabetes and other common diseases. The plant was studied because of its wide range of medicinal applications. Previous studies on *A. paniculata* afforded andrographolide [2], isoandro-grapholide [3], neoandrographolide [4], andrographiside [5], paniculides and other andrographolide derivatives [6]. We now report the identification of two diterpenes (**1** and **2**) from the same species and results of the micronucleus test on the crude extract, mixtures of natural products containing **1** and **2** and acetylated compounds (**3-5**).



1



2 R, R′ = OH
5 R, R′ = OAc



3 R, R′ = OAc
4 R = OH, R′ = OAc

EXPERIMENTAL

Sample. The plant sample was collected at Villa Angela, Angeles City, Pampanga in January, 1994. The sample was identified as *Andrographis paniculata*, Nees by the National Museum. The plant sample was deposited at the National Museum with voucher specimen no. PNH 170790.

Extraction and Isolation. The air-dried leaves (200g) were soaked in $CHCl_3$ (1.2 L) for three days. The crude extract (8.0 g) was chromatographed on a gravity column packed with dry silica gel (70-230 mesh) using Me_2CO in $CHCl_3$ (10% increment) and MeOH in Me_2CO (10% increment) as eluents. The 30% Me_2CO in $CHCl_3$ fractions afforded a mixture of diterpenes (211.7 mg, 7.06% of the crude) after recrystallization from Et_2O. Thirty mg of the crystals was acetylated with acetic anhydride (1 mL) in pyridine (1 mL) to afford **3** (12 mg) and **4** (8 mg) after rechromatography with DCM:Et_2O:acetonitrile (8:1:1) as eluent. The 10% Me_2CO in $CHCl_3$ fractions afforded a mixture of diterpenes (45.2 mg, 1.51% of the crude) after recrystallization from Et_2O. Thirty mg of the crystals was acetylated with acetic

anhydride in pyridine to afford **5** (10 mg) after rechromatography with DCM:Et_2O:Acetonitrile (9:0.5:0.5) as eluent.

Antimutagenicity Test. The samples (170 mg/kg) dissolved in DMSO (7.5 mL/kg) were orally and simultaneously administered with a known mutagen, mitomycin C (2.75 mg/kg) to mice of the Swiss strain (source: DOST). For the positive control, only mitomycin C (2.75 mg/kg) and DMSO (7.5 mg/kg) were administered to the same strain of mice. Five mice were tested for each sample and the control. The second administration was carried out after twenty-four hours. Six hours after the second administration, the mice were killed by dislocation of the neck. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with undiluted May-Grunwald, followed by 50% May-Grunwald, and finally 15% Giemsa stain solution [9]. The micronucleus stained bluish black, while the young cells which underwent mitosis stained light blue and the old cells stained pink. The numbers of MPCE/1000 PCE were counted by the use of a Zeiss microscope.

Instrumentation and General Procedures. NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AM 300 nmr spectrometer with $CHCl_3$ (δ 7.26, 77.0 ppm) as reference. Eims was carried out on a JEOL D 100 mass spectrometer. Zeiss microscope was used in counting the MPCE. Si gel type 60 (Merck) was used for column chromatography and plastic-backed plates coated with Si gel F254 (Merck) were used for thin layer chromatography. Plates were visualized by spraying with vanillin/H_2SO_4 and warming.

RESULTS AND DISCUSSION:

The chloroform extract of the leaves of *Andrographis paniculata* afforded mixtures of diterpenes containing andrographolide (**1**) and deoxyanhydroandrographolide (**2**). These compounds were obtained pure as acetates only after the mixtures of diterpenes were treated with acetic anhydride in pyridine to afford **3-5**. The structures of **3-5** were elucidated by nmr spectroscopy and mass spectrometry. The structures of the natural products (**1-2**) were deduced from the structures of the acetylated compounds (**3-5**).

The 30% acetone in chloroform fraction from the chromatography of the crude extract afforded colorless crystals. From the disparity in single hydrogen peaks in the ^{1}H nmr spectrum it was deduced that the fraction was a mixture containing hydroxyl groups as indicated by the resonances for carbinyl protons between δ 3.00 - δ 4.00. It was acetylated to obtain pure compounds (**3** and **4**) and a mixture of other diterpenes. Since the other diterpenes were not obtained pure, their structures were not elucidated.

The ^{1}H-nmr spectrum of **3** revealed four methyl groups, two of which belong to acetates at δ 2.01 (3H, s) and δ 2.00 (3H, s); carbinyl hydrogens at δ 4.58 (1H, dd, 5.4, 11.1 Hz), δ 4.12 (1H, d, 11.7 Hz), δ 4.35 (1H, d, 11.7 Hz), δ 4.75 (1H, s, br) and δ 4.77 (1H, s, br); exocyclic olefinic protons at δ 4.55 (1H, s, br) and δ 4.77 (1H, s, br); and conjugated olefinic protons at δ 6.09 (1H, d, 15.8 Hz), δ 6.89 (1H, dd, 10.0, 15.8) and δ 7.15 (1H, s, br) (Table 1).

Table 1. Comparison of 300 MHz ^{1}H NMR spectral data of compounds **3**, **4** and **5** in $CDCl_3$

	Chemical Shifts		
Protons	**3**	**4**	**5**
H1,1'	1.25 (1H, m), 1.50 (1H, m)	1.20 (2H, m)	
H2,2'	1.52 (1H, m), 1.60 (1H, m)	1.47 (1H, m), 1.60 (1H, m)	
H3	4.58 (1H, dd)	3.28 (1H, dd)	4.58 (1H, dd, 5.4, 11.1 Hz)
H5	1.25 (1H, m)		
H6,6'	1.55 (1H, m), 1.79 (1H, m)	1.47 (1H, m), 1.66 (1H, m)	
H7,7'	1.65 (1H, m), 2.35 (1H, m)	2.35 (1H, m), 2.01 (1H, m)	
H9	2.30 (1H, d , 10.0 Hz)	2.29 (1H,d, 10.1 Hz)	
H11	6.89 (1H, dd, 10.0Hz, 15.8Hz)	6.83 (1H, dd, 10.1Hz, 15.8Hz)	
H12	6.09 (1H, d, 15.8 Hz)	6.08 (1H, d, 15.8 Hz)	
H14	7.15 (1H, s, br)	7.15 (1H, s, br)	7.10 (1H, s, br)
H15,15'	4.75 (1H, s, br), 4.77 (1H, s, br)	4.75 (1H,s, br), 4.75 (1H, s, br)	4.77 (2H, s, br)
H17,17'	4.53 (1H, s, br), 4.77 (1H, s, br)	4.50 (1H, s, br), 4.77 (1H,s, br)	4.58 (1H,s,br), 4.89 (1H,s, br)
H18	0.86 (3H, s)	0.81 (3H, s)	0.71 (3H, s)
H19	1.00 (3H, s)	1.12 (3H, s)	1.01 (3H, s)
H20,20'	4.12 (1H, d, 11.7 Hz), 4.65 (1H, d, 11.7 Hz)	4.12 (1H, d, 11.6 Hz), 4.29 (1H, d, 11.6 Hz)	4.09 (1H,d, 11.6 Hz), 4.35 (1H, d, 11.6 Hz)
OAc	2.00 (3H, s), 2.01 (3H, s)	2.01 (3H, s)	2.03 (3H, s), 2.04 (3H, s)
OH		2.70 (1H,s, br)	

The COSY spectrum showed the following isolated spin systems: the methylene protons at δ 1.25 (H1) and δ 1.50 (H1') were coupled to the methylene protons at δ 1.52 (H2) and δ 1.60 (H2'), which were in turn coupled to the carbinyl hydrogen at δ 4.58 (H3) by 7.0 Hz and 9.3 Hz; the non-equivalent carbinyl hydrogens at δ 4.12 ((H20) and δ 4.35 (H20') were coupled by 11.7 Hz; the methine proton at δ 1.25 (H5) was coupled to the methylene protons at δ 1.55 (H6) and δ 1.79 (H6'), with the latter proton coupled to allylic methylene hydrogens at δ 1.65 (H7) and δ 2.35 (H7'), which were in turn coupled to olefinic methylene protons at δ 4.55 (H17) and δ 4.77 (H17'); the olefinic methine proton typical of conjugated lactones at δ 7.15 (H14) was coupled to the carbinyl hydrogens at δ 4.77 (H15) and δ 4.75 (H15'); the trans olefinic protons at δ 6.09 (H12) and δ 6.89 (H11) were coupled by 15.8 Hz, with the latter hydrogen coupled to the allylic proton at δ 2.30 (H9) by 10 Hz.

Further information was given by the ^{13}C-nmr spectrum of **3** which indicated twenty-four carbons with the following functionalities: three carbonyls of two esters and a lactone, six olefinic carbons, three carbons singly bonded to oxygens and twelve aliphatic carbons (Table 2).

Table 2. Comparison of 75 MHz ^{13}C NMR data of compounds **3**, **4** and **5** in $CDCl_3$

3 (δ)	4 (δ)	5 (δ)	FUNCTIONALITIES
171.2	172.2	174.2	C=O (lactone)
170.8, 170.5	171.0	170.8, 170.5	C=O (acetates)
143.2, 135.5, 121.3	143.3, 135.7, 121.1	143.9	CH=
147.9, 129.1	147.8, 129.1	146.6, 134.7	C=
109.4	109.1	107.5	CH_2=
80.0	79.1	79.9	CH-O
69.5, 64.7	69.6, 65.0	70.0, 64.7	CH_2-O
61.6, 54.7	61.6, 54.7	55.9, 55.4	CH
41.3, 38.2	42.4, 38.2	41.3, 39.2	C
38.6, 36.7, 24.1, 22.6	38.5, 36.6, 27.6, 24.0	38.3, 36.9, 24.8, 24.2, 22.6	CH_2
23.8, 21.1, 21.0, 15.1	23.5, 21.0, 15.4	22.6, 21.1, 21.0, 14.6	CH_3

The mass spectrum did not show a molecular ion peak. The highest mass m/z 356 resulted from the loss of acetic acid. This was concluded after considering other terpene acetates for which no molecular ion peak was observed [7]. Thus, the molecular weight of **3** is 416. The ^{13}C-nmr, DEPT and ^{1}H-nmr spectra confirmed the presence of twenty-four carbons and thirty-two protons in **3** corresponding to a mass of 320. The remaining mass of 96 would account for six oxygen atoms of two esters and a lactone. Thus, the molecular formula of **3** is $C_{24}H_{32}O_6$, indicating an index of hydrogen deficiency of nine. With six double bond equivalents (3 C=C and 3 C=O) deduced from the ^{13}C-nmr spectrum, the remainder is accounted for by a tricyclic system.

Correlation of the partial structures deduced from the COSY spectrum and the data obtained from the ^{1}H, ^{13}C-nmr and MS resulted in structure **3**. Literature search revealed that **3** is diacetylanhydroandrographolide. Confirmatory evidence is the melting point of **3** (137-138°C) which is identical to the melting point of diacetylanhydroandrographolide [2]. Although **3** was previously reported in the literature [2, 7], this is the first report on the ^{13}C nmr and the complete ^{1}H nmr data of the compound.

The natural product from which **3** was derived was andrographolide (**1**). This conclusion is supported by the ^{1}H nmr spectrum of the mixture of natural products containing **1** prior to acetylation. The spectrum did not show resonances for conjugated olefinic protons at δ 6.09 (H-12), δ 6.89 (H-11) and δ 7.15 (H-14). Instead, a resonance due to the hydrogen of an exocyclic double bond in conjugation with the lactone system was found at δ 6.51 (H-12) which is consistent with structure **1**. Furthermore, previous studies reported that acetylation of **1** with acetic anhydride in the presence of sodium acetate afforded **3** which contained an endocyclic double bond in conjugation with the unsaturated lactone system [7].

The structure of **4** was derived by analogy with **3**. Comparison of the ^{1}H-nmr spectra of **3** and **4** (Table 1) revealed the non-appearance of the acetate at δ 2.00 (3H, s) and the carbinyl proton at δ 4.58 (1H, dd, 5.4, 11.1 Hz) in **4**. The methyl group at δ 1.00 (3H, s) in **3** was also deshielded to δ 1.12 (3H, s) in **4**, suggesting the shielding effect of the acetate in **3**. A hydroxyl was assigned to the broad singlet at δ 2.70 (1H, s, br).

Comparison of the ^{13}C-nmr spectra of **3** and **4** (Table 2) showed the absence of a carbonyl carbon of an ester (170.45 ppm) and a methyl carbon (21.05 ppm) in **4** which were typical resonances for acetates. Because of the loss of one of the acetates, it was expected that **4** will only have two C-O. However, the three C-O resonances remained. This indicated that an OH (δ 2.70, s, br) which was not acetylated by acetic anhydride is present in **4**. This was supported by the mass spectrum of **4** which showed a fragment at m/z 296 resulting from the loss of acetic acid and H_2O.

The COSY spectrum of **4** was instrumental in determining the position of the hydroxyl. The methylene protons at δ 1.20 were coupled to the methylene hydrogens at δ 1.47 and δ 1.60, which were in turn coupled to the hydrogen attached to the carbon bearing the OH at δ 3.28. The other fragments of **4** deduced from COSY were similar to those of **3**.

The structure of **4** was verified by the inverse long-range heteronuclear experiment HMBC optimized for J = 8 Hz (Table 3). All long-range correlations observed were consistent with structure **4**.

Table 3. Long range ^{13}C-^{1}H correlation spectral data of **4**

Carbon Assignments	^{13}C (δ)	^{1}H (δ)	HMBC Correlation
C1	38.5	1.20 (2H, m)	H2,2', H9, H18
C2	24.0	1.60 (1H, m), 1.47 (1H, m)	H1, H20,20'
C3	79.1	3.28 (1H, dd, 5.4, 11.1 Hz)	H5, H19, H20
C4	42.4		H19
C5	54.7	1.25 (1H, m)	H2, H7, H18, H19
C6	27.6	1.47 (1H, m), 1.66 (1H, m)	H18, OAc
C7	36.5	2.35 (1H, m), 2.01 (1H, m)	H15, H17
C8	147.8		H9, H15
C9	61.6	2.29 (1H, d, 10.1 Hz)	H12, H17,17'
C10	38.2		
C11	135.7	6.83 (1H, dd, 10.1, 15.8 Hz)	H9
C12	121.1	6.08 (1H, d, 15.8 Hz)	H9, H14
C13	129.1		H11, H12, H15
C14	143.0	7.15 (1H, s, br)	H11, H12, H15
C15,15'	69.6	4.75 (2H, s br)	H14
C16	172.2		H12, H14
C17,17'	109.1	4.77 (1H, s, br), 4.50 (1H, s, br)	H9
C18	22.5	0.80 (3H, s)	H2', H9
C19	15.4	1.12 (3H, s)	H5, H20
C20,20'	65.0	4.12 (1H, d, 11.6 Hz), 4.29 (1H, d, 11.6 Hz)	H3, H5, H19
OAc	21.0	2.0 (3H, s)	
	171.0		H20,20', OAc

The structure of **4** was confirmed by NOESY which indicated correlation through space of the proton nuclei in the molecule (Fig. 1). Thus, the methyl protons at δ 0.80 (H18) are close in space to the olefinic hydrogen at δ 6.83 (H11) and the methylene protons at δ 4.12 and δ 4.29 (H20, H20'); the carbinyl hydrogen at δ 3.28 (H3) is close to the methyl protons at δ 1.12 (H19) and the acetate attached to C20; the proton at δ 1.60 (H2) is close to the hydrogen at δ 2.35 (H7) which was in turn close to the olefinic proton at δ 4.77 (H17'); the olefinic hydrogen at δ 4.50 (H17) was close to the olefinic proton at δ 6.08 (H12) which was in turn close to the allylic hydrogen at δ 2.29 (H9). These data support the relative stereochemistry of **4** shown in Fig. 1.

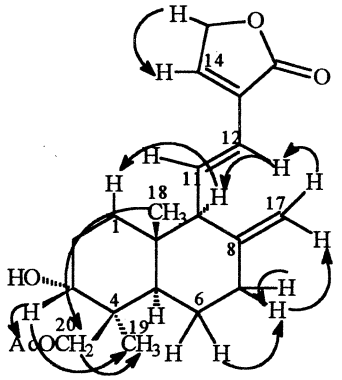


Figure 1. Correlation of ^{1}H NMR nuclei of **4** from NOESY

Thus, when the mixture of natural products containing **1** was acetylated, two products were obtained pure: the diacetylated compound **3** which was previously reported in the literature [7] and the monoacetylated compound **4**. This is the first report on the isolation and structure elucidation of **4**.

The 10% acetone in chloroform fraction from the chromatography of the crude extract afforded colorless crystals. The fraction is a mixture of compounds with hydroxyl groups as indicated by the resonances for carbinyl protons between δ 3.00-4.00 in the ^{1}H nmr spectrum. To facilitate separation and remove H-bonding, the mixture was acetylated to afford **5** and mixtures of two other diterpenes. Since the other diterpenes were not obtained pure, their structures were not elucidated.

The structure of **5** was elucidated by comparing its ^{1}H-nmr spectrum with **3** (Table 1). Although there are many similarities, there is also a difference in the spectra. The similarities are the presence of two acetates, conjugated lactones and exocyclic double bonds in both compounds. The difference is the non-appearance of the olefinic protons at δ 6.09 (1H, d) and δ 6.89 (1H, dd) in **5**, resulting in the shielding of the methyl protons at δ 0.86 (3H, s) in **3** to δ 0.65 (3H, s) in **5**. These data are consistent with structure **5**.

The structure was further supported by the comparison of the ^{13}C-nmr and DEPT spectral data of **3** and **5** (Table 2) which indicated that the only difference between the two compounds is the loss of the resonances for one of the olefins at δ 129.1 and δ 121.3 in **5**. The NOESY spectrum of **5** revealed similar relative stereochemistry to that of **3**.

The structure of the natural product **2** from which **5** was derived was supported by the ^{1}H nmr spectrum of the mixture of natural products prior to acetylation. The spectrum did not show resonances for acetyl groups at δ 2.03 and δ 2.03 and olefinic protons at δ 6.09 (H12) and δ 6.89 (H11). However, a resonance at δ 7.08 (H14) indicated the presence of an olefinic hydrogen conjugated to a lactone which is consistent with **2**.

Micronucleus test was conducted on the crude extract, mixtures of natural products and acetylated compounds to determine their potential as antimutagens. Mitomycin C, a known mutagen which causes mutation in normal cells by acting as an alkylating agent was used as the positive control.

Results of the antimutagenicity test are presented in Table 4. It indicated an 84.7% reduction in micronucleated polychromatic erythrocyte (MPCE) for the crude extract; the mixture of natural products containing **1,** mixture of natural products containing **2,** and the acetylated compounds **3**, **4**, and **5** likewise exhibited 89.8%, 86.7%, 93.2%, 94.6% and 91.1% reduction in MPCE, respectively. Statistical analysis using the t-test showed that there is a significant decrease in MPCE at $\alpha = 0.01$. Therefore, all samples tested possess high antimutagenic activity. Because of a strong correlation between antimutagenic and antitumor activities, it is highly probable that the samples tested have antitumor activity and are potential sources of antitumor drugs.

The results showing the antimutagenicity potential of the samples are very close. It is expected that since the crude extract contains only 8.57% of the mixture of antimutagenic diterpenes, the extract will give a much lower activity than the diterpenes. However, the study was not bioassay guided. Only the fractions which afforded colorless crystals in sufficient quantity were tested for their potential antimutagenic activity. In other studies conducted by our research group, common plant constituents such as stigmasterol, lupeol, β-amyrin and their acetates, β-carotene and a diadinoxanthin derivative indicated high antimutagenic activity. Even fatty acids enhance the antimutagenic activity of some triterpenes we have studied. In addition, a high dosage (170 mg/kg mouse) which is commonly employed in testing for the antimutagenicity potential of crude extracts was used. All bioassay procedures are dosage dependent. The higher the dosage, the higher the potency of the sample until the optimum dosage is reached. It is very possible that for the diterpenes tested, the optimum dosage may be significantly lower than what was used in this study.

Table 4. Effects of samples on the formation of micronucleated polychromatic erythrocytes (MPCE) induced by mitomycin C

Sample Tested	Dosage (mg isolate/ kg mouse)	Ave. No. of MPCE/1000 PCE ± σ*		% Reduction in MPCE
Crude Extract	170	1.5	0.91	84.7
Mixture of Natural Products Containing **1**	170	1.0	0.37	89.8
Mixture of Natural Products Containing **2**	170	1 3	0.98	86.7
3	170	0.67	0.61	93.2
4	170	0.53	0.51	94.6
5	170	0.87	0.61	91.1
Positive Control (mitomycin C + DMSO)	2.75 mg/kg 7.5 ml/kg	9.8	1.5	

*Average of 15 slides

CONCLUSION

Our investigation of the chloroform extract of the air dried leaves of *A. paniculata* thus afforded mixtures of diterpenes which were acetylated to obtain pure compounds (**3-5**). This is the first report on the monoacetylated compound (**4**). The crude extract, mixtures of natural products and the acetylated compounds showed high antimutagenic activity against a known mutagen, mitomycin C.

ACKNOWLEDGMENT

A research grant of the Science Foundation through the University Research Coordination Office of De La Salle University is gratefully acknowledged.

REFERENCES

1. Chopra, R.N., Chopra, I.C., Handa, K.L. and Kapur L.D. *Indigenous Drugs of India*. U.N. Dhur, Calcutta. 278 (1958).
2. Chan, W. K., Taylor, D.R. and Bodden, R.L. *Tetrahedron 27*, 5081 (1971).
3. Cava, M.P., Chan, W.R., Stein, R.P. and Willis C.R. *Tetrahedron 21*, 2617 (1965).
4. Chan, W.R., Taylor, D.R. and Willis, C.R. *Tetrahedron Letters 46*, 4803 (1968).

5. Aruna and Kapil. *Biochem Pharmacol. 46*(1), 182 (1993) (1973).

6. Balmain, A. and Connolly, J.D. *J. Chem. Soc. Perkin I 12*, 1247 (1973).

7. Bohlmann, F., Ziesche, J., King, R.M. and Robinson, H. *Phytochemistry 20*, 751 (1981).

8. Kleipool, R.J.C. and Kostermans, D.G.F.R. *Rec. Trav. Chim. 70*, 1085 (1951).

9. Schmid, W. *Mutation Research 31*, 10 (1972).