Synthesis and Cytotoxic Activity of (R)-(-)-PGME Amide of Diterpene Acid

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R)-(-)-PGME amide of diterpene acid (2) was assigned the absolute configuration from NMR correlation experiments. The compound (2) was tested for its growth inhibitory effects against tumor cell lines by the MTT method.

Key words : (R)-(-)-PGME amide of diterpene acid (2), NMR correlation experiments. growth inhibitory effects, tumor cell lines, MTT method.

Introduction

The chemistry of *A. flexuosa* was investigated after screening of the crude ethanol extract showed whole-well cytotoxicity aganinst BSC-cells[1,2]. The initial investigation also indicated that the compounds responsible for this cytotoxicity were of low to medium polarity. C-18 and silica gel column chromatography gave the main cytotoxic compound whose signals were clearly visible in the ^{1}H-NMR spectrum of the crude extract. The diterpene acid 1 (which has been named anisotomenoic acid) has also been identified in two other species of Anisotome[3]. These species are *A. lyallii* Hook. (the only lowland species of Anisotome) and *A. haastii* Hook., both of which show high levels of 1 or its derivative in their ethanolic extracts. It is interesting to note that no evidence of 1 was found in *A. aromatica* Hook., even though A. flexuosa was once regarded as a variant of this species[2]. The diterpene acid 1 and its derivative form a new class of irregular diterpenes. Their unusual structures and their presences in several Anisotome species raises many questions about the biosynthesis of these compounds and their functions in the plant. Determination of the absolute configurations of organic compounds has become an important task of the natural products chemist as well as the synthetic chemist. There are a few physical methods, e.g., exciton chirality method[4] and X-ray crystallography, that fill this need, but they have some limitations. There are also several chemical methods used to predict the absolute configurations of organic substances.[5]

In this study, the synthesis and cytotoxic activity of (R)-(-)-PGME amide of 1 (2) are reported.

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· Received : 2005/01/17 · Revised :2005/02/15 · Accepted : 2005/03/22

Experimental

1. Materials and methods

All solvents were distilled before use. Removal of solvents from chromatograph fractions were removed by rotary evaporation at temperature up to 40℃. Initial fractionation of crude *A. lyallii* extract using reverse phase column chromatography was performed with octadecyl-functionalized silica gel (C_{18} Aldrich) as the adsorbent. Further column fractionation was performed using Davisil slica 60Å (35-70 μm silica gel, Alltech) as the adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} visualized first with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. MS, UV and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra, of $CDCl_3$ solutions at 25℃, were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.27 ppm and $CDCl_3$ at 77.08 ppm. DEPT, COSY, HSQC, NOESY and CIGAR experiments were run at 45℃. Benzotriazolyloxy tri (phrrolidiryl) phosphoniumhexa - fluorophosphate and 1-hydroxy benzotriazole were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, USA). All other chemicals were of reagent grade.

2. Plant Material.

The *A. lyallii* was collected in the Dunedin Botanical Garden in December 2001. Plant was identified by a botanist and voucher specimens deposited in the PERU herbarium.

3. Extraction

A bulk extract of fresh plant material (195.1 g, collection code 011215) was prepared by blending with EtOH (1 x 500 mL, 1 x 400 mL), and then with $CHCl_3$ (300 mL). The solvent was removed from the extracts and subsamples analysed by ^{1}H-NMR, the spectra of both extracts were similar so these were combined to give a dark yellow gum (10.58 g).

4. Isolation of anisotomenoic acid.

The anisotomenoic acid - pale yellow oil; Si-gel TLC (hexane : EtOAc, 3 : 1), Rf 0.333, plus blue/green with vanillin; ^{1}H and ^{13}C-NMR, DEPT, HSQC, CIGAR, NOESY and COSY data are presented in Table 1. Anisotomenoic acid (1) thus isolated was identified by comparison of its spectral data with those published or by direct comparison with an authentic sample[6].

5. Conversion of anisotomenoic acid to the corresponding (R)-(-)-PGME amide.

To a stirred solution of a mixture of anisotomenoic acid (50 mg, 0.164 mmol) and R-PGME (38.9 mg, 0.19 mmol) in DMF (750 mL) were successively added PYBOP (97.2 mg, 0.22 mmol), HOBT (25.6 mg, 0.19 mmol) and N-methylmorpholine (60.5 μg, 0.55 mmol) at 0℃. After the mixture was stirred at room temperature for 3 hrs, ethyl acetate (13 mL) were added and the resulting diluted solution was successively washed with 5% HCl, saturated bicarbonate solution, and brine. The obtained crude product was purified using PTLC (n-hexane-ethyl acetate, 3 : 1), affording 52 mg (yield 64.8%) of (R)-(-)-PGME amide of 1 (Fig. 1)[7].

6. Cell culture.

A549 (Lung cancer cells), MDA-MB-231 (breast cancer cells), and SNU-C4 (large intestinal cancer cells) were grown at 37℃ in RPMI medium supplemented with 10% FBS penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were grown in a humidified atmosphere of 95% air / 5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

7. 4,5-Dimethylthiazol-2-yl-2,5-diphenyl-tetrazoliumbromide (MTT) assay.

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product which can be measured spectrophotometrically[4,5]. Tumor cell lines were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^5) were cultured for 48 hrs at 37℃ in a humidified 5% CO_2 incubator in the presence or absence of sample.

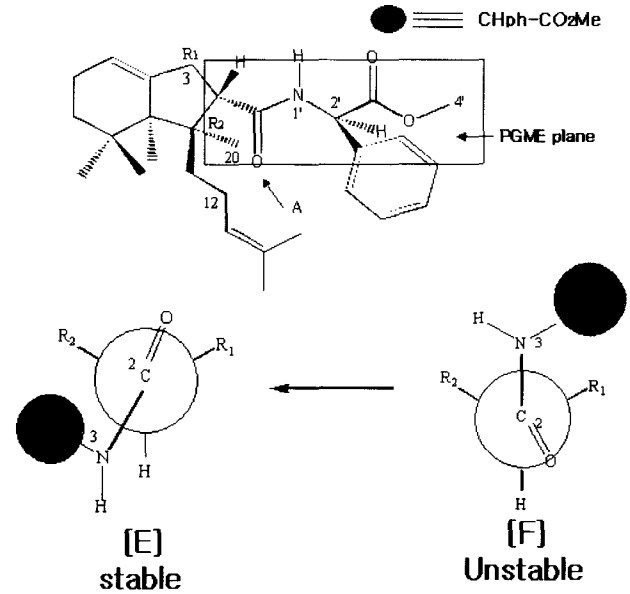

Fig. 1. Stable conformation of the PGME amide of anisotomenoic acid (2). The Newman model [E] is a view from the direction A. Rotation around C1-C2 by 180° gives another conformation, [F]. The solid circle represents the methoxy carbonyl phenyl methyl group of the PGME moiety.

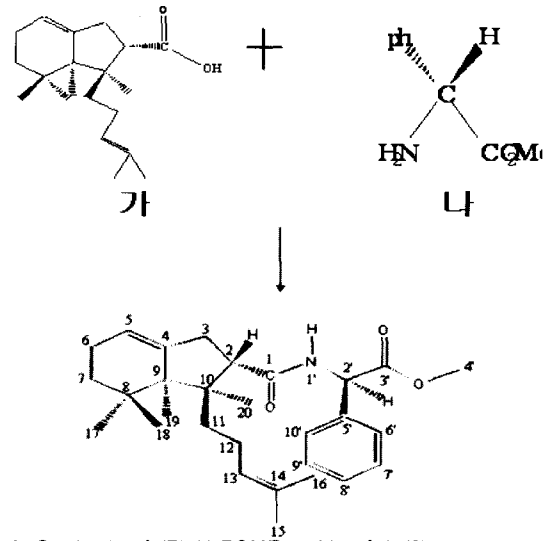

Fig. 2. Synthesis of (R)-(-)-PGME amide of 1 (2)

8. Evaluation of toxicity.

In order to determine the cytotoxicity mediated by 2, the

Table 1. Summary of NMR data for (R)-(-)-PGME amide of 1 (2)

Position	Carbon[a](45℃)	DEPT(45℃)	Proton[b]	Couplings	HMBC Correlations	1D/2D NOE Interactions	COSY
1	173.9	C					
2	51.3	CH	2.45	t, J=8.5Hz	173.9, 41.4, 33.1	2.13, 1.89, 1.37	2.13
3	33.1	CH$_2$	2.77 2.13	brm dd, J=9.5, 13.5Hz	- -	2.13, 1.04 *	2.13, 2.13
4	145.3	C					
5	116.9	CH	5.38	d, J=5.5Hz	33.1	2.13, 1.91	1.91, 2.05, 2.77
6	22.0	CH$_2$	2.05 1.91	m brm	- 116.9	0.82, 0.89	1.59, 1.91
7	36.2	CH$_2$	1.59 1.17	t, J=5.5Hz brm	- -	- *1.37, 0.89, 0.82, 0.98	
8	36.7	C			28.8, 25.7, 19.5		
9	50.5	C			28.8, 25.7, 19.5		
10	52.8	C			51.3, 41.4, 19.5, 17.7, 17.3	-	
11	41.4	CH$_2$	1.62 1.37	dd, J=5.5, 13.3Hz ddd, J=4, 12, 18.8Hz	41.4, 124.7 124.7, 24.1, 51.3	-	1.37
12	24.1	CH$_2$	2.03 1.89	br m br m	124.7, 131.6 124.7, 131.6	1.89, 1.56 2.03, 1.56, 0.82, 0.89	1.89, 1.62, 1.37, 1.62, 1.37
13	124.7	CH	5.07	dt, J=1.5, 7.0Hz	25.6, 17.7	1.67	2.03, 1.89, 1.37, 1.67, 1.56
14	131.6	C					
15	25.6	CH$_3$	1.67	d, J=1.0Hz	131.6, 17.7, 124.7	1.56	
16	17.7	CH$_3$	1.56	d, J=0.5Hz	51.3, 131.6, 25.6, 124.7	-	1.37
17	17.3	CH$_3$	0.82	s	52.8, 41.4, 50.5	-	
18	28.8	CH$_3$	0.89	s	50.5, 36.7, 25.7	-	
19	25.7	CH$_3$	0.98	s	50.5, 145.3, 28.8, 36.7, 22.0	0.89, 0.82	
20	19.5	CH$_3$	1.04	s	50.5, 52.8, 36.7, 28.8	0.89, 0.82	
1′			6.35	d, J=7.0Hz	173.9	7.34(w), 2.45, 5.57, 1.89	5.57
2′	56.6	CH	5.57	d, J=7.0Hz	173.9, 171.6, 137.0, 127.3	7.34, 6.35, 3.71 (w)	
3′	171.6	C					
4′	52.6	CH$_3$	3.71	s	171.6	7.34(w), 5.57	
5′	137.0	C					
6′	128.9	CH	7.29 -7.35	m	137.0, 128.4, 127.3, 128.9, 56.6	5.57	
7′	128.4	CH	7.29 -7.35	m	137.0, 128.9, 127.3, 128.4, 56.6	5.57	
8′	127.3	CH	7.29 -7.35	m	137.0, 128.9, 128.4, 56.6, 127.3	5.57	
9′	128.4	CH	7.29 -7.35	m	137.0 128.9, 127.3, 128.4, 56.6	5.57	
10′	128.9	CH	7.29 -7.35	m	137.0, 128.4, 127.3, 128.9, 56.6	5.57	

[a] Measured in $CDCl_3$ at 125 MHz. [b] Measured in $CDCl_3$ at 500 MHz.

colorimetric assay was used. These compounds were serially diluted in EMEM (Eagle's minimum essential medium) with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells/mL). After one hour, fresh culture medium was supplied to a total volume of 1~100 μM. On the third day of incubation at 37℃ an incubator MTT terazolium dye (5 mg/mL; 20 μL/well ; polyscience, Inc. Warrigton, PA) was added to the cells. After 3 hr, the absorbance was measured at 540 nm using ELISA reader. All experimental data were expressed as the mean ± S.D. of triplicate experiments. The 50% cytotoxic dose (IC_{50}) was calculated using the computer program.

9. Statistical analysis.

All values, expressed as the mean ± S.D., were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

Results and Discussion

Structural interchange between these conformers could explain this line broadening. Increasing numbers of NMR methods have been developed to elucidate the absolute configuration of secondary alcohols by using the anisotropy of aromatic rings of chiral auxiliaries such as MTPA[8], MPA[9] and other analogs (chiral anisotropic reagents)[10]. Nagai et al[11] described two new NMR reagents, and (S)-phenylglycine dimethylamide (PGDA) and methyl ester (PGME), which are designed to determine the absolute configuration of carboxylic acids. The principle of the PGME method is summarized in Fig. 1. a chiral α, α-disubstituted acetic acid [A] is condensed with (R)-(-)-PGME [B], now commercially available, giving the amide [C]. Coplanarity of the atoms from position 1 to position 4 is guaranteed owing to the S-trans amide linkage, which is well established in peptide chemistry. The planarity can be extended to the methoxy carbonyl group at the 5-position, because a polar ester group will prefer the conformation anti (with respect to the C3 - C4 bond) to the polar carbonyl group at the 2-position. This assumption was verified by X-ray crystallography NOE studies (Figs. 1 and 2)[11].

Fig. 3. shows the potent cytotoxic activity of (R)-(-)-PGME

amide of 1 (2) against cancer tumor cell lines. In general, the cytotoxic activity of this compound was in a dose-dependent manners, and the susceptibility of the cancer cell lines to the compound (2) was not quite sensitive. However, the compound (2) mediated cytotoxicity did not increased in the MTT assay against tumor cell lines when its concentration was increased from control to 100 μg/mL. As shown in Fig. 3, the compound (2) mediated cytotoxicity gradually increased in the MTT assay against tumor cell lines when its concentration was increased from 200 μg/mL to 400 μg/mL. The compound (2) was the most effective growth inhibitor of MDA-MB-231 breast cancer cell lines in the MTT method.

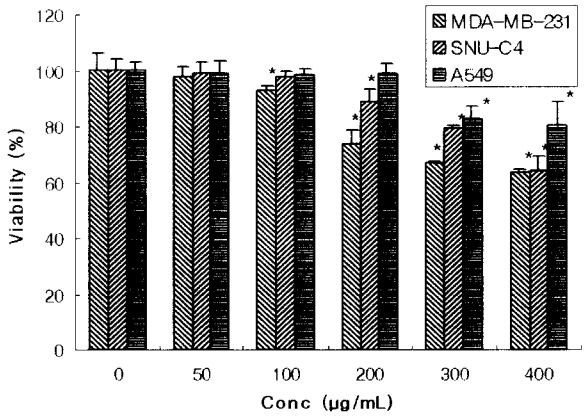

Fig. 3. In vitro cytotoxicity of (R)-(-)-PGME amide of 1 (2) by the MTT method. This compound was serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^5 cells). The colorimetric assay was performed as described in the materials and methods section. Data are mean values of results obtained from three sets of experiments. *Significantly different from the control value: **P<0.05 (Student's-test).

In conclusion, (R)-(-)-PGME amide of diterpene acid of 1 (2) was assigned the absolute configuration from NMR correlation experiments. The compound (2) was tested for its growth inhibitory effects against tumor cell lines by the MTT method.

Acknowledgements

This research was supported by Wonkwang Health Science College in 2004.

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