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# Antitumor evaluation and antimicrobial activity of geranyl phenyl ethers

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#### **SUMMARY**

Geranyl phenyl ethers **1**, **2**, **3**, **4** and **5**, 5-fluorouracil **6** and adriamycin **7** as references were tested for their growth inhibitory effects against tumor cell lines and NIH 3T3 fibroblasts using two different assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and sulforhodamine B protein (SRB) assays. These results suggest that methyl-4-[{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]-3-methoxy benzoate **1** showed growth inhibition activity against tumor cell lines. The maximum activity exhibited by methyl-4-[{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]-3hydroxybenzoate **3** against *Staphylococcus epidermidis* (MIC, 1,000 µg/ml).

Key words: Geranyl phenyl ethers; Tumor cell lines; Staphylococcus epidermidis

## **INTRODUCTION**

Chemotherapeutic agents have the capacity to inhibit both normal and malignant cell growth, with the preference to cancer cells which proliferate actively (Wertz and Michael, 1977). In this context, the cytotoxic effect of chemotherapeutic agents on normal cells, especially on mucosal fibroblasts, may be an essential factor for drug applications and for diagnosis and prevention of side effects. Recently, many investigators have studied the cytotoxic mechanism of toxic materials using various types of cultured cells (Borenfreund et al., 1988; Iijima et al., 1983; Lampidis et al., 1980). Considerable interest has focused on the development of short term in vitro cytotoxicity assays with cultured cells for the evaluation of the acute cytotoxicities of chemotherapeutic agents. We recently reported that methyl-4- [{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]-3-methoxy benzoate (DTM, 1) showed the most growth-inhibitory activity against KB (ATCC No. OCLL7) cell lines using two different assays, MTT and SRB assays. Various assay techniques have been recommended to evaluate the cytotoxicity of chemotherapeutic agents such as neutral red (NR) (Borenfreund *et al.*, 1998), 5-diphenyltetrazolium bromide (MTT), and sulforhodamine B protein amount (SRB). These are relatively simple as well as sensitive. To compare the cytotoxicities of chemotherapeutic agents on oral fibroblasts, we have used 5-diphenyltetrazolium bromide (MTT) (Borenfreund *et al.*, 1988; Borenfreund and Puerner, 1985; Babich and Borenfreund, 1987; Babich and Borenfreund, 1987; Mosmann, 1983; Carmichael *et al.*, 1987; Cole, 1986), and sulforhodamine B protein amount (SRB) (Skehan *et al.*, 1990).

In the present study, we investigated inhibitory effects of geranyl phenyl ethers against KB cell lines using two different assays, MTT and SRB assays. The effects of 5-fluorouracil were also examined for comparison.

# MATERIALS AND METHODS

Melting points were determined on a Kofler hot stage and were uncorrected. <sup>1</sup>H and <sup>13</sup>C-NMR spectra were recorded using Varian Gemini-200 and Varian VXR-300 spectrometers. When CDCl<sub>3</sub> was

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used as a solvent, CHCl<sub>3</sub> (<sup>1</sup>H,  $\delta$  H, 7.27) or CDCl<sub>3</sub> (<sup>13</sup>C,  $\delta$  C, 77.08) was used as an internal reference. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrophotometer. Mass spectra were recorded on a Varian Mat CH-5 mass spectrometer. TLC was carried out on Si gel 60 F<sub>254</sub> precoated 0.2 mm aluminium sheet (Merck 5562). Developed plates were visualized by UV light and staining with a 5% solution of anisaldehyde in ethanol. Flash chromatography was carried out with a silica gel 230-400 mesh.

#### MATERIALS AND METHODS

Adriamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide, fetal bovine serum (FBS), sulforhodamine B protein, streptomycin, and penicillin were obtained from Sigma Chemical Co. Ltd. (St. Louis, USA). Geranyl bromide, sodium hydride, and dimethylsulphate were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, USA). 3-Methyl-4hydroxybenzoic acid was obtained from Merck Chemical Co. (Germany). Methyl-4-[{(2E)-3,7dimethyl-2,6-octadienyl}oxy]-3-methoxy benzoate 1,methyl-3-[{(2*E*)-3,7-dimethyl-2,6-octadienyl}oxy]-4-methoxybenzoate 2, methyl-4-[{(2E)-3,7-dimethyl-2,6-octadi eny}oxy]-3-hydroxy benzoate 3, methyl-3-[{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]-4-hydroxybenzoate 4 and methyl-3,4-di-[{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]]benzoate 5 were prepared by using Baeks method (Perry et al., 1996; Baek et al., 1998). Tumor cells were obtained from Korean Cell Line Bank in the Seoul National University. All other chemicals were of reagent grade.

#### Tumor cell lines and culture conditions

SK-MEL-3, KB and NIH 3T3 cells were grown at  $37^{\circ}$ C 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<sub>2</sub>. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

# **Biological assay**

The microorganisms used included: *Streptococcus* aureus (ATCC 29213), *Streptococcus mutans* (JC-2), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas*  aeruginosa (KCTC 1636). Pseudomonas putida (KCTC 8729), Candida albicans (KCTC 1940).

# Antimicrobial assay

The dried plant extracts were dissolved in 10% aqueous dimethyl sulfoxide (DMSO) to a final concentration 2000 µg/ml and sterilized by filtration through a 0.45 µm membrane filter. Antimicrobial tests were then carried out by the agar serial dilution method (Han et al., You et al., 1994). Each of several concentrations of a tube of molten agar, which is then mixed, poured into a the petri plates, and allowed to solidify. The organisms containing 10<sup>6</sup> bacterial cells/ml or 10<sup>8</sup> yeast cells/ml were inoculated in to the petri plates. After the plates have been incubated for 24 h at 37°C for bacteria and for five-seven days at 22°C for fungi, the lowest concentration of 1, 2, 3, 4 and 5 that inhibit grows of the organisms, was determined as the MIC of the antimicrobial agents. Ampicillin served as positive controls for S. aureus, S. mutans, and S. epidermidis, consequently, whereas, ketoconazole served as a negative control for P. aeruginosa, and P. putida. Each test was carried out in triplicate experiments.

#### Evaluation of antitumor activity

The antitumor activities of **1**, **2**, **3**, **4**, **5**, **6** and **7** were determined by the modification of the literature methods (Mosmann, 1983; Carmichael *et al.*, 1987; Min *et al.*, 1996) All experimental data were expressed as the mean±S.D. of triplicate experiments.

# 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (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. KB cell lines were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells ( $5 \times 10^4$ ) were cultured for 48 hrs at  $37^{\circ}$ C in a humidified 5% CO<sub>2</sub> incubator in the presence or absence of **1**, **2**, **3**, **4**, **5**, **6** and **7**.

#### Sulforhodamine B protein (SRB) assay

The SRB assay was performed essentially according

to the method of Skehan *et al.* (1990) The methods of plating and incubation of cells were identical to those cells of the MTT assay.

#### Evaluation of toxicity : Cytotoxicity assay

In order to determine the cytotoxicity mediated by **1**, **2**, **3**, **4**, **5**, **6** and **7** the 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 fibroblast  $(5\times10^4 \text{ cells/ml})$ . After one hour, fresh culture medium was supplied to a total volume of 1-100  $\mu$ M. On the third day of incubation at 37°C an incubator MTT terazolium dye (5 mg/ml; 20  $\mu$ l/well; Polyscience, Inc. Warrigton, PA) was added to the cells. After 3 h, the absorbance was measured at 540 nm (ELISA reader, SPECTRAmax 250, Molecular Devices, USA). Percentage of cytotoxicity was calculated using the following formula;

 $\frac{\text{mean A with sample}}{\text{mean A with sample}} \times 100$ 

All experimental data were expressed as the mean $\pm$ S.D. of three experiments. Student's *t*-test was used for statistical analysis. The 50% cytotoxic dose (CD<sub>50</sub>) was calculated using the computer program.

#### Morphology

Changes in morphology of SK-MEL-3, KB and NIH 3T3 fibroblasts cultured in a medium with **1**, **2**, **3**, **4**, **5**, **6** and **7** were documented by microphotography.

#### Statistical analysis

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

#### RESULTS

#### In vitro growth inhibitory effects

Table 1 shows values of the antitumor activities of 1, 2, 3, 4, 5 and 6 against KB cells. In general, the antitumor activities of these compounds were in a dose-dependent, and the susceptibility of the KB cell lines to methyl-4-[(2E)-3,7-dimethyl-2,6-octadienyl}-oxy]-3-methoxybenzoate 1 was quite sensitive (Wertz

Table 1. The growth inhibitory effects of 1, 2	2, 3	3,	4,	5
and <b>6</b> on KB cell lines. Comparison of IC $_{50}$ for	1, 2	2, 3	3,	4,
5 and 6 by SRB and MTT assays.				

Compounds <sup>a</sup> -	$\mathrm{IC}_{50}\left(\mu\mathrm{M}\right)^{\mathrm{b}}$			
Compounds	MTT assay	SRB assay		
1	5.55	41.42		
2	28.84	59.21		
3	111.63	445.61		
4	113.12	718.71		
5	1173.93	1821.44		
6	44.36	45.20		

<sup>a</sup>Each compound was examined in four concentrations in triplicate experiments.

 ${}^{b}IC_{50}$  represents the concentration of a compound required for 50% inhibition of cell growth.

Table 2. The antitumor activities of 1, 2, 3, 4, 5 and 7 on SK-MEL-3 cell lines. Comparison of  $IC_{50}$  for 1, 2, 3, 4, 5 and 7 by SRB and MTT assays.

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Compounds <sup>a</sup>	$IC_{50} (\mu M)^{b}$			
Compounds	MTT assay	SRB assay		
1	42.48	49.99		
2	46.57	58.75		
3	160.68	554.12		
4	199.59	456.48		
5	1261.03	1760.79		
7	20.12	38.63		

<sup>a</sup>Each compound was examined in four concentrations in triplicate experiments.

 $^{\text{D}}\text{IC}_{50}$  represents the concentration of a compound required for 50% inhibition of cell growth.

and Michael, 1977; Borenfreund *et al.*, 1988; Oh *et al.*, 1999). The values of **1**, **2**, **3**, **4**, **5** and **6** showed that **1** and **2** exerts the potent antitumor activity. The values of MTT<sub>50</sub> and SRB<sub>50</sub> of the **1** on KB cells were determined at  $5.55 \,\mu$ M and  $41.42 \,\mu$ M, respectively. And the values of MTT<sub>50</sub> and SRB<sub>50</sub> of the **2** on KB cells were determined at 28.84  $\mu$ M and 59.21  $\mu$ M, respectively. 5-Fluorouracil **6** as a reference was evaluated for the strong antitumor activity against KB cells. However, **6** has the strong cytotoxic effect of NIH 3T3 fibroblasts, producing a CD<sub>50</sub> values of 41.27  $\mu$ M by MTT assay and 75.90  $\mu$ M by SRB assay (Table 3).

Table 2 shows the potent antitumor activities of **1**, **2**, **3**, **4**, **5** and **7** against SK-MEL-3 cells. In general, the antitumor activities of these compounds were in a dose-dependent, and the susceptibility of the

**Table 3.** The cytotoxic effects of 1, 2, 3, 4, 5, 6 and 7 onNIH 3T3 fibroblasts. Comparison of  $CD_{50}$  for 1, 2, 3, 4, 5,6 and 7 on NIH 3T3 fibroblasts by SRB and MTT assays.

Compounds <sup>a</sup>	$\mathrm{CD}_{50}\left(\mu\mathrm{M} ight)^{\mathrm{b}}$		
Compounds –	MTT assay	SRB assay	
1	56.92	102.70	
2	50.34	304.26	
3	113.71	457.55	
4	99.61	210.18	
5	837.54	9946.20	
6	41.27	75.90	
7	23.26	46.94	

<sup>a</sup>Each compound was examined in four concentrations in triplicate experiments.

<sup>b</sup>IC<sub>50</sub> represents the concentration of a compound required for 50% inhibition of cell growth.

SK-MEL-3 cell lines to methyl-4-[{(2E)-3,7-dimethyl-2,6-octadienyl} oxy]-3- methoxybenzoate **1** was quite sensitive. The values of **1**, **2**, **3**, **4**, **5** and **7** showed that methyl-4-[{(2E)-3,7-dimethyl-2,6octadienyl}oxy]-3-methoxybenzoate **1** exerts the potent antitumor activity. The values of MTT<sub>50</sub> and SRB<sub>50</sub> on SK-MEL-3 cells were determined at 42.48  $\mu$ M and 49.99  $\mu$ M, respectively. But isomer **2** of **1** showed very similar IC<sub>50</sub>. Adriamycin **6** as a reference was evaluated for the strongest antitumor activity against SK-MEL-3 cells. However, it has the strongest cytotoxic effect of NIH 3T3 fibroblasts, producing a CD<sub>50</sub> values of 23.26  $\mu$ M by MTT assay and 46.94  $\mu$ M by SRB assay (Table 3).

Compounds 1, 2, 3, 4, 5, 6 and 7 were evaluated for

# cytotoxic activity against NIH 3T3 fibroblasts. **1** and **2** has the cytotoxic effect of NIH 3T3 fibroblasts, producing a $CD_{50}$ values of 56.92 $\mu$ M, 50.34 $\mu$ M by MTT assay and 102.71 $\mu$ M, 304.26 $\mu$ M by SRB assay respectively. But 5-fluorouracil **6** has strong cytotoxic activity or NIH 3T3 fibroblasts, producing a $CD_{50}$ values of 41.27 $\mu$ M in MTT assay and 75.90 $\mu$ M in SRB assay (Table 3).

#### **Biological Activity**

In general, the growth inhibitory effects of these compounds 1, 2, 3, 4, 5 and 6 were in a dosedependent over the micromolar concentration range 1  $\mu$ M to 100  $\mu$ M, and the susceptibility of human oral epithelioids carcinoma cell lines to these compounds was quite different. The comparison of IC<sub>50</sub> values of these compounds in human oral epithelioids carcinoma cell lines shows that their susceptibility to these compounds decreases in the following order: 1>2>6>3>4>5 by the MTT assay and 1>6>2>3>4>5 by the SRB assay (Table 1). 1 and 2 were evaluated for antitumor efficacy against human oral epithelioids carcinoma cell lines. The growth inhibitory effects of 1 and 2 against the human oral epithelioids carcinoma cell lines is given in Table 1. However, 1 and 2 were the most effective growth inhibitor of human oral epithelioids carcinoma cell lines, producing an IC<sub>50</sub> of about 5.6  $\mu$ M by the MTT assay and 41  $\mu$ M by the SRB assay.

#### DISCUSSION

The sulforhodamine B protein stain assay was compared with the tetrazolium (MTT) colorimetric

Table 4. Minimum inhibitory	concentrations (MICs) of 1, 2, 3, 4, 5, ampicillin and ketoconazole for the reference
strains.	
Strains tostad	MICs $(\mu g/ml)^{b}$
Strains tested	

Studius tostad	wites (µg/ iii)						
Strains tested	1	2	3	4	5	AP	KT
S. mutans	>1,000	>1,000	>1,000	>1,000	>1,000	3.125	50
S. epidermidis	>1,000	>1,000	1,000	>1,000	>1,000	50	50
S. aureus	>1,000	>1,000	>1,000	>1,000	>1,000	3.125	200
P. aeruginosa	>1,000	>1,000	>1,000	>1,000	>1,000	50	100
P. putida	>1,000	>1,000	>1,000	>1,000	>1,000	>200	50
S. typhimurium	>1,000	>1,000	>1,000	>1,000	>1,000	-	-
C. albicans	>1,000	>1,000	>1,000	>1,000	>1,000	>200	25

<sup>a</sup>AP; Ampicillin, KT; Ketoconatole, -; not determined.

<sup>b</sup>Data are the average of three experiments.

**Cytotoxicity** 

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assay for *in vitro* chemosensitivity testing of human oral epithelioids carcinoma cell lines. The MTT assay appeared to be more sensitive than the SRB assay, with a better linearity with cell number and higher reproducibility. However, the growth inhibitory effects of **1** and **2** exhibit more active than that of **6** on human oral epithelioids carcinoma cell lines (Table 1). As shown in table 1, the growth inhibitory effects of **6** as a reference shows the effective growth inhibitor of human oral epithelioids carcinoma cell lines.

The present study also compares the in vitro growth inhibitory activities of 1, 2, 3, 4, 5 and 6 against SK-MEL-3 cells which were evaluated for antitumor efficacy. Table II shows the potent antitumor activity of these compounds against SK-MEL-3 cells. In general, the antitumor activities of these compounds 1, 2, 3, 4, 5 and 6 were in a dose-dependent over the micromolar concentration range 1 to 100  $\mu$ M, and the susceptibility of SK-MEL-3 cells to these compounds was quite different. Compounds in SK-MEL-3 cells show that susceptibility to these compounds decrease in the following order: 6>1> 2>3>4>5 by the MTT assay and 6>1>2>4>3>5 by the SRB assay. 1 was the most effective growth inhibitor of SK-MEL-3 cell lines, producing an IC<sub>50</sub> of about 42  $\mu$ M in MTT assay and 50  $\mu$ M in SRB assay. MTT assay appeared to be more sensitive than SRB assay.

Tables 3 shows the cytotoxic activities of 1, 2, 3, 4, 5, 6 and 7 against NIH 3T3 fibroblasts. In general, the cytotoxic activities of these compounds 1, 2, 3, 4, 5, 6 and 7 were in a dose-dependent manner over the concentration range 1  $\mu$ M to 100  $\mu$ M, and the susceptibility of NIH 3T3 fibroblasts to these compounds was quite different (Table 3). The comparison of CD<sub>50</sub> for 1, 2, 3, 4, 5, 6 and 7 were tested on NIH 3T3 fibroblasts by SRB and MTT assays. The comparison of CD<sub>50</sub> values of these compounds in NIH 3T3 fibroblasts shows that their susceptibility to these compounds decrease in the following order; 2>1>4>3>6>5 by MTT assay, 2>6>1>4>3> 5 by SRB assay (Table 3). The growth inhibitory effects of 1 and 2 exhibit more active than that of 6 on human oral epithelioid carcinoma cell lines. This compound 1 and 2 has been selected as therapeutic compounds on human oral epithelioid carcinoma cell lines for further examination and 3 and **4** has been selected as lead compound on human oral epithelioid carcinoma cell lines for further examination.

Geranyl phenyl ethers **1**, **2**, **3**, **4** and **5** inhibited gram-positive bacteria, gram-negative bacteria and fungus. These bioactive compounds showed weaker activity against gram-positive bacteria, gram-negative bacteria and fungus than ketoconazole and ampicillin. However, methyl-4-[{(2E)-3,7-dimethyl-2,6-octadienyl}oxy]-3-hydroxybenzoate **3** has weak activity against *S. epidermidis* (MIC, 1,000  $\mu$ g/ml).

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#### REFERENCES

- Babich H, Borenfreund E. (1987) In vitro cytotoxicity of organic pollutants to bluegill sunfish (BF-2) cells. *Envir. Res.* **42**, 229-237.
- Babich H, Borenfreund E. (1987) Polycyclic aromatic hydrocarbon in vitro cytotoxicity to bluefill BF-2 cells; mediation by S-9 microsomal fraction temperature. *Toxicology Lett.* **36**, 107-116.
- Baek SH, Perry NB, Weavers RT, Tangney RS. (1998) Geranyl phenyl ethers from the New Zealand liverwort *Trichocolea hatcheri*. J. Nat. Prod. **61**, 126-129.
- Borenfreund E, Puerner JA. (1985) Toxicity determined in vitro by morphological alterations neutral red absorption. *Toxicology Lett.* **24**, 119-124.
- Borenfreund E, Babich H, Martin-Alcuacil N. (1998) Comparison of two in vitro cytotoxicity assay: The neutral red(NR) and tetrazolium MTT tests. *Toxic. in vitro* **2**, 1-6.
- Borenfreund E, Babich H, Martin-Alcuacil N. (1988) Comparison of two in vitro cytotoxicity assays: The neutral red(NR) and tetrazolium MTT tests. *Toxic. in vitro* **2**, 1-6.
- Carmichael J, Degraff WG, Gazear AF, Minna JD, Mitchell JB. (1987) Evaluation of a tetrazoliumbased semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **47**, 936-942.
- Cole SPC. (1986) Rapid chemosensitivity tessting of human lung tumor cells using the MTT assay. *Cancer Cheomther. Pharmac.* 17, 259-263.

- Han DS, Jung KH, Jung WY, Oh IK, Kang KU and Baek SH (2000) Synthesis and cytotoxic effcts of deoxytomentellin. Arch. Pharm. Res. 23, 121-127.
- Iijima S, Spindle A, Pederson RA. (1983) Developmental cytogenic effects potassium dichromate mouse embreo in vitro. *Teratology* 27, 109-115.
- Lampidis TJ, Henderson IC, Israel M, Canellos GP. (1980) Structural and functional effects of adriamycin on cardiac cell in vitro. *Cancer Res.* **40**, 3901-3909.
- Min BS, Bang KH, Lee JS, Bae KH. (1996) Screening of the antifungal activity from natural products against *Candida albicans* and *Penicillium avellaneum*. *J. Pharm. Soc. Kor.* **40**, 582-590.
- Mosmann T. (1983) Rapid colorimetric assays for cellular growth and survival: application to proliferation and cytotoxicity assays. *Immunol. Methods* **65**, 55-63.
- Oh IK, You EA, Han DS, BAEK SH. (1999) Antitumor evaluation of methyl-4-[{(2E)-3,7-dimethyl-2,6-

octadienyl}oxy}]-3-methoxybenzoate (DTM) by colorimetric methods. *J. Toxiol. Pub. Health* **15**, 437-445.

- Perry NB, Poster LM, Lonier SD, May BCH, Weavers RT, Toyota V. (1996) Isoprenyl phenyl ethers from liverworts of the Genus *Trichocolea. J. Nat. Prod.* **50**, 729-733.
- Skehan P, Storeng S, Studiero D, Monke A, McMahon J, Vistica D, Warren JT, Bodesh H, Kenny S, Boyd MR. (1990) New colorimetric cytotoxicity assay for Anticancer-drug screening. J. Natl. Cancer Inst. 82, 1107-1112.
- You YH, Nam JP, Byung OK, Moon JC, Jeom SS. (1994) *In vitro* and *in vivo* antibacterial activities of the new quinolone, DWQ 013. *J. Pharm. Soc. Kor.* **38**, 265-273.
- Wertz RA, Michael D. (1977) Midpalatal suture opening: A normative study, *Am. J. Orthod.* **71**, 367-381.