Plant Pathol. J. 24(3) : 317-321 (2008)

The Plant Pathology Journal

Antifungal Activity of the Methanol Extract of *Myristica malabarica* Fruit Rinds and the Active Ingredients Malabaricones Against Phytopathogenic Fungi

Nam Hee Choi[1,2], Gyung Ja Choi[1], Kyoung Soo Jang[1], Yong Ho Choi[1], Sun Og Lee[1], Jae Eul Choi[2] and Jin-Cheol Kim[1*]

[1]Bio-Control Research Team, Korea Research Institute of Chemical Technology, Yuseong, P.O. Box 107, Daejeon 305-600, Korea
[2]College of Agricultural and Life Science, Chungnam National University, Daejeon 305-764, Korea

(Received on May 19, 2008; Accepted on July 8, 2008)

In a search for plant extracts with *in vivo* antifungal activity for plant diseases, we found that the methanol extract of *Myristica malabarica* fruit rinds effectively suppressed the development of several plant diseases. The methanol extract exhibited potent 1-day protective activity against rice blast, tomato late blight, wheat leaf rust and red pepper anthracnose. It also showed 7-day and 4-day protective activities against the plant diseases. Three antifungal resorcinols were isolated from the methanol extract of *M. malabarica* fruit rinds and identified as malabaricones A (MA), B (MB), and C (MC). Inhibitory activity of the three resorcinols against mycelial growth of plant pathogenic fungi varied according to compound and target species. All three compounds effectively reduced the development of rice blast, wheat leaf rust and red pepper anthracnose. In addition, MC was highly active for reducing the development of tomato late blight. This is the first report on the antifungal activities of malabaricones against filamentous fungi.

Keywords **:** antifungal activity, Bombay mace, malabaricone, *Myristica malabarica*, resorcinols

Many species of pathogenic fungi have inflicted serious damage on agricultural crops that result in severe losses to crop yields. In the past, farmers have used mainly synthetic fungicides for plant protection, but side effects of many of these fungicides, such as environmental pollution, residual toxicity, and resistance have led scientists to develop environmentally benign biopesticides such as microbial biopesticides and natural biopesticides. Since plants produce a very diverse range of secondary metabolites such as terpenoids, alkaloids, polyacetylenes, flavonoids and unusual amino acids and sugars, they can be always a large source of new agricultural chemicals.

The Bombay nutmeg tree, *Myristica malabarica* is an endemic Indian plant in the Myristicaceae family. The fruit rind of the plant, known as rampatri, Bombay mace, or false nutmeg, is used as an exotic spice in various Indian cuisines. It is also used for medical purposes such as hepatoprotective, anticarcinogenic, and antithrombotic activities (Morita et al., 2003). The superoxide-scavenging (antioxidant) and endopeptidase inhibitory activities of the methanol extract of *M. malabarica* were also reported by Khanom et al. (2000). Our research group has recently reported the nematicidal activity of the methanol extract of *M. malabarica* (Choi et al., 2008).

In the search program for antifungal compounds from plants, we have found that the methanol extract of *M. malabarica* is highly active to several fungal plant diseases. This paper reports the isolation procedures of the three resorcinol compounds, their characterization, and the *in vitro* and *in vivo* antifungal activity against various plant pathogenic fungi.

*Corresponding author.
Phone) +82-42-860-7436, FAX) +82-42-861-4913
E-mail) kjinc@krict.re.kr

Materials and Methods

Plant material. Dried fruit rinds of *M. malabarica* (Bombay mace) were imported from India by KFI Corporation (Seoul, Korea). A voucher specimen has been deposited at our laboratory.

Isolation and identification of antifungal substances. The isolation and identification procedures for antifungal substances from dried Bombay mace were previously described in detail (Choi et al., 2008). Briefly, dried Bombay mace was extracted with methanol and then concentrated. After dissolving in methanol+water (4:1, v/v), the aqueous layer was partitioned with *n*-hexane twice and then the aqueous layer was concentrated to remove methanol. Distilled water was added to give 800 ml and then the aqueous layer was successively partitioned with ethyl acetate and 1-butanol. Because 1-butanol extract was the most active, the three antifungal principles were isolated from 1-butanol extract through repeated silica gel column chromatography.

The chemical structures of the three purified compounds isolated from Bombay mace were determined using spectroscopic analyses such as mass and NMR spectrometry. Mass spectra were recorded on a double-focusing high-resolution mass spectrometer (JMS-DX303, JEOL, Tokyo, Japan). NMR spectra were recorded on a Bruker AMX-500 (500 MHz) spectrophotometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) at 500 MHz for ^1H and 125 MHz for ^{13}C in deuterochloroform with TMS as an internal standard.

Growth-inhibition assay. The *in vitro* antifungal activity of the purified compounds was evaluated against mycelial growth of eight plant pathogenic fungi such as *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum coccodes*, *C. gloeosporioides*, *Fusarium oxysporum*, *Magnaporthe grisea*, *Pythium ultimum*, and *Rhizoctonia solani*. The three substances were dissolved in dimethylsulphoxide (DMSO). After autoclaving, potato dextrose agar medium was cooled to about 50°C and then mixed with each chemical solution. Aliquots (ca 20 ml) of the mixture were poured into sterile Petri dishes (8.5 cm ID). Final concentrations of the chemicals were 100, 33, 11, 3.7, 1.2 and 0.41 µg/ml. Control dishes were treated with DMSO (10 µl/ml) alone, and three replicates of each concentration for each fungus were used. After inoculation, the dishes were incubated at 25°C (all test fungi except *B. cinerea*) or 20°C (*B. cinerea*) for 2-5 days, and then radial growth was measured. This experiment was conducted twice and the activity was expressed as IC_{50} values (concentration of the compound inhibiting radial growth by 50%).

Evaluation of *in vivo* control efficacy. The 1-day protective activities of the methanol extract of Bombay mace and the purified substances were evaluated against seven plant pathogenic fungi on plants: *M. grisea* on rice plants, *R. solani* on rice plants, *B. cinerea* on tomato plants, *Phytophthora infestans* on tomato plants, *Puccinia recondita* on wheat plants, *Blumeria graminis* f. sp. *hordei* on barley plants, and *C. coccodes* on red pepper plants. The *in vivo* antifungal bioassays were performed as described previously (Kim et al., 2001; 2004). The pots were arranged in a randomized complete block design, with three replicates per treatment. All experiments for *in vivo* antifungal activities of the purified substances were conducted twice, and the six estimates for each treatment were converted into a control percentage (±standard deviation) compared with the control treatments.

In order to investigate further 7-day and 4-day protective activities against the plant pathogenic fungi, which were highly susceptible to the methanol extract of Bombay mace in the 1-day protective assay, foliar applications of the extract were done 7 and 4 days before inoculation. Blasticidin-S (50 µg/ml) for rice blast, dimethomorph (2 µg/ml) for tomato late blight, flusilazole (2 µg/ml) for wheat leaf rust, and dithianon (50 µg/ml) for red pepper anthracnose were applied as positive controls. Pots were arranged as a randomized complete block with three replications per treatment. The experiment was conducted twice and values are expressed as percentage control (±standard deviation) compared with the control.

Results and Discussion

The methanol extract of Bombay mace effectively suppressed the development of rice blast, tomato late blight, wheat leaf rust, and red pepper anthracnose among the seven plant diseases tested (Table 1 and Fig. 1). At 1,000 and 2,000 µg/ml, it exhibited potent *in vivo* antifungal activities with control values more than 67% against the four plant diseases. Even at the low concentration of 500 µg/ml, it controlled 79% for rice blast, 87% for tomato late blight, 60% for wheat leaf rust, and 58% for red pepper anthracnose. However, the methanol extract was virtually inactive to rice sheath blight, tomato gray mold, and barley powdery mildew.

The methanol extract of Bobmbay mace at 2,000 µg/ml showed control activity of more than 70% in 4- and 1-day protective applications, but 34% in a 7-day protective application; the protective activities of the methanol extract of Bobmbay mace were similar to those of blasticidin-S (50 µg/ml) (Fig. 2). The methanol extract of Bombay mace were highly active to wheat leaf rust with control values of more than 58% in protective applications. Although the methanol extract of Bombay mace showed efficacies more or less similar to positive controls against rice blast, tomato late blight, and wheat leaf rust, the former was much less

Table 1. One-day protective activity of the methanol extract of *Myristica malabarica* fruit rinds against seven phytopathogenic fungi[a]

Conc. (µg/ml)	Control value (%)[b]						
	RCB[c]	RSB	TGM	TLB	WLR	BPM	RPA
2,000	92±2.3	35±10	7±11	89±3.5	77±12	0	91±2.5
1,000	85±3.5	35±7.1	8±22	89±22	67±10	0	67±3.5
500	79±21	30±0	0	87±3.3	60±9.4	0	58±12

[a]Seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after spraying with solutions of the plant extracts.
[b]Each value represents the mean±standard deviation of two runs with three replicates each.
[c]RCB, rice blast (*Magnaporthe grisea*); RSB, rice sheath blight (*Rhizoctonia solani*); TGM, tomato gray mold (*Botrytis cinerea*); TLB, tomato late blight (*Phytophthora infestans*); WLR, wheat leaf rust (*Puccinia recondita*); BPM, barley powdery mildew (*Blumeria graminis* f. sp. *hordei*); RPA, red pepper anthracnose (*Colletotrichum coccodes*).

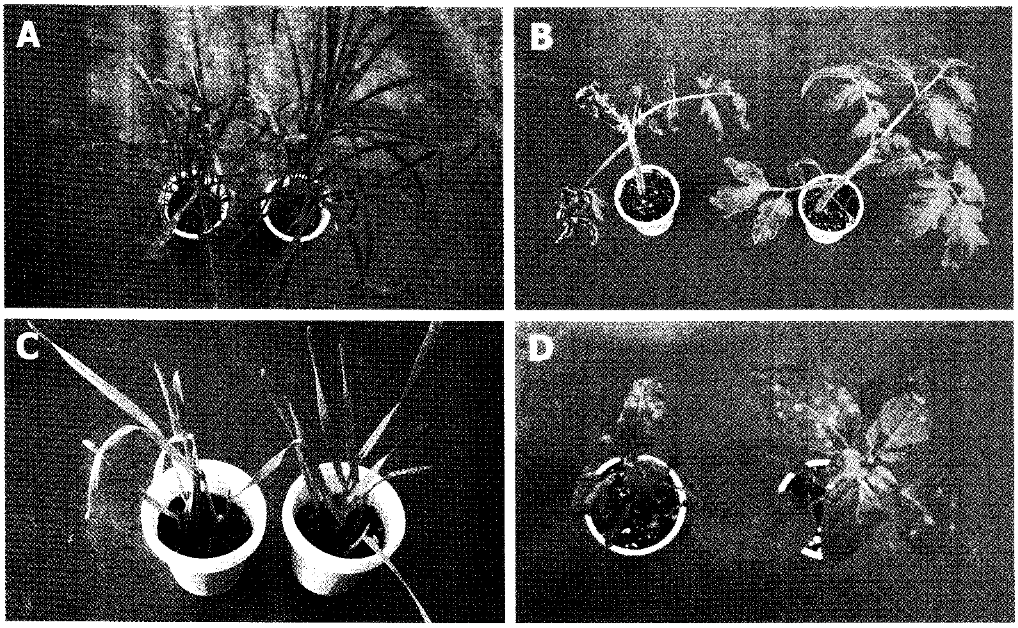


Fig. 1. *In vivo* antifungal activities of the methanol extract of *Myristica malabarica* fuit rinds against rice blast (A), tomato late blight (B), wheat leaf rust (C), and red pepper anthracnose (D). Plant seedlings were inoculated with spore suspensions of each pathogenic fungus 24 h after treatment with 2,000 μg/ml of the methanol extract of *M. malabarica* fruit rinds (right) or solvent alone (left).

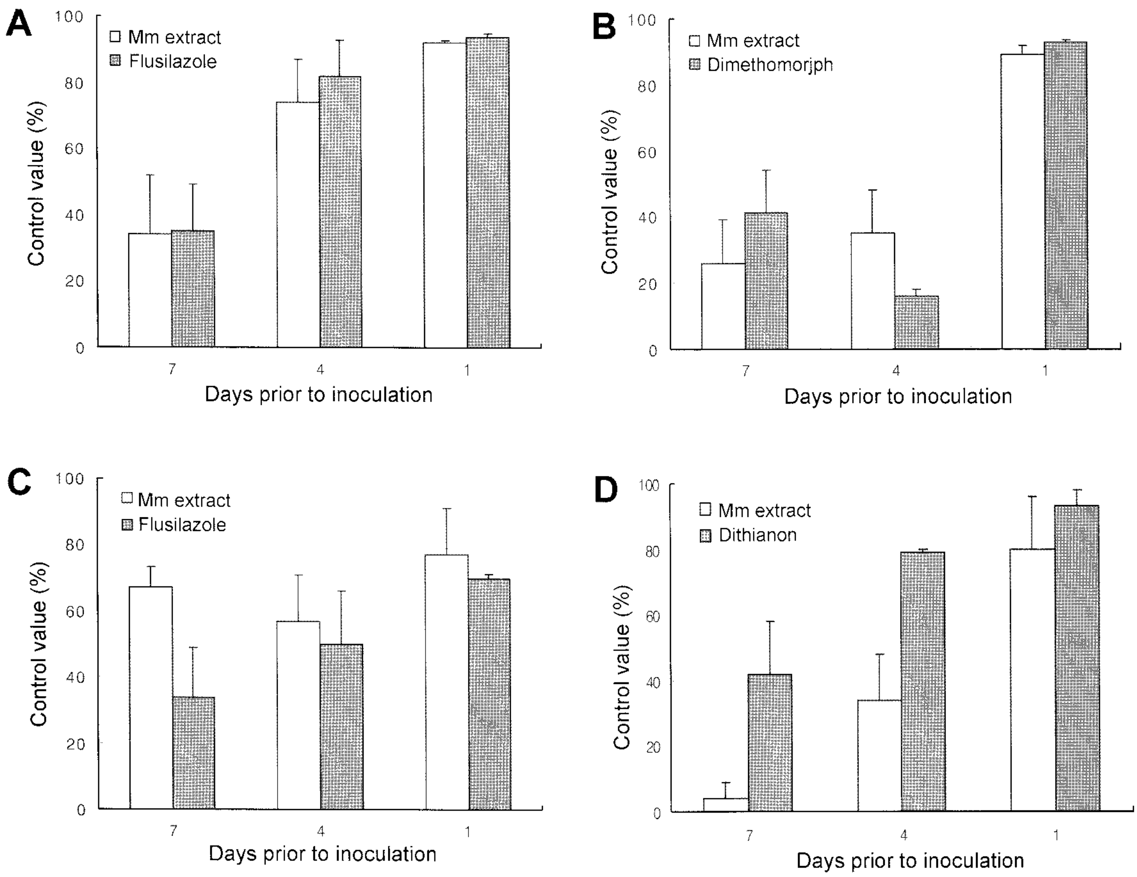
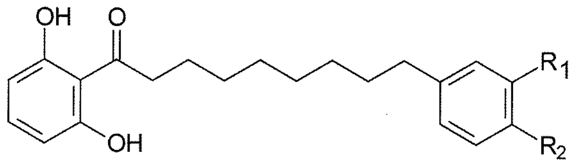


Fig. 2. *In vivo* control of rice blast (A), tomato late blight (B), wheat leaf rust (C), and red pepper anthracnose (D) with protective applications of the methanol extract of *Myristica malabarica* (Mm extract: methanol extract of *M. malabarica* fruit rinds (2,000 μg/ml). Blasticidin-S, dimethomorph, flusilazole, and dithianon were treated with 50, 2, 2, and 50 μg/ml, respectively.

effective to red pepper anthracnose than positive control, dithianon (50 μg/ml).

The three resorcinols were isolated from the methanol extract of Bombay mace by butanol partitioning and repeated silica gel column chromatography as reported previously (Choi et al., 2008). Their chemical structures were determined to be malabaricones A (MA), B (MB), and C (MC) by mass and NMR spectral data (Fig. 3).

The resorcinols have been found in various species of *Myristica* species including *M. malabarica* such as *M. cagayanesis*, *M. gigantean*, *M. maingayi*, and *M. gragrans* (Kuo et al., 1989; Orabi et al., 1991; Pharm et al., 2000; 2002; Sen et al., 2007). Malabaricones have been reported to have antioxidant, arg-gingipain inhibitory, antipromastigote, and nematicidal activities (Choi et al., 2008; Patro et al., 2005; Shinohara et al., 1999; Sen et al., 2007). As for the antimicrobial activity, Orabi et al. (1991) reported that both MB and MC exhibited a good level of antimicrobial activity when tested against a variety of microorganisms, including *Staphylococcus aureus* and *Candida albicans*; both metabolites showed minimum inhibitory concentrations at a range of 1-4 μg/ml against *S. aureus* and 4-32 μg/ml against *C. albicans*. The three malabaricones isolated from *My. malabarica* fruit rinds are similar to a number of 5-alkylresorcinols that were found to promote DNA strands scission (Scannell et al., 1988). Thus, the three malabaricones is supposed to exert their antifungal action through a similar mechanism. This is the first report on the antifungal activities of malabaricones against filamentous fungi.



Malabaricone A: R_1=R_2=H
Malabaricone B: R_1=H, R_2=OH
Malabaricone C: R_1=R_2=OH

Fig. 3. Chemical structures of malabaricones A, B, and C.

The resorcinols inhibited the mycelial growth of most of the plant pathogenic fungi tested (Table 2). Two *Colletotrichum* species such as *C. coccodes*, *C. gloeosporioides* and *R. solani* were relatively sensitive to the three resorcinols, whereas *B. cinerea* and *P. ultimum* were relatively resistant to them. *A. alternata* and *F. oxysporum* showed significantly different responses to each chemical.

In *in vivo* antifungal assays, all of the three resorcinols isolated from Bombay mace effectively suppressed the development of rice blast, wheat leaf rust, and red pepper

Table 2. IC_{50} values of three malabaricones isolated from fruit rinds of *Myristica malabarica* against eight plant pathogenic fungi

Plant pathogens	IC_{50} (μg/ml)[a]		
	MA[b]	MB	MC
Alternaria alternata	>100	22	100
Botrytis cinerea	>100	>100	NI
Colletotrichum coccodes	34	53	9.7
Colletotrichum gloeosporioides	39	17	11
Fusarium oxysporum	>100	86	33
Magnaporthe grisea	35	58	>100
Pythium ultimum	NI	84	NI
Rhizoctonia solani	71	36	87

[a]IC_{50}, concentration causing 50% growth inhibition.
[b]MA, malabaricone A; MB, malabaricone B; MC, malabaricone C
[c]NI, no inhibition

Table 3. One-day protective activity of three malabaricones isolated from fruit rinds of *Myristica malabaricones* against seven phytopathogenic fungi[a]

Chemical	Conc (μg/ml)	Control value (%)[b]						
		RCB[c]	RSB	TGM	TLB	WLR	BPM	RPA
	500	90±3.5	25±7.1	0	7±10	83±3.2	0	72±3.6
MA[d]	250	90± 14	15±1.1	0	0	75±12	0	64±7.2
	125	38±9.4	10±2.2	8±11	21±11	68±21	0	38±58
	500	94±0.9	20±2.2	15±47	29±10	90±9.4	42±59	92±3.6
MB	250	93±0	20±2.2	8±0	21±10	93±0	25±35	77±11
	125	85±14	5±1.1	0	21±10	88±7.1	8±12	74±7.2
	500	95±0	20±0	8±14	94±2.0	95±2.4	0	73±5.8
MC	250	85±14	10±0	23±12	64±10	93±0	0	54±7.2
	125	13±71	15±1.1	15±24	36±11	88±7.1	0	26±40

[a]Seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after spraying with the chemical solutions.
[b]Each value represents the mean standard deviation of two runs with three replicates each.
[c]RCB, rice blast (*Magnaporthe grisea*); RSB, rice sheath blight (*Rhizoctonia solani*); TGM, tomato gray mold (*Botrytis cinerea*), TLB; tomato late blight (*Phytophthora infestans*); WLR, wheat leaf rust (*Puccinia recondita*); BPM, barley powdery mildew (*Blumeria graminis* f. sp. *hordei*), RPA: red pepper anthracnose (*Colletotrichum coccodes*).
[d]MA, malabaricone A; MB, malabaricone B; MC, malabaricone C

anthracnose (Table 3). It is noticeable that only MC out of the three compounds showed potent *in vivo* antifungal activity against tomato late blight. They were not active virtually to rice sheath blight, tomato gray mold, and barley powdery mildew. The antifungal spectra of the three resorcinols were similar to that of the methanol extract of Bombay mace (Table 1), suggesting that the three resorcinols are mainly responsible for the antifungal activity of the extract of Bombay mace.

In this study, the methanol extract of Bombay mace and the three malabaricones purified from the plant extract showed potent antifungal activity against rice blast, tomato late blight, wheat leaf rust, and red pepper anthracnose. The metabolites also inhibited mycelial growth of pathogenic fungi. The metabolites may be used directly as fungicides or as lead molecules in the development of new fungicides. Even though Bombay mace is used for spices and medicinal purposes, the toxic effects to human beings and animals should be evaluated prior to commercial development. Phytotoxicity tests against various plants are also necessary. We are studying on establishment of optimal extraction process and formulation for the development of new natural fungicide by using Bombay mace.

Acknowledgement

This research was supported by a grant (PF06219-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of Korea.

References

Choi, N. H., Kwon, H. R., Son, S. W., Choi, G. J., Choi, Y. H., Jang, K. S., Lee, S. O., Choi, J. E., Ngoc, L. H. and Kim, J.-C. 2008. Nematicidal activity of malabaricones isolated from *Myristica malabarica* fruit rinds against *Bursaphelenchus xylophilus*. *Nematol.* (in press).

Khanom, F., Kayahara, H. and Tadasa, K. 2000. Superoxide-scavenging and prolyl endopeptidase inhibitory activities of Bangladeshi indigenous medicinal plants. *Biosci. Biotechnol. Biochem.* 64:837-840.

Kim, J.-C., Choi, G. J., Lee, S.-W., Kim, J.-S., Chung, K. Y. and Cho, K. Y. 2004. Screening for antifungal extracts against various plant pathogenic fungi and control of powdery mildew with extracts of *Achyranthes japonica* and *Rumex crispus*. *Pest Manag. Sci.* 60:803-808.

Kim, J.-C., Choi, G. J., Park, J.-H., Kim, H. T. and Cho, K. Y. 2001. Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica*. *Pest Manag. Sci.* 57:554-559.

Kuo Y.-H., Lin, S.-T. and Wu, R.-E. 1989. Three new lignans from the nutmeg of *Myristica cagayanesis*. *Chem. Pharm. Bull.* 37:2310-2312.

Morita, T., Jinno, K., Kawagishi, H., Arimoto, Y., Suganuma, H., Inakuma, T. and Sugiyama, K. 2003. Hepatoprotective effect of myristicin from nutmeg (*Myristica fragrans*) on lipopolysaccharide/D-galactosamine-induced liver injury. *J. Agric. Food Chem.* 12:1560-1565.

Orabi, K. Y., Mossa, J. S. and El-Feraly, F. S. 1991. Isolation and characterization of two antimicrobial agents from mace (*Myristica fragrans*). *J. Nat. Prod.* 54:856-859.

Patro, B. S., Bauri, A. K., Mishra, S. and Chattopadhyay, S. 2005. Antioxidant activity of *Myristica malabarica* extracts and their constituents. *J. Agric. Food Chem.* 53:6912-6918.

Pharm, V. C., Jossang, A., Sevenet, T. and Bodo, B. 2000. Cytotoxic acylphenols from *Myristica maingayi*. *Tetrahedron* 56: 1707-1713.

Pharm, V. C., Jossang, A., Sevenet, T. and Bodo, B. 2002. Novel cytotoxic acylphenol dimmers of *Myristica gigantean*; enzymatic systhesis of giganteones A and B. *Tetrahedron* 58:5709-5714.

Sen, R., Bauri, A. K., Chattopadyay, S. and Chatterjee, M. 2007. Antipromastigote activity of the malabaricones of *Myristica malabarica* (rampatri). *Phytother. Res.* 21:592-595.

Shinohara, C., Mori, S., Ando, T. and Tsuji, T. 1999. Arg-gingipain inhibition and anti-bacterial activity selective for *Porphyromonas gingivilis* by malabaricone C. *Biosci. Biotechnol. Biochem.* 63:1475-1477.

Scannell, R. T., Barr, J. R., Murty, V. S., Reddy, K. S. and Hecht, S. M. 1988. DNA strand scission by naturally occurring 5-alkylresorcinols. *J. Am. Chem. Soc.* 110:3650-3651.