Arch Pharm Res Vol 29, No 2, 140-144, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Cytotoxic Activity of Four Xanthones from *Emericella variecolor*, an Endophytic Fungus Isolated from *Croton oblongifolius*

Surachai Pornpakakul, Jatupol Liangsakul[1], Nattaya Ngamrojanavanich, Sophon Roengsumran, Prakitsin Sihanonth[2], Jittra Piapukiew[3], Ek Sangvichien[4], Songchan Puthong[5], and Amorn Petsom[5]

Research Centre for Bioorganic Chemistry, Department of Chemistry, [1]Program of Biotechnology, [2]Department of Microbiology, [3]Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, [4]Department of Biology, Faculty of Science, Ramkhamhang University, Bangkok 10240, Thailand, and [5]Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand

(Received May 18, 2005)

Four xanthones were isolated from mycelia of *Emericella variecolor*, an endophytic fungus isolated from the leaves of *Croton oblongifolius*. Their structures were elucidated by spectroscopic analysis to be shamixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone methanoate, and tajixanthone hydrate. All compounds were tested for cytotoxic activity against various human tumor cell lines including gastric carcinoma, colon carcinoma, breast carcinoma, human hepatocarcinoma, and lung carcinoma. The antitumor activities of these xanthones were compared with that of doxorubicin hydrochloride, a chemotherapeutic substance. All of them showed moderate activities and were selective against gastric carcinoma, colon carcinoma, and breast carcinoma. Only tajixanthone hydrate exhibited moderate activity against all cancer cell lines. Furthermore, under the test conditions it was found that 14-methoxytajixanthone-25-acetate and tajixanthone hydrate are almost as active as doxorubicin hydrochloride against gastric carcinoma (KATO3) and breast carcinoma (BT474).

Key words: *Emericella variecolor*, Shamixanthone, 14-Methoxytajixanthone-25-acetate, Tajixanthone methanoate, Tajixanthone hydrate, Cytotoxic activity, Endophyte

INTRODUCTION

Since their first discovery in 1904, the endophytes were isolated and subsequently examined (Tan and Zou, 2001). Bacteria and fungi are the most common microbes existing as endophytes. Endophytes have since been demonstrated to be a rich and reliable source of chemically novel compounds and/or biologically active compounds with potential medicinal or agricultural applications (Strobel, 2003; Strobel and Daisy, 2003). Some endophytes are even able to produce phytochemicals originally characteristic of their hosts. For example, the fungal endophyte *Taxomyces andreaanae* produces paclitaxel. This might be related to a genetic recombination of the endophyte with the host that occurs in evolutionary time (Tan and Zou, 2001). Thus, our research group has investigated an endophytic fungus that grows on Thai medicinal plants and produces biologically active compounds. In this work, we report the isolation and cytotoxicity of four xanthone derivatives from *Emericella variecolor* grown on leaves of *Croton oblongifolius* Roxb.

Correspondence to: Surachai Pornpakakul, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, THAILAND
Tel: 66-2-218-7637, Fax: 66-2-2187598
E-mail: psuracha@chula.ac.th

MATERIALS AND METHODS

General procedures

Specific optical rotations were measured with a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Perkin Elmer Lamda 25 UV-Vis spectrophotometer. MS spectra were measured with a Fisons Instruments Mass Spectrometer Model Trio 2000 in EI mode at 70 eV. ^{1}H- and ^{13}C- NMR were recorded with Varian Mercury+ 400 NMR spectrometer. Melting points were measured on an Electrothermal 9100 and were uncorrected.

Isolation and identification of microorganism

Emericella variecolor was obtained from mature petioles of *C. oblongifolius* Roxb., collected from Chachoengsao

Province, Thailand in June 2002. The fungus was isolated using modified Schulz's surface-sterilization technique (Schulz *et al.*, 1993). The surface-sterilized disks were placed on potato dextrose agar (PDA), incubated at room temperature and purified on PDA. During purification procedures, the strain was identified at genus level by means of light microscopy observation. *E. variecolor* was characterized by slide culture method and by ribosomal internal transcribed spacer (ITS) analysis.

Extraction and isolation

Seventy-five Erlenmeyer flaks (250 mL) containing malt extract (2 g) and water (100 mL per flask), were autoclaved twice at 121°C for 40 min. Pure culture of *E. variecolor* grown on PDA at room temperature for 7 days were cut into disks 8 mm in diameter. Two disks were transferred under sterile conditions into each Erlenmeyer flask and then statically incubated for 6 weeks at room temperature. The fermentation broth was filtered through Whatman no.1 filter paper.

The mycelium (253 g wet weight) was extracted with methanol (500 mL × 10) to yield crude a methanol extract that was a dark reddish solid (13.95 g). The dark reddish solid was re-extracted with ethyl acetate (500 mL × 10) to give 5.86 g of crude ethyl acetate extract. This extract was then separated by silica gel column chromatography (230-400 mesh, 100 g) and eluted with an *n*-hexane-ethyl acetate mixture with stepwise increasing polarity. A total of 600 fractions of 100 mL each were collected and combined on the basis of TLC profile. UV light and vanillin/H_2SO_4/EtOH reagent were used as detecting methods. Fraction A (131 mg), obtained from 10% ethyl acetate in hexane as an orange solid, was isolated by preparative thin layer chromatography using 10% EtOAc in hexane as mobile phase. This step was then followed by crystallization from $CHCl_3$ and hexane to give xanthone **1** (22 mg). Fraction B (154 mg) was obtained from Hexane-EtOAc (80:20) and crystallized from $CHCl_3$-Et_2O-Hexane to yield xanthone **2** (75 mg). Fraction C (154 mg) was obtained from Hexane-EtOAc (75:25) and was crystallized from $CHCl_3$-Et_2O-Hexane to yield xanthone **3** (120 mg). Fraction D (281 mg) was obtained from Hexane-EtOAc (60:40) and was crystallized from $CHCl_3$-Et_2O-Hexane to yield xanthone **4** (135 mg).

Shamixanthone (1)

Yellow needle crystals, m.p. 139-140°C; $[\alpha]_D^{20}$ +16° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$): λ_{max} (ε) 240 (19325), 259 (8932), 270 (11368), 278 (12992), 300 (3654), and 397 (2030) nm; IR ν_{max} (KBr) cm^{-1}: 3447 (OH), 2921, 2851, 1734 (C=O), 1645, 1567, 1478, 1419, 1240, 1116, 1022, and 820; EIMS *m/z*: 406 [M^+, 20%], 388 (11), 375 (13), 363 (25), 337 (53), 310 (34), 295 (100), 279 (12), 255 (15), 242 (20), and 67 (24); ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 1.75 (3H, s, CH_3-17), 1.79 (3H, s, CH_3-18), 1.85 (3H, s, CH_3-23), 2.36 (3H, s, CH_3-24), 2.73 (1H, s, H-20), 3.49 (1H, d, *J* = 5.5 Hz, H-14*a*), 3.50 (1H, d, *J* = 6.0 Hz, H-14*b*), 4.34 (1H, dd, *J* = 2.8, 10.8 Hz, H-19b), 4.43 (1H, dd, *J* = 2.8, 11.2 Hz, H-19a), 4.58 (1H, s, H-22b), 4.80 (1H, s, H-22a), 5.09 (1H, s, 25-OH), 5.31 (1H, dd, *J* = 7.2, 7.6 Hz, H-15), 5.41 (1H, s, H-25), 6.74 (1H, d, *J* = 8.4 Hz, H-2), 7.30 (1H, s, H-5), 7.44 (1H, d, *J* = 8.4 Hz), 12.60 (1H, s, 1-OH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C (ppm): 184.5 (C-13), 159.7 (C-1), 152.8 (C-10), 152.3 (C-11), 149.4 (C-7), 142.6 (C-21), 138.4 (C-6), 136.6 (C-3), 133.3 (C-16), 121.7 (C-15), 120.9 (C-8), 119.4 (C-5), 118.9 (C-9), 116.7 (C-12), 112.3 (C-22), 109.8 (C-2), 109.2 (C-4), 64.6 (C-19), 63.2 (C-25), 44.9 (C-20), 27.5 (C-14), 25.8 (C-17), 22.6 (C-23), 17.9 (C-18), 17.5 (C-24).

14-Methoxytajixanthone-25-acetate (2)

Yellow needle crystals, m.p. 219-220°C; $[\alpha]_D^{20}$ -38° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$): λ_{max} (ε) 387 (6916), 295 (9880), 274 (15413), 267 (30628), 253 (31616), and 237 (57304) nm; IR ν_{max} (KBr) cm^{-1}: 3447 (OH), 2921, 1746 (C=O), 1637, 1559, 1470, 1423, 1369, 1236, 1077, 1018, and 828 cm^{-1}; EIMS *m/z* 494 [M^+, 8%], 451 (6), 434 (16), 423 (16), 363 (100), 347 (12), 333 (14), 307 (10), and 293 (8); ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 1.23 (3H, s, CH_3-17), 1.31 (3H, s, CH_3-18), 1.89 (3H, s, CH_3-23), 2.08 (3H, s, OAc-25), 2.35 (3H, s, CH_3-24), 2.72 (1H, s, H-20), 3.17 (1H, d, *J* = 8.0 Hz, H-15), 4.31 (1H, dd, *J* = 3.2, 11.2 Hz, H-19b), 4.55 (1H, d, *J* = 11.2 Hz, H-19a), 4.63, (1H, d, *J* = 8.0 Hz, H-14b), 4.76 (1H, s, H-22b), 4.81 (1H, s, H-22a), 6.83 (1H, d, *J* = 8.4 Hz, H-2), 6.90 (1H, s, H-25), 7.26 (1H, s, H-5), 7.66 (1H, d, *J* = 8.4 Hz, H-3), 13.14 (1H, s, 1-OH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C (ppm):183.2 (C-13), 170.0 (-O*C*CH_3), 162.2 (C-1), 152.5 (C-10), 151.6 (C-11), 150.3 (C-7), 141.4 (C-21), 138.0 (C-6), 135.1 (C-3), 120.3 (C-5), 116.2 (C-12), 115.5 (C-9), 114.9 (C-8), 112.8 (C-22), 110.8 (C-2), 109.1 (C-4), 76.1 (C-14), 66.7 (C-15), 65.5 (C-25), 63.8 (C-19), 57.8 (C-16), 42.4 (C-20), 24.8 (C-17), 22.4 (C-23), 21.3 (-OC*C*H_3), 19.8 (C-18), 17.4 (C-24).

Tajixanthone methanoate (3)

Yellow needle crystals, m.p. 197-198°C; $[\alpha]_D^{20}$ -105° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$): λ_{max} (ε) 399 (10170), 300 (18527), 278 (72640), 268 (61744), 258 (49032), and 238 (99880) nm; IR ν_{max} (KBr) cm^{-1}: 3447 (OH), 2968, 2925, 1766 (C=O), 1641, 1567, 1466, 1477, 1345, 1240, 1190, 1092 and 1049 (C-O-C), 820 and 765 cm^{-1}; EI MS *m/z* 454 [M^+, 11%), 424 (10), 385 (10), 363 (11), 333 (28), 255 (11), and 73 (100); ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 1.28 (3H, s, CH_3-17), 1.32 (3H, s, CH_3-18), 1.84 (3H, s, CH_3-23), 2.35 (3H, s, CH_3-24), 2.69 (1H, dd, *J* = 10.4, 14.0 Hz, H-14b), 2.73 (1H, d, *J* = 1.0 Hz, H-20), 3.12 (1H, dd, *J* = 1.6,

14.0 Hz, H-14a), 3.29 (3H, s, 14-OCH_3), 3.77 (1H, d, *J* = 10.0 Hz, H-15), 4.34 (1H, dd, *J* = 3.2, 11.2 Hz, H-19b), 4.42 (1H, dd, *J* = 3.6, 11.2 Hz, H-19a), 4.57 (1H, s, H-22b), 4.80 (1H, s, H-22a), 5.06 (1H, d, *J* = 4.0 Hz, 25-OH), 5.40 (1H, s, H-25), 6.78 (1H, d, *J* = 8.4 Hz, H-2), 7.22 (1H, s, H-5), 7.57 (1H, d, *J* = 8.4 Hz, H-3), 13.14 (1H, s, 1-OH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C (ppm): 184.4 (C-13), 160.2 (C-1), 153.0 (C-10), 152.0 (C-11), 149.5 (C-7), 142.5 (C-21), 138.3 (C-6), 138.2 (C-3), 121.0 (C-8), 119.1 (C-5), 116.9 (C-12), 116.8 (C-9), 112.3 (C-22), 109.9 (C-2), 109.2 (C-4), 77.3 (C-16), 76.5 (C-15), 64.5 (C-19), 63.2 (C-25), 49.2 (-OCH_3), 44.9 (C-20), 31.2 (C-14), 22.5 (C-23), 20.9 (C-18), 19.2 (C-17), 17.4 (C-24).

Tajixanthone hydrate (4)

Yellow needle crystals, m.p. 194-195°C; $[\alpha]_D^{20}$ -76° (*c* 0.23, $CHCl_3$); UV ($CHCl_3$): λ_{max} (ε) 237 (45760), 257 (17248), 268 (29920), 278 (24640), 300 (6160), and 399 (2640) nm; IR ν_{max} (KBr) cm^{-1}: 3486 (OH), 3073, 2976, 2883, 1797 and 1738 (C=O), 1645, 1571, 1474, 1345, 1244, 1046 and 1026 (C-O-C), 898 and 820 cm^{-1}; EI MS *m/z* 440 [M^+, 14%], 409 (14), 398 (8), 371 (44), 333 (100), 283 (46), 271 (22), 255 (56), 242 (8), 225 (56), and 59 (39); ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 1.34 (3H, s, CH_3-17), 1.39 (3H, s, CH_3-18), 1.82 (3H, s, CH_3-23), 2.28 (3H, s, CH_3-24), 2.37 (1H, s, 16-OH), 2.44 (1H, s, 15-OH), 2.63 (1H, dd, *J* = 10.8, 14.0 Hz, H-14b), 2.69 (1H, s, H-20), 3.16 (1H, dd, *J* = 1.2, 14.0 Hz, H-14a), 3.70 (1H, d, *J* = 10.8 Hz, H-15), 4.31 (1H, dd, *J* = 2.8, 10.8 Hz, H-19b), 4.41 (1H, dd, *J* = 2.0, 10.8 Hz, H-19a), 4.53 (1H, s, H-22b), 4.77 (1H, s, H-22a), 4.98 (1H, d, *J* = 4.0 Hz, 25-OH), 5.34 (1H, s, H-25), 6.72 (1H, d, *J* = 8.4 Hz, H-2), 7.19 (1H, s, H-5), 7.49 (1H, d, *J* = 8.4 Hz, H-3), 12.54 (1H, s, 1-OH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C (ppm): 184.3 (C-13), 160.3 (C-1), 153.1 (C-10), 151.9 (C-11), 149.5 (C-7), 142.5 (C-21), 138.5 (C-6), 138.3 (C-3), 120.8 (C-8), 119.1 (C-5), 116.8 (C-12), 116.3 (C-9), 112.3 (C-22), 109.9 (C-2), 109.2 (C-4), 77.7 (C-15), 72.9 (C-16), 64.5 (C-19), 63.2 (C-25), 44.8 (C-20), 32.0 (C-14), 26.5 (C-17), 23.6 (C-18), 22.6 (C-23), 17.4 (C-24).

Cytotoxic assays by MTT method

Bioassay of cytotoxicity against human cell cultures *in vitro* was performed by the MTT [3-(4,5)-dimethylthiazol-2,5-diphenyltetrazolium bromide] colorimetric method (Twentyman *et al.*, 1987; Carmichael *et al.*, 1987). Each isolate was evaluated for cytotoxicity against gastric carcinoma (KATO3), colon carcinoma (SW620), breast carcinoma (BT474), human hepatocarcinoma (HEP-G2), and lung carcinoma (CHAGO). Doxorubicin hydrochloride was used as a positive control.

RESULTS AND DISCUSSION

An endophytic fungus, *E. variecolor*, was isolated from leaves of *C. oblongifolius* using modified Schulz's surface-sterilization technique (Schulz *et al.*, 1993). It was identified by slide culture method and the ribosomal internal transcribed spacer (ITS) analysis. When nucleotide sequences on ITS regions were compared with data collected in the GenBank database, it was found to be similar to *E. variecolor* with 96.064% identity (99.261% ungrapped). From a static culture of *E. variecolor* in MEB, we isolated four known xanthones (**1-4**), previously reported by Chexal *et al.* (1974, 1975).

The structures of **1-4** (Fig. 1) were assembled through 1D and 2D NMR including COSY, HSQC, HMBC, and NOESY experiments. Coupling constant (J^a 1 Hz) of H-20

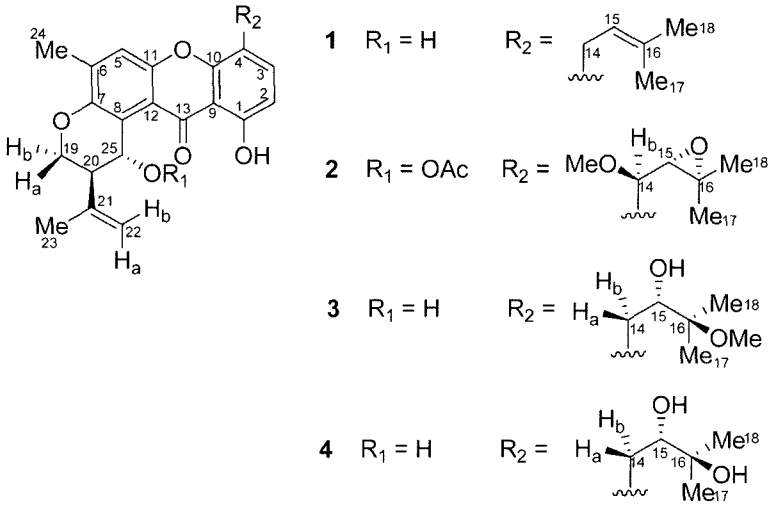

Fig. 1. Structure of xanthones

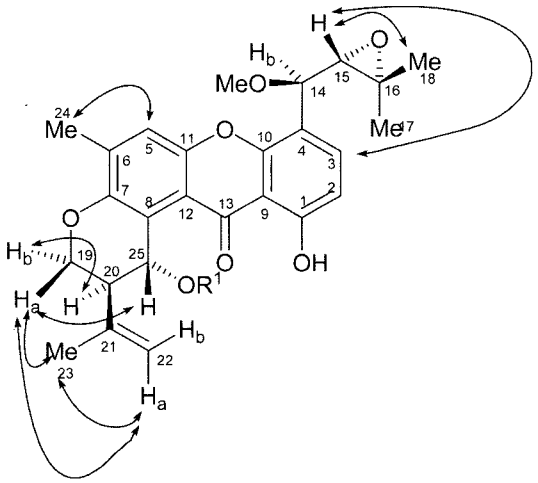

Fig. 2. Selected NOESY correlation

Table I. Cytotoxicity data of compounds **1-4**[a]

Compounds	Cell lines[b]				
	KATO3	SW620	BT474	HEP-G2	CHAGO
1	6.1 (15.0)	8.7 (21.4)	5.1 (12.5)	>10	>10
2	5.7 (11.5)	7.1 (14.4)	6.0 (12.1)	8.7 (17.6)	>10
3	9.1 (20.0)	8.7 (19.2)	6.4 (14.1)	>10	7.8 (17.2)
4	4.8 (10.9)	6.0 (13.6)	5.4 (12.3)	7.2 (16.4)	5.1 (11.6)
Doxorubicin HCl	5.9 (10.17)	0.4 (0.69)	6.2 (10.7)	0.8 (1.38)	2.6 (4.48)

[a]Results are expressed as IC_{50} in mg/mL (mM).
[b]KATO-3, human gastric carcinoma ATCC No. HTB 103
SW620, human colon adenocarcinoma ATCC No. CCL 227
BT474, human breast ductol carcinoma ATCC No. HTB20
HEP-G2, human liver hepatoblastoma ATCC No. HB 8056
CHAGO, human undifferentiated lung carcinoma

and H-25 indicated that the stereochemistry of **1-4** at C-20 and C-25 were similar. Coupling constant (J = 8.0 Hz) of H-14 and H-15 of **2** revealed alignment of both protons at approximately 180 degrees. The stereochemistry of C-14 was assigned to R configuration and C-15 was assigned to S configuration (Chexal *et al.,* 1974). Additionally the NOESY spectrum of **2** (Fig. 2) showed a correlation from H-15 to H-3 and Me-18, from Me-24 to H-5, from H_a-19 to H_b-22, Me-23 and H-25, from H_b-19 to H-20 and from Me-23 to H_a-22 and H-25, thus providing assignment of stereochemistry at 14-, 15-, 20-, and 25-position. On the basis of the coupling constants, COSY, NOESY, HSQC, and HMBC data of xanthone **2**, the stereochemistry of **1**, **3**, and **4** were therefore established as illustrated in Fig. 1.

By comparing their NMR data to that reported in the literature (Chexal *et al.*, 1974, 1975) the four compounds were identified as follows: 1 = shamixanthone, 2 = 14-methoxytajixanthone-25-acetate, 3 = tajixanthone methanoate, and 4= tajixanthone hydrate.

Compounds **1-4** were tested for their cytotoxicities against human tumor cell lines. Doxorubicin hydrochloride, a chemotherapeutic substance in clinical use having a flat aromatic moiety similar to xanthones, was used as a positive control. As seen in Table I, compounds **1-4** showed moderate activities. Only compound **4** exhibited activity against all cell lines including gastric carcinoma (KATO3), colon carcinoma (SW620), breast carcinoma (BT474), human hepatocarcinoma (HEP-G2), and lung carcinoma (CHAGO). Compounds **1-3** showed selectivity against KATO3, SW620, and BT474. Furthermore, under the test conditions, it was found that compounds **2** and **4** are almost as active as doxorubicin hydrochloride against gastric carcinoma (KATO 3) and breast carcinoma (BT474). Thus, this is the first report of cytotoxicity of these compounds. It is interesting to note that varixanthone, a structurally-related xanthone isolated from a marine-derived strain of *E. variecolor,* does not show any cytotoxicity at 1 μg/mL. However, it displays antimicrobial potency against Gram- positive and Gram-negative bacteria (Malmstrøm *et al.*, 2002).

ACKNOWLEDGEMENTS

We are thankful to the Office of the National Research Council of Thailand and the Graduate School and Department of Chemistry, Faculty of Science, Chulalongkorn University for the financial support. The Rachadapisek-sompoj Endowment, Chulalongkorn University are also gratefully acknowledged.

REFERENCES

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, B., Evaluation of a tetrazolium based semiautomated colorimetric assay; assessment of chemosensitivity testing. *Cancer Res.*, 47, 936-942 (1987).

Chexal, K. K., Fouweather, C., Holker, J. S. E., Simpson, T. J., and Young, K., The biosynthesis of fungal metobolites. Part III Structures and biosynthesis of some minor metabolites from variant strains of *Aspergillus variecolor. J. Chem. Soc. Perkin Trans. I*, 1584-1593 (1974).

Chexal, K. K., Holker, J. S. E., and Simpson, T. J., The biosynthesis of fungal metabolites. Part VI Structures and biosynthesis of some minor metabolites from variant strains of *Aspergillus variecolor. J. Chem. Soc. Perkin Trans. I*, 549-554 (1975).

Malmstrøm, J., Christophersen, C., Barrero, A.F., Oltra, J.E., Justicia, J., and Rosales, A., Bioactive metabolites from a marine-derived strain of the fungus *Emericella variecolor. J. Nat. Prod.*, 65, 364-367 (2002).

Schulz, B., Wanke, U., Draeger, S., and Aust, H.-J., Endophytes

from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol. Res.*, 97, 1447-1450 (1993).

Strobel, G. A., Endophytes as source of bioactive products. *Microbes Infect.*, 5, 535-544 (2003).

Strobel, G. A. and Daisy, B., Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiol. Mol. Biol. Rev.*, 64, 491-502 (2003).

Tan, R. X. and Zou, W. X., Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.*, 18, 448-459 (2001).

Twentyman, P. R. and Luscombe, M., A study of some variables in a tetrazolium dyme (MTT) based assay for growth and chemosensitivity. *Br. J. Cancer*, 56, 279-285 (1987).