J Nat Med
DOI 10.1007/s11418-013-0743-6

ORIGINAL PAPER

Antiviral activity of diarylheptanoid stereoisomers against respiratory syncytial virus in vitro and in vivo

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Received: 7 November 2012 / Accepted: 6 January 2013

Abstract We previously showed that (5*S*)-5-hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0011**) and (5*S*)-5-methoxy-1,7-diphenylhept-3-one (**AO-0016**) isolated from *Alpinia officinarum* exhibited stronger anti-influenza virus activity and anti-respiratory syncytial virus (RSV) activity, respectively, than the other isolated diarylheptanoids. In this study, we synthesized an enantiomer (**AO-0503**) and racemate (**AO-0504**) of **AO-0011** and an enantiomer (**AO-0514**) of **AO-0016**. The anti-RSV activities of the three stereoisomers (**AO-0503**, **AO-0504**, and **AO-0514**) and **AO-0011** were examined in vitro and in vivo to evaluate the stereoisomeric effect on anti-RSV activity. In a plaque reduction assay using human epidermoid carcinoma cells, all four diarylheptanoids significantly exhibited anti-RSV activity, and **AO-0514** and **AO-0016** exhibited stronger anti-RSV activity than **AO-0503**, **AO-0504**, and **AO-0011**. In a murine RSV infection model, all four diarylheptanoids with anti-RSV activity in vitro were also significantly effective in reducing virus titers in the lungs of RSV-infected mice. In the histopathological analysis of RSV-infected lungs, the oral administration of even **AO-0514**, which showed the lowest reduction of virus titers in the lungs, was significantly effective in reducing the infiltration of lymphocytes and in reducing the interferon-γ level, which is a marker of severity of pneumonia due to RSV infection, in bronchoalveolar lavage fluids prepared from RSV-infected mice. Although the stereoisomeric effects of diarylheptanoids on anti-RSV activity varied moderately, all four diarylheptanoids examined were suggested to ameliorate pneumonia and have a potential anti-RSV activity in vivo. They are possibly mother compounds for the development of an anti-RSV drug in the future.

Keywords *Alpinia officinarum* · Diarylheptanoid · RSV · Antiviral · Stereoisomer · Synthesis of diarylheptanoids

Introduction

Respiratory syncytial virus (RSV) is a common and worldwide pathogen, and RSV infection is a highly significant cause of bronchiolitis and pneumonia [1–3]. The infection becomes a serious cause of deadly disease not only in infants but also in

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Published online: 24 January 2013

the elderly and high-risk patients such as immunocompromised or transplanted patients [4–6]. Ribavirin has been used for the treatment of RSV infection, and palivizumab and motavizumab have been used prophylactically [7–11]. Although new candidates for anti-RSV drugs such as a small interference RNA drug (ALN-RSV01), fusion inhibitor (TMC 35311), and RNA polymerase inhibitor (RSV604) [12–14] are in development and clinical trials, there are few specific anti-RSV drugs that are effective clinically and practically.

Alpinia officinarum (*A. officinarum*), family Zingiberaceae, has been widely cultivated in Asia from ancient times and is well known as lesser galangal. Its rhizome is used in various Asian cuisines and as an important traditional medicine in Asia. Diarylheptanoids isolated from *A. officinarum* [15–20] have been shown to exhibit various bioactivities in inhibiting tumor promotion [15], inducing apoptosis [16], anti-inflammatory, anti-nociceptive, and anti-psychiatric effects [17], melanogenesis [20], and promoting an antioxidant effect [18, 19]. Recently, we showed the first evidence demonstrating the anti-influenza virus activity of eleven diarylheptanoids isolated from *A. officinarum* in vitro. Among them, influenza virus was more susceptible to (5*S*)-5-hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0011**) than to the others [21]. Influenza virus and RSV are members of the Orthomyxoviridae and Paramyxoviridae, respectively, and have similar characteristics, such as an envelope and negative-sense single-stranded RNA. Thus, **AO-0011** may be also effective against RSV. On the other hand, we demonstrated that (5*S*)-5-methoxy-1,7-diphenylhept-3-one (**AO-0016**) showed anti-RSV activity in vitro and its therapeutic index (>6.1) was the highest of nine diarylheptanoids isolated from *A. officinarum* [22]. Thus, **AO-0011** and **AO-0016** are possible candidates for mother compounds to develop anti-RSV compounds.

In this study, we synthesized the stereoisomers of **AO-0011** and **AO-0016** to evaluate the stereoisomeric effect of diarylheptanoids on anti-RSV activity and examined their anti-RSV activity in vitro and in vivo. We found that all synthesized stereoisomers of diarylheptanoids, including **AO-0011**, significantly exhibited potent anti-RSV activity in vitro and in vivo, although the anti-RSV activity of the stereoisomers varied moderately. They are suggested to be mother compounds for the development of anti-RSV drugs. The relationships between the structure and anti-RSV activity of the stereoisomers are discussed.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) and ribavirin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. **AO-0011** and **AO-0016** were isolated from the rhizome of *A. officinarum* as described previously [21, 22], and their chemical structures are given in Fig. 1. The purities of AO-0011 and AO-0016 were validated at more than 95 % by ^{1}H-NMR.

Synthesis of diarylheptanoid stereoisomers

(5*R*)-5-Hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0503**) and (5*RS*)-5-hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0504**) were synthesized as an enantiomer and racemate, respectively, of **AO-0011** (Fig. 1). (5*R*)-5-Methoxy-1,7-diphenylhept-3-one (**AO-0514**) was synthesized as an enantiomer of **AO-0016** (Fig. 1).

Each reaction with air- and moisture-sensitive components was performed under an argon atmosphere in a flame-dried reaction flask. Reaction solvents used were anhydrous grade (using solvent supply systems) tetrahydrofuran (THF) and dichloromethane (CH_2Cl_2) purchased from Kanto Chemical Co. Inc. Silica gel (F254) was used for all analytical thin layer chromatography. Flash column chromatography was carried out using Kanto silica gel 60 N (spherical, neutral, 40–50 μm), containing 0.5 % fluorescence reagent 254 and a quartz column. ^{1}H-NMR spectra were recorded at 600 MHz and ^{13}C-NMR spectra were recorded at 150 MHz, as solutions in deuteriochloroform ($CDCl_3$), on a JEOL ECA-600. Chemical shifts are reported in ppm downfield from TMS. Mass spectra were obtained on a JEOL JMS-GCMATE.

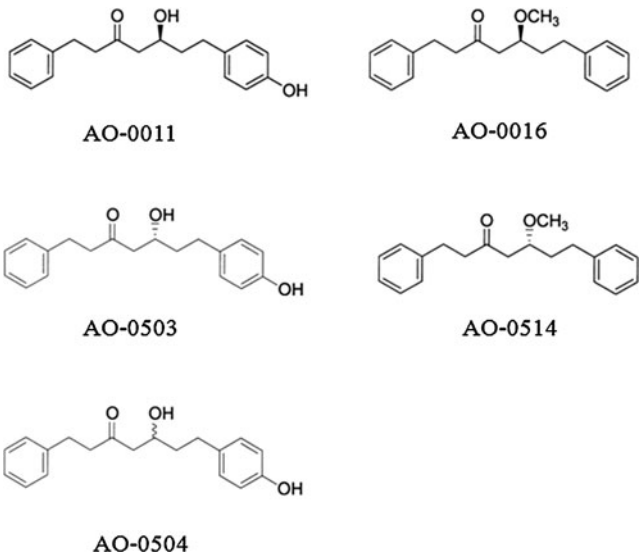

Fig. 1 Chemical structures of diarylheptanoids. Chemical structures of **AO-0011** and **AO-0016** are cited from reference [21] and [22], respectively

Cells and viruses

Human epidermoid carcinoma (HEp-2) cells (CCL-23, American Type Culture Collection, Rockville, MD, USA) were purchased from Dainippon Pharmaceutical (Osaka, Japan) and grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with heat-inactivated 10 % and 2 % fetal calf serum. The A2 strain of RSV was obtained from American Type Culture Collection and grown in HEp-2 cell cultures.

Animals

Female BALB/c mice (6 weeks old, 19–21 g) were purchased from Kyudo Animal Laboratory (Kumamoto, Japan). The mice were housed in groups of five per cage with food and water ad libitum under a 12-h light/dark diurnal cycle (light at 7:30 a.m.). The temperature in the room was kept at 23 ± 2 °C. The mice were acclimated for at least 5 days before starting experimental procedures. Animal studies followed the animal experimentation guidelines of Kyushu University of Health and Welfare (Nobeoka, Japan) and were carried out in an approved biosafety level facility there.

Antiviral and cytotoxic assays

The anti-RSV activity of diarylheptanoids was examined by a plaque reduction assay using HEp-2 cells [23]. Briefly, HEp-2 cells grown in 24-well plates were infected with 100 plaque-forming units (PFU)/0.2 ml of RSV at 37 °C for 1 h. The cells were overlaid with 1 ml of maintenance EMEM containing 0.8 % methylcellulose and various concentrations of diarylheptanoids or ribavirin, and maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C for 4–5 days. The infected cells were fixed and stained, and the number of plaques was counted. All diarylheptanoids were dissolved in DMSO and diluted with culture medium to make the various final concentrations. The concentration of DMSO in each medium was less than 1 %. Ribavirin was dissolved in DMSO and used as a control. The 50 % effective antiviral concentration (EC_{50}) was the concentration that reduced virus-induced cell destruction by 50 %, as described previously [23].

The cytotoxicity of diarylheptanoids was assessed by a Trypan blue exclusion test using mock-infected HEp-2 cells. Cells were seeded at a concentration of 5×10^4 cells/ml in 24-well plates and cultured at 37 °C for 24 h. The culture medium was then replaced with fresh medium containing diarylheptanoids at various concentrations, and the cells were further incubated for 48 h. The cells were trypsinized, and the number of viable cells was determined by a Trypan blue exclusion test. The 50 % cytotoxic concentration (CC_{50}) was determined as the concentration that reduced cell destruction by 50 % [24].

Experimental murine RSV infection

Experimental RSV infection was performed as reported previously [25]. Briefly, 6-week-old female mice were infected intranasally with 1×10^6 PFU per 0.1 ml of A2 strain of RSV under anesthesia. Diarylheptanoids (**AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514**) dissolved in 1 % DMSO were administered orally to mice by gavage at 30 mg/kg of body weight once at 4 h prior to, once at 1 h after, and once again at 6 h after virus infection on day 0, and three times daily from day 1 to day 3 after infection. A 1 % DMSO solution was used as a control. The mice were weighed daily from day 0 to day 4 through the experiment. On day 4 after infection, the lungs were removed for virus titration, immediately frozen in liquid N_2, and stored at −80 °C. Frozen lungs were homogenized with cold quartz sand in a homogenizer, and viral titers in the supernatants of the homogenates were measured by a plaque assay. For the measurement of interferon (IFN)-γ levels in bronchoalveolar lavage fluid (BALF) of mice, BALF was obtained from the mice under anesthesia by instilling 1.0 ml of cold phosphate-buffered saline into the lungs and aspirating it from the trachea using a tracheal cannula [26]. Ice-cold BALF was centrifuged at 100*g* at 4 °C for 10 min. The supernatant was stored at −80 °C until use.

Histopathological examination

For histopathological examination of the infected lungs, RSV-infected mice were killed on day 4 after infection, and the lungs were removed and placed in buffered formalin for a minimum of 24 h. The tissue was then embedded in low-melting-point paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (HE).

Pathological changes (hemorrhage, infiltration of neutrophils, hyperplasia of macrophages, infiltration of lymphocytes, edema, degeneration of tracheal epithelium, formation of hyaline membrane, and bacterial flora) in tissue sections were scored as 0, negative; 1, minimal; 2, mild; 3, moderate; 4, severe. The means of each pathological change between **AO-0514**-administered mice and control mice were compared statistically.

ELISA

IFN-γ levels in BALF were measured using a specific ELISA kit (Ready-set-go, eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions.

This product was tested and found to conform to all eBioscience Inc. quality control release specifications. The lower limit of detection sensitivity in the kit is 4 pg/ml for IFN-γ. The intra- and inter-assay coefficients of variation for the ELISA were less than 10 %.

Statistical analysis

Statistical significances of differences between the EC_{50} and CC_{50} values, and in virus titers, scores of pathological changes, and IFN-γ levels were evaluated using Student's *t* test. A *P* value of 0.05 or less was considered to be statistically significant.

Results

Synthesis of diarylheptanoid stereoisomers

As shown in Fig. 2, racemic syntheses of allylic alcohols **4a** and **4b**, as well as β-hydroxyketo **AO-0504** were carried as follows. Preparation of **4a** was initiated by lithiation of 4-phenyl-1-butyne **1** by *n*-BuLi, followed by dropwise addition of 3-phenylpropanal **2** to form alkynyl alcohol **3**. Reduction of **3** by Red-Al afforded the desired allylic alcohol **4a**.

4b and **AO-0504** were synthesized from β-hydroxyketone **7** as a common starting material, which was prepared by aldol reaction between 4-phenyl-2-butanone **5** and arylpropioaldehyde **6** (R=OTBDPS). TBAF-mediated deprotection of **7** gave racemic β-hydroketo **AO-0504**. Alternatively, dehydration of **7** with TsOH furnished the enone product **8**, which was subjected to Luche reduction to give the allylic alcohol **4b** [27].

Optically active β-methoxyketo **AO-0514** and β-hydroxyketo **AO-0503** were synthesized from β-hydroxyketones **4a** (R=H) and **4b** (R=OTBDPS) according to the following procedures. The Sharpless asymmetric epoxidation of **4a** or **4b** gave enantio-enriched anti-epoxy alcohols **9** through kinetic resolution [28], followed by Dess–Martin oxidation to afford epoxy ketones **10**. Treatment of **10** with diphenyldiselenide under reductive conditions gave the enantio-enriched β-hydroxy products **11**. Finally, β-methoxyketo **11a** was obtained by treating **AO-0514** (R=H) with MeOTf and 2,6-DTBP; deprotection of **11b** (R=OTBDPS) by TBAF yielded β-hydroxyketo **AO-0503**. Detailed spectroscopic data of the synthesized compounds can be obtained from us by request. The synthesized **AO-0503**, **AO-0504**, and **AO-0514** were

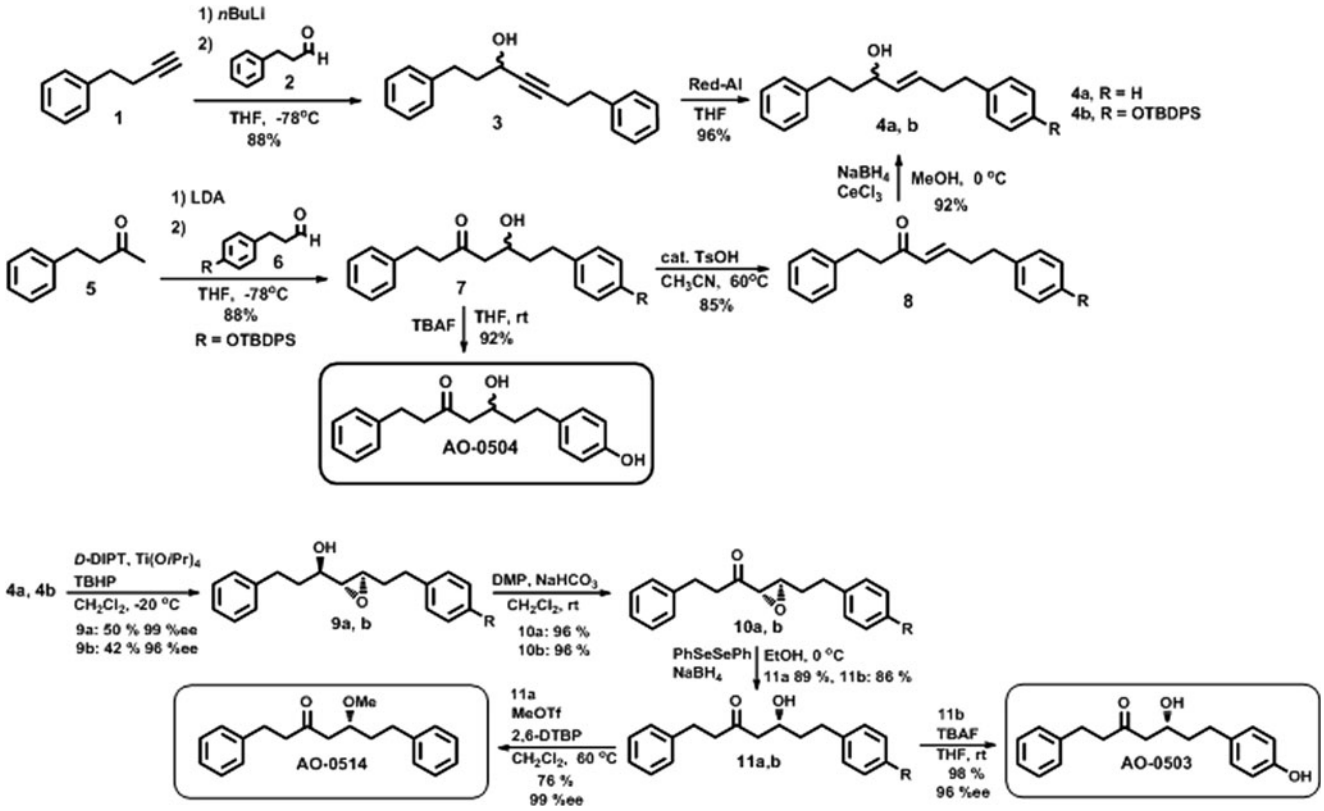

Fig. 2 Synthesis of diarylheptanoid analogues. *DIPT* diisopropyltartrate, *DMP* Dess–Martin periodinane, *2,6-DTBP* 2,6-di-*tert*-butylpyridine, *TBAF* *tert*-butylammonium fluoride, *TBDPS* *tert*-butyldiphenylsilyl, *TBHP* *tert*-butylhydroperoxide. The enantiomeric excess values of **AO-0503** and **AO-0514** were determined by HPLC equipped with chiral phase supported column (OD-3)

identified as follows. The purity of AO-0503, AO-0504, and AO-0514 were validated as more than 99 % by ^{1}H-NMR.

*(5R)-5-Hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0503**)*

$[\alpha]_D = -0.34°$ ($c = 2.30$, acetone); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.29–7.26 (m, 2 H), 7.20–7.16 (m, 3 H), 7.03 (d, 2 H, $J = 8.6$ Hz), 6.74 (d, 2 H, $J = 8.6$ Hz), 5.04 (s, 1 H), 4.05–4.02 (m, 1 H), 3.10 (m, 1 H), 2.90–2.87 (m, 2 H), 2.76–2.68 (m, 3 H), 2.62–2.51 (m, 3 H), 1.80–1.74 (m, 1 H), 1.64–1.62 ppm (m, 2 H); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 211.3, 153.8, 140.6, 133.8, 129.5, 128.6, 128.3, 126.2, 115.2, 66.9, 49.2, 45.0, 38.2, 30.8, 29.5 ppm; HRMS (EI) *m/z*: 298.15672 (calcd for $C_{19}H_{22}O_3$: 298.15688).

*(5RS)-5-Hydroxy-7-(4-hydroxyphenyl)-1-phenylhapt-3-one (**AO-0504**)*

^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.29–7.26 (m, 2 H), 7.20–7.16 (m, 3 H), 7.03 (d, 2 H, $J = 8.6$ Hz), 6.74 (d, 2 H, $J = 8.6$ Hz), 5.04 (s, 1 H), 4.05–4.02 (m, 1 H), 3.10 (m, 1 H), 2.90–2.87 (m, 2 H), 2.76–2.68 (m, 3 H), 2.62–2.51 (m, 3 H), 1.80–1.74 (m, 1 H), 1.64–1.62 ppm (m, 2 H); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 211.3, 153.8, 140.6, 133.8, 129.5, 128.6, 128.3, 126.2, 115.2, 66.9, 49.2, 45.0, 38.2, 30.8, 29.5 ppm; HRMS (EI) *m/z*: 298.15672 (calcd for $C_{19}H_{22}O_3$: 298.15688).

*(5R)-5-Methoxy-1,7-diphenylhept-3-one (**AO-0514**)*

$[\alpha]_D = -10.2°$ ($c = 0.33$; $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.28–7.26 (m, 4 H), 7.19–7.16 (m, 6 H), 3.73–3.68 (m, 1 H), 3.30 (s, 3 H), 2.90–2.88 (m, 2 H), 2.80–2.60 (m, 5 H), 2.47–2.43 (m, 1 H), 1.82–1.74 ppm (m, 2 H); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 208.5, 141.9, 141.0, 128.5, 128.4, 126.1, 125.9, 76.7, 57.0, 47.4, 45.4, 35.7, 31.4, 29.5 ppm; HRMS (EI) *m/z*: 296.17803 (calcd for $C_{20}H_{24}O_2$: 296.17762).

Anti-RSV activity of diarylheptanoids in vitro

Four diarylheptanoids (**AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514**, Fig. 1) were examined for their anti-RSV activity and cytotoxicity in vitro. **AO-0011** has been reported to have anti-influenza virus activity [21] but its anti-RSV activity was unknown. As shown in Table 1, the EC_{50} values of **AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514** were 40.7 ± 3.5, 44.7 ± 1.5, 24.3 ± 0.6, and 7.0 ± 1.4 μg/ml, respectively, and the EC_{50} values were significantly lower than their CC_{50} values (85.5 ± 26.2, 56.8 ± 6.4, 82.5 ± 28.6, and >100 μg/ml, respectively). All diarylheptanoids examined exhibited anti-RSV activity in vitro. The diarylheptanoids (**AO-0011**, **AO-0503**, and **AO-0504**) are stereoisomers and their CC_{50}/EC_{50} values were similar (2.1, 1.3, and 3.4, respectively). However, the CC_{50}/EC_{50} values of **AO-0016**, which have been reported by Konno et al. [22] and are given in Table 1, and **AO-0514** (6.1 and 14.3, respectively) were higher than those of **AO-0011**, **AO-0503**, and **AO-0504**. In particular, the EC_{50} value of **AO-0514** was much lower than that of **AO-0016,** and the CC_{50}/EC_{50} value (>14.3) of **AO-0514** was more than double that of **AO-0016**. DMSO at 1 %, which was used as a maximum concentration to dissolve diarylheptanoids in the culture medium, was not cytotoxic. In this assay, the EC_{50} value of ribavirin, used as a positive control, was similar to the results reported previously [29, 30]. **AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514** all exhibited anti-RSV activity in vitro, and, of them, **AO-0514** seemed to be most effective.

Anti-RSV activity of diarylheptanoids in mice

To assess the potential anti-RSV activity of the diarylheptanoids in vivo, virus titers in the lungs of infected mice

Table 1 Anti-RSV activity and cytotoxicity of diarylheptanoids

Compounds	EC_{50}^a (μg/ml)	CC_{50}^a (μg/ml)	CC_{50}/EC_{50}
(5*S*)-5-Hydroxy-7-(4-hydroxyphenyl)-1-phenylhept-3-one (**AO-0011**)[d]	40.7 ± 3.5[b]	85.5 ± 26.2	2.1
(5*R*)-5-Hydroxy-7-(4″-hydroxyphenyl) -1-phenylhept-3-one (**AO-0503**)	44.7 ± 1.5[b]	56.8 ± 6.4	1.3
(5*RS*)-5-Hydroxy-7-(4″-hydroxyphenyl) -1-phenylhept-3-one (**AO-0504**)	24.3 ± 0.6[b]	82.5 ± 28.6	3.4
(5*S*)-5-Methoxy-1,7-diphenylhept-3-one (**AO-0016**)[d]	16.3 ± 3.5[b,e]	>100[e]	6.1[e]
(5*R*)-5-Methoxy-1,7-diphenylhept-3-one (**AO-0514**)	7.0 ± 1.4[b]	>100	14.3
Ribavirin	0.67 ± 0.08	ND[c]	ND[c]

[a] Mean ± SD of four independent experiments

[b] $P < 0.05$ versus CC_{50} by Student's *t* test

[c] Not done

[d] **AO-0011** and **AO-0016** are reported by reference [21] and [22], respectively

[e] EC_{50}, CC_{50}, and CC_{50}/EC_{50} values are cited from reference [22]

Table 2 RSV titers of lungs of infected mice

Compounds	Titer (log_{10}PFU/ml)[a] ± SD	% of control
Experiment 1		
Control[c]	5.31 ± 0.15	100
AO-0011[b]	3.97 ± 0.33[d]	4.6
AO-0503[b]	4.45 ± 0.30[d]	13.8
AO-0504[b]	4.81 ± 0.20[d]	31.6
Experiment 2		
Control[c]	4.02 ± 0.08	100
AO-0514[b]	3.87 ± 0.13[d]	69.6

Experiments 1 and 2 were performed independently

[a] Mean ± SD for five mice

[b] Diarylheptanoids were administered daily at 30 mg/kg/mouse

[c] 1 % DMSO solution was administered daily as the control

[d] $P < 0.05$ versus control by Student's t test

administered diarylheptanoids at 30 mg/kg and 1 % DMSO as a control were compared on day 4 after infection. As shown in Table 2, **AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514** were significantly effective in reducing the virus titers of lungs of infected mice compared with the controls ($P < 0.05$). There were no significant differences between the mean ± SE of body weights of mice administered **AO-0011**, **AO-0503**, and **AO-0504** (20.2 ± 0.4, 19.5 ± 0.3, 19.4 ± 0.4 g, respectively, in experiment 1) and the control (18.2 ± 0.9 g in experiment 1) on day 4 after infection, and **AO-0514** (20.1 ± 0.4 g in experiment 2) and the control (20.4 ± 0.4 g in experiment 2). All four diarylheptanoids exhibited potential anti-RSV activity in mice.

Histopathological analysis of lungs of infected mice

The virus titers in lungs of RSV-infected mice were reduced to 4.6, 13.8, 31.6, and 69.6 % of the controls by the oral administration of **AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514**, respectively (Table 2). **AO-0514** showed the weakest antiviral activity in the reduction of virus titers of lungs. To evaluate whether the oral administration of **AO-0514** reflected the alleviation of histopathological damage of lungs, we histopathologically analyzed the lungs of infected mice administered **AO-0514**. Figure 3 shows representative HE-stained images of lungs of mice administered **AO-0514** or 1 % DMSO solution as a control. **AO-0514** administration markedly reduced infiltration of immune cells. As shown in Table 3, the hemorrhage, inflammation of neutrophils, hyperplasia of macrophages, infiltration of lymphocytes, and edema scores in control infected mice were higher than those of mice administered **AO-0514**. Administration of **AO-0514** was especially significantly effective in reducing infiltration of lymphocytes. Pathological changes such as edema, degeneration of

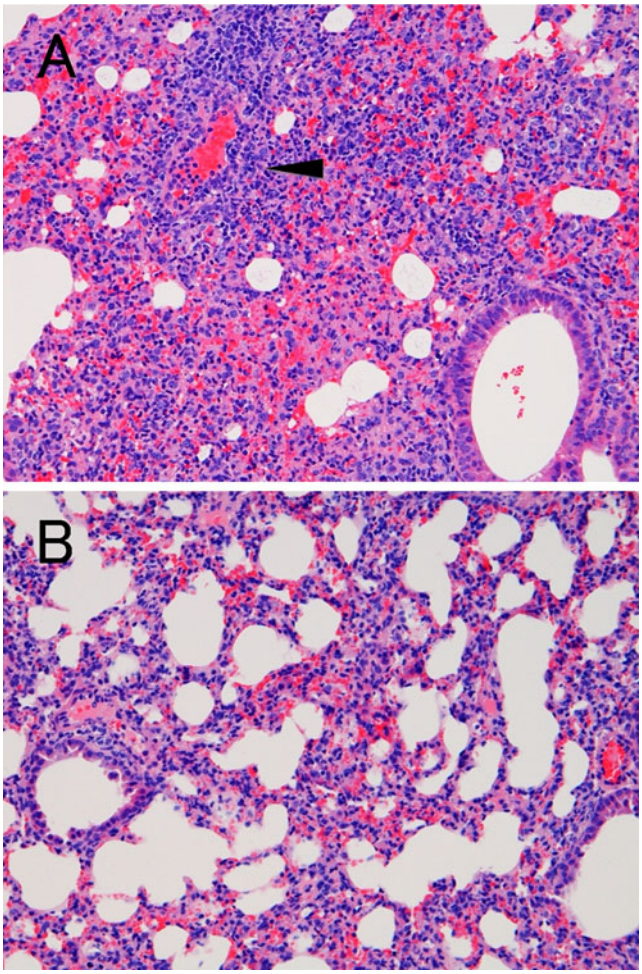

Fig. 3 Lungs of RSV-infected mice at 4 days after infection. Five mice per group were used. Representative HE-stained images (×140) are shown. The *arrowhead* indicates infiltration of lymphocytes and hemorrhage. **a** Control mouse with RSV infection. **b** **AO-0514**-administered mouse with RSV infection

Table 3 Histopathological change scores

Histopathological changes	Score[a] ± SD	
	Control[c]	AO-0514[b]
Hemorrhage	1.6 ± 1.8	0.8 ± 0.8
Infiltration of neutrophils	1.6 ± 0.9	1.2 ± 0.4
Hyperplasia of macrophages	1.6 ± 0.9	0.8 ± 0.8
Infiltration of lymphocytes	1.4 ± 0.5	0.6 ± 0.5[d]
Edema	0.4 ± 0.9	0
Degeneration of tracheal epithelium	0	0
Formation of hyaline membrane	0	0
Bacterial flora	0	0

[a] Mean ± SD for five mice

[b] Diarylheptanoids were administered daily at 30 mg/kg/mouse

[c] 1 % DMSO solution was administered daily as the control

[d] $P < 0.05$ versus control by Student's t test

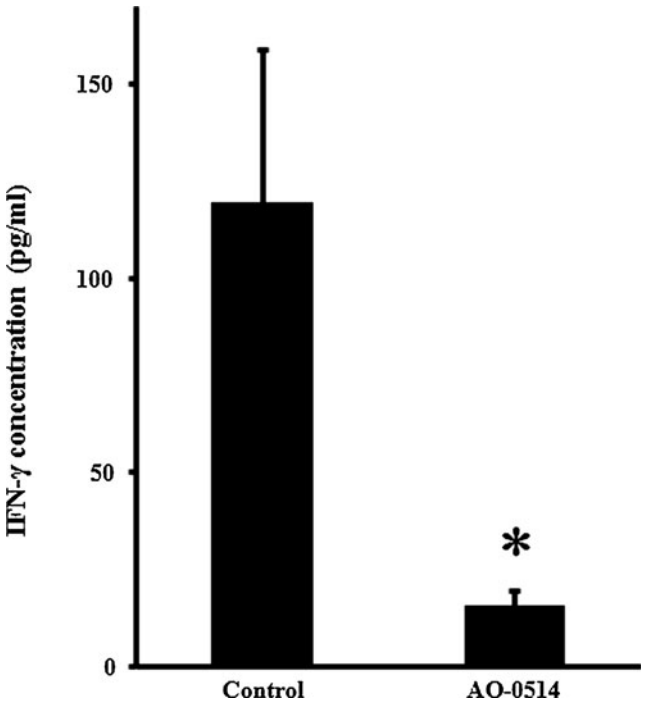

Fig. 4 Concentration of IFN-γ in BALF of RSV-infected mice. BALF was obtained from 5 mice per group at 4 days after infection. **AO-0514** at 30 mg/kg and 1 % DMSO solution as the control were administered orally to RSV-infected mice. The IFN-γ levels were expressed as the mean ± SD in each group represented. $*P < 0.05$ versus control by Student's *t* test

tracheal epithelium, formation of hyaline membrane, and bacterial flora were hardly observed in both groups.

It has been reported that the IFN-γ level in BALF of RSV-infected mice increases greatly and is a marker of severity of pneumonia due to RSV infection [31]. We compared the IFN-γ levels in BALF of RSV-infected mice administered **AO-0514** and 1 % DMSO solution as a control. As shown in Fig. 4, the oral administration of **AO-0514** significantly reduced the IFN-γ levels in BALF of RSV-infected mice ($P < 0.05$). Thus, although **AO-0514** was not very effective in reducing virus titers in the lungs of infected mice as compared with the other three diarylheptanoids, **AO-0514** administration was found to ameliorate the histopathological damage to lungs by RSV infection.

Discussion

We have previously reported that two diarylheptanoids (**AO-0011** and **AO-0016**) isolated from *A. officinarum* exhibited effective anti-influenza virus and anti-RSV activity, respectively, in vitro [21, 22]. In this study, we synthesized **AO-0503** and **AO-0504** as the enantiomer and racemate, respectively, of **AO-0011**, and **AO-0514** as the enantiomer of **AO-0016** to evaluate stereoisomeric effects on anti-RSV activity. The synthesized diarylheptanoids (**AO-0503**, **AO-0504**, and **AO-0514**) including **AO-0011** were examined for their anti-RSV activity in vitro and in vivo. All four diarylheptanoids examined exhibited significant anti-RSV activity in vitro and in vivo, although the anti-RSV activity of the four diarylheptanoids varied moderately. Thus, these diarylheptanoids may be candidates for mother compounds for the development of anti-RSV drugs.

Diarylheptanoids **AO-0011** and **AO-0503** are enantiomers of the hydroxyl group and **AO-0504** is the racemate. In Table 1, based on stoichiometric composition of enantiomers in a racemate, the EC_{50} value (24.3 ± 0.6 μg/ml) of **AO-0504** did not reflect the additive value of enantiomers **AO-0011** (40.7 ± 3.5 μg/ml) and **AO-0503** (44.7 ± 1.5 μg/ml). The CC_{50} value (82.5 ± 28.6 μg/ml) of **AO-0504** did not reflect the additive value of enantiomers **AO-0011** (85.5 ± 26.2 μg/ml) and **AO-0503** (56.8 ± 6.4 μg/ml), either. The biological activity including antiviral activity or cytotoxicity of racemate has been reported to not always reflect the additive activity of the enantiomers [32–34]. It would be interesting to analyze their differences to obtain the better anti-RSV diarylheptanoids. The CC_{50}/EC_{50} values of **AO-0011**, **AO-0503,** and **AO-0504** (2.1, 1.3, and 3.4, respectively) were lower than those (6.1 [22] and 14.3) of **AO-0016** and **AO-0514,** which are enantiomers of the methoxy group (Fig. 1). A methoxy group at position 5 of diarylheptanoids may be necessary to produce stronger anti-RSV activity than a hydroxyl group. In the former enantiomers and racemate (**AO-0011**, **AO-0503**, and **AO-0504**), anti-RSV activity in vitro was not greatly influenced by the stereoisomeric differences of the hydroxyl group. However, in the latter enantiomers (**AO-0016** and **AO-0514**), the R form (**AO-0514**) showed markedly stronger anti-RSV activity than the S form (**AO-0016**). Thus, the stereoisometric effect of a methoxy group at position 5 on anti-RSV activity may be larger than that of a hydroxyl group. The CC_{50} values (>100 μg/ml) of **AO-0016** and **AO-514** were higher than those (56.8–85.5 μg/ml) of **AO-0011**, **AO-0503**, and **AO-0504**. In our previous report [22], the cytotoxicity of (5*S*)-5-methoxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone, which has a hydroxyl group at position 4″, was shown to be higher than that of **AO-0016**, without that group. The existence of a hydroxyl group in diarylheptanoids may reflect cytotoxicity rather than anti-RSV activity. The methoxy group at position 5 of diarylheptanoids is possibly indispensable to provide effective anti-RSV activity in vitro.

In the murine RSV infection model, all four diarylheptanoids examined significantly exhibited anti-RSV activity in mice. Among them, diarylheptanoids (**AO-0011**,

AO-0503, and **AO-0504**) with a hydroxyl group were more effective in reducing virus titers (4.6, 13.8, and 31.6 %, respectively, of control) in lungs than **AO-0514** with a methoxy group (69.6 % of control) (Table 2). This is inconsistent with the in-vitro results that diarylheptanoids with a hydroxyl group were less effective than a diarylheptanoid with a methoxy group. In our murine RSV infection model, diarylheptanoids were administered orally. Thus, the direct anti-RSV action of diarylheptanoids on cells in vitro might not reflect in-vivo results. A study of the mode of antiviral action in vivo and their biological *availability* would be necessary to analyze the inconsistency. All diarylheptanoids examined were administered at 30 mg/kg and no weight loss of mice was observed compared with control mice. Therefore, the four diarylheptanoids examined were shown to be effective against RSV infection in mice without toxicity.

In the histopathological analysis of RSV-infected lungs, the oral administration of **AO-0514** obviously ameliorated pneumonia caused by the infiltration of immune cells such as lymphocytes and neutrophils and hemorrhage due to RSV infection on day 4 post-infection (Table 3; Fig 3). It has been reported that the level of IFN-γ in the BALF of mice is a representative marker of pneumonia development due to RSV infection [23, 25]. In this study, the oral administration of **AO-0514** significantly reduced the level of IFN-γ in BALF of RSV-infected mice (Fig. 4). These results indicate that **AO-0514** ameliorated pneumonia. As shown in Table 2, oral administration of **AO-0514** effectively reduced the virus titer to 69.6 % of control. Such a reduction of the RSV virus titer in lungs might be enough to ameliorate pneumonia. Because **AO-0514** has the weakest anti-RSV activity in vivo (Table 2), the other three diarylheptanoids (**AO-0011**, **AO-0503**, and **AO-0504**) examined would be expected to be more effective in ameliorating pneumonia. Thus, all four diarylheptanoids (**AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514**) were found to be effective compounds against RSV not only in vitro and but also in vivo. Although further studies analyzing the relationships between structure and antiviral activity of diarylheptanoids are needed, diarylheptanoids **AO-0011**, **AO-0503**, **AO-0504**, and **AO-0514** are probably useful as promising candidates for the development of anti-RSV drugs in the future.

Acknowledgments We thank Dr. Tomomi Takeshita, Ms. Akane Hino, and Ms. Yukiko Shimoda for their excellent technical assistance and Ms. Katherine Ono for her editorial assistance. We would also like to thank Dr. Kouichi Metori (Analytical Center, School of Pharmacy, Nihon University) for performing mass spectroscopy. This study was partly supported by a Grant-in-Aid for Scientific Research (No. 24790182) from the Japan Society for the Promotion of Science and Health and Labor Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labor and Welfare of Japan.

References

1. Leader S, Kohlhase K (2003) Recent trends in severe respiratory syncytial virus (RSV) among US infants, 1997 to 2000. J Pediatrics 143:127–132
2. Chi H, Chang IS, Tsai FY, Huang LM, Shao PL, Chiu NC, Chang LY, Huang FY (2011) Epidemiological study of hospitalization associated with respiratory syncytial virus infection in Taiwanese children between 2004 and 2007. J Form Med Assoc 110: 388–396
3. Rodríguez-Auad JP, Nava-Frías M, Casasola-Flores J, Johnson KM, Nava-Ruiz A, Pérez-Robles V, Caniza MA (2012) The epidemiology and clinical characteristics of respiratory syncytial virus infection in children at a public pediatric referral hospital in Mexico. Int J Inf Dis 16:508–513
4. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE (2005) Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med 352:1749–1759
5. Villarruel GR, Langley GE, Abedi GR, Anderson LJ (2010) Respiratory syncytial virus activity—United States, July 2008–December 2009. MMWR Morb Mortal Wkly Rep 59:230–233
6. Shah JN, Chemaly RF (2011) Management of RSV infections in adult recipients of hematopoietic stem cell transplantation. Blood 117:2755–2763
7. Weisman LE (2009) Respiratory syncytial virus (RSV) prevention and treatment: past, present, and future. Cardiovasc Hematol Agents Med Chem 7:223–233
8. Pelaez A, Lyon GM, Force SD, Ramirez AM, Neujahr DC, Foster M, Naik PM, Gal AA, Mitchell PO, Lawrence EC (2009) Efficacy of oral ribavirin in lung transplant patients with respiratory syncytial virus lower respiratory tract infection. J Heart Lung Transpl 28:67–71
9. Cingoz O (2009) Motavizumab. MAbs 1:439–442
10. Gill MA, Welliver RC (2009) Motavizumab for the prevention of respiratory syncytial virus infection in infants. Expert Opin Biol Ther 9:1335–1345
11. Fernández P, Trenholme A, Abarca K, Griffin MP, Hultquist M, Harris B, Losonsky GA (2010) A phase 2, randomized, double-blind safety and pharmacokinetic assessment of respiratory syncytial virus (RSV) prophylaxis with motavizmab and palivizumab administered in the same season. BMC Pediatrics 10:38
12. Zamora MR, Budev M, Rolfe M, Gottlieb J, Humar A, Devincenzo J, Vaishnaw A, Cehelsky J, Albert G, Nochur S, Gollob JA, Glanville AR (2011) RNA interference therapy in lung transplant patients infected with respiratory syncytial virus. Am J Respir Crit Care Med 183:531–538
13. Rouan MC, Gevers T, Roymans D, de Zwart L, Nauwelaers D, De Meulder M, van Remoortere P, Vanstockem M, Koul A, Simmen K, Andries K (2010) Pharmacokinetics-pharmacodynamics of a respiratory syncytial virus fusion inhibitor in the cotton rat model. Antimicrob Agents Chemother 54:4534–4539
14. Chapman J, Abbott E, Alber DG, Baxter RC, Bithell SK, Henderson EA, Carter MC, Chambers P, Chubb A, Cockerill GS, Collins PL, Dowdell VC, Keegan SJ, Kelsey RD, Lockyer MJ, Luongo C, Najarro P, Pickles RJ, Simmonds M, Taylor D, Tyms S, Wilson LJ, Powell KL (2007) RSV604, a novel inhibitor of respiratory syncytial virus replication. Antimicrob Agents Chemother 51:3346–3353
15. Yasukawa K, Sun Y, Kitanaka S, Tomizawa N, Miura M, Motohashi S (2008) Inhibitory effect of the rhizomes of *Alpinia officinarum* on TPA-induced inflammation and tumor promotion in two-stage carcinogenesis in mouse skin. J Nat Med 62:374–378
16. Tabata K, Yamazaki Y, Okada M, Fukumura K, Shimada A, Sun Y, Yasukawa K, Suzuki T (2009) Diarylheptanoids derived from *Alpinia officinarum* induce apoptosis, S-phase arrest and

differentiation in human neuroblastoma cells. Anticancer Res 29:4981–4988
17. Lee J, Kim KA, Jeong S, Lee S, Park HJ, Kim NJ, Lim S (2009) Anti-inflammatory, anti-nociceptive, and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. J Ethnopharmacol 126:258–264
18. Ly TN, Shimoyamada M, Kato K, Yamauchi R (2003) Isolation and characterization of some antioxidative compounds from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). J Agric Food Chem 51:4924–4929
19. Ly TN, Shimoyamada M, Kato K, Yamauchi R (2004) Antioxidative compounds isolated from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). BioFactors 21:305–308
20. Matsuda H, Nakashima S, Oda Y, Nakamura S, Yoshikawa M (2009) Melanogenesis inhibitors from the rhizomes of *Alpinia officinarum* in B16 melanoma cells. Bioorg Med Chem 17: 6048–6053
21. Sawamura R, Sun Y, Yasukawa K, Shimizu T, Watanabe W, Kurokawa M (2010) Antiviral activities of diarylheptanoids against influenza virus in vitro. J Nat Med 64:117–120
22. Konno K, Sawamura R, Sun Y, Yasukawa K, Shimizu T, Watanabe W, Kato M, Yamamoto R, Kurokawa M (2011) Antiviral activities of diarylheptanoids isolated from *Alpinia officinarum* against respiratory syncytial virus, poliovirus, measles virus, and herpes simplex virus type 1 in vitro. Nat Prod Commun 6:1881–1884
23. Watanabe W, Shimizu T, Hino A, Kurokawa M (2008) A new assay system for evaluation of developmental immunotoxicity of chemical compounds using respiratory syncytial virus infection to offspring mice. Environ Toxicol Pharmacol 25:69–74
24. Watanabe W, Konno K, Ijichi K, Inoue H, Yokota T, Shigeta S (1994) MTT colorimetric assay system for the screening of anti-orthomyxo- and anti-paramyxoviral agents. J Virol Methods 48: 257–265
25. Watanabe W, Shimizu T, Hino A, Kurokawa M (2008) Effects of decabrominated diphenyl ether (DBDE) on developmental immunotoxicity in offspring mice. Environ Toxicol Pharmacol 26:315–319
26. Sawamura R, Shimizu T, Sun Y, Yasukawa K, Miura M, Toriyama M, Motohashi S, Watanabe W, Konno K, Kurokawa M (2010) In vitro and in vivo anti-influenza virus activity of diarylheptanoids isolated from *Alpinia officinarum*. Antivir Chem Chemother 21:33–41
27. Gemal LA, Luche LJ (1981) Lanthanoids in organic synthesis. 6. The reduction of α-enones by sodium borohydride in the presence of lanthanoid chlorides: synthetic and mechanistic aspects. J Am Chem Soc 103:5454–5459
28. Katsuki T, Sharpless KB (1980) The first practical method for asymmetric epoxidation. J Am Chem Soc 102:5974–5976
29. Xie YY, Zhao XD, Jiang LP, Liu HL, Wang LJ, Fang P, Shen KL, Xie ZD, Wu YP, Yang XQ (2006) Inhibition of respiratory syncytial virus in cultured cells by nucleocapsid gene targeted deoxyribozyme (DNAzyme). Antiviral Res 71:31–41
30. Kawana F, Shigeta S, Hosoya M, Suzuki H, De Clercq E (1978) Inhibitory effects of antiviral compounds on respiratory syncytial virus replication in vitro. Antimicrob Agents Chemother 31: 1225–1230
31. Watanabe W, Shimizu T, Sawamura R, Hino A, Konno K, Hirose A, Kurokawa M (2010) Effects of tetrabromobisphenol A, a brominated flame retardant, on the immune response to respiratory syncytial virus infection in mice. Int Immunopharmacol 10:393–397
32. Comin MJ, Vu BC, Boyer PL, Liao C, Hughes SH, Marquez VE (2008) D-(+)-iso-methanocarbathymidine: a high-affinity substrate for herpes simplex virus 1 thymidine kinase. ChemMedChem 3:1129–1134
33. Barber-Rotenberg JS, Selvarnathran SP, Kong Y, Erkizan HV, Snyder TM, Hong SP, Kobs CL, South NL, Summer S, Monroe PJ, Chruszcz M, Dobrev V, Tosso PN, Scher LJ, Minor W, Brown ML, Metallo SJ, Üren A, Toretsky JA (2012) Single enantiomer of YK-4-279 demonstrates specificity in targeting the oncogene EWS-FLI1. Oncotarget 3:172–182
34. Burgess JA, Stevens CW, Fahl WE (1985) Mutation at separate gene loci in Salmonella typhimurium TA100 related to DNA nucleotide modification by stereoisomeric benzo(a)pyrene 7,8-diol-9,10-epoxides. Cancer Res 45:4257–4262