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1 Introduction

Biotransformation is superior to chemical synthesis due to superior regio- and stereo-selectivities,¹ environmentally friendly procedures,² and mild reaction conditions.³ Biocatalysts play an important role in regio- and stereo-selective reactions,^{4,5} including acetylation,⁶ hydroxylation,⁷ and epoxidation of steroids.⁸ Our previous studies have shown that many transformations have been achieved by microorganisms.⁹⁻¹²

α -Terpineol (1), a cyclic monoterpene alcohol, is widely present in many traditional Chinese medicines, such as *Thymus vulgaris*, *Annona squamosa*, and *Magnolia denudata*. α -Terpineol is also commonly used in industrial syntheses^{13,14} and certain pharmaceutical preparations.¹⁵⁻¹⁷ The *in vivo* metabolic pathways of α -terpineol were confirmed to be connected to the digestive system and the P450 enzyme system.^{18,19} It has been reported that α -terpineol is oxidized to oleuropeic acid by the larvae of common cutworms (*Spodoptera litura*).²⁰ It is reasonable to speculate that microbes possessing P450 enzyme system might facilitate above transformation. As a key raw material, oleuropeic acid (2) can be used to synthesize a variety of active substances.²¹⁻²⁵ Therefore, the investigation of the accumulation of oleuropeic acid (2) by microbial fermentation is important and prospective.

In the present study, the biotransformations of α -terpineol were screened in liquid fermentation using nine plant

endophyte fungi to confirm its biological oxidation. The results showed that *Alternaria alternata* fermentation could facilitate the biotransformation of interest. Isolation of the fermentation broth led to the identification of two oxidative products 4R-oleuropeic acid (2) and (1S,2R,4R)-*p*-menthane-1,2,8-triol (3) (Fig. 1). 7-Hydroxy- α -terpineol (2a), isolated from the fermentation broth by shortening fermentation time, was confirmed to be an oxidative intermediate. The oxidation of α -terpinol (1) to compound 2 is consistent with larvae that reported in the literature.²⁰ The conversion ratio of α -terpineol (1) to 4R-oleuropeic acid (2) was determined by high-performance liquid chromatography (HPLC) and the yield of compound 3 was determined by gas chromatography (GC). Additionally, cytochrome P450 enzymes were further confirmed to catalyze this biotransformation. Compared with feeding insects to collect their excreta, the method of liquid fermentation by fungi to convert α -terpinol is more potential for mass production. This is the first study to confirm the biotransformation of α -terpineol,

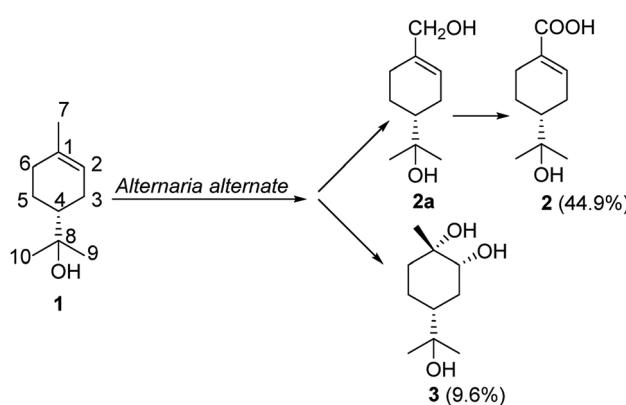


Fig. 1 The biotransformation of **1** by *Alternaria alternate*.

and this study could serve as a reference for further studies on the internal metabolism of this compound.

2 Experimental

2.1 Chemicals

α -Terpineol (compound 1, CAS: 10482-56-1) was purchased from Aladdin Industrial Corporation. Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Corporation, Qingdao, China. Acetonitrile and methanol (Hipure Chem, China) were of HPLC grade. 1-Aminobenzotriazole (ABT, 98%), piperonyl butoxide (PBO, 90%), and *n*-tetradecane (98%) were obtained from J&K Scientific Ltd. (Beijing, China). CDCl_3 (99.9 atom% D, containing 0.03% TMS) was purchased from ARMAR Chemicals (Ningbo, China) and MeOD (99.9 atom% D, contains 0.03% TMS) was purchased from Sigma-Aldrich (Shanghai, China). The water used in this study was purified using a water purification system (Chengdu, China) and had a resistivity $\geq 18.25 \text{ M}\Omega \text{ cm}^{-1}$. Other reagents were of analytical grade and were purchased from Tianjin Fuyu Fine Chemical Co. Ltd. (Tianjin, China).

2.2 Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra were obtained on a Bruker Avance 400 MHz spectrometer (400.00 MHz, ^1H ; 100.00 MHz, ^{13}C) using tetramethylsilane (TMS) as the internal reference.

2.3 Polarimeter

Optical rotation was measured with a Jasco P-1020 digital polarimeter (Jasco, Tokyo, Japan). A Shimadzu UV-vis 2550.

2.4 HPLC

An Agilent Technologies 1200 Series HPLC equipped with an Agilent dual pump, a diode array detector (DAD), an Agilent ZORBAX SB-C18 (4.6 \times 250 mm, 5 μm) and an Agilent Guard Column Hardware Kit was used for all HPLC analyses.

2.5 GC

An Agilent Technologies 7890B GC System with a flame ionization detector (FID), an Agilent 19091J-413 HP-5 (30 m \times 320 $\mu\text{m} \times 0.25 \mu\text{m}$) was used for all GC analyses.

2.6 Microorganisms and culture medium

A. alternata, *Fusarium solani*, *Fusarium avenaceum*, *Aspergillus tubingensis*, *Fusarium oxysporum*, *Myrothecium inundatum*, *Aspergillus sydowii*, *Penicillium virgatum* and *Aspergillus versicolor* were obtained from the Yunnan Institute of Microbiology, Yunnan Province, China. *A. tubingensis*, *A. sydowii*, *P. virgatum* and *A. versicolor* were separated from the root of *Panax notoginseng*. *F. oxysporum*, *M. inundatum*, *A. alternata*, *F. solani*, *F. avenaceum* were separated from the tuber of *Gastrodia elata*. Culture and biotransformation experiments used fungi grown in potato dextrose agar (PDA, 1 L of water, 200 g of potato, 20 g of dextrose and 15 g of agar) slant culture medium.

2.7 Screening of biotransformation

PDA slant culture medium was inoculated with the aforementioned fungi and kept in a constant temperature incubator at 28 °C for 7 days. Then, the mature slants were added to conical flasks (150 mL) that contained 20 mL of potato dextrose broth (PDB: 1 L of water, 200 g of potato starch, and 20 g of dextrose) seed culture medium and placed on a rotary shaker shaking at 120 rpm at 28 °C for 3 days. And then 150 mL of PDB was added to a 500 mL conical flask to serve as the fermentation culture medium. After the fermentation culture medium was sterilized in an autoclave at 121 °C and 1.06 kg cm⁻² for 30 min, the seed cultures and 1 mL (5 $\mu\text{L mL}^{-1}$) of substrate were added and the medium was incubated at room temperature for 7 days. Then, the PDB before and after fermentation was immersed in EtOAc (200 mL) and extracted with an ultrasonic cleaner three times (30 min each), extracts were analyzed with thin layer chromatography (TLC).

2.8 Biotransformation process investigation

A. alternata was grown in shaking cultures at 28 °C for 3 days in 150 mL conical flasks that contained 50 mL of sterile PDB. The α -terpineol (10.0 μL each) dissolved in MeOH (0.5 mL) was evenly distributed between two flasks containing 50 mL of the previously mentioned sterile PDB and strains that had been cultured for 3 days. The α -terpineol mixtures were incubated for a further 1, 5, 7, or 9 days each. The broth was extracted with EtOAc (200 mL) three times to afford the extract. HPLC was used to determine the content of compound 2a and 2 in samples at different times.

2.9 Purification of metabolites

The extracts of strains that had been fermented for 5 days were subjected to a Sephadex LH-20 column (CHCl_3 -MeOH (1 : 1)) and silica gel CC (PE-EA, (15 : 1, 7 : 1, and 4 : 1)) to obtain compound 2a (7.8 mg). In addition, extracts of strains that had been fermented for 9 days were subjected to a Sephadex LH-20 column (CHCl_3 -MeOH (1 : 1)) and silica gel CC (PE-EA, (4 : 1)) to obtain compound 2 (14.5 mg), or the same extract was subjected to silica gel CC (PE-EA (2 : 1)) and a Sephadex LH-20 column (MeOH) to yield compound 3 (12.3 mg).

2.10 Spectroscopic data

7-Hydroxy- α -terpineol (2a). White oil; $[\alpha]_D^{25} -25.1$ (*c* 0.020, CHCl_3); EIMS *m/z* 170 M⁺; for ^1H and ^{13}C NMR spectral data, see Table 1.

4R-Oleuropeic acid (2). White amorphous powder; $[\alpha]_D^{25} -38.9$ (*c* 0.020, MeOH); EIMS *m/z* 184 M⁺; for ^1H and ^{13}C NMR spectral data, see Table 1.

(1S,2R,4R)-*p*-Menthane-1,2,8-triol (3). Amorphous powder; $[\alpha]_D^{25} +16.3$ (*c* 0.020, CHCl_3); EIMS *m/z* 188 M⁺; for ^1H and ^{13}C NMR spectral data, see Table 1.

2.11 Determining reaction yield by HPLC and GC analysis

Standards and samples were dissolved in MeOH (1.000 mL) and filtered through a 0.45 μm filter prior to injection into the HPLC



Table 1 The NMR spectroscopic data for **2a**, **3** in CDCl_3 and **2** in MeOD

	2a		2		3	
Position	δ_{C}	δ_{H} mult. (J in Hz)	δ_{C}	δ_{H} mult. (J in Hz)	δ_{C}	δ_{H} mult. (J in Hz)
1	137.6 s		131.7 s		71.0 s	
2	122.6 d	5.69 (m, 1H)	140.8 d	6.98 (t, 1H, 2.4)	74.1 d	3.61 (brs, 1H)
3	23.7 t	2.05 (m, 1H)	24.6 t	2.02 (m, 1H)	30.0 t	1.79 (m, 2H)
		1.24 (m, 1H)		1.22 (m, 1H)		
4	45.2 d	1.54 (m, 1H)	45.6 d	1.53 (m, 1H)	41.1 d	1.63 (tt, 1H, 12.0, 6.0)
5	26.5 t	1.55 (m, 1H)	26.4 t	2.48 (br d, 1H, 16.0)	22.3 t	1.54 (t, 1H, 8.0)
		1.36 (m, 1H)		2.08 (m, 1H)		1.34 (td, 1H, 8.0, 4.0)
6	26.7 t	1.76 (m, 1H)	28.5 t	2.34 (br d, 1H, 16.0)	33.7 t	1.75 (t, 1H, 2.0)
		1.52 (m, 1H)		2.01 (m, 1H)		1.50 (t, 1H, 3.6)
7	67.2 t	4.01 (t, 2H, 12.0)	171.1 s		27.8 q	1.22 (s, 3H)
8	72.9 s		72.9 s		72.8 s	
9	27.5 q	1.19 (s, 3H)	26.4 q	1.17 (s, 3H)	26.7 q	1.18 (s, 3H)
10	26.7 q	1.24 (s, 3H)	27.0 q	1.17 (s, 3H)	27.6 q	1.14 (s, 3H)

and GC. Different concentrations of compound **2** and **3** methanol solution were injected into HPLC or GC and integrate the peak area to draw a standard working curve. Each sample was analyzed in triplicate. The contents of products were determined according to the corresponding calibration curves; further conversions were calculated on the basis of these contents.

A gradient elution system consisting of solvents A (0.1% HAc) and B (acetonitrile) was used for the HPLC analysis. The gradient program was as follows: 0–10 min, 20–70% solvent B; 10–40 min, 70–80% solvent B; 40–45 min, 80–100% solvent B; and 45–46 min, 100–20% solvent B and then held for 10 min. The detecting wavelength of the diode array detector was set to 225 nm. The flow rate was 1.0 mL min^{-1} , the column temperature was set at 25°C , and the injection volume was $10 \mu\text{L}$.

Argon was used in the mobile phase of GC analysis, argon at a flow rate of 1 mL min^{-1} was used as a carrier gas. The oven temperature was programmed from 200 to 250°C at 4°C min^{-1} . The injector and detector temperatures were 270 and 280°C , respectively.

2.12 Effects of the inhibitors ABT and PBO and of the inducer *n*-tetradecane

The inhibitors ABT and PBO ($40 \mu\text{g mL}^{-1}$, final concentration) and the inducer *n*-tetradecane (2%, v/v, final concentration) were added to the spore suspension after it had been incubated for 24 h. After an additional 48 h of culturing, 1 mL of the substrate ($10 \mu\text{L mL}^{-1}$) was added. The spore suspension without the inhibitors and inducer was used as the positive control. The concentrations of the product and reactant were determined by HPLC and GC analysis after 6 h, 24 h, 48 h, 72 h and 96 h. Each sample was repeated in triplicate.

3 Results and discussion

3.1 Screening of microorganism

Oxidation reactions caused by microbes are commonly reported in the literature;^{26,27} thus it is reasonable to propose that α -

terpineol was oxidized to *4R*-oleuropeic acid by the enteric microorganism in the larvae of common cutworms. Therefore, in the present study, the biotransformation of α -terpineol was screened in liquid fermentation using nine plant pathogenic fungi to confirm its biological oxidation process. TLC was used to monitor the transformation process during fermentation. The results showed that *A. alternata* fermentation could facilitate the biotransformation of interest.

3.2 Identification of compounds

All ^1H and ^{13}C NMR spectral data of the known compounds **2a**, **2** and **3** agreed with spectral data reported in the literature.^{20,28,29} The absolute configuration of compound **3** was determined based on the ROESY spectrum. As mentioned above, chiral center at C-4 remained unchanged during the fermentation process. The α -orientation of OH-2 was assigned on the basis of NOE correlations between H-4 β [δ_{H} 1.63 (tt, $J = 12.0, 6.0 \text{ Hz}$)] and H-2 [δ_{H} 3.61 (brs, 1H)]. The NOE correlation (Fig. 2) between CH_3 -7 [δ_{H} 1.22 (s, 3H)] and H-2 indicated that they were oriented in the same direction. Thus, the chiral centers in **3** were assigned as 1*S*, 2*R* and 4*R*. Compound **3** was chemically synthesized by a previously reported method,³⁰ and this is the first report of its synthesis *via* microbial fermentation.

3.3 *A. alternata* fermentation of α -terpineol

Fermentation of α -terpineol by *A. alternata* for seven days allowed the isolation of two oxidative products, *4R*-oleuropeic acid (**2**) and (*1S,2R,4R*)-*p*-menthane-1,2,8-triol (**3**). After reducing fermentation time to 5 days, we isolated 7-hydroxy- α -terpineol (**2a**) as a key oxidative intermediate, which confirmed the oxidation process of α -terpineol (**1**) to oleuropeic acid (**2**). The fermentation itself process was also investigated. As shown in Fig. 3, no absorption peak was detected at the beginning (Fig. 3a), when the fermentation time increased, compound **2a** gradually appeared, reached a peak at 5 days (Fig. 3b), then decreased quickly (Fig. 3c) and finally disappeared (Fig. 3d). In



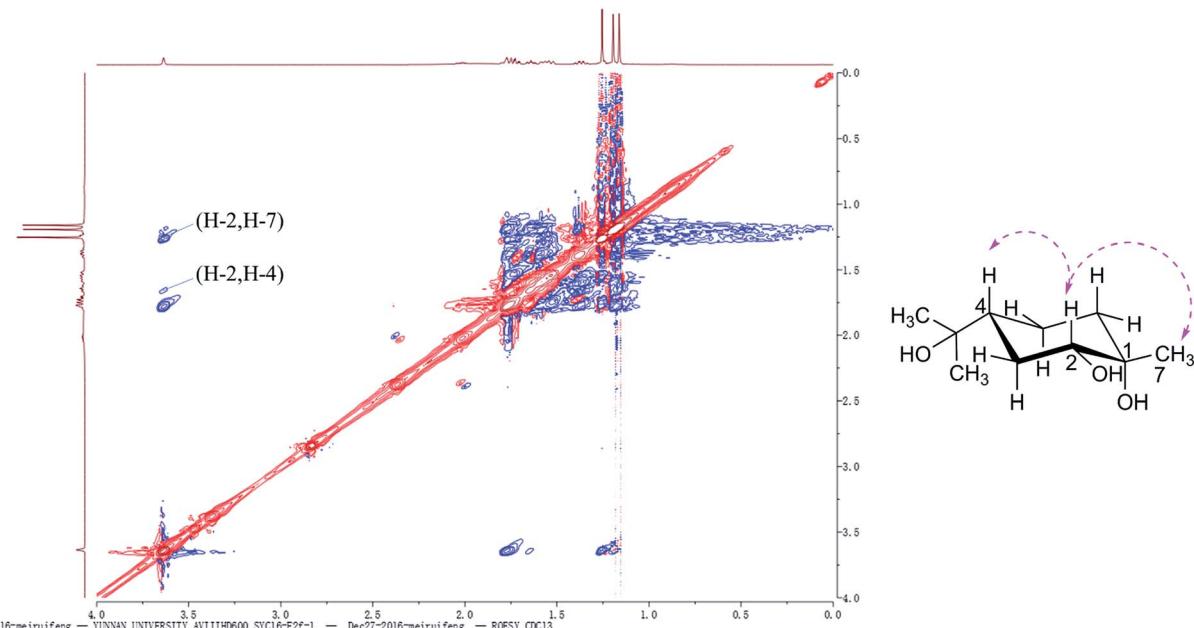


Fig. 2 ROESY spectrum and Key NOE (✓) correlations of 3.

addition, the content of compound 2 gradually increased and achieved its highest level after 9 days of fermentation (Fig. 3d), corresponding to a maximum conversion ratio of $44.9\% \pm 2.4\%$. This biotransformation facilitates the possibility of mass production of 4R-oleuropeic acid (2). The isolation of 3 indicated that *A. alternata* fermentation could facilitate *cis*-dihydroxylation of the C₍₁₎–C₍₂₎ double bond in α -terpineol, which is the same as the reported in the literature.^{31–33} Compound 3 could not be detected by FID until four days fermentation. The yield of compound 3 after 9 days of fermentation was $9.6\% \pm 0.8\%$.

3.4 Effects of the inhibitors ABT and PBO and the inducer *n*-tetradecane

Cytochrome P450 monooxygenases catalyze the oxidation of a wide variety of endogenous and exogenous organic substrates typically by inserting an oxygen atom from atmospheric dioxygen into a carbon–hydrogen bond to produce the corresponding alcohol^{34,35} or carboxylic acid.³⁶ In this study, compound 2 was proposed to be derived from α -terpineol *via* cytochrome P450 oxidative catalysis.

To determine the enzyme system involved in this biotransformation, special inhibition and induction experiments were performed. PBO (piperonyl butoxide, which contains a methylenedioxophenyl (MDP) or benzodioxole moiety) is used to estimate the inhibition and induction of reactions mediated by cytochrome P450.^{37,38} ABT is a triazole P450-14DM inhibitor that is widely used as an inactivator of many P450 isozymes.³⁹ *n*-Tetradecane is an effective inducer of cytochrome P450 enzymes.⁴⁰ As shown in Fig. 4a and b, ABT and PBO exhibited a significant inhibitory effect on the conversion of α -terpineol to compound 2a at a concentration of $40 \mu\text{g mL}^{-1}$. Under such

conditions, the production of compound 2 was very slow and we did not detect compound 2 by HPLC in these cases. In contrast, this transformation could be significantly induced by 2% (v/v) *n*-tetradecane (Fig. 4c). In the early stage of the fermentation, compound 2a was produced much more quickly than it was in

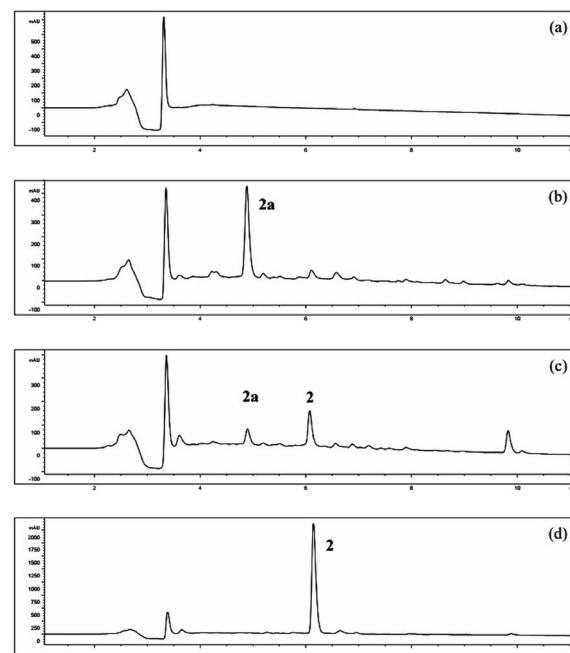


Fig. 3 Detection of the biotransformation process of compound 2a to 2 by HPLC. Figure (a–d) shows the fermentation time: 1, 5, 7, 9 days. α -Terpineol was first oxidized to compound 2a then decreased quickly and finally oxidized to 2.

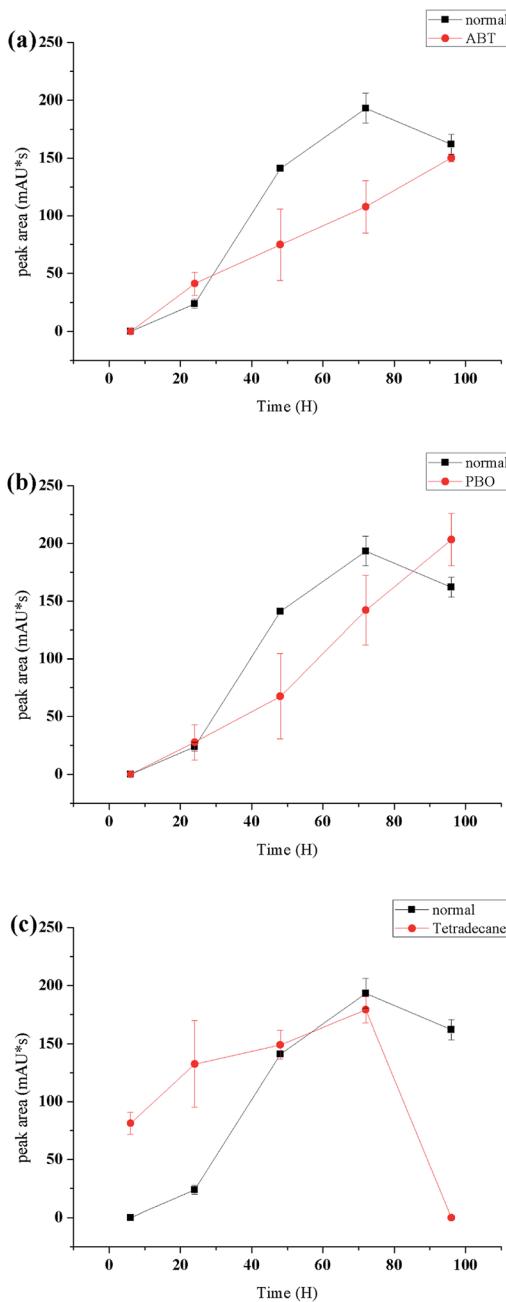


Fig. 4 Effects of ABT ((a), $40 \mu\text{g mL}^{-1}$), PBO ((b), $40 \mu\text{g mL}^{-1}$) and *n*-tetradecane ((c), 2% v/v) on the biotransformation of 1 to 2a.

the absence of the inducer. After 90 hours of fermentation, the content of **2a** decreased quickly to produce the final oxidation product **2**, indicating that *n*-tetradecane could promote the biotransformation from α -terpineol to **2**. The results of the inhibition and induction experiments indicated that cytochrome P450 enzymes serve as the catalysis for this biotransformation. The same approach was tried to accumulate compound **3**, but the results of the experiment showed that the yield of compound **3** had no relationship with P450 enzyme inhibitors and inducers. Through literature review,^{41–44} compound **3** is more likely to be catalyzed by dioxygenase.

4 Conclusions

In summary, the present study verified that α -terpineol could be converted to *4R*-oleuropeic acid (**2**) and a *cis*-dihydroxyl derivative (**3**) by *A. alternata*. 7-Hydroxy- α -terpineol (**2a**) was shown to be an intermediate in the biotransformation of α -terpineol to **2**. Cytochrome P450 enzymes were confirmed to catalyse this biotransformation. This is the first study to clarify the biotransformation of α -terpineol, and this study could serve as a reference for further investigations on the internal metabolism of this compound.

Conflicts of interest

There are no conflicts to declare.

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