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ORIGINAL ARTICLE

Stereo-selective oxidations of terpinolene by cytochrome P450 monooxygenases in the microsomal fraction of *Cupressus lusitanica* cultured cells

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Abstract We showed previously that in *Cupressus lusitanica* (Mexican cypress), the first two steps of terpinolene oxidation, beginning at hydroxylation at the aryl position and then forming epoxide at the double bond, were driven by cytochrome P450s. The significance of enantio differences, in general, has received attention because those enantiomers often have their own biological activities. We, therefore, investigated the stereo-specificity of the substrate and the enantio-selectivity of these reactions. The hydroxylation of terpinolene by cytochrome P450 in the microsomal fraction from *C. lusitanica* cells gave a single product with an *S* configuration of 5-isopropylidene-2-methylcyclohex-2-enol. Next, epoxidizing enzyme accepted only the *S*-configuration substrate and produced a single enantiomer product, (1*R*, 2*S*, 6*S*)-(+)-1,6-epoxy-4(8)-*p*-menthen-2-ol. No isomer was detected at our gas chromatography/mass spectrometry sensitivity; therefore, the calculated enantiomeric excess values were 100 %. These results indicate that the cytochrome P450s involved in terpinolene metabolism in *C. lusitanica* cells have very strict stereo-selective ability. Our findings may be helpful in the stereo-selective synthesis of fine chemicals, although the physiological meanings of these chiral products are still not unclear.

Keywords Cytochrome P450 · *Cupressus lusitanica* · Microsomal fraction · Stereo-selective oxidation · Terpene

Introduction

Cytochrome P450s are ubiquitous in all kingdoms [1]. They have the functions of activating molecular oxygen and introducing one oxygen atom into the substrate [2]. In plants, this group of enzymes is involved in many secondary metabolisms, and all of the cytochrome-P450-produced compounds can be expected to have significant roles; for example, in helping plants survive or be competitive in nature. Some of the plant secondary metabolites are also commercially attractive, and the details of cytochrome P450 concerning biosynthesis have thus been eagerly investigated [3].

In particular, the significance of enantio differences has received increasing attention over the past three decades [4]. Each enantiomer has its own biological activities such as vasodilatory activity [5], antimicrobial activities [6], and more as reviewed by Fassihi [7]. The topic of stereochemistry in the fields of chemical ecology and medical and biomedical studies has become important, and investigations of substrate stereo-selectivity and enantio-selective products are now essential for plant metabolism research and for use in daily life. It is for these reasons that plant cytochrome P450s have been examined for their specific substrate stereo-selectivity and enantio-selective products, although animal and fungal cytochrome P450s have relatively broader selectivities and reactivities [8].

The lifespan of many woody plants should be tens or hundreds of years or more, and their stems are tall and sound. These plants must keep fighting against many potential pests and pathogens, and they accumulate many

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antibiotics in their tissue [9]. We have been investigating the biosynthesis of β-thujaplicin (hinokitiol) [10, 11] as a monoterpene in Cupressaceae trees, which have a strong and a wide spectrum of antimicrobial activity [12]. However, woody plants are not good research material for the study of metabolism and enzymes because they have very hard tissue and almost no living cells except at only the cambium and parenchyma.

Therefore, our group has established an experimental system using cultured cells obtained from *Cupressus lusitanica* (Mexican cypress) [13]. The elicitor-treated *C. lusitanica* cell cultures produce many types of olefine and oxygenated monoterpenes with a significant amount of β-thujaplicin [14, 15]. In this cell line, a ^{13}C-glucose feeding experiment demonstrated that β-thujaplicin was biosynthesized via an ordinary six-membered ring intermediate, i.e., a menthane-type skeleton produced by terpene synthase and followed by ring expansion to a unique conjugated seven-membered ring, tropolone [16]. Assays of terpinolene and terpinolene synthase suggested that terpinolene is the first olefin monoterpene intermediate in β-thujaplicin production [15].

In fact, this speculation was confirmed by the results of a feeding experiment using deuterized terpinolene added to the cell culture [17]. It was also shown that the microsomal fraction from the cells oxidized terpinolene into the hydroxylated compound, 5-isopropylidene-2-methylcyclohex-2-enol (IME), and then IME was further oxidized by the microsomal fraction to the epoxidized compound, 1,6-epoxy-4(8)-*p*-menthen-2-ol (EMO) [18]. Experiments on kinetics and with specific inhibitors confirmed that these reactions were caused by cytochrome P450 monooxygenases [18]. Typical cytochrome P450s can oxidize their substrate with strict stereo-control as mentioned above, and the EMO in the cultured cells was enantio-pure [14]. In the present study, we investigated the substrate stereo-specificity and enantio-selectivity of products on this metabolic pathway.

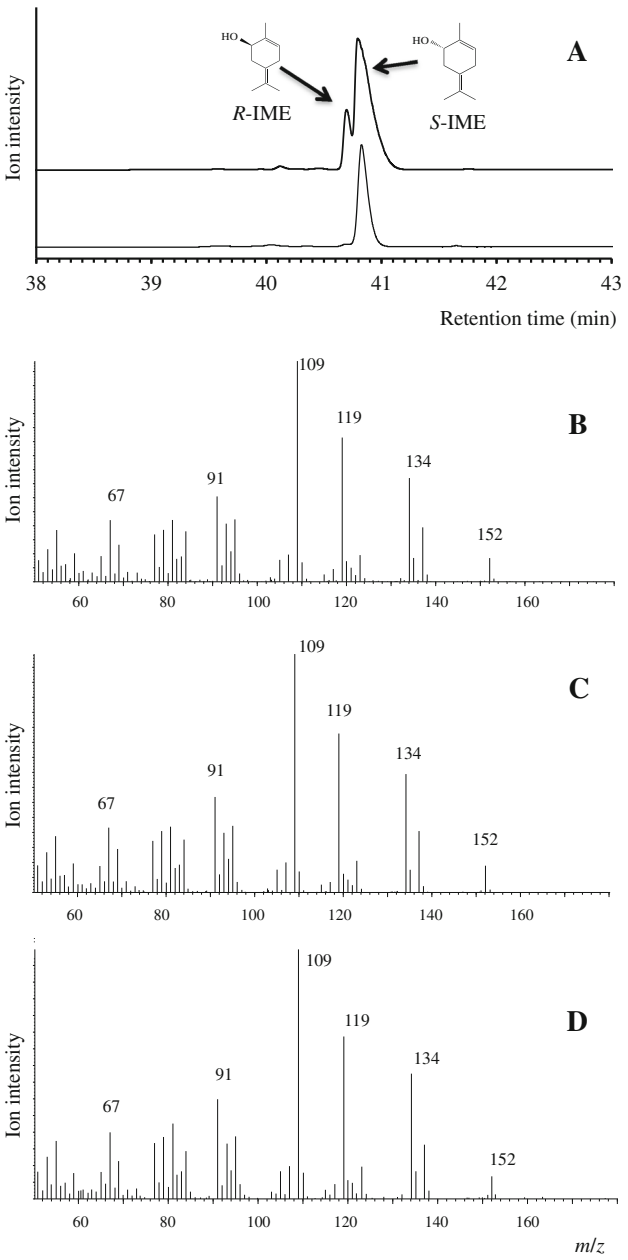

Fig. 1 Results of the chiral GC/MS of chemical synthesized IMEs and enzymatic produced IME. **a** Total ion chromatogram. *Upper line* chemically synthesized IME before chiral HPLC purification. *Lower line* enzyme product from terpinolene and the microsomal fraction prepared from *C. lusitanica* cultured cells. **b** The EI mass spectrum of the peak at retention time (Rt) = 40.7 of panel A upper. **c** The EI mass spectrum of the peak at Rt = 40.8 of panel A upper. **d** The EI mass spectrum of the peak at Rt = 40.8 of panel A lower

Materials and methods

Cell culture and microsome preparation

Callus cultures of *C. lusitanica* were maintained in Gamborg B5 medium [19] supplemented with 0.01 mM benzyl aminopurine, 10 mM naphthyl acetic acid, 20 g/L sucrose and 2.7 g/L gellan gum at pH 5.5 for more than 10 yrs. No terpene including β-thujaplicin was produced under this growth condition. For enzyme induction, approx. 5 g of *C. lusitanica* cells growing in solid B5 medium for 4 weeks was transferred to 30 mL of liquid β-thujaplicin production medium [20]. To initiate terpene production, we added 2 mL of partially purified yeast extract solution as an elicitor [21]. After 24 h of 25 °C, 70 rpm shaking, and dark incubation, cells were separated from the medium and stored at −80 °C.

For the preparation of the microsomal fraction, we ground 8 g of the cells in a pre-chilled mortar and pestle

with liquid nitrogen, and suspended the powder in 200 mL of pre-chilled buffer as described by Bouwmeester et al. [22]. The homogenate was transferred to a small bottle, sonicated for 3 min in 10-s pulses at 35 % power (USP-400A, Shimadzu, Kyoto, Japan) and stirred for 12 min. The homogenate was centrifuged at 20000 g for 20 min, followed by filtration with a glass fiber filter, and the supernatant was then centrifuged at 150000 g for 90 min. Microsomal pellets were either used directly for the assays or stored at −80 °C.

Preparation of authentic IMEs

We chemically synthesized authentic IME by Motherwell's method [23] as described by Harada et al. [18]. The purity and structure of the synthesized IME was confirmed by gas chromatography–mass spectrometry (GC/MS) (HP 5890 series II and HP 5972; Hewlett Packard, Palo Alto, CA) with the fused silica capillary column InertCap 5 (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm; GL Sciences, Tokyo) was used. Helium was used as the carrier gas with a column head pressure of 100 kPa. The oven temperature was kept at 70 °C for 3 min and increased with a gradient of 10 °C/min up to 250 °C. The temperature of the inlet port was 220 °C and that of the interface to MS was 250 °C. The purified products' structures were determined by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy (AL-400; JEOL, Akishima, Japan). Each of the chiral compounds were enantio-purified by a chiral high-performance liquid chromatography (HPLC) system equipped with a CHIRALPAK IC 4.6 × 250 mm column (Daicel, Osaka, Japan). The enantio-purity was confirmed by chiral GC/MS with a GL Sciences InertCap CHIRAMIX 30 m × 0.25 mm column and 0.25 μm film. Helium was used as the carrier gas with a column head pressure of 100 kPa. The oven temperature was kept at 40 °C and increased with a gradient of 2.5 °C/min up to 180 °C. The chromatogram obtained with this analysis was shown in Fig. 1. The absolute configurations of the secondary hydroxyl group of IME were determined by a modified-Mosher method [24]. The results of this analysis are shown in Fig. 2.

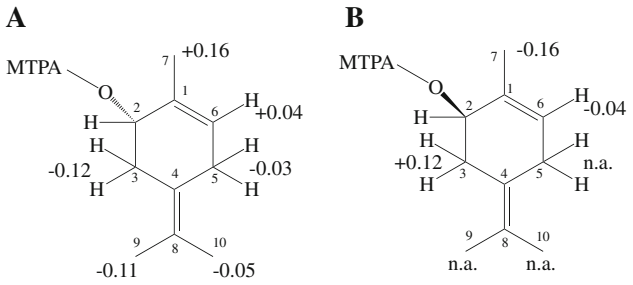

Fig. 2 Determination of absolute configuration of the IME hydroxyl group that appeared at Rt = 40.8 min in Fig. 1 by the modification of Mosher's method [24]. Δδ values are displayed beside the protons. Some protons in **b** were not assigned and displayed as *n.a.*

Preparation of authentic EMO

Because EMO has already been discovered in extracts of *C. lusitanica* cells and medium [14], we obtained authentic EMO by the extraction and purification of the cells and medium. We isolated EMO from the extracts by silica gel column chromatography. The purity of the obtained EMO was confirmed by chiral and achiral GC/MS analysis as described above.

Microsomal reaction and enzyme product assay

We conducted an assay of cytochrome P450s in microsomal fraction as reported [18]. Microsomal pellets were resuspended in assay buffer [22]. One milliliter of microsomal suspension was incubated in a 20-mL Teflon-lined screw cap vial flushed with O_2 gas, and the reaction was started by the addition of 1 mM NADPH, an NADPH-regenerating system (5 mM glucose-6-phosphate, 1 i.u. mL^{-1} glucose-6-phosphate dehydrogenase) and 550 nmol of terpinolene or 315 nmol of IME (10 μL of acetone stock, respectively). After incubation for 4 h at 30 °C, reactions were stopped by the addition of Et_2O or ethyl acetate. As an internal standard, 1.5 nmol of dodecyl alcohol was added to the reaction mixtures, and then the mixtures were extracted twice with Et_2O or ethyl acetate. The reaction products were concentrated by a stream of N_2 and analyzed by chiral and achiral GC/MS analyses as described above.

Results

Chemical synthesis, chiral purification and determination of the absolute conformation of the hydroxyl group of IME

Because the levels of the crude enzymatic products were too low to determine their enantiomeric properties by NMR, we compared the synthesized IME and enzyme products. IME was synthesized by Motherwell's method [23] with slight modification. The aluminum column chromatography gave pure IME confirmed by normal GC, but it was shown by chiral GC that this fraction contained two enantiomers (Fig. 1a upper). Each of the chiral compounds was enantio-purified by chiral HPLC. The absolute configurations of the secondary hydroxyl group of IME shown as a retention time of 40.8 min in Fig. 1 were determined. The Δδ values of positions 7 and 6 were plus values, and those of positions 3,

9, and 10 were minus values (Fig. 2a). Therefore, the absolute configuration of the hydroxyl group of this compound was determined as having an *S* configuration (*S*-IME). Other enantiomer, Rt = 40.7 in Fig. 1, was also confirmed as having a *R* configuration (*R*-IME) (Fig. 2b).

Enantio-selective formation of IME by crude enzyme solution from *C. lusitanica* cultured cells

Chemically synthesized IME has two enantio configurations on its hydroxyl group. To determine the enantiomeric purity of enzymatic products, we performed an assay of the terpinolene reaction with the crude microsomal fraction, and it was observed that the enzyme product gave a single peak at the same retention time as the *S* configuration (Fig. 1a) of the authentic IME, which was purified enantiomerically and whose absolute configuration was determined as described above. Their mass spectra were also identical (Fig. 1c, d). Another configuration product, *R*-IME, was not detected at this GC sensitivity; i.e., the enantiomeric excess value of this enzyme product was almost 100 %. It should thus be noted that the enantio-regulation by this enzyme was very strict.

Stereo substrate specificity of IME during EMO formation

The subsequent metabolic step after IME formation was epoxidation from IME to EMO [18]. The production of only an *S* configuration by cytochrome P450 monooxygenase reaction with the *C. lusitanica* microsomal fraction raised the next question about the substrate specificity of another cytochrome P450, which oxidize IME. We, therefore, used enantio-pure synthesized IMEs as the next microsomal reaction.

EMO was not detected on GC/MS with the substrate of *R*-IME at the level of our detection limit, but the *C. lusitanica* microsomal reaction with *S*-IME gave the product, EMO (Fig. 3a). The substrate specificity of this reaction was thus very strict stereo-selective, accepting only *S*-IME. In this experiment, no EMO produced by two succeeding enzyme reactions was observed, probably because the shorter reaction time we used in this experiment was not enough to detect EMO from the enzyme-produced IME.

Enantio-purity of EMO produced by cytochrome P450 reaction

We also investigated the absolute configuration of EMO produced by this cytochrome P450-catalyzed oxidation. The crude enzyme product was compared on the chiral GC analysis with authentic EMO, which was obtained from the *C. lusitanica* cells. The enantio-purity and the configuration of authentic EMO had already been confirmed by NMR spectrum obtained with the modified-Mosher method [14]. As shown in Fig. 4, the retention time on chiral GC of the authentic enantio-pure EMO and those of the crude enzyme-produced EMO were identical. And mass spectra of both were also identical (data not shown). No other peak with the same or similar mass spectrum as EMO was observed on chiral GC. It was, therefore, shown that the crude enzyme product was enantio-pure (1*R*, 2*S*, 6*S*)-(+)-1,6-epoxy-4(8)-*p*-menthen-2-ol (*RSS*-EMO).

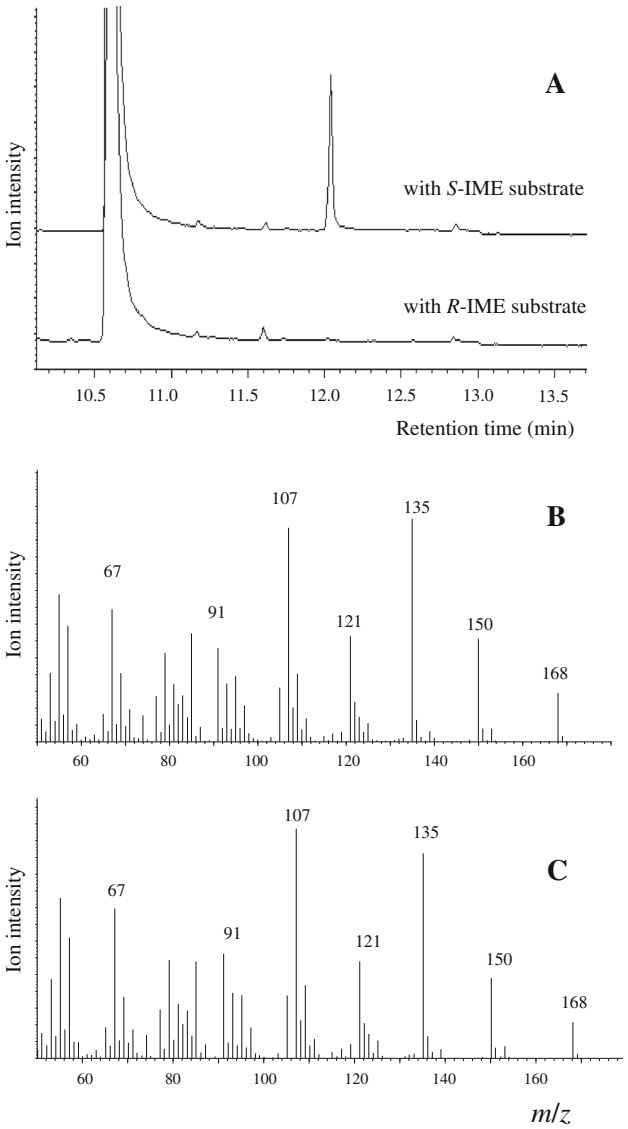

Fig. 3 GC/MS analysis of EMO produced by the microsomal reaction with enantio-pure IME. The peak at 10.6 min is IME fed as a substrate. **a** Total ion chromatogram. *Upper line* the product with *S*-configuration IME. *Lower line* the product with *R*-configuration IME. The standard EMO appeared at 12.1 min. **b** The EI mass spectrum of standard EMO. **c** The EI mass spectrum of the peak at 12.1 min of the enzyme product

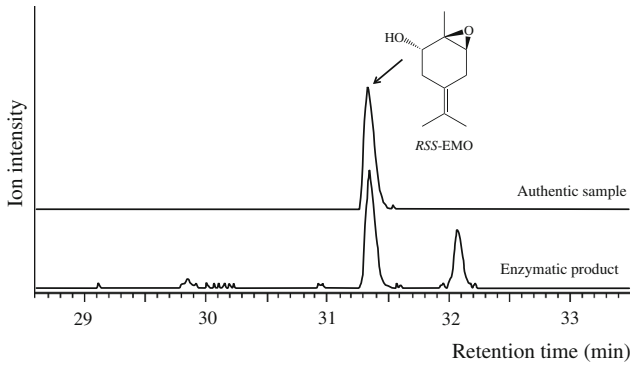

Fig. 4 Chiral GC/MS analysis of EMO produced by the microsomal cytochrome P450 prepared from *C. lusitanica* cells by selected ion monitoring on $m/z = 168$. *Upper line* selected ion chromatogram ($m/z = 168$) of standard EMO obtained from cultured cell. Enantiopurity and configuration had been confirmed by Matsunaga et al. [14]. *Lower line* selected ion chromatogram ($m/z = 168$) of products by the microsomal cytochrome P450 from *C. lusitanica* cells. Any other peak than Rt = 31.35, including Rt = 32.1, did not have the same or similar mass spectrum as EMO, indicating production of a single chiral product configuration by this assay

Discussion

There is a large family of a variety of cytochrome P450s [1], and the variety is one of the reasons for the huge diversity of plant secondary metabolites. Among the secondary metabolites, terpenoids are the largest group of compounds; therefore, the research on the cytochrome P450s for plant terpenoids metabolism is keenly ongoing [25]. The first step of terpenoid synthesis is conducted by terpene synthases, which produce olefin terpenes and determine the basic skeletons. After that, skeletal intermediates are oxidized by cytochrome P450s [26, 27], and the biological activities of the final products strongly depend on how the molecule was modified. Moreover, enantio differences present a critical issue in light of their biological activities for plant survival strategies and potential clinical uses in humans [4].

In our previous report [18], terpinolene was oxidized to IME and EMO by the cytochrome P450 in microsomal fraction of *C. lusitanica* cells. Details of the stereo-selectivity of these terpinolene metabolisms were investigated in the present study. We found that IME produced by the microsomal fraction with terpinolene had only an *S* configuration (Figs. 1, 2). The enantiomeric excess (e.e.) value was 100 % at our detection level; i.e., very strict stereo-selectivity was observed in the formation of IME. *S*-IME may have a special role in *C. lusitanica* survival in nature, considering the examples of the different biological activities of each enantiomer [28]. However, this idea conflicts with the finding that no IME was detected in the cells, medium, or headspace air of cultures [14, 15]. Another possibility is that the system might be exclusive to exogenous compounds including *R*-IME, but further observation is required to identify the role(s) of this stereo-selectivity.

There are reports in which multiple products were generated in succeeding reactions by one cytochrome P450 [29–31] and in which regio-isomers were produced from multiple substrates [32]). The possibility of succeeding two-step reactions with one enzyme in our cell line was low because only IME (no EMO) was observed in our in vitro enzyme reaction.

It has been reported that no products other than EMO and no EMO diastereomer were found during EMO formation by the crude enzyme [18]. In addition, we did not observe enantio-isomers other than *RSS*-EMO, and *R*-IME substrate was not accepted in this EMO-forming reaction. The conformation of the hydroxyl group in EMO seems no difference from that of the original IME, and epoxy oxygen was inserted from a specific direction; thus, the configuration of the hydroxyl group of IME is a very important property for substrate recognition.

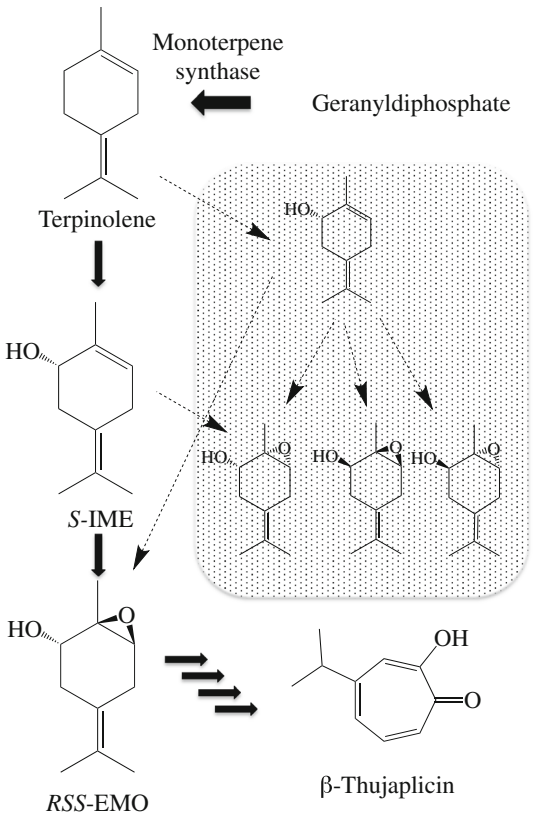

Fig. 5 Summary of stereo-selective oxidations from terpinolene by cytochrome P450s in *C. lusitanica* cultured cells. The pathway with *solid arrows* shows the metabolic pathway deduced by the experiments described in the main text. Others pathways in shaded area are reactions not observed in the present study

As described above and previously reported, this terpinolene metabolism in *C. lusitanica* shows very strict substrate-, regio- and stereo-selective reactions as summarized in Fig. 5.

One of the future goals of our project is to find a novel pathway to form a tropolone ring such as β-thujaplicin. We have already established a feeding experiment system with d6-terpinolene [17]. This system will reveal intermediates after EMO of d-labeled compounds and the biosynthetic pathway. The ring opening by cleavage of the C–C bond and re-closing the ring would be required for ring expansion enzyme(s). However, it seems nonsense that intermediates keep their strict stereo-selectivity, which will be lost by ring cleavage, although the enantio-pure structure of a putative triol intermediate may be required for ring expansion enzyme(s). Alternatively, EMO may have unknown biological and/or physiological role(s) in the cell or in the pathway to β-thujaplicin due to its specific conformation and accumulation in the cell. Feeding experiments of EMO will also be a significant method to clarify the pathway to β-thujaplicin.

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