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A 37-kDa peroxidase secreted from liverworts in response to chemical stress

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Abstract

A peroxidase was purified from the culture medium of a suspension culture of *Marchantia polymorpha* (liverwort) after treatment with bornyl acetate, which acts as a chemical stress agent to the cells. The peroxidase was characterised as a glycoprotein of molecular mass 37-kDa having a pI of about 10 and an optimal pH of 6.5. The peroxidase was thermally stable at 50°C for up to 60 min. The partial amino acid sequence of the peroxidase was determined and found to be dissimilar to the amino acid sequences of other higher plant peroxidases. The oxidative polymerization of lunularin by this peroxidase was examined and the formation of a dimer, a trimer and a tetramer was demonstrated by negative ion Fast Atom Bombardment (FAB)-mass spectroscopy of the reaction products. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Marchantia polymorpha*; Hepaticae; Liverworts; Chemical stress; Secretion; Cationic peroxidase; Bornyl acetate

1. Introduction

It is well known that the attack on higher plants by many phytopathogens can lead to induction of formation of biologically active substances (phytoalexins) and the secretion of enzymes such as peroxidase (Chibbar et al., 1984), phosphatase (Nishizaki et al., 1996) and esterase (Izumi et al., 1995). Ishida et al. (1985, 1987) reported that most of the peroxidase activity was found in the cell walls of cultured cells of *Marchantia polymorpha* and peroxidase was released into the cultured medium from the cell. On the other hand, we recently found that cultured cells of *M. polymorpha* secreted lunularin (**1**) and hydrogen peroxide when the cells were treated with bornyl acetate as a chemical stress agent (Chen et al., 1996). The peroxidase activity in the cell walls of plants is presumed to aid resistance against disease and help wound healing. The primary function of this peroxidase may be to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. In this paper we describe the purification of a peroxidase secreted from cells of *M. polymorpha* cultured with the chemical stress agent bornyl acetate and the oxidative coupling of lunularin (**1**) with the peroxidase, in the presence of H_2O_2.

2. Results and discussion

2.1. Purification and characterization of Marchantia polymorpha peroxidase

In order to demonstrate the secretion of peroxidase by cells of *M. polymorpha* cultured under chemical stress, the suspension cultures of *M. polymorpha* were treated with bornyl acetate and peroxidase activity in the culture medium was measured at regular time intervals. When the cells were cultured with 1.3 mM bornyl acetate the peroxidase activity (measured using guaiacol as the substrate) was observed after 24 h cultivation which corresponded to the start of exponential phase of the cell growth. The peroxidase activity in the culture medium

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subsequently increased during the culture period. The presence of several peroxidase isoenzymes in the medium of the 4-day old cultures, with or without bornyl acetate treatment, was revealed by the analysis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and ion exchange chromatography.

The bornyl acetate-induced peroxidase was further purified using the procedures indicated in Table 1. The

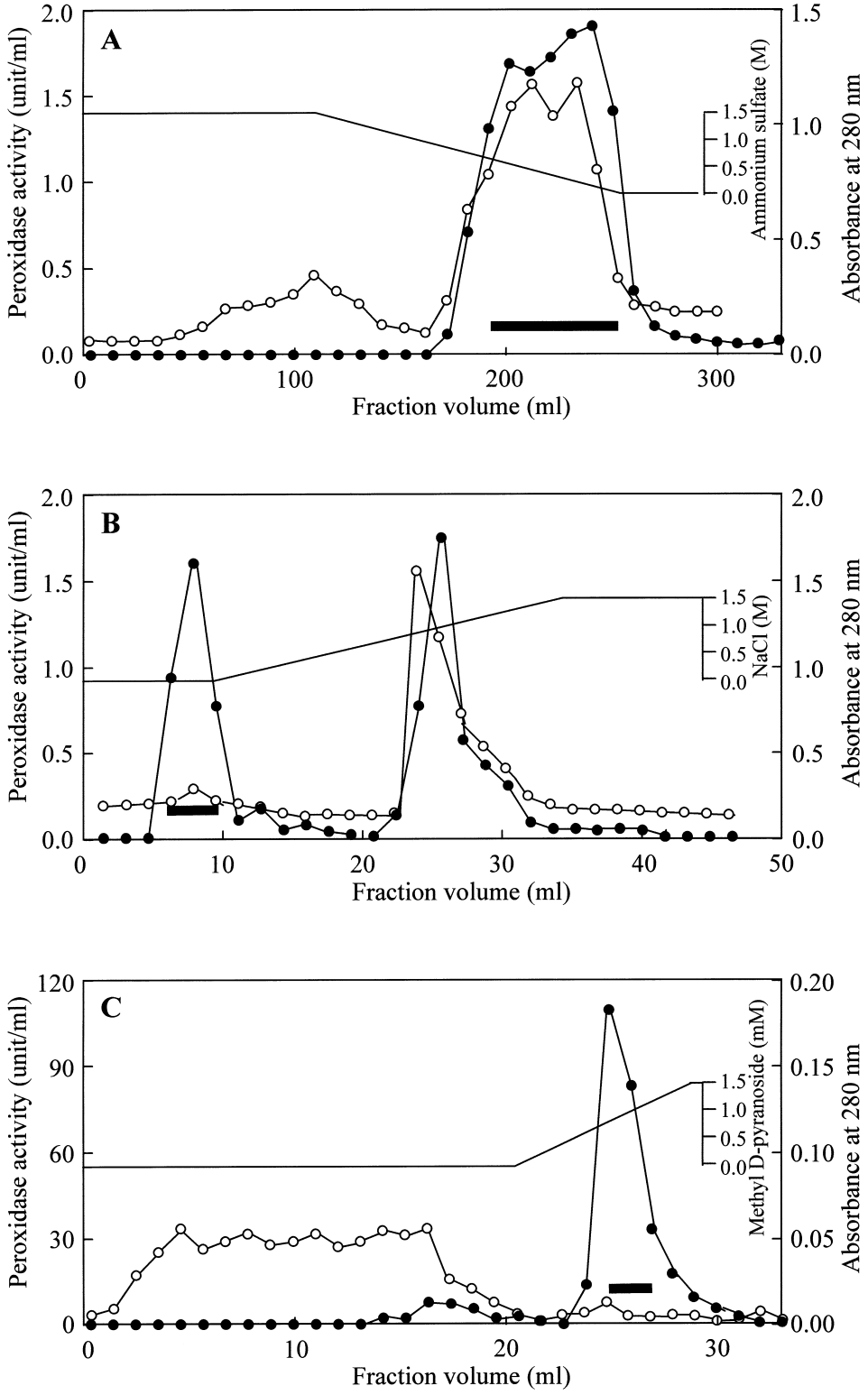


Fig. 1. Purification of peroxidase secreted from *Marchantia polymorpha*. Chromatography profiles of butyl-Toyopearl (A), DEAE-Toyopearl (B), and Con-A Sepharose (C) columns used for the purification. Closed and open circles indicate the peroxidase activity and protein concentration profile, respectively.

combination of the purification steps resulted in a further 69-fold purification of the initial ammonium sulfate fraction, as shown in Fig. 1 and Table 1. SDS–PAGE analysis of the purified peroxidase showed a single band corresponding to a molecular mass of 37-kDa when stained with guaiacol as a substrate or Coomassie Blue R-250 (Fig. 2). Gel filtration analysis showed that the peroxidase was eluted in a volume corresponding to a molecular mass of about 35–40 kDa, indicating that the native enzyme consisted of a monomer. Isoelectric focusing revealed ~pI 10 for the peroxidase. The presence of other isoenzymes was excluded after isoelectric focusing, because only one narrow colored band was seen after guaiacol staining. The peroxidase showed a minimal loss of activity by treatment at 50°C for 2 h, indicating the enzyme to be very thermally stable. The peroxidase showed an optimum pH of 6.5 in phosphate and Tris–HCl buffers with guaiacol as substrate. The peroxidase was stable at acidic pH; the activity at pH 4 was about 80% of the maximal activity, whereas the activity at pH 8 was about 50%.

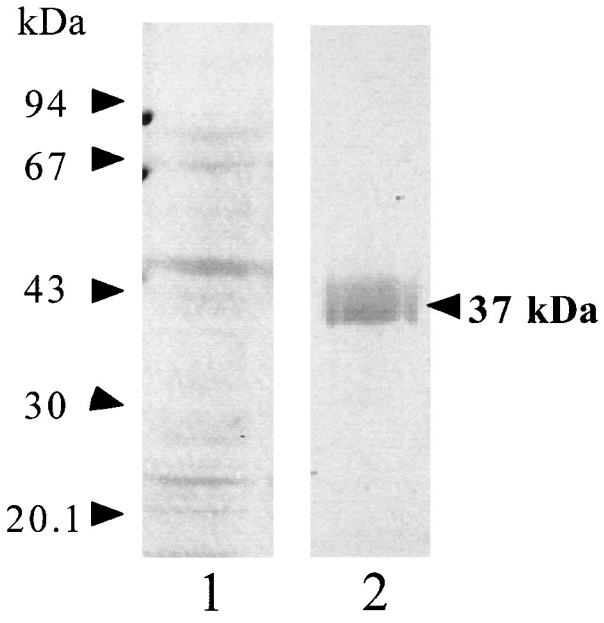


Fig. 2. SDS–PAGE of the secreted proteins from *Marchantia polymorpha*. SDS–PAGE: lane 1, ammonium sulfate precipitation stained by Coomassie Blue; lane 2, purified peroxidase stained by Ag (Silver Stain II kit, Wako). Molecular mass values are shown in the left margin.

Since it has been reported that most of the plant peroxidases have oligosaccharide chains linked to asparagine (Wan and van Huystee, 1993; Yang et al., 1996), the peroxidase was examined by both SDS–PAGE and native PAGE with periodate/Schiff staining to determine the presence of a sugar component. The analyses showed that the peroxidase was indeed a glycoprotein. The characterization of the glycoprotein was confirmed by the purification step employing a concanavalin-A column; the peroxidase was released with a methyl D-glucopyranoside solution (Fig. 1C). Furthermore, when the peroxidase bound to the concanavalin-A column was treated with α-mannosidase (O'Donnell et al., 1992) there was a loss of molecular mass unit of about 2-kDa, whilst the enzyme activity was retained completely. These observations suggested that the 37-kDa peroxidase is a glycoprotein, and the linked oligosaccharide in the peroxidase is not essential for peroxidase activity.

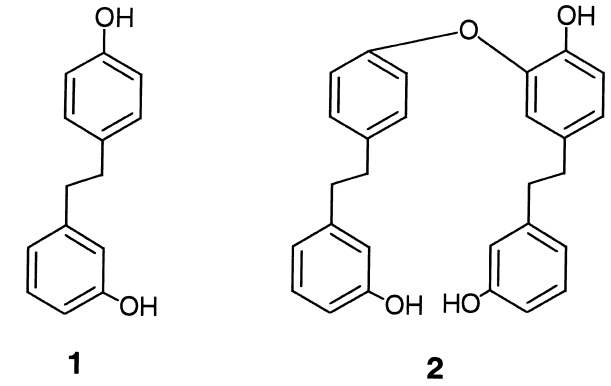
The peroxidase was subjected to micro-sequencing analysis without any treatment for removing the linked saccharides, but it was found that the N-terminal amino acid of the peroxidase was blocked. However, the analysis of a peptide obtained by digestion of the enzyme with V8 endopeptidase gave some structural information of the following partial amino acid sequence: Ala-Gln-Leu-Leu-Ala-Thr-Leu-Lys-Lys-Thr-X-Pro-Asn-Gly-Gly-Gly. This sequence shows a 50% identity to bovine glutathione peroxidase (Martin-Alonso et al., 1993). However, this sequence has no similarity with the sequences of other glutathione peroxidases from higher plants, such as *Arabidopsis thaliana* (Bartling et al., 1993; Mullineaux et al., 1998; Sugimoto and Sakamoto, 1997), *Helianthus annuus* (Roeckel-Drevet et al., 1998), *Pisum sativum* (Mullineaux et al., 1998), *Spinacia oleracea* (Sugimoto et al., 1997), *Lycopersicon esculentum* (Depege et al., 1998), *Nicotiana sylvestris* (Criqui et al., 1992), and *Zantedeschia aethiopica* L. (Lino-Neto et al., 1998).

2.2. Oxidation of lunularin (*1*) with the peroxidase

To investigate whether lunularin (**1**) is a substrate of the purified 37-kDa peroxidase, oxidation of **1** with the peroxidase in the presence of hydrogen peroxide was examined. Negative FAB-mass spectrum of the reaction products showed the peaks at *m*/*z* 213, 425, 637, and 849, corresponding to lunularin (**1**) (MW 214), lunularin

Table 1
Purification of the peroxidase from suspension culture of *M. polymorpha*

Step	Total actitivy (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
$(NH_4)_2SO_4$Fr.	77600	2480	32.3	(100)	(1.0)
Butyl Toyopearl	14300	72.8	196	18.4	6.1
DEAE Toyopearl	7710	22.6	341	9.9	11
ConA Sepharose	4130	1.8	2240	5.3	69



dimer (MW 426), trimer (MW 638), and tetramer (MW 850), respectively, as shown in Fig. 3. This indicates that lunularin (**1**) was converted into a dimer, a trimer and a tetramer, and some presumed polymers were also produced. One of the dimeric compounds was confirmed to be perrottetin E (**2**) (Asakawa, 1995) by ^{1}H NMR analysis of its trimethyl ether. This observation indicates that the 37-kDa peroxidase has an activity for oxidative phenol-coupling of lunularin (**1**). Asakawa et al. (1987, 1990; Asakawa, 1995) have reported that cyclic *bis*(bibenzyl) compounds such as marchantin C (**3**; MW 424) were isolated from *M. polymorpha*. Although it was considered that the dimer (**2**) obtained in the coupling reaction of lunuralin (**1**) catalyzed by the 37-kDa peroxidase may be an intermediate for the biosynthesis of the cyclic *bis*(bibenzyl) compounds such as marchantin C (**3**) in *M. polymorpha* (Friederich et al., 1999a,b), the presence of **3** could not be detected in the enzymatic oxidation. Recently, it has been reported that the specific cytochrome *P*-450 enzymes from *M. polymorpha* catalyzed the biosynthesis of marchantins A and C from phenylalanine via lunularic acid (**4**), but not lunularin (**1**) (Friederich et al., 1999a,b).

The purified peroxidase from *M. polymorpha* may allow us to shed some light on the biogenetic pathway that leads to oligomers from phenolic compounds and presumed cell wall constituents as a defense reaction against chemical stress. Further work is necessary to clarify the physiological functions of the secreted peroxidase in *M. polymorpha*.

3. Experimental

3.1. Materials

The cultured suspension cells of *M. polymorpha* (liverwort) were prepared by the culture of *M. polymorpha* cells in MSK-II medium with continuous shaking for two weeks (Chen et al., 1996).

Lunularin (**1**; 4-[2-(3-hydroxyphenyl)ethyl]phenol) [^{1}H NMR ($CDCl_3$) δ 2.83 (4H, *s* 1′-H_2 and 2′-H_2), 6.64 (1H, *s*, 2″-H), 6.65 (1H, *d*, $J=7.8$ Hz, 4″-H), 6.75 (1H, *d*, $J=7.6$ Hz, 6″-H), 6.76 (2H, *d*, $J=8.3$ Hz, 2-H and 6-H), 7.07 (2H, *d*, $J=8.3$ Hz, 3-H and 5-H), and 7.14 (1H, *t*, $J=7.8$ Hz, 5″-H)] was synthesized from *p*-methoxybenzyl chloride and triphenyl phosphine via 3,4′-dimethoxystilbene. Concanavalin-A Sepharose and low range molecular weight protein standard were obtained from Amersham Pharmacia Biotech. All other chemicals were from Wako Pure Chemical Ind. and Sigma.

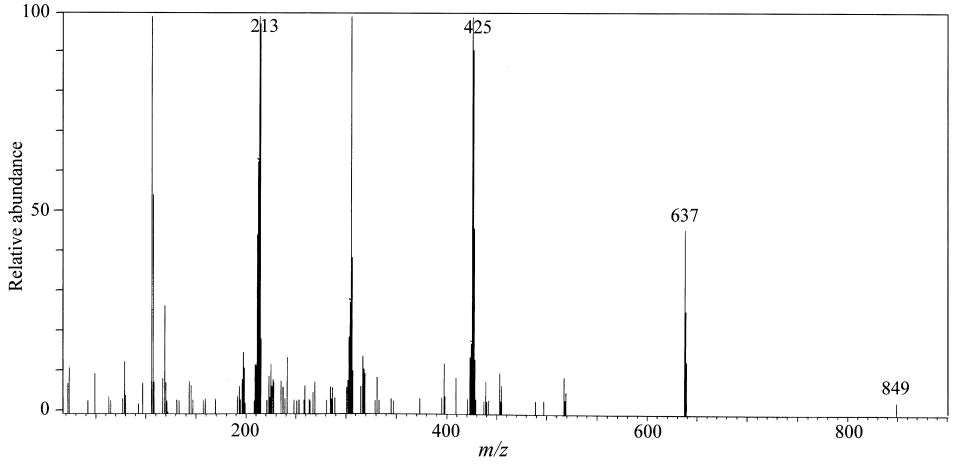


Fig. 3. Negative FAB-mass spectrum of the reaction products in the oxidation of lunularin (**1**) with the 37-kDa peroxidase.

3.2. Enzyme and protein assay

The activity of the enzyme during the purification was monitored by spectrophotometric measurements of the oxidation products of guaiacol (*o*-methoxyphenol) at 470 nm, according to the reported methods (Hendriks et al., 1991; Miller et al., 1992). The reaction was performed at 25°C with a reaction mixture containing 31 μM H_2O_2, 12.5 mM guaiacol and 200 μl of enzyme preparation in 3 ml of 3.1 mM sodium phosphate buffer (pH 7.0). One unit of peroxidase activity is defined as the amount of enzyme that catalyzed the oxidation of 1.0 μmol of guaiacol per min.

Protein concentration was determined by the method of Bradford, with bovine serum albumin as a standard protein (Bradford, 1976). The protein concentration during the enzyme purification process was determined by measurement of the absorbance at 280 nm.

3.3. Purification steps

The suspension cells (1380 g) of *M. polymorpha* were cultured for two weeks in 5.5 l of fresh MSK-II medium (Ono et al., 1979) at 25°C. Bornyl acetate (6.9 mmol) was added to the culture and it was allowed to grow for a further four days. The culture medium was collected by filtration and then fractionated with 40–80% ammonium sulfate. The precipitated proteins were resuspended in buffer A (10 mM sodium phosphate buffer, pH 7.0) and dialyzed against water. The solution was loaded onto a butyl-Toyopearl 650 M column equilibrated with buffer A containing 1.5 M ammonium sulfate and eluted with the ammonium sulfate gradient decreasing from 1.5 to 0 M in buffer A. The peroxidase was released from the column at an ammonium sulfate concentration of about 0.5 M. After the active fraction was dialyzed against buffer B (5 mM Tris–HCl, pH 8.0), the fraction was loaded onto a DEAE-Toyopearl 650 M column equilibrated with buffer B and eluted with a NaCl gradient from 0 to 1.5 M in buffer B. A cationic peroxidase fraction was eluted from the column, and then anionic peroxidases were released at about 1 M NaCl concentration. The cationic peroxidase fraction was further loaded onto a concanavalin-A Sepharose column equilibrated with buffer C (100 mM acetate buffer, pH 6.0 containing 1 M NaCl, 1 mM $MnCl_2$ and 1 mM $MgCl_2$) and eluted with a methyl D-glucopyranoside gradient from 0 to 100 mM in buffer C. A cationic peroxidase was eluted from the column at 60 mM methyl D-glucopyranoside.

3.4. Electrophoresis and molecular mass assay

SDS–PAGE was performed with a 12.5% gel. Isoelectric focusing was performed with an Ampholine PAG plate range between 3.5 and 9.5. These gels were stained with guaiacol in sodium acetate buffer (pH 5) for peroxidase activity and with Coomassie Brilliant Blue R-250 for total protein concentration. Periodate/Schiff staining was used for detection of glycosidic residues. The molecular mass of the peroxidase was estimated by SDS–PAGE and native PAGE with the low range molecular weight standards, and by gel filtration using a Tosoh G3000SW HPLC column.

3.5. N-Terminal amino acid sequencing

Purified peroxidase on a 12.5% SDS–PAGE gel was degraded partially by V8 endopeptidase and the peptide fragment on the SDS–PAGE gel was blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ, Millipore). The Coomassie stained peptide band was excised. Sequence analysis was performed with an online phenylthiohydantoin amino acids analyzer (Applied Biosystems Model 473A pulsed liquid sequencer).

3.6. Thermal stability

The 37-kDa peroxidase was incubated in 2 ml of 10 mM sodium phosphate buffer (pH 7.0) at 60°C for 20 h. An aliquot (0.2 ml) was taken out at 0.5, 1, 2, 6, 12, and 20 h, and assayed for peroxidase activity with guaiacol as substrate.

3.7. Oxidation of lunularin (*1*) with the peroxidase

To a solution (50 ml) of the 37-kDa peroxidase (150 units) in 50 mM sodium phosphate buffer (pH 5.0), 10 mg of lunularin (**1**) and 0.3 ml of 10% hydrogen peroxide were added and the mixture was incubated at 37°C for 10 min. After the incubation, the reaction mixture was extracted with EtOAc to give a crude product (8 mg). The crude products were analyzed by negative FAB-mass spectroscopy, which was recorded by the negative ion mode with a glycerol matrix on a JEOL SX-102A mass spectrometer equipped with a Xenon FAB gun (Fig. 3). The negative FAB-mass spectrum showed the molecular ion peaks at *m*/*z* 213, 425, 637, and 849, corresponding to lunularin (**1**) (MW 214), lunularin dimer (MW 426), trimer (MW 638), and tetramer (MW 850), respectively. However, a control experiment using a thermally denatured enzyme preparation did not result in formation of any oligomeric products.

Furthermore, the crude products for the enzymatic reaction were subjected to reversed-phase high performance liquid chromatography (HPLC) on an ODS column (Inertsil ODS-2, 4.6×250 mm, GL Sciences Inc.), eluted with H_2O–CH_3CN–2-propanol (4:5:1, v/v/v) at a flow rate of 0.3 ml/min with monitoring at 210 nm. HPLC analysis showed three peaks at retention time (Rt) 16.4 (85%), 25.0 (11%), and 33.9 (3%) min.

Products at Rt 16.4 and 25.0 were isolated by HPLC. The negative FAB-mass spectra of the isolated products at Rt 16.4 and 25.0 exhibited a molecular ion peak at *m/z* 213 and 425, respectively, which indicated lunularin (**1**) and lunularin dimer (**2**). The product at Rt 25.0 was further methylated with diazomethane and purified by thin layer chromatography on silica gel with hexane–EtOAc (4:1, v/v) to give a trimethyl ether: MS (EI) *m/z* 468 (M^+); ^{1}H NMR ($CDCl_3$) δ2.82 and 2.87 (8H, $-CH_2-$), 3.76 (3H, *s*, –OMe), 3.77 (3H, *s*, –OMe), 3.82 (3H, *s*, –OMe), and 6.7–7.2 (*m*, 14H, phenyl H), identical to that of authentic perrottetin E trimethyl ether.

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