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Novel, Unnatural Benzo-1,2,3-thiadiazole-7-carboxylate Elicitors of Taxoid Biosynthesis

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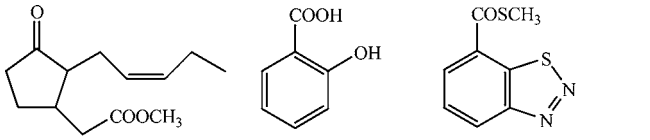
In order to establish the chemical biological technology for production of valuable secondary metabolites, a novel family of unnatural elicitors derived from the plant activator benzo-1,2,3-thiadiazole-7-carboxylic acid were designed and synthesized. New synthetic elicitors that showed powerful eliciting activities upon taxoid biosynthesis by *Taxus chinensis* suspension cells were obtained. For example, benzo-1,2,3-thiadiazole-7-carboxylic acid 2-(2-hydroxybenzoxyl)ethyl ester was more effective and resulted in nearly 40% increase in taxuyunnanine C content and production in comparison with methyl jasmonate, which was previously reported as the most powerful chemical elicitor for taxoid biosynthesis. The novel class of elicitors was found to induce plant defense responses, including promotion of H_2O_2 levels originating from oxidative burst and activation of phenylalanine ammonia lyase. Interestingly the plant defense responses induced corresponded well to the superior stimulating activity in *T. chinensis* cell cultures. The work indicates that the newly synthesized benzothiadiazoles can act as a new family of elicitors for taxoid biosynthesis in plant cells.

KEYWORDS: Benzo-1,2,3-thiadiazole-7-carboxylate derivatives; chemically synthesized elicitors; plant defense; plant cell culture; taxoid induction; secondary metabolites

INTRODUCTION

Plant cell culture is a promising alternative for mass production of valuable plant products. Among the manipulative techniques available to promote the productivity of useful secondary metabolites from plant cell cultures, the use of elicitors has been one of the best approaches for dramatically increasing product yields (*1*−*3*). Jasmonic acid and its methyl ester (methyl jasmonate) (**Figure 1**) are important members of the family of jasmonates that are linolenic acid-derived cyclopentanone-based compounds of wide distribution in higher plants. Exogenously applied methyl jasmonate was shown to result in enhanced production of secondary metabolites by a variety of plant species, and it was especially demonstrated as the most effective abiotic elicitors in *Taxus* cell cultures to produce the effective anticancer drug taxol and the neuron growth factor (NGF) active material taxuyunnanine C (*4*−*8*). Our recent work showed that new methyl jasmonate derivatives were more efficient than methyl jasmonate (*9*−*13*). However, it is very difficult to isolate, purify, and synthesize methyl jasmonate because of the two chiral carbons in the methyl jasmonate molecule (*14*, *15*). Therefore, the design and synthesis of novel unnatural elicitors are a great challenge to chemists. As a long-term interest, it is very important to establish the chemical biological technology for valuable secondary metabolites through the design and application of elicitors with new backbone structure.



methyl jasmonate salicylic acid *S*-methyl-benzo-1,2,3-thiadiazole-7-carboxylate

Figure 1. Three inducers of plant SAR (systemic acquired resistance).

Salicylic acid (**Figure 1**) is also an important signal molecule to cause systemic acquired resistance (SAR) in plants to pathogens and pests (*16*). Salicylic acid and its derivatives could also elicit secondary metabolism in *Taxus* cell cultures as with methyl jasmonate (*17*, *18*). It was noticed that *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate, the first commercial SAR activator for plants, could induce the same set of defense responses as salicylic acid in signal transduction (*19*). Therefore, it was interesting to prove whether or not the *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives could also induce secondary metabolism in plant cell cultures besides their effect on crop protection as reported.

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Systemic acquired resistance occurs during stress environment stimuli, including addition of elicitors, and leads to the increased amount of secondary metabolites such as phytoalexins (*20*). It is reasonable to assume that identical characteristics for defense responses exist in cells in vitro as in plants. Enhanced levels of H_2O_2 production and phenylalanine ammonia lyase (PAL) activity are two typical events related to defense responses. The production of H_2O_2 originating from oxidative burst is considered to be one important event in plant cell defense response (*21*). Our previous work proved that eliciting efficiency of methyl jasmonate derivatives in *Taxus chinensis* cell cultures corresponded well to the H_2O_2 level (*22*). As a key enzyme required for biosynthesis of phenolic defense compounds, PAL was reported to be a plant cell defense response marker caused by specific external stimuli, including additions of elicitors (*23*, *24*). *S*-Methyl benzo-1,2,3-thiadiazole-7-carboxylate as a SAR activator enhanced the elicitation of PAL mRNA and the induction of coumarin secretion in parsley cells (*25*). However, few reports have been published about *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate as elicitor in the production of secondary metabolites. When *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate was added to medium on which *Amni majus* cultures were planted, the growth rates of the elicited and the nonelicited tissues were not significantly different, suggesting that *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate was an activator of SAR but not an elicitor (*26*). However, a mixture of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate and yeast enhanced rosmarinic acid production in a suspension culture of *Agastache rugosa* (*27*).

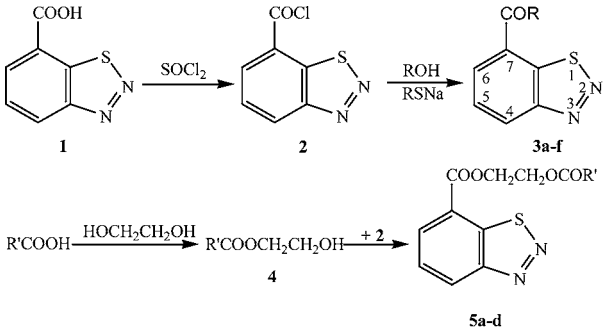
In this study of the important anticancer drug taxol and other taxoids, a *T. chinensis* cell system was selected to evaluate the eliciting efficiency of new synthetic *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives and the relationship between the defense response and metabolite production. A series of novel benzo-1,2,3-thiadiazole-7-carboxylate derivatives was designed and synthesized, and their eliciting activities in suspension cultures of *T. chinensis* were evaluated. In addition, two early and important events in plant defense responses, oxidative burst and activation of PAL, were also evaluated to investigate the relationship between the defense response and the metabolite production.

MATERIALS AND METHODS

Triethylamine was dried over KOH and distilled. Methyl jasmonate was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Trifluoroethanol, pentafluoropropanol, and pentafluorobenzyl alcohol were purchased from Fluka Chem. Co. All other solvents and chemicals were reagent grade and used without further purification. Melting Points were recorded by an electrothermal digital apparatus and are uncorrected. The ^{1}H NMR spectra were recorded with a Brucker AM-500 spectrometer. The MS spectra were measured with a HRMS Micromass GCT CA 055 spectrometer. The elemental analysis data was measured by an Elementar vario EL III analyzer.

Syntheses of New Elicitors. The compound **3c (Figure 2**) was prepared from benzo-1,2,3-thiadiazole-7-carboxylic acid chloride **2** and CH_3SNa solution instead of CH_3SH as in the literature (*28*−*30*). **3d**−**f** were obtained by the acylation of fluorine-containing alcohols and **2**. **5a**−**d** were prepared from benzo-1,2,3-thiadiazole-7-carboxylic acid chloride **2** and corresponding monoesterification of aromatic acids and diols (**4**) (*31*). Specific detailed data for each of the new compounds are given below.

Synthesis of *S*-Methyl Benzo-1,2,3-thiadiazole-7-carboxylate (3c) (*28*). A mixture of 0.3 g (1.7mmol) of benzo-1,2,3-thiadiazole-7-carboxylic acid and thionyl chloride (5 mL) was heated and maintained at refluxing temperature of 90 °C for 8 h. The resulting oil was solidified when the excess thionyl chloride was removed by vacuum distillation.



Compounds	R	compounds	R′
3a	OH	5a	C_6H_5
3b	OCH_3	5b	C_6F_5
3c	SCH_3	5c	$C_6H_4Cl(2\text{-})$
3d	OCH_2CF_3	5d	$C_6H_4OH(2\text{-})$
3e	$OCH_2CF_2CF_3$		
3f	$OCH_2C_6F_5$		

Figure 2. Preparation of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives.

The benzo-1,2,3-thiadiazole-7-carboxylic acid chloride was dissolved in methylene chloride (20 mL) and cooled by ice−water bath, 15% methyl methyl mercaptan sodium solution (3 mL) was added dropwise with stirring during 0.5 h, and the mixture stirred at room temperature for another 5 h. The mixture was washed with water three times and dried with anhydrous $MgSO_4$ overnight. The filtrate was concentrated by vacuum distillation and the brown oil obtained was purified by silica gel column chromatography and eluted with ethyl acetate/petroleum (1:4). Light yellow crystals (0.12 g, 34% yield) were obtained. Mp: 134−135 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.59 (s, 3H, SCH_3), 7.98 (dd, 1H, J = 7.26 and 7.89 Hz, 5-Ar-H), 8.60 (d, 1H, J = 7.26 Hz, 4-Ar-H), 9.08 (d, 1H, J = 7.88 Hz, 6-Ar-H). IR (KBr): ν 3000, 1625, 1524, 1470, 1398, 1087, 1071, 908, 780, 730 cm^{-1}.

Synthesis of 2,2,2-Trifluoroethyl Benzo-1,2,3-thiadiazole-7-carboxylate (3d) (General Procedures for 3e and 3f) (*28*). Benzo-1,2,3-thiadiazole-7-carboxylic acid chloride prepared as above was dissolved in dry toluene (4 mL) and then dropped into to a mixture of trifluoroethanol (2 mL), toluene (4 mL), and triethylamine (0.8 mL) with stirring at room temperature. After 4 h, the mixture was poured into water (20 mL), extracted with ethyl acetate, washed with water, and dried with anhydrous $MgSO_4$ overnight. The filtrate obtained was concentrated by vacuum distillation and the brown solid obtained was purified by silica gel column chromatography and eluted with benzene. Light yellow crystals (0.24 g, 54%) were obtained. Mp: 119−121 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.16 (q, 2H, J = 9.0 Hz, CH_2CF_3), 7.98 (dd, 1H, J = 8.35 and 7.36 Hz, 5-Ar-H), 8.49 (d, 1H, J = 7.37 Hz, 4-Ar-H), 9.10 (d, 1H, J = 8.35 Hz, 6-Ar-H). IR (KBr): ν 3074, 2978, 1718, 1558, 1410, 1305, 1180, 1135, 1050, 980, 820, 753 cm^{-1}. HRMS (EI): *m/z*: 262.0026 [M$^+$], $C_9H_5F_3N_2O_2S$ requires 262.0024.

3e: ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.25 (t, 2H, J = 9.0 Hz, $CH_2CF_2CF_3$), 8.00 (dd, 1H, J = 8.13 Hz and 7.32 Hz, 5-Ar-H), 8.45 (d, 1H, J = 7.32 Hz, 4-Ar-H), 9.11 (d, 1H, J = 8.12 Hz, 6-Ar-H). IR (KBr): ν 3081, 2963, 1710, 1558, 1454, 1298, 1190, 1130, 1070, 870, 830, 764 cm^{-1}. HRMS (EI): *m/z* 311.9613 [M$^+$], $C_{10}H_5F_5N_2O_2S$ requires 311.9963.

3f: ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.62 (s, 2H, CH_2), 7.93 (dd, 1H, J = 7.35 and 8.11 Hz, 5-Ar-H), 8.40 (d, 1H, J = 7.35 Hz, 4-Ar-H), 9.04 (d, 1H, J = 8.10 Hz, 6-Ar-H). IR (KBr): ν 3059, 2978, 1707, 1506, 1410, 1291, 1135, 1040, 935, 870, 770, 615 cm^{-1}. HRMS (EI): *m/z* 359.9983 [M$^+$], $C_{14}H_5F_5N_2O_2S$ requires 359.9992.

Synthesis of Benzo-1,2,3-thiadiazole-7-carboxylic Acid 2-Benzoyloxyethyl Ester (5a) (General Procedures for 5b, 5c and 5d) (*28*). Sulfuric acid (98%, 0.5 mL) was added to a mixture of benzoic acid

(2.4 g, 0.02 mol) and ethylene glycol (6 mL). The mixture was heated to 120 °C with stirring for 2.5 h. After cooling, water (40 mL) was added and the mixture was neutralized with $NaHCO_3$. The separated oil was washed with $NaHCO_3$ solution and water and then dissolved in ethyl acetate and dried with $MgSO_4$. The 2-hydroxyethyl benzoate was obtained as a colorless oil after concentration (87%) (*31*).

Benzo-1,2,3-thiadiazole-7-carboxylic acid chloride was dissolved in dry toluene (4 mL) and then dropped into a mixture of 2-hydroxyethyl benzoate (0.3 g, 1.7 mmol), toluene (6 mL), and triethylamine (0.36 mL) with stirring at room temperature. After 16 h of stirring, the mixture was poured into water (20 mL), extracted with ethyl acetate, washed with water, and dried with anhydrous $MgSO_4$ overnight. The filtrate was concentrated by vacuum distillation and the brown solid obtained was purified by silica gel column chromatography, eluted with ethyl acetate/hexane (2:1) as white crystals (0.24 g, 55%). Mp: 79−82 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 4.67−4.78 (m, 4H, OCH_2CH_2O), 7.48 (dd, 2H, J = 7.77 Hz and 7.38 Hz, 3′,5′-Ar-H), 7.63 (t, 1H, J = 7.39 Hz, 4′-Ar-H), 7.92 (dd, 1H, J = 7.35 and 7.87 Hz, 5-Ar-H), 7.95 (d, 2H, J = 7.78 Hz, 2′, 6′-Ar-H), 8.42 (d, 1H, J = 7.30 Hz, 4-Ar-H), 9.01 (d, 1H, J = 7.99 Hz, 6-Ar-H). IR (KBr): ν 3070, 2945, 1720, 1570, 1460, 1270, 1120, 1070, 860, 760, 720 cm^{-1}. HRMS (EI): *m/z* 328.0503 [M$^+$], $C_{16}H_{12}N_2O_4S$ requires 328.0518. Elemental analysis (%) found: C 58.66, H 3.54, N 8.49. Requires: C 58.53, H 3.68, N 8.53.

5b: ^{1}H NMR (500 MHz, DMSO-d_6): δ 4.75−4.80 (m, 4H, OCH_2-CH_2O), 7.96 (dd, 1H, J = 7.31 and 8.37 Hz, 5-Ar-H), 8.42 (d, 1H, J = 7.30 Hz, 4-Ar-H), 9.0 (d, 1H, J = 8.37 Hz, 6-Ar-H). HRMS (EI): *m/z* 418.0373 [M$^+$], $C_{16}H_7F_5N_2O_4S$ requires 418.0358.

5c: ^{1}H NMR (500 MHz, DMSO-d_6): δ 4.69−4.77 (m, 4H, OCH_2-CH_2O), 7.40−7.44 (m, 1H, Ar-H), 7.53−7.55 (m, 2H, Ar-H), 7.82 (d, 1H, J = 8.53 Hz, Ar-H), 7.94 (dd, 1H, J = 7.29 and 7.88 Hz, 5-Ar-H), 8.43 (d, 1H, J = 7.28 Hz, 4-Ar-H), 9.03 (d, 1H, J = 7.90 Hz, 6-Ar-H). IR (KBr): ν 3067, 2956, 1703, 1558, 1436, 1284, 1135, 1060, 860, 749, 695 cm^{-1}. HRMS (EI): *m/z* 362.0092 [M$^+$], $C_{16}H_{11}ClN_2O_4S$ requires 362.0128. Elemental analysis (%) found: C 53.20, H 3.06, N 7.59. Requires: C 52.97, H 3.06, N 7.72.

5d: ^{1}H NMR (500 MHz, DMSO-d_6): δ 4.71−4.80 (m, 4H, OCH_2-CH_2O), 6.88−6.91 (m, 1H, Ar-H), 6.95−6.97 (m, 1H, Ar-H), 7.48−7.51 (m, 1H, Ar-H), 7.77−7.80 (m, 1H, Ar-H), 7.94 (dd, 1H, J = 7.34 and 8.34 Hz, 5-Ar-H), 8.45 (d, 1H, J = 7.33 Hz, 4-Ar-H), 9.04 (d, 1H, J = 8.34 Hz, 6-Ar-H), 10.38 (s, 1H, OH). IR (KBr): ν 3280 (OH), 3059, 2963, 1722, 1666, 1606, 1580, 1480, 1400, 1290, 1250, 1161, 1080, 860, 756, 700 cm^{-1}. HRMS (EI): *m/z* 344.0451 [M$^+$], $C_{17}H_{12}$-$Cl_2N_2O_5S$ requires 344.0467. Elemental analysis (%) found: C 55.88, H 3.53, N 7.99. Requires: C 55.81, H 3.51, N 8.14.

Cell Subculture Conditions. The *T. chinensis* cell culture was maintained on Murashige and Skoog medium supplemented with 0.5 mg/L of 6-benzyladenine, 0.2 mg/L of 2,4-dichlorophenoxy acetic acid, 0.5 mg of naphthalene acetic acid, 100 mg/L of ascorbic acid, and 30 g/L of sucrose. The pH was adjusted to 5.8 before autoclaving. The cells were subcultured at an interval of 2 weeks in a 500-mL Erlenmeyer flask containing 200 mL of medium on a rotary shaker at 110 rpm and 25 °C in the dark.

Elicitation Study. For elicitation experiments, ca. 5 g of fresh cell aggregates were incubated into a 250-mL Erlenmeyer flask containing 50 mL of medium with the same culture conditions as in subcultures. All elicitors were added to the cultures in 1 μL of ethanol per 1 mL of culture medium, sterilized by filtering through 0.22 μm polyvinylidene-difluoride (PVDF) syringe filters (Millipore) and finally added to the culture medium at day 7 of the subculturing period. The experiments were performed in triplicate.

Measurement of Cell Mass. The samples from flasks were filtered under vacuum and washed with several volumes of distilled water to remove residual medium. The cells were weighed to obtain the fresh weight, and 5 g was dried at 50 °C to a constant weight for the measurement of cell dry weight (DW).

Taxane Extraction and Analysis. For taxane extraction, 100 mg of powdered dry cells was soaked in 2 mL of methanol for 2 days and then the mixture was ultrasonicated twice for 40 min. After centrifugation at 4000*g* for 10 min, the extract was removed and the cell debris was extracted once more with 2 mL of methanol. The combined extracts were evaporated to dryness at 25 °C. The residue was dissolved in 2 mL of dichloromethane and 2 mL of distilled water. After sufficient mixing, the mixture was centrifuged at 4000*g* for 10 min. The organic phase was collected and evaporated to dryness at 25 °C. The residue was dissolved in 1 mL of methanol and filtered through a 0.22 μm PVDF syringe filter (Millipore); 20 μL was analyzed by reverse phase HPLC, using a Hewlett-Packard series 1100 HPLC system (Agilent, Palo Alto, CA). A 250 × 4.6 mm i.d. 5 μm Zorbax Phenyl column (Agilent) with a Zorbax Phenyl guard column was used at 25 °C. The mobile phase consisted of acetonitrile and water (58:42, v/v), and the flow rate was 1 mL/min. Taxane was monitored at the wavelength of 227 nm by using authentic standards as the reference.

Assay of Oxidative Burst. H_2O_2, associated with the so-called oxidative burst, originates from superoxide generated by a plasma membrane-associated NADH oxidase in challenged plant cells. H_2O_2 produced by the cells and released into the medium was determined by the scopoletin fluorescence oxidative quenching method (excitation wavelength, 350 nm; emission, 460 nm) according to the literature (*32*). To measure H_2O_2 accumulation, samples were taken at various intervals over the 180-min period following elicitation. Aliquots of 4 mL of extracellular medium were mixed with 40 μL of 5 mM stock solution of scopoletin in DMSO and 40 μL of 1 mg/mL stock solution of peroxidase (Sino-American Biotechnology Co., Shanghai), respectively. The concentration of H_2O_2 in the medium was calculated from the fluorescence decrease using a calibration curve established in the presence of H_2O_2. A standard curve by adding scopoletin to the solutions at different H_2O_2 concentrations was prepared by using cell-free medium.

Effects of various jasmonate elicitors on peroxidase-dependent assay for H_2O_2 determination were tested. Various jasmonates were added to cell-free medium to obtain final concentrations of 10, 50, or 100 μM, respectively. In the assay conditions, an addition of jasmonate elicitors had no obvious effect on the decrease of scopoletin fluorescence because of H_2O_2 addition.

Enzyme Extraction and PAL Activity Analysis. Cells were harvested as described above, and then samples of 1 g of fresh cells were frozen in liquid nitrogen. After grinding the cells with a mortar and pestle, crude enzymes in the frozen powder were extracted by adding 50 mg of polyvinypyrrolidone and 2 mL of prechilled buffer of pH 7.2 (0.1 M phosphate buffer, 2 mM ethylenediaminetetraacetic acid, 4 mM dithiothreitol), and then the mixture was homogenized at 4 °C. The mixture was centrifuged at 10 000 *g* for 30 min at 4 °C. The supernatant was used directly for PAL assay using a method slightly modified from a previous report (*33*): 200 μL of the protein extracts was incubated with 120 μL of 0.1 M L-phenylalanine dissolved in 280 μL of 0.1 M borate buffer of pH 8.8 at 30 °C for 60 min. The reaction was stopped by adding 50 μL of 5 N trichloroacetic acid. After centrifugation at 10 000*g* for 30 min, the supernatant was analyzed by HPLC under the following conditions: solvent, water:methanol:acetic acid (40:60:1, v/v/ v); detection, 280 nm; flow, 1 mL/min; column, 250 × 4.6 mm i.d., 5 μm Zorbax ODS (Agilent); injection volume, 20 μL. Genuine *trans*-cinnamic acid (Sigma) was used as an external standard. One unit (U) of enzyme activity is defined as the amount of enzyme forming 1 pmol of *trans*-cinnamic acid from the substrate L-phenylalanine per min.

RESULTS AND DISCUSSION

Preparation of *S*-Methyl Benzo-1,2,3-thiadiazole-7-carboxylate and Its Derivatives (Figure 2). *S*-Methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives (**1**, **3a**, and **3b**) were synthesized according to the literature (*28*). Target compounds **3c**−**f** and **5a**−**d** were prepared as in **Figure 2**. Compounds **3d**−**5d** have not been previously reported. All compounds were separated and purified by recrystallization or silica gel chromatography. Their structures were identified by ^{1}H NMR, HRMS, and elemental analyses.

Compound **3c** was prepared by acylation of benzothiadiazole-7-carboxylic acid chloride and CH_3SNa solution instead of CH_3SH (*29*, *30*), which avoids the odor and volatility of CH_3SH.

Table 1. Effects on the Maximum Cell Concentration of Compound 3a on the Taxuyunnanine C Content and Production in Cell Cultures of *T. chinensis*

concn of **3a** (μM)	cell concn (g DW/L) (14 d)	taxuyunnanine C (21 d)[a] content (mg/g DW)	taxuyunnanine C (21 d)[a] production (mg/L)
0	15.7 ± 0.5	15.3 ± 0.1	160 ± 3.8
1	15.5 ± 0.6	16.2 ± 0.3	226 ± 7
10	16.1 ± 0.6	18.5 ± 1.1	240 ± 15
100	10.9 ± 0.6	18.7 ± 0.5	255 ± 8
200	8.5 ± 0.3	19.4 ± 0.1	151 ± 14

[a] Data are the means with standard deviations of three flasks.

Table 2. Compounds Prepared and Their Eliciting Activity Comparison with Controls, Methyl Jasmonate, and Salicylic Acid in *Taxus chinensis* Cell Suspension Culture

compd	R	cell growth (elicitor/control)	taxuyunnanine C content (elicitor/control)	taxuyunnanine C production (elicitor/control)	activity score
3a	OH	0.81	1.40	1.52	1.00
3b	OCH_3	0.86	1.48	1.72	1.13
3c	SCH_3	0.85	1.44	1.62	1.07
3d	OCH_2CF_3	0.96	2.07	1.87	1.23
3e	$OCH_2CF_2CF_3$	0.83	1.39	1.35	0.89
3f	$OCH_2C_6F_5$	1.01	1.28	1.25	0.82
5a	C_6H_5	1.07	1.26	1.13	0.74
5b	C_6F_5	0.95	1.38	1.34	0.88
5c	$C_6H_4Cl(2\text{-})$	1.11	1.96	1.90	1.25
5d	$C_6H_4OH(2\text{-})$	1.04	3.37	3.36	2.21
MJA	–	0.95	2.44	2.44	1.60
SA	–	0.98	1.48	1.68	1.10

However, esterification of benzothiadiazole-7-carboxylic acid and trifluoroethanol did not work in the preparation of **3d** under some conditions, because of the acidity of trifluoroethanol, so condensation of benzothiadiazole-7-carboxylic acid chloride and trifluoroethanol in the presence of triethylamine was adopted. In the process of preparing compound **5d**, the protection of hydroxyl group with acetic anhydride was not required. Because of steric factors and intramolecular H-bond (*34*), the free phenolic hydroxyl group would not participate in the reaction as long as no excess acid chloride was added.

Biological Activity of *S*-Methyl Benzo-1,2,3-thiadiazole-7-carboxylate and Its Derivatives. For the evaluation of the newly synthesized derivatives of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate, a suspension culture of *T. chinensis* cell was used. Under the same experiment conditions, no elicitor was introduced in the culture system as control. To compare the elicitation efficiency of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives, methyl jasmonate, which was the best chemical elicitor reported earlier, was also used (*35*, *36*). The optimal concentration and culture time experiments of compound **3a** as example are shown in **Table 1**. The optimal concentration was 100 μm and the maximal taxuyunnanine C content was reached in 21 days. The taxuyunnanine C content and production of all the new elicitors are listed in **Table 2**.

Compounds **3a**−**c** are known or commercial SAR activators for plants. It was shown in our experiments that although *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate (**3c**) was the most effective in causing SAR in plants (*30*), it was not the most effective elicitor in the cell culture among its derivatives. Its eliciting activity was a little lower than that of methyl benzo-1,2,3-thiadiazole-7-carboxylate (**3b**).

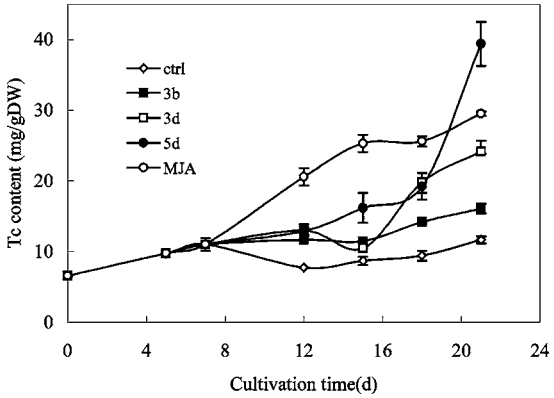


Figure 3. Eliciting activity of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives on taxuyunnanine C content (control, methyl jasmonate, **3b**, **3d**, and **5d**).

As shown in **Figure 3**, the *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives showed different dynamic profiles of taxuyunnanine C content from methyl jasmonate. Generally, an increase in taxuyunnanine C content was observed after the addition of methyl jasmonate at 7 day, but it did not appear in the case of *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives. After 15 days, there was an obvious increase in taxuyunnanine C content for *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives. During the cultivation, at 15 days the taxuyunnanine C content for **5d** was 16.2 mg/g dry weight (DW), and 6 days later, their taxuyunnanine C content increased rapidly to 39.4 mg/g DW.

By comparison of **3d** with **3b**, it was found that the presence of a trifluoromethyl group (**3d**) produced nearly a 40% increase in taxuyunnanine C content, but the eliciting activities of **3e** and **3f**, which have a pentafluoroethyl or pentafluorophenyl group, were lower than that of **3b**. Fluorine-containing compounds usually have higher lipophilicity, and it seemed that too high a lipophilicity (*A* log P_{98} of **3d**, **3e** and **3f** is estimated to be 2.82, 3.31, and 4.42, respectively), might be not beneficial to their interaction with their receptors in plant cell membranes.

Introduction of aromatic acids to benzo-1,2,3-thiadiazole-7-carboxylates gave better results. Activities of **5a**, **5c** and **5d** seemed to relate to 2-position substituents on the benzoic acid moiety, and the introduction of 2-Cl and 2-OH contributed remarkably to the eliciting activity. The compound **5d** is the only one of the benzo-1,2,3-thiadiazole-7-carboxylates whose eliciting activity surpassed that of methyl jasmonate, which has been considered to be one of the most effective abiotic elicitors in *T. chinensis* cell cultures. Its bioactivity was also more than that of two parts of benzo-1,2,3-thiadiazole-7-carboxylic acid and salicylic acid combined.

To find some biological evidence for our results, **3c** and **3d** were selected to demonstrate typical examples of the investigation of the elicitor-induced plant defense responses, while the cultures with nonelicitor addition was used as controls.

Figure 4 shows that increased levels of H_2O_2 production followed by increased PAL activity and taxoid overproduction was observed in *T. chinensis* suspension cultures elicited with *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives. There is an increasing body of evidence that H_2O_2 can act as a diffusible signal to activate defense genes and the biosynthesis of plant secondary metabolites (*37*−*39*). The increased PAL activity provided additional evidence for occurrence of the oxidative burst in elicited cell cultures. In addition, the time courses of H_2O_2 production and PAL activity of cells elicited

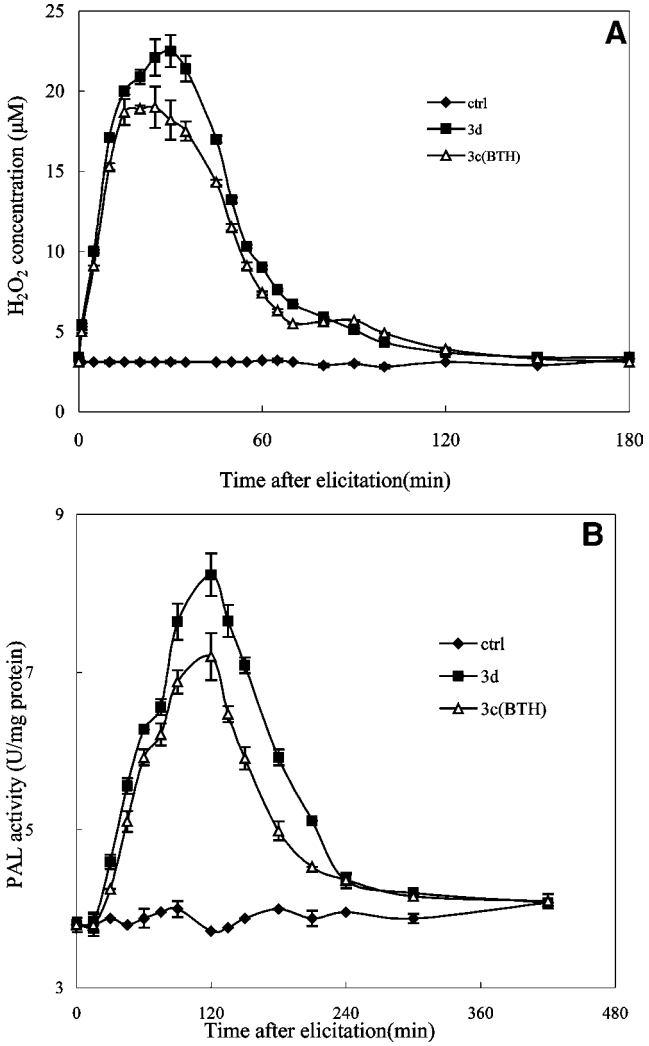


Figure 4. Comparison of (**A**) **3d** and *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate-induced H_2O_2 production and (**B**) PAL activity in *T. chinensis* suspension cultures.

with synthetic *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives were almost the same as those of methyl jasmonate-treated cells in our previous work (data not shown). Our results are also different from other reports. Thaler et al. (*19*) claimed that *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate could attenuate the methyl jasmonate-related expression of the anti-herbivore defense-related enzyme polyphenol oxidase in plants, while Kim et al. (*27*) reported that in elicited secondary metabolites, the addition of methyl jasmonate could increase rosmarinic acid accumulation in *Coleus blumei*, but *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate alone did not. This suggests that methyl jasmonate and *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate may act in different ways in plant cells, but they displayed almost similar activities in eliciting taxuyunnanine C production in *T. chinensis* cell cultures in our work.

Interestingly, the H_2O_2 level and PAL activity were induced to a higher level in the cells treated with **3d** than those in *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate-treated cells, which corresponded well to the superior stimulating activity for the former versus the latter. It indicates that the synthetic *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivative (**3d**) can act as a more powerful inducing signal than *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate for secondary metabolite production by plant cell cultures. The results also implied that the measurement of H_2O_2 level and PAL activity of elicitors might provide another method to evaluate efficiency of new elicitors in plant cell cultures.

Compounds **3d**, **5c**, and **5d** had excellent eliciting activities. By comparison with methyl jasmonate, **5d** was more effective and resulted in 38% increase in taxuyunnanine C content and production. It is much easier to synthesize these compounds than to synthesize methyl jasmonate derivatives (*40*−*45*). To the best of our knowledge, this is the first report that new *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives effectively enhanced both taxuyunnanine C content and production. In addition, *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives were found to induce plant defense responses as effectively as methyl jasmonate, including oxidative burst and activation of PAL. The work indicates that the newly synthesized *S*-methyl benzo-1,2,3-thiadiazole-7-carboxylate derivatives may act as powerful inducing signals for secondary metabolite biosynthesis in plant cell cultures.

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