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Flavone production in transformed root cultures of *Scutellaria baicalensis* Georgi

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Abstract

A flavone derivative 5,2′-dihydroxy-6,7,8,3′-tetramethoxyflavone identified by ^{1}H-, ^{13}C-NMR, FABMS, together with two known phenolics skullcapflavone I and acteoside (**3**), were isolated from *Scutellaria baicalensis* transformed roots (clone C) into which the β-glucuronidase gene had been integrated by the infection with *Agrobacterium rhizogenes* A13. Another clone of transformed roots (clone W) was also induced by infection with *A. rhizogenes* ATCC 15834 (wild type). Both clones C and W, cultured in phytohormone-free BF liquid medium, produced **3** at a high content (maximum; clone C: 1.81% and clone W: 2.96% of tissue dry weight) under the light and dark conditions. The contents of glucuronide-type flavonoids, such as baicalin and wogonin 7-*O*-glucuronide in clone W, were almost three times higher than those in clone C. Compound **3**, which was not detected in the intact plant roots, also accumulated in leaf (0.23% dry weight) and root (0.68% dry weight) parts of in vitro cultured plantlets.

Keywords: *Scutellaria baicalensis*; Labiatae; Hairy root; *Agrobacterium rhizogenes*; β-glucuronidase; Flavone; 5,2′-dihydroxy-6,7,8,3′-tetramethoxyflavone; Acteoside

1. Introduction

The roots of *Scutellaria baicalensis* Georgi (Labiatae), which contain a variety of flavones, phenylethanoids, amino acids, sterols and essential oils (Takido, 1987), has been used as an important Chinese crude drug ('Wogon' in Japanese) for treatment of hepatitis, tumor, diarrhea and inflammatory diseases (Chiang Su New Medical College, 1977). This plant is particularly rich in flavonoid derivatives, most of which are glucuronides, having methoxyl and/or hydroxyl groups at various positions of the aromatic rings. In a previous paper (Nishikawa & Ishimaru, 1997), we reported the modification of biosynthesis of glucuronide-type flavonoids in transformed roots, into which the β-glucuronidase (GUS) gene had been integrated by infection with *Agrobacterium rhizogenes* A13. In the present study, we report the isolation of a new flavone derivative together with two known phenolics, skullcapflavone I (Takido, Yasukawa, Matsuura & Iinuma, 1979) and acteoside (Sasaki, Nishimura, Chin & Mitsuhashi, 1989) from the root cultures and the chemical structure of the new flavone was elucidated based on spectroscopic evidence. Another transformed root culture of this species, induced by *A. rhizogenes* ATCC 15834 (wild type, without GUS gene integration), was also established and the phenolic (flavonoid and acteoside) content of the transformed roots was compared with those induced by *A. rhizogenes* A13.

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Table 1
^{1}H- and ^{13}C-NMR spectral data of **1** (in DMSOd_6, δ values)[a]

	^{13}C	^{1}H
2	161.95	–
3	111.98	6.39 s
4	182.49	–
5	148.55	–
6	135.85	–
7	152.64	–
8	132.70	–
9	146.30	–
10	106.40	–
1′	114.08	–
2′	152.30	–
3′	157.78	–
4′	106.65	6.93 d (8)
5′	131.94	7.47 t (8)
6′	113.79	7.26 d (8)
5-OH	–	12.64
6-OMe	60.62	3.84 s
7-OMe	61.50	4.02 s
8-OMe	61.89	3.81 s
3′-OMe	56.17	3.79 s

[a] Coupling constants (J in Hz) in parentheses.

2. Results and discussion

2.1. Isolation of phenolics from transformed roots

Of the various clones (A–F) isolated from *S. baicalensis* transformed roots (Nishikawa & Ishimaru, 1997), into which GUS gene had been integrated by infection with *A. rhizogenes* A13, clone C was selected for these experiments. Firstly, for the investigation of new metabolites in transformed roots of *S. baicalensis*, the secondary metabolites (except nine phenolics, previously isolated from *S. baicalensis* roots (Ishimaru et al., 1995)) of clone C were determined; the methanol extract of the roots was applied to Sephadex LH-20 and preparative C18 columns to afford three phenolics **1**–**3**. Of these, compounds **2** and **3** were identified as skullcapflavone I (Takido et al., 1979) and acteoside (Sasaki et al., 1989), respectively, by comparison of the spectral data (^{1}H- and ^{13}C-NMR) with those of reference compounds.

2.2. Characterization of a new phenolic compound

A new compound **1**, obtained as a pale yellow amorphous powder, exhibited $[M]^+$ peak at m/z 374 ($C_{19}H_{18}O_8$) in the positive FABMS. The ^{1}H-NMR spectrum (Table 1) of **1** showed four methoxyl (δ 3.79, 3.81, 3.84 and 4.02), a non-coupled olefinic (δ 6.39), ABC-type aromatic (δ 6.93, 7.26 and 7.47) and a chelated hydroxyl (δ 12.64) proton signals. The ^{13}C-NMR spectrum of **1** (Table 1) exhibited 15 carbons arising from a flavone moiety (C-2 ∽ 10 and C-1′ ∽ 6′) whose

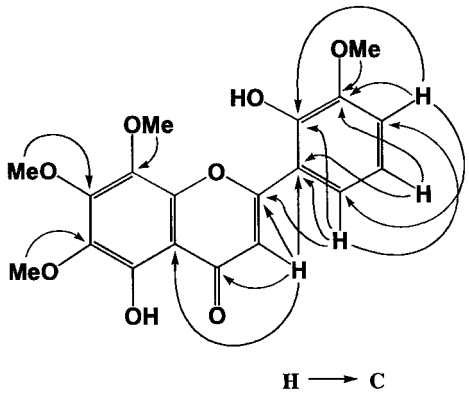

Fig. 1. Long-range ^{1}H–^{13}C correlations observed in the HMBC spectrum of **1**.

A-ring carbon chemical shifts were in good agreement with those of 5-hydroxy-6,7,8-trimethoxyflavone (Ishimaru et al., 1995). The existence of a characteristic signal (δ 12.64) of the chelated hydroxyl in the ^{1}H-NMR spectrum and the bathochromic shift observed in UV spectrum of **1** on the addition of $AlCl_3$ and $AlCl_3$ + HCl also supported the position of the hydroxyl group at C-5. The substitution pattern of B-ring (ABC-type) of **1** was concluded to be 2′-hydroxy-3′-methoxyl type by spectral evidences of the ^{1}H–^{13}, C-NMR (Table 1), HMBC (Fig. 1) and ^{1}H–^{1}H NOESY spectrum which showed a NOE correlation between a methoxyl (δ 3.79, H-3′-OMe) and an aromatic (δ 6.93, H-4′) proton. Therefore, from the data mentioned above, **1** was characterized as 5,2′-dihydroxy-6,7,8,3′-tetramethoxyflavone.

2.3. Phenolic production by transformed roots

Another transformed root clone W was also induced by infection with *A. rhizogenes* ATCC 15834 (wild type) without GUS gene integration. To determine the phenolic production in clones C and W in detail, a time course experiment on the growth and the phenolic content of these clones was carried out. Both clones grew well in a phytohormone-free BF liquid medium showing a maximum biomass (0.6–0.7 g dry weight/flask) of the roots at the later phase of culture growth under light or dark conditions (Fig. 2).

The production of fourteen phenolics, i.e. **1**–**3** and related flavonoids such as 5,2′,6′-trihydroxy-6,7-dimethoxyflavone 2′-*O*-β-D-glucopyranoside (**4**) (Ishimaru et al., 1995), 5,2′,6′-trihydroxy-6,7,8-trimethoxyflavone 2′-*O*-β-D-glucopyranoside (**5**) (Ishimaru et al., 1995), baicalin (**6**) (Takido, 1973), baicalin methyl ester (**7**) (Tomimori, Miyaichi & Kizu, 1982), wogonin 7-*O*-glucuronide (**8**) (Takido, 1973), 5,7,2′,6′-tetrahydroxyflavone (**9**) (Tomimori et al., 1982), 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone (**10**) (Tomimori, Miyaichi, Imoto, Kizu & Tanabe,

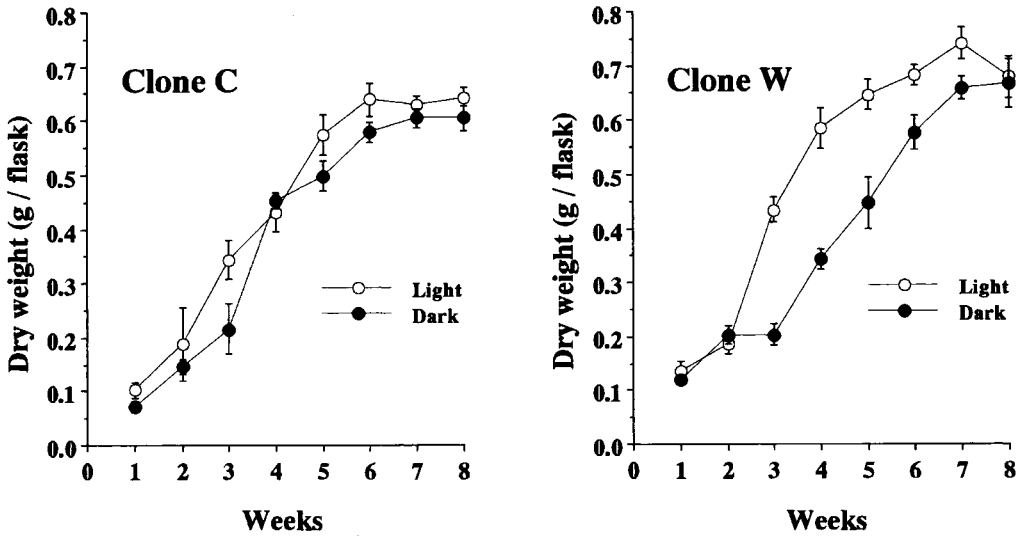

Fig. 2. Growth of *S. baicalensis* transformed roots (clones C and W) cultured in BF liquid medium in the light or dark.

1984), skullcapflavone II (**11**) (Takido et al., 1975), 3,5,7,2′,6′-pentahydroxyflavanone (**12**) (Takagi, Yamaki & Inoue, 1981), baicalein (**13**) (Takido, 1987) and wogonin (**14**) (Takido, 1987) in the transformed roots was examined (Figs. 3 and 4). In clone C, ten phenolics (**1**–**6**, **8**, **11** and **13**,**14**) were detected and the major phenolic accumulated in the tissues was **3** (1.74% in the light and 1.81% in the dark, on dry weight basis at week 8) (Fig. 3). The contents of glucuronic acid-conjugated (glucuronide-type) flavonoids such as **6** and **8**, which were the major phenolics in the roots of the intact plant (Table 2), were also high. Under dark condition, the amount of **1** gradually increased reaching a maximum level (1.07% dry weight) at the end of the culture period (week 8). Clone W also produced high amounts of **3** both in the light (2.95% as dry weight, at week 7) and the dark (2.96% as dry weight, at week 8) (Fig. 4). As the major difference in flavonoid accumulation in both clones C and W, higher contents of **6** and **8** (glucuronide-type flavonoids) were observed in clone W, which levels were almost three times higher those in clone C. This observation might suggest that the expression of exogenous GUS gene integrated by *A. rhizogenes* A13 caused the decrease of glucuronide-type flavonoids (**6** and **8**) accumulation in clone C. In a previous paper (Nishikawa & Ishimaru, 1997), we also reported on the decrease of glucuronide-type flavonoids in some

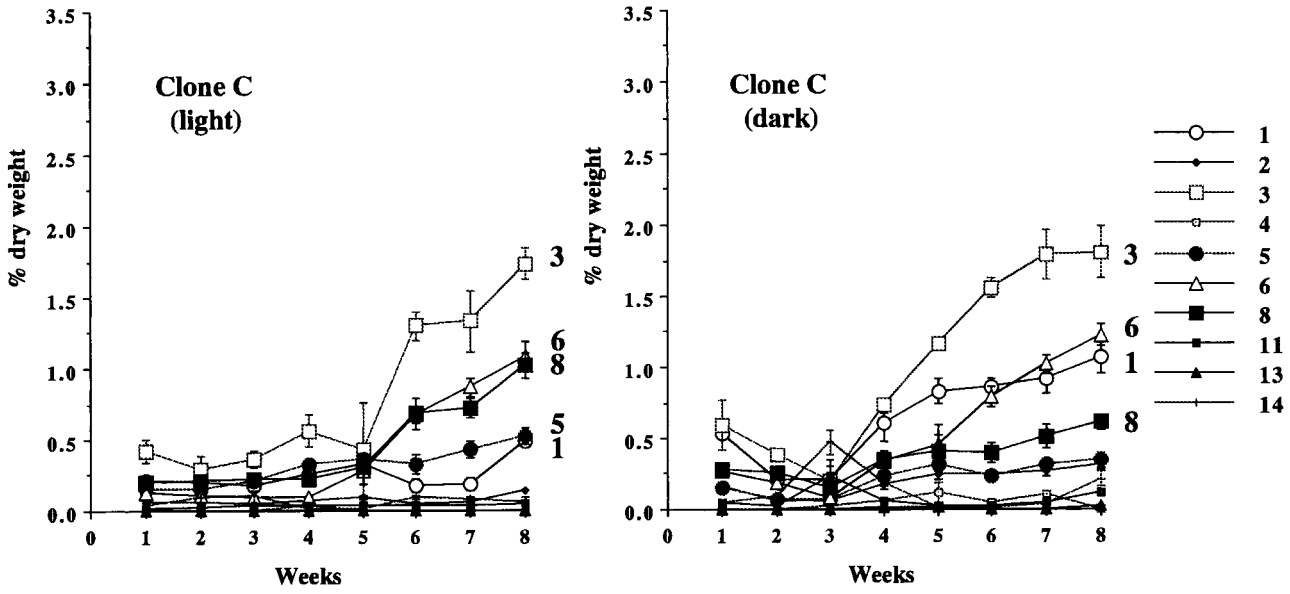

Fig. 3. Phenolics contents of *S. baicalensis* transformed roots (clone C) cultured in BF liquid medium in the light or dark.

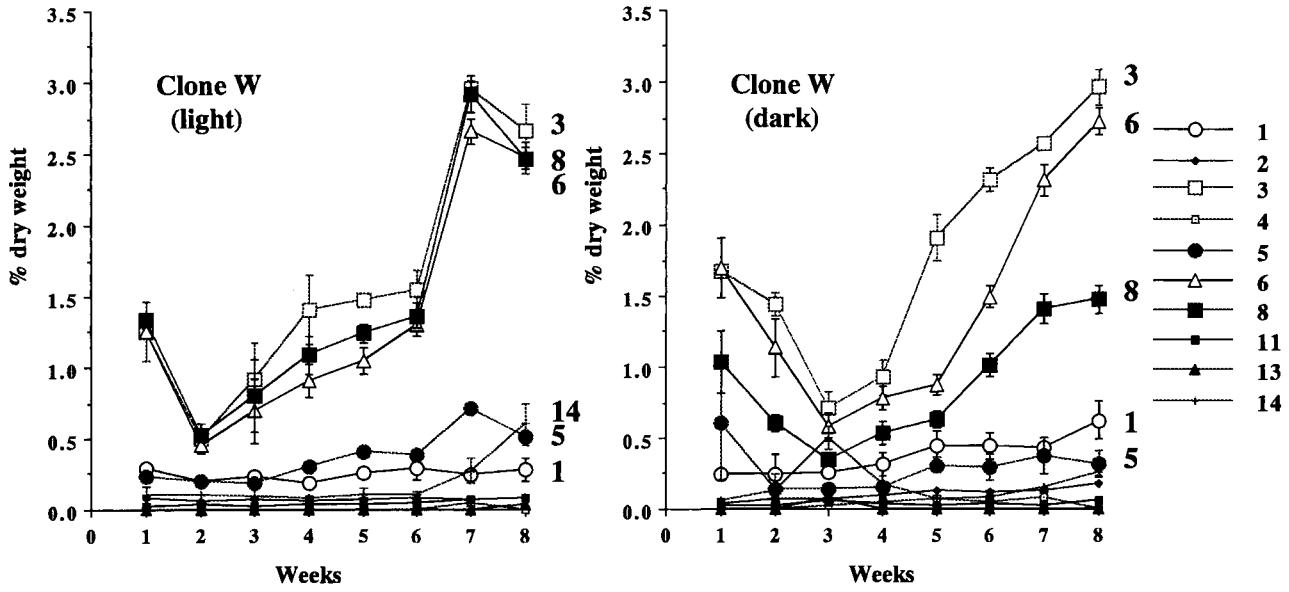

Fig. 4. Phenolics contents of *S. baicalensis* transformed roots (clone W) cultured in BF liquid medium in the light or dark.

transformed root cultures which contained exogenous GUS genes. To clarify this presumption, more detailed experiments on the gene and enzyme (GUS) expression in these clones are necessary. Illumination showed little effect on flavonoid accumulation in *S. baicalensis* root cultures.

It is noteworthy that compound **3**, which did not accumulate in the root portion of the field-grown intact plant (Table 2), was produced at a very high level in the transformed root cultures (Fig. 3). Accordingly, the phenolic content in shoot cultures (in vitro) of this plant, whose morphological traits were similar to those of the in vivo plants, were also determined (Table 2).

Table 2
Phenolic content of *S. baicalensis* (% dry weight)[a]

Compound	In vivo plant	In vitro plant		
	dried root	leaf	stem	root
1	–	–	–	–
2	–	–	–	–
3	–	0.23 ± 0.01	–	0.68 ± 0.02
4	0.05 ± 0.02	–	–	–
5	0.05 ± 0.01	–	–	0.23 ± 0.07
6	5.45 ± 0.20	–	–	0.80 ± 0.10
7	0.08 ± 0.01	–	–	–
8	2.56 ± 0.08	–	–	0.79 ± 0.02
9	0.07 ± 0.01	–	–	–
10	0.12 ± 0.02	–	–	0.01 ± 0.01
11	0.11 ± 0.01	–	–	0.24 ± 0.06
12	0.09 ± 0.01	–	–	–
13	0.77 ± 0.12	–	–	0.25 ± 0.15
14	2.04 ± 0.12	–	–	0.78 ± 0.31

[a] –: not detected.

In the aerial part of the in vitro plantlets, amongst fourteen phenolics (**1**–**14**) investigated, only compound **3** was detected (0.23% as dry weight) in the leaf portion and other flavonoids were not accumulated (in stem portion, no phenolics were detected). On the contrary, in the root portion of the shoot cultures, seven flavonoids (the major compounds were **6**, **8** and **14**) were detected together with **3**. Therefore, phenolic constituent **3** might be biosynthesized particularly in tissue cultures of this plant. Although the detailed data are not shown here, various tissue cultures (particularly callus cultures) of *Scutellaria* plants such as *S. incana*, *S. iyoensis*, *S. galericulata*, *S. pontica*, *S. taurica*, *S. ventenatii* etc. showed high accumulation of **3**. The results, together with the identification of new metabolites in tissue cultures of *Scutellaria* will be reported in near future.

3. Experimental

^{1}H- and ^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively, locked to the major deuterium resonance of the solvent [$(CD_3)_2SO$]. TLC was performed on silica gel and spots were detected by UV illumination and visualized by spraying with 2% $FeCl_3$ and/or 5% H_2SO_4. Half-strength Murashige–Skoog (MS) (Murashige & Skoog, 1962) and BF media used for the experiments contained 30 g/l sucrose. BF medium is a modified MS medium, containing half the amount of KNO_3, NH_4NO_3 and $CaCl_2$ and double the amount of KH_2PO_4 and $MgSO_4$ compared to those in MS basal medium. All media were adjusted to pH 5.7 before autoclaving at 121°C for 15 min. Cultures were

placed in continuous light (16 h photoperiod per day, ca. 3000 lux) or dark, at 25°C. Voucher specimens are deposited at the Faculty of Agriculture, Saga University.

3.1. Scutellaria baicalensis hairy root cultures

Among 6 clones (A–F) of *S. baicalensis* hairy roots (Nishikawa & Ishimaru, 1997) into which the GUS gene has been introduced by the infection with *Agrobacterium rhizogenes* A13, clone C was selected and used for the experiments. Another hairy root clone W was also induced by the infection with *A. rhizogenes* ATCC 15834 (wild type) using the same infection procedures as described (Nishikawa & Ishimaru, 1997). Clones C and W were subcultured in phytohormone-free BF liquid medium at two-month intervals for over a year in the dark, prior to use in the experiments.

3.2. Extraction and isolation of phenolics

Clone C cultured for 6 weeks in the dark was harvested and used for the extraction of the secondary metabolites. The lyophilized roots (21 g) were extracted with MeOH (three times; 750, 500 and 300 ml) at room temp. After concn under red. pres., the extract was chromatographed on Sephadex LH-20 (Pharmacia) CC and eluted by H_2O with an increasing amount of MeOH and then by EtOH to afford eight frs (Frs 1 ∽ 8). Fr. 1 was applied to Preparative C18 125 Å (Waters, H_2O–MeOH; 1:0–2:3) CC to give **1** (60 mg) and **2** (9 mg). Fr. 3 was separated by preparative C18 125 Å (H_2O–MeOH; 1:0–1:4) and Sephadex LH-20 (H_2O–EtOH; 1:0–0:1) CC to afford **3** (97 mg). **2** and **3** were characterized as skullcapflavone I (Takido et al., 1979) and acteoside (Sasaki et al., 1989), respectively, by the comparison of the spectroscopic data (^{1}H- and ^{13}C-NMR) with those in the refs.

3.3. 5,2′-dihydroxy-6,7,8,3′-tetramethoxyflavone (*1*)

A pale yellow amorphous powder, $[\alpha]_D^{28}$ +35.4° (MeOH; *c* 0.1), ^{1}H- and ^{13}C-NMR data: see Table 1; Negative FAB MS *m/z* (rel. int): 373 $[M–H]^-$ (93), Positive FAB MS *m/z* (rel. int): 374 $[M]^+$ (28), 375 $[M+H]^+$ (36),397 $[M+Na]^+$ (29), UV λ_{max}^{MeOH} nm (log ε): 270.0 (4.18); $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 404.0 (3.54), 283.0 (4.20); $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm (log ε): 403.0 (3.54), 281.0 (4.19); $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 268.0 (4.17); $\lambda_{max}^{MeOH+NaOAc+H_3BO_3}$ nm (log ε): 270.0 (4.17).

3.4. Time course experiment of transformed root cultures of Scutellaria baicalensis

Clones C and W (each ca. 50 mg, fresh wt) were inoculated into phytohormone-free BF liquid medium (50 ml medium in 100 ml Erlenmeyer flask) and cultured on a rotary shaker (100 rpm) in the dark or the light (16 h photoperiod per day, ca. 3000 lux). These roots were harvested at weekly intervals up to 8 weeks and the growth and phenolic production were determined.

3.5. Quantitative determination of phenolics by HPLC

Lyophilized samples (ca. 20–30 mg) were extracted with MeOH (1–2 ml) for 16 h at room temperature. After filtration through a millipore filter (0.5 μm), each extract was subjected to HPLC analysis; column: CAPCELL PAK C18 (4.6 × 250 mm), mobile phase; 1 mM tetrabutylammonium chloride (adjusted to pH 2.8 with AcOH)-CH_3CN (4:1 → 1:4, in 30 min), flow rate: 0.6 ml/min, column temperature: 40°C, detection: 280 nm (UV). Fourteen phenolic compounds **1**–**14** were used for the analysis. **4**–**12** have been isolated from the roots of *S. baicalensis* intact plant (Ishimaru et al., 1995) and **13** and **14** have been isolated from commercial dried roots of this species (not published data). Rt (min) were as follows: **3** (9.18), **12** (9.69), **4** (12.62), **5** (14.22), **9** (15.84), **10** (17.15), **6** (19.62), **7** (21.99), **8** (22.44), **13** (25.86), **2** (26.35), **1** (26.40), **14** (29.63) and **11** (30.03). Dried roots of the intact plant of *S. baicalensis* which were a gift from Kanebo Co. Ltd. and in vitro *S. baicalensis* plantlets (Nishikawa, Shimomura, Kayano, Yoshihira & Ishimaru, 1996), cultured for 4 weeks on phytohormone-free 1/2 MS solid medium, were also used as samples for the analysis.

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