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青阳参组织培养及愈伤组织的成分分析①

赵沛基 甘烦远 珠 娜 沈月毛②
(中国科学院昆明植物研究所 昆明 650204)

摘要 用青阳参(*Cynanchum otophyllum*)的嫩枝和芽在MS + 2.0 mg/L 2,4-D + 0.1 mg/L KIN的培养基上诱导愈伤组织。通过不同的培养基和激素配比实验,发现6,7-V + 2.0 mg/L 2,4-D + 0.3 mg/L KIN最适合愈伤组织的生长。但在6,7-V + 1.0 mg/L 2,4-D + 0.1 mg/L KIN培养基中的愈伤组织次生代谢物含量最高。愈伤组织的生长周期为27 d,但在33 d时次生代谢产物的含量最高。从愈伤组织中分离到7个化合物:(1)9,10,11-三羟基-十八碳-12(Z)-烯酸甲酯(methyl 9,10,11-trihydroxy-12-octadecenoate),(2)胡萝卜甙(daucosterol),(3)β-谷甾醇(β-sitosterol),(4)华木酸(betulinic acid),(5)齐端果酸(oleanolic acid),(6)棕榈酸(hexadecanoic acid),(7)十八碳-9-烯酸(9-octadecenoic acid)。首次报道从植物愈伤组织中分离到多羟基十八碳烯酸,并讨论了化合物(1)对植物细胞生长的可能影响。

关键词 9,10,11-三羟基-十八碳-12(Z)-烯酸甲酯,青阳参,愈伤组织,萝藦科

Studies on the Tissue Culture of *Cynanchum otophyllum* and Calli Chemical Constituents

ZHAO Pei-Ji GAN Fan-Yuan ZHU Na SHEN Yue-Mao②
(*Kunming Institute of Botany, the Chinese Academy of Sciences*, Kunming 650204)

Abstract The calli of *Cynanchum otophyllum* were induced from the tender stems and young buds on MS medium with the supplement of 2.0 mg/L 2,4-D and 0.1 mg/L KIN. The result showed that 6,7-V medium was the optimal medium for callus growth. The tests using plant growth regulators with various concentrations showed that 6,7-V medium plus 2.0 mg/L 2,4-D and 0.3 mg/L KIN was the best combination for callus growth, whereas the production of secondary metabolites was higher on 6,7-V medium plus 1.0 mg/L 2,4-D and 0.1 mg/L KIN. The best period of culture was 27 d for callus growth, and 33 d for the maximal production of secondary metabolites. The isolation of nature products from the callus provided seven compounds including (1) fatty acid methyl ester named methyl 9, 10, 11-trihydroxy-12-octadecenoate and (2) daucosterol, (3) β-sitosterol, (4) betulinic acid, (5) oleanolic acid, (6) hexadecanoic acid, (7) 9-octadecenoic acid. This is the first report of the isolation of poly-hydroxyl octadecenoic acid from a callus, and its proven biological function on the growth of plant cells was discussed.

Key words Methyl 9, 10, 11-trihydroxy-12-octadecenoate, *Cynanchum otophyllum*, Callus, Asclepiadaceae

Cynanchum otophyllum (Asclepiadaceae) mainly distributed in southwest of China is used as a

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②通讯作者。Author for correspondence. E-mail: yshen@public.km.yn.cn
作者简介:赵沛基,男,1974年2月生,1997年毕业于云南大学生物系,现为中国科学院昆明植物研究所在职研究生,主要从事植物次生代谢的研究。沈月毛,研究员,主要从事次生代谢产物的生物合成。
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folk herb medicine. Its roots can be used as nourishment and muscularity, and in the treatment of arthralgia of rheumatism (Mu and Zhou, 1983a). The isolation and bioactivities of steroid compounds from *C. otophyllum* were reported previously (Mu *et al*, 1986). Some C21 steroids from the root and stem of *C. otophyllum* were shown pharmacologically and clinically active against chronic hepatitis (Mu and Zhou, 1983b). The anticonvulsive activity of some steroids from the root of *C. otophyllum* was observed as well (Pei *et al*, 1981). However, the tissue cultures and callus chemical constituents were not reported before this work.

1 Materials and methods

1.1 Callus tissue induction and culture

The callus was induced from the explants of *C. otophyllum* tender stems and young buds. The explants were sterilized by normal method (30 s soaked in 75% ethanol and 7 min soaked in 0.12% $HgCl_2$). The basic induction medium was MS medium with the supplement of 2.0 mg/L 2,4-D and 0.1 mg/L KIN. The culture was incubated at 25℃ in the dark. The explants obviously expended after one week, and the nascent calli appeared two weeks later. The calli were transferred to fresh media after four weeks, and the cultures were subcultivated every four weeks.

1.2 Callus culture

The calli were inoculated on different media, with different concentrations and various combinations of plant growth regulators, and the growth rate and the production of secondary metabolites were compared to find out the optimum culture condition.

1.3 The curves of growth rate and the production of secondary metabolites

The calli were cultivated under the optimum conditions. Three flasks of callus cultures were collected every three days, for measuring the callus growth amount and the yield of ethyl acetate extract.

1.4 Chemical constituents of the callus

The callus (103 g, biomass dry weight DW) were collected and extracted three times with 95% ethanol under refluxing to afford 20.9 g of crude extracts. The extracts were extracted by chloroform at first, and then by methanol. The chloroform part was named to be fraction A. The methanol part was refluxed in 0.5% H_2SO_4 methanol solution for 1 h followed by being neutralized by 5% NaOH aqueous solution. After the removal of methanol under vacuum, the aqueous solution was extracted with ethyl acetate to afford fraction B. Fraction B (0.72 g) was subjected to column chromatography over reversed-phase C_{18} Si gel (80 g) eluted with methanol-water (1:1, 3:2, 7:3, 8:2, v/v), and further purified with column chromatography over Si gel to give compounds (1) (17 mg) and (2) (12 mg), respectively. Fraction A (3.92 g) was subjected to column chromatography over Si gel (100 g) eluting with chloroform containing increasing amount of methanol to produce 6 fractions: Aa, Ab, Ac, Ad, Ae and Af. Fraction Ab (0.5 g) was further subjected to column chromatography over Si gel eluting with chloroform containing increasing amount of ethyl acetate (100:2, 100:5, 9:1, 8:2, v/v) to give compound (3) (10 mg) and the mixture of (6) and (7)

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(40 mg), respectively. Fraction Ac (1.30 g) was chromatographed over Si gel column eluted with chloroform-methanol (100:1, 100:2, v/v), and further purified with column chromatography over Si gel to give compounds (4) (20 mg) and (5) (13 mg), respectively.

2 Results and discussion

2.1 Induction of callus

Through comparing the dedifferentiation of explants on different media and different combinations of plant growth regulators (Table 1), MS medium plus 2.0 mg/L 2,4-D and 0.1 mg/L KIN were selected to be the optimum for callus induction. The induction period was as long as two weeks, and the induction rate was over 90%.

Table 1 Induction rate in different media and different combinations of growth regulators

	MS	B5	6,7-V
2.0 mg/L 2,4-D, 0.1 mg/L KIN	93%	60%	75%
2.0 mg/L 2,4-D, 0.3 mg/L KIN	82%	56%	70%

2.2 The optimization of culture conditions

To search the ideal combination of plant growth regulators in 6,7-V media (Table 2), two groups of experiments were carried out. In one group, different KIN concentrations with constant amount of 2,4-D were added, in another group different amounts of 2,4-D with the selected KIN concentration were applied into the media. Results showed that the combination of 2.0 mg/L 2,4-D and 0.3 mg/L KIN was the best for callus growth. The growth rate was 22.03 g FW·d^{-1}·L^{-1}. However, 1.0 mg/L 2,4-D plus 0.1 mg/L KIN were better for the production of secondary metabolites. Additionally, the growth rate was slower on MS than on 6,7-V, but the yield of ethyl acetate extract was higher.

Table 2 Constitutent of 6,7-V medium

Major element		Minor element	
KCl	200 mg/L	$MnSO_4 \cdot 5H_2O$	4.3 mg/L
$MgSO_4 \cdot 7H_2O$	250 mg/L	$ZnSO_4 \cdot 7H_2O$	1.5 mg/L
$NaH_2PO_4 \cdot 2H_2O$	170 mg/L	H_3BO_4	5 mg/L
$CaCl_2$	150 mg/L	$NaMoO_4 \cdot 2H_2O$	0.25 mg/L
KNO_3	800 mg/L	KI	0.05 mg/L
$(NH_4)_2SO_4$	100 mg/L	$CoCL_2 \cdot 6H_2O$	0.025 mg/L
Organic compounds		Ferric salt	
$VitB_1$	0.5 mg/L	$FeSO_4 \cdot 7H_2O$	27.8 mg/L
$VitB_6$	0.5 mg/L	Na_2EDTA	37.3 mg/L
Nicotinic acid	0.5 mg/L	pH	5.8~6.0 (before sterilization)
Glycine	0.5 mg/L	Agar	7 g/L
Inositol	100 mg/L	Sucrose	30 g/L

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2.3 Growth curve and the production of secondary metabolites

In the process of tissue culture, its lag phase was very short, only 3 days. The growth curve and the yield of crude extract of ethyl acetate showed in Figure 1, which revealed that the best culture period was 21 days for cell growth, but at 33 days the production of secondary metabolites was maximal.

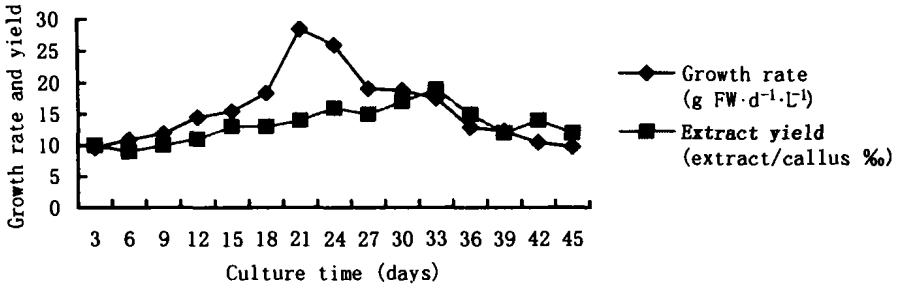

Fig.1 The curves of cell growth rate and production of secondary metabolites

2.4 Chemical constituents

Compound (1), colorless powder, EIMS (70 eV) *m/z* (%): 308 (1), 295 (15), 277 (7), 217 (48), 200 (35), 185 (59), 168 (34), 155 (46), 140 (40), 121 (33), 109 (49), 97 (50), 83 (76), 69 (74), 55 (100); $[\alpha]_D^{23}$ + 5.9° (c 0.02, MeOH); UV (MeOH): 202 nm; IR (KBr)·cm^{-1}: 3400 (OH) (s), 3002, 2930, 2856 (C-H), 1739 (ester carbonyl), 1628 (C = C), 1466, 1437, 1257, 1217, 1174, 1061 (C-O), 722 (nCH_2); the negative high resolution fast atom bombardment-mass spectrometry (HRFABMS): *m/z* 343.2494 (calc. for $C_{19}H_{35}O_5$ 343.2486); FABMS *m/z* (%): 343 (M-H) (37), 329 (M-CH_3) (8), 311 (M-CH_3-H_2O) (5), 255 (7); see Table 3 for the NMR data. Compound (1) had anti-tuberculosis activity on *Mycobacterium tuberculosis* and anti-fungal activity on *Penicillium avellaneum* UC-4376. The molecular formula was determined to be $C_{19}H_{36}O_5$ base on the HRFABMS *m/z*:343.2494(M-H). The IR spectrum showed the presence of hydroxyl (3400), carbonyl (1739), double bond (1628), which together with the absorptions at 2930, 2856, 1466, 1437/cm illuminated that (1) was a polyhydroxyl-substituted long chain alkene compound. The ^{13}C-NMR (DEPT) showed nineteen signals for two methyls, ten methylenes, six methenyls and one quaternary carbon, including two methylenes of one double bond at δ_c 132.7 and 137.3, and three oxygen-substituted methylenes at δ 76.9 (δ_H5.19), 71.3 (δ_H 4.47) and 69.1 (δ_H 5.19).

The HMBC experiments showed the ^{1}H-^{13}C long-range correlations between the methyl protons at δ 0.77 (H-18) and the carbons at δ 22.8 (C-17) and 31.7 (C-16), and between the methylene protons at δ 2.22 (H-14) and the carbons at δ 29.3 (C-15), 31.7 (C-16), 132.3 (C-13) and 132.7 (C-12), determining the structure of fragment 1a and indicating the location of C-12/C-13 double bond (Figure 2). The ^{1}H-^{1}H COSY experiment showed the correlations between the protons at δ 2.22, 5.72, 6.11, 5.19, 3.95, 4.47 and 2.04, sequentially, indicating the structure of

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fragment 1b (Figure 2). The methoxyl substitution at C-1 carbonyl was readily determined by the analysis of HMBC correlation. Therefore, compound (1) was determinate to be methyl 9,10,11-trihydroxy-12-octadecenoate.

Table 3 The data of compound 1[a]

Position	^{13}C	^{1}H[b]	HMBC
1	173.9s	/	/
2	34.1t	2.28 (2H, t, 7.2)	C-1, C-4
3	25.3t	1.49-1.58 (2H, m)	c
4	29.7t	1.25-1.32 (2H, m)	c
5	29.8t	1.19-1.21 (2H, m)	c
6	26.6t	1.49-1.58 (m) 1.77 (m)	c C-8, C-9
7	30.0t	1.25-1.32 (2H, m)	c
8	34.8d	1.92 (m) 2.04 (m)	C-6, C-7, C-9, C-10
9	71.3d	4.47 (dt, 4.4, 7.2)	C-7, C-10, C-11
10	76.9d	3.95 (dd, 2.8, 6.4)	C-8, C-12
11	69.1d	5.19 (dd, 6.4, 8.8)	C-9, C-10, C-13
12	132.7d	6.11 (dd, 8.8, 10.8)	C-10, C-14
13	132.3d	5.70 (dt, 7.2, 10.8)	C-11, C-14, C-15
14	28.4 t	2.22 (2H, m)	C-12, C-13, C-15, C-16
15	29.3t	1.19-1.21 (2H, m)	c
16	31.7t	1.11-1.17 (2H, m)	c
17	22.8t	1.11-1.17 (2H, m)	c
18	14.2q	0.77 (3H, t, 6.8)	C-17, C-16
MeO-1	50.8q	3.60 (3H, s)	C-1, C-2d

a, ^{1}H-, ^{13}C-NMR and HMBC spectra were obtained at 400 MHz, 100 MHz and 500 MHz, and recorded in C_5D_5N at room temperature, respectively. b, Coupling constants are presented in Hz. Unless otherwise indicated, all proton signals integrate to 1H. c, The ^{1}H-^{13}C long-range correlation were not readily observed because of the overlap of proton signals. d, The four-bond ^{1}H-^{13}C long-range correlation was observed.

Compound (2), colorless needles, EIMS (70 eV) *m/z* (%): 414 (M-162, 8), 396 (100), 382 (46), 357 (5), 303 (5), 255 (12), 213 (7); ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.67 (3H, s, H-26), 0.87 (3H, s, H-28), 0.93 (3H, s, H-27), 0.94 (3H, d, J = 6.5 Hz, H-21), 1.01 (3H, s, H-19), 1.27 (3H, s, H-20), 1.70 (3H, s, H-18), 2.28 (1H, m, H-4), 3.28 (1H, m, H-2′), 3.40 (2H, m, H-3′, H-4′), 3.97 (1H, m, H-3β), 3.98 (1H, m, H-5′), 4.41 (1H, d, J = 7.6 Hz, H-1′), 4.42 (1H, dd, J = 4.9, 11.9 Hz, H-6′), 4.57 (1H, d, J = 11.9 Hz, H-6′), 5.36 (1H, d, J = 5.1 Hz, H-6). Those data were consistent with literature (Xie *et al*, 1994), therefore, compound (2) was determined to be daucosterol.

Compound (3), colorless needles, EIMS (70 eV) *m/z* (%): 414 ($[M]^+$, 100), 396 (M-H_2O, 27), 381 (18), 367 (37), 354 (5), 329 (20), 303 (34), 289 (5), 273 (15), 255 (15), 231 (12), 213 (16), 145 (17), 109 (14) and 69 (25). Comparing with standard sample on TLC over Si gel, compound (3) was determined to be β-sitosterol.

Compound (4), colorless needles, EIMS (70 eV) *m/z* (%): 456 ($[M]^+$, 43), 438 (M-H_2O, 12), 423 (M-H_2O-CH_3, 8), 410 (M-CO_2, 10), 248 (43), 207 (68), 203 (37), 189

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(100); ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.80-1.26 (15H, s, 5 × Me), 1.71 (3H, s, H-30), 3.47 (1H, d, J = 8), 3.56 (1H, m, H-3), 4.93 (1H, br s, $W_{1/2}$ = 5 Hz, H-29a), 4.76 (1H, br s, $W_{1/2}$ = 5 Hz, H-29b). The data were consistent with those reported in literature (Srivatava and Jain, 1989) for 3-hydroxy-20(29)-lupen-28-oic acid.

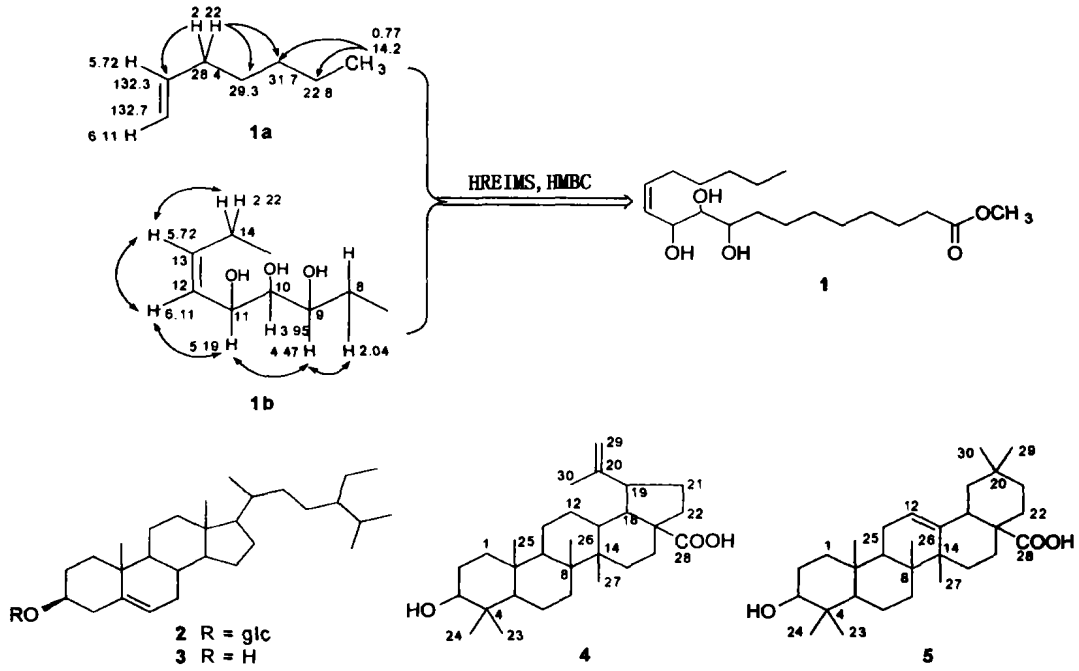

Fig.2 Structures of compounds 1 ~ 5, and structural fragments of 1 and selected ^{1}H-^{1}H COSY (dashed ↔) and ^{1}H-^{13}C long-range (H-C) correlations

Compound (5), colorless needles, EIMS (70 eV) *m/z* (%): 456 ([M]$^+$, 12), 438 (M - H_2O, 5), 423 (3), 410 (M-CO_2, 5), 392 (M-CO_2-H_2O, 7), 248 (100), 203 (61), 189 (27), 133 (44); ^{1}H-NMR (500 MHz, C_5D_5N) δ: 0.83-1.19 (21H, s, 7 × Me), 3.33 (1H, t, J = 8.17, H-3), 5.29 (1H, d, J = 16.8, H-12). Those data were consistent with literature (Voutquenne *et al*, 1999), therefore, compound 5 was determined to be 3-hydroxy-12-oleanen-28-oic acid.

Compounds (6) and (7) were obtained in a mixture and refluxed in 0.5% H_2SO_4 methanol solution for two hours. After being neutralized with 5% NaOH aqueous solution, the reaction mixture was extracted by petroleum ether. The methyl esters of hexadecanoic acid (6) and 9-octadecenoic acid (7) were separated and identified by GC-MS, respectively.

Trihydroxyl octadecenoic acid was reported as defense responsive component against pathogen attack and wounding (Hou and Forman, 2000; Göbel *et al*, 2001). In the trihydroxyl octadecenoic acid family, 9,12,13-trihydroxystearic acid and 9,10,18-trihydroxystearic acid together with their $\triangle^{12}$ and $\triangle^{10}$ unsaturated analogs are the most common components. In plants, trihydroxy

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octadecenoic acid was regard as middle signal molecular and as biosynthetic precursor of singal compound jasmonic acid (Abian *et al*, 1990). Trihydroxy octadecenic acid oxidized from linoleic acid or linolenic acid catalyzing by lipoxygenase. When potato cells were treated by elicitor from phtophthora infestans, lipoxygenase was accumulated (Göbel *et al*, 2001). On the other hand, increase susceptibility to insect attack was observed in transgenic potato plants with reduced lipoxygenase level (Royo *et al*, 1999) and *Arabidopsis* plants that were deficient in the lipoxygenase substrate linolenic acid (McConn *et al*, 1997). In *C. otophyllum* tissue cultures, the callus was germ-free, the production of abundant trihydroxy octadecenoic acid may be related to the rapid growth of calli. In the later stage of culture, part of calli died for completing nutrition and exuded cell components after cracking, which may act as wounding signals and received by other live cells to induce the biosynthesis of defense compounds such as trihydroxy octadecenoic acid.

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