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Studies on the Non-anthraquinones of Hotao Rhubarb (*Rheum hotaoense*)

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Abstract From the methanolic extract of stem and root of *Rheum hotaoense* C. Y. Cheng *et* C. T. Kao, eight non-anthraquinones were isolated. Their structures were identified by chemical and spectroscopic methods, as: β-sitosterol(Ⅰ), rhapontigenin(Ⅱ), gallic acid(Ⅲ), 3-(3′, 5′-dihydroxy-trans-cinnamoyl)-5-hydroxy-$\triangle^5$-α-pyranone(Ⅳ), daucosterol(Ⅴ), piceatannol-3′-O-β-D-glucopyranoside(Ⅵ), rhaponticin(Ⅶ) and sucrose(Ⅷ). Ⅰ、Ⅲ、Ⅴ、Ⅵ、Ⅷ were obtained from this plant for the first time and Ⅳ is a new compound and named rheumin.

Key words *Polygonaceae* rheumin *Rheum hotaoense* C. Y. Cheng *et* C. T. Kao

Sterol Composition and Biosynthesis in Hairy Root Cultures of *Cassia obtusifolia*

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Abstract Hairy root cultures of *Cassia obtusifolia* induced by *Agribacterum rhizogens* were established to investigate the composition and biosynthesis of phytosterols. Effects of rare earth element Eu on the growth of hairy root were also investigated. Twelve different sterols were isolated from the hairy roots. They were identified by chromatographic (TLC, GLC and HPLC) and spectral methods (MS). The biosynthetic pathways leading to the production of major sterols were proposed based on the results of this study.

Key words *Cassia obtusifolia* hairy root culture sterol biosynthesis

Extensive investigations on the production of secondary metabolites by hairy root cultures (transformed by *Agrobacterium rhizogens*) were conducted in arrays of plant species[1~8]. It was found that the majority of hairy root clones generated same or similar chemical constituent profiles when compared with the intact plants. Therefore hairy root cultures are viewed as a promising potential source to produce active secondary metabolites without growing the original plant in the field. Recently, Kyung

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Soo Ko *et al.* reported the production of polyketide pigments in hairy root cultures of *Cassia* L. plants[9]. In the course of our biogenetic studies on anthraquinones and sterols in traditional Chinese crude drugs Semen Cassiae and Rhubarb, hairy root cultures (transformed with *Agribacterium rhizogens*) of *Cassia obtusifolia* were established.

Phytosterols are widely distributed in plant kindom and now known to play multiple physiological roles in growth, development and reproduction of living organisms. They not only serve as cell membrane insert but also as hormonal and regulatory factors[10]. The composition of phytosterols and their biogenetic pathways were extensively approached in the original plant species[11~13]. However studies on the sterol composition and biosynthetic pathways in the cultured hairy roots were rarely investigated. We wish to report, in the present paper, the results of our study on sterol composition in the cultured hairy root. The biosynthetic pathways leading to the production of major sterols were proposed based on the results of sterol analysis.

1 Materials and Methods

1.1 Plant materials: Seeds of *Cassia obtusifolia* were obtained from Nanyang city, Henan provinec. They were washed with tap water and soaked in 75% alcohol for 2 min. After rinsing with sterile distilled water, they were soaked in 0.1% $HgCl_2$ for 15 min and rinsed repeatedly with sterile distilled water. The sterilized seeds were then inserted into solid MS basic medium in culture boxes. Germinated seedlings were then kept under 12 h light and 12 h dark conditions at 25℃. For induction of hairy root the seedlings were allowed to grow for 2 weeks ~3 weeks.

1.2 Induction and culture of hairy roots: the bacterium strain used in this study was *Agrobacterium rhizogens* 9402, grown on YMB medium containing mannitol 10 g/L, yeast extract 0.4 g/L, K_2HPO_4 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L and NaCl 0.1 g/L kept in dark places at 0℃~4℃ and activited at 28℃. Four days' culture of the above strain was used for the infection experiments. The cotyledons of aseptically grown plantlets were cut into 0.25 cm^2 pieces and soaked in activiated bacterial culture diluted with MS liquid medium for 30 minutes. The superfluous bacterial liquid was filtered with suction on sterilized filter paper. The infected cotyledons were transfered to MS basic medium and cultivated at 25℃ for 3 to 4 days and then transfered to MS medium containing claforan (250 mg/L). After 6 days' cultivation growth of fine white hairy roots were observed and allowed to grow for a week.

To establish a liquid culture, segments of hairy root free of bacterium were excised and transfered into the MS medium in Erlenmeyer flasks and cultivated at 25 ℃ with 130 rpm under light. The growing hairy roots in liquid medium were subcultured every 5 weeks. The materials from these cultivation were used for analysis.

1.3 Effects of rare earth element on growth: Different concentrations of Eu^{3+} ion (0.001, 0.01, 0.1, 1.0, 10 mg/L) were added to the medium to cultivate hairy roots. Control culture was paralleled to observe the effects of Eu^{3+} on growth of hairy roots.

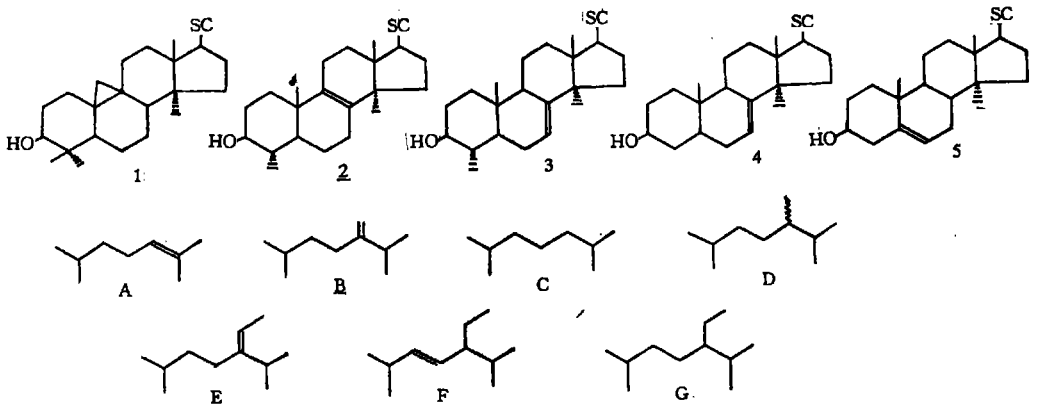

Figure 1 Nuclei and side chains (sc) of sterol structures in *Cassia obtusifolia* hairy root cultures

Table 1 Chromatographic and spectral properties of sterols in *Cassia obtusifolia* hairy root cultures

sterol	structure	TLC (R_f)	HPLC (α_c)	MS (M^+)	rel. content (%)
cycloartenol	1A	0.29	1.05	426	0.8
24-methylenecycloartenol	1B	0.29	1.12	440	0.2
obtusifoliol	2B	0.25	0.94	426	0.2
24-methylenelo-phenol	3B	0.25	0.98	412	0.4
citrostadienol	3F	0.25	1.14	426	0.3
cholesterol	5C	0.17	1.00	386	1.2
24-methylene cholesterol	5B	0.17	0.96	398	0.2
avenasterol	4E	0.17	1.04	412	0.4
isofucosterol	5E	0.17	1.00	412	0.8
24-methyl cholesterol	5D	0.17	1.14	400	4.0
sitosterol	5G	0.17	1.19	414	30.0
stigmasterol	5F	0.17	1.11	412	61.0

1.4 Sterol analysis: Sterols were isolated from the *Cassia obtusifolia* hairy roots with or without the addition of rare earth element Eu^{3+} respectively by saponitying the sliced roots in an aqueous solution of 10% KOH in methanol containing 10% water at reflux for 30 min, then extracting the neutral lipid with petroleum ether. The extracts were pooled, the solvent evaporated and the residues subjected to TLC plates eluting with benzene/diethylether (85 : 15). Sterols were separated by the degree of substitution at C-4 (4, 4-dimethyl, 4-monomethyl and 4, 4-desmethyl). The bands corresponding to each class of sterols were scraped off the plates and removed from silica gel by eluting with acetone. HPLC was applied to obtain pure sterol fractions for further analysis. GLC was performed using a 3% SE-30 packed column and HPLC using a Prodigy ODS reversed phase column connected to a Waters 996 variable wavelength set at 205 nm with pure methanol as eluating solvent at ambient temperature. GC-MS was performed on Hewlett — Packard 5890 Series Ⅱ gas chromatograph coupled to a 5970 Mass detector at 70 e. The capillary column for GLC was a 15-m DB5MS column. The temperature program was 170℃ for 1 minute increasing to 270℃ at 20℃/min, isothermal at 270℃ for 3 min and increasing to 280℃ at 2℃/min, then isothermal at 280℃ for 6 min. Sterols were identified by their behaviors in TLC, GLC, and HPLC, experessed as Rf values (TLC) or retention times (RRTc in GLC and α_c in HPLC) relative to cholesterol, and by comparison with mass spectra of authentic samples available to us.

2 Results and Discussion

Hairy root cultures of *Cassia obtusifolia* were established as described in Materials and Methods. They were pale colored at the initial stage of culturing, but gradually turning to dark brown after culturing for

about 20 days. Influences of antibiotic concentration, pH values, culture media, carbon sources and rare earth element on the growth of hairy roots were investigated. It was found that concentration of antibiotic kanamycin at 25 mg/L was suitable for screening the transformed roots. 1/10 MS medium at pH 5.8～6.5 was most favorable for the growth and biomass of hairy roots. The addition of rare earth element Eu^{3+} at 10 mg/L can effectively increase the biomass of hairy roots and content of anthraquinones in the hairy roots. However, a lower concentration of Eu^{3+} (<0.1 mg/L) can inhibit the hairy root growth and biosynthesis of anthraquinones (data not shown).

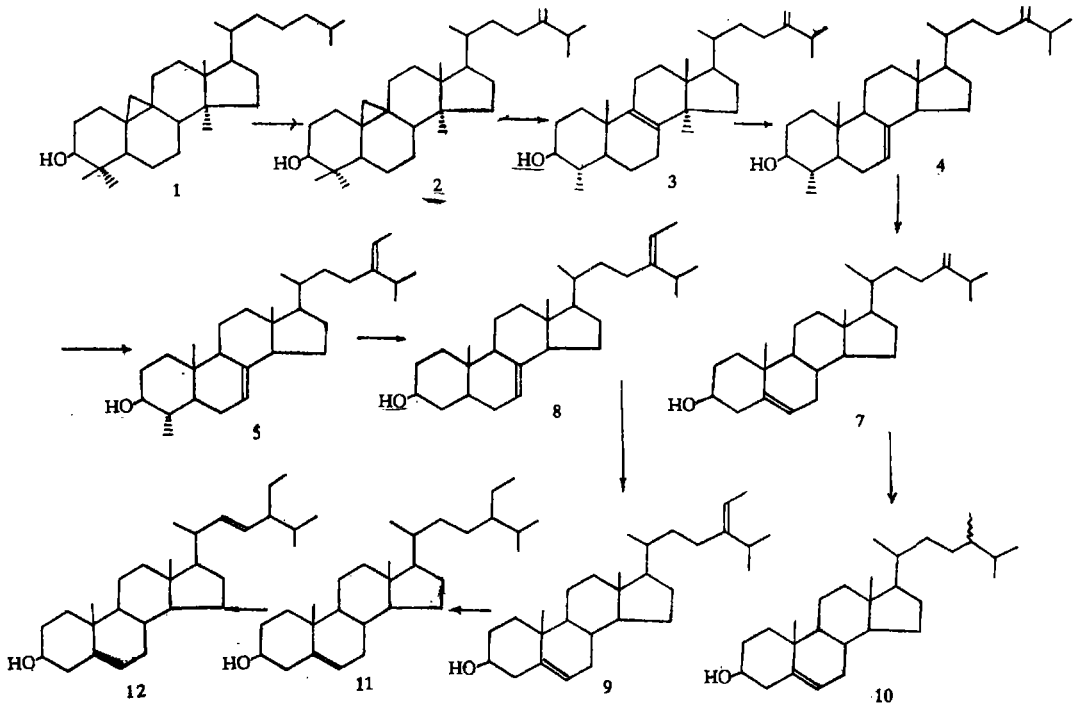

Figure 2 Proposed biosynthetic pathways of major sterois in *Cassia obtusifolia* hairy root cultures

The sterol composition in hairy root cultures of *Cassia obtusifolia* was analysed by using chromatographic and spectral methods. As shown in Table 1, twelve sterols were isolated and identified. The identity of each sterol was confirmed by matching their GLC behaviors and mass spectra with data obtained on authentic samples. These sterols were determined as cycloartenol, 24-methylenecycloartenol, obtusifoliol, 24-methylenelophenol, citrostadienol, cholesterol, 24-methylenecholesterol, avenasterol, isofucosterol, 24-methylcholesterol, sitosterol and stigmasterol respectively. The major sterols occured in the hairy roots were sitosterol and stigmasterol, which proved to be the two end products in the sterol biosynthetic pathways. Several trace sterols with substitution at C-4 were detected, e.g. cycloartenol, 24-methylenecycloartenol, obtusifoliol and 24-methylenelophenol. These are the intermediates in the pathway. The sequence of the sterols detectedfrom the hairy roots in the pathway

were arranged on the basis of degrees of substitution at C-4 position of A-ring in sterol nucleus and reference on the commonly recognized sterol pathways in higher plants. We did not foumd any major variance on the sterol composition in the hairy roots when compared with that of majority of higher plants. These also showed that the cell membranes of *Cassia obtusifolia* hairy roots kept the similar properties with those of untransformed plant roots concerning their membrane composition[14].

The influence of rare earth element Eu^{3+} on the sterol composition was also investigated. It was found that Eu^{3+} has no influence either on the sterol composition or on the content of each sterol, in contrast with the observation that Eu^{3+} has determinable influence on the contents of anthraquinones in the hairy roots.

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摘　要　应用发根农杆菌(*Agrobacteriium rhizogens*)感染决明无菌苗建立了决明毛状根培养体系。分析了决明毛状根中的甾醇类化学成分并初步探讨了它们的生物合成途径。用薄层色谱(TLC)、气相色谱(GLC)、高效液相色谱(HPLC)和质谱(MS)等方法从毛状根中分离鉴定了12个甾醇类成分。根据研究结果提出了其中主要甾醇类成分的生物合成途径。

关键词　决明　毛状根培养　甾醇　生物合成

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接骨木化学成分的研究

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摘　要　从忍冬科接骨木属植物接骨木 *Sambucus williamsii* 干燥茎枝乙醇提取物的石油醚和氯仿部分中分得6个单体化合物。通过化学方法和波谱分析鉴定其结构分别为:棕榈酸蛇麻脂醇酯(Ⅰ)、熊果酸(Ⅱ)、β-谷甾醇(Ⅲ)、α-香树脂醇(Ⅳ)、三十烷酸(Ⅴ)和β-谷甾醇-β-D-葡萄糖苷。以上化合物均为首次从该植物中分得。

关键词　接骨木　棕榈酸蛇麻脂醇酯　熊果酸　α-香树脂醇

接骨木为忍冬科接骨木属植物接骨木 *Sambucus williamsii* Hance. 的茎枝。《本草新编》记载:"接骨木,入骨节,专续筋接骨。临床上用接骨木酊剂治疗骨折,取得了显著的疗效。动物实验表明[1],接骨木可明显促进骨折的愈合。家兔用人工方法造成骨折模型,用

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