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A novel long-chain acyl-derivative of epigallocatechin-3-*O*-gallate prepared and purified from green tea polyphenols*

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Abstract: Lipophilic tea polyphenols (LTP) were prepared by catalytic esterification of green tea polyphenols (GTP) with hexadecanoyl chloride. A novel long-chain acyl-derivative of epigallocatechin-3-*o*-gallate (EGCG) was first isolated from purification of LTP by high-speed countercurrent chromatography (HSCCC) using a solvent system composed of n-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v). The molecular structure of the acyl-derivative, Epigallocatechin-3-*O*-gallate-4′-*O*-hexadecanate , was elucidated by means of elemental analysis, IR, ^{1}H-NMR and MS spectra.

Key words: Green tea polyphenols, Lipophilic tea polyphenols, Epigallocatechin-3-*O*-gallate, Epigallocatechin-3-*O*-gallate-4′-*O*-hexadecanate, Isolation, High-speed countercurrent chromatography
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INTRODUCTION

Green tea polyphenols (GTP) from the leaves of green tea (*Camellia sinensis L.*) is used as a kind of food antioxidant and dietary supplement as epigallocatechin-3-*O*-gallate (EGCG), epicatechin (EC), epigallocatechin (EGC) and epicatechin-3-*O*-gallate (ECG; for structures see Fig.1), these principal green tea catechins have been known to possess anti-arteriosclerosis, resistance to oxidation and anticancer characteristics (Hollman *et al.*, 1999; Dreosti, 1996; Zhu *et al.*, 2001; Johnson and Loo, 2000; Li *et al.*, 2000). However, the use of GTP is greatly limited because of its poor solubility in lipid medium. Therefore, it is important to properly prepare lipophilic tea polyphenols (LTP) for use in lipid-soluble medium. Recent results indicated that solubility of LTP in salad oil increased more than 2000 times when aliphatic acyl chlorides having 10 or more carbon atoms were used (Wang *et al.*, 2002). Although the structure of LTP was not determined, the ability to scavenge hydroxyl radicals (·OH), and reactive oxygen species generated by macrophage's breathe burst in biological system decreased in the order GTP > LTP > V_E (Vitamin E), but the inhibitory activity on the lipid peroxidation initiated by 2, 2'-azobis(2, 4-dimethylvaleronitrile) (AMVN) is decreased in order V_E > LTP ≈ GTP, when the concentration is more than 50 μg/mL (Liang *et al.*, 1999). The present paper first describes isolation and purification of long-chain acyl-derivative of EGCG from LTP prepared by catalytic esterification between GTP and C_{16}-fatty acid using high-speed countercurrent chromatography separation and then elucidates the chemical structure of the acyl-derivative.

EXPERIMENTAL

Apparatus

A J-type high-speed countercurrent chromatography (HSCCC) instrument was employed in

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the present study. It was fabricated at the Zhejiang University, Hangzhou, China. The type J multilayer coil planet centrifuge with a 10 cm revolution radius induces a synchronous planetary motion of the column holder. The separation column revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction (Ito and Conway, 1996). The column holder hub was 25 cm in length and 6 cm in OD. The multilayer coil was prepared by winding 48 m of 5.5 mm I.D PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) around the holder hub. The capacity of the column was 1100 mL. The experiment was performed at a revolution speed of 750 rev/min. The mobile phase was delivered using a Waters 510 HPLC pump (Millipore Corporation, Milford, MA, U.S.A.). An injection loop was used for sample loading and a UV-VIS detector (Model UV-752, Shanghai Instrument Factory, Shanghai, China) was used for monitoring the effluent.

Reagents

GTP (quality specifications: tea polyphenols: minimum 95%, EGCG: minimum 50%) used for preparation of LTP was supplied by Zhejiang Yuyao Siming Tea Biological Products Co., Ltd, and hexadecanoyl chloride was fresh prepared by collecting 152 ℃ – 156 ℃/267 Pa fractions of distillation of reaction product between hexadecanoic acid and thionyl chloride. epigallocatechin-3-*O*-gallate (EGCG) was purchased from Sigma, n-hexane, methanol and ethyl acetate used for high-speed countercurrent chromatography were analytical grade solvents. Methanol used for HPLC analysis was HPLC grade reagent.

Preparation of two-phase solvent system

In order to achieve effective separation by HSCCC, one must first determine a suitable solvent system which provides an optimum range of partition coefficient ($0.5 < K < 2.0$) for the target compounds. This can be done according to the following two methods: a simple and most effective method is to find a previous report on CCC experiments for similar compounds. If such data are not available, one may conduct a systematic search for the solvent system as described elsewhere (Oka *et al.*, 1991) although this trial and error procedure may require several hours. In the present study, the preparative HSCCC separation and purification was performed with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v). After thoroughly equilibrating the mixture of the solvents in a separatory funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as mobile phase, and the lower aqueous phase as stationary phase.

Preparation of LTP

The catalytic esterification was carried out according to a patented method (Zhong *et al.*, 1999). LTP was prepared using 4.0 g of GTP and 6.5 g of hexadecanoyl chloride in 50 mL of ethyl acetate in the presence of a catalyst at 40 ℃. The reaction was kept for 3 hours under stirring. Then the reaction solution obtained by filtration was washed with deionized water (30 mL × 3), and the upper organic layer was evaporated and dried in vacuum at 40 ℃ to yield 8.7 g of the light-yellow powdery product. A likely reaction can be as indicated in Fig.1.

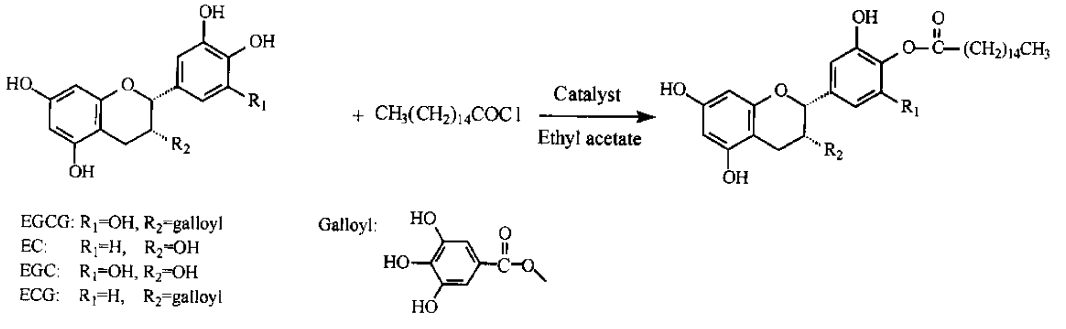

Fig.1 A likely esterification reaction scheme between GTP and hexadecanoyl chloride

Separation procedure

The multilayer coiled column was first entirely filled with the lower aqueous phase as the stationary phase. Then the apparatus was started. The HSCCC sample solution was prepared by dissolving 5.0 g of LTP in 50 mL of upper phase, and injected through the injection loop before the mobile phase was pumped into the column at a flow rate of 3.2 mL/min after running to 750 rev/min. The effluent was monitored at 280 nm and collected with a fraction collector.

HPLC analysis and identification of the acyl-derivative

An Agilent 1100 HPLC system composed of quaternary pump with degasser, thermostated column compartment, variable wavelength detector, manual injector and 1100 ChemStation software. The HPLC separation was performed on a Zorbax-ODS column (5 μm, 4.6 mm I.D. × 25cm) eluted with 85% of methanol in water from 0 – 30 min at a flow rate of 1.0 mL/min and monitored at 280 nm and column temperature of 25 ℃. Identification of the acyl-derivative was carried out by Bruker Vector-22 infrared (IR) spectrometer, Esquire-LC electrospray ionization (ESI) Mass spectrometry (MS), AC-400 nuclear magnetic resonance (NMR) spectrometer and Carlo-Erba EA1110 Elemental analyzer.

RESULTS AND DISCUSSION

Isolation and purification of lipophilic acyl-derivative of EGCG

In the molecules of catechins many hydroxyl groups may react with acyl chloride. Therefore, the reaction products were complicated (Fig.2) though the reaction condition was strictly controlled. However, it is interesting that the main products were isolated by the separation of HSCCC (Fig.3). The fraction corresponding to peak 3 was collected and the solvent evaporated to yield 1.4 g of white powder from 5.0 g of LTP. HPLC analysis of the powder showed it was very pure (Fig.4). The result of the elemental analysis (calcd. for $C_{38}H_{48}O_{12}$: C65.52%, H6.90%; found: C65.38%, H6.85%) was in good agreement with that of the EGCG acyl-derivative.

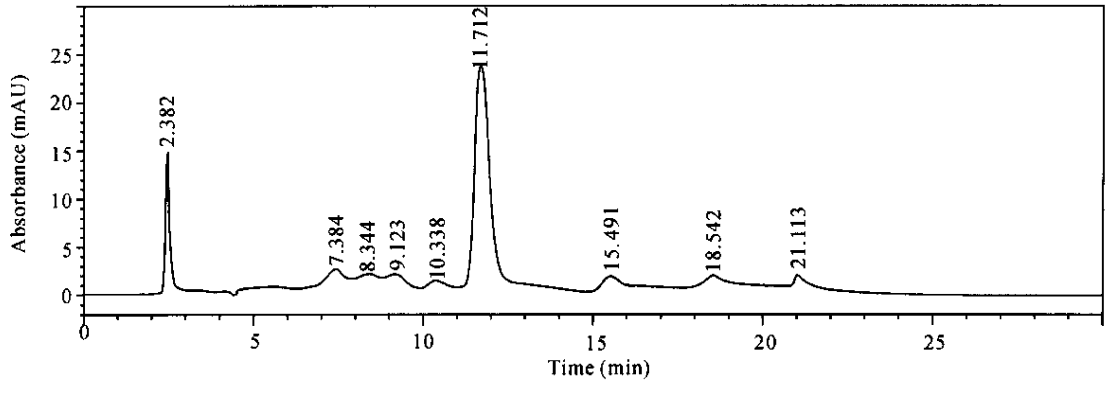

Fig.2 HPLC analysis of the reaction products between GTP and hexadecanoyl chloride. Zorbax-ODS column (5 μm, 4.6 mm I.D. × 25 cm); column temperature: 25 ℃; mobile phase: 85% of methanol in water; flow rate: 1.0 mL/min; wavelength: 280 nm

Elucidation of the acyl-derivative structure

1. Infrared spectrum (IR) analysis

Fig.5 shows the IR spectra of EGCG and its acyl-derivative (KBr), in which there were two strong absorption peaks at 2924 cm^{-1} (ν_{as}) and 2853 cm^{-1} (ν_s) that were the characteristic absorption peaks of long chain aliphatic saturated hydrocarbon while the other parts were similar to that of the EGCG. There was another characteristic absorption peak at 1735 cm^{-1} ($\nu_{c=o}$) which indicated the saturated fatty acid ester linkage between long fatty chain and EGCG.

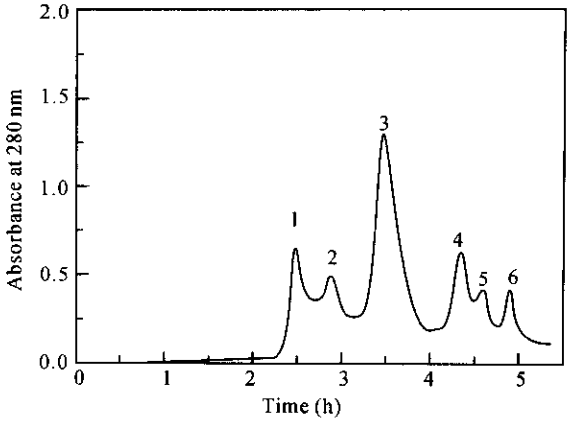

Fig.3 HSCCC chromatogram of LTP. Solvent system: n-hexane-ethyl acetate-methanol-water (1∶1∶1∶1, v/v); mobile phase: upper organic phase; flow rate: 3.2 mL/min; rotating speed of the column: 750 rev/min; sample size: 5.0g; retention of stationary phase: 69%

2. Mass spectrometry (MS) analysis

Electrospray ionization (ESI) MS was adopted to analyze the EGCG acyl-derivative. The data showed that m/z 695.4 is the negative ion of the EGCG acyl-derivative, which is the calculated molecular mass 696.8 of the derived EGCG by 16-C fatty acid chain. The main fragment m/z 457.1 is the negative ion of EGCG (molecular mass 458.4). Therefore, it can be inferred from the MS analysis that the acyl-derivative should be EGCG-$COOC_{15}H_{31}$.

3. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) analysis

Table 1 lists the ^{1}H-NMR chemical shifts (δ) of EGCG and the acyl-derivative of EGCG in DMSO-d_6. The δ values of the acyl-derivative were close to the corresponding protons in EGCG while one proton (δ 8.01) missed and three kinds of protons [δ0.85(3H, t, J = 6.72 Hz),

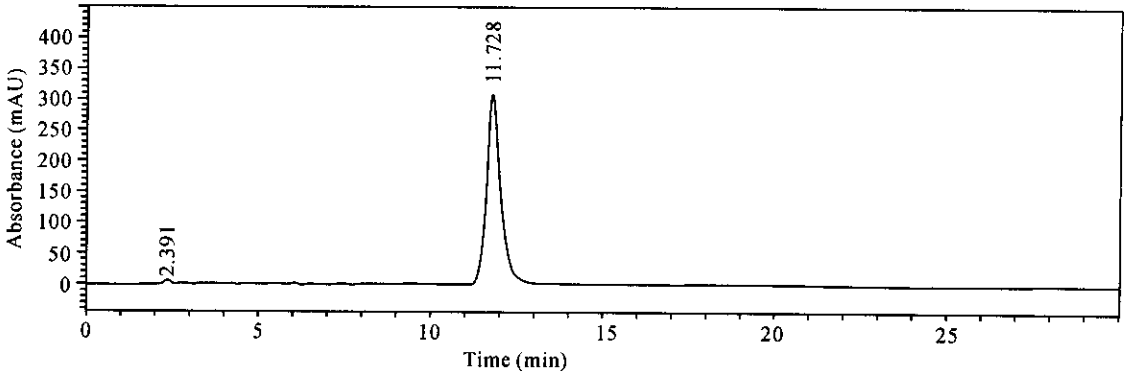

Fig.4 HPLC analysis of the fraction corresponding to peak 3 in HSCCC chromatogram (Analysis conditions: the same as above)

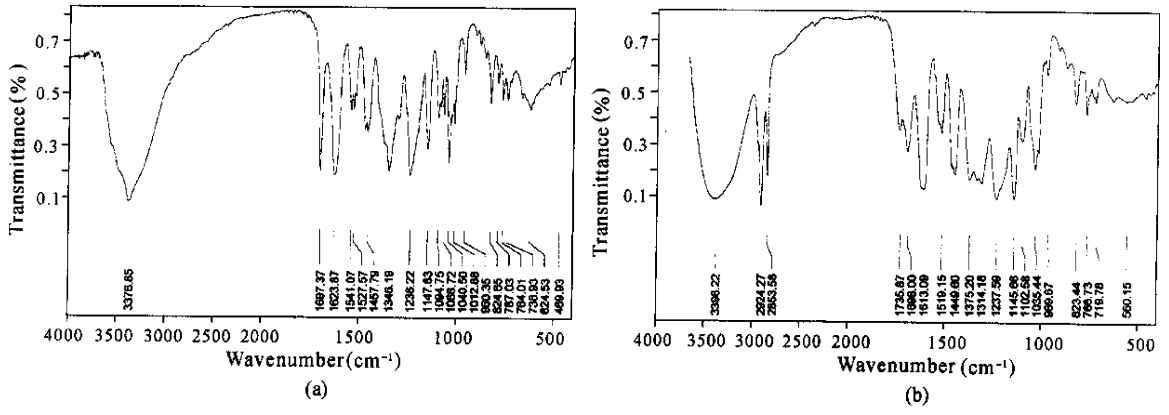

Fig.5 The IR spectra of EGCG and its acyl-derivative (KBr)

(a) EGCG; (b) EGCG Acyl-derivative

δ1.24(26H, m), δ1.58(2H, m)] increased. Proton corresponding δ8.01 is the proton at 4′-OH on the B-ring of the EGCG structure (Jia *et al*., 1998), and respective protons corresponding δ0.85(3H, t, J = 6.72 Hz), δ1.24(26H, m) and δ1.58(2H, m) are protons in the groups of $-CH_3$, $-(CH_2)_{13}-$ and $-CO-CH_2-$. Consequently, It is clear that the acyl-derivative is epigallocatechin-3-*O*-gallate-4′-*O*-hexadecanate, a single-substitution acyl-derivative at 4′ on the B-ring of the EGCG structure (Fig. 6) based on the above elemental analysis, IR, ^{1}H-NMR and MS spectra.

Table 1 ^{1}H-NMR data on EGCG and the EGCG acyl-derivative(δ_H in 10^{-6})

Position	EGCG	EGCG acyl-derivative
$-CH_3$		0.85 (3H, t)
$-(CH_2)_{13}-$		1.24 (26H, m)
$-CO-CH_2-$		1.58 (2H, m)
2	4.95 (1H, s)	5.04 (1H, s)
3	5.36 (1H, s)	5.37 (1H, m)
4a	2.92 (1H, m)	2.90 (1H, m)
4e	2.65 (1H, d)	2.61 (1H, d)
6	5.93 (1H, dd)	5.94 (1H, d)
8	5.82 (1H, dd)	5.83 (1H, d)
2′,6′	6.40 (2H, s)	6.49 (2H, s)
2″,6″	6.81 (2H, s)	6.81 (2H, d)
HO-5	9.27 (1H, s)	9.30 (1H, d)
HO-7	9.03 (1H, s)	9.06 (1H, s)
HO-3′, 5′	8.70 (2H, s)	8.70 (2H, s)
HO-4′	8.01 (1H, s)	
HO-3″, 5″	9.18 (2H, s)	9.18 (2H, s)
HO-4″	8.89 (1H, s)	8.89 (1H, s)

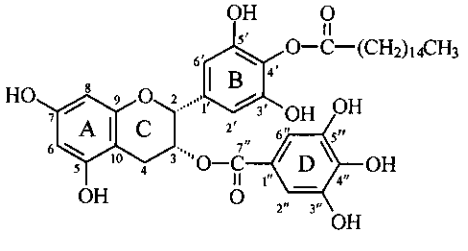

Fig.6 The structure of the EGCG acyl-derivative

CONCLUSIONS

The molecular structure of the catechins in green tea polyphenols (GTP) can be modified to prepare lipophilic tea polyphenols (LTP) by catalytic esterification. The results showed that the main acyl-derivatives of the catechins were isolated and purified by the separation of high-speed countercurrent chromatography (HSCCC), and a novel long-chain single-substitution acyl-derivative of epigallocatechin-3-*O*-gallate (EGCG), epigallocatechin-3-*O*-gallate-4′-*O*-hexadecanate, was obtained from lipophilic tea polyphenols (LTP). This means that structural modification can significantly improve some properties of natural products, and that HSCCC is indeed a versatile tool in separation science.

References

Dreosti, I.E., 1996. Bioactive ingredients: antioxidants and polyphenols in tea. *Nutr. Rev.*, **54**(11): S51 – S58.

Hollman, P.C.H., Feskens, E.J.M. and Katan, M.B., 1999. Tea flavonols in cardiovascular disease and cancer epidemiology. *Proc. Soc. Exp. Biol. Med.*, **220**: 198 – 202.

Ito, Y. and Conway, W.D.(Editors), 1996. High-Speed Countercurrent Chromatography. Wiley-Interscience, New York.

Jia, Z.S., Zhou, B., Yang, L., Wu, L.M. and Liu, Z.L., 1998. 2D NMR study on tea polyphenols. *Chin. J. Mag. Resonance*, **15**(1): 23 – 30(in Chinese).

Johnson, M.K. and Loo, G., 2000. Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat. Res.*, **459**: 211 – 218.

Li, H.C., Yashiki, S., Sonoda, J., Lou, H., Ghosh, S.K., Byrnes, J.J., Lema, C., Fujiyoshi, T., Karasuyama, M. and Sonoda, S., 2000. Green tea polyphenols induce apoptosis in vitro in peripheral blood T lymphocytes of adult T-cell leukemia patients. *Jpn. J. Cancer. Res.*, **91**: 34 – 40.

Liang, Y., Shen, S.R., Yang, X.Q., Zhao, B.L. and Hou, J.W., 1999. Antioxidant properties of lipophilic tea polyphenols. *J. Zhejiang University(Agric. & Life Sci.)*, **25**(5): 529 – 534(in Chinese).

Oka, F., Oka, H. and Ito, Y., 1991. Systematic search for suitable two-phase solvent systems for high-speed counter-current chromatography. *J. Chromatogr.*, **538**(1): 99 – 108.

Wang, Q.E., Tang, A.B., Ma, Z.H., Huang, W. and Shi, B., 2002. Synthesis of liposoluble tea polyphenols with varying aliphatic groups and their antioxidarion activity. *Chin. Fine Chemicals*, **19**(2): 86 – 89(in Chinese).

Zhong, J.H., Chen, P., Zhang, G.Y., Wang, L.S. and Qian, J., 1999. A method of esterification production for lipophilic tea polyphenols. CN 1231277A(in Chinese).

Zhu, N., Wang, M., Wei, G.J., Lin, J.K., Yang, C.S. and Ho, C.T., 2001. Identification of reaction products of (-)-epigallocatechin, (-)-epigallocatechin gallate and pyrogallol with 2,2-diphenyl-1-picrylhydrazyl radical. *Food Chemistry*, **73**: 345 – 349.