Pharmacological properties of galenical preparations (XX)[1]: Screening of natural prodrugs in Polygalae Radix

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Abstract

We isolated and identified natural prodrugs of 3,4-5-trimethoxycinnamic acid (TMCA) and methyl 3,4,5-trimethoxycinnamate (M-TMCA) from the water extract of Polygalae Radix. After oral administration of oligosaccharide fraction and onjisaponin fraction to rats, the time courses of plasma concentration of TMCA were studied, and then it was found that TMCA was kept in a constant plasma concentration from 0.5 hr to 3 hr after oral administration of the oligosaccharide fraction. In this way, we furthermore isolated and identified sucrose derivatives of TMCA as prodrugs from the oligosaccharide fraction. After oral administration of the sucrose derivatives, tenuifolisides A and C, the plasma concentrations of TMCA were low at 1 and 2 hr, but became higher from 3 hr and were kept at a constant level until 5 hr. After oral administration of tenuifoliside A to kanamycin-treated rats the plasma concentrations of TMCA and M-TMCA were significantly reduced, showing that the intestinal bacteria play a role in the conversion of tenuifoliside A to TMCA in the gastrointestinal tract. The above findings indicated that tenuifolisides A and C were natural prodrugs of TMCA. In addition, it was suggested that sucrose derivatives possessing a 3,4,5-trimethoxycinnamoyl moiety in their structures may be natural prodrugs of TMCA.

Key words intestinal bacteria, Polygalae Radix, prodrug, sucrose derivative, 3,4,5-trimethoxycinnamic acid, tenuifolisides A and C.

Abbreviations 3D-HPLC, three dimensional high performance liquid chromatography; M-TMCA, methyl 3,4,5-trimethoxycinnamate; PMCA, p-methoxycinnamic acid; TMCA, 3,4,5-trimethoxycinnamic acid.

Introduction

Onji (遠志), Polygalae Radix, *Polygala tenuifolia* WILLDENOW (Yuan-zhi in Chinese), is a well known Chinese traditional medicine used as a sedative, expectorant and tonic agent. In our previous study, we made a trial searching for bioactive substances in blood and bile samples of rats after oral administration of the extracts of Polygalae Radix and found 3,4,5-trimethoxycinnamic acid (TMCA), methyl 3,4,5-trimethoxycinnamate (M-TMCA) and p-methoxycinnamic acid (PMCA) which induced the prolongation of hexobarbital sleeping time in mice.[2] Pharmacokinetics of TMCA and the time course of M-TMCA concentration in rat plasma after oral administration of the water extract of Polygalae Radix have been reported.[3] After oral administration of TMCA, the plasma concentration of TMCA rapidly decreased; but TMCA in the plasma was kept in a constant concentration after oral administration of the water extract of Polygalae Radix; it was suggested that the water extract contained some natural prodrugs for TMCA.[3] In this paper, we studied with the purpose of isolation and identification of the natural prodrugs in the water extract of Polygalae Radix.

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Materials and Methods

Crude drug : Polygalae Radix, the dried root of *Polygala tenuifolia* WILLD., was commercially obtained from the Japanese market, Mikuni Co., Ltd. in Osaka. Polygalae Radix was cut into small pieces and used for this experiment.

Chemicals : 3,4,5 - trimethoxycinnamic acid (TMCA) and kanamycin sulfate were purchased from Wako Pure Chemical I dustries Ltd. in Japan. Methyl 3,4,5-trimethoxycinnamate (M-TMCA) was isolated from the bile of rats after administration of TMCA in our laboratory and the details for the methods of isolation and identification have been reported in our previous report.[2] Tenuifolisides A and C were isolated from Polygalae Radix and identified by direct comparison with authentic samples.[1,5]

Animals : Male Wistar/ST rats, weighing 180-220 g, used in this experiment were purchased from Nihon SLC Co., Ltd. (Hamamatsu, Japan). They were housed under conditions of 24±1°C and 12 hr light (from 6 a.m. to 6 p.m.) and fed a commercial diet (MF, Oriental Yeast Co., Tokyo) and allowed tap water *ad libitum* before the experiments.

Screening of natural prodrugs in Polygalae Radix : The procedures of extraction and separation of the constituents in Polygalae Radix were shown in Scheme 1. 1) Water extract ; 250 g of Polygalae Radix was mixed with 5 l of distilled water, and the mixture was boiled until the volume was reduced to 2.5 l. The filtered decoction was freeze-dried and the obtained powder (1.0 g corresponds to 4.0 g of the crude drug) was kept in a refrigerator. Water extract was dissolved and/or suspended in distilled water to make a solution of 0.33 g/ml just before oral administration to rats and was administered at 5.0 g/kg, and then the blood sample was collected at 30 min, 1 hr, 2 hr and 3 hr after the administration. 2) Butanol layer ; 750 ml of Water extract (corresponding to 250 g of the crude drug) was extracted 5 times with the same volume of water-saturated n-butanol and the butanol solution was evaporated to dryness under reduced pressure, and then the resulting powder (1.0 g corresponds to 6.8 g of the crude drug) was kept in a refrigerator. The butanol extract was dissolved and/or suspended in distilled water to make a solution of 0.20 g/ml just before oral administration and was administered to rats at 3.0 g/kg, and then the blood sample was collected at 30 min, 1 hr, 2 hr and 3 hr after the administration. 3) Oligosaccharide and onjisaponin fractions ; 150 g of butanol layer (corresponding to 1.0 kg of the crude drug) was chromatographed on a silica gel column with ethyl acetate-methanol (90 : 10 →0 : 100), and then the oligosaccharide fraction (58.0 g) and onjisaponin fraction (58.0 g) were obtained. Oligosaccharide and onjisaponin fractions were dissolved and/or suspended in distilled water to make the respective solutions of 0.10 g/ml and were orally administered to rats at 1.47 g/kg of oligosaccharide fraction and onjisaponin fraction. The blood samples were collected at 30 min, 1 hr, 2 hr and 3 hr after the administration. 4) Fractions I and II obtained from oligosaccharide fraction ; 14.5 g of oligosaccharide fraction (corresponding to 250 g of the crude drug) was chromatographed on a silica gel column with a mixture of ethyl acetate-methanol to give Fractions I (3.26 g) and II (10.95 g). Fractions I and II were suspended in distilled water with 0.5 % Tween 80 and 2 % ethanol to make solutions of 0.026 g/ml and 0.084 g/ml, respectively, and were orally administered to rats at 0.26 g/kg of Fraction I and 0.85 g/kg of Fraction II. The blood samples were collected at 30 min, 1 hr, 2 hr, 3 hr and 4 hr after the administration. 5) Tenuifolisides A and C ; Fraction I from oligosaccharide fraction was chromatographed on a silica gel column with chloroform-methanol (85 : 15) and the obtained compounds were purified by preparative HPLC with acetonitrile-water (1 : 5→1 : 4). The purified compounds were identified by direct comparisons with authentic samples, tenuifolisides A and C.[1,5] Tenuifolisides A and C were suspended in distilled water with 0.5 % Tween 80 and 2 % ethanol to make solutions of 0.026 g/ml and were orally administered to rats at 0.26 g/kg. The blood samples were collected at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr after the administrations.

All the blood samples in the above items 1)-5) were collected from the portal vein and treated with methanol, chloroform - methanol (4 : 1) and acetonitrile-water (4 : 1) for three dimensional high performance liquid chromatography (3D-HPLC)

analysis, and the details for the method of quantitative analysis have been reported in the previous paper.[3]

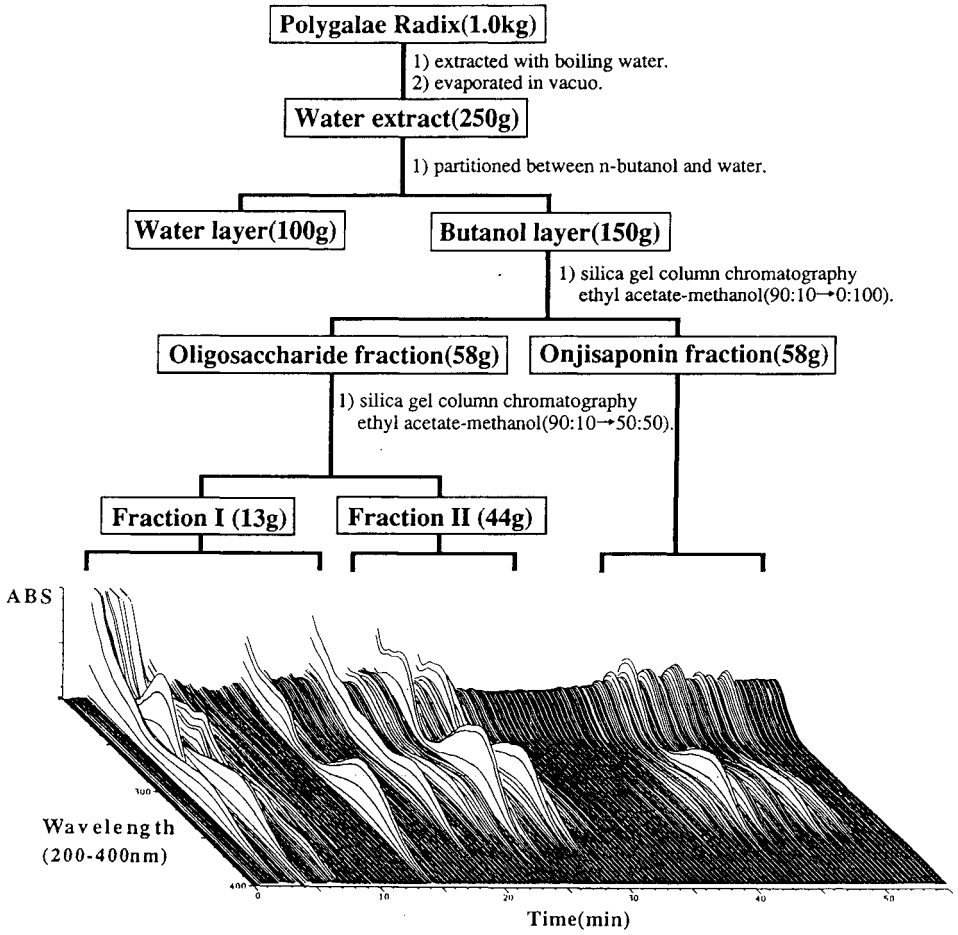
The effect of kanamycin on the plasma concentration of TMCA and M-TMCA after oral administration of tenuifoliside A : Kanamycin sulfate was given orally twice daily for two days at a dose of 200 mg per rat,[6] thereafter tenuifoliside A was orally administered to rats at a dose of 0.26 g/kg. The blood samples were collected at 3 hr and 4 hr after the administration of tenuifoliside A and treated with the same method mentioned above.

Results

Screening of natural prodrugs in Polygalae Radix

Scheme 1 shows the procedures of extraction and separation of constituents in Polygalae Radix and the 3D-HPLC profile[2] for water extract. Almost all the constituents which had U.V. absorptions in Water extract were extracted with n-butanol. Butanol layer was successfully divided into oligosaccharide and onjisaponin fractions by chromatography on a silica gel column. Furthermore, oligosaccharide fraction was divided into Fractions I and II. Fractions I and II mainly contained sucrose derivatives and pentasaccharide derivatives, respectively.[4,5]

1) *Water extract and butanol layer* : Fig. 1 shows the time profile of plasma concentrations of TMCA and M-TMCA after oral administration of water extract and butanol layer, respectively. The time profile of butanol layer was almost the same with that of water extract. It was found that almost all the



Scheme 1 The procedures of extraction and separation of the constituents in Polygalae Radix and the 3D-HPLC profile for water extract.

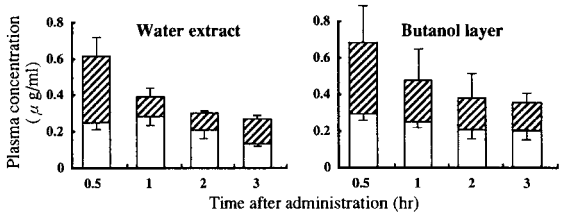


Fig. 1 Time profiles of plasma concentrations of TMCA and M-TMCA after oral administration of water extract (5.0 g/kg) and butanol layer (3.0 g/kg) to rats. (see Scheme 1)

Each column and vertical bar represent the mean and S.D. of 3 rats.
□: TMCA ▨: M-TMCA

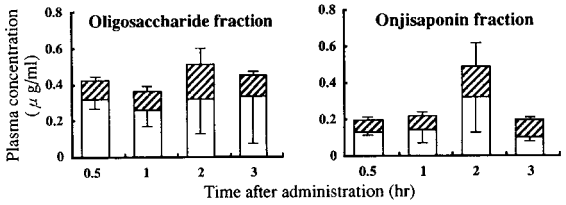


Fig. 2 Time profiles of plasma concentrations of TMCA and M-TMCA after oral administration of oligosaccharide fraction (1.47 g/kg) and onjisaponin fraction (1.47 g/kg) to rats. (see Scheme 1)

Each column and vertical bar represent the mean and S.D. of 3 rats.
□: TMCA ▨: M-TMCA

natural prodrugs in water extract were extracted into butanol layer because the doses of water extract and butanol layer correspond to the same dose (20.0 g/kg) calculated on the dry weight basis of the crude drug.

2) *Oligosaccharide and onjisaponin fractions* : Fig. 2 shows the time profiles of plasma concentrations of TMCA and M-TMCA after oral administration of oligosaccharide and onjisaponin fractions at the each dose of 1.47 g/kg. The time profile of the oligosaccharide fraction showed a higher plasma concentration of TMCA than that of the onjisaponin fraction during all the experiment periods. There was a strong possibility that the prodrugs were fractionated into oligosaccharide fraction more than into onjisaponin fraction. According to the above findings we further divided oligosaccharide fraction into Fractions I and II.

3) *Fractions I and II from oligosaccharide fraction* : Fig. 3 shows the time profiles of plasma concentrations of TMCA and M-TMCA after the administration of Fractions I and II at the dose of 0.26 g/kg and 0.85 g/kg, respectively. The plasma concentrations of TMCA and M-TMCA were kept in almost constant concentrations from 0.5 hr to 4 hr after oral administration of Fraction I. After oral administration of Fraction II, TMCA in the plasma reached the highest concentration at the first 0.5 hr ; and then it rapidly decreased, which was very similar to the findings[3] after oral administration of TMCA.

4) *Tenuifolisides A and C* : Fig. 4 shows a HPLC elution profile of Fraction I from oligosaccharide fraction. Many sucrose derivatives of TMCA (C1, C2, C3, C4 and C5 in Fig. 4) were identified by direct comparison with authentic samples on 3D-HPLC, and the main compounds, tenuifolisides A and C, were isolated from Fraction I. Fig. 5 shows the time profiles

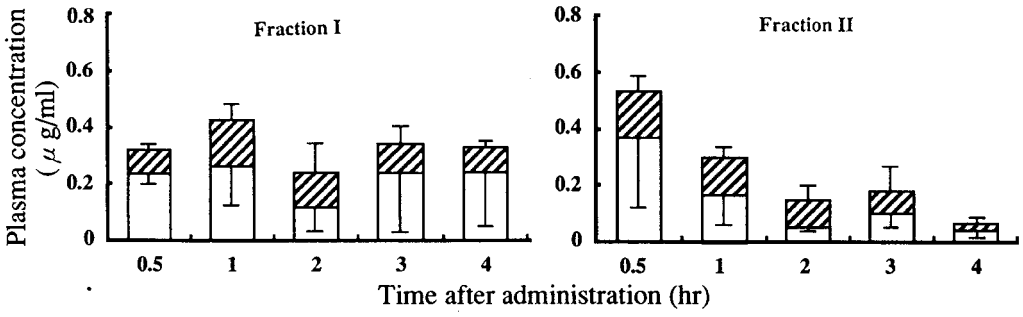


Fig. 3 Time profiles of plasma concentrations of TMCA and M-TMCA after oral administration of Fraction I (0.26 g/kg) and Fraction II (0.85 g/kg) to rats. (see Scheme 1)

Each column and vertical bar represent the mean and S.D. of 3 rats.
□: TMCA ▨: M-TMCA

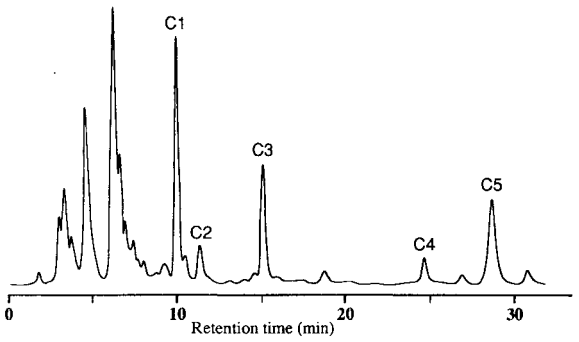


Fig. 4 HPLC elution profile of Fraction I. (see Scheme 1)
Analytical conditions : A Waters 600 multisolvent delivery system equipped with Waters 991 J photodiode array detector and its data processor. Column : Inertsil ODS-2. Column temperature : 40°C. Detection wavelength : 300 nm. Mobile phase : water (0.1 % acetic acid)-acetonitrile (0.1 % acetic acid)-23 : 77. Flow rate : 1 ml/min.
C1 : tenuifoliside A. C2 : tenuifoliside D.
C3 : tenuifoliside C.
C4 : β-D-[3-O-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-[6-O-(benzoyl)]-glucopyranoside.
C5 : β-D-[3-O-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-[6-O-(p-methoxybenzoyl)]-glucopyranoside.

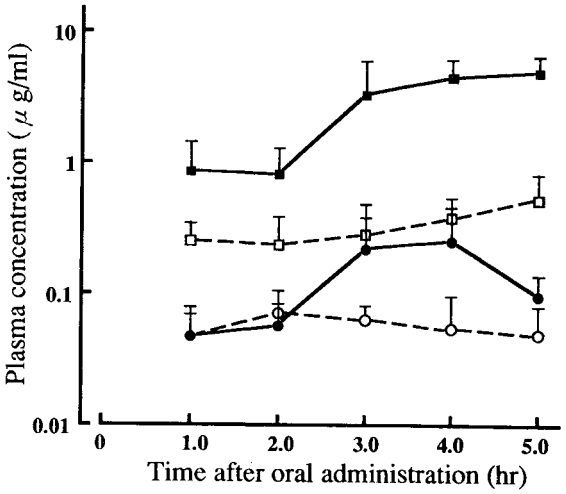


Fig. 5 Time profiles of plasma concentrations of TMCA and M-TMCA after oral administration of tenuifolisides A and C at each dose of 0.26 g/kg to rats.
Each point and vertical bar represent the mean and S.D. of 3 or 4 rats.
●—●: TMCA; ○···○, M-TMCA: plasma concentration after oral administration of tenuifoliside A.
■—■: TMCA; □···□, M-TMCA: plasma concentration after oral administration of tenuifoliside C.

of plasma concentrations of TMCA and M-TMCA after oral administration of tenuifolisides A and C at each dose of 0.26 g/kg. None of tenuifolisides A and C were detected in rat plasma but their metabolites, TMCA and M-TMCA, were detected from 2 hr to 5 hr after the administration. The plasma concentration of TMCA was comparatively lower in the first 2 hr, but 2 hr later it increased until 4 hr or 5 hr after the administration. It was found that tenuifolisides A and C were prodrugs of TMCA and M-TMCA. The plasma concentrations of TMCA and M-TMCA derived from tenuifoliside C were much higher than those from tenuifoliside A.

5) *The effect of Kanamycin on the plasma concentrations of TMCA and M-TMCA after oral administration of tenuifoliside A*: In order to prove the metabolism of the prodrugs by intestinal bacteria, we investigated the effect of kanamycin on the plasma

Table I Effects of kanamycin on the plasma concentrations of TMCA and M-TMCA after oral administration of tenuifoliside A.

Time	Rat	TMCA[a] mean±S.D.	M-TMCA[a] mean±S.D.	Total[b] mean±S.D.
3 hr	Normal	0.298±0.136	0.069±0.009	0.374±0.150
	Treatment[c]	0.056±0.016*	0.120±0.046*	0.185±0.030
4 hr	Normal	0.423±0.074	0.145±0.023	0.569±0.050
	Treatment[c]	0.093±0.020**	0.101±0.011*	0.200±0.035**

Each datum represents the mean(μg/ml) and S.D. of 3 or 4 rats.
a) After oral administration of tenuifoliside A at a dose of 0.26 g/kg.
b) Total plasma concentration of TMCA and M-TMCA.
c) The rats were given kanamycin sulfate orally twice daily for two days at a dose of 200 mg per rat.
*: $p<0.05$ and **: $p<0.01$ vs normal rats.

concentrations of TMCA and M-TMCA after oral administration of tenuifoliside A. The plasma concentrations of TMCA and M-TMCA in kanamycin-treated rats were significantly reduced in comparison with those in non-treated rats at 3 hr and 4 hr after oral administration of tenuifoliside A (Table I).

Discussion

In our studies on pharmacological properties of galenical preparations, we have found bioactive compounds, TMCA, M-TMCA and PMCA, present in the blood and bile after oral administration of a water extract of Polygalae Radix to rats.[2] In order to elucidate and evaluate the clinical effects of the traditional Chinese medicine, we have studied the pharmacokinetic properties of these bioactive compounds and the water extract of Polygalae Radix in rat body.[3] These studies gave us the idea that precursors of bioactive compounds (prodrugs) might be contained in the water extract of Polygalae Radix. In this paper, we tried to isolate and identify the prodrugs present in the water extract. The water extract was divided into several fractions and they were administered to rats for the screening of the prodrugs at the same dose calculated on the dry weight basis of the crude drug. From the findings of the fractions in Fig. 1-3, it was suggested that sucrose derivatives of TMCA in Fraction I might be prodrugs of TMCA and M-TMCA; and then tenuifolisides A and C were isolated from Fraction I. It was proved that tenuifolisides A and C were the prodrugs for TMCA and M-TMCA in the rat blood. The plasma concentrations of TMCA and M-TMCA after oral administration of tenuifoliside A in kanamycin-treated rats were significantly reduced in comparison with those in non-treated rats, showing that the intestinal bacteria play a role in the conversion of the prodrug, tenuifoliside A, to TMCA in the gastrointestinal bacteria. All the above-mentioned findings indicate that tenuifolisides A and C are natural prodrugs for TMCA and M-TMCA in the rat blood after oral administration of the water extract of Polygalae Radix. And it is also suggested that all the sucrose derivatives of TMCA have a possibility as prodrugs of TMCA and they will be metabolized by gastrointestinal bacteria in various degrees according to the difference in their chemical structure. We consider that after oral administration of the water extract, TMCA in the plasma within the first 2 hr have its origins in free TMCA and some prodrugs which are hydrolyzed and/or metabolized rapidly, and from 2 hr to 5 hr it is derived from tenuifolisides A and C and other sucrose derivatives of TMCA. The studies on the absorption, metabolism and excretion of these prodrugs in Polygalae Radix will provide us with a lot of important information for the activity and toxicity of this crude drug which are pharmacological and pharmaceutical properties of galenical preparations. The method in this paper, the biopharmaceutical procedure applied to the discovering prodrugs in Polygalae Radix, will be applicable to the searching of other crude drugs for their bioactive substances in a view of elucidating and evaluating the clinical effects.

Further studies on other prodrugs for TMCA in Polygalae Radix are in progress and will be reported in another paper.

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和文抄録

著者らは，TMCA と M-TMCA の天然プロドラッグを遠志水エキスから単離，同定した。Oligosaccharide 分画と onjisaponin 分画をラットに経口投与した後，血漿中の TMCA 濃度の経時変化を調べたところ，oligosaccharide 分画の経口投与後 30 分から 3 時間まで TMCA は一定の血漿濃度を保つことが明らかとなった。この方法によって，さらに oligosaccharide 分画からプロドラッグとして TMCA の sucrose 誘導体類を単離・同定した。Sucrose 誘導体である tenuifoliside A と C の経口投与後，TMCA の血漿濃度は 1，2 時間目では低く，3 時間目から高くなり 5 時間目まで一定濃度に持続された。

Kanamycin 処理を施したラットに tenuifoliside A を経口投与したところ，その後の血漿中 TMCA と M-TMCA の濃度は有意に減少した。このことは，消化管内において腸内細菌が tenuifoliside A を TMCA に変換することを示している。以上から，tenuifoliside A と C は TMCA の天然プロドラッグであり，またさらに 3,4,5-trimethoxycinnamoyl を化学構造式内に持つ sucrose 誘導体類は，TMCA の天然プロドラッグであることが示唆された。

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