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ACCUMULATION AND ENZYMATIC SYNTHESIS OF 2-*O*-ACETYL-3-*O*-(*p*-COUMAROYL)-*meso*-TARTARIC ACID IN SPINACH COTYLEDONS

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Key Word Index *Spinacia oleracea*; Chenopodiaceae; spinach; hydroxycinnamic acid; tartaric acid; phenylpropanoid metabolism; accumulation; 1-*O*-acylglucose; acetyl-CoA; enzymatic synthesis.

Abstract—2-*O*-Acetyl-3-*O*-[(*E*)-*p*-coumaroyl]-*meso*-tartaric acid was isolated from cotyledons of *Spinacia oleracea* and its structure elucidated and characterized with the aid of TLC, HPLC, FAB MS and ^{1}H NMR. Accumulation and enzymatic synthesis of the diester are described, proceeding first via 1-*O*-(*p*-coumaroyl)-β-glucose in the formation of *p*-coumaroyltartaric acid and second via acetyl-CoA in the formation of 2-*O*-acetyl-3-*O*-[(*E*)-*p*-coumaroyl]-*meso*-tartaric acid. Some properties of the CoA-thioester-dependent acyltransferase activity were studied.

INTRODUCTION

Higher plants accumulate the common hydroxylated cinnamic acids *p*-coumarate, caffeate, ferulate and sinapate mainly in conjugated forms as esters with a vast array of different compounds [1–3]. Hydroxycinnamic acid esters with aliphatic (hydroxy) carboxylic acids, so far detected, include conjugates with tartaric [e.g. 4], malic [e.g. 5], hydroxycitric [6], glucaric [7, 8], dihydroxypropanedicarboxylic [9], tartronic [10], and gluconic acids [11].

Tartaric acid has repeatedly been described as the dicarboxylic acid moiety of hydroxycinnamic acid esters in various plants, e.g. in *Cichorium intybus* [4, 12], *C. endivia* [13], *Vitis vinifera* [14], *Phaseolus vulgaris* [15] which lacks definite proof, *Spinacia oleracea* [16–18], *Lactuca sativa* [19], and *Echinacea angustifolia* and *E. purpurea* [20].

Biosyntheses of these conjugates proceed either through acylcoenzyme A or acylglucose mediated carboxyl group activation [21] of the phenolic acid. The physiological role of these alternative pathways is presently under study in our laboratory. Since nothing is known about the enzymatic synthesis of phenolic acid tartrate esters we investigated cotyledons of spinach seedlings and found a rapidly accumulating *p*-coumaric acid ester, which is the major phenylpropanoid constituent. We isolated this ester and determined its structure to be 2-*O*-acetyl-3-*O*-[(*E*)-*p*-coumaroyl]-*meso*-tartaric acid (**1**). This is in agreement with the structure proposed by Tadera and Mitsuda [17] of the compound present in spinach leaves. We report here on the structural elucidation of this major *p*-coumaric acid ester, its accumulation and enzymatic synthesis in spinach cotyledons.

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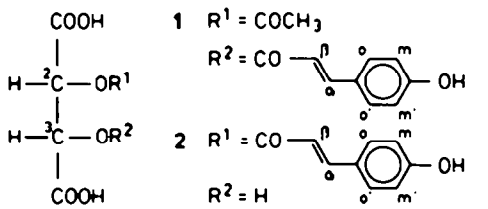
RESULTS AND DISCUSSION

Structure elucidation and accumulation of 1

Chromatographic analyses (TLC, HPLC) of methanolic extracts from cotyledons of spinach showed a complex pattern of phenolic compounds, among which a *p*-coumaric acid derivative was found to be the major constituent, i.e. 2-*O*-acetyl-3-*O*-[(*E*)-*p*-coumaroyl]-*meso*-tartaric acid (**1**). For isolation of this compound the crude extracts were pre-fractionated on polyamide columns with H_2O, MeOH and 0.03% NH_4OH in MeOH as solvents. Compound **1** and a minor second *p*-coumaric acid conjugate, i.e. 2-*O*-[(*E*)-*p*-coumaroyl]-*meso*-tartaric acid, were present in the ammoniacal fraction (TLC in SS1 and HPLC). The latter was identified by co-chromatography with **2**, which was prepared through partial hydrolysis of **1** (see below). In addition, 1-*O*-(*p*-coumaroyl)-β-glucose was detected (co-chromatography with reference material) [*cf.* 22] as a minor compound in the H_2O fraction. Compound **1** was isolated by TLC in SS1 and further purified by re-chromatography on a polyamide column and finally by CC on Sephadex LH-20 (MeOH as solvent). The structures of compounds **1** and its partial hydrolysis product **2** were assigned on the basis of the data presented below.

The UV spectral data of **1** (λ_{max}^{MeOH} 226, 312; $\lambda_{max}^{MeOH\ NH_4OH}$ 222, 353 nm) and its colour reaction on TLC (under UV at 350 nm changing from absorbing to blue fluorescence when treated with NH_3 vapour) are consistent with those of *p*-coumaric acid esters. **1** and **2**



exhibited the following R_f values in SS1: 0.78, 0.66; SS2: 0.55, 0.29; SS3: 0.58, 0.54. Both compounds gave *p*-coumaric acid (co-chromatography with authentic *p*-coumaric acid in SS4: 0.58) upon alkaline hydrolysis (30 min at room temp. in 1 N aq. NaOH). Tartaric acid liberated from both compounds was identified by co-chromatography with reference compounds on SS5 (0.12) and SS6 (0.62) and was found to be *meso*-tartrate. The acids were visualized by spraying the dried chromatograms with bromocresol green in alkaline (NaOH) EtOH. The presence of tartrate as an ester moiety was expected from other studies on spinach [16–18].

The ^{1}H NMR spectrum unambiguously identified **1** as a *p*-coumaric acid tartrate conjugate. However, in addition a vicinal acetyl group was detected. This is in agreement with the structure proposed by Tadera and Mitsuda [17] of the compound which is present in spinach leaves. All signals were doubled due to the presence of both *Z* and *E* isomers of the *p*-coumaroyl moiety, which were identified by their characteristic vicinal coupling constant. The *E*-isomer was the naturally occurring one, which was found by HPLC with freshly prepared alcoholic extracts from spinach cotyledons. The molecular ion $[C_{15}H_{14}O_9 - H]^-$ at 337 d and fragmentation pattern in the negative ion FAB MS of **1** confirmed the structure.

Partial hydrolysis with 0.1 N NaOH at 56° for 8 min caused quantitative loss of the acetyl group to give **2**, as expected for bimolecular basic hydrolysis with acyl-oxygen fission [23]. Complete hydrolysis of the diester was achieved after *ca* 90 min. The structure of **2** was confirmed by ^{1}H NMR.

Detailed quantitative HPLC of methanolic extracts from cotyledons of developing spinach seedlings revealed a rapid accumulation of **1** as the major soluble secondary product of phenylpropanoid metabolism. The highest concentration of *ca* 30 nmol per cotyledon pair was reached on the 9–10th day of culture (Fig. 1). In addition, **2** and 1-(*p*-coumaroyl)-β-glucose were detected. The former was identified by co-chromatography with the partial hydrolysis product (**2**) from compound **1** and the latter with a reference compound isolated from petals of *Antirrhinum majus* [24]. The glucose derivative showed a transient accumulation reaching *ca* 1.4 nmol at the 5th day and was later detected in trace amounts. The tartaric acid monoester, initially accumulated concomitantly with *p*-coumaroylglucose, showed a second phase of accumulation (max. *ca* 0.5 nmol) after 2 weeks of development. Analyses of other organs from spinach (e.g. adult leaves) also showed **1** as the major secondary constituent.

Enzymatic synthesis of **1**

Protein preparations from cotyledons of spinach seedlings were assayed for the enzymatic synthesis of **1**. Both possible mechanisms of synthesis via coenzyme-A and glucose-carboxyl group activation, so far detected [21], were tested. It was found that the initial reaction was *p*-coumaroyltartaric acid monoester formation which proceeds exclusively via 1-*O*-(*p*-coumaroyl)-β-glucose as the activated donor. Heat-denatured (5 min at 80°) protein showed no enzymatic activity. The product was identified by co-chromatography (TLC in SS1 and SS2, HPLC) with **2**. No product was formed when *p*-coumaroyl-CoA was tested as a possible acyl donor according to methods developed by Zenk and co-workers [25, 26] and as

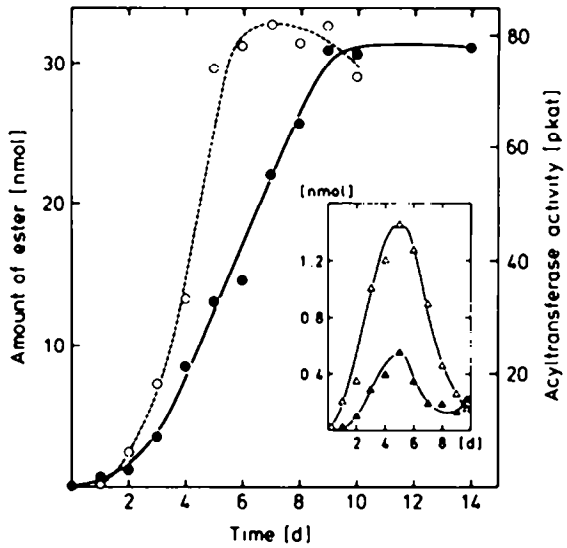


Fig. 1. Accumulation patterns of *p*-coumaric acid esters (nmol per pair of cotyledons) and development of CoA-dependent acyltransferase activity (○) (pkat per pair of cotyledons at 0.33 mM acetyl-CoA and 0.52 mM *p*-coumaroyltartaric acid, **2**) in cotyledons of spinach; mean data from two separate experiments and duplicate determinations; 1-(*p*-coumaroyl)-glucose (△), *p*-coumaroyltartaric acid (**2**) (▲), 2-acetyl-3-(*p*-coumaroyl)-tartaric acid (**1**) (●). At the stage of maximal enzyme activity V_{max} was 210 pkat/pair of cotyledons and 620 pkat/mg protein (see text). The specific enzyme activity (protein as parameter) showed the same rapid increase as that seen here. Mixed assays from low and high enzymatic activities showed strict linearities.

described by Strack *et al.* [27]. We were also unable to show the possible CoA-dependent back reaction using **2** or **1** as donors and free CoA as acceptor. This acylglucose-dependent reaction accepted only *meso*-tartaric acid. D- or L-Tartaric acid were not accepted. Unfortunately the rate of formation of **2** was very low (*ca* 0.1 pkat/mg protein) and could not be appreciably increased, thus precluding further characterization. Also the enzyme preparation was very labile and activity could only be observed with freshly prepared protein. Although the partially purified enzymatic activity (DEAE-cellulose) showed a slightly higher activity, various attempts to optimize the enzyme assay, including the use of proteinase inhibitors, were unsuccessful (data not shown).

The subsequent second ester formation, leading to the diester **1**, proceeds via acetyl-CoA (see Fig. 2). The reaction product was identified by co-chromatography (TLC in SS1 and SS2, HPLC) with **1**. The enzyme(s) involved showed high activities and this made possible a study of some properties of this ester formation (see below). The reaction is dependent on the presence of the *p*-coumaroyl moiety of the acceptor molecule. No product was formed when free tartaric acid was tested as a possible acceptor molecule (incubation with ^{14}C-labelled acetyl-CoA). This was investigated by means of TLC in SS6 (radioscanner, Berthold, Wildbad, West Germany) and radio-HPLC [isocratic with 1.5% aq. H_3PO_4 as solvent or 30–40% solvent B in (A + B), see below]. We observed, however, an appreciable amount of liberated acetic acid, which is probably due to rapid hydrolysis of the CoA thioester.

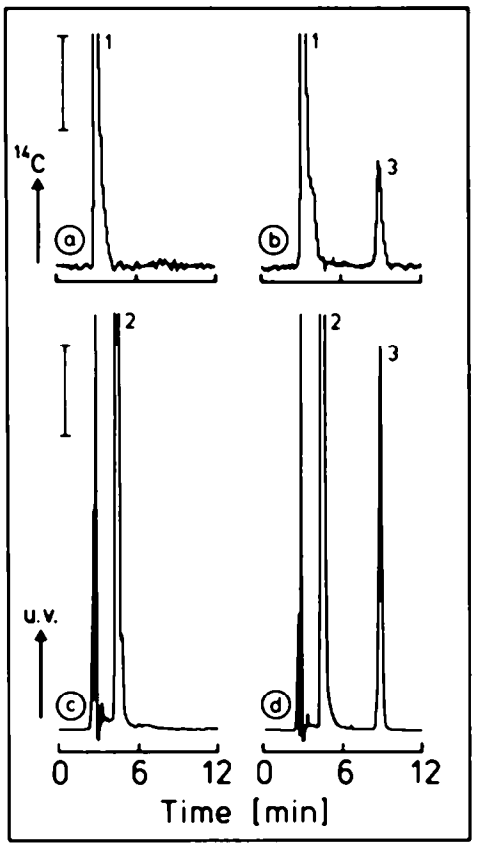


Fig. 2. HPLC analyses of an acyltransferase (CoA dependent) assay immediately after introduction of enzyme (a, c) and after 20 min reaction time (b, d). Lower graphs show HPLC profiles of UV detection, the upper graphs on-line scintillation counter tracing of ^{14}C-labelled components. Peak identification: 1 = [1-^{14}C]acetyl-CoA; 2 = *p*-coumaroyltartaric acid (**2**); 3 = 2-acetyl-3-(*p*-coumaroyl)-tartaric acid (**1**) and 2-[1-^{14}C]acetyl-3-(*p*-coumaroyl)-tartaric acid in graph b. Bars represent 40 cps (a, b) and 4.7×10^{3} absorbance unit at 310 nm (c, d), respectively.

In summary we deduced from these results that biosynthesis of **1** proceeds as follows:

(1) 1-(*p*-coumaroyl)-β-glucose + *meso*-tartaric acid → *p*-coumaroyl-*meso*-tartaric acid + glucose

(2) acetyl-CoA + *p*-coumaroyl-*meso*-tartaric acid → 2-acetyl-3-(*p*-coumaroyl)-*meso*-tartaric acid + HS-CoA.

This pathway is compatible with the accumulation pattern of the spinach *p*-coumaroyl conjugates (Fig. 1).

Characterization of CoA-dependent acyltransferase

General properties. The formation of **1** was positively correlated with the amount of protein, and was linear for 10 min. The greatest enzyme activity was found at pH 7.6 in KPi buffer showing 50% of activity near pH 6.5 (KPi) and 9.0 (Tricine). A study of the ion strength dependency (KPi) showed maximal activity at 70 mM (78 μS) with 50% activity near 20 and 220 mM, respectively. There was no requirement of divalent cations or thiols. The crude protein preparation showed no loss of enzyme activity when stored at −20° for several weeks. The frozen protein showed appreciably greater enzyme activity (*ca* 2-fold) after thawing than fresh preparations.

Kinetics, substrate specificity and development of CoA-dependent acyltransferase. The reaction showed a typical saturation curve with increasing substrate concentrations (**2**, acetyl-CoA). Double reciprocal plots were linear and gave apparent K_m values of 63 and 670 μM for acetyl-CoA (at 0.52 mM **2**) and **2** (at 0.33 mM acetyl-CoA), respectively. However, Lineweaver–Burk plots demonstrated that the K_ms for acetyl-CoA and **2** were dependent on the concentration of the second substrate. This observation will be the basis of a detailed study of the kinetic properties of this enzyme whose purification is presently in progress. As we had a limited amount of *p*-coumaroyltartrate, it was used at suboptimal concentrations which gave *ca* 40% of the maximal reaction velocity.

We could not study the acceptor specificity of the acyltransferase since no tartaric acid conjugate other than **2** was available. However, it was unambiguously shown that free tartaric acid was not acetylated (see above). We tested various CoA-thioesters as donors (Table 1) and found that the enzyme showed a wide range of specificity. With valeryl-CoA the enzyme showed highest reaction rate, followed by acetyl-, butyryl-, hexanoyl- and propionyl-CoA with K_ms of 80, 63, 23, 133 and 33 μM (at 0.52 mM **2**), respectively. There was no difference in this specificity when protein from adult leaves or from cotyledons at different developmental stages were used (data not shown). Butyryl-CoA was the best donor as the relative ratios of the V_{max}/K_m values for butyryl-, valeryl-, acetyl-, propionyl- and hexanoyl-CoA were found to be 100:54:52:45:29, respectively. Dicarboxylic acids, branched-chain and unsaturated carboxylic acids were poor substrates (see Table 1). In summary, the best substrates were straight chain, saturated monocarboxylic acids up to a C_6-chain length. We assume that the described acyltransferase activity is specifically involved in the formation of **1** in spinach. Protein preparations from primary leaves of mung bean [27] or from cotyledons of radish [22] were inactive with **2** and acetyl-CoA as substrates. However, further studies are needed to show whether or not this activity is due to one specific enzyme.

Figure 1 illustrates the development of the CoA-dependent acyltransferase activities during cotyledon growth. As seen there is a rapid increase in activity reaching *ca* 80 pkat per pair of cotyledons at 0.52 mM **2** (V_{max} at this stage was 210 pkat/pair of cotyledons and 620 pkat/mg protein). This pattern of activity development correlated well with the accumulation pattern of **1**. In later stages this enzyme activity remained at a high level, showing only a continuous slight activity decrease (data not shown). At the 20th day of cotyledon development there was still *ca* 60% of the maximal activity.

EXPERIMENTAL

Plant material. Seeds of spinach (*Spinacia oleracea* L. var. *monopa*) were purchased from Albert Treppens, Berlin, West Germany. Seedlings and young plants were grown in a defined

Table 1. Donor specificity of the CoA-dependent acyltransferase preparation from 5-day-old spinach seedlings. Mean data from two separate protein preparations and duplicate enzyme activity determinations

Donor (0.33 mM)	% Relative activity*	Donor (0.33 mM)	% Relative activity
Acetyl-CoA	100	Malonyl-CoA	4
Propionyl-CoA	58	Succinyl-CoA	—†
Butyryl-CoA	99	Glutaryl-CoA	
Valeryl-CoA	121	Isobutyryl-CoA	9
Hexanoyl-CoA	90	Isovaleryl-CoA	13
Heptanoyl-CoA	15	Crotonyl-CoA	10

*Formation of acyl-(*p*-coumaroyl)-tartaric acid relative to the formation of 1 with acetyl-CoA; 100 = *ca* 70 pkat per pair of cotyledons at 0.52 mM 2. Reaction products other than 1 were tentatively identified by their chromatographic behaviour in HPLC relative to that of the native product 1 and the applied acceptor molecule (2).

†No product detected.

soil (type T, Balster, Fröndenberg, West Germany, mixed 1:1 with peat) in a phytotron under fluorescent light (*ca* 8000 lux) with an 11 hr day at 21° in light and 18° in the dark and 60% relative humidity.

Substrates. 1-*O*-(*p*-Coumaroyl)-β-glucopyranose was isolated from petals of *Antirrhinum majus* [24]; its structure was proved by NMR spectroscopy (data not shown). 1 was isolated from spinach leaves and 2 was prepared through partial alkaline hydrolysis (see text). Purification of 2 was carried out as described for 1 (see below). Various acyl-CoA thioesters came from Sigma, München, West Germany, and [1-^{14}C]acetyl-CoA (2 GBq/mmol) from Amersham Buchler, Braunschweig, West Germany. *p*-Coumaroyl-CoA was chemically prepared according to ref. [25]. Coenzyme A (free acid) was purchased from Serva (Heidelberg, West Germany). *meso*-Tartaric acid came from Riedel de Haen (Seelze, West Germany), D- and L-tartaric acid from Merck (Darmstadt, West Germany).

Standard enzyme preparation. Cotyledons from 40 spinach seedlings were ground in a pre-cooled (0–4°) mortar in the presence of 0.2 g insoluble polyclar AT, quartz sand and 10 ml KPi buffer (100 mM, pH 7.6). The homogenate was poured into a pre-cooled beaker, allowed to stand for 30 min with continuous stirring, then passed through Miracloth, and the filtrate centrifuged at 48 000 *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 30% satn. The ppt was removed by centrifugation and the supernatant was raised to 80% satn. The pptd protein was collected by centrifugation and was finally filtered through Sephadex G-25 (Pharmacia PD-10 columns). Protein contents were determined by the method described in ref. [28] using bovine serum albumin as standard.

Enzyme assay. Unless otherwise stated the reaction mixtures contained the following components. Formation of 2: 20 mM *meso*-tartaric acid, 1 mM 1-*O*-(*p*-coumaroyl)-β-glucose, 100 mM KPi buffer (pH 7) and 10 μl protein solution in a total vol. of 100 μl. Formation of 1: 0.52 mM 2, 0.33 mM acetyl-CoA, 70 mM KPi buffer (pH 7.6) and 10 μl protein solution in a total vol. of 100 μl. The reactions were started by the introduction of protein soln and were stopped after incubation at 30° for 10 min by transferring the mixtures to a freezer (−20°) or by immediate HPLC analyses. Enzyme assay with ^{14}C-labelled donor (see Fig. 2) contained 0.18 mM 2, 0.15 mM acetyl-CoA, 1.8×10^3 cps [1-^{14}C]acetyl-CoA, 70 mM KPi (pH 7.6) and 25 μl protein solution in a total vol. of 145 μl.

Determination of enzyme activities. This was performed with HPLC as described below and in Fig. 2.

pH *Optimum.* The following buffer systems (0.1 M each) were used to determine the pH optimum of the acyltransferase activity: MES, 2-(*N*-morpholino)ethane sulphonic acid (pH 5–7); KPi (pH 6–8); Tricine, *N*-tris-(hydroxymethyl)-methylglycine (pH 7.5–9.5); glycine (pH 9–11).

Kinetic properties. Apparent K_m and V_{max} values were graphically estimated according to Lineweaver and Burk [29].

Isolation and purification of 1. Cotyledons and leaves from 7-day-old and 4-week-old spinach plants, respectively, were cut into small pieces and extracted with 80% aq. MeOH with an Ultra-Turrax homogenizer. After filtration, the extract was concd under vacuum to a vol. of *ca* 10 ml and was fractionated on a polyamide column (CC6, 4 × 78 cm; Macherey-Nagel, Düren, West Germany) using H_2O, MeOH and 0.03% NH_4OH in MeOH. Elution was monitored continuously by UV absorption at 254 nm and fractions of the eluent showing high UV absorption were collected and examined by TLC (SS1 and SS2). From the ammoniacal fraction one major absorbing compound (1), which changed to blue fluorescence under UV light (350 nm) after treatment with ammonia vapour, was separated from other phenolics by TLC in SS1. Purification was then achieved initially with polyamide CC as described above and finally with Sephadex LH-20 CC (2.5 × 105 cm; Pharmacia, Uppsala, Sweden) twice using MeOH as solvent.

TLC. On microcrystalline cellulose (Avicel, Macherey-Nagel): (SS1) *n*-BuOH–HOAc–H_2O (6:1:2), (SS2) $CHCl_3$–HOAc–H_2O (3:2, H_2O saturated), (SS3) *n*-BuOH–EtOH–H_2O (4:1:2.2), (SS4) toluene–HOAc–H_2O (2:1, H_2O saturated), (SS5) *n*-BuOH–formic acid–H_2O (6:1:2), (SS6) acetone–MeOH–HOAc–H_2O (4:2:1:1).

HPLC. The HPLC apparatus (LKB) and the data processor (Shimadzu) are described elsewhere [27]. The chromatographic column (250 mm long, 4.6 mm inner diameter) was prepacked with Shandon ODS (Hypersil 5 μm) (Bischoff Analysentechnik und -Geräte, Leonberg, West Germany); detection at 310 nm; solvents: (A) 1.5% H_3PO_4 in H_2O, (B) 1.5% H_3PO_4, 20% HOAc, 25% MeCN in water; elution systems for quantification of *p*-coumaric acid esters: linear gradient within 35 min from 30% solvent B in (A + B) to solvent B (*p*-coumaroylglucose, R_t 6.7 min; 2, R_t 9.6 min; 1, R_t 17.1 min); elution systems for enzymatically formed products: *p*-coumaroyltartrate (2) with a linear gradient as described above, 1 with 60% solvent B in (A + B), propionyl-, butyryl- and isobutyryl-(*p*-coumaroyl)-tartaric acid with 80% B in (A + B), other products (see Table 1) with solvent B; flow rate at 1 ml/min. The HPLC radioactivity monitor

(LB 503, Berthold, Wildbad, West Germany) was equipped with a 400 μl Cer-activated glass scintillator cell. ^{14}C-Chromatograms and calculations of cps values were obtained with an Apple computer system (Apple Computer International, France) and a radio-HPLC processing program (Berthold LB 500 M Chromatographie-System, Nuclear Interface GmbH). *p*-Coumaroylglucose and [1-^{14}C]acetyl-CoA were used as reference compounds for calculating amounts (mol) and radioactivity (^{14}C), respectively.

NMR and MS. ^{1}H NMR spectra were recorded at ambient temperature at 400 MHz on a Bruker WM-400 NMR spectrometer locked to the deuterium resonance of the solvent. Chemical shifts are reported in ppm relative to TMS. Negative-ion FAB MS was recorded on a Kratos MS 50 S mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

2-*O*-*Acetyl*-3-*O*-(p-*coumaroyl*)-meso-*tartaric acid* (1). ^{1}H NMR ($CDCl_3$): *E* (57%): δ = 7.716 [*d*; H_α; *J* ($\alpha - \beta$) 15.8 Hz]; 7.507 [*d*; H_o; *J* (o – nm + *J* (o – m') 8.7]; 6.847 [*d*; H_m]; 6.432 [*d*; H_β]; 5.689, 5.645 [*d* × 2; H-2, H-3; *J* (2–3) 2.4]; 2.187 [*s*; $COCH_3$]. *Z* (43%): δ = 7.723 [*d*; H_o; *J* (o – m) + *J* (o – m') 8.8] 6.955 [*d*; H_α; ($\alpha - \beta$) 12.8]; 6.783 [*d*; H_m]; 5.892 [*d*, H_β]; 5.665, 5.593 [*d* × 2; H-2, H-3; *J* (2–3) 2.4]; 2.158 [*s*; $COCH_3$]. FAB MS *m/z*: 337 $[M-H]^-$, 277 $[M - H - CH_3CO_2H]^-$, 191 $[M - COCH = CHC_6H_4OH]^-$, 163 $[OCOCH = CHC_6H_4OH]$.

2-*O*-p-*Coumaroyl*-meso-*tartaric acid* (2). ^{1}H NMR (DMSO-d_6): *E*: δ = 7.545 [*d*; H_o; *J* (o – m) + *J*(o – m) 8.5 Hz]; 7.540 [*d*; H_α; *J* (α-β) 15.9]; 6.812 [*d*; H_m]; 6.370 [*d*; H_β]; 5.198[broad *s*; H – 2]; 4.066 [broad *s*; H – 3]. *Z*: δ = 7.692 [*d*; H_o; *J* (o – m) + *J* (o – m') 8.5]; 6.852 [*d*; H_α; *J* (α-β) 12.6]; 6.753 [*d*; H_m]; 5.761 [*d*; H_β]; 5.148 [broad *s*; H – 2]; 4.047 [broad *s*; H – 3].

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