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CESTRIC ACID, A CAFFEIC ACID ESTER FROM *CESTRUM EUANTHES*

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Key Word Index—*Cestrum euanthes*; Solanaceae; leaves; caffeic acid; glucaric acid; phenolic esters; isomers.

Abstract—Cestric acid, a new phenolic ester was isolated from leaves of *Cestrum euanthes*. By means of GC, HPLC, mass spectroscopy, GC/MS, and ^{13}C NMR, it was shown to be an ester of caffeic acid with glucaric acid. The ester occurs as an equilibrium mixture of four isomers.

INTRODUCTION

Caffeic acid occurs abundantly in plant materials, mostly in an esterified form. Very often, the non-phenolic part of these esters are polyhydroxy compounds such as quinic acid, shikimic acid, glucose, 3,4-dihydroxyphenyllactic acid, tartaric acid or malic acid. The biosynthetic role of these esters is unknown. At least some of them appear to be in active turn-over, which is an indication that they may be intermediates in certain biosynthetic pathways. In our experiments on the metabolic fate of such esters we detected an unknown ester of caffeic acid with glucaric acid in leaves of *Cestrum euanthes* [1].

RESULTS AND DISCUSSION

The caffeylglucaric acid was present in 80% ethanol extracts of *C. euanthes* leaves as an equilibrium mixture of four isomers. Compounds **1**, **2**, **3** and **4** amounted to 55, 82, 165 and 160 μg/g fresh plant material, respectively, measured using caffeic acid as reference. After purification using preparative reverse phase HPLC, it was found that only isomer **4** was relatively stable towards isomerization. Isomers **1**, **2** and **3** were not obtained pure as they were quickly interconverted to the equilibrium mixture of the four isomers.

When **4** was treated with sodium methoxide in dry methanol, we obtained methyl caffeate as the reaction product, which proved that caffeic acid was esterified to the non-aromatic moiety. Ester **4** also possessed one or more acidic functions: its R_t on reverse phase HPLC depended strongly on the pH of the eluant, and it was retained by an anion exchanger of the carbohydrate type (see Experimental, and ref. [2]). This indicated that the alcoholic moiety also contained one or more acidic functions. Ester **4** was hydrolysed with 0.1 N NaOH and after removal of the sodium ions (cation exchangers), the aromatic and non aromatic parts of the molecule were separated by H_2O–EtOAc extraction. The organic phase contained caffeic acid as shown by HPLC, UV spectroscopy and GC/MS.

The polyhydroxy moiety was present mainly in the H_2O fraction. As this product also contained acidic functions, the residue was treated in such a way as to avoid lactone formation (see Experimental). EI GC/MS analysis of the derivatized sample indicated a MW of 642; its fragmentation pattern corresponded to that of a hexaric acid. GC retention indices were available in the literature for the six possible hexaric acids [3].

With the help of these data and reference compounds, the open form could be assigned to glucaric acid. (The capillary GC technique used resolved all six possible isomers.) When no precautions were taken to avoid lactone formation, the GC analysis was more complex. GC/MS measurements of this mixture showed it to be composed of three lactone forms (two mono- and one di-lactone) and an open form. When reference glucaric acid was treated in the same way, the same GC elution pattern was produced. From GC/MS measurements on the intact isomer **4** (after derivatization) we obtained the fragmentation patterns shown in Table 1. They correspond to a lactone form of the molecule shown in Fig. 1. This lactone could have been formed in the pyridine solution that was used for the derivatization prior to GC. It seemed very likely that in aqueous solution, isomers **1**, **2**, **3** and **4** were esters of caffeic acid with the open form of glucaric acid because each isomer was easily converted to an equilibrium mixture of the four at slightly basic pH (10 min at pH 10). At this pH, lactone forms would be opened irreversibly, i.e. they should not give rise to an equilibrium mixture. The interconversion of positional isomers at slightly basic pH is a well-known phenomenon which occurs with all esters of phenolic acids with polyhydroxy compounds.

A mass spectrum of the non-derivatized isomer **4** is also given in Table 1. MW information was obtained from CI/D mass spectroscopy; MH^+ and MNH_4^+ peaks were obtained with ammonia as reactant gas. The M^+ peak also corresponded to a lactone form. The latter could have been formed under the conditions used for mass spectroscopy, so that it is no proof for the existence of isomer **4** as a lactone in aqueous solutions. EI spectra were not informative as the compound decomposed in the conditions used for this technique.

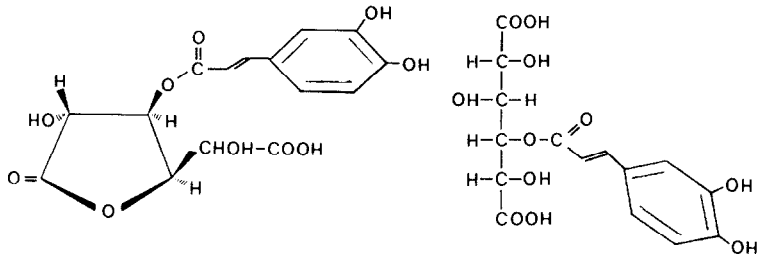

Fig. 1. Possible structures for the caffeylglucaric acid ester, i.e. one possible lactone form (left) and one of the four possible open forms (right).

Table 1. MS data of **4** and its non-phenolic moiety

Compound	Technique	Reactant gas	*m/z* (% relative abundance)
Caffeylglucaric acid lactone (MW = 354)	D/CI	NH_3	184–100; 191(39); 192(33); 198(83); 225(26); 310(4); 355(85); 356(3); 372(3).
Caffeylglucaric acid lactone penta TMSi (MW = 714)	GC/MS; CI	NH_3	555(14); 556(10); 581(12); 715(16); 716(10); 732(100); 733(44); 734(70); 735(38); 736(16).
	GC/MS; CI	Methane	565(18); 699(100); 700(66); 701(52); 702(22); 703(10); 714(26); 715(60); 716(30); 717(22).
	GC/MS; CI	*iso*-Butane	555(20); 581(24); 582(14); 699(18); 700(14); 709(32); 710(100); 711(68); 712(60); 713(24).
	GC/MS; EI		657(10); 699(32); 700(34); 701(24); 702(8); 714(100); 715(52); 716(44); 717(16); 718(8).
Glucaric acid hexa TMSi (from hydrolysis of **4**) (MW = 642)	GC/MS; EI		117(41); 147(40); 204(17); 217(16); 292(36); 305(14); 333(100); 334(35); 423(11); 627(7, 1).

The ^{13}C NMR spectrum of **4** was the sum of the spectra of caffeic acid and glucaric acid (Table 2). The resonance signals from the four carbon atoms of glucaric acid which bear a hydroxyl group, were not resolved (Table 2). This was in accordance with the ^{13}C NMR spectrum of a hexaric acid which served as reference substance, i.e. galactaric acid (Table 2). In this spectrum, the hydroxyl bearing carbon atoms gave two signals, but they were not completely resolved (72.37 and 72.66 ppm respectively). The off-resonance spectrum (Table 2) was also in accordance with the proposed structure.

The four isomers were labelled when [3-^{14}C]cinnamic acid was fed to plant leaves. They were labelled more slowly than other caffeic acid esters such as chlorogenic acid and caffeylglucose [4]. This could mean that they were formed from caffeylglucose, a biochemically active compound, present in relatively large amounts in *C. euanthes* [4]. The latter hypothesis corresponds well with the recent experiences of Tkotz and Strack [5] who have shown that 1-sinapylglucose was converted to the malic acid ester by an enzyme preparation from *Raphanus sativus* cotyledons. Whether the described caffeylglucaric acid can be formed by *C. euanthes* extracts via an analogous transesterification reaction will be investigated.

EXPERIMENTAL

Extraction. An 80% EtOH extract (5 l.) was taken from leaves of *C. euanthes* (*ca* 300 g fr.wt.). The extract was concd *in vacuo* to *ca* 150 ml and the resulting H_2O phase

Table 2. ^{13}C NMR data of **4**, caffeic acid and galactaric acid

Compound	Solvent	Resonance lines-ppm (splitting pattern with off-resonance)
Caffeylglucaric acid	H_2O	72.1(*d*); 114(*d*); 116(*d*); 117(*d*); 124(*d*); 127 (*s*); 145(*s*); 148(*d*); 148(*d*); 169(*s*); 179(*s*).
Caffeic acid*	Hexadeutero DMSO	114; 115; 116; 126; 144; 145; 148; 168.
Galactaric acid	H_2O	72.37; 72.66; 180.

*The resonance lines of the caffeic acid spectrum were in accordance with the data of ref. [7].

was centrifuged at 25 000 *g* for 10 min. The phenolics were present in the supernatant fraction which was used for further chromatographic purification.

Purification. A quarter of the above-obtained H_2O extract was used in the following purification steps. (1) *Pre-purification on a PVP column.* The H_2O extract was injected onto a preparative column (45 cm × i.d. 2.5 cm) containing *ca* 50 g PVP. The column was eluted with H_2O (2 ml/min) and the eluant monitored with a UV detector. The mixture of isomers **1**, **2**, **3** and **4** was collected in the fraction from 300 ml up to 480 ml. Other phenolic acids such as chlorogenic acid had higher retention vols. The collected fraction was concd to 15 ml. Preparation of PVP material: a 100 g amount of PVP was boiled in 500 ml 10% HCl for 10 min. The resulting material was filtered off, rinsed with H_2O, washed with Me_2CO and dried.

(2) *Removal of basic compounds.* The concd fraction from the PVP column was injected on a small column (10 cm × i.d. 3.5 cm) filled with Sephadex SP (Pharmacia, H^+ form). Acidic and phenolic compounds were eluted with H_2O (the eluant was monitored for UV absorbance and pH). The phenolic fraction was sampled and concd to 10 ml.

(3) *Prep. RP HPLC column.* The column (25 cm × i.d. 2.2 cm) was filled with Lichrosorb 10 RP 8 (Merck) and eluted with H_2O–MeOH–HOAc (4% MeOH in H_2O pH adjusted to 3 with HOAc). Repetitive injections were performed yielding three fractions which contained isomer **1**, a mixture of isomers **2** and **3** and isomer **4**. These fractions were concd to dryness under vacuum and the resulting material dissolved in a few ml H_2O. Analytical RP HPLC showed that only **4** had not isomerized in these conditions. This isomer was therefore used in further expts.

(4) *Prep. TLC.* For some expts (e.g. spectroscopy) **4** was further purified on prep. cellulose TLC plates (20 × 20 cm, Merck). The compound (3 mg in H_2O) was applied to each plate (line application). The plates were eluted with *n*-BuOH–H_2O–HCO_2H (5:4:1, organic phase). After elution, the zone containing **4** was scraped off, the scrapings shaken with a few ml H_2O and the cellulose removed by centrifugation. RP PHLC analysis showed the compound to be 97% pure (UV). It was stored at −18°.

Analytical HPLC. Separations were performed as described in ref. [6]. The R_ts of isomers **1**, **2**, **3** amd **4** were 1.7; 1.8; 2.6 and 3.5 min, respectively, on a reverse phase column (30 cm × i.d. 4.6 mm) which was eluted with a gradient from 5% B to 18% B in 34 min (solvent A = 8×10^{-3} N H_3PO_4; solvent B = MeOH).

GC and GC/MS of **4**. A few mg of purified isomer were derivatized by dissolving in 200 μl pyridine, 200 μl BSTFA + 1% TMCS soln and heating at 100° for 10 min. The derivatized sample was injected on a GC column (0.5 m, i.d. 2 mm) filled with 5% OV 101 on Chromosorb G. N_2 carrier was at 20 ml/min and the oven temp. programmed from 180° to 300° at 15°/min. The same column and conditions were used in the GC/MS system. Spectra were obtained with EI and with CI techniques (NH_3 as reactant gas).

Cleavage of **4**. (1) NaOH. A few mg of **4** were dissolved in 1 ml 0.1 N NaOH at 20°. After 2 hr, the soln was injected on a small cation exchange column (Sephadex SP, Pharmacia, H^+ form) containing *ca* 1 meq H^+. The hydrolysis products were eluted from the column with H_2O. This fraction was evaporated, dried under vacuum and derivatized for GC analysis.

(2) *Opening of lactone forms.* In the above treatment, several lactone forms were produced from the hexaric acid during concn of the eluted fraction. As these forms complicated the qualitative identification of the hexaric acid, they were converted to the open form: a few mg of the polyhydroxy compounds (a mixture of lactones + open form) were treated with 0.1 N NaOH at 20° for 2 hr. The pH was adjusted to 7 with 0.1 N HOAc. This soln was injected on a cation exchanger (Sephadex SP, Pharmacia, NH_4^+ form), and the glucaric acid eluted as its NH_4^+ salt with H_2O as eluant. The sample was vacuum-dried and the residue derivatized for GC and GC/MS analysis.

GC and GC/MS of non-aromatic moiety. Three different columns were used: (1) 5% OV-101 on Chromosorb G, 0.5 m × i.d. 2 mm, N_2 flow 20 ml/min, isothermal at 160°. (2) 3% OV-17 on Chromosorb W, 1.5 m, N_2 flow 30 ml/min, isothermal at 200°. (3) OV-101 WCOT Duran 50, 50 m × i.d. 0.5 mm, N_2 flow 3 ml/min, isothermal at 200°. Quinic acid, sorbitol, glucaric acid and galactaric acid were used as ref. substances for calibration of the separation as described in ref. [3].

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