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SYNTHESIS OF ELLAGIC ACID *O*-ALKYL DERIVATIVES AND ISOLATION OF ELLAGIC ACID AS A TETRAHEXANOYL DERIVATIVE FROM *FRAGARIA ANANASSA*

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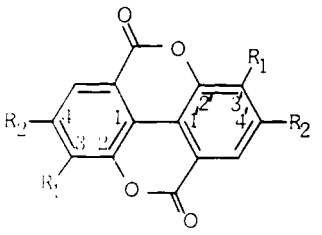
ABSTRACT.—Ellagic acid [**1**] is a gallic acid dimer that occurs in plants, fruits, and nuts, either in its free form, or in a series of ellagitannins, or as a glucoside. It has been shown to inhibit cancer induced by several types of chemical carcinogens including polycyclic aromatic hydrocarbons, *N*-nitrosamines, aflatoxin, and aromatic amines. It has been extracted from a number of fruits, including strawberries; however, its presence in the extracts was determined only by hplc connected with a diode array detector. In the present report, ellagic acid was isolated as a tetrahexanoyl derivative **2** from *Fragaria ananassa* and identified by ^{13}C and ^{1}H nmr and ms. The ^{13}C-nmr shifts of the aromatic carbons adjacent to a hexanoyloxy group were assigned using two new synthetic model compounds: 3,3′-dihexanoyloxydiphenic-2,2′,6,6′-dilactone [**3**] and 4,4′-dihexanoyloxydiphenic-2,2′,6,6′-dilactone [**4**].

Two new derivatives of ellagic acid [**1**], 3,3′-di-β-D-glucopyranosylellagic acid decaacetate [**5**] and 3,3′-di-*n*-octyl-4,4′-dihexanoylellagic acid [**7**], were also synthesized. Both derivatives were less effective as inhibitors of benzo[*a*]pyrene tumorigenesis in the lungs of strain A/J mice than ellagic acid.

Ellagic acid [**1**], a gallic acid dimer, has exhibited anticarcinogenic activity in both in vitro and in vivo systems (1–7). Because of its inhibitory effect on chemically induced cancer, we determined the content of ellagic acid in various nuts and fruits including strawberries by hplc (8). However, the identity of ellagic acid in the strawberry extracts could not be confirmed by ms, gc-ms, or lc-ms.

In this report, the presence of ellagic acid [**1**] in strawberries (*Fragaria ananassa*) was confirmed by extracting 1.1 kg of the frozen fruit, isolating ellagic acid as a tetrahexanoyl derivative **2**, and identifying the derivative by nmr, ms, and mmp using synthetic standards. In addition, the structure of the derivative **2** was determined by ^{13}C nmr. In the ^{13}C-nmr study, chemical shifts of the four aromatic carbons at the 3, 3′, 4, and 4′ positions, each of which bears a hexanoyloxy group, experienced similar electron withdrawing or anisotropic properties which made it difficult to assign ^{13}C-nmr shifts. Therefore, 3,3′-dihexanoyloxydiphenic-2,2′,6,6′-dilactone [**3**] and 4,4′-dihexanoyloxydiphenic-2,2′,6,6′-dilactone [**4**] were synthesized as model compounds, and the chemical shifts of the aromatic carbons of the derivative **2** were assigned by comparing them to those of the model compounds.

Ellagic acid [**1**] is relatively insoluble in H_2O and organic solvents and has low bioavailability (9). Therefore, in attempts to improve its bioavailability, we synthesized two additional new derivatives of ellagic acid, 3,3′-di-β-D-glucopyranosylellagic acid decaacetate [**5**] and 3,3′-di-*n*-octyl-4,4′-dihexanoylellagic acid [**7**]. The regiochemistry of these derivatives **5** and **7** was determined by comparing their ^{13}C-nmr shifts to those of tetrahexanoylellagic acid [**2**].



1 $R_1=R_2=OH$
2 $R_1=R_2=$hexanoyloxy
3 $R_1=$hexanoyloxy, $R_2=H$
4 $R_1=H$, $R_2=$hexanoyloxy
5 $R_1=$-*O*-tetra-*O*-acetyl-β-D-glucopyranosyl
$R_2=OAc$
6 $R_1=OAc$, $R_2=OH$
7 $R_1=$-*O*-*n*-octyl, $R_2=$hexanoyloxy
8 $R_1=OH$, $R_2=$hexanoyloxy
9 $R_1=OMe$, $R_2=OH$
10 $R_1=$-*O*-Benzyl, $R_2=OH$
11 $R_1=OH$, $R_2=OMe$

RESULTS AND DISCUSSION

Ellagic acid [**1**] is a symmetrical molecule with two orthodihydroxy groups on each side. The compound is very polar and poorly soluble in H_2O and in organic solvents, and it has an mp higher than 360°. However, the tetrahexanoyl derivative **2** was found to be more soluble than ellagic acid [**1**] and its previously characterized derivatives. In addition, a lower mp (208–210°) permitted its identification by mmp using a synthetic standard. Finally, the derivative **2** was sufficiently soluble in $CHCl_3$ to permit nmr experiments.

^1H nmr of ellagic acid tetrahexanoylate [**2**] in $CDCl_3$ showed only one peak in the aromatic region and provided very little information on its structure. However, in ^{13}C nmr, the derivative was found to have 10 aliphatic, 6 aromatic, 2 carbonyl, and 1 lactone carbon signals. The four aromatic carbons with hexanoyloxy groups had a similar anisotropy; therefore, it was difficult to assign the chemical shifts. Accordingly, we assigned the chemical shifts of the derivative **2** as follows:

The lactone carbon appeared at 157.4 ppm in **3**, at 158.1 ppm in **4**, and at 156.8 ppm in **2**, indicating that the carbonyl groups of the hexanoyl moiety cause a diamagnetic anisotropy of 0.6–1.3 ppm. The chemical shift increment caused by the carbonyloxy group (acetoxy) is −6.4 ppm for the ortho carbons (10). The expected total diamagnetic shift for the carbons adjacent to the carbon bearing a hexanoyloxy group in **2** is 7.0–7.7 ppm. In compounds **3** and **4**, the carbon atoms bearing a hexanoyloxy group appear at the highest field in the aromatic region with a chemical shift of C-3 at 142.2 ppm in **3** and of C-4 at 152.6 ppm in **4**. When the total diamagnetic increment of −7.0 is added to the chemical shift of C-3 in **3** and of C-4 in **4**, the resulting chemical shifts should be those of C-3 and C-4 in **2**. Indeed, C-3 (C-3′) of **2** appears at 135.6 ppm and C-4 (C-4′) appears at 145.2 ppm with diamagnetic shifts of 7.4 ppm and 7.0 ppm. Other aromatic ^{13}C shifts were calculated according to equation 1 (10) using Model I (Figure 1) for **3**, Model II for **4**, and Model III for **2**:

$$d_k=d_{k(RH)}+\Sigma Z_{ik} \quad \text{(eq. 1)}$$

where d_k is the ^{13}C shift of carbon k bearing the substituent, $d_{k(RH)}$ is the ^{13}C shift of the corresponding carbon in parent compound RH, and Z_{ik} is a ^{13}C shift increment of the substituent. The results are summarized in Table 1. ^{13}C shifts of ellagic acid [**1**] in DMSO-d_6 were reported without comparing to model compounds (11). Although the shifts of C-5 and C-6 of **1** were unresolved, C-4 appeared at the highest field and C-3 at the next highest field among aromatic carbons, which is in agreement with our data.

The regiochemistry of ellagic acid dimethyl ether is well established (12). Oxidative coupling of methyl 4-*O*-methylgallate yields 3,3′-dimethylellagic acid [**9**] (mp 330–

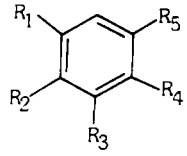


FIGURE 1. Structures of Model Compounds I, II, and III.
(R_1=OAc, R_2=phenyl, R_3=OAc)
Model I R_4=OAc, R_5=H
Model II R_4=H, R_5=OAc
Model III R_4=OAc, R_5=OAc

331°). Methylation of 3,3′-dibenzylellagic acid [**10**] and hydrolysis of the product yields 4,4′-dimethylellagic acid [**11**] (mp >360°). The two dimethylellagic acids are poorly soluble in nmr solvents, and no ^1H- or ^{13}C-nmr data are available.

Sayer *et al.* (13) elucidated the regiochemistry of the reaction product between ellagic acid and B[*a*]P-7,8-diol-9,10-epoxide by permethylation of the product and hydrolysis of the B[*a*]P moiety with dilute HCO_2H. The conversion product was found by chromatography and uv spectroscopy to be 3,3′,4-trimethylellagic acid, with the B[*a*]P moiety attached to the 4 position of ellagic acid. Chang *et al.* (7) synthesized 3-*O*-decylellagic acid, the regiochemistry of which was determined by pK_a measurements on the compounds.

In ^{13}C nmr, all shift patterns are very similar except C-3 of **5** and **8**, suggesting that the substituents are connected to C-3 carbons (Table 1). The anomeric carbon of **1** appears at 100.75 ppm, showing that the glucose moiety has a β configuration.

Since both 3,3′-di-β-D-glucopyranosylellagic acid decaacetate [**5**] and 3,3′-di-*n*-octyl-4,4′-dihexanoylellagic acid [**7**] are more soluble than ellagic acid [**1**], studies are underway to compare their pharmacokinetic and pharmacodynamic characteristics in

TABLE 1. Aromatic ^{13}C Shifts of Ellagic Acid Derivatives.

Carbon	Compound							
	2[a]	**2**[b]	**3**[a]	**3**[b]	**4**[a]	**4**[b]	**5**[a]	**7**[a]
C-6	115.9	123.7	118.7	128.8	120.4	134.6	114.1	112.2
C-5	116.2	124.9	125.9	129.2	116.3	121.5	116.1	117.1
C-1	120.6	135.3	126.5	137.6	119.1	124.6	120.1	120.3
C-3	135.6	143.6	142.6	150.0	114.3	116.2	139.7	145.3
C-2	142.4	145.3	141.2	143.9	149.8	151.7	141.4	141.4
C-4	145.6	145.4	117.1	122.3	152.6	151.8	145.0	144.5

[a]Shifts in ppm from TMS.
[b]Expected ^{13}C shifts calculated (see Results and Discussion).

animals and their anticarcinogenic activity with those of ellagic acid [**1**]. Preliminary results indicate that both derivatives are less effective in inhibiting B[*a*]P tumorigenesis in strain A/J mouse lung than ellagic acid [**1**] itself (5).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—*Chemicals.*—Ac_2O, Br_2, *n*-BuOH, $CDCl_3$, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, ellagic acid [**1**], Et_2O, EtOH, α-D-glucose, hexanoyl chloride, MeOH, Me_2CO, *n*-octyl iodide, pyridine, trifluoroacetic acid, and TMS were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Frozen strawberries (*Fragaria ananassa*, Kent variety) (4 kg) grown on the farm of Mr. Harold Creque in Holland, Ohio and harvested in the summer of 1990 were used in this study.

^{13}C and ^1H nmr and ms.—Nmr spectra were obtained on either a Varian VXR-400 or a Varian Gemini-200 (Varian Instruments, Palo Alto, CA). Samples were dissolved in $CDCl_3$ or DMSO-d_6, and chemical shifts were referenced to internal TMS unless otherwise stated. All spectra were collected in the time domain and signals were averaged to produce spectra with adequate signal-to-noise ratios. Ms spectra were obtained on a Hewlett-Packard 5988 GC/MS (Varian Instruments, Palo Alto, CA). Samples were introduced via a direct insertion probe and volatilized by heating at a rate of 60°/min to 300°. Ms of 5 was obtained on a VG 70-250 (VG Analytical, Manchester, England), either on glycerol-DMF (1:1) or magic bullet matrix.

ISOLATION OF ELLAGIC ACID [**1**] AS A TETRAHEXANOYL DERIVATIVE **2**.—Frozen strawberries (1.1 kg) were homogenized, and the homogenate was lyophilized. The dried residue (108 g) was extracted 3 times with 700 ml of Me_2CO-H_2O (80:20). The combined extracts were evaporated to dryness. The residue was suspended in 500 ml of H_2O and extracted 3 times with 500 ml of *n*-BuOH. The combined extracts were dried over anhydrous Na_2SO_4 and filtered. After evaporation to dryness, the residue was hydrolyzed in 200 ml of 1 N trifluoroacetic acid in 50% MeOH at 100° for 1 h. After drying in a P_2O_5 vacuum desiccator for 6 h, the residue was reacted with 20 ml of *n*-hexanoyl chloride at 110° for 3 h. After cooling to room temperature, precipitates were collected and extracted with 50 ml of $CHCl_3$. The extract was evaporated to dryness to produce a dark black mass of 2.8 g, which was purified on a Si gel column (120 g, 3×52 cm) using $CHCl_3$. The combined fractions yielded 33 mg of tetrahexanoylellagic acid [**2**]: mp 208–210°; ^{1}Hnmr (δ) 0.9 (6H, m), 1.4 (12H, m), 2.6 (2H, t), 2.7 (4H, t), (aliphatic protons), 8.0 (H, s, aromatic); ^{13}C nmr (δ) 13.89, 22.30, 24.49, 24.52, 31.13, 31.15, 31.18, 33.66, 33.88 (aliphatic carbons), 115.94, 116.20, 120.68, 135.68, 142.46, 145.66 (aromatic carbons), 156.86 (lactone carbonyl), 169.30 (hexanoyl carbonyl), 170.41 (hexanoyl carbonyl); ms *m/z* $[M]^+$ 596 (2%), 498 (25%), 401 (21%), 400 (100%), 303 (13%), 302 (85%), 99 (59%).

SYNTHESIS OF 3,3′-DIHEXANOYLOXY-2,2′,6,6′-DIPHENICDILACTONE [**3**].—3,4-Dihydroxybenzoic acid (5.0 g, 31.5 mmol) was dissolved in 10 ml of concentrated H_2SO_4, and the solution was brought to room temperature. $K_2S_2O_8$ (5.0 g, 18.5 mmol) was added in an icebath at a rate such that the reaction temperature did not exceed 45°. The dark green reaction mixture was stirred at room temperature for 12 h. The mixture was poured into 80 ml of ice-H_2O.

Precipitates were collected and washed with 10 ml of hot EtOH and 20 ml of Et_2O. After drying, the solid (1.2 g) was reacted with 10 ml of hexanoyl chloride at 110° for 3 h. After the reaction mixture was cooled, precipitates were collected and purified on a Si gel column to yield **3** (619 mg): mp 148–151°; ^{1}H nmr (δ) 0.9 (m, 3H), 1.4 (m, 8H), 1.9 (m, 4H), 2.7 (t, 2H), 7.5 (d, 2H), 8.2 (d, 2H); ^{13}C nmr (δ) 13.91, 22.32, 24.48, 31.12, 33.88, 117.17, 118.77, 125.92, 126.50, 141.21, 142.25, 157.40, 170.58; ms *m/z* $[M]^+$ 466 (6.4%), 369 (665.8%), 368 (16.5%), 312 (3.5%), 271 (70.1%), 270 (65.6%), 99 (100%).

SYNTHESIS OF 4,4′-DIHEXANOYLOXY-2,2′,6,6′-DIPHENICDILACTONE [**4**].—3,5-Dihydroxybenzoic acid (8 g, 52 mmol) was dissolved in 100 ml of HOAc mixed with 5 ml of H_2SO_4 by heating at 90°. K_2SO_4 (8.0 g, 29.6 mmol) was added in portions. The blood-red reaction mixture was stirred at 90° for 2 h. After the reaction mixture was cooled to room temperature, precipitates were collected and washed with 300 ml of hot H_2O and 20 ml of Et_2O. The product was dried in a vacuum desiccator for 6 h, and the residue (0.6 g) was reacted with 10 ml of *n*-hexanoyl chloride at 110° for 3 h and cooled to room temperature. Precipitates were collected and purified on a Si gel column to yield **4** (420 mg): mp 244–248°; ^{1}H nmr (δ) 0.9 (3H, m), 1.4 (4H, m), 1.8 (2H, m) 2.6 (2H, t), 7.5 (1H, d), 7.9 (1H, m); ^{13}C nmr (δ) 13.90, 22.30, 24.44, 31.20, 34.21, (aliphatic carbons), 114.83, 116.29, 119.08, 120.24, 149.88, 152.6 (aromatic carbons), 158.19 (lactone carbonyl), 171.48 (hexanoyl carbonyl); ms *m/z* $[M]^-$ 466 (2.4%), 369. (5.2%), 368 (21.4%), 312 (2.1%), 271 (34.8%), 270 (100%), 99 (16.9%).

SYNTHESIS OF 4,4′-DIACETYLELLAGIC ACID [**6**].—Compound **6** was synthesized according to the method of Jurd (12). Briefly, ellagic acid [**1**] (10 g) was reacted with 100 ml of Ac_2O and 2 drops of H_2SO_4 at 100° for 1 h. After cooling to room temperature, the reaction mixture was filtered and the filter cake was

washed with 300 ml of Me_2CO to remove unreacted Ac_2O. The residue (mp higher than 360°) was dried and hydrolyzed with 200 ml of pyridine/H_2O mixture by heating briefly at 115°. After cooling the reaction mixture, the precipitates were collected, washed with 500 ml of H_2O and 300 ml of Me_2CO, and dried. The residue was further dried in a P_2O_5 vacuum desiccator for 6 h to obtain 5.2 g of **6**; mp 323–328°. The product was used for synthesis without further purification.

2,3,4,6-TETRA-*O*-ACETYL-α-D-GLUCOPYRANOSYL BROMIDE (TAGB).—TAGB was synthesized as described (14) starting from 50 g of α-D-glucose to obtain 92 g (mp 86–88°).

3,3′-DI-β-D-GLUCOPYRANOSYLELLAGIC ACID DECAACETATE [**5**].—To 80 ml of dry Me_2CO was added 5 g of **6**, 16 g of Drierite, and 3.3 g of Ag_2CO_3. The reaction mixture was stirred for 30 min while protected from moisture and light at room temperature. TAGB (15 g) was added, and the mixture was stirred overnight. The dark brown reaction mixture was filtered through a pad of Celite filter-aid, and the filtrate was evaporated to dryness. The residue was recrystallized in a mixture of $CHCl_3$ and EtOH to obtain 1.2 g of **5**: mp 230–231°; ^{1}H nmr (δ) 1.96 (3H, s, OAc), 1.99 (6H, s, OAc), 2.13 (3H, s, OAc), 2.33 (3H, s, OAc), 3.95 (1H, d, J=9.9, G-6), 4.03–4.09 (1H, m, G-5), 4.01–4.25 (1H, m, G-4), 5.04 (1H, d, J=9.6, G-6′), 5.16 (1H, dd, J=8, 9.6, G-2), 5.46 (1H, t, J=9.6, G-3), 5.68 (1H, d, J=8.0, G-1); ^{13}C nmr (δ) 20.27, 20.34, 20.38, 20.45 (Me carbons), 61.27 (G-6), 67.79 (G-4), 70.93 (G-2), 71.17 (G-5), 71.72 (G-3), 100.75 (G-1), 116.60, 120.16, 139.78, 141.47, 145.07 (aromatic carbons), 157.10 (lactone carbonyl), 168.22, 169.23, 169.34, 169.38 (acetate carbonyl); elemental analysis C 52.77% (calcd 53.22%), H 4.39% (calcd 4.40); ir 1743 (CO), 1771 (CO), cm^{-1}; ms *m/z* [M+H]$^+$ 1047 (2%), 331 (49%), 169 (100%), 109 (62%).

SYNTHESIS OF 3,3′,4,4′-TETRAHEXANOYLELLAGIC ACID [**2**].—Ellagic acid [**1**] (5 g) was reacted with 20 ml of *n*-hexanoyl chloride at 110° for 3 h. After the reaction mixture was cooled, the precipitates were recrystallized with 600 ml of boiling Me_2CO 3 times to obtain 4.2 g of **2**: mp 207–210°; ^{1}H nmr (δ) 0.9 (6H, m), 1.4 (12H, m), 2.6 (2H, t, $OCOCH_2$), 2.7 (2H, t, $OCOCH_2$), 8.0 (1H, s, aromatic proton); ^{13}C nmr (δ) 13.89, 22.30, 24.49, 24.52, 31.13, 31.15, 31.18, 33.66, 33.88 (hexanoyl aliphatic carbons), 115.94, 116.20, 120.68, 135.68, 142.46, 145.66, 156.86 (aromatic carbons), 169.30 (lactone carbonyl), 170.41 (hexanoyl carbonyl); ms *m/z* [M]$^+$ 596 (2%), 498 (25%), 401 (21%), 400 (100%), 303 (13%), 302 (85%), 99 (59%).

4,4′-DIHEXANOYLELLAGIC ACID [**8**].—Compound **2** (4 g) was partially hydrolyzed in 40 ml of pyridine-H_2O (1:1) by boiling briefly. After cooling to room temperature, precipitates were collected and washed with 300 ml of H_2O and 100 ml of Me_2CO. The residue was dried to obtain 1.5 g of **8**. The semipure product was used for synthesis without further purification.

3,3′-DI-*n*-OCTYL-4,4′-DIHEXANOYLELLAGIC ACID [**7**].—Compound **8** (1 g), 4 g of Drierite and 3.3 g of Ag_2CO_3 were suspended in 100 ml of Me_2CO dried over $CaCO_3$ and stirred for 30 min. To the reaction mixture 10 ml of *n*-octyl iodide was added, and the reaction mixture was stirred at room temperature for 48 h. The dark brown reaction mixture was filtered through a pad of Celite filter-aid, and the filter cake was washed with 300 ml of $CHCl_3$. The combined filtrates were evaporated to dryness. The residue (0.4 g) was purified on a Si gel column (120 g, 3×52 cm) with $CHCl_3$. The fractions with an R_f value of approximately 0.5 on Si gel tlc with $CHCl_3$ were combined and evaporated. The residue was recrystallized in 200 ml of boiling Me_2CO to obtain 210 mg of product: mp 118–120°; ^{1}H nmr (δ) 0.88 (3H, t, J=6.62), 0.94 (3H, t, J=7.0), 1.29 (6H, m), 1.42 (6H, m), 1.78 (6H, m), 2.62 (2H, t, J=7.72), 4.50 (2H, t, J=6.62), 7.87 (H, s, aromatic proton); ^{13}C nmr (δ) 171.2 (hexanoyl carbonyl), 157.6 (lactone carbonyl), 145.39, 144.50, 141.43, 120.31, 117.12, 112.27 (aromatic carbons), 74.96, 33.84, 31.79, 31.26, 30.29, 29.34, 29.24, 25.68, 24.49, 22.63, 22.31, 14.06, 13.87 (aliphatic carbons); ms *m/z* [M−97]$^+$ 625 (1.1%), 527 (100%), 415 (7.1%), 302 (8.3%), 99 (4.9%).

MOUSE LUNG TUMOR INHIBITION STUDY.—Ellagic acid [**1**], 3,3′-di-β-D-glucopyranosylellagic acid decaacetate [**5**], and 3,3-di-*n*-octyl-4,4′-dihexanoylellagic acid [**7**] were tested for their ability to inhibit B[*a*]P tumorigenesis in strain A/J mouse lung as described by Lesca (5). All three compounds were administered in the diet (0.4 g/kg) to 6–8-week-old mice (15 mice per experimental group) for a period of 21 days. On the fourteenth day of feeding of the putative inhibitors, B[*a*]P was given in a single ip injection of 100 mg/kg body wt to each mouse. Control mice were treated with B[*a*]P only. Seven months after exposure to the carcinogen, control and treated mice were killed, their lungs removed, and the surface lung tumors quantitated as described (5). The incidence (percentage of mice with tumors) and multiplicity (number of tumors per mouse) of lung tumors in experimental and control (B[*a*]P-treated) mice were compared by analysis of variance (ANOVA).

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