SYNTHESIS OF ^{13}C- AND ^{14}C-LABELED ELLAGIC ACID

by

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SUMMARY

   The syntheses of [^{13}C]- and [^{14}C]ellagic acid (**EA**) were accomplished by reacting [^{13}C]- or [^{14}C]CO$_2$ with metalated 3,4,5-trimethoxybenzene to afford trimethylgallic acid which was O-demethylated with hydroiodic acid. Oxidation of the resulting labeled gallic acid with potassium persulfate produced **EA**. The yields of trimethylgallic acid and **EA** based on [^{14}C]BaCO$_3$ are 48 and 1 %, respectively. Final purification of **EA** involved the use of preparative reversed-phase HPLC and removal of the eluent buffer salts on a C$_{18}$ Sep-Pak column. The isotopic purity of [^{13}C]**EA** was determined by ^{13}C NMR. The [^{14}C]**EA** had 99% radiochemical purity as determined by analytical HPLC and a specific activity of ca. 110 mCi/mmol.


Key words: [^{13}C]- and [^{14}C]ellagic acid, gallic acid, K$_2$S$_2$O$_8$, [^{13}C]- and [^{14}C]BaCO$_3$, HPLC, antimutagenicity and antitumorigenicity.

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INTRODUCTION

   Ellagic acid (**EA**) (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-

cde][1]benzopyran-5,10-dione) (Scheme 1) is a natural product

shikimate derivative present in soft fruits, nuts and vegetables

(1-3). It has proven to be an effective inhibiting agent against

the *in vitro* and *in vivo* activity of several chemical carcino-

gens, including polycyclic aromatic hydrocarbons (4-7) and the

direct-acting mutagens N-methyl-N-nitrosourea (**MNU**) (8) and N-

methyl-N'-nitro-N-nitrosoguanidine (9). *In vitro* DNA methyla-

tion studies with **MNU** (8) showed that **EA** selectively inhibited

the formation of O^6-methylguanine, while little effect was seen

in the extent of methylation at N7-guanine. This selective

inhibition of methylation at O^6-guanine was more recently ob-

served *in vivo* with nitrosomethylbenzylamine, which both methyl-

ates DNA and induces cancer in the esophagus (10). The formation

of O^6-methylguanine by **MNU** has been suggested to play a potential

role in the *in vitro* mutagenicity and carcinogenicity of **MNU** and

related DNA methylating agents (11). It was proposed that the

inhibitory action of **EA** is related to its ability to form an

affinity binding complex with DNA (8,12) that masks or protects

the O^6-guanine site from methylation.

   To qualitatively and quantitatively address the DNA affinity

binding properties of **EA**, and to study its *in vivo* bioavail-

ability, the synthesis of radiolabeled **EA** was initiated. Pre-

vious experience with commercially prepared [4,9-^{3}H]**EA** suggested

that the ^{3}H-label would not be sufficiently stable for *in vivo*

experimentation. We describe herein the synthesis of [^{14}C]**EA** with

the label in the carbonyl groups of the dilactone moiety starting

from economic materials.
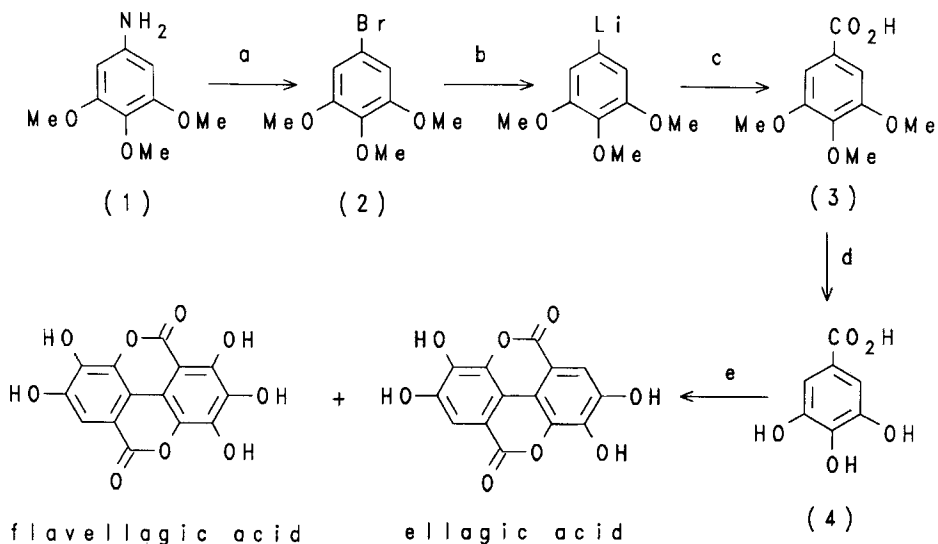
Scheme 1. Synthesis of ellagic acid: a, HNO_2, HBr, Cu_2Br_2; b, $\underline{n}$-BuLi/ether; c, $BaCO_3$, H_2SO_4; d, HI, HOAc; e, $K_2S_2O_8$, H_2SO_4, HOAc.

EXPERIMENTAL

Materials

The 3,4,5-trimethoxyaniline, HBr, Cu_2Br_2, $\underline{n}$-BuLi, $BaCO_3$, [^{13}C]-$BaCO_3$ (99 atom % ^{13}C), HI, $K_2S_2O_8$, isobutyryl chloride, 3,4,5-trimethoxybenzoic acid, 3,4,5-trimethoxyphenol, gallic acid, and **EA** were obtained from Aldrich (Milwaukee, WI). [^{14}C]-$BaCO_3$ (specific activity, 58 mCi/mmol) was purchased from New England Nuclear (Boston, MA). The **EA** was crystallized several times from pyridine and dried *in vacuo*. All other chemicals used were of reagent grade. Diethyl ether and pyridine were dried over $LiAlH_4$ and BaO, respectively. UV-VIS, FT-IR and NMR spectra were obtained on Varian DMS 300, Mattson Alfa Centauri and Varian XL-300 spectrometers, respectively. ^{14}C-Radioactivity was measured by scintillation counting on a Beckman model LS 3801 instrument. For NMR studies tetramethylsilane (TMS) was used as the internal standard. High resolution mass spectral data were obtained on an AEI MS-9 spectrometer in the electron-impact (EI) mode using a solid probe at 39.7 eV. The percentage of ^{13}C incorporation was determined by EI mass spectroscopy. High performance liquid

chromatographic (HPLC) separations were performed on a Beckman
model 110 A gradient system with UV detection at 254 nm, unless
specified otherwise. Peak areas were quantitated using a Hew-
lett-Packard 3380A integrator with external standardization.
Radioactivity detection of HPLC peaks employed a FLO-ONE/Beta
series A-200 radioactivity flow detector (Radiomatic Instruments
& Chemical Co., Inc., Tampa, Florida). Thin layer chromatography
(TLC) analyses employed 0.25 mm Kieselgel 60 F-254 plates (E. M.
Merck). Flash chromatography used 40 μM silica gel (J.T. Baker).
Preparation of Unlabeled Materials

1-Bromo-3,4,5-trimethoxybenzene (2)

3,4,5-Trimethoxyaniline (**1**) (4.17 g, 24 mmol) was dissolved
in a mixture of H_2O (150 ml) and 48% HBr (55 ml). The resulting
mixture was initially heated to 60 °C and then cooled to -5 °C in
an ice-salt bath. $NaNO_2$ (10.68 g, 150 mmol) in water (50 ml) was
slowly added. The reaction mixture was stirred for 15 min at -5
°C to ensure complete diazotization, and then the excess HNO_2 was
decomposed by a slow batchwise addition of urea until the deep
brown color changed to yellow. Destruction of the HNO_2 was
confirmed by a negative test with a KI-starch paper. A cold
solution of Cu_2Br_2 (30 g, 200 mmol) in HBr (100 ml) was added to
the above arenediazonium ion solution. The diazonium salt was
decomposed at 60 °C for 1 h at which time the evolution of N_2
ceased. The mixture was then extracted with $CHCl_3$ (3 x 50 ml)
and the combined organic extracts washed with 10% aqueous NaOH
(100 ml) and dried ($MgSO_4$). The solution was filtered and the
solvent removed under reduced pressure to afford a yellow solid.
This crude product was purified by flash column chromatography on
silica with CH_2Cl_2 as the eluent. The product obtained was
further purified by crystallization from petroleum ether to give
3.18 g (56%) of white crystalline material; mp 80-81 °C [Lit.
79-80 °C, (14)]; TLC, (silica gel, CH_2Cl_2) R_f = 0.28; ^{1}H-NMR (DMSO-

d_6) δ 3.65 (s, 3H, OCH$_3$), 3.79 (s, 6H, OCH$_3$'s), 6.87 (s, 2H,

aryl); ^{13}C-NMR (DMSO-d_6) δ 56.20 (OCH$_3$'s), 59.90 (OCH$_3$), 108.90 (C$_2$

and C$_6$), 115.50 (C$_1$), 137 (C$_4$), 153.70 (C$_3$ and C$_5$); IR (KBr disc)

3099, 2934, 1589, 1500, 1451, 1403, 1306, 1280, 1124, 994, 813,

767 cm^{-1}.

3,4,5-Trimethoxybenzoic Acid (3)

Bromide (2) (0.25 g, 1.01 mmol) was dissolved in dry diethyl

ether (10 ml) and cooled to -5 °C in an ice-salt bath. After Ar

purging of the flask containing the ethereal bromide solution, n-

BuLi in hexane (1.04 mmol, 0.4 ml of 2.6 M) was introduced. The

reaction mixture was stirred at -5 °C for 15 min. The cooling

was discontinued and the flask again flushed with Ar. Another 10

ml round-bottomed flask containing BaCO$_3$ (0.17 g, 0.89 mmol) was

connected to this flask by teflon tubing while maintaining an Ar

purge on the entire system. Concentrated H$_2$SO$_4$ (1 ml) was in-

jected into the flask containing the BaCO$_3$, and the attached

flask containing the organolithium compound in ether was immedi-

ately cooled in a liquid N$_2$ bath to condense the generated CO$_2$.

The liquid N$_2$ bath was removed after 0.5 h and the flask contain-

ing the organolithium salt and the CO$_2$ was allowed to warm to the

room temperature. Any unreacted CO$_2$ remaining was vented

through a trap containing a methanolic solution of Ba(OH)$_2$ by

flushing with Ar. A 10% aqueous NaHCO$_3$ solution (10 ml) was

injected into the ethereal reaction mixture to extract the acidic

product. This basic aqueous extract was washed once with diethyl

ether (10 ml). The pH of the aqueous extract was then adjusted

to 2.0 with H$_2$SO$_4$ and the aqueous solution extracted with diethyl

ether (3 x 25 ml). The combined organic extracts were dried over

MgSO$_4$. After filtering, the ether was concentrated under reduced

pressure and the yellow product was purified by flash silica

column chromatography with CH$_2$Cl$_2$/CH$_3$OH/AcOH (95:5:1) as the

eluent. Fractions containing the desired product were pooled

together, concentrated under reduced pressure, and the residue
crystallized from petroleum ether to afford 104 mg (48%) of
product; mp 170-173 °C [Lit. 167-169 °C (14)]; TLC (silica gel,
$CH_2Cl_2/CH_3OH/AcOH$, 95:5:1) R_f = 0.29; ^1H-NMR (DMSO-d_6) δ 3.75 (s,
3H, OCH_3), 3.85 (s, 6H, OCH_3's), 7.258 (s, 2H, aryl); ^{13}C-NMR
(DMSO-d_6) δ 55.92 (OCH_3's), 60.07 (OCH_3), 106.65 (C_2 and C_6),
125.96 (C_1), 141.48 (C_4), 152.67 (C_3 and C_5), and 166.90 (CO_2H); IR
(KBr disc) 2961, 2647, 1686, 1587, 1470, 1422, 1417, 1326, 1227,
1224, 1123, 1001, 939, 858, 761, 715 cm^{-1}.

Gallic Acid (4)

Acid **3** (104 mg, 0.49 mmol) was treated with a mixture of
glacial AcOH (3 ml) and HI (4 ml of 57% HI in H_2O). The above
solution was refluxed under Ar for 8 h and then concentrated to
dryness in vacuo at 40 °C. The resulting deep brown residue was
triturated with water (20 ml). The mixture was again concen-
trated to dryness. This procedure was repeated at least two more
times until the residue assumed a light brown color. Gallic acid
(87 mg) prepared in this manner contained traces of iodine, but
was used without further purification. Yield 87 mg; TLC (silica
gel, $CHCl_3/CH_3OH/AcOH$, 80:20:5) R_f = 0.23; ^1H-NMR (DMSO-d_6) δ 6.96
(s, 2H, aryl); ^{13}C-NMR (DMSO-d_6) δ 108.70 (C_2 and C_6), 120.42 (C_1),
137.90 (C_4), 145.32 (C_3 and C_5), and 167.43 (COOH).

Ellagic Acid

The gallic acid (87 mg) was dissolved in glacial AcOH (1 ml)
and concentrated H_2SO_4 (0.05 ml) at 100 °C. $K_2S_2O_8$ (88 mg, 0.32
mmol) was added to the mixture in small amounts so the reaction
did not become highly exothermic. The reaction mixture was
stirred at 100 °C for 5 min and then allowed to cool for 0.5 h.
H_2O (20 ml) was added to the reaction mixture to precipitate the
crude product. The precipitated material was triturated with
CH_3OH (10 ml), filtered and the filtrate purified by reverse
phase HPLC (column, 4.6 mm X 25 cm 10μ Econosil C_{18}; solvent, 1.5

ml/min, 40% CH$_3$OH/60% 0.1 M NaH$_2$PO$_4$ buffer-pH 3.0) R$_t$ = 14.33.
Fractions corresponding to **EA** were collected, pooled together,
and concentrated under reduced pressure at 40 °C. The residue
was desalted using a Sep-Pak C$_{18}$ cartridge (Waters) and elution
with CH$_3$OH. The CH$_3$OH effluent was concentrated and dried <u>in</u>
<u>vacuo</u> to yield 3 mg (1% overall yield based on BaCO$_3$) of product;
UV (CH$_3$OH) 364 (log ϵ, 3.93) and 256 nm (log ϵ, 4.59); ^{1}H-NMR
(DMSO-d$_6$) δ 7.47 (s, 2H, aryl); ^{13}C-NMR (DMSO-d$_6$) δ 159.14 (CO),
148.14 , 136.41, 139.66, 112.34, 110.27 (C$_4$ and C$_9$) and 107.64;
EI/MS m/z (relative intensity) 303 (M+1)$^{\cdot}$ (7.37), 302 (M$^{\cdot}$)
(45.23); Mass for C$_{14}$ H$_6$O$_8$: calculated, 302.0063; found 302.0062.

EA-tetraisobutyrate

EA (100 mg, 0.29 mmol) was dissolved in refluxing pyridine
(10 ml) and treated with an excess of isobutyryl chloride (5 ml,
40 mmol) for 24 h. The reaction mixture was cooled, concentrated
<u>in</u> <u>vacuo</u> and the resulting residue extracted with CHCl$_3$ (3 x 50
ml). The combined organic extract was filtered to remove any
solid matter and the filtrate washed sequentially with 1 N HCl
(100 ml) and 10% aqueous NaHCO$_3$ (100 ml). The CHCl$_3$ solution was
dried over anhydrous MgSO$_4$, filtered and concentrated to afford a
black residue. The residue was purified by flash column chroma-
tography on silica using a solvent mixture of CHCl$_3$ and EtOAc
(97:3, v/v) as the eluent. The fractions containing product were
combined and concentrated, and the resulting residue crystallized
from CH$_3$OH to yield 150 mg (80%) of white solid; mp 270 °C (dec);
TLC (silica gel, EtOAc) R$_f$ = 0.69; HPLC (column, 4.6 mm x 25 cm
10μ Econosil C$_{18}$; solvent, 1 ml/min of 80% CH$_3$CN and 20% H$_2$O) R$_t$,
12.32; UV (CHCl$_3$) 339 (log ϵ, 4.21), and 355 (log ϵ, 4.27); ^{1}H-
NMR (CDCl$_3$) δ 1.4 (2d, 24H, CH(C<u>H</u>$_3$)$_2$), 2.9 (m, 4H, C<u>H</u>(CH$_3$)$_2$) and
8.04 (s, 2H, aryl); ^{13}C-NMR (CDCl$_3$) δ 173.68, 172.65, 156.83 (CO),
145.94, 142.51, 135.95, 120.61 (C$_4$ and C$_9$), 116.37, 34.11, and
18.93; IR (KBr disc) 3062, 2979, 2927, 1758, 1616, 1465, 1411,

1341, 122, 1155, 1075, 915, 835, 762 cm⁻¹; EI/MS m/z (relative %
intensity) 582 (M⁺) (2), 512 (M-70)⁺ (6), 442 (M-140)⁺ (13), 372
(M-210)⁺ (18), 302 (M-280)⁺ (12), and 71 (100). Mass for $C_{30}H_{30}$
O_{12}: calculated, 582.1737; found, 582.1744.

Preparation of Labeled Materials:

[¹³C]-**EA**

    [¹³C-Carboxy]trimethoxybenzoic acid (0.18 g) was obtained in
43 % yield from 1-bromo-3,4,5-trimethoxybenzene (0.490 g, 1.97
mmol), <u>n</u>-BuLi (0.8 ml, 2.5 M in hexane), and [¹³C]-BaCO₃ (0.344 g,
1.74 mmol) according to the procedure described above. Without
being purified, this product was treated with a mixture of
glacial AcOH (6 ml) and HI (8 ml) and refluxed for 8 h. The
reaction mixture was processed as before by successive evapora-
tion from H_2O to remove excess HI and I_2. The crude [¹³C]-gallic
acid (0.28 g) was oxidized with $K_2S_2O_8$ (0.17 g, 0.62 mmol) in a
solvent mixture of glacial AcOH (2 ml) and conc. H_2SO_4 (0.1 ml) at
100-105 °C as described above. The precipitated crude [¹³C]-**EA**
was dissolved in CH₃OH (10 ml), filtered and the filtrate puri-
fied by HPLC (column, 9.4 mm x 25 cm 10μ Magnum 9 Partisil 10
ODS-3; solvent, 3 ml/min, 50% CH₃OH/50% 0.1M NaH_2PO_4 buffer–pH
3.0) R_t = 11.10. Fractions corresponding to the peak of authen-
tic unlabeled **EA** were pooled together, concentrated, and the
sample desalted using a Sep-Pak C₁₈ solid phase extraction column.
The CH₃OH extract was concentrated and dried <u>in vacuo</u> to yield 4
mg (0.77% overall yield) of product; UV (CH₃OH) 367 and 256 nm;
¹H-NMR (CD₃OD) δ 7.47 and 7.48 (d, 2H, aryl, J=4 Hz, split by ¹³C
of CO); ¹³C-NMR (DMSO-d₆) δ 159.5 (CO); ¹³C-NMR (CD₃OD) δ 161.5 (CO)
and 112 (C_4 and C_9); EI/MS m/z (relative % intensity) 305 (M+1)⁺
(9), 304 (M⁺) (69); Mass for $^{12}C_{12}{}^{13}C_2H_6O_8$: calculated, 304.0129;
found 304.0134 (¹³C enrichment at CO = 97%).

[¹⁴C]-**EA**

    [¹⁴C]**EA** was prepared from the same starting materials except

[14C]-BaCO$_3$ (0.341 g, 1.72 mmol; specific activity, 58 mCi/mmol) was used in the synthesis. The [14C]EA was purified by HPLC (column, 10 mm x 25 cm 10 μ YMC [YMC Inc., Morris Plains, NJ]; solvent, 3 ml/min, 50% CH$_3$OH/ 50% 0.1M NaH$_2$PO$_4$ buffer-pH 3.0) R$_t$ = 15.16. The purified product was obtained (3 mg) by removal of salts with a C$_{18}$ Sep-Pak cartridge using CH$_3$OH. The overall yield was 0.6% as determined by HPLC. Radiochemical purity was checked using analytical HPLC (column, 10 μ Econosil C$_{18}$, R$_t$, 10.60) coupled with a radioactive detector for 14C measurement (Figure 2) with background substraction. The [14C]EA in methanol (20 ml, 0.5 mM concentration) was transferred into glass amber reaction vials and stored over argon at -80 °C.

RESULTS AND DISCUSSION

The synthesis of 13C- and 14C-labeled EA (Scheme 1) is based on the preparation of EA by K$_2$S$_2$O$_8$ oxidation of gallic acid in AcOH/ H$_2$SO$_4$ (20:1, v/v) (13). Although the yield for this reaction is only ~10%, the synthetic scheme appeared to be the shortest possible route to product, and allows the introduction of the label from an inexpensive source late in the synthesis. It also involves the facile preparation of carboxy-13C- and -14C-gallic acid from 3,4,5-trimethoxybenzene utilizing a previously reported synthesis of [14C]-gallic acid with only minor modifications (14).

The 1-bromo-3,4,5-trimethoxybenzene was obtained by the diazotization of the commercially available 3,4,5-trimethoxy- aniline followed by the Sandmeyer reaction involving the decom- position of 3,4,5-trimethoxybenzenediazonium salt in the presence of Cu$_2$Br$_2$ and HBr. Attempts to prepare the 1-halo-3,4,5-tri- methoxybenzene by other methods (15-17) were unsuccessful. Iodination of trimethoxybenzene in the presence of I$_2$ and AgOCOCF$_3$ yielded exclusively the undesired isomeric product, 1-iodo- 2,3,4-trimethoxybenzene. Bromination of trimethoxybenzene with N-bromosuccinimide in CCl$_4$ also afforded the undesired positional

bromo isomer as determined by ^{1}H-NMR, which showed the presence of a pair of doublets in the aromatic region, one centered around 6.7 and another at 7.3 ppm, with a 8-9 Hz coupling characteristic of ortho protons. Bromination of trimethoxybenzene in CCl_4 with Br_2 and Fe yielded a complex mixture of products. Only two components were purified, and while they showed a single ^{1}H-NMR resonance in the aromatic region, the integration of aromatic to aliphatic protons was 1:9. Presumably they were isomeric dibromo substituted trimethoxybenzene derivatives. Aromatic decarbonylation (17) of the commercially available 3,4,5-trimethoxybenzoyl chloride by Wilkinson's catalyst $[(Ph)_3P]_3 \cdot Rh(I)Cl$ also did not provide the desired product.

The organolithium salt of 3,4,5-trimethoxybenzene, which was prepared *in situ* by halogen-metal exchange on treatment of 1-bromo-3,4,5-trimethoxybenzene with n-BuLi at low temperature, was quenched with labeled-CO_2 generated from $BaCO_3$ to afford the labeled 3,4,5-trimethoxybenzoic acid. This product had a light yellow color that was due to the presence of a minor byproduct, 3,4,5-trimethoxyphenol. This may be produced by the quenching of the 3,4,5-trimethoxybenzene lithium salt with oxygen by the inadvertent introduction of air into the reaction flask. The presence of this phenol was readily identified with 1H-NMR by the presence of a downfield singlet near 6.0 ppm, and by comparison on TLC with an authentic sample. Rigorous exclusion of air avoided this impurity.

The O-demethylation of 3,4,5-trimethoxybenzoic acid was achieved with 57% HI in glacial AcOH. Attempts to use BBr_3 in CH_2Cl_2 to demethylate the trimethylgallic acid gave a mixture of products. A complication in using HI to remove the aromatic O-CH_3 groups is the inability to separate the I_2 byproduct from the gallic acid product. Since **EA** has been previously prepared by heating gallic acid in an aqueous solution of I_2 (13), it was

anticipated that the removal of the I_2 impurity in the gallic acid would not compromise its oxidation to **EA** in the next step.

The detailed mechanism by which **EA** is obtained from the $K_2S_2O_8$ mediated oxidation of gallic acid is not well understood. The reaction may proceed via a 1-e⁻ oxidation of gallic acid to a phenoxy radical species which can dimerize to give the biphenyl system. The hydroxyl and carboxyl groups in the resulting biphenyl intermediate are oriented such that they will lactonize to afford **EA** (Scheme 2). The requirement for dimerization of such reactive species may account for the low yield of this reaction. Consistent with the proposed mechanism, several attempts to optimize the yield of **EA** through variation of reaction parameters, viz., ratio of concentration of reactant to the oxidizing agent, temperature and time, did not yield any significant positive improvement. However, in agreement with a previous report (18), the use of 96% H_2SO_4 as the medium gave
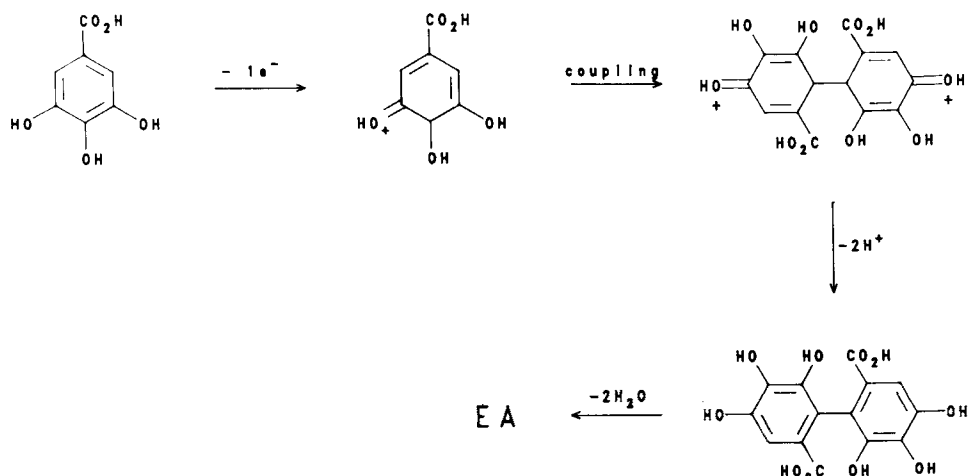
Scheme 2. Proposed mechanism for the formation of ellagic acid from gallic acid.

flavellagic acid (**FA**) (Figure 1) as the major product (~ 90%) and **EA** as the minor (~10%). A ratio of glacial AcOH and conc. H_2SO_4 of 20:1 was found essential to generate **EA** in acceptable yield as determined from HPLC, ^{13}C-NMR and EI/MS studies. The observation

that **EA** was not readily oxidized to **FA** by K₂S₂O₈ under these
conditions suggests that the latter was probably derived from the
initial oxidation of gallic acid prior to biphenyl formation. **FA**
was detected as an impurity by reverse phase HPLC as an earlier
eluting peak that runs very close to **EA** (R$_t$: 13.23 vs 14.29 min,
respectively). Accurate EI mass measurement of the HPLC peak
corresponding to that of **FA** gave a mass of 317.9994 amu as the
parent molecular ion which is consistent with the calculated mass
of C₁₄ H₆O₉. Derivatization of the mixture of **EA** and **FA** with
isobutyryl chloride yielded the corresponding derivatives, **EA**-
tetraisobutyrate and **FA**-pentaisobutyrate, respectively, with
clear separation on HPLC (R$_t$: 12.32 vs 17.66 min). The struc-
ture of **FA** pentaisobutyrate was confirmed from the fragmentation
pattern in EI/MS showing peaks at m/z 668 (M⁺), 598, 528, 458,
388, and 318 due to the sequential cleavage of five isobutyrate
ester groups. The tetraisobutyrate derivative of **EA** is very
soluble in EtOAc and CHCl₃ and is mobile on normal phase silica
TLC. In contrast, **EA** is insoluble in most organic solvents with
exception of polar viscous solvents like DMSO and polyethylene
glycol. **EA** also tails heavily on normal phase TLC even with
polar solvent mixtures. The tetraisobutyrate derivative offered
another attractive advantage of being deprotected very easily
with methanolic ammonia to give **EA**. Based on these findings, the
crude K₂S₂O₈ oxidation mixture containing [¹³C]-**EA** was derivatized
with isobutyryl chloride. Attempts to purify [¹³C]-**EA** tetraiso-
butyrate were made by normal phase chromatography using CHCl₃-
EtOAc (97:3) as the eluent. However, examination of the isolated
¹³C-labeled **EA** derivative by ¹H-NMR revealed the presence of
impurities containing aryl peaks at 7.7 ppm, in addition to the
EA isobutyrate peaks at 1.3 ppm and the expected split aromatic
signal at 8.05 ppm (d, J = 4 Hz, due to ¹³C of CO). The ¹³C-NMR
showed other CO peaks at 192 and 201 ppm in addition to the

enhanced 156.83 ppm signal of the ester ¹³C-labeled CO. EI/MS of
the ¹³C-labeled **EA** tetraisobutyrate sample, further purified by
HPLC, showed peaks at m/z 364, 294, 224, and 154, coming from a
volatile impurity, and the expected peaks at 584 (M⁺), 514, 444,
374, and 304. All these studies conclusively established that
another ¹³C-labeled CO containing impurity with a triisobutyryl-
oxybenzoyl skeleton was co-eluting on HPLC with the desired [¹³C]-
EA tetraisobutyrate derivative. Therefore, the strategy to
derivatize the **EA** prior to HPLC purification was abandoned and **EA**
from the crude oxidation reaction mixture was directly purified
by preparative reversed-phase HPLC.

The purity of the synthesized [¹³C]**EA** was checked by analyti-
cal HPLC and ¹³C-NMR (Figure 1). The [¹⁴C]**EA** was found to have 99%
radiochemical purity after preparative reversed-phase HPLC
(Figure 2) with a calculated specific activity of 110 mCi/mmol.
Approximately 1 % of the radioactivity of [¹⁴C]-BaCO₃ used in the
experiment was incorporated in the product (1.1 mCi in 20 ml of
CH₃OH, 0.5 mM).

The [¹⁴C]**EA** is currently being used to examine its affinity
binding to DNA, and the mechanism by which **EA** inhibits methyla-
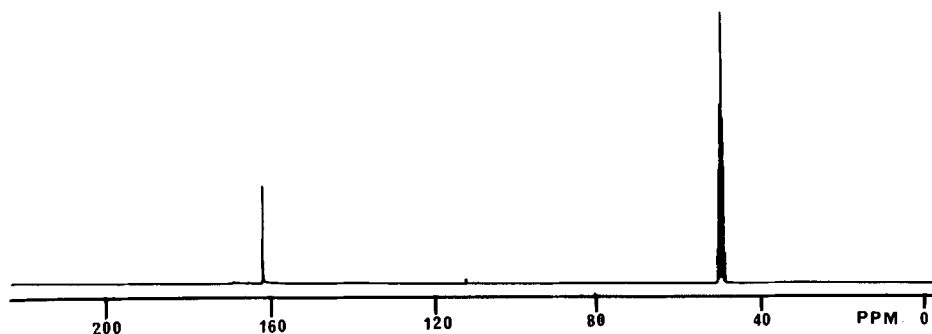tion of DNA by **MNU**.

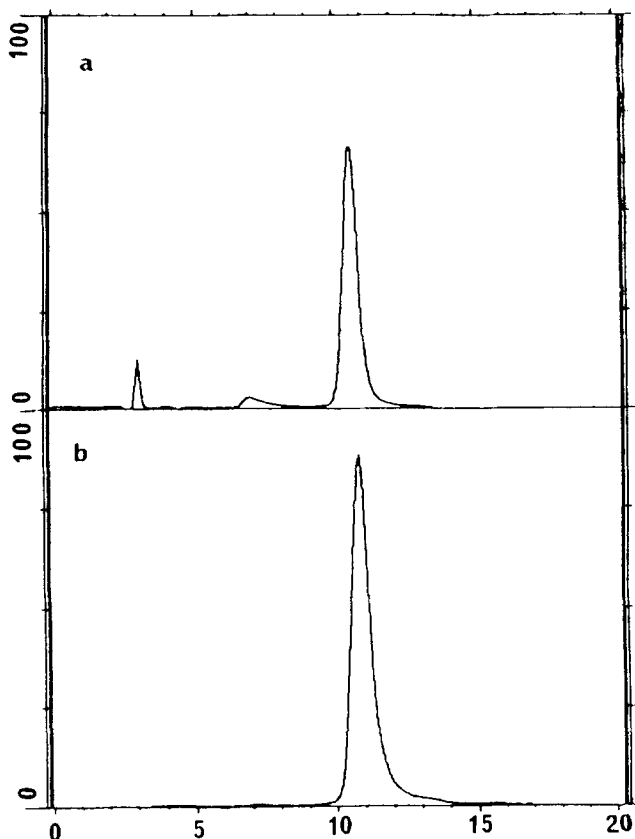**Figure 1.** ¹³C NMR (CD₃OD) of [¹³C]ellagic acid.

Figure 2. HPLC of [^{14}C]ellagic acid: a, UV at 254 nm; b, net CPM.

CONCLUSIONS

The synthesis of [^{14}C]EA (specific activity, 110 mCi/mmol) in 1 % yield starting from [^{14}C]BaCO$_3$ is reported. The final product was purified by preparative reverse phase HPLC.

ACKNOWLEDGMENT

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