SYNTHESIS OF [^{14}CO]ELLAGIC ACID

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SUMMARY

[^{14}CO]Ellagic acid with a chemical purity of 98.9% and radiochemical purity of 99.9% was synthesized with an overall yield of 16% (both chemically and radiochemically). Reaction of $^{14}CO_2$ with lithiated 3,4,5-trimethoxybenzene and demethylation of the resulting 3,4,5-trimethoxybenzoic acid was followed by esterification and coupling of methyl gallate into ellagic acid. Two efficient coupling methods were employed: direct aeration and aeration of methyl gallate in the presence of the phenolic oxidase, tyrosinase. The latter method produced the highest yield and purity. This preparation produced [^{14}CO]ellagic acid with a specific activity of 20 mCi/mmol. The yields of labeled 3,4,5-trimethoxybenzoic acid and ellagic acid based on $Ba^{14}CO_3$ were 65% and 16%, respectively.

Key words: ellagic acid, enzymatic oxidative coupling, anticarcinogenesis, antimutagenesis

INTRODUCTION

Ellagic acid (2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3, cde][1]-benzopyran-5,10-dione) is a naturally occurring phenol that is widely distributed in plants.[1,2,3] In nature, ellagic acid is found conjugated with glucose in the form of ellagitannins. Metabolism and synthetic studies have shown that ellagic acid and ellagitannins are formed by the oxidative coupling of gallate, and the ease of oxidative coupling of gallate ensures the abundance of ellagic acid in the plant kingdom.[4]

The antitumor properties of ellagic acid have been investigated in several laboratories. To date, ellagic acid has been shown to exhibit antimutagenic and anticarcinogenic effects against four groups of chemical carcinogens: polycyclic aromatic hydrocarbons[5], N-nitrosamines[6], aflatoxin B_1[7] and reactive amines[8]. Several possible mechanisms for the antimutagenic and anticarcinogenic

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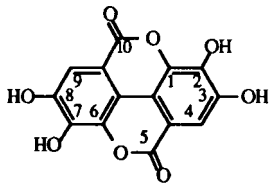


Figure 1. The Structure of Ellagic Acid

effects of this compound have been postulated.[9,10] These include inhibition of the metabolism and DNA damage of carcinogens[7], scavenging of the DNA reactive metabolites of carcinogens[10,11], and by shielding the DNA bases from electrophilic attack[12].

Data regarding the distribution and metabolism of ellagic acid is very sparse. Doyle and Griffiths[13] studied the metabolism of ellagic acid and the distribution of its metabolites in the rat. The major metabolite was identified as 3,8-dihydroxy-6H-dibenzo[b,d]-pyran-6-one, with other metabolites being unidentified. Recent studies in mice[14,15] also detected several structurally unidentified metabolites of [^{3}H]ellagic acid(generally labeled). These studies, as well as those of Smart[16], demonstrated that ellagic acid was poorly absorbed and most of it was excreted in the feces and urine.

Since ellagic acid is only slightly soluble, a sensitive marker molecule is needed for use in studies to clarify its uptake and distribution in the body. Since tritium exchange of [^{3}H]ellagic acid (generally labeled) with other biomolecules via ketonization $\rightleftharpoons$ enolization[17] followed by exchange of the phenolic ^{3}H occurs[19] *in vivo* , previous studies using tritiated ellagic acid[14,15] are somewhat ambiguous. Selectively labeled [^{14}C]ellagic acid would be better suited for studies of uptake and biodistribution.

Kozak, Kronrad and Prochazka[18] have chemically synthesized carboxyl labeled [^{14}C]gallic acid with a high specific radioactivity; i.e., 24 mCi/mmol. Since gallic acid is a biosynthetic precursor of ellagic acid, this study provided a possible means for the synthesis of [^{14}CO]ellagic acid. Recently, Mishra and Gold[19] reported the synthesis of carbonyl labeled [^{14}C]ellagic acid in 0.6% yield and 99% radiochemical purity. In the present study, we employed a modified ^{14}C incorporation and a very different coupling methodology from that used by Mishra and Gold to produce larger quantities of carbonyl labeled ellagic acid in considerably higher yield. The synthetic sequence used in the present study is outlined in figure 2.

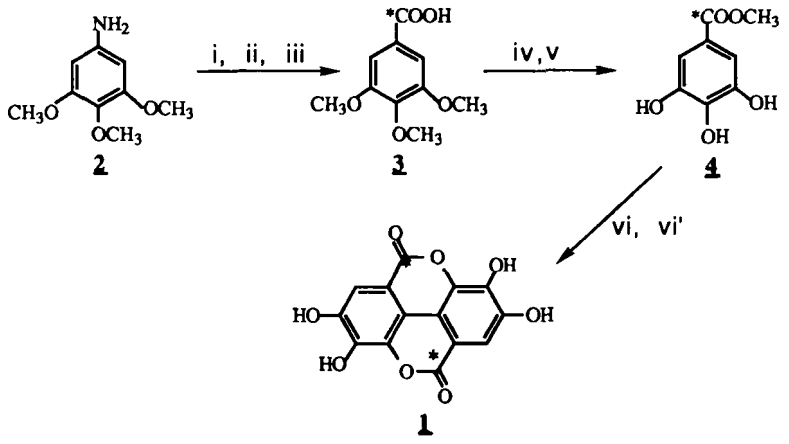


Figure 2. Synthetic Pathway of [^{14}C]Ellagic Acid

i: HNO_2/CuBr; ii: n-BuLi; iii: $Ba^{14}CO_3/H_2SO_4$; iv: HI/HOAc; v:MeOH/H^+; vi: Air/2N NH_4OH; vi': Air/Tyrosinase, 0.75 M $NaHCO_3$

EXPERIMENTAL

General

3,4,5-Trimethoxyaniline, n-butyl lithium (n-BuLi), barium carbonate ($BaCO_3$), hydroiodic acid (HI) and all other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). $Ba^{14}CO_3$ (specific activity, 58 mCi/mmol) was purchased from ICN Biochemicals, Inc. (Irvine, CA). Tyrosinase was purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of reagent grade. Scintillation cocktail (Flo-Scint II) was purchased from Radiomatic Instruments & Chemical Co, Inc. (Tampa, FL). Proton nuclear magnetic resonance (NMR) spectra were determined in the solvent specified on a Varian XL-200 NMR spectrometer operating at 200 MHz. Chemical shifts are reported in ppm relative to an internal tetramethylsilane (TMS) reference. Mass spectra were obtained on a Hewlett Packard 5987A GC/MS/DS system. Infrared spectra were obtained using a Nicolet DX/FT IR-20. High performance liquid chromatography (HPLC) was performed using two Waters pumps (model 510) and an automated gradient controller (model 680) with UV absorbance detector (model 440) recorded on a Waters data module (model 730) and radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL), using an ODS column, 4.6 x 250 mm (Beckman Instruments, Inc., San Ramon, CA). Two HPLC solvent systems involved in this study were: A. 10 mM $(NH_4)_2HPO_4$ water solution; B. 30 mM $(NH_4)_2HPO_4$ water/methanol (1:1) containing 0.5% of 80% H_3PO_4. Radioactivity was determined using a Beckman LS 7800 liquid scintillation counter

(Beckman Scientific Instruments Division, Irvine, CA). Thin layer chromatography (TLC) analyses employed 0.2 mm Silica Gel 60 F_{254} plates (E. Merk, Darmstadt, Germany). Radio-TLC analyses employed the same plates and same developing solvents, except that the plates were sliced in a distance of 6 mm and the radioactivity of each slice was counted with a scintillation counter.

Synthesis of 3,4,5-Trimethoxybenzoic Acid

3,4,5-Trimethoxybenzoic acid was prepared from 3,4,5-trimethoxybromobenzene following Kozak, Kronrad and Prochazka's synthetic route[18] using a modified incorporation methodology[20,21] on a 3 mmole scale. A yield of 57% was achieved. TLC showed one spot with an Rf of 0.52 upon development with chloroform: methanol: formic acid (95:5:0.1). HPLC (solvent B at a flow rate of 1 ml/min.) analysis showed a purity of higher than 96%. ^{1}H NMR and melting point matched literature[18,19] data. MS, m/e (relative intensity): 212 (M^+, 100), 197 (51), 182 (4), 169 (10), 154 (13), 152 (8), 151 (7), 141 (18), 139 (9), 137 (5), 126 (6), 111 (10), 109 (5), 93 (12), 81 (6), 77 (5), 69 (7), 66 (6), 65 (7), 44 (5).

Synthesis of Gallic Acid

Demethylation of 3,4,5-trimethoxybenzoic acid (1.74 mmol) was accomplished following Kozak, Kronrad and Prochazka's method[18]. The product was recrystallized twice from 10% methanol/water to afford a white crystal, which had an Rf of 0.62 in TLC upon development with chloroform: methanol: formic acid (85:15:1) in a yield of 90%. HPLC (linear gradient program from 100% B to 30% B and 70% A in 60 min. at a flow rate of 1 ml/min.) showed a single peak. ^{1}H NMR (in DMSO-d6) matched the literature data[19]. MS, m/e (relative intensity): 170 (M^+, 100), 153 (80), 135 (9), 125 (17), 107 (5), 79 (15), 53 (10), 51 (13).

Synthesis of Methyl Gallate

Gallic acid (0.267 g, 1.57 mmol) was esterified in the usual manner using methanol and concentrated sulphuric acid to produce 73% of yield of methyl gallate. The product had an Rf of 0.65 on TLC (development with chloroform: methanol: formic acid 85:15:0.1). HPLC (linear gradient program from 100% B to 30% B and 70% A in 60 min. at a flow rate of 1 ml/min.) analysis showed a purity of 98%. ^{1}H NMR (DMSO-d6): d3.72 (s, 3H), d6.84 (s, 2H), d9.08 (b, 3H). MS, m/e (relative intensity): 184 (M^+, 51), 153 (100), 125 (21), 107 (5),79 (14), 67 (6), 53 (10), 51 (11).

Synthesis of Ellagic Acid

A. Direct Oxidative Coupling.

Methyl gallate (92 mg, 0.5 mmol) was dissolved in 2 ml of 2N ammonium hydroxide. As the solution was aerated EA precipitated as it was formed. After 24 hours of room temperature aeration, the product was filtered, washed with a large excess of water, ethanol, and dried over P_2O_5 under vacuum. The final product was pale yellowish and amounted to 40 mg (47% yield). HPLC (linear gradient program from 100% B to 30% B and 70% A in 60 min. at a flow rate of 1 ml/min.) analysis showed a purity of 88%.

B. Enzymatic Oxidative Coupling.

Methyl gallate (92 mg, 0.5 mmol) was dissolved in 150 ml of 0.75 N sodium bicarbonate. To this solution, 0.03 E.U. of tyrosinase was added. The mixture was aerated for 5 hours at room temperature. When the mixture was acidified with concentrated hydrochloric acid, ellagic acid precipitated out as a white powder and was separated by centrifugation. The solid was washed with water and ethanol and dried under vacuum to give 52 mg (62%) of ellagic acid. TLC showed one spot with an Rf of 0.65 (development with chloroform, methanol, formic acid, 2:1:1). HPLC (linear gradient program from 100% B to 30% B and 70% A in 60 min. at a flow rate of 1 ml/min.) analysis showed the purity to be greater than 98%. ^{1}H NMR (DMSO-d6): d7.43 (s, 2H). MS, m/e (relative intensity): 302 (M+,100), 273 (4), 228 (5), 218 (4), 190 (7), 162 (12), 151 (12), 133 (4), 128 (5), 123 (5), 117 (4), 105 (6), 87 (9), 77 (12), 59 (12). IR (KBr plate): 3078 cm^{-1} (b), 1700 cm^{-1} (s).

Synthesis of [^{14}CO]Ellagic Acid

In the low activity synthesis, the above reaction was carried out employing the same conditions as described above using $Ba^{14}CO_3$ with specific radioactivity of 36 μCi/mmol. The total yield was 12% ellagic acid, when methyl gallate was coupled in the presence of tyrosinase. The intermediates and the final product were analyzed by radio-TLC and HPLC (employing both UV and radioactive detector) to verify the purities. The results showed that the compounds were both chemically and radiochemically pure; i.e., the purities were higher than 98%.

Following the above sequence, higher radioactivity (10 mCi/mmol) was used in the final synthesis. The reactions and results are described below:

1. 3,4,5,-Trimethoxy[^{14}CO]benzoic Acid

A mixture of 484.2 mg $BaCO_3$ and 106.8 mg of 55.3 mCi/mmol of $Ba^{14}CO_3$ (30 mCi) (giving a final specific activity of 10 mCi/mmol) was used to generate carbon dioxide. The product was recrystallized from hot water to give 412 mg (65%) long needles melting at 168-170^0 C. Radio-TLC showed one spot which represented 99.9% of the radioactivity. HPLC analysis showed that the

product had a chemical purity of 99.8% and a radiochemical purity of 99.9% . The specific activity of the product was 10 mCi/mmol. ^{1}H NMR (in DMSO-d_6): d3.7O (s, 3H), d3.80 (s, 6H),d7.21 (s, 2H). MS (relative intensity): 214 (M^++2, 20), 214 (M^++1, 13), 212(M^+, 100), 199(8), 187(54), 195(4), 182(3), 181(3), 171(2), 169(12),154(12), 152(10), 141(28), 139(11), 137(7), 126(11), 123(6), 111(11), 95(9), 93(18), 81(10), 77(9), 69(17), 66(19), 44(33).

2. [^{14}CO]Gallic Acid

3,4,5-Trimethoxy[^{14}CO]benzoic acid was demethylated to provide 258 mg (73%) of gallic acid. Radio-TLC showed one spot which represented 99.8% of the radioactivity. HPLC analysis proved that the product was both chemically pure (99.9%) and radiochemically pure (99.8%). MS (relative intensity): 172(M^++2, 13), 171(M^++1, 14), 170(M^+,100), 155(20), 154(8), 153(99), 142(3), 141(7), 140(2), 137(5), 135(14), 127(3), 126(4), 125(18), 96(7), 95(7), 79(29), 77(10), 71(19), 53(37).

3. Methyl [^{14}CO]Gallate

[^{14}CO]Gallic acid was esterified to produce 182 mg (65%) of yellowish methyl [^{14}CO]gallate. Radio-TLC showed one spot which represented 99.8% of the radioactivity. HPLC analysis showed one peak with 99.8% chemical purity and 99.9% radiochemical purity. MS(relative intensity): 186 (M^++2, 9), 185(M^++1, 5), 184(M^+, 53), 183(M^+-1, 4), 155(16), 154(7), 153(100), 135(1), 127(2), 126(3), 125(20), 79(13), 51(4).

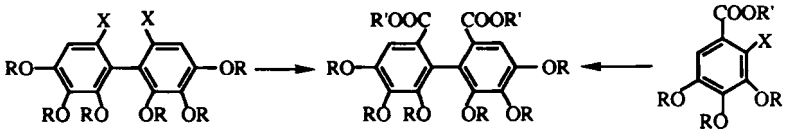
4. [^{14}CO]Ellagic Acid

Upon coupling, the methyl [^{14}CO]gallate was converted into 85 mg (51%) of ellagic acid. Radio-TLC showed one spot containing 99.9% of the radioactivity. HPLC analysis showed one minor peak and one major peak with both the UV 254 detector and radioactive detector. The major peak had a chemical purity of 98.9% and a radioactive purity of 99.9%. The specific activity of the ellagic acid was 20 mCi/mmol. MS (relative intensity): 305(M^++3, 5), 304(M+2, 27), 303(M+1, 23), 302(M, 100), 226(4), 219(7), 218(8), 190(13), 163(6), 162(21), 151(11), 145(5), 144(10), 126(5), 117(6), 105(24), 104(10), 103(9), 91(28), 87(20), 86(20), 79(17), 74(25), 59(28), 53(24), 45(31).

RESULTS AND DISCUSSION

The goal of this project was to synthesize [^{14}C]-labeled ellagic acid. A desirable approach in the synthesis of labeled compounds is to introduce the label as late in the synthetic sequence as

possible. As mentioned above, the immediate precursor of ellagic acid, in nature and in several synthetic schemes, is hexahydroxydiphenic acid(**5b**). Therefore, it seemed reasonable to synthesize this labeled precursor. We successfully synthesized, 2,2'3,3'4,4'-hexahydroxy-6,6'-dibromo-biphenyl(**6a**) by an appropriate Ullmann coupling[22], and dilithiation using n-butyl lithium was accomplished (from D_2O quenching studies). However, all reactions of **6b** with CO_2 produced **5a** in <5% yield. Reactions of **6a** with CuCN in DMF were also unsuccessful[23].



6a: R=CH_3, X=Br
6b: R=CH_3, X=Li

5a: R=CH_3, R'=H
5b: R=R'=H
5c: R=R'=CH_3

7a: R=R'=CH_3 X=Br
7b: R=X=H R'=CH_3

Figure 3. Possible Pathways of Synthesizing Ellagic Acid Precursors

Any alternative pathway to ellagic acid requires the incorporation of radiolabel at an early stage in the synthesis, since these approaches utilize coupling of 3,4,5-trimethoxybenzoic acid or 3,4,5-trihydroxybenzoic acid derivatives. The gallate ester(**7a**) underwent Ullmann reaction to give dimethyl 2,2',3,3',4,4'-hexamethoxy-6,6'-diphenate successfully in the present study. Demethylation of **5d** was carried out in acetic acid with 48% hydroiodic acid to give ellagic acid as a yellowish powder, but the overall yield was less than 10%, primarily due to the low yield in the coupling reaction. Therefore, this route was not considered effective for synthesizing carbonyl labeled ellagic acid.

Finally, we were lead to mimic nature's method of making ellagic acid by the oxidative coupling of gallic acid esters (**7b**). Gallic acid itself can be oxidatively coupled to ellagic acid by a variety of methods[24], but these also produce overoxidation products such as flavellagic acid, sometimes as the major products.[19,25] Esters of gallic acid have also been coupled by several methods[26,27,28] and two of these methods[27,28] were adapted in the present work. Aeration of aqueous ammonium hydroxide solutions, in our hands, produced a 48% conversion of labeled methyl gallate to carbonyl labeled ellagic acid. The pale yellow product was determined to have an 88%

chemical purity and an 85% radiochemical purity by HPLC. The contaminants were quinones (mass spectrometry). Purification by recrystallization or by preparative HPLC was unsatisfactory.

However, coupling of methyl gallate in aqueous sodium bicarbonate solutions in the presence of tyrosinase produced ellagic acid in a good yield (62%) and excellent purity (99%). The aeration at 25^0 C formed ellagic acid as a fine white (not yellow) powder which was precipitated by

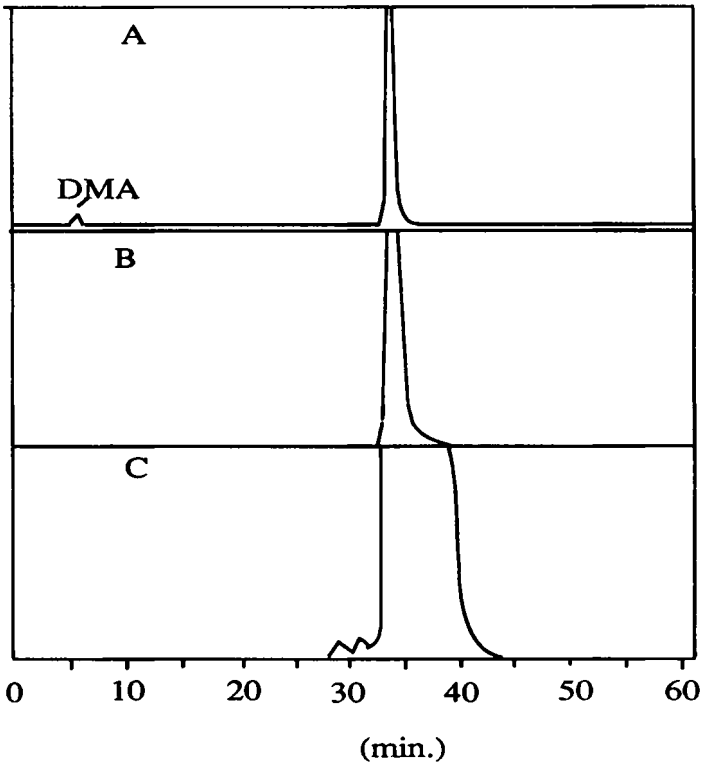


Figure 4. HPLC Chromatography of [^{14}C]Ellagic Acid

A: Recorded from UV 254 nm absorption with a scale of 0.0-1.0.
B: Recorded from radioactive detector with a scale of 0-50,000.
C: Recorded from radioactive detector with a scale of 0-250.
DMA: N,N-Dimethyl acetyamide

centrifugation. Decantation and washing of the products gave a white powder of ellagic acid, pure without recrystallization. Inevitably, the heating process required to dissolve ellagic acid in any solvent produced a slight coloration in the recrystallized product. When using methyl [^{14}CO]gallate, [^{14}CO]ellagic acid was produced in 51% yield with 98.9% chemical purity and 99.9% radiochemical

purity (for its HPLC, see Fig. 4). The overall chemical and radiochemical yield of the complete sequence in the labeled synthesis was 16%.

The biosynthesis studies of Ishikura and Hayashida[29] produced very small quantities of randomly labeled ellagic acid, and in a recent report,[19] [^{14}CO]ellagic acid was produced as a solution in methanol with a yield of 0.6%. The present report describes the first synthesis of fully characterized [^{14}CO]ellagic acid (for its mass spectrum see Fig. 5), as well as all of its [^{14}C]-labeled synthetic precursors. This newly available material is presently being employed in experiments designed to elucidate the disposition and distribution of ellagic acid *in vivo*. Metabolism and pharmacokinetic studies will follow.

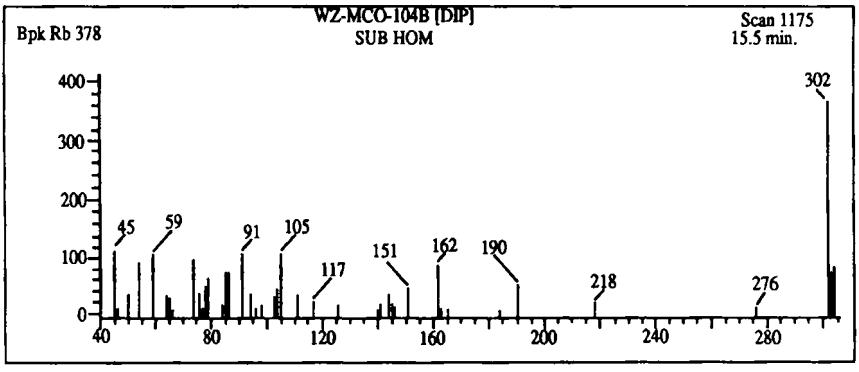


Figure 5. Mass Spectrum of [^{14}C]Ellagic Acid

CONCLUSIONS

[^{14}CO]Ellagic acid with a specific activity of 20 mCi/mmol was synthesized in 16% radiochemical and chemical yield starting from $Ba^{14}CO_3$. Because of the specificity of the enzymatic reaction, the final product did not require further purification.

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REFERENCES

1. Daniel, E. M., Krupnick, A. S., Heur, Y. H., Blinzler, J. A., Nims, R. and Stoner, G. D. - **J. of Food Composition and Analysis 2**: 338 (1989)
2. Bate-Smith, E. C.- **Chem. and Ind. BIF Review** R32 (1956)

3. Hillis, W. E. and Carle, A.- **Biochem. J.** 74: 607 (1960)

4. Nonaka, G. I., Ishimatsu, M., Ageta, M. and Nishioka, I.- **Chem. Pharm. Bull.** 31(1): 50 (1989)

5. Wood, A. W., Huang, M. T., Chang, R. L., Newmark, H. L., Lehr, R. E., Yagi, A., Sayer, J. M. and Conney, A. H.- **Proc. Natl. Acad. Sci. USA** 79: 5512 (1982)

6. Mandal, S. and Stoner, G. D.- **Carcinogenesis** 11: 55 (1990)

7. Mandal, S., Ahuja, A., Shivapurkar, N. M., Cheng. S.-J., Groopman, J. D. and Stoner, G. D.- **Carcinogenesis** 8: 1651 (1987)

8. Tanaka, T., Inata, H., Niwa, K., Mori, Y and Mori, H.- **Jpn J. Cancer Res.** 79: 1279 (1988)

9. Dixit, R. and Gold, B.- **Proc. Natl. Acad. Sci. USA.** 83: 8039 (1986)

10. Sayer, M., Yagi, H., Wood, A. W., Conney, A. H. and Jerina, D. M.- **J. Amer. Chem. Soc.** 104: 5562 (1982)

11. Newmark, H. L.- **Can. J. Physiol. Pharmacol.** 65: 461 (1987)

12. Teel, R. W, Martin, R. M. and Allahyari, R.- **Cancer Lett.** 36: 203 (1987)

13. Doyle, B. and Griffiths, L. A.- **Xenobiotica** 10: 248 (1980)

14. Teel, R. W.- **Cancer Lett.** 34: 165 (1987)

15. Teel, R. W. and Martin, R. M.- **Xenobiotica** 18(4): 397 (1988)

16. Smart, R. C. , Huong, M. T., Chang, R., , Sayer, J. M. , Jerina, D. M. and Conney, A. H. - **Carcinogenesis** 7(10): 1663 (1986)

17. Hart, H.- **Chem. Rev.** 79(6): 515 (1979)

18. Kozak, I., Kronrad, L. and Prochazka, M.- **J. Labelled Compds. Radiopharm.** 15: 401 (1978)

19. Mishra, N. C. and Gold B. - **J. Labelled Compds. Radiopharm.** 28: 927 (1990)

20. Tolbert, B. M.- **J. Biol. Chem.** 173: 205 (1948)

21. Ostwald, R.- **ibid** 173: 207 (19480

22. Fanta, P. E.- **Synthesis** 1: 9 (1974)

23. These results will be reported in detail in a subsequent publication in preparation.

24. Perkin, A. G. and Nierenxtein, M.- **J. Chem. Soc.** 87: 1412 (1905)

25. Perkin, A. G.- **J. Chem. Soc.** 89: 251 (1906)

26. Ernst, S. and Zwenger, P.- **Ann.** 159: 32 (1871)

27. Hathway, D. E.-**J. Chem. Soc.** 519 (1957)

28. Hathway, D. E.-**J. Biochem.** 67: 445 (1957)

29. Ishikra, N., Hayashida, S. and Tazaki, K., **Bot. Mag., Tokyo** 97: 355 (1984)