SYNTHESIS OF [21-14C]-FUSARIN C BY ENZYMIC DEMETHYLATION
AND REMETHYLATION WITH [14C]-DIAZOMETHANE

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SUMMARY

Fusarin C, a potent mutagen isolated from <u>Fusarium moniliforme</u> culture
extracts, has been prepared radiolabeled in two steps by enzymic hydrolysis of
the 21-methyl ester group, using phenobarbital induced microsomal preparations,
followed by remethylation using [14C]-diazomethane. Yields, based upon fusarin
C, were essentially quantitative and approximately 10% of the [14C]-methyl-
nitrosourea, converted to diazomethane, reacted to yield [14C]-fusarin C.

Keywords: [14C]-diazomethane, [21-14C]-fusarin C, carboxyesterase

INTRODUCTION

Fusarin C, <u>1</u>, isolated from cornmeal cultures of <u>Fusarium moniliforme</u>, is
highly mutagenic and toxic to bacteria and mammalian cells when metabolized by
microsomal enzymes [1,2,3,4]. Since <u>1</u> often contaminates corn in China and
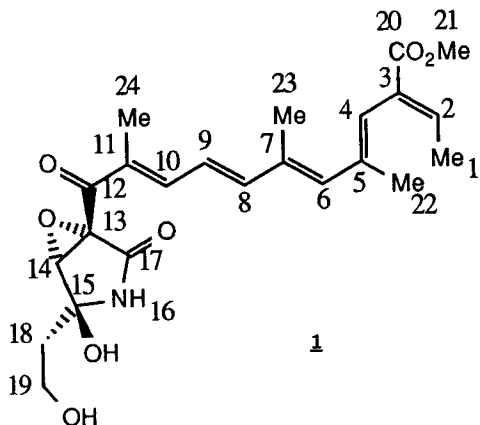
Southern Africa, two of the highest incidence areas of esophageal cancer in the world, it has been suggested that it may play a role in the development of esophageal cancer in these areas [1,3]. In order to investigate the metabolism and mechanism by which 1 acts as a mutagen, and possibly a carcinogen, radiolabeled material is needed. Any changes in the characteristic polyene structure would significantly alter both the λ_{max} and extinction coefficient of 1 in which case it would be difficult to detect.

MATERIALS AND METHODS

Alcohol dehydrogenases, isolated from horse and Thermoanaerobium brockii, NAD and NADP were obtained from Sigma Chemical Co. Sodium borotritide and cyanoborotritide were obtained from Amersham Corp. NAD³H was prepared by reduction of NAD with sodium cyanoborotritide [5]. NADP³H was prepared by using [2-³H]-propan-2-ol, prepared by reduction of acetone with sodium borotritide, in the presence of T. brockii alcohol dehydrogenase [6]. The microsomes were from rats induced with phenobarbital [4]. Esterase activity was measured using p-nitrophenyl acetate as substrate [7].

Fusarin C was isolated as previously described [8]. Since 1 is light sensitive all experiments were performed under yellow light or in the dark. Fusarin PM1 [9,10] was prepared from 443 nmol 1 in 100 µl DMSO by incubation at 37° for 2 hr with 5 ml microsomes, containing 4 units of esterase, suspended in 0.1 M phosphate buffer, pH 7.4, from which the standard NADPH generating system incorporated with microsomes was omitted. The reaction mixture was then extracted with 2 x 10 ml chloroform, with centrifugation to separate the phases. The aqueous layer was transferred to a C_{18} Sep-Pak Cartridge (Waters Assoc.), previously washed with methanol followed by water. The column was washed with 20 ml water and then the PM1 was eluted with 2 ml methanol and concentrated by rotary evaporation.

Diazomethane was prepared from [¹⁴C]-methyl-N-nitrosourea as previously reported [11] but with some modification. PM1 (2.3 µmol), dissolved in 200 µl methanol, was transferred to a 5 ml round bottomed flask and cooled to -70°. [¹⁴C]-methyl-N-nitrosourea (4.46 µmol, 250 µCi, 56mCi/mmol, 97% pure, Amersham

Corp.) was dissolved in 400 μl ether, and transferred to another 5 ml flask at 0°. KOH (50 μl, 50% w/v in water) was added to this flask which was immediately connected with the PM1 flask by a glass tube. The [^{14}C]-methyl-N-nitrosourea reaction mixture was warmed to room temperature and the diazomethane generated was transferred with a gentle stream of argon over the next 45 min.

After the transfer was finished, the PM1 reaction was brought to 0° and stirred for an additional 45 min. The methanol and ether were then evaporated by a stream of argon, 2 ml water added, and the 1 extracted with 3 x 2 ml chloroform. The chloroform layers were combined and evaporated under argon.

Acetylation of the 19-hydroxyl group of 1 (464 nmol) was achieved by reacting 1.3 μmol acetic anhydride and 250 μmol pyridine in dioxan for 5 hr at room temperature.

HPLC separations were made using a Dupont 850 instrument at a flow rate of 1 ml/min using Zorbax ODS-reverse phase columns, (3.9 mm x 300 mm, Phenomenex, Rancho Palos Verdes, CA), and a LKB 2140 Rapid Spectral diode array detector. Radioactivity was measured with a LKB 1215 Rackbeta scintillation counter.

RESULTS AND DISCUSSION

We tried several approaches to prepare radiolabeled 1 based upon the presence of the 19 primary hydroxyl group. Horse alcohol dehydrogenase has a broad substrate specificity [12] but although benzyl alcohol, as a positive control, underwent exchange with this enzyme, no radioactivity was associated with 1 when separated by hplc (Zorbax ODS, 60% methanol in water, retention time 21 min). We also tested an alcohol dehydrogenase isolated from Thermoanaerobium brockii [6] with NADP^{3}H as coenzyme. Again, benzyl alcohol and butan-1-ol underwent exchange with this enzyme but no tritium was transferred to 1. Another possibility for labeling 1 was by chemical oxidation of the 19-hydroxyl group followed by reduction back to 1. All model compounds, such as benzyl alcohol and butanol could be oxidized to their corresponding aldehydes by fresh manganese dioxide [13] and Swern's reagent [14]. 1 was not oxidized by the former reagent and gave a single product with the latter which, based upon its 500 MHz nmr and mass spectra, was not the corresponding aldehyde. Acetylation of the 19-hydroxyl group with acetic anhydride gave a single product by tlc (chloroform:methanol::20:1; R_f 1, 0.16; 1 acetate, 0.46). The 500 MHz nmr spectrum (CD$_2$Cl$_2$) of the acetate was similar to 1, [15] with the following exceptions: 19H, 4.38; 14H, 4.0; 18H 2.23 and 2.1 and an additional three protons at 2.08 ppm. This compound was however unsuitable for biological experiments since it underwent 20% reaction (10 mM phosphate buffer, pH 7.4, 37°) in 2 hr to a product indistinguishable from 1 by hplc analysis.

Fusarin A, a closely related structure [16] has been labeled using [^{13}C]-acetate supplements to cultures of F. moniliforme. Although this is a potential method for obtaining [^{14}C]-1, the yields, based upon added label, would be very low and the final product would have a very low specific activity. Recently, 1 was found to be a substrate of carboxy esterases present in liver microsomes [9]. The methyl ester was hydrolyzed to form its free carboxylic acid derivative, designated PM1, which is very water soluble. Our recent studies [10] have confirmed that fusarin PM1 is the major metabolite formed by incubation of 1 with microsomes in the absence of NADPH. Treatment of PM1 with diazomethane converted it back to a compound identical to 1 by hplc retention time and its uv spectrum. Owing to the lability of 1 in microsomal systems it may be considered that [21-^{14}C]-1 is not a suitable radiolabeled derivative of 1 for metabolic studies. However, we have shown that this esterase activity can be inhibited by low concentrations of di-isopropyl fluorophosphate which does not interfere with the metabolic activation of 1 mutagenic metabolites [10]. 1, in the presence of microsomes from rats previously induced with phenobarbital but absence of NADPH, is converted to PM1 in 67% yield. This value was calculated assuming the same molar coefficient ($\lambda_{358} = 32,000$) [15] for PM1 as 1, despite the λ_{max} for 1 being 368 nm. PM1 was >90% pure when analyzed by hplc using a linear gradient from 40 to 100% methanol in water over 20 min. Retention times were 11.5 min and 19.5 min for PM1 and 1 respectively.

The purity of the [^{14}C]-1, prepared from [^{14}C]-diazomethane and PM1, was determined by hplc as described above and 1 min fractions were collected for scintillation counting. The radiochemical and chemical purities based upon absorption at 368 nm were both found to be 94% and similar to the starting material. The product (0.33 μmol, 20 μCi) had the same uv spectrum as 1 and essentially the same specific activity (60 mCi/mmol) as the starting [^{14}C]-methyl nitrosourea and was produced in 8% yield based on the nitrosourea. Typically about 60% yields are obtained in the preparation of diazomethane when undertaken a larger scale.

These procedures, therefore, provide a convenient method to prepare [21-^{14}C]-1 for studies of its microsomal metabolism and binding to DNA.

Hazardous materials: 1 is mutagenic and possibly carcinogenic. Diazomethane is toxic and potentially explosive [17].

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