SYNTHESIS OF [16-^{14}C]TRICHODIENE THE PRECURSOR TO TRICHOTHECENES

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SUMMARY

The synthesis of radiolabelled trichodiene with ^{14}C at the olefinic methyl (C-16) is described. The isotope was introduced using a ^{14}C-Wittig reagent prepared *in situ* from triphenyl phosphine and ^{14}C-methyliodide.

Key words: ^{14}C, [16-^{14}C] Trichodiene, Trichothecenes.

INTRODUCTION

Bu'Lock first suggested in 1965 that trichodiene (1, Fig. 1) could be the precursor to trichothecenes.(1) Trichodiene was then found to be a natural product(2) and existed in all the fungal species which also synthesized trichothecenes. Inhibition of oxygenases with an inhibitor, ancymidol led to its accumulation.(3) The first experimental rigorous proof of its intermediacy in trichothecene biosynthesis was done in 1989 by feeding experiments with [15-$^{2}H_2$]trichodiene and [15-^{3}H]trichodiene.(4,5) [15-^{3}H]Trichodiene was prepared in a 60:40 mixture with bazzanene (2, Fig. 1) its stereoisomer differing in the stereochemistry of the methyl at position 15. It was possible to improve the separation of the two stereoisomers (to 90:10), however, this required several tedious repeated HPLC purifications. [15-^{2}H]Trichodiene with the tracer [15-^{3}H]trichodiene were incorporated (10%) into the main trichothecene of Fusarium culmorum: 3-acetyl-

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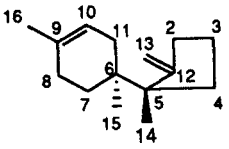
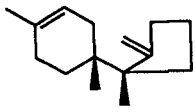
deoxynivalenol (3, Fig. 1).[4] [13-^{13}C]Trichodiene was also shown to be converted to trichothecenes.[6] In order to analyze the metabolic fate of trichodiene as well as assaying this enzyme, a radiolabel was essential. The purity of trichodiene was important (no formation of bazzanene) and ^{14}C was preferred over ^{3}H since it is less labile.

In this work, [16-^{14}C]trichodiene was synthesized with a specific radioactivity of 86.74 μCi/mmole. The radiolabel was incorporated using the Wittig reagent obtained *in situ* from triphenylphosphine and ^{14}C-methyl iodide.



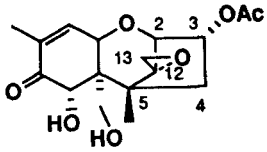
Trichodiene

1



Bazzanene

2



3-Acetyldeoxynivalenol

3

Figure 1. The precursor trichodiene 1, its stereoisomer bazzanene 2 and a trichothecene 3-acetyldeoxynivalenol. The numbering in trichodiene is given by analogy to trichothecenes to emphasize the precursor-product relationship.

RESULTS AND DISCUSSION

There have been various efficient syntheses of trichodiene[7] most of them result in a mixture of trichodiene and bazzanene. The elegant synthesis of Pearson and O'Brien[7] was selected since the

synthetic precursors of trichodiene and bazzanene are complexed with $Fe(CO)_3$ (6 and 7, Fig. 2) and are very easily separated by flash chromatography and recrystallization. In addition, this choice allowed the radiolabel to be conveniently introduced in very high yield at a very late step via a Wittig reaction. The reaction conditions were modified to enable scaling up the experiments and to improve the yield of some steps. The synthetic steps followed are shown in Figure 2.

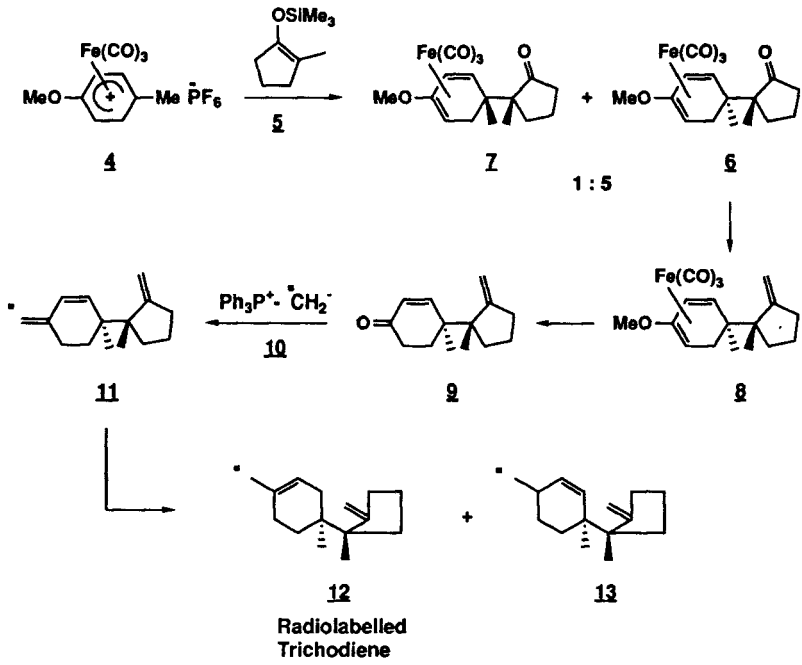


Figure 2. Synthesis of radiolabelled [16-^{14}C]trichodiene. The reagents and reaction conditions involved in the preparation of 6 and 7 are: 1) MeLi, DME, r.t; 2) n-Bu_3SnCl, -78°C; 3) of 5: CH_3CN, -78°C r.t.; of 8: 6 equiv. Ph_3PCH_3Br, t-BuOK/t-BuOH, ether, 55°C, 3 days; of 9: sat. $CuCl_2$/EtOH, 20 min; of 11: Ph_3CH_3I, t-BuOK/t-BuOH, ether, 55°C; of 12: Na, NH_3(l), ether, -78°C.

The modifications to the procedure of Pearson and O'Brien,[7] and the introduction of the radiolabel will be discussed. Unlabelled trichodiene was also prepared by this procedure as a starting material for other syntheses. When large amounts of the starting materials (4 and 5, Fig. 2) were used the desired product (6, Fig. 2), obtained after purification by flash chromatography (compound 6 R_f = 0.14, compound 5 R_f = 0.12 in ethyl acetate: hexane 1:9) had to be recrystallized from a mixture of ethyl acetate and hexane (5:95). Compound 6 was then converted to 8 by a Wittig reaction. The yield of this reaction was increased (61% to 82%) by changing the experimental conditions: six equivalents of the Wittig reagent were added in two portions within a 36-hour interval instead of six portions in 12-hour intervals. In the deprotection of compound 8, satisfactory results were not obtained with literature conditions (saturated $CuCl_2$/ethanol for 8 hours). However, when the reaction time was shortened to twenty minutes the dienone 9 was obtained in 86% yield. In order to prepare ^{14}C-labelled trichodiene from compound 9 the conditions for the Wittig reaction had to be modified. Excess of compound 9 was necessary for optimum incorporation of radiolabel into 11. Birch reduction gave two geometrical isomers 12 and 13 differing by the position of one double bond. Unlabeled pure trichodiene synthesized by the scaling up of this procedure was added to the radiolabeled compounds 12 and 13 in order to enable the purification. Flash chromatography on silver nitrate impregnated silica gel, using pentane as eluent enabled the recovery of pure [^{14}C]trichodiene. The purity was checked by HPLC using a Berthold radioactivity monitor and a UV detector. The ratio of the radioactive peak to the UV peak remained constant in three different flow conditions. In addition the silver nitrate - silica gel TLC plate showed a symmetrical peak (Fig. 3) on the Bioscan radioactivity imaging scanner with an R_f of 0.23 (pentane). Due to the extreme volatility of trichodiene, care was taken to evaporate solvents at room temperature.

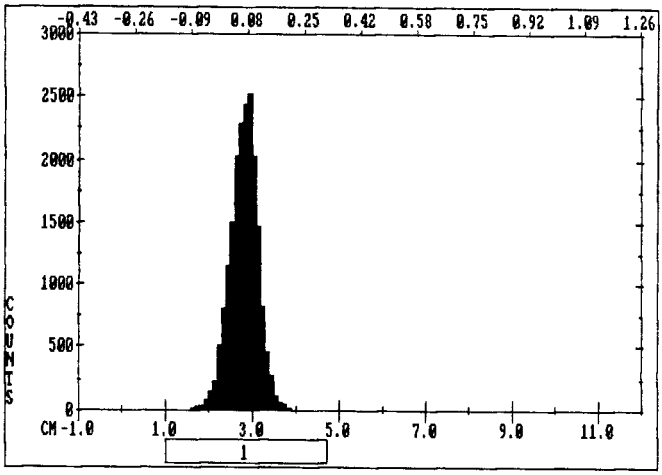


Figure 3. Radioactive scanning of a TLC plate with [16-^{14}C]trichodiene.

EXPERIMENTAL

Materials

[^{14}C]CH_3I was purchased from Amersham Co. 1.4 M Methyl lithium (MeLi) solution in dry ethyl ether, unlabelled methyl iodide, tributyltin chloride, triphenyl phosphine, methyltriphenyl-phosphonium bromide, and copper(II) chloride were purchased from Aldrich. Tricarbonyl-(4-methoxy-1-methylcyclohexadienylium) iron hexafluorophosphate[7g] and 2-methyl-1-(trimethylsilyloxy)cyclopent-1-ene[8] were prepared according to the literature procedure. The potassium t-butoxide solution in t-butanol was prepared by reacting potassium with t-butanol under a nitrogen atmosphere. Anhydrous ammonia was purchased from Union Carbide Canada Ltd. All solvents were distilled under a nitrogen atmosphere and over a drying agent as shown below (solvent/drying agent) : acetonitrile (CH_3CN)/calcium hydride; 1,2-dimethoxyethane (DME)/calcium hydride and then K, acetophenone; tetrahydrofuran (THF) and diethyl ether (Et_2O)/Na, acetophenone. Flash chromatography was

done using BDH silica gel (230-400 mesh). Silver nitrate impregnated silica gel was prepared by mixing silica gel (7 g) with silver nitrate (1 g) solubilized in 30 ml acetonitrile. The solvent was evaporated *in vacuo* and the silica gel was then dried at 100°C overnight. Silver nitrate ($AgNO_3$) coated TLC plates were prepared by rinsing with $AgNO_3$ solution in acetonitrile (1 g/15 ml) and drying the plates in the dark first at room temperature then at 100°C overnight. All reactions were conducted in flame or oven-dried glassware under a nitrogen atmosphere.

Instrumentation

The NMR spectra were recorded on a Varian XL-200 or XL-300 instrument and deuteriated chloroform was used as the solvent with tetramethylsilane as an internal standard. HPLC analysis was performed on a Perkin-Elmer series 3B liquid chromatograph attached to a Berthold LB505 radioactivity detector and a UV detector (Perkin Elmer LC-75). The mode employed was isocratic. Radioactivity scanning of TLC plates was done using a Bioscan system 200 imaging scanner. Melting points were measured on a Büchi 510 instrument.

Tricarbonyl (4-methoxy-1-methyl-1-(1-methyl-2-oxocyclopentyl)-(2-5-η)-cyclohexa-2,4-dienyl)iron 6

2-Methyl-1-(trimethylsilyloxy)cyclopent-1-ene (6.88 ml, 35.4 mmol) was reacted with a solution of 1.4 M MeLi in dry diethyl ether (27.76 ml, 35.4 mmol), Bu_3SnCl (9.6 ml) and compound 4 (12.0 g, 29.1 mmol) as described in the literature, leading to a golden-brown solid. After most of the isomer 7 (fast running band) was removed by careful flash chromatography on silica gel eluting with ethyl acetate/hexane (5:95), the yellowish crystal was dissolved in 15 ml of hot ethyl acetate/hexane (5:95). Upon cooling, the solution was allowed to stand in the

refrigerator overnight. Compound 6 was obtained as pale yellow crystals (mp 68.5-69.0°C). The infrared and ^{1}H-NMR spectra were identical to the literature.

Tricarbonyl(4-methoxy-1-methyl-1-(1-methyl-2-methylenecyclopentyl)-(2-5-η)-cyclohexa-2,4-dienyl)iron 8

To a 200 ml three-necked flask equipped with a reflux condenser provided with a nitrogen balloon, magnetic stirrer, and a rubber septum, was added Ph_3PCH_3Br (12.25 g, 28.88 mmol) and 20 ml of dry ethyl ether. A solution of potassium t-butoxide (1.09 g/mmol potassium t-butoxide) (31.59 g, 28.9 mmol) was added via syringe. The resulting yellow suspension was stirred under nitrogen for 1 hour at a gentle reflux before cooling to room temperature and adding the carbonyl compound 6 (3.15 g, 8.75 mmol) dissolved in 20 ml of dry diethyl ether. The suspension was stirred at 55°C. After 36h, the mixture was cooled, the insolubles were allowed to settle, the supernatant was added via cannula to a freshly prepared 3.3 equiv. of ylide suspension (prepared as described above) and the mixture was heated to 55°C. After 36h (a total of 6.6 quivalents of ylide were used), the mixture was cooled and filtered through celite. The filtrate was poured into 200 ml of water, extracted with diethyl ether (3 x 50 ml), concentrated *in vacuo* and flash chromatographed on silica eluting with a gradient of ethyl acetate/hexane 5%-20% to yield 2.6 g of compound 8 (82%) as a pale yellow oil and no starting material. The spectroscopic data (IR, NMR) were identical to the literature.

1-Methyl-1-(1-methyl-2-methylenecyclopentyl)-2-cyclohexen-4-one 9

Diene complex 8 (3.58 g, 10 mmol) dissolved in 60 ml of ethanol was added to 60 ml of a saturated $CuCl_2$-ethanol solution and stirred for 20 min. The mixture was then poured into 200 ml of water,

extracted with diethyl ether (4 x 100 ml), washed with liberal amounts of water, dried ($MgSO_4$) and concentrated to yield a pale yellow oil. Flash chromatography on silica with an ethyl acetate/hexane gradient (10%-20%) as eluent afforded 1.75 g (86%) of 9 as a pale yellow oil. R_f: 0.125 (ethylacetate/hexane = 10/90). The IR and ^{1}H-NMR spectra were identical with that of the literature.

1-Methyl-1-(1-methyl-2-methylenecyclopentyl)-4-(^{14}C-methylene-2-cyclohexene 11

A 25 ml two-necked flask equipped with a three-way stopcock, fitted with a nitrogen balloon and a ^{14}C-CH_3I ampoule (100 μCi) with a magnetic hammer (all the system must be well sealed), was evacuated and sealed by turning the stopcock. The flask was turned upside down and the magnetic hammer was released to break the ampoule seal. The flask was immediately immersed in a -78°C bath for 3 min and then nitrogen was allowed to leak into the flask. Subsequently, Ph_3P (43.9 mg, 0.158 mmol) in 0.35 ml of THF was added via syringe and the mixture was stirred first for 1h at -78°C then at r.t. for 2h. Unlabelled CH_3I (10 μl, 22.4 mg) in 0.6 ml of THF was added via syringe. The system was sealed again and stirred at r.t. for 42h. Upon evaporation, the white solid was washed with anhydrous toluene (3 x 0.5 ml) and dried *in vacuo* for 3.5 h. After 0.35 ml of diethyl ether and 258 mg of potassium t-butoxide/butanol solution (1.09 g/1 mmol potassium t-butoxide) were introduced, the suspension was heated at 55°C for 10 min. Upon cooling, compound 9 (38.7 mg ,0.19 mmol) in 0.3 ml of ether was added and reaction was heated at 55°C for 1.5 h. Upon cooling, the mixture was diluted with 5 ml of pentane, filtered through celite, washed with water (2 x 5 ml), dried ($MgSO_4$) and evaporated under a stream of nitrogen. Flash chromatography on silica with pentane as eluent gave 10 mg of the title compound as a colorless

oil. The product has the same R_f (0.54, pentane) with unlabelled 11 prepared by the same procedure.

[16-^{14}C]-Trichodiene 12

To a 25 ml three-necked flask equipped with a gas inlet, a stopper and a vent topped with a KOH drying tube, cooled within a dry ice-acetone bath, 0.3 ml of THF was added. Ammonia gas was introduced until about 10 ml of liquid ammonia had accumulated. At -78°C sodium (7 mg) was added and a few minutes later, compound 11 (10 mg) in 0.2 ml of THF was added. The mixture was stirred at -78°C for 45 min and was quenched with 0.5 ml of ethanol. After evaporation of the ammonia at r.t., the mixture was dissolved in 5 ml of pentane, washed with water and dried ($MgSO_4$). The concentrated crude trichodiene was mixed with 10 mg of unlabelled trichodiene (prepared by the same method) and was purified by flash chromatography on silica gel impregnated with $AgNO_3$ using pentane as eluent. The purity was checked by HPLC and by radioactivity imaging on a Bioscan 200 imaging scanner. (Fig. 3). Under two different isocratic HPLC conditions [i) methanol 90%, water 10% flow 1 ml/min, retention time 50 min; ii) methanol 85%, water 15% flow 1 ml/min, retention time 102 minutes] we obtained a symmetrical peak with constant specific activity. A high resolution TLC plate (LHP-KF, Whatman) was rinsed with a solution of 1 g $AgNO_3$ in 15 ml acetonitrile, dried in the dark at r.t. overnight and then at 100°C overnight. [16-^{14}C]Trichodiene was spotted on this plate and chromatographed with pentane as solvent, R_f = 0.23. The radioactivity scan is shown in Fig. 3. Thus 6.88 mg of radiolabelled (2.87 μCi) [16-^{14}C] trichodiene was obtained with a specific activity of 86.74 μCi per mmol.

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