# Preparation of (+)-[$^3$H]-pisatin with high specific radioactivity

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## Summary

(+)-Pisatin, the phytoalexin produced by pea (*Pisum sativum*) tissues on fungal infection or by abiotic treatment, has been tritiated with high specific radioactivity (23.9 Ci.mmol$^{-1}$) by treatment with n-butyllithium and $T_2O$.

## Introduction

The pterocarpan (+)-pisatin **1** is the only phytoalexin synthesized by pea (*Pisum sativum*) as a result of infection by fungus such as *Nectria haematococca* (1). In the disease of *P.sativum* caused by *N.haematococca*, genetic studies on the fungus suggested that the ability of fungal isolates to detoxify (+)-pisatin is necessary for high virulence on pea plants (2). The detoxification of (+)-pisatin involves a demethylation reaction catalyzed by pisatin demethylase, a microsomal cytochrome P450 (Scheme 1). To establish whether the pathogenicity of fungus is directly related to the demethylation reaction step, a convenient and sensitive pisatin demethylase test was necessary and required the synthesis of high specific radioactivity (+)-pisatin. The accurate determination of the (+)-6a-hydroxymaackiain **2** based on radioactivity precluded a localization of the label on the methyl group of (+)-pisatin since it is lost during the reaction.

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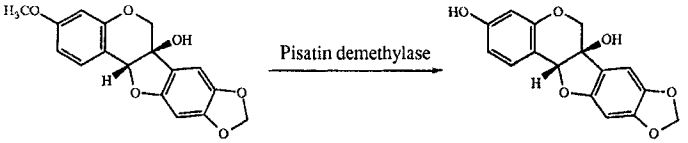



(+)-pisatin **1** (+)-6a-hydroxymaackiain **2**

Scheme 1 : demethylation of pisatin by pisatin demethylase

Up to now, [$^{14}$C] or [$^{2}$H] labelled pisatin has mainly been obtained by feeding chemically induced pea pods with radiolabelled precursors. However, use of [$^{14}$C(U)]-L-phenylalanine (3,4), [$^{14}$C]-formononetin (5) and [$^{14}$C]-maackiain (6) yields (+)-[$^{14}$C]-pisatin with low specific radioactivities. The selective methylation reaction of (+)-6a-hydroxymaackain **2** leads to (+)-[$^{14}$C]-pisatin with a rather high specific activity (13 mCi.mmol$^{-1}$)(7) but is not well adapted to monitor pisatin demethylation (*vide supra*).

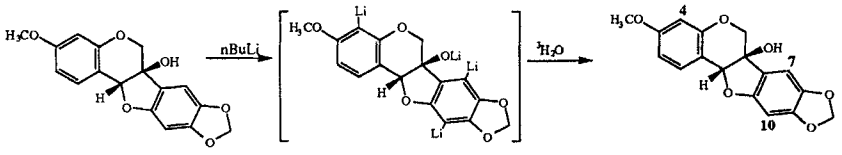
[$^{3}$H] labelled (+)-pisatin has not been described yet but again two strategies could be considered. The first one, based on incorporation of [$^{3}$H] labelled precursors into (+)-pisatin by pea was not adaptated because of the dilution of labelled material as demonstrated with [$^{2}$H]-formononetin (5). A direct [$^{1}$H]-[$^{3}$H] base catalyzed exchange could also have been considered by analogy with the incorporation of [$^{2}$H] into maackiain (8). However, this technique is not realistic because of the cost and contamination problems linked to the use of high specific radioactive tritiated water as solvent.

We wish to report here a convenient method for the preparation radiolabelled (+)-pisatin on aromatic positions having a high specific radioactivity.

## Results and discussion

Tritiation procedures of aromatic sites utilizing acidic catalysts (9,10,11) were to be avoided as (+)-pisatin easily undergoes dehydration under acidic conditions (12). Orthometallation of aromatic ethers appeared as a good way for introducing the label since the metal hydrogen exchange can be achieved either by hydrolysis of ortholithiated derivative or by hydrogenolysis with hydrogen gas of the potassium derivative (13). To avoid the lithium potassium exchange step which can be tedious to run on small amounts of product we decided to hydrolyze the lithiated intermediate compound with $T_2O$ (prepared for tritium gas).

The first step of the reaction (Scheme 2) is directed orthometallation (14) by metal hydrogen exchange which is carried out by treatment of (+)-pisatin with n-butyllithium in anhydrous ether (-15°C to room temp. 15 min). The addition of $^{3}H_2O$ at room



Scheme 2 : Tritiation of pisatin

temperature (15 min) afforded tritiated (+)-pisatin as predominantly a single component (UV coupled scintillation counter) which was purified by HPLC. Potential metallation/reduction sites on (+)-pisatin are C-2, C-4 ortho to the methoxy group and C-7, C-10 ortho to the methylene dioxy group. Tritium NMR spectroscopy of the product demonstrated unambiguously the sites of labelling as C-4,C-7 and C-10 aromatic positions. The presence of the three labelled sites ensures the maintenance of label in the metabolite (+)-6a-hydroxymaakain **2** while a specific activity of 23.9 Ci.mmol$^{-1}$ enables a variety of enzymatic studies.

The C-4 and C-10 incorporation demonstrates the directing effect of the two ether groups ortho to these positions.The proton at C-7 which is ortho to a methylene dioxy group and surrounded by a hydroxyl group is acidic enough to be exchanged by a metal such as lithium. The lack of label on C-2 is attributed to a poorer acidity of that hydrogen by comparison with C-7.

This is, to the best of our knowledge, the first report of (+)-[$^3$H]-pisatin on aryl positions. This procedure is very efficient and mild and is applicable to both pterocarpanol and pterocarpan derivatives.

## Experimental

$^1$H NMR and $^{13}$C NMR spectra were recorded respectively at 200 MHz and 50 MHz on a Brucker AC200 spectrometer in $CDCl_3$. Chemical shifts $\delta$ are expressed in ppm relative to trimethylsilane. $^3$H NMR spectrum was recorded at 320 MHz on a Brucker AC300 spectrometer.

**Chemicals**.n-Buthyllithium was purchased from Aldrich. Palladium oxide (PdO) was from Lancaster. All other chemicals and solvents were of the highest purity available.

**(+)-Pisatin 1 isolation and purification.** (+)-Pisatin was obtained from pea (Senior strain, Pioneer Genetique) using a modified procedure of R.E. Carlson (15)

to induced phenylpropanoid biosynthesis : The pea sprout were stressed by an asparagine-copper(II) chloride-phenylalanine solution (40 mM/5 mM/1 mM) for one day. The solution was drained off and the peas were rinsed with water and incubated at 24°C for two more days.

Treated pea seedlings were homogenized with ethanol 95% in a blender. After being allowed to stand overnight in the refrigerator at 4°C, the homogenate was filtred through Whatman No 1 filter paper. The EtOH extract was concentrated to give a thick aqueous solution and was then partitioned with chloroform. The solvent was evaporated to dryness, providing a crude syrup. The syrup was chromatographed on silica gel (eluent with cyclohexane/ethyl acetate/methanol 70/30/1). The fractions containing (+)-pisatin were pooled and concentrated.

TLC($SiO_2$) cyclohexane/ethyl acetate 7/3:Rf: 0.5
M.P.=67°C, (Litt.: M.P.=60-61°C (12))

RMN $^1H$ ($CDCl_3$, 200 MHz) : 2.51 (s,1H,OH); 3.78 (s,3H,$OCH_3$); 4.01 (d,J=11.8,1H,$H_{6r}$); 4.20 (d,J=11.8,1H,$H_{6s}$); 5.29 (d,J=0,7,1H,$H_{11a}$); 5.91 (d,J=1.4,1H, OCHHO); 5.94 (d,J=1.4,1H,OCHHO); 6.40 (s,1H,$H_{10}$); 6.46 (d,J=2.5,1H,$H_4$); 6.66 (dxd,$J_{2,1}$=8.6,$J_{2,4}$=2.5,1H,$H_2$); 6.81 (s,1H,$H_7$); 7.38 (d,J=8.6,1H,$H_1$)
RMN $^{13}C$ ( $CDCl_3$, 50 MHz) : 55.35 ($OCH_3$); 69.50 ($C_6$); 77.16 ($C_{6a}$); 84.85 ($C_{11a}$); 94.17 ($C_{10}$); 101.52 ($OCH_2O$); 101.60 ($C_4$); 103.01 ($C_7$); 109.77 ($C_2$); 112.37 ($C_{1a}$); 128.93 ($C_{7a}$) ; 131.79 ($C_1$); 142.38 ($C_8$); 149.79 ($C_9$); 154.47 ($C_{10a}$); 155.67 ($C_{4a}$); 161.01 ($C_3$)
Masse (I.C.,$NH_3$) 332 ($M^+NH_4^+$,15%); 315 ($MH^+$,8%); 297 ($MH^+$-$H_2O$,100%)

**(+)-Pisatin tritiation.** In a dry two neck reduction round bottom flask is placed a dry stirring bar and PdO (52.3 mg). The apparatus is placed on Toeppler pump under dynamic vacuum and dried with a heat gun. After closing the stopcocks, dry THF (1 ml) is added and the mixture is frozen. 20 Ci tritium is then added and the absorption is done at atmospheric pressure during 90 minutes. Meanwhile, in an other dry round bottom flask, (+)-pisatine (5 mg) is dissolved in dry ether (1.5 ml). The temperature is brought down to -15°C and n-butyllithium (58 µl, 8.6 eq) is added. The mixture is stirred for 15 min. and the filtered solution of $^3H_2O$ is added. The stirring is continued for 15 more min. The solvent is then evaporated under vacuum. The residue is taken up in EtOH (100 ml). Purification using HPLC (column Nucleosil C18 9.6*250 mm. Eluent : water/acetonitrile 58/42, 2 ml/min , λ=309 nm. Retention time : 6.3 min) yielded (+)-[$^3$H]-pisatin (75.5 mCi). Radiochemical purity (100%) was checked by HPLC (column Nucleosil C18 4.6*250 mm. Eluent : water/acetonitrile 58/42, 1 ml/min , λ=309 nm. Retention time : 10.7 min) and thin layer chromatography (Kieselgel F254 Merck, eluent

cyclohexane/ethyl acetate/methanol 75/25/1. Rf = 0.3). Specific radioactivity (23.9 Ci/mmol) was determinated by UV spectroscopy ($\lambda$=309 nm, log $\varepsilon$=3.86). The location of the labelled positions was confirmed by $^3$H NMR ($CDCl_3$, 320 MHz) : 6.4 (s,$^3$H, $H_{10}$); 6.46 (s,$^3$H, $H_4$); 6.81 (s,$^3$H, $H_7$)

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