Phytochemistry, Vol. 26, No. 4, pp. 987–989, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

THE SYNTHESIS OF TRITIATED RIBOSYLZEATIN WITH HIGH SPECIFIC ACTIVITY

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(*Received* 7 *April* 1986)

Key Word Index—Tritiated N^6-(Δ^2-isopentenyl)adenosine; tritiated ribosylzeatin; high specific activity cytokinins; allylic oxidation.

Abstract—Ribosylzeatin tritiated on the purine ring with a specific activity of 829×10^{10} Bq (22 mCi) per millimole was easily prepared by allylic oxidation of N^6-(Δ^2-isopentenyl)[2,8-^{3}H]adenosine which had been synthesized by alkylation of commercially available [2,8-^{3}H]adenosine. This allylic oxidation gave mainly the *trans*-isomer which was obtained free of the *cis*-isomer and with a radiochemical purity of 99.8% by a one-step purification using reversed-phase HPLC. This simple procedure yields ribosylzeatin, the most common naturally occurring cytokinin, labelled with tritium at near maximum specific activity.

INTRODUCTION

Plant hormones labelled with radioisotopes at high specific activities are necessary for metabolic studies, the isolation of receptors and quantitation by radioimmunoassay.

Among plant hormones of the cytokinin type, tritiated N^6-benzyladenine (bz^{6}Ade) and N^6-(Δ^2-isopentenyl)adenine (i^{6}Ade) of high specific activities have been described and used to isolate and characterize high affinity binding protein moieties [1–3]. Bz^{6}Ade labelled on the benzyl group, having specific activity in the range of 10–26 Ci/mmol has been prepared by the elegant method of Sussman and Firn [4] which uses the metal-catalysed dehalogenation (hydrogenation) of a halogenated precursor, *p*-bromo-bz^{6}Ade or *m*-iodo-bz^{6}Ade [5]. This method, unfortunately, is not applicable to the isopentenylated cytokinins N^6-(Δ^2-isopentenyl)adenine (i^{6}Ade) and zeatin (io^{6}Ade), due to the presence of the allylic double bond.

I^{6}Ade labelled on the purine ring with a specific activity of 39.9 Ci/mmol has been reported, without details, by Chen *et al.* [2]. It was prepared according to ref. [6], by periodate oxidation of N^6-(Δ^2-isopentenyl)[2,8-^{3}H]adenosine (i^{6}Ado), synthesized from [2,8-^{3}H]adenosine (Ado) according to Pacès *et al.* [7].

Zeatin has been labelled with tritium, either by exchange or by condensation of 6-chloro[2,8-^{3}H]purine with 4-amino-2-methylbut-*trans*-2-en-1-ol [8]. Tritium-labelled zeatin riboside has similarly been prepared by Summons *et al.* [9] by selective exchange of the C-8 hydrogen in the presence of ^3H_2O [10]. However, the specific activities obtained were rather limited, in the 100–400 mCi/mmol range.

We report here a new, easy and convenient method of preparation of tritium labelled ribosylzeatin of high specific activity from [2,8-^{3}H]Ado. It uses the stereospecific allylic oxidation of i^{6}Ado recently described by David *et al.* [11]. The synthesis of [2,8-^{3}H]i^{6}Ado, adapted from [7], is also described.

RESULTS AND DISCUSSION

Synthesis and purification of [2,8-^{3}H]i^6*Ado*

The procedure used to alkylate Ado with 4-bromo-3-methyl-2-butene was basically that of Pacès *et al.* [7] adapted to an extremely small scale reaction due to the high specific activity of the tritiated Ado. Preliminary attempts at alkylation of 0.5 μmol of Ado (containing 3 μCi of [2,8-^{3}H]Ado to monitor the reaction) in the presence of 1–3 μmoles of isopentenylbromide added as a 1% solution in DMF resulted in very limited alkylation of Ado, perhaps due to contaminants in the DMF. We therefore chose to use an excess of the alkylating bromide, with close monitoring of the reaction. Under these conditions, yields of 40–50% based upon the conversion of Ado to i^{6}Ado were constantly obtained.

Alkylation of [2,8-^{3}H]Ado was stopped after 9 hr, when TLC analysis (solvent A) and radioactivity monitoring indicated that 51% of the radioactivity on the chromatogram was associated with N^1-(Δ^2-isopentenyl)adenosine (i^{1}Ado) at R_f = 0.07. About 20% of the radioactivity was located at R_f 0.25 and 21% corresponded to Ado. N^1–N^6 transposition monitored by TLC (solvent A) was complete after 16 hr. The product was purified by Sephadex LH-20 chromatography as described [7], except that elution was with 25% aqueous ethanol. The UV absorbing fractions at the expected elution volume of i^{6}Ado were highly radioactive, and analytical HPLC on the Ultrasphere ODS 5 μm column confirmed that 99.8% of the radioactivity was associated with authentic i^{6}Ado (retention time 5.4 min, isocratic elution with 60% aqueous methanol at a flow rate of 1 ml/min). Identity was confirmed by the UV spectrum of

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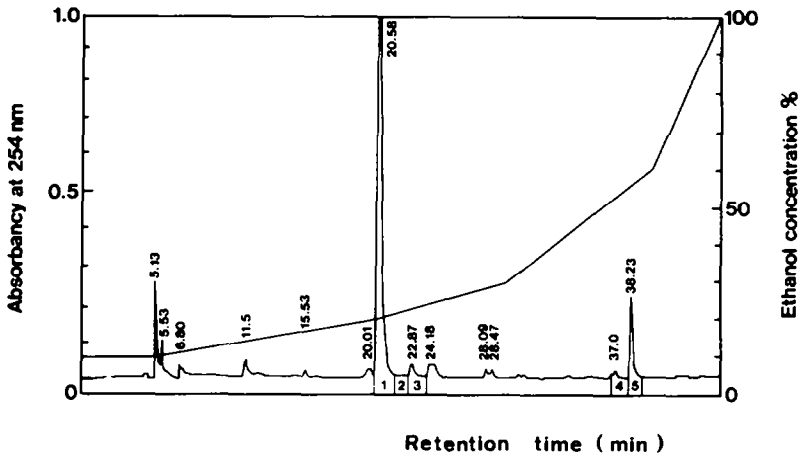

Fig. 1. Purification of *trans*-[2,8^{3}H]ribosylzeatin by reversed-phase chromatography on an Ultrasphere ODS 5 μm column (25 × 4.5 mm) equilibrated with 10% aqueous EtOH and eluted with an H_2O–EtOH gradient as shown. Fractions 1–5 were collected.

the sample [12]. Its specific activity was calculated from several radioactivity determinations and found to be 22 Ci/mmol, as compared to 31 Ci/mmol for the starting [2,8-^{3}H]Ado. This was attributed to some loss of tritium from C-8 during the N^1–N^6 transposition step in the presence of dimethylamine. The proton in this position has been shown to be exchangeable in basic solution [10]. Therefore, to avoid this problem use of [2-^{3}H]Ado would be preferable in further syntheses.

Synthesis and purification of trans-[2,8-^{3}H]*ribosylzeatin*

Allylic oxidation of i^{6}Ado to *trans*-io^{6}Ado was performed according to David *et al.* [11], using *tert*-butylhydroperoxide and SeO_2 in CH_2Cl_2 solution. Progress of the hydroxylation was monitored by rapid TLC analysis (solvent B) and radioactivity detection. The reaction time was shortened to a few hours in order to avoid excessive oxidation which resulted in lower yields.

The actual synthesis was conducted with 74 nmol tritiated i^{6}Ado ($\simeq$ 25 μg) and stopped after 3.5 hr when TLC analysis indicated that only 22% of the initial [^{3}H]i^{6}Ado remained and that about 49% of the radioactivity on the silica gel strip was located at the R_f of io^{6}Ado. These data were confirmed by analytical HPLC and radioactivity monitoring which further indicated that the *trans*- and the *cis*-isomers were in a 42:1 ratio. The two isomers are well separated on both C_{18} columns used.

The tritium-labelled *trans*-ribosylzeatin was readily purified by reversed-phase HPLC. First, solid-phase extraction with a disposable ODS column removed about 54% of the total radioactivity, 34% of which was not retained and was discarded. The remaining 20% appeared in the combined second fraction (0.5 ml 50% aqueous methanol eluate) and the following third fraction (0.5 ml methanol) which were saved, being mostly the remaining tritiated i^{6}Ado. The first fraction (0.5 ml 50% aqueous methanol eluate) contained most of the radioactivity (about 2 × 10^9 cpm), and it was chromatographed on the Ultrasphere ODS 5 μm column (Fig. 1). A water–ethanol gradient was used in order to obtain the desired labelled trans-ribosylzeatin as an aqueous ethanol solution which did not require any further treatment. About 19.5 nmol were recovered, based on the UV absorption spectrum of the fraction (ε_{268} of 20000 M^{-1}). This represented an overall yield of 26%. This purification scheme is therefore simple and very efficient since the tritiated *trans*-zeatin riboside was found absolutely free of its *cis*-isomer and with a radiochemical purity greater than 99.8%. Its specific activity was calculated and found to be the same as the specific activity of the starting [2,8-^{3}H]i^{6}Ado, i.e. 22 Ci/mmol.

Both tritiated i^{6}Ado and *trans*-io^{6}Ado were stored at −20° as 20 and 10 μM stock solutions in 50% and 20% aqueous ethanol, respectively. Under these conditions, they appeared quite stable. An average decomposition rate of 0.6%/month was observed over a period of 15 months, the major decomposition product being tritiated Ado.

The allylic oxidation of [2,8-^{3}H]i^{6}Ado of high specific activity allowed us to prepare tritium labelled *trans*-ribosylzeatin with high specific activity. This reaction constitutes a new and convenient route to isotopically labelled ribosylzeatin. It requires only the preparation of labelled i^{6}Ado, another important naturally occurring cytokinin, which is readily synthesized from commerically available tritiated Ado of high specific activity. Yields are satisfactory, given the availability of tritiated Ado. The specific activity obtained is about two orders of magnitude higher than that previously obtained by selective tritium exchange [9]. This feature should prove useful in future studies of the metabolism and of a mode of action of this major naturally occurring cytokinin, i.e. the search for cytokinin receptors. With such high specific activity, it should also advantageously replace its tritiated tri-alcohol derivative in radioimmunoassays of *trans*-ribosylzeatin

since this derivative is not stable enough to be used as an internal standard [Morris, R., personal communication] to estimate extraction and purification procedures.

EXPERIMENTAL

Chemicals. [2,8-^{3}H]Ado (31 Ci/mmol) was obtained from New England Nuclear. 4-Bromo-2-methyl-2-butene was from Columbia Organic Chemicals Co, 70% *tert*-butylhydroperoxide and SeO_2 from Aldrich Chemical Co. Authentic i^{6}Ado and io^{6}Ado were obtained from Sigma. Pure *trans*- and *cis*-io^{6}Ado were also obtained by allylic oxidation of i^{6}Ado as described [11]. A reference sample of i^{1}Ado was synthesized according to ref. [13].

Chromatographic procedures. TLC on 30 × 80 mm strips of 6060 silica gel Eastman chromatogram sheets. Reference compounds were visualized under UV light. Solvent systems: (A) CH_3Cl–MeOH–HOAc (80:19:1), (B) CH_3Cl–MeOH (75:25), R_f values: 0.33, 0.07 and 0.65 for Ado, i^{1}Ado and i^{6}Ado, respectively (A); 0.44, 0.78 and 0.60 for Ado, i^{6}Ado and *t*-io^{6}Ado, respectively (B). HPLC: Beckman system equipped with a model 165 variable wavelength UV detector and a model 421 controller. Samples were injected via an Altex injector fitted with either a 100 μl or a 2 ml loop. Analyses were performed with either an Ultrasphere ODS 5 μm column (250 × 4.5 mm i.d.) or a Partisil 10 ODS 2 column (250 × 4.5 mm i.d.), both protected with a precolumn (70 × 4.5 mm i.d.) filled with CoPell ODS (Whatman). Elutions were as specified.

Radioactivity detection. Radioactivity of high activity samples analyzed by TLC was directly monitored with a Berthold LB 2832 automatic TLC Linear analyser. In the case of HPLC, radioactivity was monitored on line with a Flo-One HP liquid scintillation detector (Radiomatic Instruments & Chemicals, Tampa, FL, U.S.A.) fitted with a 0.5 ml cell and operated with Flo-Scint II (also from Radiomatic Instruments & Chemicals) at a flow rate of 3 ml/min. Otherwise, radioactivity was monitored by liquid scintillation spectrometry.

Synthesis of N^6-(Δ^2-*isopentenyl*)[2,8-^{3}H]*adenosine.* An aq. soln (15 ml) of [2,8-^{3}H]Ado (0.48 μmol) was evaporated to dryness by rotary film evaporation (RFE) in *vacuo*. The residue was dried over P_2O_5 and dissolved in 0.5 ml dry DMF. This soln was stirred at room temp. with an excess of freshly distilled 4-bromo-2-methyl-2-butene (4 μl, 35 μmol, added every 1.5 hr). After 9 hr, MeOH (1 ml) and 40% aq. dimethylamine (0.2 ml) were added and stirring was continued for 16 hr. The soln was then brought to dryness by RFE *in vacuo*. The residue was evaporated 3 × with 0.5 ml MeOH and finally dissolved in 2 ml H_2O. The product was extracted with EtOAc (2 × 2 ml) and purified on a Sephadex LH20 column (30 × 2.5 cm) eluted with 25% aq. EtOH. Fractions containing i^{6}Ado (elution volume 350 ml) were pooled and concentrated by RFE *in vacuo*. The residue was dissolved in 11 ml of 50% aq. EtOH. A UV spectrum of this soln indicated the recovery of 0.203 μmol of tritiated i^{6}Ado (ε_{269} of 20000 M^{-1}).

Synthesis of trans-[2,8-^{3}H]*zeatin riboside.* The above aq. EtOH soln (4 ml) of [^{3}H]i^{6}Ado (74 nmol, 1.63 mCi) was evaporated to dryness by RFE in a 5 ml round bottom flask and the residue dissolved in 400 μl CH_2Cl_2. Then 100 μl of a CH_2Cl_2 solution of *tert*-butylhydroperoxide (prepared by partitioning 70% *tert*-butylhydroperoxide with an equal volume of CH_2Cl_2) and 10 μl (200 nmol) of a freshly prepared CH_2Cl_2 soln of SeO_2 (2.2 mg/ml) were added. This soln, protected from light, was stirred at room temp. At intervals, aliquots (≃ 1 μl) were removed to monitor the reaction which was terminated after 3.5 hr by addition of 2 ml MeOH. After RFE to dryness, the residue was taken up in 1 ml H_2O and this soln was solid-phase extracted with a 1 ml C18 Baker-10 SPE disposable column which was further rinsed with H_2O (2 × ml) prior to sequential elution with 50% aq. MeOH (2 × 0.5 ml) and MeOH (2 × 0.5 ml). The first 0.5 ml of the 50% MeOH eluate was diluted to 2 ml with H_2O and the soln injected via the 2 ml loop on the Ultrasphere ODS 5 μm column previously equilibrated with 10% aq. EtOH. The column was then eluted (Fig. 1). The main UV absorbing fraction (fraction 1, 1.3 ml) was collected and diluted to 3 ml with 20% aq. EtOH. Analytical HPLC with radioactivity monitoring confirmed that it was *trans*-io^{6}Ado free of its *cis*-isomer. A UV spectrum of the sample was then recorded and its radioactivity measured.

Fraction 3 (retention time 22.87 min) was also collected. Analytical HPLC with radioactivity monitoring indicated that it was *cis*-ribosylzeatin which was obtained free of the *trans*-isomer after a second purification in the same system. Radioactivity recovered indicated that the sample contained 0.78 nmol, assuming the same sp. act. as that of the *trans*-isomer.

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