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THE SYNTHESIS OF RADIOISOTOPICALLY LABELLED ZEATIN*

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Abstract—The synthesis of ^{14}C- and ^{3}H-labelled zeatin is described.

INTRODUCTION

THE PLANT hormone zeatin [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine (I)], a naturally occurring cytokinin,[1,2] has been synthesized by condensation of 6-chloropurine with 4-amino-2-methylbut-*trans*-2-en-1-ol[3,4] (IIa). Although radioisotopically labelled zeatin would be of great value in physiological studies, its synthesis has not been reported. This communication reports the synthesis of both ^{14}C- and ^{3}H-labelled zeatin.

Zeatin-8-^{14}C could be readily prepared by condensing 4-amino-2-methylbut-*trans*-2-en-1-ol with 6-chloropurine-8-^{14}C, a commercially available product. The high cost of the latter, however, necessitates that the reaction be normally carried out with a maximum of only a few milligrams of the labelled purine. This precludes recrystallization of the product and purification becomes dependent on paper or thin-layer chromatography (TLC). Hence the amine for use in the synthesis must be of high purity and free from isomeric impurities which would yield 6-(substituted amino)purines difficult to separate from zeatin by chromatographic methods. 4-Amino-2-methylbut-*trans*-2-en-1-ol has been synthesized by three methods.[3-5] It appeared that reduction[3] of methyl γ-azidotiglate (IIc) synthesized from methyl tiglate was the method which would most readily yield high purity amine. Investigation revealed, however, that formation of isomeric compounds occurred to a marked degree during the synthesis which failed to yield amine of sufficient purity. A modified synthesis, which yielded amine of the desired purity, was therefore devised. This amine was used to synthesize zeatin labelled in the purine ring with either ^{14}C or tritium. A procedure for preparing generally tritiated zeatin is also reported.

* Part XI in the series "Regulators of Cell Division in Plant Tissues". For Part X see D. S. LETHAM and H. YOUNG, *Phytochem.* **10,** 23 (1971).

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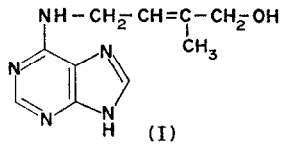
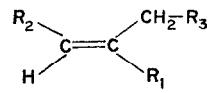


(I)



(II)

	R_1	R_2	R_3
(IIa)	$-CH_2OH$	$-CH_2NH_2$	H
(IIb)	$-COOCH_3$	$-CH_2Br$	H
(IIc)	$-COOCH_3$	$-CH_2N_3$	H
(IId)	$-COOH$	$-CH_2Br$	H
(IIe)	$-COOCH_3$	$-CH_3$	Br

RESULTS AND DISCUSSION

Methyl tiglate was brominated with *N*-bromosuccinimide using incandescent light as free radical initiator under conditions almost identical to those used by Shaw *et al.*[3] in their synthesis of zeatin, Inhoffen *et al.*,[6] and Ratney and English.[7] Gas chromatography of the product, which the above investigators regarded as methyl γ-bromotiglate, revealed the presence of two major components. The NMR spectrum indicated that these were methyl γ-bromotiglate (IIb) and methyl 2-bromomethyl-but-2-enoate (IIe) in the ratio of 3:2 respectively. Repetition of the bromination using azobisisobutyronitrile as free radical initiator yielded a similar mixture of the two bromides.

Since a satisfactory separation of these isomeric bromides was not achieved, a different synthetic procedure which would yield pure methyl γ-bromotiglate was sought. *t*-Butyl tiglate was reacted with *N*-bromosuccinimide under conditions similar to those employed above with methyl tiglate. It was hoped that the *t*-butyl group would sterically hinder bromination of the adjacent methyl group; this did not occur to a significant degree and bromination yielded a mixture of the two isomeric allylic bromides. However, it was found that reaction of free tiglic acid with *N*-bromosuccinimide and crystallization of the crude product readily yielded pure γ-bromotiglic acid (IId). Esterification with diazomethane gave pure methyl γ-bromotiglate. Reaction of this with sodium azide under the conditions used by Shaw *et al.*[3] for preparing 'methyl γ-azidotiglate' from 'methyl γ-bromotiglate' yielded surprisingly a mixture of the *cis* and *trans* azido esters (methyl γ-azidoangelate and methyl γ-azidotiglate (IIc) respectively) which were separated by preparative TLC. Reduction of the *trans* isomer yielded pure 4-amino-2-methylbut-*trans*-2-en-1-ol which was condensed with 6-chloropurine-8-^{14}C and 6-chloropurine-2,8-^{3}H to yield zeatin-8-^{14}C and zeatin-2,8-^{3}H respectively. Since the proton at C_8 of the purine ring is very readily exchangeable in the presence of base at elevated temperature,[8] the latter condensation was conducted in dry 1,2-dimethoxyethane, an aprotic solvent.

For preparation of zeatin-2,8-^{3}H, 6-chloropurine-2,8-^{3}H was required. An attempt to prepare this by irradiation of 6-chloropurine with tritium gas (Wilzbach method) was not successful. Autoradiography of thin-layer chromatograms indicated that very little of the radioactivity in the crude product was attributable to labelled 6-chloropurine. Numerous degradation products, some apparently of high specific activity, appeared to have been formed. In a further attempt to prepare 6-chloropurine-2,8-^{3}H, 6-chloropurine and a platinum catalyst were stirred with tritiated water under reflux. Surprisingly the main product was tritiated hypoxanthine which was purified by preparative TLC. This was then reacted with phosphoryl chloride to yield 6-chloropurine-2,8-^{3}H (443 mc/mM).

To prepare generally tritiated zeatin, the hormone and a platinum catalyst were stirred with tritiated water under reflux. Purification of the crude product yielded pure tritiated

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zeatin (231 mc/mM). GLC[9] is the only known method for separating zeatin from dihydrozeatin. This technique established that dihydrozeatin was not formed to a significant extent during labelling. Oxidative degradation of the labelled zeatin yielded adenine (210 mc/mM) indicating that approximately 91 per cent of the label was at positions 2 and 8 of the purine ring. 6-(Substituted amino)purines can be readily converted into ribosides enzymically.[10] By use of this reaction, zeatin riboside labelled in the purine moiety could be conveniently synthesized from the tritiated zeatin prepared as described in the present paper.

Reduction of 6-(3-formylbut-*trans*-2-enylamino)purine with tritiated sodium borohydride would be expected to yield zeatin specifically labelled in the side chain. Attempts to prepare this aldehyde by oxidation of zeatin with each of the following reagents were unsuccessful: activated manganese dioxide, Jones reagent, chromium trioxide–pyridine complex, dimethylsulphoxide (in the presence of dicyclohexylcarbodiimide and pyridinium trifluoroacetate), and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. It is possible that the aldehyde formed in these reactions underwent cyclization. Chromatography of the reaction mixture obtained by oxidation of zeatin with activated manganese dioxide yielded a compound formed by cyclization. The IR spectrum indicated the absence of an aldehyde group; non-reduction by sodium borohydride confirmed this. The UV spectrum was divergent from the spectra of 6-(substituted amino)purines,[1] 1,N_6-disubstituted adenines,[11,12] 7,7-dimethyl-7,8,9-trihydropyrimido[2,1-*i*]purine[13] and 8-ethyl-7,8-dihydroimidazo[2,1-*i*]purine.[14] The two last mentioned compounds were obtained by cyclization of the substituent of 6-(substituted amino)purines to position 1. The absence of peaks at *m/e* 108, 135, 148 and 149 in the mass spectrum confirmed that the oxidation product was not a 6-(substituted amino)-purine.[15] Mass spectrometry indicated that the compound was 6-(3-methylpyrrol-l-yl)-purine.

The synthesis of zeatin devised by Shaw *et al.*[3] has been regarded as unequivocal. Since the starting 'compound' has now been shown to be a mixture of two allylic bromides which are structural isomers, and since geometrical isomerization accompanies conversion of the desired bromide into the corresponding azide, the synthesis of Shaw *et al.*[3] can no longer be regarded as unequivocal. However this does not cast doubt on the structure of zeatin which has been confirmed by other syntheses[4,16] known to be unambiguous.

EXPERIMENTAL

Bromination of methyl tiglate. A suspension of *N*-bromosuccinimide (15·5 g) in a mixture of CCl_4 (18 ml) and methyl tiglate (10 g) was heated under reflux with an incandescent lamp (250 W) for 2·5 hr. Succinimide was removed by filtration and the filtrate diluted to 120 ml with CCl_4 before being cooled to 0°. The precipitate which formed was filtered off. The residue obtained by evaporation of the CCl_4 under reduced pressure was distilled rapidly to yield a liquid (9·7 g; b.p. 99–108°/17–20 mm) which was fractionally distilled. An ether solution of the fraction (7·3 g) with b.p. 100–102°/20 mm was extracted first with dilute NaOH and then with saturated NaCl solution. This extraction removed an impurity which gave a peak at 1760 cm^{-1} in the IR spectrum. Ether was evaporated from the dried ($MgSO_4$) solution and the residue distilled to yield a colourless liquid, a mixture of methyl γ-bromotiglate (IIb) and methyl 2-bromomethylbut-2-enoate (IIe) (3:2 from integral of NMR spectrum and GLC), b.p. 100–102°/20 mm; ν_{max}^{film} 1720, 1647, 1277 cm^{-1}. NMR ($CDCl_3$): δ 1·94 (doublet, J = 7 cycles/sec, $C\underline{H}_3$—CH=C), 1·97 (doublet broadened by homoallylic coupling, J = 1·25 cycles/sec, CH=C($C\underline{H}_3$)—$COOCH_3$), 3·76 (singlet,—$COOC\underline{H}_3$), 3·79 (singlet,—$COOC\underline{H}_3$), 4·05 (doublet,

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J = 8·5 cycles/sec, Br—C$\underline{H}_2$—CH=C), 4·25 (singlet, CH=C(COOC$\underline{H}_3$)—C$\underline{H}_2$—Br), 7·00 (broad multiplet, —C$\underline{H}$=C of both isomers) ppm.

A solution of methyl tiglate (51 g) in CCl_4 (300 ml) was heated under reflux with *N*-bromosuccinimide (70 g) and azobisisobutyronitrile (250 mg) for 2 hr in N_2. The reaction mixture was then cooled to − 10° and the succinimide filtered off. The oil obtained by evaporation of the solvent was vacuum distilled rapidly to yield a liquid (42 7 g), b.p. 100–111°/27 mm. GLC and NMR spectroscopy indicated that this was a mixture of methyl γ-bromotiglate and methyl 2-bromomethylbut-2-enoate (ratio 2:1 respectively). Attempts to separate these isomers on a preparative scale were not successful. Thus the slow take-off rate required to achieve a reasonable separation by fractional distillation with a spinning-band column resulted in extensive decomposition. Although a good separation of the two isomers was possible by analytical GLC (4% SE30 on H.P. Chromosorb W at 75°), the more severe conditions required for preparative work at a practical through-put caused excessive decomposition.

γ-Bromotiglic acid (*IId*). A solution of tiglic acid (100 g) in CCl_4 (500 ml) was refluxed under N_2 with *N*-bromosuccinimide (174 g). The reaction flask was irradiated with a 250-W incandescent lamp. When no *N*-bromosuccinimide remained (*ca.* 1 hr), the reaction mixture was kept at 0° for 12 hr, and then filtered to remove succinimide. Evaporation of the solvent yielded an oil which was kept at 0° overnight yielding a semicrystalline product. The crystals were filtered off and recrystallized from hexane–Et_2O and then hexane to give pure *γ-bromotiglic acid* (10·9 g), m.p. 93–94°; ν_{max}^{mull} 1680 (broad), 1640, 1292 cm^{-1} (Found: C, 33·7; H, 3 9; O, 18·3; Br, 44·8. $C_5H_7O_2Br$ required: C, 33 5; H, 3·9; O, 17·9; Br, 44·6%). NMR spectrum ($CDCl_3$): δ 1 91 (3H, doublet, J = 1·5 cycles/sec, CH= C—C$\underline{H}_3$), 4 03 (2H, doublet, J = 8 cycles/sec, Br—C$\underline{H}_2$—CH= C), 7·04 (1H; triplet further split by allylic coupling; J_{vic} = 8 cycles/sec, $J_{allylic}$ = 1·5 cycles/sec; —C$\underline{H}$= C) ppm. The homogeneity of this product was further established by GLC.

The oil obtained above as a filtrate was dissolved in petroleum–Et_2O. The resulting solution was concentrated and kept at 0° yielding a crystalline fraction which was decolourized with charcoal (in CH_2Cl_2) and recrystallized from petroleum to give a further 7 9 g of pure γ-bromotiglic acid.

Methyl γ-bromotiglate (*IIb*). An ether solution of CH_2N_2 was added in portions to a solution of γ-bromotiglic acid (18·8 g) in methanol (40 ml). To avoid an excess of CH_2N_2, the reaction was monitored by GLC. Evaporation of the solvent and fractional distillation of the residue yielded *methyl γ-bromotiglate* (14·9 g, homogeneous by GLC), b.p. 110–112°/24 mm; ν_{max}^{film} 1720, 1620, 1272 cm^{-1}. NMR spectrum ($CDCl_3$): δ 1·92 (3H, singlet broadened by allylic coupling, CH= C—C$\underline{H}_3$), 3 75 (3H, singlet, —COOC$\underline{H}_3$), 4 04 (2H, doublet, J = 8 cycles/sec, Br—C$\underline{H}_2$—CH= C), 6·92 (1H; triplet further split by allylic coupling; J_{vic} = 8 cycles/sec, $J_{allylic}$ = 2 cycles/sec; —C$\underline{H}$= C) ppm.

Methyl γ-azidotiglate (*IIc*) *and methyl γ-azidoangelate.* A mixture of methyl γ-bromotiglate (15·4 g), NaN_3 (25·8 g) and dry acetonitrile (63 ml) was heated under reflux in N_2 for 2 hr. Insoluble inorganic salt was filtered off and the filtrate evaporated yielding a dark yellow oil (11·8 g).

To separate the two components present, 1-g amounts of this crude azide were subjected to preparative TLC on silica gel (Merck PF_{254}; plate developed twice with benzene–petroleum ether 1:1, v/v). Elution yielded azide A (720 mg; component of lower R_f) and azide B (100 mg; component of higher R_f). Both azides were distilled at < 10^{-2} mm with a bath temp. of < 50°. Azide A (*methyl γ-azidotiglate*) exhibited ν_{max}^{film} 2100 (azide), 1720 (ester), 1657 cm^{-1} (trisubstituted double bond); NMR ($CDCl_3$): δ 1·93 (3H, partially resolved multiplet, CH = C—C$\underline{H}_3$), 3 75 (3H, singlet, —COOC$\underline{H}_3$), 3·96 (2H, doublet broadened by homoallylic coupling, J = 6·5 cycles/sec, N_3—C$\underline{H}_2$—CH= C), 6·73 (1H; triplet further split into quartets; J_{vic} = 6·5 cycles/sec, $J_{allylic}$ = 1·5 cycles/sec; —C$\underline{H}$= C) ppm. Azide B (*methyl γ-azidoangelate*) exhibited ν_{max}^{film} 2100, 1720, 1652 cm^{-1}; NMR ($CDCl_3$): δ 1·97 (3H, partially resolved multiplet, CH= C—C$\underline{H}_3$), 3·76 (3H, singlet, —COOC$\underline{H}_3$), 4·27 (2H, doublet broadened by homoallylic coupling, J = 6 cycles/sec, N_3—C$\underline{H}_2$—CH= C), 5 98 (1H; triplet further split into quartets, J_{vic} = 6 cycles/sec, $J_{allylic}$ = 1·5 cycles/sec; —C$\underline{H}$= C) ppm.

4-*Amino*-2-*methylbut*-trans-2-*en*-1-*ol* (*IIa*). A solution of methyl γ-azidotiglate (2 30 g) in dry ether (82 ml) was added slowly to a stirred suspension of $LiAlH_4$ (0·91 g) in dry ether (126 ml) under N_2. After the addition of all the azide, stirring was continued for 1 hr and then 10% aqueous NH_4Cl (7 ml) was added slowly. The inorganic salts were filtered off and the ether was evaporated from the dried (Na_2SO_4) filtrate The residue was dissolved in CH_2Cl_2; the solution was dried (Na_2SO_4) and evaporated to yield 4-amino-2-methylbut-*trans*-2-en-1-ol (0 73 g, 49%). TLC revealed only one ninhydrin-reacting component which co-chromatographed with authentic amine.[4] Further purification of the amine prepared above is unnecessary and tends to cause decomposition.

6-*Chloropurine*-2,8-^3H. 6-Chloropurine (0 40 g) and a Pt catalyst (200 mg) were stirred with tritiated water (3 ml, > 100 c) under reflux for about 20 hr. The catalyst, tritiated water and labile tritium were then removed. The above procedures were carried out at the Radiochemical Centre (Amersham, England). TLC indicated that the crude product was mainly tritiated hypoxanthine and contained only traces of 6-chloropurine. The crude product was subject to preparative TLC on silica gel (Merck PF_{254}) with water-saturated ethyl methyl ketone Each plate was developed twice and the desired zone then eluted with methanol–water–conc. NH_4OH (20 20:1, by vol.). The residue obtained by evaporation of the eluate was

extracted with 2 N NH_4OH and insoluble material discarded. To completely free the hypoxanthine from an impurity, the above chromatography and extraction were repeated. Crystallization from water yielded hypoxanthine-2,8-^{3}H (80 mg: 470 mc/mM); λ_{max} 249·5 nm (pH 6), 258·5 nm (pH 11).

The foregoing hypoxathine (20 mg) was refluxed for 5 hr with phosphoryl chloride (0·92 ml) in the presence of *N,N*-dimethylaniline (0·08 ml). Evaporation of the reaction mixture at 40° yielded a syrup to which water (4 ml) at 0° was added. The resulting solution was diluted to 10 ml, adjusted to pH 1·5 and extracted with ethyl acetate (5 times with 20 ml). The extracted fraction was subjected to preparative TLC (Merck silica gel PF_{254}) using water-saturated ethyl methyl ketone. The zone of 6-chloropurine was eluted with methanol and the evaporated eluate rechromatographed on a silica gel preparative layer with water-saturated *n*-butanol. Elution yielded chromatographically homogeneous 6-chloropurine-2,8-^{3}H (11 mg; 443 mc/mM); λ_{max} 266 nm (pH 6), 274 nm (pH 12).

Zeatin-2,8-^{3}*H* (*I*). A mixture of the foregoing 6-chloropurine (5·0 mg), chromatographically pure 4-amino-2-methylbut-*trans*-2-en-1-ol (9·9 mg), NEt_3 (10 μl), and 1,2-dimethoxyethane (0·3 ml; dried by standing over CaH_2 and then by distillation from $LiAlH_4$) was heated under reflux under anhydrous conditions for 5 hr. The resulting solution was applied to two layers (20 × 20 × 0·25 mm) of Merck silica gel PF_{254}. The chromatograms were developed with water-saturated ethyl methyl ketone and the UV-absorbing zones (R_f 0·35) eluted with methanol to yield chromatographically homogeneous zeatin-2,8-^{3}H (3·5 mg; 407 mc/mM); λ_{max}^{EtOH} 269 nm.

Zeatin-8-^{14}C (*I*). A mixture of 6-chloropurine-8-^{14}C (1 0 mg; 3·6 mc/mM; source Calbiochem), 6-chloropurine (1·0 mg), chromatographically pure 4-amino-2-methylbut-*trans*-2-en-1-ol (4·0 mg), NEt_3 (5 μl), and isopropanol (0·20 ml) was heated under reflux for 5 hr. The product was purified by the method used for zeatin-2,8-^{3}H yielding zeatin-8-^{14}C (1·3 mg; 1·8 mc/mM); λ_{max}^{EtOH} 269 nm.

Zeatin-^{3}H (*G*). Zeatin (150 mg) and a Pt catalyst (200 mg) were stirred with tritiated water (3 ml, > 100 c) under reflux for about 20 hr. Tritiated water, labile tritium and the Pt catalyst were then removed. The catalyst was extracted with hot water followed by boiling methanol. The resulting solutions were combined and evaporated; the residue (158 mc) was dissolved in ethanol (25 ml). The above procedure was carried out at the Radiochemical Centre (Amersham). Autoradiography of TLC indicated that the crude product (110 mg) contained at least 10 compounds in addition to zeatin. The product was chromatographed on four layers (20 × 20 × 2 mm) of Merck silica gel PF_{254} using water-saturated ethyl methyl ketone. The zeatin zone was eluted with methanol and the eluate rechromatographed on three of the above layers in EtOAc–HOAc–H_2O (10:3:4, by vol.) as solvent. The zeatin zone was again eluted with methanol which was then evaporated. The residue was extracted with 0·5 N acetic acid (20 ml) and the insoluble material discarded. Evaporation of the acetic acid yielded a residue which TLC (silica gel, 8 solvents) indicated was pure tritiated zeatin. This was crystallized from water to yield zeatin-^{3}H (G) (35 mg; 231 mc/mM). $\lambda_{max}^{H_2O}$: pH 1, 273·5 nm (ϵ = 17,010); pH 13, 275 nm (ϵ = 17,220) with shoulder 283 nm. λ_{max}^{EtOH} 269 nm (ϵ = 17,140).

The purity of this product was further established by the following observations: (1) Ion-exchange TLC on cellulose phosphate (H^+ form) revealed only one component; (2) recrystallization from two other solvents did not change the specific activity; (3) GLC of the trimethylsilyl derivative indicated homogeneity and the absence of dihydrozeatin. The trimethylsilyl derivative was prepared by reacting *N,O*-bis(trimethylsilyl)acetamide (43 μl) with zeatin-^{3}H (43 μg) in dry pyridine (22 μl) at 100° for 30 min. A method reported for the preparation of trimethylsilyl derivatives of cytokinins[9] was found unsatisfactory.

Zeatin-^{3}H (G) (0·3 mg) was oxidised to adenine with $KMnO_4$ under conditions similar to those previously employed.[1] The reaction mixture was chromatographed on paper (solvent water-saturated ethyl methyl ketone) and the adenine spot eluted. UV spectroscopy (to determine concentration) and liquid scintillation counting established that the specific activity of the adenine was 210 mc/mM. The eluate was subjected to TLC on silica gel (3 solvents) and on cellulose phosphate (1 solvent); the specific activity of the adenine was not changed significantly by any of these procedures.

Oxidation of zeatin with manganese dioxide. Zeatin (50 mg), activated MnO_2[17] (0·50 g) and dry dimethylformamide (5 ml) were stirred at 90° for 1·25 hr. TLC on silica gel using water-saturated ethyl methyl ketone revealed two minor components, adenine and zeatin, and a major component, a compound of higher R_f. Preparative TLC yielded 10 mg of this compound in crude form. Recrystallization from $CHCl_3$ gave colourless crystals of 6-(3-*methylpyrrol*-1-*yl*)*purine* which sublimed on heating at about 250° (Found by MS: mol. wt. 199 0852; $C_{10}H_9N_5$ required 199·0858); ν_{max}^{mull} 1592 cm^{-1}. $\lambda_{max}^{H_2O}$ 300 nm (pH 1), 294 nm (pH 7), 220 and 300 nm with shoulder at 310 nm (pH 13). λ_{max}^{EtOH} 301 nm. The mass spectrum showed only three prominent peaks—*m/e* 199 (parent and base peak), 198 and 80. Weak peaks (less than 10 per cent of base) occurred at *m/e* 171, 120, 119, 99, 93 and 92. Metastable ion peaks were observed for the transitions 199 → 198 and 199 → 80. The peaks at *m/e* 80 and 119 were ascribed to the 3-methylpyrrolyl ion (found *m/e* 80·0501; C_5H_6N required 80 0500) and the purinyl ion respectively.

When the oxidation with activated MnO_2 was conducted at room temp., only zeatin was recovered.

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