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N^6-ALKYLADENOSINES AND ADENINES LABELLED WITH TRITIUM

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SUMMARY

Catalytic dehalogenation of 2-chloroadenosine with tritium gas led to tritium labelled adenosine which was subsequently alkylated with (*E*)-4-*t*-butoxy-3-methylbut-2-enyl bromide or with 3-methylbut-2-enyl bromide to yield labelled (*E*)-N^6-(4-*t*-butoxy-3--methylbut-2-enyl)adenosine (**2a**) and N^6-(3-methylbut-2-enyl)adenosine (**2b**). Acidic hydrolysis of **2b** gave N^6-(3-methylbut-2-enyl)adenine while acidolysis of **2a** with trifluoroacetic acid led to a mixture of (*E*)-zeatin riboside and (*E*)-zeatin. Hydrogenation of (*Z/E*)-zeatin riboside ($^3H_2$, $PdO/BaSO_4$) afforded labelled dihydrozeatin riboside and, after hydrolysis, dihydrozeatin.

Key words: Tritium labelling; adenosine; (*E*)-zeatin riboside and (*E*)-zeatin; N^6-isopentenyladenosine.

INTRODUCTION

Alkylation of adenosine with alkyl bromides has been occasionally used for the synthesis of N^6-alkyladenosines [1-3]. The method is also applicable to the preparation of labelled N^6-(3-methylbut-2-enyl)adenosine (**2b**) and (*E*)-zeatin riboside (**2c**). Both compounds belong to the cytokinin group of plant hormones. We found that the alkylation yield with 3-methylbut-2-enyl bromide (**1b**) depends strongly on the chemical and radiochemical purity of the starting [^{3}H]adenosine.

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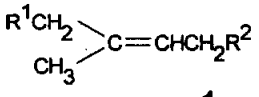
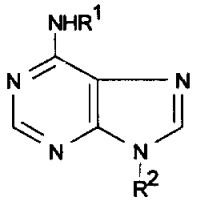
RESULTS AND DISSCUSION

In the preparation of labelled adenosine we followed the published procedure based on catalytic dehalogenation of 2-chloroadenosine with $Pd/BaSO_4$ in a strongly alkaline solution [4]. In the course of the dehalogenation considerable exchange into position 8 (purine ring) occurred as was shown by ^3H NMR [5] and the specific radioactivity of adenosine reached 2 TBq/mmol when carrier-free tritium gas was used. A reverse exchange ($Pd/BaSO_4$ in acetic acid) was performed to remove most of the less stable label from position 8. The adenosine was then desalted, freeze-dried and stored in dry dimethylformamide.



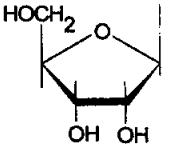
1

1a: R^1 = *t*-butoxy; R^2 = Br (*E*)
1b: R^1 = H; R^2 = Br
1c: R^1 = ThpO; R^2 = Br
1d: R^1 = Br; R^2 = *t*-butoxy (*E/Z*)



2

2a: R^1 = (*E*)-4-*t*-butoxy-3-methylbut-2-enyl
2b: R^1 = 3-methylbut-2-enyl
2c: R^1 = (*E*)-4-hydroxy-3-methylbut-2-enyl
2d: R^1 = 4-hydroxy-3-methylbutyl
2e: R^1 = (*E*)-4-hydroxy-3-methylbut-2-enyl
2f: R^1 = 3-methylbut-2-enyl
2g: R^1 = 4-hydroxy-3-methylbutyl

2a-d: R^2 = 

2e-g: R^2 = H

This procedure yielded 95-97 % radiochemically pure adenosine. The specific radioactivity of the product (1.05–1.1 TBq/mmol) indicated that almost all the radioactivity from position 8 was removed and adenosine was selectively labelled in position 2 (position 2 is stable to reverse exchange on Pd catalyst).

A special alkylating agent (*E*)-3-methyl-4-(tetrahydropyran-2-yloxy)but-2-enyl bromide (**1c**) was used as a synthon in the preparation of labelled (*E*)-zeatin [6]. The tedious synthesis and low chemical stability of **1c** are the principal disadvantages of this approach. We tried to prepare another alkylating agent, (*E*)-4-*t*-butoxy-3-methylbut-2-enyl bromide, by modification of the method published for the preparation of (*E/Z*)-4-*t*-butoxy-3-methylbut-2-enyl chloride [7].

1,4-Addition of *t*-butyl hypobromite on isoprene gave a mixture of bromides (GC-MS, see below) that showed chromatographic behaviour (TLC, HPLC) similar to the mixture of chlorides prepared analogously using *t*-butylhypochlorite [7]. After purification on silica gel column, the bromides having a similar retention time were collected as a single fraction, evaporated *in vacuo* and stored at –30 °C (anhydrous conditions). This mixture of crude unsaturated bromides was stable for years without substantial decomposition. HPLC of the mixture and GC-MS analysis of the HPLC peaks were performed to characterise these bromides. The analytical results were compared with those obtained for (*E*)-4-*t*-butoxy-3-methylbut-2-enyl chloride and (*E*)-4-*t*-butoxy-2-methylbut-2-enyl chloride, respectively [8]. The largest HPLC peak in the mixture of bromides was isolated by preparative HPLC and transformed into its N-phthaloyl derivative by reaction with potassium phthalimide. The IR spectrum of the N-phthaloyl derivative was identical with that of (*E*)-N-(4-*t*-butoxy-3-methylbut-2-enyl)phthalimide, which was prepared as described [11] from 4-*t*-butoxy-3-methylbut-2-enyl chloride [8]. The other bromides, separated as HPLC peaks, represented probably the *Z*-isomer of **1a** and *E* or/and *Z* isomers of **1d**. Just before alkylation, the mixture of bromides was separated by preparative HPLC and pure **1a** was used for alkylation.

Alkylation of [2-^{3}H]adenosine with excess of bromides **1a**, **1b** was accomplished in dry dimethylformamide under optimised reaction conditions. Dimroth rearrangement of the crude alkylation mixture yielded N^6-alkyladenosines **2a** and **2b**, respectively. The radiochemical yield varied from 35 to 45 %. Purification by reversed phase HPLC gave (*E*)-N^6-(4-*t*-butoxy-3-methylbut-2-enyl)adenosine (**2a**) with ca. 80 % radiochemical purity, while N^6-(3-methylbut-2-enyl)adenosine (**2b**) was pure.

Acidolysis of the *t*-butyl group of **2a** was accompanied by partial cleavage of the 9-ribosyl bond. The procedure gave a mixture of (*E*)-zeatin (**2e**) and (*E*)-zeatin riboside (**2c**) (total yield 85 %) and these two compounds were separated and purified by RP HPLC. Hydrolysis of N^6- (3-methylbut-2-enyl)adenosine (**2b**) yielded the appropriate N^6-alkyladenine **2f**.

The hydrogenation of zeatin is currently used for the preparation of dihydrozeatin [9,10]. $Pd/BaSO_4$ was not sufficiently active for the hydrogenation of zeatin or zeatin riboside, while with $PdO/BaSO_4$ the hydrogenation of the two compounds proceeded in alkaline solutions. Such conditions suppress the exchange of tritium into the solvent and diminish the hydrogenolysis of the 4-hydroxy group. After hydrogenation of (*E/Z*)- or (*E*)-zeatin riboside ca. 80 % of the radioactivity was found associated with the peak of dihydrozeatin riboside (**2d**) by radio-HPLC. As $Pd/BaSO_4$ is the catalyst of choice currently used for the catalytic exchange reaction of tritium gas to position 8 of the purine ring, we concluded that the product of hydrogenation **2d** is labelled both in the N^6-side chain and in the purine ring. When tritium gas with 70 % of carrier-free activity was used for hydrogenation, the specific radioactivity found for **2d** was 1.8 TBq/mmol. Hydrolysis of **2d** yielded [^{3}H]dihydrozeatin (**2g**) almost quantitatively.

EXPERIMENTAL

The separation of labelled cytokinins was performed by reversed phase HPLC with a flow rate of 0.4 mL/min. UV (270 nm) detector and radio flow detector (non-commercial product) were used simultaneously. Column: RP Select B, Superspher (Merck), 4 x 250 mm, MeOH - 40 mM buffer $AcOH/NH_4OH$, pH 3.4. Mobile phases: **A** = 50 : 50 (15 min), 60 : 40 (10 min); **B** = 50 : 50 (10 min), linear gradient to 80 : 20 (10 min), 80 : 20 (10 min); **C** = 30 : 70 ; **D** = 55 : 45. **E** = 30 : 70 (15 min), 50 : 50 (20 min). Column: Superspher 100, RP 18e (Merck), 4 x 250 mm. Mobile phase as above: **F** = 30 : 70.

Silica gel plates 60 F_{254} (20 cm, Merck) were used for TLC. Mobile phase: **G** = EtOAc- n-PrOH - conc. NH_4OH (5 : 5 : 1); **H** = $CHCl_3$ (satd. with NH_3)-MeOH- conc. NH_4OH (8 : 2 : 0.2); **I** = n-heptane - EtOAc (9 : 1).

GC-MS analysis was carried out on a Varian 3400 spectrometer combined with ion trap detector Finnigan ITD 800 equipped with fused silica capillary BP-5 (25 m x 0.25 mm, df 0.25 μm, J&W) using carrier gas flow rate 1.0 mL/min, split ratio 1 : 50, inlet temperature 150 °C and temperature program 2 min at 80 °C, then 4 °C/min to 150 °C and held for 10 minutes. EI mass spectra were scanned from m/z 40 to 260.

[2-^{3}H]Adenosine.

An amount of 3 mg (9.7 μmol) of 2-chloroadenosine (Aldrich) was stirred with 50 μL of 2 N KOH until a clear solution was obtained. 250 μL of water and 15 mg of $Pd/BaSO_4$ (10 %) were added and the mixture was hydrogenated with tritium gas (80% of carrier-free activity, 720 Torr) for 1.5 hours. The water was then lyophilised off, the rest dissolved in 200 μL water and 100 μL acetic acid and stirred under an atmosphere of hydrogen for 2 hours. The mixture was freeze-dried and dissolved in water. After centrifugation of the catalyst the supernatant was applied on a small column (0,3 x 2 cm) of Dowex 50 (H^+), the column was washed with water and tritiated adenosine eluted with 3% NH_4OH (inert atmosphere was used during the collecting of adenosine to diminish the formation of ammonium carbonate). 12.95 GBq (350 mCi) of [2-^{3}H]adenosine was prepared with a specific activity of 0.9-1.0 TBq/mmol (UV 270 nm). The radiochemical purity (TLC, system **H**, $\mathbf{R_f}$ = 0.23) was 95 - 97 % (2,0-2,5% of [^{3}H]adenine was found as the principal radiochemical impurity).

Alkylation of adenosine with 3-methylbut-2-enyl bromide.

7.4 GBq (200 mCi, ca. 7.4 μmol) of [2-^{3}H]adenosine, dissolved in 0.6 mL of dry dimethylformamide, was stirred with a molecular sieve 3A (2 beads, 3 mm) for 2 hours. 18.5 μmol (2.15 μL) distilled 3-methylbut-2-enyl bromide (**1b**) was added and the mixture was stirred for 1 day at ambient temperature. 7.4 μmol (1.25 μL) of N-ethyldiisopropylamine and 14.8 μmol (1.7 μL) of **1b** were then added and the stirring continued for 1 day. The progress of the alkylation was monitored by radio-TLC (system G); (10 μL of the reaction mixture was taken off, mixed with 100 μL of dimethylamine solution and heated (see below)). The reaction mixture was then

treated with dimethylamine solution (30 % solution of dimethylamine in MeOH/H_2O, 1 : 1 v/v) for 1 day at ambient temperature (or 4 hours at 50° C) to effect the Dimroth rearrangement. The molecular sieve was removed and the solution freeze-dried. The residue was dissolved in 200 µL of MeOH, acidified with 10 µL of glacial acetic acid and purified by HPLC (system **A**, 11 runs). 2.63 GBq of N^6-(3-methylbut-2-enyl)-[2-^{3}H]adenosine (**2b**) was collected with a specific radioactivity of 0.95 TBq/mmol (as determined by UV spectroscopy). Radiochemical purity (TLC, system **G**, R_f = 0.45) was >98 %.

Hydrolysis of N^6-(3-methylbut-2-enyl)[2-^{3}H]adenosine (**2b**).

Dry **2b** (1.4 GBq) was dissolved in 0.2 mL of cold trifluoroacetic acid (100 %) and then diluted with 0.2 mL of water. The clear solution was left overnight, then freeze-dried and purified by HPLC, system D (5 runs). 0.63 GBq of N^6-(3-methylbut-2-enyl)[2-^{3}H]adenine (**2f**) and 0.26 GBq of starting **2b** were isolated. The radiochemical purity of **2f** was > 99 % (TLC, system **H**, R_f = 0.61). The specific activity was the same as for **2b**.

Preparation of (*E*)-4-*t*-butyl-3-methylbut-2-enylbromide (**1a**).

An amount of 65 g of *t*-butylhypobromite was added to the mixture of 58 mL of isoprene and 175 mL of t-butanol over the course of 4 hours (stirring, ambient temperature). The excess of solvents was removed *in vacuo* and the residue (ca. 33 g) was purified on a silica gel column; 10 g was applied on a 4 x 30 cm column (silica gel 60, Merck, 40-60 mesh) and eluted with n-hexane - dry EtOAc (95 : 5). A group of compounds with similar retention time and showing R_f 0.4-0.6 on TLC (system **I**, detection UV 254 nm and $KMnO_4$) was collected and evaporated to dryness *in vacuo* (anhydrous conditions). HPLC analysis of this fraction (silica gel, 5µm, n-hexane-dry EtOAc (99 : 0,5), UV 254 nm) revealed 5 dominant peaks. The peaks were separated and analysed by GC MS. HPLC peak 1 (the shortest retention time, R_t = 9 min) contained two compounds with GC retention times 692 and 870 sec. HPLC peaks 2 (R_t = 10,7 min), 3 (R_t = 14,3 min), and 4 (R_t = 15,3 min), (GC retention times 692, 767 and 772 sec, respectively) gave surprisingly the same EI mass spectrum:

MS[m/z(%)]: 149 (4), 147 (4), 141 (2), 123 (11), 85 (3), 57 (100), 41 (48). The mass spectrum was very similar to that of (*E*)-4-*t*-butoxy-3-methylbut-2-enyl chloride or (*E*)-4-*t*-butoxy-2-methylbut-2-enyl chloride, prepared as described [7]. No molecular peak was found even if chemical ionisation was used. The largest HPLC peak from the mixture of bromides (HPLC peak 2) proved to be (*E*)-4-*t*-butoxy-3-methylbut-2-enyl bromide (**1a**). The above mentioned mixture contains ca. 20 % of **1a**, which is well separated on HPLC from other bromides (peaks 1, 3 and 4). The bromide **1a** was separated by preparative HPLC [silica gel, 25 x 250 mm, 10 μm, n-hexane - dry EtOAc (99 : 0.5)]. After evaporation of the HPLC solvents, the colourless oil was dried in a desiccator for 3 hours (15 Torr); ca. 40 mg of **1a** was separated in one run. The pure bromide **1a** was dissolved in n-hexane and the solution was immediately used for alkylation of adenosine.

Alkylation of [2-^{3}H]adenosine with (*E*)-4-*t*-butoxy-3-methylbut-2-enyl bromide (**1a**).

Starting from 9.6 GBq (9.6 μmol) of [2-^{3}H]adenosine, the alkylation with **1a** was performed as described for 3-methylbut-2-enyl bromide. Purification by HPLC (system **B**, 15 runs) gave 2.7 GBq of crude (*E*)-N^6-(4-*t*-butoxyisopent-2-enyl)[2-^{3}H]-adenosine. The radiochemical purity (TLC, system **H**, $\mathbf{R_f}$ = 0.6) was 80-85 %. This product was used for subsequent acidolysis without further purification.

Acidolysis of (*E*)-N^6-(*4-t*-butoxy-3-methylbut-2-enyl)[2-^{3}H]adenosine (**2a**).

2.59 GBq of crude **2a** was dried by evaporation with abs. EtOH. The rest was treated with 1 mL of cold 100 % trifluoroacetic acid and the solution was kept in a refrigerator (17 hours, +5 °C). Trifluoroacetic acid was lyophilised off, the rest dissolved in 100 μL of MeOH and the products **2e** and **2c** were purified by HPLC (system **C**) in 5 runs. (*E*)-[2-^{3}H]zeatin (0.63 GBq) and (*E*)-[2-^{3}H]zeatin riboside (0.48 GBq) were separated with a specific activity of 1.0 TBq/mmol (as determined by UV spectroscopy). The radiochemical purity [TLC, system **G**, $\mathbf{R_f}$ (zeatin) = 0.48, $\mathbf{R_f}$ (zeatin riboside) = 0.38] was >98 % for both compounds. HPLC [Superspher 100 RP 18e, 4 x 250 mm (Merck), MeOH - 40 mM AcOH/NH_4OH, pH 3.4, (20 : 80),

linear gradient to 30 : 70 (15 min), then isocratic 30 : 70 (10 min)] proved that the (*E*)-zeatin/(*Z*)-zeatin ratio was >99 %.

Hydrogenation of zeatin riboside with tritium gas.

An amount of 1.5 mg of (*E*)- or (*E/Z*)-zeatin riboside was hydrogenated with tritium gas (70% of carrier-free activity, 700 Torr) in 0.3 mL water containing 50 μL of 1 N KOH and 8 mg $PdO/BaSO_4$ (10 %) for 0.5 hours. The reaction mixture was freeze-dried and the rest extracted with 4 x 0.2 mL of MeOH. The collected extracts were centrifuged and separated from the catalyst and the MeOH was evaporated. The rest was dissolved in 100 μL of MeOH, acidified with 10 μL of glacial acetic acid and purified by HPLC, system **E** (7 runs). 2.85 GBq of [^{3}H]dihydrozeatin riboside (**2d**) was isolated with a radiochemical purity > 98 % (TLC, system **H**, R_f = 0.21). The specific radioactivity was 1.8 TBq/mmol (UV 272 nm).

Hydrolysis of [^{3}H]dihydrozeatin riboside (**2d**) to [^{3}H]dihydrozeatin (**2g**).

1.48 GBq of **2d** was hydrolysed in 50 % trifluoroacetic acid (1 day, ambient temperature). The reaction mixture was freeze-dried, the rest dissolved in 100 μL of MeOH and made alkaline with 5 μL of conc. NH_4OH. Purification was done by HPLC, system **E** (5 runs). 0.9 GBq of [^{3}H]dihydrozeatin (**2g**) was separated with a radiochemical purity >98 % (TLC, system **H**, R_f = 0.42). The specific radioactivity was the same as for the starting riboside.

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5. ^{3}H NMR (266.8 MHz, MeOD): 8.26 s (1 H, C8); 8.36 s (1 H, C2). The authors are grateful to T. Elbert, Fac. of Science, Charles University, Prague, for measurement of ^{3}H NMR spectra during his fellowship in CNRS, France

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8. The mixture of chlorides prepared according to the published method [7] contained two principial components, viz. (*E*)-4-*t*-butoxy-3-methylbut-2-enyl chloride and (*E*)-4-*t*-butoxy-2-methylbut-2-enyl chloride. The mixture of the two chlorides was separated by distilllation (61,5 - 62,5 °C/ 1,7 Torr) as a single fraction and subsequently transformed into appropriate N-alkylphthalimides (potassium phthalimide in dimethylformamide, 100 °C, 2 hours). After evaporation of dimethylformamide (*E*)-N-(4-*t*-butoxy-3-methylbut-2-enyl)phthalimide crystallised from ethanol. Re-crystallisation from ethanol gave the pure product (mp 73 - 73,5 °C, lit. [11], mp 74 - 75 °C) .

^{1}H NMR(400 MHz, $CDCl_3$): 1.180 (9H, s, t-Bu), 1.849 (3H, dtt, J = 1.3, 0.7, 0.6 Hz, olef. Me), 3.768 (2H, dtq, J = 1.3, 1.0, 0.6 Hz, OCH_2), 4.322 (2H, dtq, J = 7.2, 1.0, 0.7 Hz, NCH_2), 5.575 (1H, ttq, J = 7.2, 1.0, 0.7 Hz, =CH), 7.696 & 7.829 (4H, AA'BB', phthalimide). ^{13}C NMR (100 MHz, $CDCl_3$): 14,12 q (Me), 27.50 q (3C, t-Bu), 35.43 t (NCH_2), 66.86 t (OCH_2), 73.14 s (CMe_3), 118.94 d (=CH), 123.10 d (2C), 136.56 s (2C), 168.01 s (2C, 2 x C=O).

Proton at 4.322, coupled to a carbon at 35.43 ppm, represents a NCH_2 group. According to J_{vic} = 7.2 Hz, it is vicinal to olefinic proton. The olefinic proton exhibits NOE to both OCH_2 and NCH_2, the olefinic methyl follows the same pattern. Therefore, =CH and methyl are trans- disposed. Unpublished results.

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