

N⁶-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*)-N⁶-(4-*t*-butoxy-3-methylbut-2-enyl)adenosine (**2a**) and N⁶-(3-methylbut-2-enyl)adenosine (**2b**). Acidic hydrolysis of **2b** gave N⁶-(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 (³H₂, PdO/BaSO₄) afforded labelled dihydrozeatin riboside and, after hydrolysis, dihydrozeatin.

Key words: Tritium labelling; adenosine; (*E*)-zeatin riboside and (*E*)-zeatin; N⁶-isopentenyladenosine.

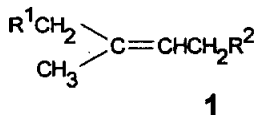
INTRODUCTION

Alkylation of adenosine with alkyl bromides has been occasionally used for the synthesis of N⁶-alkyladenosines [1-3]. The method is also applicable to the preparation of labelled N⁶-(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 [³H]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/BaSO₄ 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/BaSO₄ 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.

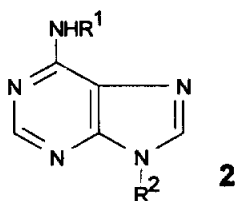


1a: R¹ = *t*-butoxy; R² = Br (*E*)

1b: R¹ = H; R² = Br

1c: R¹ = ThpO; R² = Br

1d: R¹ = Br; R² = *t*-butoxy (*E/Z*)



2a: R¹ = (*E*)-4-*t*-butoxy-3-methylbut-2-enyl

2b: R¹ = 3-methylbut-2-enyl

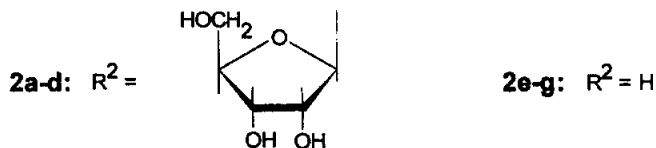
2c: R¹ = (*E*)-4-hydroxy-3-methylbut-2-enyl

2d: R¹ = 4-hydroxy-3-methylbutyl

2e: R¹ = (*E*)-4-hydroxy-3-methylbut-2-enyl

2f: R¹ = 3-methylbut-2-enyl

2g: R¹ = 4-hydroxy-3-methylbutyl



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 [2-³H]adenosine with excess of bromides **1a**, **1b** was accomplished in dry dimethylformamide under optimised reaction conditions. Dimroth rearrangement of the crude alkylation mixture yielded N⁶-alkyladenosines **2a** and **2b**, respectively. The radiochemical yield varied from 35 to 45 %. Purification by reversed phase HPLC gave (*E*)-N⁶-(4-*t*-butoxy-3-methylbut-2-enyl)adenosine (**2a**) with ca. 80 % radiochemical purity, while N⁶-(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 N⁶- (3-methylbut-2-enyl)adenosine (**2b**) yielded the appropriate N⁶-alkyladenine **2f**.

The hydrogenation of zeatin is currently used for the preparation of dihydrozeatin [9,10]. Pd/BaSO₄ was not sufficiently active for the hydrogenation of zeatin or zeatin riboside, while with PdO/BaSO₄ 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/BaSO₄ 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 N⁶-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 [³H]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/NH₄OH, 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 F₂₅₄ (20 cm, Merck) were used for TLC. Mobile phase: **G** = EtOAc- *n*-PrOH - conc. NH₄OH (5 : 5 : 1); **H** = CHCl₃ (satd. with NH₃)-MeOH- conc. NH₄OH (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 μ m, 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.

[2-³H]Adenosine.

An amount of 3 mg (9.7 μ mol) of 2-chloroadenosine (Aldrich) was stirred with 50 μ L of 2 N KOH until a clear solution was obtained. 250 μ L of water and 15 mg of Pd/BaSO₄ (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 μ L water and 100 μ L 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% NH₄OH (inert atmosphere was used during the collecting of adenosine to diminish the formation of ammonium carbonate). 12.95 GBq (350 mCi) of [2-³H]adenosine was prepared with a specific activity of 0.9-1.0 TBq/mmol (UV 270 nm). The radiochemical purity (TLC, system H, R_f = 0.23) was 95 - 97 % (2,0-2,5% of [³H]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 μ mol) of [2-³H]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 μ mol (2.15 μ L) distilled 3-methylbut-2-enyl bromide (**1b**) was added and the mixture was stirred for 1 day at ambient temperature. 7.4 μ mol (1.25 μ L) of N-ethyl-diisopropylamine and 14.8 μ mol (1.7 μ L) 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 μ L of the reaction mixture was taken off, mixed with 100 μ L of dimethylamine solution and heated (see below)). The reaction mixture was then

treated with dimethylamine solution (30 % solution of dimethylamine in MeOH/H₂O, 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 N⁶-(3-methylbut-2-enyl)-[2-³H]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 N⁶-(3-methylbut-2-enyl)[2-³H]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 N⁶-(3-methylbut-2-enyl)[2-³H]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 KMnO₄) 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 μ m, 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 [2-³H]adenosine with (*E*)-4-*t*-butoxy-3-methylbut-2-enyl bromide (**1a**).

Starting from 9.6 GBq (9.6 μ mol) of [2-³H]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*)-N⁶-(4-*t*-butoxyisopent-2-enyl)[2-³H]-adenosine. The radiochemical purity (TLC, system H, R_f = 0.6) was 80-85 %. This product was used for subsequent acidolysis without further purification.

Acidolysis of (*E*)-N⁶-(4-*t*-butoxy-3-methylbut-2-enyl)[2-³H]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 μ L of MeOH and the products **2e** and **2c** were purified by HPLC (system C) in 5 runs. (*E*)-[2-³H]zeatin (0.63 GBq) and (*E*)-[2-³H]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, R_f (zeatin) = 0.48, 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/NH₄OH, 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 μ L of 1 N KOH and 8 mg PdO/BaSO₄ (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 μ L of MeOH, acidified with 10 μ L of glacial acetic acid and purified by HPLC, system E (7 runs). 2.85 GBq of [³H]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 [³H]dihydrozeatin riboside (**2d**) to [³H]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 μ L of MeOH and made alkaline with 5 μ L of conc. NH₄OH. Purification was done by HPLC, system E (5 runs). 0.9 GBq of [³H]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. ³H 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 ³H 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 principal 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 distillation (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).
¹H NMR(400 MHz, CDCl₃): 1.180 (9H, s, *t*-Bu), 1.849 (3H, dt, J = 1.3, 0.7, 0.6 Hz, olef. Me), 3.768 (2H, dtq, J = 1.3, 1.0, 0.6 Hz, OCH₂), 4.322 (2H, dtq, J = 7.2, 1.0, 0.7 Hz, NCH₂), 5.575 (1H, ttq, J = 7.2, 1.0, 0.7 Hz, =CH), 7.696 & 7.829 (4H, AA'BB', phthalimide). ¹³C NMR (100 MHz, CDCl₃): 14,12 q (Me), 27.50 q (3C, *t*-Bu), 35.43 t (NCH₂), 66.86 t (OCH₂), 73.14 s (CMe₃), 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 NCH₂ group. According to J_{vic} = 7.2 Hz, it is vicinal to olefinic proton. The olefinic proton exhibits NOE to both OCH₂ and NCH₂, the olefinic methyl follows the same pattern. Therefore, =CH and methyl are trans- disposed. Unpublished results.
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