SYNTHESIS OF GUANOSINE-3′-(5-BROMO-4-CHLOROINDOL-3-YL)-PHOSPHATE (G-3′-BCIP)

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N-2-Benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranylguanosine (**5**) and 1-acetyl-5-bromo-4-chloroindol-3-yl-3-phosphorodichloridate (**7**) were synthesized and coupled to give the title compound **9**.
Key words: Identification of ribonuclease activity.

Chromogenic substrates are routinely used for spectroscopic analysis of enzyme activity[1,2]. The 5-bromo-4-chloroindol-3-yl group may be a particularly useful group in identifying ribonuclease activity. Specific ribonucleases should cleave the bond between the phosphate and the chromophore. The oxidation of the resulting 5-bromo-4-chloroindol-3-ol to a dimeric product results[3] in an important absorbance increase between 600 and 700 nm. For this reason a synthetic route to the title compound has been developed.

Synthesis of G-3′-BCIP was performed as illustrated in Scheme 1. *N*-2-Benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranylguanosine **5** was synthesized starting from *N*-2-benzoylguanosine[4] **1** by protecting the 3′,5′ hydroxyl groups using 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane[5] in the presence of anhydrous pyridine, to produce product **2** in good yield after workup and silica gel chromatography. Compound **2** was treated with 3,4-dihydro-2*H*-pyran (DHP) in anhydrous dioxane in the presence of *p*-toluenesulfonic acid[6] yielding a mixture of the tetrahydropyranyl isomers **3.** This mixture was used in the next step without purification. It was treated with potassium fluoride–tetraethylammonium bromide mixture to cleave the 3′,5′ silyl protecting group as described by Kamimura et al.[7]. After workup and silica gel chromatography compound **4** was obtained in a good yield. Treatment of product **4** with 4,4′-dimethoxytrityl chloride (DMTCl) in the presence of anhydrous pyridine[7] gave the product **5** after purification by silica gel chromatography. 3-Acetoxy-1-acetyl-5-bromo-4-chloroindole was selectively deacetylated at the 3-position[8] with 80% sulfuric acid to give **6** which was

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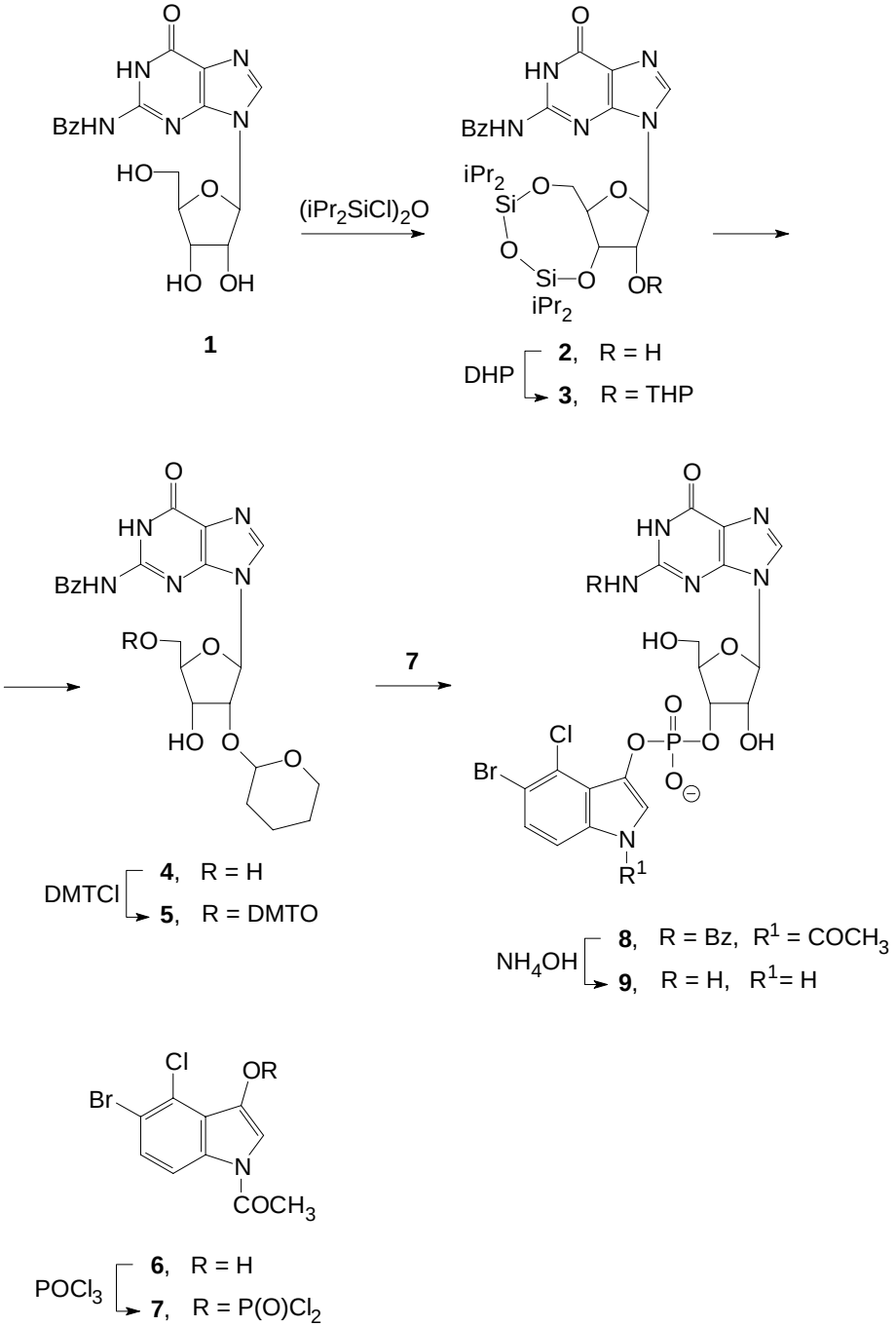
BzHN
HO
HO OH
1
(iPr2SiCl)2O
iPr2
Si
OR
iPr2
DHP
2, R = H
3, R = THP
RO
4, R = H
DMTCl
5, R = DMTO
7
RHN
Cl
Br
OH
R1
NH4OH
8, R = Bz, R1 = COCH3
9, R = H, R1= H
COCH3
6, R = H
POCl3
7, R = P(O)Cl2

SCHEME 1

phosphorylated as described[3] yielding compound **7**. Coupling of **7** with **5** in dry pyridine catalyzed by 1,2,4-triazole at room temperature was monitored by TLC and after 40 min quenched by triethylammonium hydrogencarbonate (TEAB). The crude product was subjected to silica gel chromatography but a partial decomposition of the trityl group during the column chromatography was observed, so all the fractions containing the required product were collected together, evaporated to dryness and treated with 80% acetic acid to affect detritylation and to cleave the tetrahydropyranyl group[9]. Finally the benzoyl and the acetyl groups were cleaved using concentrated ammonium hydroxide[9–11] to give compound **9**. All the synthesized products were characterized using ^{1}H and ^{31}P NMR spectra and mass spectra, and treatment with ribonuclease T1 for the final product.

EXPERIMENTAL

^{1}H NMR spectra were measured on Bruker AC (250 MHz) spectrometer, chemical shift values (δ, ppm; *J*, Hz) were reported relative to $CDCl_3$ (7.30), $(CD_3)_2SO$ (2.50) or D_2O (4.81) signals. Proton decoupled ^{31}P NMR spectra were measured on Bruker WP 80 (32.37 MHz). Chemical shifts are given in ppm downfield from 85% aqueous H_3PO_4 as an external standard. Fast Atom Bombardment (FAB) mass spectra were recorded on a Kratos instrument Model MS-890. TLC was performed on silica gel F254 (Merck). Detection of spots was effected by UV light, then charring with 10% sulfuric acid in 95% ethanol. Preparative HPLC was carried out using a VYDAC C_{18} column (1 × 25 cm). Pyridine was distilled from CaH_2 and stored over potassium hydroxide.

N-2-Benzoyl-3′,5′-di-*O*-(tetraisopropyldisiloxane-1,3-diyl)guanosine (**2**)

1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (2.71 g, 8.6 mmol) was added to a solution of *N*-2-benzoylguanosine (3 g, 7.75 mmol) in anhydrous pyridine (25 ml). The reaction mixture was stirred at room temperature overnight, then evaporated under reduced pressure. The residue was partitioned between chloroform (60 ml) and the same volume of saturated aqueous sodium hydrogencarbonate, the chloroform layer was washed with water (2 × 30 ml), dried over anhydrous sodium sulfate and finally purified over silica gel column chromatography using chloroform–methanol (97 : 3 v/v), giving the compound **2** (3.8 g, 78%), R_F = 0.54 (chloroform–methanol 9.5 : 0.5). Mass spectrum, *m/z*: 629. ^{1}H NMR spectrum: 8.06–7.51 m, 6 H (arom.); 5.91 s, 1 H (H-1′); 4.48 q, 1 H (H-3′); 4.33 dd, 1 H (H-2′); 4.03 m, 3 H (H-4′,H-5′,H-5″); 1.06 m, 28 H (TIPDS protons). For $C_{29}H_{43}N_5O_7Si_2$ (629.3) calculated: 55.30% C, 6.88% H, 11.12% N; found: 55.45% C, 6.82% H, 11.23% N.

N-2-Benzoyl-2′-*O*-tetrahydropyranylguanosine (**4**)

To a solution of compound **2** (1.5 g, 2.4 mmol) in dioxane (15 ml) containing *p*-toluenesulfonic acid monohydrate (0.12 g), 3,4-dihydro-2*H*-pyran (4.05 ml) was added and stirred for 6 h at room temperature. After neutralization with concentrated ammonium hydroxide, filtration of ammonium tosylate, and washing with dichloromethane, the washings and filtrate were evaporated under reduced pressure. To the residue (compound **3**) dissolved in acetonitrile (40 ml), KF (1.1 g, 18.7 mmol), Et_4NBr (3.9 g, 18.7 mmol) and H_2O (1 ml) were added and the mixture was stirred for 45 min at 50 °C and then concentrated. The successive extractive workup and silica gel chromatography gave **4** (1.02 g, 91%) as a mixture of two diastereoisomers. R_F 0.30 and 0.40 (chloroform–methanol 9 : 1). Mass spectrum, *m/z*: 471. ^{1}H NMR spectrum of the fraction with lower R_F: 8.2 s, 1 H (H-8); 7.98–7.47 m, 5 H

(arom.); 5.99 d, 1 H, $J(1',2')$ = 6.65 (H-1′); and the characteristic pattern of ribofuranose. For $C_{22}H_{25}N_5O_7$ (471.2) calculated: 56.05% C, 5.34% H, 14.85% N; found: 56.25% C, 5.42% H, 14.73% N.

N-2-Benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranylguanosine (**5**)

Compound **4** (0.8 g, 1.7 mmol) was co-evaporated with anhydrous pyridine (3 × 10 ml), dissolved in anhydrous pyridine (15 ml), to this solution 4,4′-dimethoxytrityl chloride (0.68 g, 2.03 mmol) was added and the reaction mixture was stirred at room temperature and monitored by TLC. When no starting material was present (after one hour), water (3 ml) was added followed by extraction with chloroform; the chloroform layer was dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on a silica gel column eluted with chloroform–methanol (9.7 : 0.3 v/v) giving compound **5** (1.1 g, 84%, two diastereoisomers) with R_F 0.43 and 0.33 (chloroform–methanol 9.5 : 0.5). Mass spectrum, *m/z*: 773. ^{1}H NMR spectrum for the isomer with lower R_F: 8.0 s, 1 H (H-8); 7.69–6.92 m, 18 H (arom.); 5.91 d, 1 H, $J(1',2')$ = 7.19 (H-1′); 3.60 s, 6 H (2 × OCH_3).

1-Acetyl-5-bromo-4-chloro-3-hydroxyindole (**6**)

Sulfuric acid (80%, 2.5 ml) was added dropwise into a beaker containing 3-acetoxy-1-acetyl-5-bromo-4-chloroindole (0.5 g, 1.5 mmol), then stirred at room temperature for one hour and poured into ice-water, stirring for 30 min, the solid was filtered, washed with a solution of 0.1 M sodium acetate until neutral and then with water and dried over P_2O_5 giving compound **6** (0.4 g, 92%). Mass spectrum, *m/z*: 288. ^{1}H NMR spectrum: 8.38 d, 1 H; 7.81 d, 1 H; 4.34 s, 2 H (keto-enolic protons); 2.31 s, 3 H.

1-Acetyl-5-bromo-4-chloroindol-3-yl-3-phosphorodichloridate (**7**)

This product was synthesized from 3-hydroxyindole **6** exactly as described before[3] giving the same physical data.

Guanosine 3′-*O*-(5-Bromo-4-chloroindol-3-yl)phosphate (G-3′-BCIP, **9**)

To a solution of compound **5** (0.35 g, 0.48 mmol) and 1,2,4-triazole (0.164 g, 2.4 mmol) in dry pyridine (3 ml), compound **7** (0.588 g, 1.43 mmol) was added and the reaction was stirred under argon for 40 min and quenched with 1 M TEAB (2 ml) and evaporated to dryness under reduced pressure. The residue was applied to a silica gel column eluted with chloroform–methanol–triethylamine (93 : 4 : 3). All the fractions containing the product (even partially detritylated) were collected together, evaporated and dissolved in 80% acetic acid (15 ml), stirred 1 h at room temperature, evaporated to dryness and co-evaporated with water (3 × 10 ml) to eliminate all traces of acetic acid, followed by dissolving the residue in concentrated ammonium hydroxide (15 ml). Then it was left at 3 °C for 15 h and evaporated. The product was purified by flash chromatography on silica gel column using mixture of butanol–water–methanol (75 : 15 : 10) and re-purified by preparative HPLC using a linear gradient of 1 M ammonium acetate (pH 6) (A) and acetonitrile (B) (from 100% of A to 100% of B in 25 min). The ammonium acetate which contaminated the product was removed by dissolving the product in water (3 ml) and applied to a column of Sephadex A-25 (20 ml), washed with water (60 ml) and the product eluted with TEAB (0.3 mol l^{-1}, 50 ml), evaporated under reduced pressure and co-evaporated with water (3 × 10 ml) to eliminate traces of TEAB. The residue was dissolved in water (3 ml) and lyophilized to give the product **9** (30 mg, 10%). ^{31}P NMR spectrum (D_2O): 1.6. ^{1}H NMR spectrum (D_2O): 7.97 s, 1 H (H-8); 7.38–7.23 m, 3 H (arom.); 5.76 d, 1 H, $J(1',2')$ = 3.32 (H-1′); 4.26 m, 1 H (H-3′); 4.08 t, 1 H (H-2′); 3.95 dd, 1 H (H-4′); 3.86 dd, 2 H (H-5′ and H-5″). The

triethylammonium salt of **9** was submitted to ion exchange chromatography using a column (15 ml) of Dowex 50W-X4 (Na^+ form, 200–400 mesh), using water (30 ml) as eluent followed by lyophilization of the water phase. Mass spectrum (high resolution, *m/z*): For $C_{18}H_{16}BrClN_6NaO_8P$ calculated: 611.9537; found: 611.9539. This product was further characterized by incubation with ribonuclease T1 for 3 h giving a blue pigments as expected.

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