U-3′-BCIP: a chromogenic substrate for the detection of RNase A in recombinant DNA expression systems

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ABSTRACT

The synthesis of the bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5) chromogenic substrate uridinine-3′-(5-bromo-4-chloroindol-3-yl)-phosphate (U-3′-BCIP) is described. RNase A catalyzes the hydrolysis of U-3′-BCIP to release a halogenated indol-3-ol that undergoes rapid aerobic oxidation to the dark blue 5,5′-dibromo-4,4′-dichloroindigo. Preliminary kinetic studies indicate that this compound may have practical use for assaying RNase A activity both *in vitro* and *in vivo*, e.g. in screening bacterial colonies for RNase A produced by recombinant DNA methods.

INTRODUCTION

Chromogenic substrates are routinely used for the histologic, cytologic and spectroscopic analysis of enzyme activity. Among the several commonly used chromophores, the 5-bromo-4-chloroindol-3-yl group is a particularly useful one for identifying enzyme activity. It is used in immunological studies as an indolyl-phosphate for the detection of antibodies in enzyme-linked antibody techniques when the antibody protein is covalently linked to alkaline phosphatase (1). Another common derivative is 5-bromo-4-chloroindol-3-yl-galactose (X-gal) which is used as a substrate for β-galactosidase (2). When X-gal is spread on an agar medium at an appropriate concentration, bacterial colonies expressing active β-galactosidase will cleave the bond between the sugar and the chromophore. Due to the oxidation of the resulting 5-bromo-4-chloroindol-3-ol to a dimeric product, the colonies turn blue (2). Colonies that are deficient in this enzymatic activity remain white.

Bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5) is an enzyme which has been of long-standing interest in our research group, and we are currently cloning it into *E.coli*. To facilitate the screening of bacterial colonies expressing wild-type and mutant pyridine-specific phosphodiesterase activity, we have developed a reliable synthetic route to uridine-3′-(5-bromo-4-chloroindol-3-yl)-phosphate (U-3′-BCIP, compound **1** of Figure 1) and tested it as a chromogenic substrate for purified RNase A. The use of U-3′-BCIP (as a mixture of 3′,5′ and 2′,5′ phosphodiesters) for the localization of RNase activity in tissues has been reported (3). However, it was not explicitly demonstrated that **1** is a substrate for RNase, nor was the synthesis described in any useful detail.

This paper describes the synthesis of U-3′-BCIP and its use as a chromogenic substrate for RNase A. U-3′-BCIP may be used to screen for bacterial-encoded enzyme production directly on agar plates.

RESULTS AND DISCUSSION

Synthesi oμ ·-3′-BCIP was achieved as outlined in Figure 2. The 5-bromo-4-chloroindol-3-yl-1,3-diacetate (**5**) was selectively deacetylated at the 3-position in 80% sulfuric acid. Phosphorylation of **6** was achieved with phosphorous oxychloride in toluene containing pyridine following the procedure of Horwitz and Freisler (4). The 5′-O-(4,4′-dimethoxytrityl)-2′-tert-butyldimethylsilyl-uridine (**8**) was then coupled to 5-bromo-4-chloro-1-acetylindol-3-yl-phosphorodichloridate (**7**) by using 1,2,4-triazole in pyridine (5). Following completion of the coupling step, the reaction was quenched with water, and then immediately acidified with acetic acid to cleave the 4,4′-dimethoxytrityl protecting group, yielding the phosphodiester **9**. We found that purification of the fully protected nucleotide by flash chromatography resulted in partial detritylation to form the triethylammonium salt of **9** even when triethylamine was added to the solvent system of chloroform/methanol. Since the purpose of the dimethoxytrityl group was to block phosphorylation of the 5′-hydroxyl group, we therefore chose to remove it during the workup *after* the coupling step. Removal of the acetyl group from the indolyl moiety, by using ammonium hydroxide in methanol, gave the penultimate intermediate **10**. The tert-butyldimethylsilyl group was then removed under mild conditions with tetra-n-butylammonium fluoride (6) to give the final product, U-3′-BCIP (**1**), obtained as the tetra-n-butylammonium salt. Attempts to remove the silyl protecting group under acidic conditions (e.g. HCl, HOAc or HF) resulted in decomposition of the phosphodiester. In order to obtain a high resolution FAB mass spectrum of U-3′-BCIP, the tetra-n-butylammonium counterion was exchanged for sodium by using an ion-exchange resin in the Na^+ form.

Figure 3 shows a composite time-dependent UV-visible absorption spectrum demonstrating the utility of this compound

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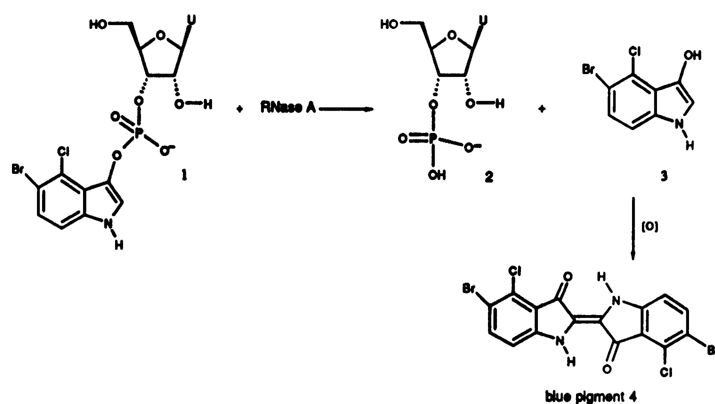

Figure 1. Proposed hydrolysis of U-3′-BCIP by RNase A. The hydrolysis product of RNase action, compound **3**, undergoes rapid aerobic oxidation to form compound **4**.

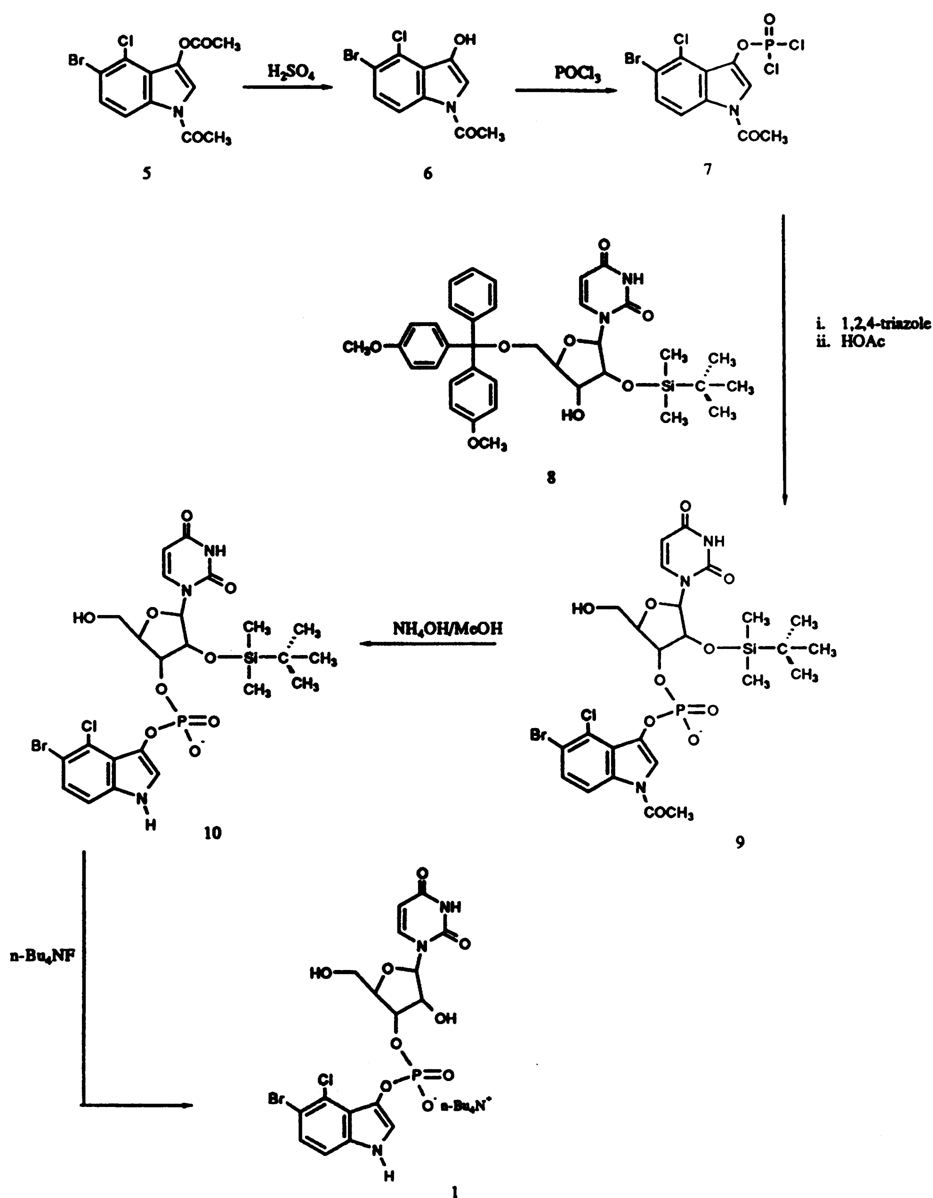

Figure 2. Synthetic scheme for the preparation of U-3′-BCIP.

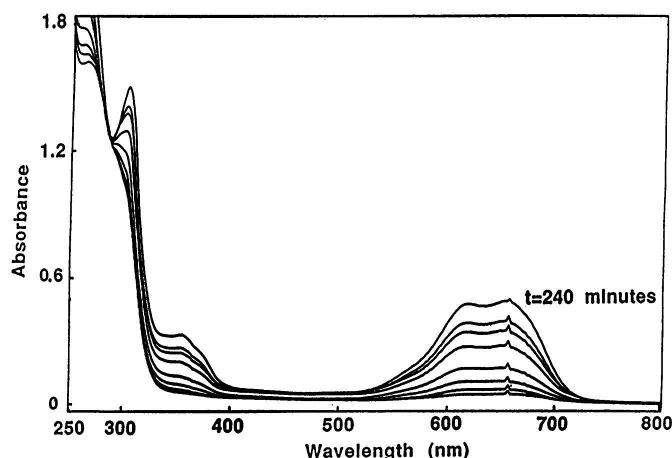

Figure 3. Composite time-dependent UV-Visible absorption spectrum obtained during the hydrolysis of U-3′-BCIP (150 mg) by RNase A (approx. 0.05 Kunitz units) in 50 mM Tris, pH 7.5, in 1.0 mL in a 1.0 cm cell. Scans were recorded over a period of 4 hours at room temperature. The lowest curve was obtained at t=0.

when tested with purified RNase A in 50 mM Tris buffer (pH 7.5). TLC analysis of the reaction mixture showed the complete disappearance of U-3′-BCIP and the appearance of a new blue compound as well as a colorless fluorescent one which co-migrated with authentic uridine-3′-phosphate (**2**), the expected product of enzymatic turnover. Control experiments, run in the same buffer in the absence of RNase A, showed <2% (detection limit) conversion to products after 26 hours at room temperature. Preliminary kinetic studies show that U-3′-BCIP is suitable for use in determinations of RNase A activity. The K_m and turnover number of U-3′-BCIP (approximately 7 mM and 8 s^{-1} respectively) are comparable with those of cCMP (0.54 mM and 1.4 s^{-1} respectively) (7). We are now testing this compound in an *in vivo* assay for identifying bacterial clones expressing active RNase A, and in ELISA-type analyses using RNase-linked antibodies.

EXPERIMENTAL

Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The ^{1}H NMR and ^{13}C NMR spectra were obtained by using a Varian XL-200 or a Varian XL-400 instrument. FAB mass spectra were obtained in 3-nitrobenzyl-alcohol as a matrix on a Kratos instrument Model MS-890. UV-visible absorption spectra were obtained on an HP-8451A Diode Array spectrophotometer. Analytical RP-HPLC was carried out on an LKB 2249 Gradient Pump coupled to an LKB 2140 Radial Spectral Detector. A Waters Nova-Pak C_{18} column (0.8×10 cm) was used in combination with a Waters RCM 100 Radial Compression Module. The optical rotation was measured on a Perkin-Elmer Model 241 polarimeter. The 5-bromo-4-chloro-indol-3-yl-1,3-diacetate (**5**) was purchased from Sigma Chemical Company, St Louis, MO. The 5′-O-(4,4′-dimethoxytrityl)-2′-tert-butyldimethylsilyl-uridine (**8**) was obtained from Peninsula Labs, Inc., Belmont, CA or synthesized following the procedure of Ogilvie *et al.* (8). Pyridine and toluene were distilled from CaH_2 under argon. Purified bovine pancreatic RNase A was a gift from D.M.Rothwarf.

1-acetyl-5-Bromo-4-chloro-3-indolol (6)

Sulfuric-acid (5 mL,80%) was added dropwise into a beaker containing 1-acetyl-5-bromo-4-chloroindoxyl acetate (**5**) (1.00 g, 3.0 mmol) while maintaining the temperature below 25°C. The solution was stirred at room temperature for 45 minutes and then poured onto ice and stirred until all of the ice had melted. The bluish-white solid was collected by filtration and washed with sodium acetate (0.1 M) until the washings were neutral, and then with water and ice cold ethanol. The product was then dried in vacuo over P_2O_5, yielding **6** (0.79 g, 91%): mp165−168°C (lit.[8] 177°C); ^{1}H NMR ($CDCl_3$, 200 MHz), δ 8.38 (1H,d), 7.78 (1H,d), 4.33 (2H, s, keto-enolic protons), 2.29 (3H, s).

5-Bromo-4-chloroindol-3-yl-1-acetyl-3-phosphorodichloridate (7)

$POCl_3$ (1.62 mL, 17.3 mmol, 2.5 eq) was added dropwise to a stirred suspension of **6** (2.00 g, 6.9 mmol) in freshly distilled toluene (35 ml) under argon. The resulting suspension was refluxed under argon, and pyridine (0.65 mL, 8.1 mmol, 1.2 eq) was added dropwise while refluxing over a period of 30 minutes. The solution was refluxed for an additional 15 minutes. The blue-green solution was then cooled to room temperature, filtered, and the filtrate was evaporated using a Kügelrohr distillation apparatus to give a greenish granular solid which was used without further purification: mp 107−108°C; ^{1}H NMR ($CDCl_3$, 200 MHz), δ 8.29 (1H, d),7.57 (1H,d), 7.53 (1H, d), 3.93 (3H, s), 3.87 (3H, s), 2.58 (3H, s).

2′-tert-Butyldimethylsilyl-uridine-3′-(5-bromo-4-chloroindol-3-yl-1-acetyl)-phosphate (9)

A mixture of **8** (0.25 g, 0.38 mmol) and 1,2,4-triazole (0.13 g, 1.90 mmol, 5 eq) was dissolved in freshly distilled pyridine (0.8 mL) under argon, and **7** was then added rapidly (0.25 g initially; then after 15 minutes an additional 0.20 g; 1.11 mmol, 2.9 eq was added). The green mixture was stirred at room temperature for 30 minutes. The reaction was quenched with 20 mL of water, and acidified with acetic acid to effect detritylation. After 30 minutes at room temperature, the product was extracted with $CHCl_3$. The organic layers were combined, dried (Na_2SO_4), and the solvent removed. The resulting solid was purified by flash chromatography ($CHCl_3$/methanol gradient, starting with 2% methanol and increasing to 20% methanol). The fractions containing the pure product were combined, evaporated and dried in vacuo over P_2O_5 yielding 0.16 g of **9** (60%): mp 211−212°C;^{1}H NMR (DMSO-d_6, 400 MHz), δ 11.32 (1H, s), 8.21 (1H, d), 7.97 (1H, d), 7.74 (1H, s), 7.61 (1H, d), 5.78 (1H, b), 5.66 (1H, s), 5.51 (1H, s), 4.51 (1H, b), 4.10 (1H, b), 2.48 (3H, s), 0.66 (9H, s), −0.28 (3H, s), −0.45 (3H, s); ^{13}C NMR (100 MHz, CD_3OD), δ 170.65, 166.23, 151.96, 141.92, 136.32, 134.50, 131.02, 125.48, 124.31, 119.09, 117.36, 115.40, 102.35, 90.33, 84.37, 76.73, 74.68, 61.36, 26.05, 23.97, 18.79, −5.05, −5.28; HRMS (FAB) m/e (M^+) calcd. 730.0353, obsd. 730.0330.

2′-tert-Butyldimethylsilyl-uridine-3′-(5-bromo-4-chloroindol-3-yl)-phosphate (10)

A solution of **9** (0.15 g, 0.21 mmol) in MeOH (5 mL) was cooled to 0°C. Concentrated aqueous NH_4OH (5 mL) was added dropwise to this solution, and the reaction was stirred at 3°C for 15 hours. The solution was evaporated in vacuo and the product dried over P_2O_5, yielding 0.13 g (89%): mp (dec.) 205°C; ^{1}H NMR (DMSO-d_6, 200 MHz), δ 10.95 (1H, s), 7.96

(1H, d), 7.35−7.11 (4H, m), 6.03 (1H, d), 5.61 (1H, d), 5.42 (1H, s), 4.50 (1H, b), 4.00 (1H, d), 3.81 (1H, d), 3.68 (2H, d), 0.68 (9 H, s), −0.36 (3H, s), −0.46 (3H, s); ^{13}C NMR (100 MHz, CD_3OD), δ 166.42, 151.89, 141.93, 134.81, 132.04, 127.01, 124.44, 119.78, 115.98, 113.78, 112.97, 102.07, 91.09, 83.77, 76.97, 73.41, 60.96, 26.76, 18.82, −5.25; HRMS (FAB) m/e (M^+) calcd. 688.0247, obsd. 688.0238.

Uridine-3′-(5-bromo-4-chloroindol-3-yl)-phosphate (U-3′-BCIP) (1)

To a solution of **10** (0.19 mmol) in THF (10 mL) was added tetra-n-butylammonium fluoride trihydrate (0.60 g, 1.9 mmol, 10 eq). The reaction was stirred at room temperature for 40 hours and the solvent was then evaporated. The product was purified by flash chromatography (n-butanol/water/methanol, 80/20/7) and crystallized from cyclohexane to give **1** (105 mg, 70%). The purity of **1** was demonstrated by analytical RP-HPLC using a linear gradient of 0.09% trifluoroacetic acid/CH_3CN (A) and 0.09% trifluoroacetic acid/H_2O (B) (from 100% of A to 50% of A in 40 minutes): mp dec. 125°C; ^{1}H NMR (DMSO-d_6, 400 MHz), δ 11.31 (1H, s), 10.90 (1H, s), 7.84 (1H, d), 7.27−7.16 (3H, m), 6.78 (1H, b), 5.75 (1H, d), 5.63 (1H, d), 5.35 (1H, m), 4.53 (1H, m), 4.04 (1H, m), 3.93 (1H, m), 3.49 (2H, m), 3.15 (2H, m), 1.55 (2H, m), 1.30 (2H, m), 0.92 (3H, M); ^{13}C NMR (100 MHz, CD_3OD) δ 166.19, 152.34, 142.29, 134.79, 132.16, 127.07, 124.90, 119.89, 115.82, 113.67, 112.81, 102.74, 90.09, 85.04, 75.48 (2), 61.85, 59.42, 24.75, 20.69, 13.96; $[\alpha]^{25}_D = -3.2°$ (c 1.27, ethanol).

U-3′-BCIP sodium salt

The tetra-butylammonium salt of U-3′-BCIP was exchanged by ion exchange chromatography using Dowex 50W-X4 (200−400 mesh), Na^+ form, and eluted with water. mp dec. 1 70°C; HRMS (FAB) m/e (M^+) calcd. 573.9395, obsd. 573.9398.

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REFERENCES

1. Blake,M.S., Johnston,K.H., Russell-Jones,G.J. and Gotschlich,E.C. (1984) *Analytical Biochemistry* **136**, 175−179.
2. Horwitz,J.P., Chua,J., Curby,R.J., Tomson,A.J., Da Rooge,M.A., Fisher,B.E.,Mauricio,J. and Klundt,I. (1964) *J. Med. Chem.* **7**, 574−575.
3. Wolf,P.L., Horwitz,J.P., Freisler,J., Vazquez,J. and Van der Muehll,E. (1968) *Experientia* **24**, 1290−1291.
4. Horwitz,J.P. and Freisler,J.V. (1970) *J. Med. Chem.*, **13**, 1024−1025.
5. Sung,W.L. and Narang,S.A. (1982) *Can. J. Chem.* **60**, 111−120.
6. Ogilvie,K.K., Beaucage,S.L., Schifman,A.L., Theriault,N.Y. and Sadana,K.L. (1978) *Can. J. Chem.* **56**, 2768−2780.
7. Eftink,M.R. and Biltonen,R.L. (1983) *Biochemistry* **22**, 5123−5134.
8. Holt,S.J., Kellie,A.E., O'Sullivan,D.G. and Sadler,P.W. (1958) *J. Chem. Soc.* 1217−1223.