Tetrahedron Letters 50 (2009) 793–795

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Solid-phase synthesis of new ribo and deoxyribo BrdU probes for labeling and detection of nucleic acids

Anilkumar R. Kore *

Life Technologies Inc., Bioorganic Chemistry Division, 2130 Woodward Street, Austin, TX 78744-1832, USA

ARTICLE INFO

Article history:
Received 12 November 2008
Revised 26 November 2008
Accepted 29 November 2008
Available online 6 December 2008

Keywords:
BrdU-14-UTP
BrdU-14-dUTP
Allylamine-UTP/dUTP
Labeling
Detection

ABSTRACT

A facile and efficient solid-phase synthesis of BrdU-14-UTP and BrdU-14-dUTP is reported. The method involves a nucleophilic addition of CPG-supported Br-dU with a 5′-carboxy modifier amidite, followed by the coupling with AA-UTP or AA-dUTP and finally the solid-support is cleaved under basic conditions. The newly prepared non-radioactive probes may have important applications in immunocytochemistry spatially in labeling and detection assays. BrdU moiety is coupled with nucleotides like dUTP and UTP and incorporated into nucleic acids using generally available polymerase like Taq polymerase or RNA polymerase. The probes thus generated can be used in standard blotting and hybridization procedures including in situ hybridization and detection with anti-BrdU conjugates.

One of the most commonly-used molecular biological techniques is the use of synthetic RNA or DNA molecules to function as probes for their complementary nucleic acid sequences. The ribo- or deoxyribo-nucleic acid probe is labeled and brought into contact with a target population of nucleic acid (RNA or DNA) to be investigated. Some routine procedures fix this target population onto a filter, sometimes after partial separation prior to transfer. The target can also be fixed cells, which are permeabilized to allow the probes to penetrate inside them. With all these techniques, the probe and target are allowed to hybridize under appropriate conditions, causing a double strand hybrid between the natural RNA or DNA and the synthetic probe to form. After excess probe is washed away, these hybrids are subsequently detected, usually by using antibodies to novel moieties located in the probe.[1–4] BrdU is often used as one of these moieties, serving as a substitute for thymidine in DNA probes, since it is both antigenically distinct and able to be incorporated by standard DNA polymerases.[5]

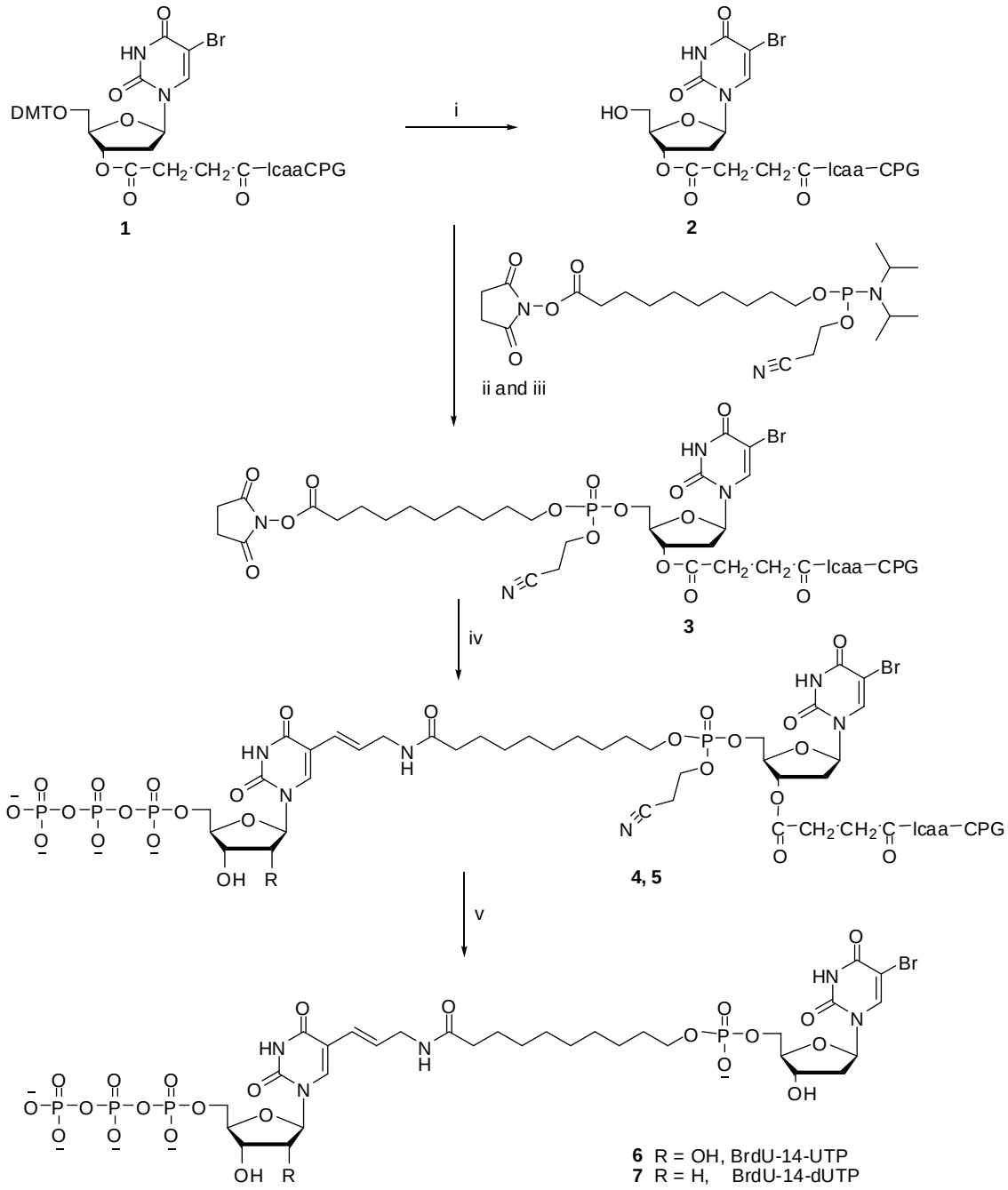
BrdU is also incorporated into the DNA of proliferating cells to study cell-cycle status or viability of cultured or harvested cells using different methods, such as image cytometry, HCA, and FCM.[6,7] A drawback of this technique is that the detection of BrdU requires harsh acidic conditions to denature the DNA and allow the BrdU to be recognized by detection antibodies, causing many epitopes to become modified or destroyed, thereby rendering them unrecognizable to the antibody and substantially reducing sensitivity.[8,9] We have designed a BrdU probe for in vitro studies which does not require this denaturation step but leaves the BrdU still available for detection and localization with alkaline phosphatase linked to the detecting antibody. In this design, the BrdU moiety is coupled to nucleotides like dUTP and UTP and incorporated into nucleic acids using generally available polymerases like Taq DNA polymerase or T7 RNA polymerase. The probes thus generated can be used in standard blotting and hybridization procedures, including in situ hybridization, and detected with standard anti-BrdU conjugates, for instance alkaline phosphatase-labeled antibodies against BrdU.

The essence of our BrdU probe is that the actual BrdU moiety is coupled to the C5 group of rUTP or dUTP through an amide linkage with a C14 carbon chain (Scheme 1). This BrdU-14-UTP probe can be incorporated effeciently during in vitro transcription with various phage-derived RNA polymerases used in molecular biology to generate RNA probes. The resulting RNA transcript will have incorporated this BrdU probe, providing an RNA molecule that readily hybridizes with target sequences. Post hybridization, these duplexes are decorated with BrdU that can be readily detected by antibodies without denaturation of the double strand. This is due to the presence of the C14 linker arm, which distances the BrdU moiety far enough from the main RNA chain that it is sterically available and efficiently recognized by the antibody during detection. Similarly, the deoxy analog of this probe can be used during PCR in the presence of Taq DNA polymerase, resulting in DNA that could be used in a similar manner.

In this Letter, we report the synthesis of new ribo- and deoxyribo-BrdU probes of high purities for use in in situ hybridization studies, using a solid-support synthesis methodology. The solid-

* Tel.: +1 512 721 3589; fax: +1 512 651 0201.
E-mail address: anil.kore@appliedbiosystems.com.

0040-4039/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2008.11.122



Scheme 1. Reagents and conditions: (i) detritylation with 3% TCA in CH_2Cl_2; (ii) coupling in CH_3CN, rt, 20 min; (iii) oxidation with I_2 in THF/H_2O/Py, rt, 20 min; (iv) reaction with AA-dUTP/AA-UTP in $NaBO_4$ buffer, pH 8.5, rt, 4 h; (v) deprotection with MeOH/NH_4OH (1:4), 55 °C, 3 h.

phase reaction pathway, using conventional syringe techniques, which leads to the formation of the desired BrdU-14-UTP **6** and BrdU-14-dUTP **7** probes is summarized in (Scheme 1).[10,11] The commercially-available resin-bound 5′-DMT-BrdU loaded CPG **1** (Glen Research, 80–100 mesh, 500 Å) was detritylated using 3% trichloroacetic acid in dichloromethane to give resin-bound 5′-OH-BrdU **2**.[12] The nucleophilic reaction of **2** with a solution of 10-carboxy-decyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite, *N*-hydroxysuccinimide ester in the presence of 5-ethylthio-1*H*-tetrazole activator, followed by oxidation with I_2, in THF/H_2O/pyridine (7:2:1) for 20 min afforded resin-bound product **3**. Next, the coupling reaction of **3** with 5-[3-aminoallyl]uridine 5′-triphosphate or 5-[3-aminoallyl]2′-deoxy-uridine 5′-triphosphate in the presence of sodium borate buffer, pH 8.5, afforded the corresponding resin-bound product **4** or **5**, respectively. Finally, the basic cleavage of the resin-bound product **4** or **5** using methanolic ammonia at 55 °C for 3 h afforded the corresponding products, BrdU-14-UTP **6** or BrdU-14-dUTP **7**, in 70% and 72% yields, respectively. Isolated overall yields were calculated based on the loading of 5′-DMT-BrdU loaded CPG (66 μmol/g). The structure of **6** and **7** was thoroughly characterized by ^1H and ^{31}P NMR and mass spectral data.[13,14]

Syringe technique for solid-phase preparation of BrdU-14-UTP ***6*** *and BrdU-14-dUTP* **7**: Probes were synthesized on a long chain alkylamine controlled-pore glass support loaded with BrdU (80–100 mesh, 500 Å) by using the conventional syringe technique with

little modification. The 5′-DMT-BrdU loaded CPG **1** (Glen Research), 66 μmol/g (0.5 g, 33.03 μmol), was poured into a 50 mL gas-tight syringe with a glass wool plug at the inlet. The support was washed initially with anhydrous CH_3CN (4 × 35 mL). The 5′-dimethoxytrityl (DMT) protecting group was then removed by washing with 3% trichloroacetic acid in dichloromethane (5 × 40 mL, 5 min). The support was then washed with anhydrous CH_3CN (4 × 40 mL) to form compound **2**. The coupling of 10-carboxydecyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite, *N*-hydroxysuccinimide ester solution (20 mL, 0.1 M solution in CH_3CN) was drawn into the syringe followed by 5-ethylthio-1*H*-tetrazole activator in anhydrous acetonitrile solution (20 mL, 0.3 M in CH_3CN). It was agitated for 20 min and the solution was ejected. The support was washed with anhydrous CH_3CN (4 × 40 mL) and oxidized with a (0.05 M, 40 mL) I_2 in THF/H_2O/Pyridine (7:2:1) for 20 min, then washed with anhydrous acetonitrile (5 × 40 mL), until no more brown color was observed coming from the support. At this point, the intermediate compound **3** was formed. Then the support was air-dried and transferred into a vial containing 5 mL of 50 mM sodium borate buffer, pH 8.5. To this, a solution of 5-[3-aminoallyl]uridine 5′-triphosphate or 5-[3-aminoallyl]2′-deoxy uridine 5′-triphosphate (3 mL, 0.25 M) in sodium borate buffer pH 8.5 was added. The reaction mixture was slowly agitated for 4 h at room temperature, after which the supernatant was removed from the support. The support was then washed with deionized water (6 × 15 mL) in order to remove any traces of uncoupled aminoallyl UTP or aminoallyl dUTP, followed by washes with anhydrous acetonitrile (2 × 15 mL) to afford the corresponding resin-bound product **4** and **5**. To cleave the product from the support, it was transferred into a 50 mL sealable vial and treated with a concentrated solution of methanol and NH_4OH in a 1:4 ratio (20 mL). The vial was sealed and incubated at 55 °C for 3 h in water bath. After the vial was removed from the water bath and cooled to room temperature, the methanolic ammonia solution containing the majority of the product was transferred to a larger vial. The support was rinsed twice with distilled water (2 × 6 mL) to obtain any remaining product, and this was added to the product vial. The product was completely dried down by lyophilization. The resulting desired products **6** and **7** were found to be >98% pure on analytical HPLC. There are several interesting features that are noteworthy from the solid-supported methodology presented here. First, the present methodology is simple and straightforward and no side products were observed in any of the steps. Second, the unreacted excess starting materials such as amidite, AA-UTP, and AA-dUTP can be easily removed from the reaction mixture by simply filtering the solid support. Another contrast to the standard solution-phase chemistry is that the final purification procedure for the products as described here require no column chromatography, since the final desired probes **6** and **7** are afforded in >98% purity by performing a basic cleavage of the final resin-bound product, followed by the evaporation of basic solution by lyophilization.

To sum up, short, efficient, high yielding, and very practical solid-phase syntheses of new non-radioactive probes, BrdU-14-UTP **6** and BrdU-14-dUTP **7** have been developed. These new probes would be useful in immunocytochemistry spatially in labeling and detection assays. Further work is in progress to evaluate its biological applications, and the outcome will be reported elsewhere.

Acknowledgments

The author thanks S. Muthian, R. Conrad, and Shirley A. Receipon (Life Technologies Inc.) for commenting and critical reading of this manuscript.

References and notes

1. Saxinger, W. C.; Ponnamperuma, C.; Gillespie, D. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2975–2978.
2. Bolton, E. T.; McCarthy, B. J. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1390–1397.
3. Gillespie, D.; Spiegelman, S. *J. Mol. Biol.* **1965**, *12*, 829–842.
4. Leary, J. J.; Brigtai, D. J.; Ward, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4045–4049.
5. Jirikowski, G. F.; Ramalho-Ortigao, J. F.; Lindl, T.; Seliger, H. *Histochemistry* **1989**, *91*, 51–53.
6. Leif, R. C.; Stein, J. H.; Zucker, R. M. *Cytometry A* **2004**, *58A*, 45–52.
7. Terry, N. H.; White, R. A. *Methods Cell Biol.* **2001**, *63*, 355–374.
8. Tang, X.; Falls, D. L.; Li, X.; Lane, T.; Luskin, M. B. *J. Neurosci.* **2007**, *27*, 5837–5844.
9. Jacobberger, J. W.; Frisa, P. S.; Sramkoski, R. M.; Stefan, T.; Shults, K. E.; Soni, D. V. *Cytometry A* **2008**, *73A*, 5–15.
10. Tanaka, T.; Letsinger, R. L. *Nucleic Acids Res.* **1982**, *10*, 3249–3260.
11. Seliger, H.; Scalfi, C.; Eisenbeiss, F. *Tetrahedron Lett.* **1983**, *24*, 4963–4966.
12. Septak, M. *Nucleic Acids Res.* **1996**, *24*, 3053–3058.
13. *Spectral data for BrdU-14-UTP* **6**: ^{1}H NMR (D_2O, 400 MHz) δ 8.12 (s, 1H), 7.90 (s, 1H), 6.46 (s, 1H), 6.33 (m, 3H), 4.71 (s, 1H), 4.56 (s, 1H), 4.33–3.88 (m, 10H), 2.30 (m, 4H), 1.60–1.21 (m, 16H); ^{31}P NMR (D_2O, 162 MHz) δ 1.74 (s), −4.71 (d, J = 19.3 Hz), −10.03 (d, J = 19.8 Hz), −20.48 (d, J = 19.3 Hz); MS (m/z): 1072 $[M]^+$.
14. *Spectral data for BrdU-14-dUTP* **7**: ^{1}H NMR (D_2O, 400 MHz) δ 8.10 (s, 1H), 7.94 (s, 1H), 6.46 (m, 1H), 6.33 (m, 2H), 5.99 (d, J = 5.2 Hz, 1H), 4.56 (m, 1H), 4.48–3.87 (m, 9H), 2.40–2.26 (m, 4H), 1.59 (m, 4H), 1.35–1.21 (m, 14H); ^{31}P NMR (D_2O, 162 MHz) δ 1.75 (s), −4.59 (d, J = 19.8 Hz), −10.14 (d, J = 20.1 Hz), −20.49 (d, J = 19.8 Hz); MS (m/z): 1056 $[M]^+$.