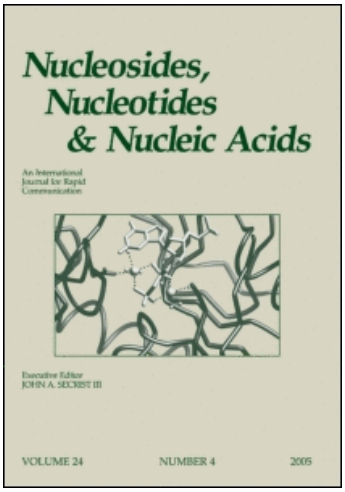

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Synthesis of a Novel Biotinyl Derivative and its Application to Non-Radioactive Labeling of DNA

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SYNTHESIS OF A NOVEL BIOTINYL DERIVATIVE AND ITS APPLICATION TO NON-RADIOACTIVE LABELING OF DNA

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ABSTRACT: A novel biotinyl derivative containing a carbodiimide group (Carb-Bio) has been newly synthesized and this compound has been applied for non-radioactive labeling of DNA. The result suggests that this reagent can be used for labeling a probe DNA for Southern analysis.

The biotinylated nucleotides are widely used as non-radioactive labeled probes (1). It is known that 1-cyclohexyl-3-{2-[4-(4-methyl)morpholinyl]ethyl}carbodiimide(water-soluble carbodiimide) specifically reacts with guanine and thymine in single-stranded DNAs (FIG. 1) (2, 3). There is a strong and specific noncovalent interaction between biotin and avidin which leads to the formation of a high affinity complex and it can be easily visualized by enzymatic methods (1). So we synthesized a novel biotinyl derivative containing a carbodiimide group (Carb-Bio). We can biotinylate single-stranded DNA with Carb-Bio and the biotinylated single-stranded DNA can be used as a Southern hybridization probe.

MATERIALS AND METHODS

Spectral measurements. ^{1}H-NMR spectra were recorded at 270 MHz on a JEOL JNM-GX270 FT NMR spectrometer and ^{13}C-NMR spectra were recorded at 67.8 MHz on a JEOL-EX 270 spectrometer. IR spectra were recorded on a Nicolet 5DX.

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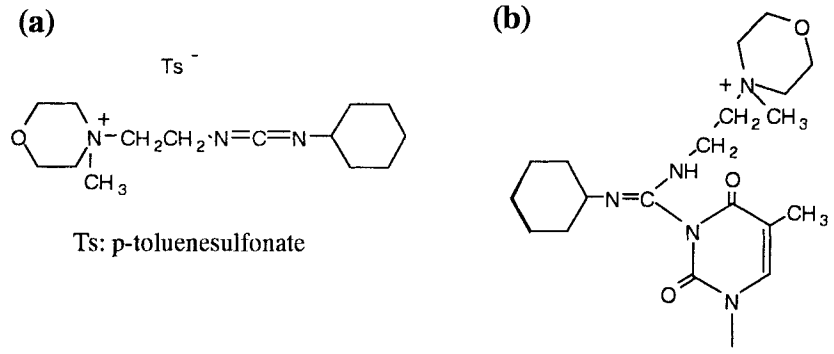

FIG. 1. (a) The water-soluble carbodiimide, 1-cyclohexyl-3-{2-[4-(4-methyl)morpholinyl] ethyl} carbodiimide. (b) The reaction product of this water-soluble carbodiimide with thymine.

Synthesis of 1-ethyl-3-{3-[(biotinylhydrazinocarbonylmethyl)dimethylammonio]propyl}carbodiimide bromide (Carb-Bio, III) (FIG. 2). Following Wilchek (4), we synthesized biotinyl-bromoacetyl hydrazide (II), and followed Sheehan (5) to synthesize 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (I). Biotinyl-bromoacetyl hydrazide (0.758 g, 2m mole) was suspended in 15 ml of dry DMF, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.47 g, 3m mole) was then dropped into the suspension. The mixture was stirred for 1h at room temperature until it became clear, then poured into 60 ml of ether, forming a gummy oil. This product was rinsed three times with 30 ml of ether, and dried under vacuum. An amorphous and strongly hygroscopic powder was obtained (0.96g, 90%): ^{1}H-NMR(DMSO-d_6) δ 10.50 (1 H, s, NHCO), 10.05 (1 H, s, NHCO), 6.40 (2 H, m, NHCONH), 4.25 (2 H, m, CH-CH), 4.15 (2 H, s, N-CH_2-CO), 3.50 (2 H, m, C-CH_2-N), 3.25 (4 H, m, CH_2-N=C=N-CH_2), 3.20 (6 H, s, CH_3-N-CH_3), 3.10 (1 H, m , -CH-S), 2.70 (2 H, m, S-CH_2-C), 2.15 (2 H, m, CO-CH_2-C), 1.90(2 H, m, =N-C-CH_2-C-N), 1.50(6 H, m, CO-C-$CH_2CH_2CH_2$-C-), 1.15(3 H, t, J=10Hz CH_3-C-N=); ^{13}C NMR (CD_3OD) δ 175.32 (C=O), 166.74 (C=O), 164.41 (C=O), 141.96 (-N=C=N-), 65.95, 63.92, 63.37, 62.36, 57.69, 53.75, 50.66, 45.34, 43.04, 41.91, 35.10, 30.12, 27.14, 26.44, 17.91; IR(liquid paraffin) 2130cm^{-1} (-N=C=N-); Anal. Calcd. for C_{20} H_{36} Br N_7 O_3 S : C, 44.94; H, 6.79; N, 18.34. Found : C, 44.72; H, 7.29; N, 18.21.

Oligonucleotide synthesis. Oligo 1, 5'-d(CCCCCCCCCCTCCCCCCCCCC), oligo 2, 5'-d(AAAAAAAAAGAAAAAAAAAA), and oligo 3, 5'-d(ACACACACACACACACAC) were synthesized using an Applied Biosystem DNA/RNA synthesizer according to the manufacturer's protocols.

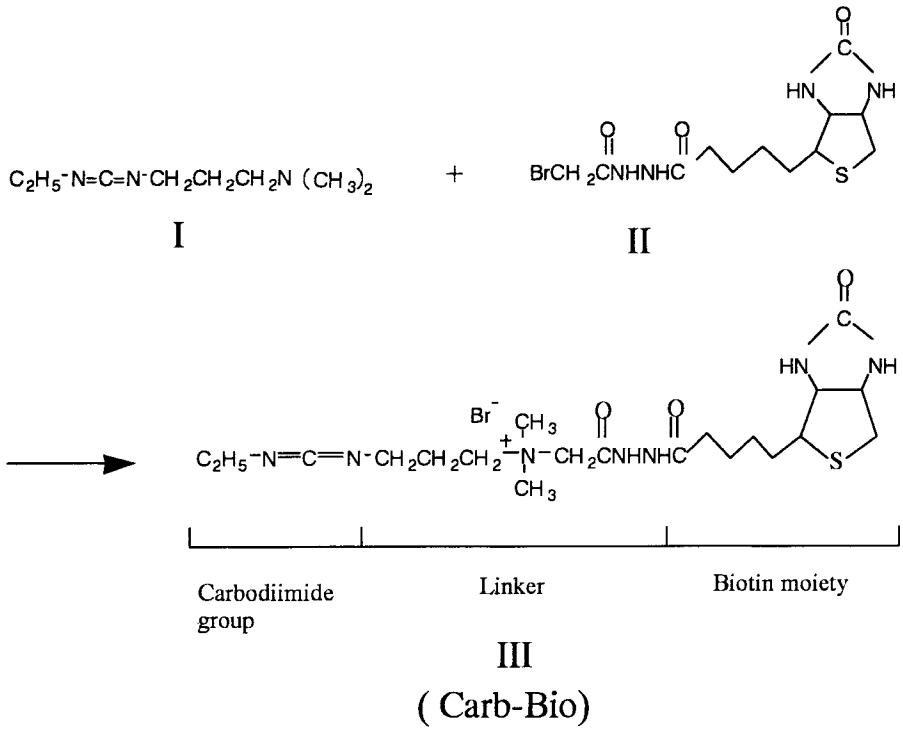

FIG. 2. Synthesis and structure of Carb-Bio. See MATERIALS AND METHODS for detailed procedures

General procedure for the reaction of Carb-Bio with DNA fragments. Carb-Bio (10-50 mg/ml) in 0.1 M borate buffer (pH 8.0) was prepared freshly. The reaction was carried out by mixing equal volume of Carb-Bio and DNA in aqueous solution (0.05-1 mg/ml) at 30 ℃ or 37 ℃ for 1.5-2.0 h. After the reaction, an equal volume of 5.0 M ammonium acetate solution (pH 7.5) was added, followed by adding two volumes of ethanol for the precipitation of DNA, and the precipitate was washed once with 70% ethanol. The resulting pellet was dissolved in water, and the amount of biotinylated DNA was measured by UV absorption at 260 nm in the same manner as for DNA. The dissolved DNA was subjected to polyacrylamide gel electrophoresis, after which, the DNA fragments were electrophoretically transferred on nitrocellulose filters (Schleicher and Schuell, pore size 0.45 μm) using Phoriz Blott (ATTO Corporation). After electrotransfer, the filters were baked for 1h at 80℃ under vacuum. Color detection was performed as described previously(1). Filters were floated in a small volume of AP7.5 buffer [0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2

mM $MgCl_2$] for 1 min for rehydration, and then incubated at 37°C for 60 min in a blocking buffer [AP7.5 buffer containing 3% (w/v) BSA (FractionV)]. Filters were then placed in AP7.5 buffer containing streptavidin-alkaline phosphatase conjugate (1 μg/ml) (GIBCO BRL) at room temperature for 10 min, washed three times for 10 min with gentle agitation in AP7.5 buffer and once for 10 min in AP9.5 buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$]. For color development, filters were incubated at room temperature in the dark with substrates solution [AP9.5 buffer containing 0.33 mg/ml nitro blue tetrazolium (NBT), 0.17 mg/ml 5-bromo-4-chlolo-3-indolyl phosphate (BCIP)]. The reaction was terminated by washing the filters with TE buffer [10 mM Tris-HCl, 1mM EDTA (pH 8.0)].

Plasmid DNA digestion. Plasmid pHAB240 carrying a mutant aldolase B gene (6) was digested with *Hae* III or *Hinf* I and used as samples of DNA fragments carrying either blunt or staggered ends.

Single-stranded DNA formation. To obtain a pair of single-stranded DNAs containing complementary sequence, plasmid pHAA47 carrying normal aldolase A gene (7) was digested with *Pst* I and subcloned into the M13mp18RF vector. We obtained single-stranded phage DNAs containing an insert of a plus or minus strand by usual manner.

Dot-Blot Hybridization. Serially diluted single-stranded M13 DNA carrying the *Pst* I fragment (786 bp) of the aldolase A gene (plus strand) was spotted to nitrocellulose filters as a target DNA (1-100 pg/spot) (Schleicher & Schuell, pore size 0.45 μm) and baked at 80 °C for 1 hr under vacuum. The filters were prehybridized at 42 °C for 4 h in prehybridization buffer [50 % deionized formamide (V/V), 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate), 50mM sodium phosphate (pH 6.5), 0.25 mg/ml sonicated denatured salmon sperm DNA, 0.2 % SDS, 5 mM EDTA and 0.2 mg/ml each of bovine serum albumin, Ficoll 400 and polyvinylpyrrolidone, Mr 40,000]. Hybridization was carried out in a shaking water bath at 55 °C for 20 h in a buffer containing four volumes of prehybridization buffer, one volume of a 0.5 g/ml solution of dextran sulfate, and Carb-Bio-labeled M13 DNA carrying the aldolase A gene (150 ng/ml, minus strand) as a labeled probe. The filters were then washed three times for 15 min at room temperature with frequent agitation in 2 × SSC, 0.1 % SDS, and another three times for 20 min at 55°C in 0.1 × SSC, 0.1 % SDS. Color detection was performed as described previously (1).

RESULTS

Rationale for the design of Carb-Bio and its synthesis. Color detection of DNA would be achieved if a new biotinyl derivative containing a carbodiimide group was synthesized, because carbodiimide directly modifies thymine and guanine in single-stranded DNA, while biotin interacting with avidin leads to the formation of a high affinity complex detectable by enzymatic methods. It is necessary for the compound to have a

reactive carbodiimide group and a linker of sufficient length for steric reasons. For this purpose, the carbodiimide group in Carb-Bio is joined to a small ethyl group for a good reactivity and there are ten atoms between the nucleotide base reacting with Carb-Bio and the biotin moiety. In addition, a quaternary ammonium group is also added to Carb-Bio for water solubility.

The synthetic pathway is shown in FIG. 2. An important feature of this pathway is that the compound containing a biotin moiety (II) is readily coupled to the tertiary amine (I) because compound II has a reactive bromoacetyl group.

Reaction of Carb-Bio with single-stranded oligonucleotides. First, we examined whether Carb-Bio would react the four different deoxynucleotides (dNMPs). The results were as expected; dGMP and TMP reacted but dAMP and dCMP did not react with this compound (data not shown). Three oligomers were examined similarly. Carb-Bio-treated oligomers were detected by UV shadows as an intense band after electrophoresis (FIG. 3a). FIG. 3b indicates that the single thymine(T) and guanine (G) were reactive with this compound (see lane 1 and lane 2), but adenine (A) and cytosine (C) were not (lane 3). When compared lane 1 with lane 2 in FIG3b, T was more reactive than G.

Labeling of single-stranded DNA with Carb-Bio. The procedure for labeling DNA probes is very simple because Carb-Bio reacts with single-stranded DNA to biotinylate under mild conditions. To examine the labeling specific activity of this compound, single-stranded M13 DNA labeled with Carb-Bio was spotted to a nitrocellulose filter, and subjected to color detection as described in MATERALS AND METHODS. One picogram of labeled DNA could be detected by this method as shown in FIG. 4A.

We then studied the usefulness of this compound for labeling DNA probes for Southern analysis. Single-stranded M13 DNA carrying the *Pst* I fragment of the aldolase A gene (plus strand) was spotted to a nitrocellulose filter and hybridized with Carb-Bio-labeled probe DNA (minus strand). Three pg of target DNA could be detected by this method (see FIG. 4B).

Reaction of Carb-Bio with double-stranded DNA fragments carrying either blunt or staggered ends. To determine whether only single- stranded portion of DNA reacts with Carb-Bio and double-stranded DNA does not, we prepared double-stranded DNA carrying either blunt or staggered ends. Plasmid pHAB240 (6) was digested with *Hae* III which generates blunt ends, while the same DNA was also digested with *Hinf* I which gives rise to staggered ends carrying a T residue in its cleavage site. As can be seen in FIG. 5 blunt ends did not react with Carb-Bio, while staggered ends tailed with a single-stranded T were reactive (compare lane 1 with lane 2 in FIG. 5b). These results indicate that completely matched, double-stranded DNA dose not react with Carb-Bio, but instead the double-stranded DNA even having the protruded T or G residue is reactive with this compound.

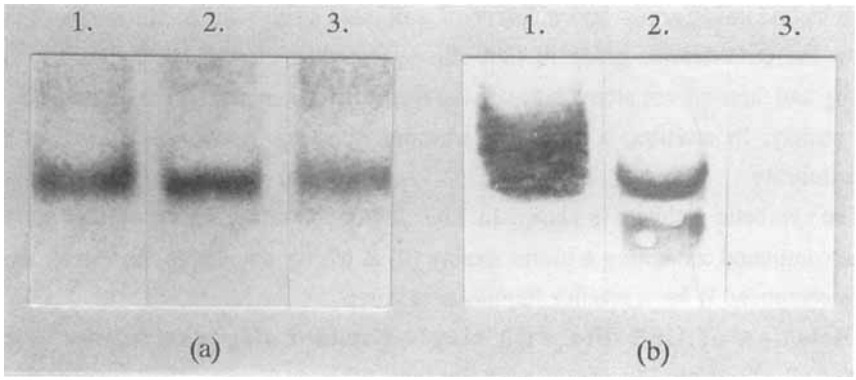

FIG. 3. Reaction of Carb-Bio with single-stranded oligonucleotides. 20μg of different oligonucleotides reacted with Carb-Bio were electrophoresed in polyacrylamide gels and blotted on a nitrocellulose filter. Color detection of this compound was performed as described in MATERIALS AND METHODS. lane 1: 5'-d(CCCCCCCCCCTCCCCCC-CCCC), lane 2: 5'-d(AAAAAAAAAGAAAAAAAAAA), lane 3: 5-d(ACACACAC-ACACACACACAC). (a) UV shadows of Carb-Bio-reacted oligonucleotides. (b) Color detection of Carb-Bio-reacted oligonucleotides. A unidentified artifactual band was seen below the main band in lane 2.

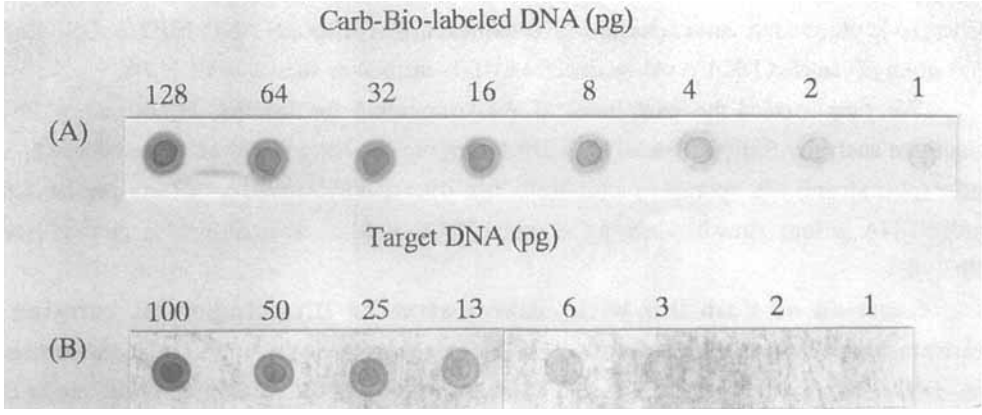

FIG4. Color detection of Carb-Bio-labeled DNA. (A) Direct detection of Carb-Bio-labeled DNA. Serially diluted Carb-Bio-labeled single stranded M13mp18 DNA was spotted to a nitrocellulose filter (1-128 pg/spot) and the filter was baked. Color detection was then performed as described in MATERIALS AND METHODS. (B) Color detection of Carb-Bio-labeled M13mp18 DNA after hybridization to target DNA. Single-stranded M13mp18 DNA carrying the *Pst* I fragment of the aldolase A gene (plus strand) was spotted to a nitrocellulose filter and the filter was baked. Single stranded M13mp18 DNA (minus strand) labeled with Carb-Bio as described was used as a probe. Prehybridization, hybridization and detection were performed as described in MATERIALS AND METHODS.

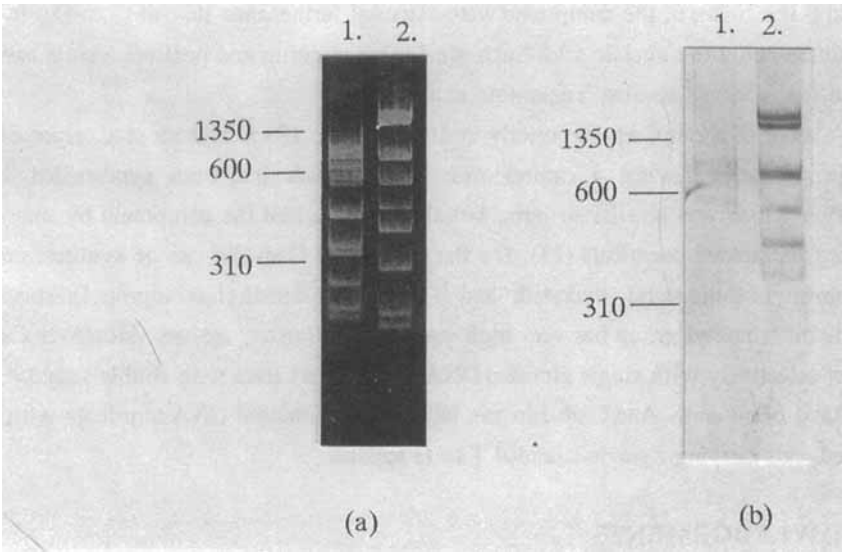

FIG. 5. Reaction of Carb-Bio with double-stranded DNA fragments carrying either blunt or staggered ends. Carb-Bio-reacted DNA fragments were electrophoresed and treated as described in MATERIALS AND METHODS. (a) Ethidium bromide staining. (b) Color detection on nitrocellulose filters. lane 1: pHAB240 plasmid DNA (6) digested with *Hae* III, lane 2: pHAB240 plasmid DNA digested with *Hinf* I, which generates 5'-ANT-3' of single-stranded residue at one end. DNA fragments shorter than 300bp fragment were not detected in lane 2, because the shorter DNA fragments were not retained on the filters.

DISCUSSION

A biotinyl derivative containing a carbodiimide group was synthesized from biotinyl-bromoacetyl hydrazide and carbodiimide containing a tertiary amino group. This new compound was reactive with single-stranded G(s) and T(s) as was water-soluble carbodiimide, and in addition made it possible to visualize the reaction products by enzymatic methods. Although Carb-Bio is a bulkier molecule than water-soluble carbodiimide, their reactvities are similar. We demonstrated this compound to be useful for labeling DNA probes for Southern analysis.

A number of methods have been developed to use biotinylated DNA as probes. Biotin-11-dUTP was used as a substrate for nick translation instead of TTP in a standard nick translation reaction mixture (8, 9). A photoreactive biotin derivative containing a azido group was also used for the biotinylation of DNA and RNA (10). 5'-Biotin-labeled oligonucleotides were also synthesized by chemical methods (11). Compared with above methods, our method is simpler and safer with almost equivalent sensivity. It is, however, still necessary to raise the sensitivity for the detection of this compound. This would be

achieved if the linker of the compound were extended further than that of Carb-Bio to reduce steric hindrance by the nucleic acid. Such studies are ongoing and positive results have been obtained (unpublished results; Yamamoto et al.).

We reported about Carb-Bio briefly in 1994 (12). In 1995, Masuda et al. reported about a biotin derivative having a carbodiimide group which had been synthesized by the conception which was similar to ours, but they synthesized the compound by many steps and using harzardous chemicals (13). On the other hand Carb-Bio can be synthesized easily from biotinyl-bromoacetyl hydrazide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, since the bromoacetyl group has very high reactivity for tertiary amines. Moreover Carb-Bio can react selectively with single stranded DNAs and dosen't react with double stranded DNAs which have blunt ends. And Carb-Bio can label double stranded DNA terminals which have staggered ends carrying a single-stranded T or G residue.

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