Photocleavable biotin derivatives: A versatile approach for the isolation of biomolecules

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ABSTRACT While the strong biotin–avidin interaction has been widely used for the detection of biomolecules, its irreversibility complicates their isolation. We report the synthesis of a photocleavable biotin derivative (PCB) which eliminates many limitations of existing methods. This reagent contains a biotin moiety linked through a spacer arm to a photocleavable moiety, which reacts selectively with primary amino groups on any substrate. In experiments using [leucine]-enkephalin as a model substrate, we show that PCB retains its high affinity toward avidin/streptavidin and allows rapid (<5 min) and efficient (>99%) photorelease of the substrate in a completely unaltered form. Photocleavable biotins should be useful in numerous applications involving the isolation of proteins, nucleic acids, lipids, and cells.

The use of avidin–biotin technology has become increasingly important in numerous areas of biochemistry, molecular biology, and medicine (1–3), including detection of proteins by nonradioactive immunoassays (4), cytochemical staining (5), cell separation (6), and isolation of nucleic acids and detection of specific DNA/RNA sequences by hybridization (7). The technique derives its usefulness from the extremely high affinity of the avidin–biotin interaction (association constant 10^15 M^{-1}) and the ability to biotinylate a wide range of target biomolecules such as antibodies, nucleic acids, and lipids.

The first step in the isolation of a target molecule is its biotinylation or the biotinylation of a biomolecule which ultimately binds to the target molecule (e.g., an antibody or hybridization probe that forms a target complex). The biotinylated molecule or the target complex is then separated from other molecules in a heterogeneous mixture by using affinity media based on the avidin–biotin interaction (1). Once the target molecule is bound through the avidin–biotin interaction, it is often important to recover it in an unmodified and biologically functional form. Existing methods for release of the target include (i) dissociation of the biotin–avidin complex (6–8 M guanidinium chloride) (3); (ii) utilization of biotin analogs with decreased affinity towards avidin (e.g., renoavidin) (8); and (iii) use of biotinylation reagents with cleavable spacer arms [e.g., S-S-biotin (9)]. However, none of these methods allows release of the target molecule or the target complex in an unaltered form, since the biotin or part of the cleavable spacer arm remains attached. In addition, these procedures involve conditions which often denature the target biomolecule.

In this study we report synthesis, characterization, and application of a photocleavable biotin derivative (PCB), which eliminates many limitations of existing methods. This reagent, termed PCB-NHS ester, contains a biotin moiety linked through a spacer arm to a photocleavable 1-(2-nitrophenyl)ethyl moiety derivatized with N-hydroxysuccinimidyl mixed carbonate, which reacts selectively with primary amino groups on the target molecule to form a stable carbamate. The PCB-target biomolecule conjugate undergoes an efficient photorelease upon 300-nm illumination, resulting in the rapid and complete release of the target molecule in an unmodified form.

MATERIALS AND METHODS

All chemicals were purchased from Aldrich unless otherwise indicated. 1H NMR spectra were recorded on a Varian XL-400 spectrometer at 400 MHz in perdeuterated dimethyl sulfoxide solution. Emission spectra were measured on an SLM 48000 fluorimeter using 380-nm excitation. IR spectra were recorded on a Nicolet 740 Fourier transform IR spectrometer.

Synthesis of PCB-NHS Ester. Synthesis of PCB-NHS ester was accomplished as shown in Scheme I. 5-Bromomethyl-2-nitroacetophenone (1) (10, 11) (2.0 g; 7.75 mmol) was added to a solution of hexamethylenetetramine (1.14 g; 8.13 mmol) in 15 ml of chlorobenzene (12). The mixture was stirred overnight and the resulting precipitate was filtered off and washed.

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washed with chlorobenzene (10 ml) and diethyl ether (20 ml). The intermediate 2 (2.93 g; 7.36 mmol) was suspended in 35 ml of 95% (vol/vol) ethanol and then concentrated HCl (3.12 ml; 5 eq) was added. The mixture was stirred overnight and then evaporated to dryness. Dimethylformamide (10 ml) was added to the residue, containing 5-aminomethyl-2-nitroacetophenone hydrochloride (3) and ammonium chloride (which remains as a precipitate), followed by 6-biotinamidocaproic acid (3.29 g; 1.25 eq, in 35 ml of N-methylpyrrolidone), dicyclohexyl carbodiimide (DCC; 2.28 g; 1.5 eq), and triethylamine (1.28 ml; 1.25 eq). The solution was stirred overnight at room temperature, the precipitate was filtered off, and the filtrate was added to 700 ml of diethyl ether. The resulting precipitate was washed with water (three times, 5 eq) was added, followed by 6-biotinamidocaproic acid (0.81 ml; 3 eq). After stirring overnight and then concentrated to about 1 ml of acetone, and the mixture was neutralized with 0.1 M sodium carbonate (Fluka) (1 g; 1.5 eq) was added, followed by triethylamine (1.28 ml; 1.25 eq). The solution was stirred at 0°C for 30 min and at room temperature for an additional 2 hr. The reaction was quenched by addition of 1 ml of acetone, and the mixture was neutralized with 0.1 M HCl and concentrated to about 5 ml. The aqueous layer was decanted and the residue was washed with water (three times, 5 ml each) and dried to give 5-(6-biotinamidocaproamidoethyl)-1-(2-nitrophenyl)ethanol (5) (0.71 g; 71% yield).

Intermediate 4 (1 g; 1.88 mmol) was dissolved in 15 ml of dimethylformamide. To this solution N,N′-disuccinimidyl carbonate (Fluka) (1 g; 1.5 eq) was added, followed by triethylamine (0.81 ml; 3 eq). After stirring at room temperature for 5 hr, solvents were evaporated to dryness. The residue was washed consecutively with 0.1 M NaHCO3, water, dioxane, and diethyl ether, and then dried to give 1.04 g (69% yield) of 5-(6-biotinamidocaproamidoethyl)-1-(2-nitrophenyl)ethyl N-hydroxysuccinimidyl carbonate (PCB-NHS) ester 6. This product was characterized as follows: mp = 113–114°C (uncorrected); chemical ionization MS: M+ = 676.5; UV–visible (in phosphate buffer, pH = 7.4) λ1 = 204 nm, ε1 = 19,190 M−1 cm−1; λ2 = 272 nm, ε2 = 6350 M−1 cm−1; 1H NMR (8, ppm): 8.48 (t, 1H), 8.05–8.03 (d, 1H), 7.75–7.71 (t, 1H, 7.66 (s, 1H), 7.35–7.45 (s, 1H), 6.44 (s, 1H), 6.37 (s, 1H), 6.28–6.27 (m, 1H), 4.39 (m, 2H), 4.30 (m, 1H), 4.12 (m, 1H), 3.57 (d, 2H), 3.09 (m, 1H), 3.01–2.99 (m, 2H), 2.79 (m, 5H), 2.58–2.55 (m, 1H), 2.17–2.15 (m, 2H), 2.04–2.02 (m, 2H), 1.72–1.71 (m, 2H), 1.66–1.43 (br, m, 6H), 1.38–1.36 (br, m, 2H), 1.26–1.25 (br, m, 3H); IR (KBr): νCO = 1815 and 1790 cm−1.

**Reaction and Photocleavage of PCB with [Leu5]Enkephalin ([Leu5]Enk).** Reaction between PCB-NHS ester and [Leu5]Enk. [Leu5]Enk (Sigma) (3.1 μmol in 200 μl of 0.1 M NaHCO3, pH 8.0) and PCB-NHS ester (3.4 μmol in 200 μl of dimethylformamide) were mixed and stirred overnight at room temperature and used without further purification.

**Photobysis of PCB-[Leu5]Enk.** PCB-[Leu5]Enk solution was diluted with 25 mM sodium phosphate buffer (pH = 7.4) to a final concentration of 1.93 μmol/ml and was irradiated with a long-wavelength UV lamp (Blak Ray XX-15 UV lamp, Ultraviolet Products, San Gabriel, CA) at a distance of 15 cm (emission peak 365 nm, lamp intensity = 1.1 mW/cm2) at a distance of 31 cm. The time dependence for photocleavage of PCB-[Leu5]Enk was studied by removing small aliquots from the photoreaction at various times of irradiation, and the concentration of released PCB-[Leu5]Enk was determined by the fluorescamine assay.

**RESULTS**

The synthesized PCB 6 referred to here as PCB-NHS ester (see Scheme I) consists of a biotinyl moiety linked through a spacer arm (6-aminoacapric acid) to an α-substituted 2-nitrobenzyl nucleus bearing an N-hydroxysuccinimidyl reactive group (NHS carbonate). The spacer was introduced to allow for effective binding to streptavidin. The α-substituted 2-nitrobenzyl moiety was selected because its derivatives, such as esters and amides, are known to exhibit highly efficient and fast photocleavages (14, 15). NHS esters and carbonates react selectively under mild conditions (pH = 8) with primary amino groups to give amides and carboxamides, respectively (1, 13).

The properties of the PCB-NHS ester 6 were studied with PCB-[Leu5]Enk, a pentapeptide (Tyr-Gly-Gly-Phe-Leu), as a model substrate (Scheme II).

![Scheme II](image-url)
First, PCB-NHS ester was allowed to react with [Leu]Enk to yield PCB-[Leu]Enk (7). HPLC traces of [Leu]Enk (trace a), the PCB-NHS ester (trace b) and PCB-[Leu]Enk (trace c) are shown in Fig. 1. Trace c shows that formation of PCB-[Leu]Enk is complete, since the peaks of [Leu]Enk (trace a) and PCB-NHS ester (trace b) are absent. Instead, two new peaks (peaks 1 and 2) are seen, which have identical UV-visible absorbance spectra (Fig. 1 Inset). These two peaks can be attributed to PCB-[Leu]Enk, with the separation most likely due to its two diastereoisomeric forms (16).

Fig. 1 also shows that illumination of PCB-[Leu]Enk (λ > 300 nm) results in almost complete photolysis in less than 5 min. Along with the decrease in intensity of peaks 1 and 2, attributed to PCB-[Leu]Enk, there is a parallel appearance of a photoproduct as indicated by the increase in the intensity of peak 3 (traces d and e). The identical retention times and similar absorbance spectra of peak 3 and the [Leu]Enk control (peak 4) establish this photolysis product as [Leu]Enk. Thus, we conclude that [Leu]Enk is photorelease from the PCB-[Leu]Enk in less than 5 min of illumination in a completely unaltered form. Other peaks (labeled with asterisks) which appear during photolysis are most likely due to the 2-nitrosoacetophenone derivative and other minor photolysis products similar to those observed in the photolysis of α-substituted 2-nitrobenzyl compounds (14, 15).

The time dependence for photolysis of PCB-[Leu]Enk in solution was also measured with fluorescamine. Importantly, fluorescamine reacts only with the free amino group (17) of [Leu]Enk to form a fluorophore (λex = 383 nm, λem = 488 nm) and does not react with PCB-[Leu]Enk, which lacks any free amino group. Fig. 2 Inset shows the time course for release of [Leu]Enk upon photolysis of the PCB-[Leu]Enk in solution. No fluorescence is detected before illumination (0 min), confirming that there exists no unreacted [Leu]Enk in solution. The amount of [Leu]Enk photorelease at different times was also calculated by using the fluorescamine assay. These data confirm that [Leu]Enk is almost completely released from PCB-[Leu]Enk in solution within 5 min of UV illumination.

To determine if the biotinyl moiety of PCB-[Leu]Enk retains its affinity to avidin, we complexed the PCB-[Leu]Enk with monomeric avidin-coated agarose beads. For this purpose, the PCB-[Leu]Enk was incubated for 30 min in a suspension of these beads at a PCB-[Leu]Enk-to-avidin ratio of 1:1.5. The suspension was then spin-filtered for 3 min (16,000 × g) and the filtrate was analyzed for unchanged PCB-[Leu]Enk by illuminating it for 6 min, which is sufficient time to release [Leu]Enk as shown by the HPLC experiments (Fig. 1). As seen in Fig. 2, only a small amount of [Leu]Enk is present in the filtrate (~6%) after spin-filtering the beads. This indicates that the free PCB-[Leu]Enk binds efficiently to the monomeric avidin-coated agarose beads.

It was also established that [Leu]Enk is released completely from PCB-[Leu]Enk even when complexed with monomeric avidin-coated agarose. For this experiment, the avidin-bound PCB-[Leu]Enk was photocleaved by illumination of the resuspended agarose beads. As seen in Fig. 1, the fluorescamine-based assay shows that approximately 9.7 nmol of [Leu]Enk is released into solution in approximately 5 min. This is close (within the estimated error) to the amount of PCB-[Leu]Enk (9.4 nmol) immobilized on monomeric avidin beads. Additionally, the small drop in measured fluorescence after 5 min is most likely due to the presence of the PCB photoproduct, which may act as either a quenching agent or an inner filter. In the case of PCB-[Leu]Enk complexed to beads, such a drop is not observed because the PCB photoproduct remains bound to the avidin.
FIG. 2. Fluorescamine assay of photorelease [Leu]Enk. PCB-[Leu]Enk complexed with agarose beads coated with monomeric avidin or tetrameric streptavidin was photolyzed, and the concentration of the released [Leu]Enk was measured by using a fluorescamine assay. Extent of unbound PCB-[Leu]Enk was determined by assaying for released [Leu]Enk after 6 min of illumination, which was calibrated using 10 nmol of [Leu]Enk standard. (Inset) Time dependence of [Leu]Enk photorelease from PCB-[Leu]Enk in solution (●); from PCB-[Leu]Enk complexed with agarose beads coated with monomeric avidin (○); and from PCB-[Leu]Enk complexed with agarose beads coated with streptavidin (△).

more, HPLC analysis confirmed that the released photoproduc-
t is [Leu]Enk (data not shown). A similar experiment (Fig. 2 Inset) in which streptavidin-coated agarose beads were used also showed that PCB-[Leu]Enk is efficiently bound by strepta-
vidin and that [Leu]Enk is completely released upon illumin-
ation in less than 4 min.

DISCUSSION

In this work we have designed and synthesized a PCB that can be
used as a reagent to label biomolecules. There are several
properties that are important for a PCB to be useful in the
isolation of biomolecules. They include the following:

(i) Reactivity. The PCB should react selectively and effi-
ciently with a wide range of target molecules under mild
conditions.

(ii) Complexation. The PCB should retain biotin’s high
affinity toward (strept)avidin.

(iii) Photocleavage. The intensity and exposure time of light
required for complete photocleavage should be minimal.

(iv) Substrate release. Photocleavage should result in
the release of the substrate in an unaltered form.

The PCB-NHS ester reported here satisfies all of these
criteria. It reacts with any substrate having a primary amino
group under mild conditions, exhibits a high affinity towards
(strept)avidin, and allows fast (~4 min) and efficient (~99%)
photorelease of the substrate in a completely unaltered form.
In addition, biotin-NHS ester and its analogs have been widely
used as biotinylation reagents for a variety of biomolecules,
including antibodies (3), hormones (1), amino-derivatized
nucleic acids (7), and lipids (18). Similar protocols should be
applicable for the biotinylation of these biomolecules with the

PCB-NHS ester, thereby expanding the range of applications
for biotin–avidin technology.

Importantly, the synthetic strategy employed here for the
synthesis of PCB-NHS ester can also be readily adapted for the
synthesis of other PCB compounds. For example, by using
the current synthetic approach the specific photoreactive moiety,
reactive group, and spacer arm can be replaced easily to create
a variety of different PCB reagents. Recently, a different
approach was reported (19) that did not satisfy requirements
i, iii, and iv listed above. In this case, a peptide hormone
(cholecystokinin) was synthesized containing biotin linked via
a 2-nitrobenzyl ester moiety. Thus, this approach did not
involve synthesis of a PCB reagent (property i). In addition,
photocleavage required a long time (40 min) at a high intensity
of UV light (200-W mercury arc lamp) (property iii) and
resulted in only 46% recovery of a modified peptide hormone
having the spacer arm and 2-nitrosobenzaldehyde moiety still
covalently attached (property iv).

In general, photocleavable biotin reagents such as PCB-
NHS ester overcome one of the most critical limitations of
avidin–biotin technology—i.e., the irreversibility of the avidin–
biotin interaction—without sacrificing the high affinity of
this interaction. PCBs are expected to be useful in a variety
of applications relating to the isolation of biological mole-
cules. Suitable target biomolecules include proteins, nucleic
cacids, carbohydrates, lipids, and macromolecular assemblies.
PCB should also be useful in the isolation of cells expressing unique
cell-surface antigen which are targets of PCB-labeled antibodies.
In addition to reacting directly with biomolecules, PCBs can also
be incorporated during their synthesis. For example, PCBs can be
introduced into nascent proteins during in vitro synthesis by using
suitable aminocetyl-riRNAs, such as lysyl-riRNA, that will select-
ively react with PCB-NHS ester. This will be especially useful
for the isolation of nascent proteins modified by site-directed isotope
labeling (20) or site-specific nonnative amino acid replacement
(21, 22).

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