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Synthesis and application of a photoaffinity analog of dehydroepiandrosterone (DHEA)

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ABSTRACT

We have synthesized an analog of dehydroepiandrosterone (DHEA, **1**) containing both a benzophenone (BP) and a biotin (Bt) group (DHEA–BP–Bt, **8**). Compound **8** was prepared by functionalization on C-17 of **1**. Biocytin was reacted with 4-benzoylbenzoic acid and the product was condensed with **1** containing a diamine–hexane linker. We detected specific protein bands of approximately 55, 80, and 150 kDa by SDS–PAGE analysis of vascular endothelial cell plasma membranes which had been photoirradiated in the presence of **8**.

There is extensive epidemiological evidence that plasma concentrations of the adrenal steroid dehydroepiandrosterone (5-androsten-3β-ol-17-one, DHEA, **1**) are related to vascular function.[1–5] Hormonal steroids activate well characterized intracellular receptors, but may also activate cellular signaling by binding to receptors expressed at the plasma membrane.[6] However, there is no known receptor for **1** and the molecular mechanisms underlying its putative vascular benefits are unknown.[7] We and others have shown that **1** binds to specific, high affinity, plasma membrane sites on vascular endothelial and other cells, to activate cellular signaling in a G-protein-dependent manner.[8–10] Interaction of **1** with this plasma membrane receptor has been proposed as a potential mechanism of its vascular effects.[8] Characterization of the putative receptor involved in this pathway is crucial to advance these studies.

Many investigators have successfully used the high affinity of avidin for biotin in protocols to affinity purify hormonal receptors by initially biotinylating the cognate ligand. Thus, biotinylated derivatives of the natural ligands have been extensively used to characterize receptors expressed in the plasma membrane, including both G-protein coupled receptors[11,12] and receptor tyrosine kinases.[13,14] Furthermore, biotin modified steroids have been used to study intracellular receptors for these hormones.[15–18] The biotinylated ligands rapidly bind to both their cognate receptor and to immobilized avidin, allowing efficient affinity capture of the receptor–ligand complex by avidin chromatography. To date there are few publications detailing the use of biotinylated steroids to identify plasma membrane bound steroid receptors.[19]

While the use of biotinylated ligands for receptor isolation has been a great advance in this field, the solubilization of plasma membrane proteins, required for receptor purification, can significantly decrease the affinity of the receptor for the biotinylated ligand and impair the ability to isolate low abundance receptors by avidin chromatography. Modification of ligands to include photoirradiation-activated cross-linking groups can facilitate extensive solubilization without loss of ligand receptor interaction after cross-linking.[20,21] We therefore took advantage of photoaffinity cross-linking and avidin–biotin affinity techniques, to design and synthesize a novel photoactivatable biotinylated analog of **1**, in order to isolate the high affinity plasma membrane DHEA binding site.

A convergent approach was designed to prepare the DHEA ligand carrying a benzophenone-containing photoaffinity label and biotin,[22] attached at the 17 position of **1** based on our previous structure–activity data.[8] A six-carbon linker was attached to **1** by

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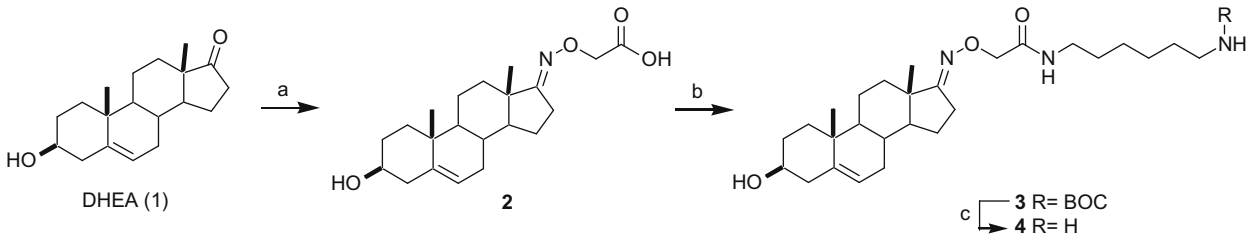

Figure 1. Synthesis of 17-(*O*-carboxymethyl)oxime derivative of **1**. Reagents and conditions: (a) carboxymethylamine, pyridine, 60 °C; (b) *N*-Boc-diaminohexane, DEAC, diisopropylethylamine, DMF; (c) HCl in dioxane.

a carboxymethyl oxime group. Treatment of **1** with carboxymethylamine in pyridine delivered the 17-(*O*-carboxymethyl)oxime derivative **2**, Figure 1. The carboxylic acid **2** was then coupled with *N*-Boc-hexyldiamine to give amide **3**. *N*-Boc de-protection was carried out under acidic conditions to deliver amine hydrochloride **4**. 4-Benzoylbenzoic acid **5** was activated with *N*-hydroxysuccinimide to give the *O*-succinimide ester **6**, Figure 2. Biocytin was then treated with sodium hydroxide and the activated benzophenone **6** was added to give compound **7** (designated benzophenone–biotin or BP–Bt).[22] Finally, coupling of the DHEA amine **4** and the biotinylated benzophenone **7** gave the desired photoaffinity ligand **8**, (designated DHEA–benzophenone–biotin or DHEA–BP–Bt), Figure 3. Characterization of all intermediates by ^{1}H and ^{13}C NMR spectroscopy confirmed the structure of the compounds.

We next tested this novel analog of **1** in a photolabeling strategy to detect the plasma membrane DHEA binding protein by PAGE and chemiluminescence. Solubilized plasma membranes, prepared from bovine aortic endothelial cells, were incubated with **8**, exposed to UV light for 15 min, and separated by SDS-PAGE. Protein bands of approximately 55, 80, and 150 kDa were seen in samples incubated with **8**. This labeling was DHEA specific since it was competed by **1** and no such labeling was seen in the absence of UV cross linking or when samples were incubated with **7** rather than **8**, Figure 4. These data demonstrate that compound **8** can efficiently and specifically identify the DHEA binding protein(s) and suggest that **8** will be suitable for isolating the DHEA receptor by avidin–agarose chromatography.

In conclusion, the aim of this study was to develop a reagent to facilitate isolation of the putative plasma membrane DHEA receptor. We and others have shown that this receptor has high affinity for DHEA (K_d = 48.7 pM), is expressed on plasma membranes, and is linked to the activation of G_i proteins.[8,10] We have synthesized a novel analog of **1** containing both a Bt group and a photoactivatable BP group. This novel photoaffinity ligand (**8**) was prepared by functionalization on C-17 of **1**. Biocytin was reacted with 4-benzoylbenzoic acid and the product was condensed with **1** containing

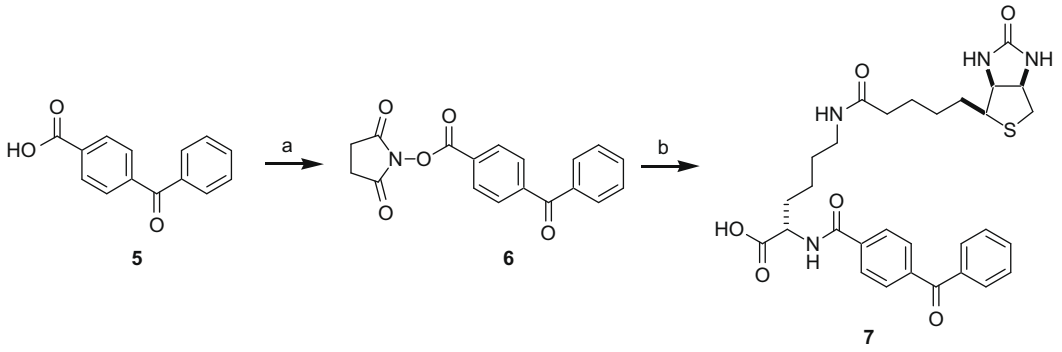

Figure 2. Synthesis of compound **7**. Reagents and conditions: (a) *N*-hydroxysuccinimide, DEAC, CH_2Cl_2, 93%; (b) biocytin, NaOH, H_2O–DMF, 82%.

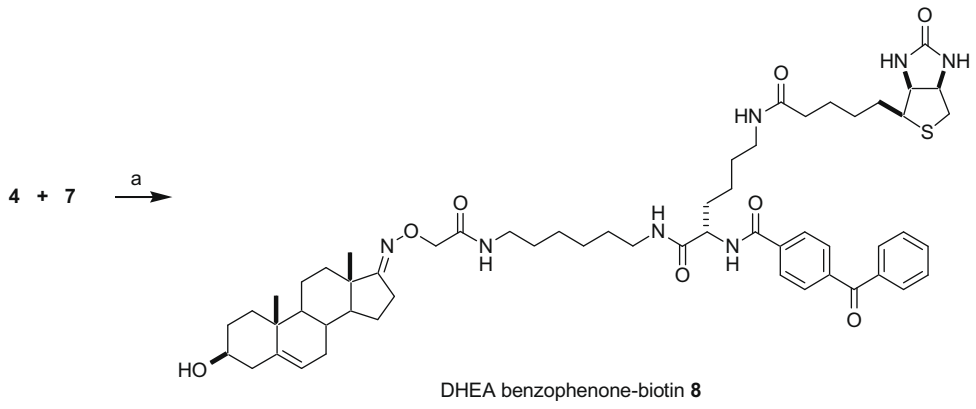

Figure 3. Synthesis of compound **8**. Reagents and conditions: (a) HOBt, Hunig's base, EDAC, DMF, 40%.

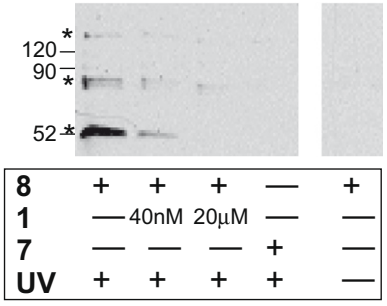

Figure 4. Compound **8** binds specifically to plasma membrane proteins of BAEC. Plasma membranes were photoirradiated in the presence of **8**, **7**, and **1**, as indicated. Membranes were separated by SDS–PAGE, transferred to nitrocellulose, incubated with avidin-conjugated horse-radish peroxidase, and the labeled proteins visualized by chemiluminescence. Specific molecular weights are indicated in kDa. Proteins binding to **8** in a DHEA specific manner are indicated with *.

a diamine–hexane linker. The structure and purity of the photoaffinity ligand were determined by nuclear magnetic resonance and mass spectroscopy. We have demonstrated that **8** can be used in strategies to identify the plasma membrane DHEA receptor. We plan to use **8** as an immobilized affinity reagent to capture DHEA binding proteins from plasma membrane preparations of vascular endothelial cells and to identify the bound proteins by mass spectrometry. Apart from the identification of the plasma membrane DHEA receptor, this work may provide a general strategy to facilitate the identification and characterization of other cell surface-expressed steroid hormone receptors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.019.

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