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Membrane Receptor Probes: Solid-Phase Synthesis of Biotin-Asp-PEG-arvanil Derivatives

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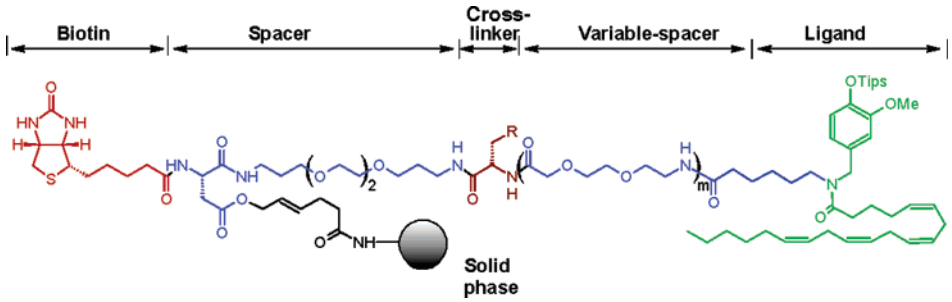
ABSTRACT



A modular, flexible solid-phase synthetic route for the preparation of biotinylated cross-linking probes of membrane receptors is described. The route utilizes an orthogonal protection strategy employing a Pd[0] cleavable allyl linker attached to the probe via an aspartate residue. The versatility of the method is illustrated through the synthesis of a number of arvanil-derived cannabinoid receptor ligands displaying either a photoaffinity or a chemical cross-linking group.

The biotinylation of small molecules exploits the uniquely tight biotin−avidin interaction for diverse applications such as detection, visualization, and purification/isolation of proteins. Innovative technological developments continue to appear, including the Scavidin gene therapy system for targeting of biotinylated therapeutics,[1,2] extracorporeal affinity adsorption[3] (for removal of radiolabeled antibodies), and biotinylated activity-based probes for chemical proteomics.[4] In this letter, we describe a flexible solid-phase method for the synthesis of biotinylated, cross-linking, small-molecule probes. The synthetic strategy is modular, based on Fmoc peptide synthesis to allow simple variation of subunits. The chemistry is conducted manually, using readily available plastics, and can be carried out in most chemistry laboratories. The methodology should be applicable to a wide variety of biotinylation problems. Previously, biotinylated adenosine 5-triphosphate analogues have been utilized for the enrichment of membranes carrying the P2Y(1) receptor,[5] while bifunctional small-molecule probes (containing biotin and a cross-linking group) have been used to identify binding sites in proteins.[6] We sought to identify a small-molecule probe capable of cross-linking partially characterized can-

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nabinoid receptors.[7,8] The endocannabinoid anandamide **1**, SR141716A **2**, and WIN55,212-2 **3** have affinity[9] for these novel receptors (Figure 1), as does arvanil **4**, an anandamide-

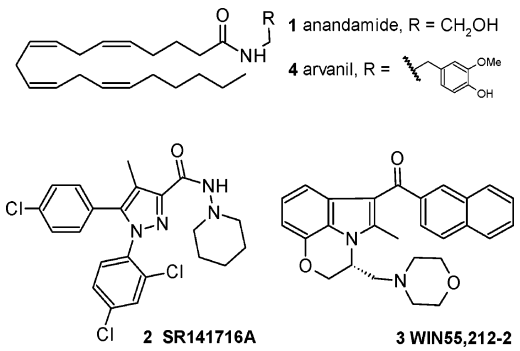
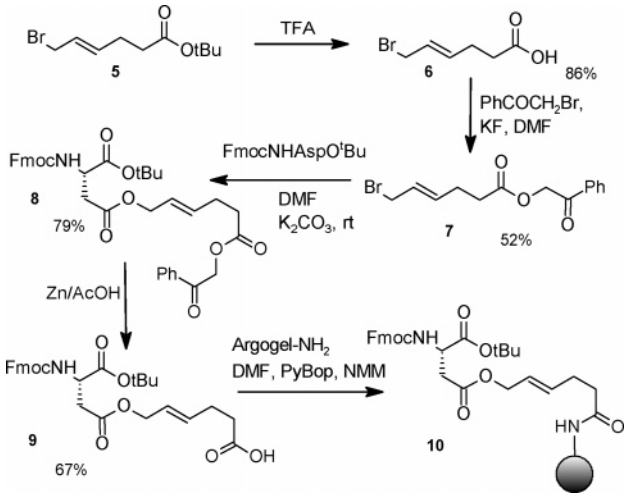


Figure 1. Cannabinoid ligands.

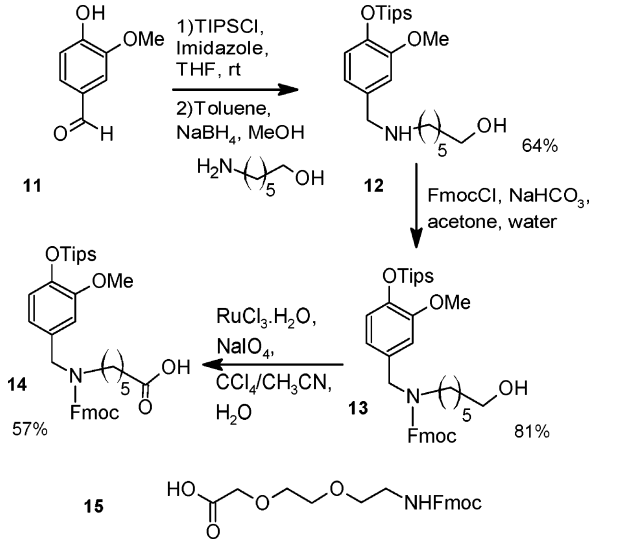
capsaicin hybrid that activates non-CB_1, non-CB_2, and non-VR_1 receptors.[8] Thus, we chose arvanil as the initial ligand for tagging. We identified the 6-hydroxy-4-hexenoate allyl ester linker[10] of Nakahara as being acid and base stable and cleavable under nearly neutral Pd[0] catalysis conditions. The stability of the arachidonate double bonds in arvanil was tested under the Pd[0] cleavage conditions; no isomerization was detected in the olefinic region by NMR (see Supporting Information). In work on the use of biotin-linked reagents for antibody pretargeting, Wilbur[11] established that a proximal aspartate gave molecules resistance to biotinidases in vivo. Incorporation of a proximal aspartate is therefore a desirable feature and also provides a convenient attachment point to the solid phase. The synthesis of the complete linker is shown in Scheme 1. Thus, 6-bromo-hex-4-enoic acid *tert*-

Scheme 1. Synthesis of the Allyl Aspartate Linker



butyl ester **5** was cleaved to the acid with TFA and reacted with phenacyl bromide to obtain the ester **7**. The coupling reaction between **7** and FmocNH−Asp-O^{t}Bu in the presence of K_2CO_3 in DMF gave, in good yield, the orthogonally protected aspartate **8**. After removal of the phenacyl group by treatment with zinc in acetic acid, the acid **9** was reacted with Argogel-NH_2 resin to give the desired aspartate-loaded resin **10**. The synthesis of the arvanil precursor is shown in Scheme 2. We chose the amide NH as the connection point

Scheme 2. Synthesis of Arvanil Precursor



to biotin, as it was straightforward synthetically and there was evidence that substitution at this point would not abolish the biological activity.[8] In contrast, the hydroxyl moiety on the aromatic ring seems to be required. Tips protection of *o*-vanillin, **11**, followed by reductive amination with amino-hexan-6-ol provided **12**, Fmoc protection to **13**, and oxidation with $RuCl_3/NaIO_4$ gave the required intermediate **14**. To establish the optimum linker length,[12] we needed to be able to vary the distance between the biotin and ligand easily. Fmoc-8-amino-3,6-dioxaoctanoic acid **15** is a convenient PEG spacer molecule. It was easy to synthesize and could be inserted using standard peptide synthesis protocols. In the first instance we prepared three molecular probes, which differed in the number of spacer units **15** used (Scheme 3). Thus, the resin-linked aspartate **10** was treated with 20% piperidine in DMF to remove the Fmoc protection. Then,

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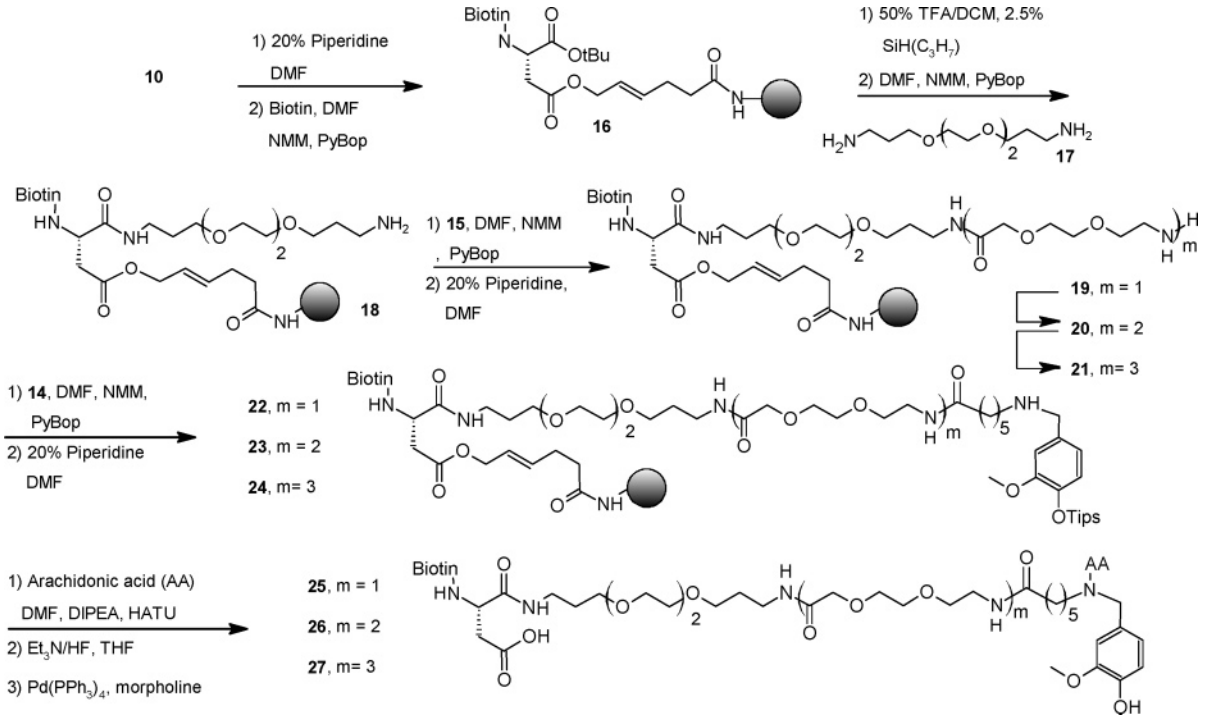
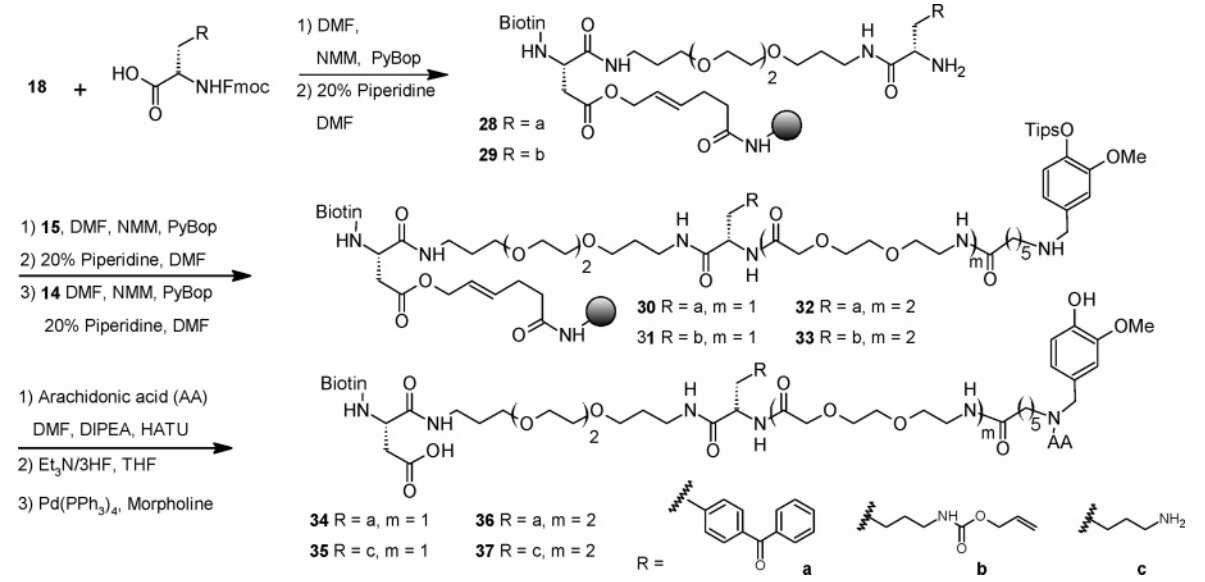
Scheme 3. Synthesis of the Biotin-PEG Ligands without Cross-Linkers



the free amine compound was coupled with biotin using PyBop and *N*-methyl morpholine in DMF. From the resin **16**, the *tert*-butyl protection was removed by treatment with 50% TFA in CH_2Cl_2, containing 2.5% of tri-*n*-propylsilane as scavenger. The resin was then reacted with the PEG-diamine **17** in the presence of PyBop and *N*-methyl morpholine in DMF. The intermediate **18** was then coupled with the spacer **15** using the above coupling conditions to give **19**. Appropriate repetition of this coupling procedure gave, after Fmoc deprotection, the resin-linked compounds **20** and **21**. Resins **19**−**21**, were coupled under the usual conditions with the acid **14** to give, after Fmoc deprotection, the intermediates **22**−**24**, respectively. Two cycles of the last coupling reaction, with arachidonic acid, were necessary, using HATU as a coupling reagent in the presence of DIPEA, in DMF. Tips deprotection with triethylamine trihydrofluoride in THF followed by cleavage from the solid support using $Pd(Ph_3P)_4$ and morpholine in $CHCl_3$ gave **25**−**27**. These molecular probes were purified by HPLC and characterized using mass spectrometry techniques. For **26**, the

Scheme 4. Synthesis of Biotin-PEG Ligands with a Cross-Linking Group



structure was confirmed by two-dimensional ^{1}H NMR (see Supporting Information). To consolidate the binding between a small-molecule ligand and its receptor, a covalent cross-link is frequently required.[13] We designed a series of compounds containing a lysine unit, allowing attachment of a thiol-specific group such as malemide (for cross-linking to a cysteine residue). Alternatively, a benzophenone unit, which is commonly employed for photoaffinity labeling, was incorporated.[14,15] The optimal distance of the cross-linking group from the ligand was explored with a series of four molecules using the spacer **15** as a repeating unit (Scheme 4).

Coupling of the amino acids with **18** gave, following the route described above, four potential cross-linking ligands: **34**−**37**. Preliminary biological evaluation used a standard cannabinoid CB_1 binding assay employing displacement of [^{3}H] SR141716A **2** in rat brain membranes. In this case, we are utilizing CB_1 as a model for the novel cannabinoid receptors. When no cross-linker was present, the binding was relatively insensitive to the number of units of **15** (see Table 1 compounds **25**−**27**). With either the 4-PhCO-phenylalanine or lysine cross-linking groups (compounds **34** and **35**) one unit of **15** was optimal.

36 also relaxed precontracted rat small mesenteric artery (EC_{50} 30 nM), which was in part endothelium-dependent, sensitive to both capsaicin and capsazepine (showing interaction with vanilloid receptors), and in some preparations it was SR141716A **2** sensitive, indicating interaction with the abnormal cannabidiol receptor.

Table 1. Radioligand CB_1 Binding Assay

probe	units of **15**	cross-linker	IC_{50} μM
25	1	none	0.7
26	2	none	3.3
27	3	none	1.0
34	1	4PhCOPh	4.0
35	1	Lys	21.4
36	2	4PhCOPh	2.6
37	2	Lys	ND[a]

[a] ND not determined.

In summary, we have developed a flexible solid-phase synthesis for biotinylation utilizing a stable allyl-Asp linker. The synthetic method is compatible with a wide range of reaction conditions, including Fmoc and Boc peptide chemistry, and should have wide applicability.

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Supporting Information Available: Detailed synthetic methods and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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