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Positional linker effects in haptens for cocaine immunopharmacotherapy

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Abstract—Cocaine use remains a serious problem, despite intensive efforts to curb abuse. Given the lack of effective pharmacotherapeutics for the treatment of cocaine addiction, research groups have targeted immunopharmacotherapy in which the drug user's immune system is trained to recognize and remove cocaine prior to entry into the central nervous system. Antibody cocaine esterases and simple binders have been procured, however, rates and/or affinities still need improvement before clinical trials are warranted. Herein, we report the synthesis and testing of two new haptens for the procurement of cocaine binding antibodies and cocaine esterase catalytic antibodies. Central in the design of these haptens was the placement of the linker functionality distal from the anticipated cocaine epitopes in an attempt to bury the hapten deep within an antibody combining site to gain possible entropic and enthalpic advantages.

Abuse of the psychostimulant cocaine (**1**), one of the most addictive stimulants that directly affects the brain, is a continuing medical, social, and political problem in many areas of the world. According to the 2005 National Survey on Drug Use and Health (NSDUH), an estimated 2.4 million people are current cocaine users, an increase over the 2 million users in 2004.[1] Despite intensive research efforts, there are no medications with proven efficacy for the treatment of cocaine addiction. Pharmacotherapies that are typically designed to block the central neurochemical effects of cocaine have had limited success, because their actions are nonselective and thus often generate adverse side effects. Furthermore, given the mechanism of action of cocaine as a dopamine transport blocker, the inherent difficulty in antagonizing an interaction such as this has led many laboratories, including our own, to explore protein-based therapeutics for the treatment of cocaine addiction.

Recently, immunopharmacotherapy for cocaine abuse has been explored as a possible alternative approach.[2] This strategy is based on the synthesis of a cocaine vaccine that induces the production of anti-cocaine antibodies. The antibodies bind the cocaine specifically to inhibit the penetration into the CNS, thus this approach can avoid the side effects of traditional pharmacotherapy. In a series of investigations from our laboratory, we have reported two effective haptens GNC (**2**)[3] and GND (**3**)[4] that resemble the molecular structure of cocaine (Fig. 1). Active immunization of rodents with a GNC-keyhole limpet hemocyanin (KLH) conjugate reduced the psychoactive effects of cocaine, and lowered the cocaine levels in the CNS of treated rodents,[2] while immunization with GND–KLH resulted in a dramatic suppression of the psychomotor stimulant effect of cocaine.[3]

These, and other, previously reported haptens[4] have been designed with a linker moiety positioned at the cocaine methyl ester functionality, primarily for ease of preparation. However, placing the linker here also may decrease efficient antibody binding as it may compromise the burying of key epitopes, that is, the benzoyl and methyl esters within the antibody combining site.[5] Herein, we report the synthesis of haptens AI1 (**4**) and

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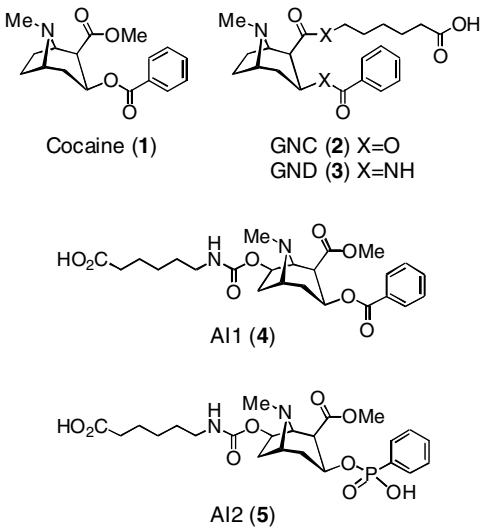
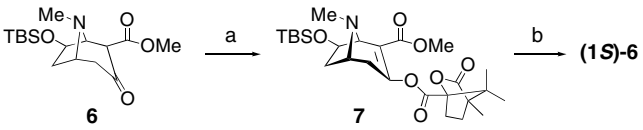


Figure 1. Structures of cocaine and haptens GNC, GND, AI1, and AI2.

AI2 (**5**), designed as optimized haptens for immunopharmacotherapy of cocaine abuse, and the ability of antibodies induced from these haptens to bind cocaine.

We envisioned that by positioning the linker distal from the native ester functionalities present in cocaine, an improved immune response would be obtained by allowing the cocaine molecule to be further sequestered within the antibody binding site. This hypothesis is grounded upon crystallographic analysis of a cocaine esterase[6] and an antibody-cocaine complex.[5] Both GNC and GND have a hexanoic acid linker for conjugation with a carrier protein at the C-2 methyl ester moiety. Alternatively, haptens **4** and **5** have the linker attached at the C-7 position by means of a carbamate spacer. In essence, this tact places the carrier protein a greater distance away from the three anticipated epitopes of cocaine, that is, the methyl ester, benzoate, and *N*-methyl amino moieties. Although the importance of the two-carbon bridge region for cocaine binding receptor topography has been suggested,[7] it is expected that antibodies induced from haptens **4** and **5** will recognize cocaine better than antibodies from GNC or GND.

Although a large number of cocaine derivatives have been synthesized, only a few chiral derivatives that possess substituents at the C-7 position have been reported. We began the synthesis of hapten **4** from known racemic 7β-(*tert*-butyldimethylsilyloxy)-2β-(methoxycarbonyl)-tropan-3-one (**6**), which can be prepared from 2,5-dimethoxy-2,5-dihydrofuran.[8] In our previous immunopharmacotherapy programs, a critical feature of all haptens has been the presence of the natural stereochemistry. In order to obtain enantiopure **6**, we performed an optical resolution of the racemic compound and achieved separation of the derived diastereomers, following the method of Meltzer et al. (Scheme 1).[9] Racemic **6** was reacted with (1′*S*)-camphanic chloride to give diastereomeric esters that could be separated by column chromatography and subsequent recrystallization. The stereochemistry was confirmed by single-crystal X-ray analysis (Fig. 2).[10] Meltzer et al. obtained the (1*R*,1′*S*) diastereomer and abandoned the isolation of (1*S*,1′*S*) diastereomer by this procedure in which the protecting group of C-7 alcohol was a MOM group instead of TBS.[9] It is interesting to note that by changing the nature of the protecting group, a reversal in the crystalline isomer was observed.



Scheme 1. Optical resolution of compound **6**. Reagents and conditions: (a) i—(1*S*)-(−)-camphanic chloride, Et_3N, CH_2Cl_2, rt; ii—diastereomer separation, 38%; (b) LiOH, quant.

To complete the synthesis of the desired hapten, the synthetic route of Kozikowski et al. used for the synthesis of racemic 7-hydroxylated cocaine was employed (Scheme 2).[11] Thus, Na–Hg reduction of (1*S*)-**6** gave (1*S*)-**8** as a minor product along with major 2α-isomer. Benzoylation and subsequent cleavage of the silyl ether gave chiral (1*S*)-**9** in good yield. Introduction of the linker moiety was achieved by activation with 4-nitrophenyl chloroformate, followed by reaction with benzyl 6-aminohexanoate. Finally, the benzyl group was removed to give the target hapten **4** which subsequently was coupled to the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). Coupling efficiency for the BSA immunoconjugate was estimated to be 27:1 by MALDI-TOF analysis.

The KLH conjugate was then used to immunize 129GIX+ mice followed by a booster injection after 1 week. Mice were then bled after an additional week and antibody titers measured by ELISA using the **4**-BSA immunoconjugate. For comparison, the titer of a comparable immunization of GNC–KLH also was measured. Both haptens resulted in robust immune responses with measured titers of greater than 25,600.

In an alternative pharmacotherapeutic approach, cocaine esterase catalytic antibodies have been envisioned as a potentially powerful tool in treating cocaine addiction.[12] These antibodies would then overcome the requisite stoichiometry of cocaine binding antibodies, and

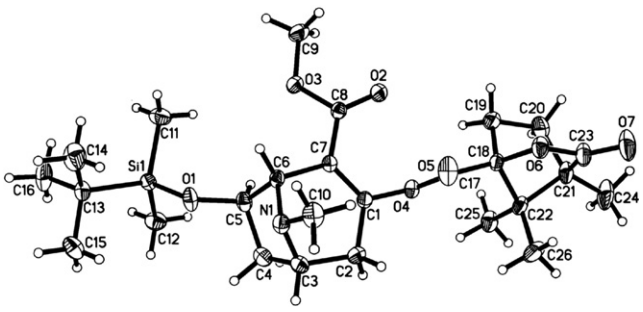
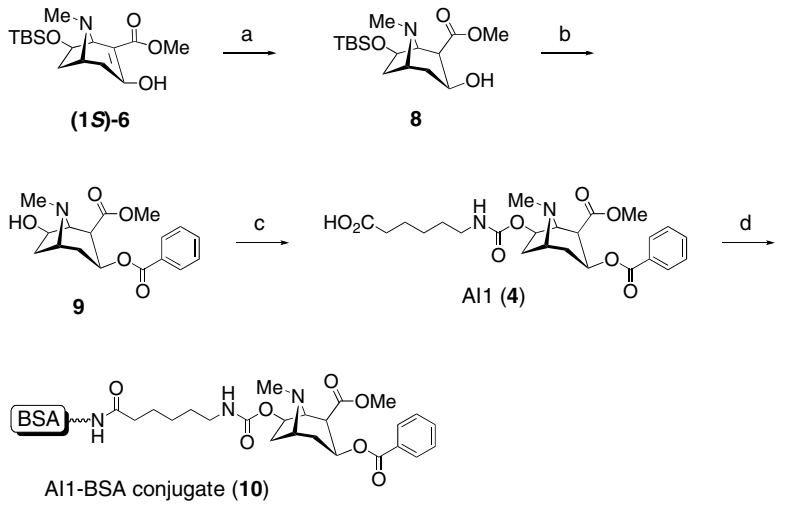
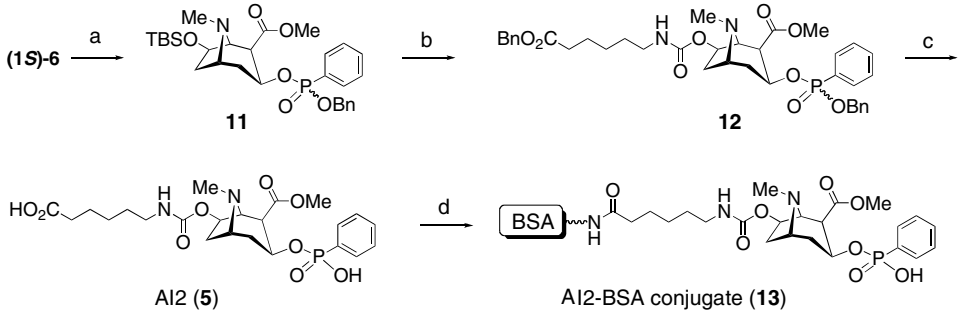


Figure 2. X-ray crystallographic structure of compound **7**.



Scheme 2. Synthesis of hapten AI1 immunoconjugate. Reagents and conditions: (a) Na–Hg, aq H_2SO_4, 9%; (b) i—BzCl, Et_3N, DMAP; ii—TBAF, 84%; (c) i—4-nitrophenoxy chloroformate, Et_3N, DMAP; ii—benzyl 6-aminohexanoate; iii—H_2, Pd/C, 70%; (d) i—Sulfo-NHS, EDC; ii—BSA, PBS, coupling efficiency = 27:1.



Scheme 3. Synthesis of AI2 immunoconjugate. Reagents and conditions: (a) i—$PhPOCl_2$, 1*H*-Tetrazole, *i*-Pr_2NEt then BnOH; ii—TBAF, 89%, (b) i—4-nitrophenoxy chloroformate, Et_3N, DMAP; ii—benzyl 6-aminohexanoate, 59%; (c) H_2, Pd/C, quant; (d) i—Sulfo-NHS, EDC; ii—BSA, PBS, coupling efficiency = 7:1.

thus provide a mechanism to avoid saturation at larger drug doses. However, much like previously developed cocaine binding antibodies, the haptens used in these studies have linkers positioned on either the cocaine methyl ester or the tropane nitrogen atom.[12] Antibodies generated using this tact, while viable cocaine esterases, still fall well short of the needed pharmacokinetic rates for potential clinical utility. The most efficient cocaine esterases are of bacterial origin and place the two cocaine esters deep within the enzyme binding cleft. In an attempt to model such interactions in an antibody binding site, repositioning of the linker was needed. Hence, we envisioned that by moving the linker distal to the 3′-transition state moiety, better presentation of the hapten may be achieved, resulting in more efficient catalysis.

Our synthesis utilized the same enantiopure intermediate **6** as was used in the preparation of **4**. Installation of the benzyl phosphonate moiety,[13] followed by TBS deprotection and linker installation proceeded in acceptable yield to give protected hapten **12**, which was then hydrogenated to yield the desired hapten **5** (Scheme 3). This compound was then coupled to BSA and KLH, and the KLH immunoconjugate injected into mice. Again, good antibody titer was observed after immunization followed by a single booster injection, as measured by ELISA.

Using the sera from both AI1 and AI2 mice, competition studies were performed to assess antibody recognition of free cocaine. Unfortunately, these studies revealed that both haptens bound cocaine rather poorly ($K_{d,app} \geqslant 400$ μM) relative to GNC ($K_{d,app} \sim 12.5$ μM). Further competition experiments demonstrated that the antibodies resulting from immunization with either hapten more efficiently bound benzoyl ecgonine ($K_{d,app} \sim 25$ μM), the product of cocaine methyl ester hydrolysis and primary cocaine metabolite. While this compound is also psychoactive[14] and thus could be viewed as a target for immunotherapy, a viable vaccine must be able to bind the parent drug prior to CNS entry and any subsequent neurochemical effects. From a mechanistic standpoint, the lability of this ester is presumed to stem from neighboring-group participation by the tropane nitrogen, as previously characterized with cocaine, but not pseudococaine or *N*-acylnorcocaine.[15]

In light of these results, it is apparent that the characteristics of our previously reported GNC hapten are uniquely optimized for vaccine development in the context of stability and immunogenicity. Future efforts to increase sequestration of cocaine within an antibody binding site for increased binding affinity or catalytic degradation will require the replacement of the cocaine methyl ester with more stable functionalities that do not compromise the molecular recognition (e.g., amides). Studies along these lines will be reported in due course.

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