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Synthesis of a Deuterated 6-AmHap Internal Standard for the Determination of Hapten Density in a Heroin Vaccine Drug Product

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Abstract

A deuterated hapten was designed and synthesized that will be essential for a future study of residual hapten and stability of a hapten-protein conjugate. This hapten, 6-AmHap, was chosen for a heroin vaccine that is now slated for a Phase 1 clinical trial. A maleimide-thiol bioconjugation strategy was successfully applied to our heroin vaccine to connect the hapten 6-AmHap with an immunogenic carrier protein (tetanus toxoid, TT) through a trityl-protected 3-mercaptopropanamide linker. The antibodies induced by the vaccine have been found to have activity against several opioids, including heroin and its metabolites, and, importantly, leave alternate pain treatment medications such as methadone untouched. To the best of our knowledge, no other hapten for a heroin vaccine has been deuterated, yet this tool may prove to be of great importance in the study of residual hapten during product release and the long-term stability program of a hapten-protein conjugate as part of FDA regulatory requirements. Hydrocodone was the starting material for the synthesis of the deuterated 6-AmHap, with a stable amide at C6 and a 3-mercaptopropanamide linker attached at C3. The desired deuterated product was prepared as the

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Author Contributions

The manuscript was written with contributions from all the authors, and all authors have given approval of the final version of the manuscript.

Disclosures

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disulfide, 3,3'-disulfanediyldis(*N*-((7*S*,7*aR*,12*bS*)-7-acetamido-3-[²H₃]methyl)-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)propanamide), that could be easily reduced to form the needed hapten, *N*-((4*aR*,7*S*,7*aR*,12*bS*)-7-acetamido-3-[²H₃]methyl)-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)-3-mercaptopropanamide.

Keywords

Deuterated hapten; heroin vaccine; 6-AmHap; hapten-protein conjugate; preclinical study

1 INTRODUCTION

The misuse of drugs like heroin and fentanyl has been the cause of an extraordinary number of deaths. In 2017, the Centers for Disease Control and Prevention noted that 68% of the 70,000+ overdose deaths involved an opioid.¹ Continued misuse of opioids has been found, by brain imaging studies, to cause physical changes in brain areas critical for judgment, decision-making, learning, memory, and behavior control. Addiction to heroin, or other opioids, is a chronic disorder that is treatable using combinations of medications and behavioral interventions.² Medications such as methadone and buprenorphine have had success and, unfortunately, many failures. Too frequently, recidivism occurs,³ and although drug relapse has been treated with extended-release naltrexone,⁴ alternate methodologies have been sought.

A relatively new methodology based on immunopharmacology is currently under investigation as a potential modality for abuse disorder treatment or intervention for drug overdose. This has been considered since 1974,⁵ and has culminated in the preclinical exploration of a heroin vaccine that utilizes the hapten, 6-AmHap.^{6–9} This vaccine has been found, in rodents, to be active against several opioids, including heroin and its metabolites. However, alternate pain treatment medications, e.g., methadone, are unaffected by this vaccine. The vaccine is composed of a hapten (6-AmHap), a linker, an immunogenic carrier and an adjuvant. The 6-AmHap contains a hydrolytically stable amide at C6 and a trityl-protected 3-mercaptopropanamide linker for bioconjugation at the C-3 position. The hapten was covalently attached to an immunogenic carrier protein (tetanus toxoid, TT) to form the TT-6-AmHap conjugate and to it was added an adjuvant (Army Liposome Formulation with 43% cholesterol and aluminum hydroxide, ALF43A).^{10, 11}

The amount of residual haptens—which may serve as a measure of purity and stability—is an important parameter to consider in developing a drug product.¹² Residual haptens may result from 1) the incomplete removal of excess haptens during the conjugation process, and 2) the cleavage of attached haptens during the long-term storage of the protein-hapten conjugate. Based on our stability study, the hapten-protein conjugate had good stability, losing only about 1–2 haptens/year after storage at 2 – 8 °C. Hapten density, or the number of haptens covalently attached to a protein molecule, is an essential determinant of vaccine efficacy.¹⁰ Because the conjugate is slated for a Phase 1 clinical trial, it is essential to develop a quantitative method for determining the stability of the TT-6-AmHap over time to comply

with the standard regulatory requirements of the U.S. Food and Drug Administration (FDA) on the stability of a drug product formulation.¹²

Hapten density of a protein-hapten conjugate is typically estimated using either spectrophotometry or mass spectrometry.^{13, 14} The change in hapten density over time can be determined via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) using a method of mass difference.¹³ However, because the molecular weight of the hapten is too small compared to the average weight of the conjugate, minimal loss of hapten could not be measured accurately. Alternatively, the cleaved haptens during storage can be directly measured. Measurement of these cleaved haptens via a spectrophotometric-based method is challenging due to the low limit of detection (typically in μM levels). In general, quantification of small molecules (e.g., hapten, ligand) in biological samples is performed using liquid chromatography tandem mass spectrometry (LC-MS/MS).¹⁵ Stable isotope labeled versions of the analyte are often used as internal standards in quantitative LC-MS/MS.¹⁵ These standards are spiked to samples and calibration standards to reduce analytical variability and to enable accurate determination of absolute concentration. We report herein the synthesis of deuterium-labeled 6-AmHap for use as a suitable internal standard for LC-MS/MS analysis.

2 RESULTS AND DISCUSSION

In order to obtain a suitable deuterated hapten, we have designed and synthesized an *N*-[²H₃]methyl relative of 6-AmHap. Theoretically, two of the potential sites for [²H₃]-methylation on our hapten might be at C6, or at C17 on the tertiary nitrogen atom (carbon numbering for opiates, Scheme 1). The former position is susceptible to hydrolysis and is a labile functional group which is cleaved off as acetic acid during MS/MS fragmentation.¹⁶ The *N*-methyl moiety of 6-AmHap was well suited for deuteration.

The [²H₃]methyl group was conveniently introduced in a sequence of an *N*-demethylation reaction and subsequent alkylation of the resulted *N*-nor intermediate with iodo[²H₃]methane. Furthermore, deuterium labelling of the chemically stable *N*-methyl group assured retention of the isotopic enrichment of the final product and thus usefulness of the deuterated 6-AmHap in the envisioned stability studies. The target compound was the disulfide (**9**), 3,3'-disulfanediybis(*N*-((7*S*,7*aR*,12*bS*)-7-acetamido-3-[²H₃]methyl-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)propanamide). It was chosen for its stability and its ability to be easily converted to the deuterated 6-AmHap (**10**), *N*-((4*aR*,7*S*,7*aR*,12*bS*)-7-acetamido-3-[²H₃]methyl-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)-3-mercaptopropanamide. 6-AmHap **10** was expected to be less stable than the disulfide **9** for long-term storage, based on studies with the non-deuterated compound that was synthesized under Good Manufacturing Practices (GMP) conditions.

As shown in Scheme 1, hydrocodone (**1**), was subjected to *N*-demethylation to obtain norhydrocodone (**2**)¹⁷ in 70% yield over 2 steps. Deuterium was introduced by methylation using iodo[²H₃]methane.^{18, 19} Reaction proceeded with a 56% yield of desired product **3** and the starting norhydrocodone (**2**) was isolated and recycled. Deuterium incorporation was

determined by ^1H NMR spectroscopy and ESI-MS. The ^1H NMR spectrum of **3** revealed disappearance of the singlet at 2.26 ppm corresponding to the *N*-methyl group of hydrocodone (Fig. S1, Supporting Information). ESI-MS analysis of **3** displayed $[\text{M} + \text{H}]^+$ peaks at m/z 303.2, 302.2, 301.2 and 300.2, designating $(\text{C}_{18}\text{H}_{19}^2\text{H}_3\text{NO}_3)$, $(\text{C}_{18}\text{H}_{20}^2\text{H}_2\text{NO}_3)$, $(\text{C}_{18}\text{H}_{21}^2\text{H}_1\text{NO}_3)$ and $(\text{C}_{18}\text{H}_{22}\text{NO}_3)$, respectively (Fig. S2, Supporting Information). The ion intensities observed for these peaks were 2,476,456; 5,089; 1,053 and 675, correspondingly. The intensity of ion designated as $(\text{C}_{18}\text{H}_{19}^2\text{H}_3\text{NO}_3)$ divided by the sum of intensity of ions $(\text{C}_{18}\text{H}_{19}^2\text{H}_3\text{NO}_3)$, $(\text{C}_{18}\text{H}_{20}^2\text{H}_2\text{NO}_3)$, $(\text{C}_{18}\text{H}_{21}^2\text{H}_1\text{NO}_3)$ and $(\text{C}_{18}\text{H}_{22}\text{NO}_3)$ and multiplication of the quotient by 100% indicated 99.5% deuterium incorporation. *O*-Demethylation of **3** provided the phenol **4**. The further steps of the synthesis were accomplished based on that of heroin vaccine hapten reported previously.⁷ The triflate **5** was formed in the reaction of **4** with *N*-phenyl-bis(trifluoromethanesulfonylimide). Amidation was performed by applying palladium-mediated Buchwald-Hartwig type coupling conditions and yielded the C3-amide **6** which was subjected to hydrolysis with subsequent Boc protection of the C3-amino moiety to give **7**. Reductive amination allowed the transformation of C6-oxo functionality to the C6-amide **8**. Boc deprotection was followed by coupling with freshly prepared 3,3'-disulfanediyl dipropionyl chloride to furnish the stable target disulfide **9** in 68% yield, overall yield 10%, and 97.6% deuterium incorporation (Fig. S3, Supporting Information), which could be further reduced to obtain the deuterated analog of 6-AmHap (**10**). The analytical data of compounds **5-9** were in agreement with the literature values⁷ and with the expected differences resulting from deuterium-labelling.

3 CONCLUSION

An eight-step synthesis provided the stable target disulfide **9** with an overall yield of 10% and 97.6% deuterium incorporation that will be used to study the stability of a protein-hapten conjugate. Few, if any, other haptens have been deuterated for use as an internal standard in assays needed for FDA approval of the vaccine before clinical introduction. Future work in vivo will determine the possible utility of this and other deuterated haptens that may be prepared.

4 EXPERIMENTAL

Iodo[$^2\text{H}_3$]methane (99.5% isotopic purity) was purchased from Sigma-Aldrich. All commercial reagents and solvents were used as received without further purification. Proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded on a Varian Gemini-400 spectrometer in CDCl_3 (unless otherwise noted), with the values given in ppm (TMS as internal standard) and J (Hz) assignments of ^1H resonance coupling. Mass spectra (HRMS) were recorded on a VG 7070E spectrometer or a JEOL SX102a mass spectrometer using an electrospray ionization (ESI) source. Thin-layer chromatography (TLC) analyses were carried out on prepackaged plates using various gradients of $\text{CHCl}_3/\text{MeOH}$ containing 1% of 28% NH_4OH (CMA). Visualization was accomplished under UV light or by staining in an iodine chamber. Flash column chromatography was performed using RediSep Rf normal phase silica gel cartridges. Robertson Microlit Analytical

Laboratories, Ledgewood, NJ 07852 performed elemental analysis and the results were within $\pm 0.4\%$ of the theoretical values.

4.1 (7aR,12bS)-9-Methoxy-2,3,4,4a,5,6-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7(7aH)-one (2).

Hydrocodone (**1**) (7 g, 23 mmol) was dissolved in anhydrous acetonitrile (70 mL) followed by the addition of K_2CO_3 (9.7 g, 69 mmol). After cooling the reaction mixture to 0 °C, a cyanogen bromide (5M, 8.3 mL, 41 mmol) solution in acetonitrile was added dropwise over 30 min. The reaction mixture was left to stir for 3 h allowing to warm up to room temperature. After the reaction was completed based on TLC, the reaction mixture was filtered and concentrated under vacuum. The resulting solid was dissolved in a minimum volume of boiling MeOH and cooled at +4 °C overnight to afford *N*-cyano-intermediate as a crystalline material. A solution of the *N*-cyano-intermediate in 2 *N*HCl (140 mL) was heated at reflux overnight. The solvent was removed in *vacuo* and the residue was triturated with EtOH yielding norhydrocodone hydrochloride salt. Norhydrocodone base (**2**) was isolated from its hydrochloric salt by the treatment with 2 *N*KOH and was additionally purified by flash chromatography using a gradient of 8–20% MeOH/5% NH_4OH in $CHCl_3$, yielding **2** as white solid (4.6 g, 16 mmol, 70%). 1H NMR (400 MHz; $DMSO-d_6$) δ : 6.72 (d, $J = 8.2$ Hz, 1H), 6.63 (d, $J = 8.2$ Hz, 1H), 4.84 (s, 1H), 3.78 (s, 3H), 3.27 (bs, 1H, under H_2O peak), 2.77–2.71 (m, 1H), 2.66–2.61 (m, 2H), 2.56–2.51 (m, 1H), 2.44–2.39 (m, 2H), 2.16–2.12 (m, 1H), 1.87 (ddd, $J = 14.6, 9.9, 4.8$ Hz, 1H), 1.80–1.74 (m, 1H), 1.49–1.45 (m, 1H), 0.95–0.84 (m, 1H). ^{13}C NMR (101 MHz; $DMSO-d_6$) δ : 208.5, 145.0, 141.8, 127.9, 127.2, 119.3, 114.4, 91.1, 56.3, 51.4, 47.2, 41.8, 39.5, 38.4, 35.4, 29.9, 25.4. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{17}H_{20}NO_3$ 286.1443, found 286.1446.

4.2 (7aR,12bS)-9-Methoxy-3-[2H_3]methyl-2,3,4,4a,5,6-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7(7aH)-one (3).

Norhydrocodone (**2**) (390 mg, 1.37 mmol) was dissolved in anhydrous THF (12 mL) under an atmosphere of nitrogen. The reaction mixture was cooled to 0 °C and sodium hydride (36 mg, 1.5 mmol) was added. After stirring for 15 min the solution of iodo[2H_3]methyl (0.09 mL, 1.37 mmol) in anhydrous THF (7 mL) was added dropwise at 0 °C over 1 h. The reaction mixture was allowed to warm up to room temperature while being stirred for additional 2 h. The reaction mixture was quenched with H_2O at 0 °C and the pH was adjusted to basic using NH_4OH . The aqueous layer was extracted 3x with a mixture of $CHCl_3$ /isopropanol (4:1). Organic layers were combined and dried over $MgSO_4$. After the filtration of drying agent, the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography using a gradient of 8–20% MeOH/5% NH_4OH in $CHCl_3$ to afford *N*-[2H_3]methyl-hydrocodone (**3**) as a white solid (233 mg, 0.77 mmol, 56%). 1H NMR (400 MHz; $CDCl_3$) δ : 6.69 (d, $J = 8.2$ Hz, 1H), 6.63 (d, $J = 8.2$ Hz, 1H), 4.65 (s, 1H), 3.90 (s, 3H), 3.18–3.16 (m, 1H), 3.01 (d, $J = 18.5$ Hz, 1H), 2.60–2.53 (m, 2H), 2.45–2.36 (m, 2H), 2.33–2.27 (m, 1H), 2.22–2.15 (m, 1H), 2.10–2.02 (m, 1H), 1.87–1.79 (m, 2H), 1.31–1.20 (m, 1H). ^{13}C NMR (101 MHz; $CDCl_3$) δ : 208.0, 145.5, 143.0, 127.4, 126.4, 119.9, 114.6, 91.6, 59.3, 56.9, 47.06, 46.90, 42.9, 40.4, 35.7, 25.7, 20.1. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{18}H_{19}[^2H_3]NO_3$ 303.1788, found 303.1791. Deuterium incorporation 99.5%.

4.3 (7aR,12bS)-9-Hydroxy-3-[²H₃]methyl--2,3,4,4a,5,6-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7(7aH)-one (4).

N-[²H₃]Methyl-hydrocodone (**3**) (460 mg, 1.52 mmol) was dissolved in anhydrous dichloromethane (5 mL) followed by the dropwise addition of boron tribromide (0.72 mL, 7.61 mmol) at -78 °C. The reaction mixture was left to warm to room temperature while stirring. After the reaction was completed based on TLC, the reaction mixture was quenched with H₂O at 0 °C, dichloromethane was removed under reduced pressure and the reaction mixture was set up to reflux overnight. After cooling, the pH was adjusted using NH₄OH to 9. The aqueous layer was extracted 5x with EtOAc/isopropanol (10:1). The organic layers were combined and dried over MgSO₄. The filtration of drying agent was followed by the evaporation of solvent. The residue was purified *via* flash chromatography using gradient of 8–30% MeOH/5% NH₄OH in CHCl₃ providing **4** as white solid (342 mg, 1.18 mmol, 78%). ¹H NMR (400 MHz; CD₃OD) δ 7.90 (s, 1H), 6.62 (d, *J* = 8.1 Hz, 1H), 6.59 (d, *J* = 8.1 Hz, 1H), 4.79 (s, 1H), 3.22–3.20 (m, 1H), 3.03 (d, *J* = 18.7 Hz, 1H), 2.64–2.51 (m, 3H), 2.41–2.29 (m, 2H), 2.26–2.20 (m, 1H), 2.17–2.09 (m, 1H), 1.92–1.85 (m, 1H), 1.72–1.68 (m, 1H), 1.23–1.12 (m, 1H). ¹³C NMR (101 MHz; CD₃OD) δ 211.7, 145.5, 140.7, 128.2, 126.1, 121.1, 118.8, 92.4, 79.5, 60.5, 48.1, 47.8, 42.8, 35.9, 26.5, 21.1. HRMS-ESI (*m/z*): [M + H]⁺ calcd for C₁₇H₁₇[²H₃]NO₃ 289.1631, found 289.1633.

4.4 (7aR,12bS)-3-[²H₃]Methyl-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl trifluoromethanesulfonate (5).

N-Phenyl-bis(trifluoromethanesulfonimide) (1.37 g, 3.83 mmol) was added in small portions at room temperature to a suspension of **4** (920 mg, 3.19 mmol) and Et₃N (1.11 mL, 7.98 mmol) in anhydrous dichloromethane (10 mL). The reaction mixture was continued stirring overnight. The solvent was evaporated, and the residue was dissolved in 2 M HCl. The resulting solution was washed with ether, after which the pH was adjusted to 9 using NH₄OH. The aqueous layer was extracted with DCM. The organic extracts were combined, washed with brine and dried over MgSO₄. After the filtration of drying agent, the solvent was evaporated to afford triflate **5** as a white solid (1.23 g, 2.93 mmol, 92%). Triflate **5** was used directly without further purification. ¹H NMR (400 MHz; CDCl₃) δ 7.00 (d, *J* = 8.4 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 1H), 4.78 (s, 1H), 3.23–3.21 (m, 1H), 3.06 (d, *J* = 18.9 Hz, 1H), 2.66–2.57 (m, 2H), 2.47–2.33 (m, 3H), 2.17–2.07 (m, 2H), 1.91–1.84 (m, 1H), 1.80–1.74 (m, 1H), 1.27–1.16 (m, 1H). ¹³C NMR (101 MHz; CDCl₃) δ 205.7, 148.2, 134.7, 131.3, 129.6, 122.6, 120.4, 92.4, 59.0, 47.2, 46.6, 42.5, 40.2, 35.6, 25.5, 20.6. HRMS-ESI (*m/z*): [M + H]⁺ calcd for C₁₈H₁₆[²H₃]F₃NO₅S 421.1124, found 421.1124.

4.5 *N*-((7aR,12bS)-3-[²H₃]Methyl-7-oxo-2,3,4,4a,5,6,7,7a-octahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-9-yl)acetamide (6).

To a triflate **5** (1.42 g, 3.37 mmol), acetamide (0.44 g, 7.43 mmol) and potassium phosphate (0.94 g, 4.38 mmol) were added. After 15 min of the purging with argon, *tert*-ButylBrettPhos palladacycle catalyst (0.29 g, 0.34 mmol) was added to the same flask. The solids were purged with argon for additional 15 min followed by the addition of prior degassed *tert*-butanol (20 mL) and H₂O (0.07 mL) under an atmosphere of argon. The reaction mixture was heated to 90 °C for 4 h. After cooling the solvent was evaporated, and

the residue was partitioned with a mixture of 10% isopropanol in CHCl_3 and water and separated. The aqueous layer was extracted with a mixture of 10% isopropanol in CHCl_3 . The combined organic layers were dried over MgSO_4 . The drying agent was filtered off and the solvent was removed in *vacuo*. The residue was purified by column chromatography using gradient of 0–15% $\text{MeOH}/5\% \text{NH}_4\text{OH}$ in CHCl_3 , yielding amide **6** as white solid (0.97 g, 2.96 mmol, 87%). ^1H NMR (400 MHz; CDCl_3) δ : 7.92 (d, $J = 8.2$ Hz, 1H), 7.44 (s, 1H), 6.69 (d, $J = 8.3$ Hz, 1H), 4.65 (s, 1H), 3.19–3.18 (m, 1H), 3.03 (d, $J = 18.7$ Hz, 1H), 2.60–2.53 (m, 2H), 2.42–2.29 (m, 4H), 2.22–2.15 (m, 4H), 2.10–2.03 (m, 1H), 1.86–1.82 (m, 1H), 1.76–1.73 (m, 1H), 1.27–1.21 (m, 1H). ^{13}C NMR (101 MHz; CDCl_3) δ : 208.4, 168.4, 146.6, 129.7, 125.8, 121.4, 120.7, 119.9, 91.7, 59.2, 47.0, 46.7, 42.9, 40.4, 35.6, 25.4, 24.6, 20.4. HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{20}[^2\text{H}_3]\text{N}_2\text{O}_3$ 330.1897, found 330.1896.

4.6 *tert*-Butyl ((7*aR*,12*bS*)-3- $^2\text{H}_3$]methyl-7-oxo-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)carbamate (**7**).

Amide **6** (650 mg, 1.97 mmol) was dissolved in 1 NHCl (15 mL) and heated at 60 °C for 48 h. After cooling to room temperature, the pH was adjusted carefully to 6.5–7.0 first with 20% NaOH and then with 5% NaOH . Di-*tert*-butyldicarbonate (1.13 mL, 4.93 mmol) was added to the solution and the reaction mixture was left to stir overnight at room temperature. The aqueous layer was extracted with CHCl_3 . The organic layers were combined and washed with 1% NaOH , dried with MgSO_4 and concentrated providing ketone **7** as a light-yellow foam (733 mg, 1.89 mmol, 96%), which was used further in the synthesis without additional purification. ^1H NMR (400 MHz; CDCl_3) δ : 7.73 (dd, $J = 7.6, 0.5$ Hz, 1H), 6.69–6.67 (m, 2H), 4.62 (s, 1H), 3.17 (dd, $J = 5.3, 2.8$ Hz, 1H), 3.02 (d, $J = 18.6$ Hz, 1H), 2.60–2.53 (m, 2H), 2.46–2.28 (m, 3H), 2.23–2.16 (m, 1H), 2.09–2.02 (m, 1H), 1.86–1.80 (m, 1H), 1.77–1.73 (m, 1H), 1.50 (s, 9H), 1.30–1.19 (m, 1H). ^{13}C NMR (101 MHz; CDCl_3) δ : 208.4, 152.9, 146.1, 128.3, 125.7, 121.2, 119.9, 119.6, 91.6, 80.5, 59.3, 46.95, 46.81, 43.0, 40.5, 35.7, 28.5, 25.4, 20.3. HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{22}\text{H}_{26}[^2\text{H}_3]\text{N}_2\text{O}_4$ 388.2310, found 388.2316.

4.7 *tert*-Butyl ((7*S*,7*aR*,12*bS*)-7-acetamido-3- $^2\text{H}_3$]methyl-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)carbamate (**8**).

To a mixture of ketone **7** (500 mg, 1.29 mmol) and ammonia solution in EtOH (2M, 6.5 mL, 12.9 mmol) was added titanium(IV) isopropoxide (0.76 mL, 2.58 mmol) at room temperature under an atmosphere of argon. The reaction mixture was left to stir overnight and after cooling to 8 °C, sodium borohydride (73 mg, 1.94 mmol) was added. While stirring for another 3 h, the reaction mixture was allowed to warm to room temperature. The reaction was quenched using NH_4OH (2M, 8 mL). The solid material was removed by filtration through Celite (1.5 g) and the solids were washed excessively with dichloromethane. The organic layer was separated, and the aqueous layer was extracted with dichloromethane. The combined organic extracts were dried over MgSO_4 . After the filtration of drying agent, the solvent was removed in *vacuo* to yield a light-yellow foam. The foam was dissolved in anhydrous EtOAc (6 mL) followed by the addition of acetic anhydride (0.18 mL, 1.94 mmol). After 15 min the formation of a white precipitate was observed. The mixture was stirred for an additional hour, and the white precipitate was filtered off, washed

with Et₂O, and dried under high vacuum. The remaining mother liquor was basified with saturated NaHCO₃, and the aqueous layer was extracted with CHCl₃. The organic layers were combined and dried over MgSO₄. The solvent was evaporated, and the residue was purified by column chromatography using a gradient of 0–10% MeOH/5% NH₄OH in CHCl₃ to afford amide **8** as a white solid (394 mg, 0.92 mmol, 71%). ¹H NMR (400 MHz; CDCl₃) δ: 7.31 (d, *J* = 8.1 Hz, 1H), 6.65 (d, *J* = 8.1 Hz, 1H), 6.35 (s, 1H), 6.23–6.21 (m, 1H), 4.63 (d, *J* = 4.7 Hz, 1H), 4.28–4.22 (m, 1H), 3.10 (dd, *J* = 6.0, 2.8 Hz, 1H), 2.97 (d, *J* = 18.9 Hz, 1H), 2.51–2.47 (m, 1H), 2.44–2.38 (m, 1H), 2.32–2.20 (m, 2H), 1.98 (s, 3H), 1.98–1.87 (m, 1H), 1.78–1.66 (m, 2H), 1.52 (s, 9H), 1.48–1.41 (m, 1H), 1.05–0.94 (m, 1H), 0.91–0.82 (m, 1H). ¹³C NMR (101 MHz; CDCl₃) δ 169.6, 153.2, 149.5, 131.7, 129.7, 122.0, 119.4, 118.3, 90.7, 80.6, 59.8, 46.6, 46.2, 42.8, 37.2, 36.5, 28.5, 23.6, 23.0, 20.6, 20.3. HRMS-ESI (*m/z*): [M + H]⁺ calcd for C₂₄H₃₁[²H₃]N₃O₄ 431.2738, found 431.2730.

4.8 3,3'-Disulfanediylbis(*N*-(7*S*,7*aR*,12*bS*)-7-acetamido-3-[²H₃]methyl-2,3,4,4*a*,5,6,7,7*a*-octahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-9-yl)propanamide) (**9**).

Preparation of 3,3'-disulfanediyl dipropionyl chloride: 3,3'-Dithiodipropionic acid (0.5 g, 2.37 mmol) was suspended in anhydrous dichloromethane (10 mL) at 0 °C under an atmosphere of nitrogen. To the mixture was added a catalytic amount of DMF (<4 μL) followed by a freshly distilled oxalyl chloride (0.6 mL, 7.13 mmol). The reaction mixture was allowed to warm to room temperature and the stirring was continued overnight. The resulting homogeneous solution was concentrated on a rotavap yielding 3,3'-disulfanediyl dipropionyl chloride as a light-yellow oil, quantitatively. The crude acid chloride was directly used in the preparation of **9**. Formation of 3,3'-disulfanediyl dipropionyl chloride was confirmed by an NMR analysis of its dimethyl ester²⁰ performed after quenching of an aliquot with anhydrous methanol. ¹H NMR (CDCl₃) δ 3.68 (s, 3H), 2.91 (t, 2H, *J* = 7.33 Hz), 2.72 (t, 2H, *J* = 7.33); ¹³C NMR (CDCl₃): δ 172.1, 51.8, 33.9, 33.1.

Amide **8** (410 mg, 0.95 mmol) was dissolved in CHCl₃ (5 mL) followed by the addition of trifluoroacetic acid (1.5 mL). After stirring for 3 h the mixture was concentrated, and the residue was partitioned with CHCl₃ and saturated NaHCO₃. The layers were separated, and the aqueous layer was extracted with CHCl₃. The combined organic layers were dried over MgSO₄ and the solvent was removed in *vacuo*. To the resulted residue (290 mg, 0.88 mmol) in anhydrous dichloromethane (11 mL) was added dropwise a solution of freshly prepared 3,3'-disulfanediyl dipropionyl chloride (109 mg, 0.44 mmol) in anhydrous dichloromethane (0.5 mL) at 0 °C. The formation of a white precipitate was observed. A solution of triethylamine (0.12 mL, 0.88 mmol) in anhydrous dichloromethane (0.5 mL) was added dropwise to the mixture, and the precipitate dissolved to obtain a clear solution. After 30 min of additional stirring, the reaction mixture was partitioned with 1 *N*NaOH. The layers were separated, and the aqueous layer was extracted with dichloromethane. The combined organic extracts were dried over MgSO₄ and the solvent was evaporated after the filtration of drying agent. The residue was purified by column chromatography using a gradient of 0–15% MeOH/5% NH₄OH in CHCl₃ providing target compound **9** as a white solid (220 mg, 0.26 mmol, 60%). ¹H NMR (400 MHz; CDCl₃) δ: 7.85 (s, 2H), 7.38 (d, *J* = 8.1 Hz, 2H), 6.64 (d, *J* = 8.2 Hz, 2H), 6.42 (d, *J* = 9.1 Hz, 2H), 4.56 (d, *J* = 4.8 Hz, 2H), 4.23–4.16 (m, 2H), 3.18–3.03 (m, 6H), 2.99–2.94 (m, 2H), 2.92–2.85 (m, 2H), 2.82–2.74 (m, 2H), 2.47–

2.37 (m, 4H), 2.29–2.24 (m, 2H), 2.21–2.14 (m, 2H), 1.95 (s, 6H), 1.88–1.81 (m, 2H), 1.74–1.65 (m, 2H), 1.57–1.54 (m, 2H), 1.43–1.36 (m, 2H), 1.09–0.98 (m, 2H), 0.89–0.80 (m, 2H). ¹³C NMR (101 MHz; CDCl₃) δ: 169.9, 169.4, 150.5, 133.1, 129.8, 123.4, 119.4, 117.7, 90.6, 59.8, 46.7, 46.2, 42.7, 37.1, 36.7, 36.4, 34.6, 23.6, 23.1, 20.51, 20.40. HRMS-ESI (*m/z*): [M + H]⁺ calcd for C₄₄H₅₁[²H₃]N₆O₆S₂ 835.4158, found 835.4154. Anal. Calcd. for C₄₄H₅₁[²H₃]N₆O₆S₂ • 1.45 CHCl₃: C, 54.46; H, 5.72; N, 8.39. Found: C, 54.46; H, 5.72; N, 8.35. Deuterium incorporation 97.6%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

6-AmHap	<i>N</i> -((4 <i>aR</i> ,7 <i>S</i> ,7 <i>aR</i> ,12 <i>bS</i>)-7-acetamido-3-[² H ₃]methyl-2,3,4,4 <i>a</i> ,5,6,7,7 <i>a</i> -octahydro-1 <i>H</i> -4,12-methanobenzofuro[3,2- <i>e</i>]isoquinolin-9-yl)-3-mercaptopropanamide
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

REFERENCES

1. Wilson N, Kariisa M, Seth P, Smith IV H, Davis NL. Drug and opioid-involved overdose deaths—United States, 2017–2018. *MMWR* 2020;69:290–297. [PubMed: 32191688]
2. NIDA. The science of drug use and addiction: The basics. 2020 p. 1–30. <https://www.drugabuse.gov/publications/media-guide/science-drug-use-addiction-basics>.
3. Kadam M, Sinha A, Nimkar S, Matcheswalla Y, De Sousa A. A comparative study of factors associated with relapse in alcohol dependence and opioid dependence. *Indian J. Psychol. Med* 2017;39:627–633. [PubMed: 29200559]
4. Lee JD, Friedmann PD, Kinlock TW, Nunes EV, Boney TY, Hoskinson RA Jr., Wilson D, McDonald R, Rotrosen J, Gourevitch MN, Gordon M, Fishman M, Chen DT, Bonnie RJ, Cornish JW, Murphy SM, O'Brien CP. Extended-release naltrexone to prevent opioid relapse in criminal justice offenders. *N. Engl. J. Med* 2016;374:1232–1242. [PubMed: 27028913]
5. Bonese KF, Wainer BH, Fitch FW, Rothberg RM, Schuster CR. Changes in heroin self-administration by a rhesus monkey after morphine immunisation. *Nature*. 1974;252:708–710. [PubMed: 4474602]
6. Li F, Cheng K, Antoline JFG, Iyer MR, Matyas GR, Torres OB, Jalah R, Beck Z, Alving CR, Parrish DA, Deschamps JR, Jacobson AE, Rice KC. Synthesis and immunological effects of heroin vaccines. *Org Biomol. Chem* 2014;12:7211–7232. [PubMed: 24995943]
7. Sulima A, Jalah R, Antoline JFG, Torres OB, Imler GH, Deschamps JR, Beck Z, Alving CR, Jacobson AE, Rice KC, Matyas GR. A stable heroin analogue that can serve as a vaccine hapten to induce antibodies that block the effects of heroin and its metabolites in rodents and that cross-react

immunologically with related drugs of abuse. *J. Med. Chem* 2018;61:329–343. [PubMed: 29236495]

8. Matyas GR, Jalah R, Antoline JFG, Torres O, Li F, Jacobson AE, Rice KC, Beck Z, Alving CR, unpublished work.
9. Matyas GR, Rice KC, Cheng K, Li F, Antoline JFG, Iyer MR, Jacobson AE, Mayorov AV, Beck Z, Torres OB, Alving CR. Facial recognition of heroin vaccine opiates: Type 1 cross-reactivities of antibodies induced by hydrolytically stable haptenic surrogates of heroin, 6-acetylmorphine, and morphine. *Vaccine*. 2014;32:1473–1479. [PubMed: 24486371]
10. Jalah R, Torres OB, Mayorov AV, Li F, Antoline JFG, Jacobson AE, Rice KC, Deschamps JR, Beck Z, Alving CR, Matyas GR. Efficacy, but not antibody titer or affinity, of a heroin hapten conjugate vaccine correlates with increasing hapten densities on tetanus toxoid, but not on CRM197 carriers. *Bioconjug. Chem* 2015;26:1041–1053. [PubMed: 25970207]
11. Suh HW, Song DK, Kim YH, Choi YS, Yoo JS, Tseng LF. Differential modulation by muscimol and baclofen on antinociception induced by morphine, β -endorphin, D-Pen2,5-enkephalin and U50,488H administered intracerebroventricularly in the mouse. *Naunyn-Schmied. Arch. Pharmacol* 1995;352:614–619.
12. Q1A(R2) stability testing of new drug substances and products | FDA. url: <https://www.FDA.Gov/regulatory-information/search-FDA-guidance-documents/Q1AR2-stability-testing-new-drug-substances-and-products> (accessed June 29, 2020 from text captured on Dec 12, 2019).
13. Torres OB, Jalah R, Rice KC, Li F, Antoline JFG, Iyer MR, Jacobson AE, Boutaghou MN, Alving CR, Matyas GR. Characterization and optimization of heroin hapten-BSA conjugates: Method development for the synthesis of reproducible hapten-based vaccines. *Anal. Bioanal. Chem* 2014;406:5927–5937. [PubMed: 25084736]
14. Singh KV, Kaur J, Varshney GC, Raje M, Suri CR. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjug. Chem* 2004;15:168–173. [PubMed: 14733597]
15. Guide to achieving reliable quantitative LC-MS measurements, RSC Analytical Methods Committee 2013.
16. Bijlsma L, Sancho JV, Hernández F, Niessen WMA. Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS(E) accurate-mass spectra. *J. Mass Spectrom* 2011;46:865–875. [PubMed: 21915950]
17. Clark RL, Pessolano AA, Weijlard J, Pfister K. N-Substituted epoxymorphinans. *J. Am. Chem. Soc* 1953;75:4963–4967.
18. Manley PW, Blasco F, Mestan J, Aichholz R. The kinetic deuterium isotope effect as applied to metabolic deactivation of imatinib to the des-methyl metabolite, CGP74588. *Bioorg. Med. Chem* 2013;21:3231–3239. [PubMed: 23611771]
19. Crooks PA, Ravard A, Byrd GD. Synthesis of (E)-N-[methyl-d3]-4-(3-pyridinyl)-3-buten-1-amine, a deuterated analogue of the nicotinic agonist RJR2403. *J Labelled Cpd. Radiopharm* 1998;41:1165–1171.
20. Dethe DH, Srivastava A, Dherange BD, Kumar BV. Unsymmetrical disulfide synthesis through photoredox catalysis. *Adv. Synth. Catal* 2018;360:3020–3025.

Scheme 1.

Synthesis of deuterated disulfide 6-AmHap (**9**) and deuterated 6-AmHap (**10**).