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Chemical and Enzyme-Assisted Syntheses of Norbuprenorphine-3- β -D-Glucuronide

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Abstract

Norbuprenorphine-3- β -D-glucuronide (nBPN-3- β -D-G, **1**) is a major phase II metabolite of buprenorphine, a pharmaceutical used for the treatment of opioid addiction. The pharmacological activity of compound **1** is not clear because investigations have been limited by the lack of chemically pure, well characterized **1** in sufficient quantities for *in vitro* and *in vivo* experiments. This work describes two concise, new methods of synthesis of **1**, a chemical and an enzyme-assisted synthesis. The chemical synthesis used a strategy based on a combination of Koenig-Knorr coupling and amino-silyl protection. The enzyme-assisted synthesis used dog liver to convert substrate norbuprenorphine (nBPN, **2**) to **1**. Both methods provided **1**, characterized by ¹H NMR and tandem mass spectrometry, with purity >96%. The fractional yield of the enzyme-assisted synthesis was greater than that of the chemical synthesis (67% vs 5.3%), but due to larger reaction volumes, the chemical synthesis afforded greater amounts of total **1**.

Drugs are metabolized in a two-phase biotransformation process that renders the drug more readily excreted in urine or bile. The first phase often produces a dealkylated or a hydroxylated metabolite that may then also undergo conjugation to a polar group such as glucuronic or sulfonic acid in the second phase. Phase I and phase II metabolites can circulate in concentrations equal to or greater than the parent drug, and in many instances have pharmacological activity that contributes significantly to the overall effects of the parent drug.^{1,2} Thus, drug metabolites have the potential to be clinically important, and indeed this is true in numerous instances. Many active phase I metabolites have been developed as new drugs. Some well-known examples are acetaminophen, a metabolite of phenacetin, fexofenadine, a metabolite of terfenadine, and nortriptyline, a metabolite of amitriptyline.¹ The opioid drug class has over 10 clinically important metabolites, including the phase I metabolites hydrocodone and hydromorphone.^{2,3} This drug class also has important active phase II metabolites, the best described being morphine-6-glucuronide (M6G), which has greater pharmacological activity than the parent drug.^{3,4} M6G has undergone phase III clinical trials as a drug for treatment of post-operative pain,^{5,6} showing benefit over the parent drug for producing analgesia with significantly fewer negative side effects (nausea, etc), and less risk of respiratory depression.⁷ The high potential for contribution of drug metabolites to the overall effect of the parent drug has led to a recent

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guidance from the FDA wherein any major metabolite, defined as one comprising >10% of parent drug systemic exposure at steady-state, be considered for safety assessment (Guidance for Industry: Safety Testing of Drug Metabolites, Food and Drug Administration, Center for Drug Evaluation and Research, <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf>).

Buprenorphine (BPN) is a phenanthrene opioid used in treatment of opioid addiction, and to a lesser extent, pain.⁸ It is extensively metabolized to 3 main metabolites: an N-dealkylated product, norbuprenorphine (nBPN), and two O-glucuronides, buprenorphine-3- β -D-glucuronide (BPN-3- β -D-G) and norbuprenorphine-3- β -D-glucuronide (nBPN-3- β -D-G).⁹ These metabolites are present in the circulation at concentrations greater than or equal to that of BPN. Plasma concentrations of nBPN and nBPN-3- β -D-G, specifically, are typically great than that of BPN following an oral dose, and the area under the plasma concentration versus time curve for nBPN and nBPN-3- β -D-G is approximately 2 and 10 times greater, respectively, than that of the parent drug.^{10–13} While there are some data regarding the pharmacological activity of nBPN,^{12, 14} the biological properties of the glucuronides and their contribution to the pharmacology of the parent drug remain unknown. The high concentration of nBPN-3- β -D-G and the precedent for active glucuronides warrant the investigation of the biological activity of this metabolite. However, such investigation has been inhibited by the limited availability of the metabolites in quantities necessary for *in vitro* and *in vivo* studies. Thus, development of an efficient means of nBPN-3- β -D-G synthesis is of considerable importance.

Glucuronides can be synthesized chemically, usually by the Koenigs–Knorr method.¹⁵ However, glucuronides are notoriously more difficult to prepare than the corresponding glucopyranoside. Schmidt and coworkers have shown a difficulty scale: 3-deoxy-2-glycosyluronates < ketoses < deoxy sugars < aldoses < glycuronates.¹⁶ Of all common sugars, glucuronides require the highest activation for a given aglycone. Alternatively, glucuronide conjugates can be prepared through enzyme-assisted synthesis using liver microsomal enzymes.^{17–20} The purpose of this investigation was to evaluate the synthesis of nBPN-3-D-G by two routes: enzyme-assisted synthesis using dog liver, and chemical synthesis involving the development of silyl as a protective group.

Experimental

Chemicals and general methods

All reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Norbuprenorphine glucuronide was purchased from Cerillant (Round Rock, TX) for use as an analytical standard. Norbuprenorphine was a generous gift from the National Institute on Drug Abuse. All organic solvents were distilled before use. After drying over Na₂SO₄, organic solvents concentrated under diminished pressure at <40 °C (bath temperature), NMR spectra were recorded at 300 MHz (¹H), in CDCl₃ or DMSO. Tetramethylsilane (TMS) was used as internal standard (δ 0) for ¹H spectra. Chemical shifts are expressed in ppm. Preparative chromatography was performed using Merck 60 PF₂₅₄ silica gel. Analytical thin layer chromatography (TLC) was performed on glass TLC plates coated with silica gel (Analtech) and visualized with UV light.

Formation of 1-[2-(trimethylsilyl)ethoxycarbonyloxy]benzotriazole (TeocOBt) (3)

Synthesis of Teoc-OBt was modified from published methods.²¹ Briefly, to a solution of 2-trimethylsilyletanol (1.0 g, 8.5 mmol) in toluene (5 mL) at –10 to –5 °C was added powdered anhydrous potassium carbonate (1 g). To this vigorously stirred suspension a 20% solution of phosgene in toluene (6.25 mL) was added dropwise over 14–30 minutes. On

completion of the addition, the mixture was stirred over ice bath for 30 min, followed by 1 h at room temperature. After stirring, nitrogen was bubbled through the mixture with the hood closed to remove the majority of the excess phosgene. The last traces of phosgene were removed from the mixture by rotary evaporation at a temperature $<40^{\circ}\text{C}$. The solids were then filtered off through a pad of anhydrous magnesium sulfate, and washed with dry ether. The combined filtrate and washings were evaporated at a temperature not higher than 50°C . The resultant oily carbonochloridate was used without further purification.

2-trimethylsilylethyl carbonochloridate (1.54 g, 8.5 mmol) was taken up in acetonitrile (25 mL). The solution was cooled to 0°C and solid 1-hydroxy-benzotriazole monohydrate (1.5 g, 11 mmol) was added with vigorous stirring followed by a solution of triethylamine (1.0 g, 10 mmol) in acetonitrile (2.5 mL). The mixture was stirred at low temperature for 15 min, then at room temperature overnight. The mixture was poured into water (100 mL) and extracted with ether (3×40 mL). The organic extracts were combined, washed with water (2×40 mL), 1 N hydrochloric acid (40 mL), and again water (2×40 mL), dried with sodium sulfate, and evaporated to dryness. The residue was taken up in boiling hexane (150–200 mL), and the solution allowed to cool. Crystallization, completed by storage at -15°C , yielded a white solid (1.31 g, 58.7% for the two-step synthesis). ^1H NMR (CDCl_3): δ 0.12 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 1.29 (t, 2H, J 9.0 Hz, SiCH_2), 4.65 (t, 2H, J 9.0 Hz, OCH_2), 7.54 (t, 1H, J 7.5 Hz, PhH), 7.77 (t, 1H, J 7.5 Hz, PhH), 8.02 (d, 1H, J 7.5 Hz, PhH), 8.22 (d, 1H, J 7.5 Hz, PhH). MS (ESI) $^+$ $[\text{M}+\text{H}]^+$ calcd 264.1, found 264.2.

Formation of *N*-(trimethylsilyl)ethoxycarbonyl-norbuprenorphine (Teoc-nBPN) (4)

To a 1,4-dioxane (2 mL) solution of nBPN (109 mg, 0.26 mMol) (2) and TeocOBt (69.6 mg, 0.26 mMol) (3), diisopropylethylamine (55 μL , 0.32 mMol) was added. The mixture was stirred at ambient temperature overnight. Solvent was removed under vacuum, and the residues were dissolved in ethyl acetate (10 mL), then washed with water (2×10 mL) and saline (10 mL), and finally dried over Na_2CO_3 . The crude product was purified by flash-chromatography (25:75 ethyl acetate: hexanes) to produce Teoc-nBPN (4) as a white solid (118.3 mg, 80.4%). ^1H NMR (CDCl_3): δ 0.05 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.99 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.26 (m, 2H, SiCH_2), 1.28 (m, 2H, CHCH_2C), 1.34 (s, 3H, CCH_3), 1.57 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}$), 1.84 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}$), 1.90 (m, 1H, CH), 2.05 (m, 2H, CCH_2CH_2), 2.84 (m, 2H, PhCH_2CH), 2.97 (m, 1H, NCHC), 3.53 (s, 3H, OCH_3), 4.14 (m, 2H, NCH_2C), 4.21 (m, 2H, OCH_2CH_2), 4.46 (s, 1H, OCHC_2), 5.76 (s, 1H, OH), 6.54 (d, 1H, J 5.1 Hz, PhH), 6.73 (d, 1H, J 5.1 Hz, PhH). MS (ESI) $^+$ $[\text{M}+\text{H}]^+$ calcd 558.3, found 558.4.

Formation of *N*-(trimethylsilyl)ethoxycarbonyl-norbuprenorphine-3- β -D-acetoglucuronide (Teoc-nBPN-3- β -D-AcG) (6)

Teoc-nBPN (4) (116.3 mg, 0.21 mmol), Ag_2CO_3 (58 mg, 0.21 mmol) and 4 Å molecular sieves (200 mg) were dispersed in freshly distilled quinoline (2 mL, 17 mmol). Acetobromo- α -D-glucuronic acid methyl ester (167 mg, 0.42 mmol) was added to this mixture with stirring at 0°C under argon. The mixture was allowed to warm to ambient temperature over an hour, and was kept at ambient temperature overnight. Molecular sieves were removed by filtration. The precipitate was thoroughly washed with ethyl acetate (3×5 mL). The organic phase was combined, washed with brine (10 mL), dried over Na_2SO_4 , and concentrated. The crude product was purified by flash-chromatography with gradient eluents (from 25:75 to 50:50 ethyl acetate:hexanes) to produce Teoc-nBPN-3- β -D-G (5) as a white solid (33.1 mg, 18.0 %). ^1H NMR (CDCl_3): δ 0.05 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.99 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.26 (m, 2H, SiCH_2), 1.28 (m, 2H, CHCH_2C), 1.34 (s, 3H, CCH_3), 1.56 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}$), 1.72 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}$), 1.84 (m, 1H, CH), 2.04 (s, 9H, CH_3COO), 2.10 (m, 2H, CCH_2CH_2), 2.85 (m, 2H, PhCH_2CH), 2.97 (m, 1H, NCHC), 3.50 (s, 3H, OCH_3), 3.71 (s, 3H, COOCH_3), 4.14 (m, 2H, NCH_2C), 4.21 (m, 2H, OCH_2CH_2), 4.42 (s, 1H, OCHC_2), 5.29 (m, 4H, CH on

glucuronide), 5.71 (s, 1H, OCH), 6.54 (D, 1H, *J* 7.8 Hz, PhH), 6.73 (D, 1H, *J* 7.8 Hz, PhH). MS (ESI)⁺ [M+Na]⁺ calcd 896.4, found 896.5.

Chemical synthesis of nBPN-3-β-D-G (1)

N-(Trimethylsilyl)ethoxycarbonyl-norbuprenorphine-3-β-D-acetoglucuronide (**5**) (65.6 mg, 0.075 mmol) was dissolved in THF/H₂O (v/v 4:1 6.5 mL). 2 M KOH solution (225 μL, 0.45 mmol) was added with stirring at 0°C. The mixture was then allowed to warm to ambient temperature and then kept at ambient temperature for two hours. After 2 hours, acetic acid (8.6 μL, 0.17 mL) was added and the mixture was extracted with dichloromethane (3 × 15 mL). The combined organic phase was washed with saline (5 mL), dried over Na₂SO₄ and concentrated. This produced Teoc-nBPN-3-β-D-G (**7**) as a light yellow solid (54.7 mg 98%) which was used for next step reaction without purification. To a solution of *N*-(Trimethylsilyl)ethoxycarbonyl-norbuprenorphine-3-β-D-glucuronide (Teoc-nBPN-3-β-D-G) (**7**) (55 mg, 0.075 mmol) in THF (4 mL), TBAF (71 mg, 0.225 mmol) was added and stirred at ambient temperature overnight. The crude product was partially purified by flash-chromatography (20:80 methanol:dichloromethane), following by purification with a preparative HPLC system to nBPN-3-β-D-G (**1**) as a white solid (16.1 mg, 36.4% for two steps).

Preparation of S9

Fresh liver was obtained from a beagle dog that had been euthanized under a Washington University Animal Studies Committee approved protocol. The tissue was cut in to approximately 30 g pieces, with the removal of as much connective tissue removed as possible. The tissue was washed with ice-cold phosphate buffered saline (PBS) with stirring, with 6 changes of PBS. The washed tissue pieces were reduced in 3 volumes (by weight) of buffer (10 mM potassium phosphate, pH 7.4, with 0.25 M sucrose) in a chilled Waring blender, and then homogenized on ice using a motorized glass-Teflon homogenizer. The homogenate was transferred to polystyrene tubes and centrifuged at 9,000Xg for 20 minutes, and the supernatant collected as the “S9 fraction”, containing both cytosolic proteins and microsomes. Protein content of the S9 was quantified by Bradford assay.

Enzyme-assisted synthesis

In a typical 1 ml incubation, 640 μL of reaction buffer (100 mM potassium phosphate, pH 7.1, 1 mM MgCl, 5 mM saccharic acid lactone) was added to 200 μL of S9 (10 ug/ul protein) in a glass test tube and the mixture sonicated for 1 minute. After sonication, 10 mM nBPN (4 mg/mL), 10, 20, 30 or 50 mM UDPGA and 150 mL of an NADPH-generating system (1.7 mg/mL β-NADP⁺, 7.8 mg/mL glucose-6-phosphate, and 6 units/mL glucose-6-phosphate dehydrogenase, prewarmed to 37°C) were added. The mixture was vortexed and then incubated overnight in a 37°C water bath with gentle shaking. Larger reaction volumes (up to 5 mL without a decrease in yield) were also occasionally used. 10 mM aminobenzotriazole was added prior to the addition of nBPN to three of the 1 ml reaction tubes.

Partial purification of nBPN-3-β-D-G from biocatalyzed reaction

The reaction was terminated by protein precipitation with three volumes of ice-cold acetonitrile. The supernatant was evaporated under nitrogen to approximately 1 mL, and then loaded onto a C18 solid phase extraction column (Sep-Pak C18 Plus 10 g, Waters Co. Massachusetts USA), preconditioned by washing with 3 column volumes of methanol followed by 3 column volumes of water. After loading the sample, the column was washed with 10 ml of water. The sample was eluted with 3 column volumes of methanol, and then concentrated by centrifugation under vacuum.

HPLC Purification of nBPN-3- β -D-G

nBPN-3- β -D-G synthesized using either method was purified by preparative HPLC. The compound of interest was separated from impurities over a Sunfire Prep C18 OBD™ 19 mm $\times$ 150 mm $\times$ 5 μ m column (Waters) using an 85% A (H₂O with 0.1% formic acid)/15% B (acetonitrile with 0.1% formic acid) isocratic method, with a 10 minute run time on an Agilent 1100 series LC system. Fractions were collected every minute and analyzed by selected ion monitoring on an Agilent 1100 MSD instrument for the presence of nBPN-3- β -D-G (*m/z* 590). Fractions containing nBPN-3- β -D-G were pooled and dried by lyophilization. Purity of the synthesized compound was determined by calibration curve. A 1 mg/mL solution of the synthesized compound was prepared in 0.1% formic acid. A 6-point calibration curve of the purchased standard, ranging from 0.1 mg/mL to 2 mg/mL was prepared. The synthesized compound, at 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL was analyzed with the calibration curve by selected ion monitoring on the Agilent 1100 LC-MS. The purity was determined as >95% based on comparison to the equation of the calibration curve.

Mass spectral characterization

Fragmentation was characterized by product ion scan in positive electrospray mode on an 4000 QTRAP MS/MS (Applied Biosystems). The compound in solution (H₂O with 0.1% formic acid) was introduced to the source by direct infusion at a rate of 20 μ L/min. MSD parameters: collision energy, 52 eV; declustering potential, 92 eV; exit cell potential, 10 eV; curtain gas, 15 eV; ion spray voltage, 5200 eV, collision gas, medium; ion source gas 1, 40; ion source gas 2, 50.0. Fragmentation of the reference material was compared to that of the chemically and biologically synthesized compound.

NMR of nBPN-3- β -D-G

¹H NMR (CDCl₃): δ 0.97 (s, 9H, C(CH₃)₃), 1.28 (m, 2H, CHCH₂C), 1.34 (s, 3H, CCH₃), 1.56 (m, 2H, CH₂CH₂C), 1.72, (m, 2H, CH₂CH₂C), 1.84 (m, 1H, CH), 2.05 (m, 2H, CCH₂CH₂), 2.78 (m, 2H, PhCH₂CH), 2.84 (m, 1H, NCHC), 3.42 (s, 3H, OCH₃), 3.48 (m, 1H, CHOH), 4.43 (s, 1H, OCH), 4.94 (m, 4H, CHOH), 5.06 (m, 1H, CHOH), 5.18 (m, 1H, CHOH), 5.46 (s, 1H, OCH), 6.55 (d, 1H, *J* 8.4 Hz, PhH), 6.87 (d, 1H, *J* 8.4 Hz, PhH). MS (ESI)⁺ [M+H]⁺ calcd 590.3, found 590.2.

Results and Discussion

Chemical glucuronidation of nBPN with **5** under nonaqueous conditions was performed using the synthetic scheme shown (Scheme 1). While glycosylation of nBPN with protected, simple aldopyranosyl bromide to give its glucuronide directly has been reported, the reactions were performed in aqueous conditions with hydroxide catalysis,²² and work from other groups suggests that C-ring cleavage is in fact the predominant reaction under these conditions²³. The presence of two competitive glucuronic acid acceptors on nBPN, the phenol and secondary amine functional groups, necessitated protection of the amine group before the addition of glucuronic acid to prevent the formation of *N*-glucuronide. The amine group was protected with trimethylsilylethoxycarbonyl (Teoc), as it is stable to mild acid and base, and can be removed with fluoride ion.^{24, 25} The use of a benzyl group as the amine protecting group was also tested, and although the benzyl groups were cleanly removed from *N*-benzyl protected nBPN (**2**) under relatively mild conditions such as palladium-carbon catalyzed hydrogenolysis, under the same conditions the deprotection of *N*-Bn-nBPN-3- β -D-G (**8**) was not successful because the glucuronic bond could not withstand even these mild hydrogenolysis conditions. A variety of other hydrogenolysis conditions were tried without success, including Pd/C catalyzed hydrogenolysis with H₂ in tetrahydrofuran (THF) or methanol/ethyl acetate at room temperature and Pd/C catalyzed

hydrogenolysis with ammonium formate in THF, refluxed at 70°C. TeocOBt, synthesized by modifying a reported procedure,²¹ was used to protect nBPN, giving Teoc-nBPN in good yield. The coupling of Teoc-nBPN (**4**) with **5** to generate Teoc-nBPN-3-β-D-AcG (**6**) under the Koenigs-Knorr reaction conditions was the most challenging step of the synthesis. The commercially available glucuronidation intermediate,^{26, 27} 1α-bromo sugar **5**, was selected as the sugar source for this synthesis. The most common solvents used for the coupling reaction have been benzene, toluene and quinoline; quinoline was used as both a base and solvent. Removal of any water produced is critical for the coupling reaction; 4 Å molecular sieves were used for the removal of water. A persistent by-product from such coupling reactions is the 'glycosene' or 2-acetoxyglycal **9**, formed by HBr elimination. A variety of salts can provide silver ions for the coupling reaction, and although silver trifluoromethanesulfonate (triflate) was used for the synthesis of retinyl glucuronide²⁸ to give a markedly better yield than Ag₂CO₃, we found that using silver triflate produced the dehydrated product **8** as the major product. Hence, Ag₂CO₃ was used. Following the success of the coupling reaction, the acetyl protecting groups and the Teoc protecting group were removed sequentially with KOH in H₂O/THF and TBAF in THF respectively. During our synthesis, formation of Δ_{4,5}-glucuronides (dehydroglucuronides, **10**) was observed. Dehydroglucuronides are a particularly relevant byproduct of this step, as they may be formed in trace or even significant amounts during any basic hydrolysis. Similar results were obtained by Tajima,²⁹ who showed that the elimination was also facilitated by a 1β-substituent.

Enzyme-assisted synthesis was accomplished via incubation of nBPN, UDP-glucuronic acid, and an NADPH generating system with a fraction of liver homogenate. *In vivo*, norbuprenorphine is glucuronidated by hepatic uridine-5'-diphosphoglucuronosyltransferases (UGT).³⁰ These enzymes are located in the endoplasmic reticulum, which are obtained as microsomes by differential ultracentrifugation of liver homogenate. An alternative is to use S9 – a fraction of liver homogenate containing both microsomes and cytosol. The benefit to using S9 is the ease of preparation; instead of multiple ultracentrifugation steps, it is obtained by collecting the supernatant following one 9,000Xg spin of total homogenate. Dog liver was used as it is more readily available than human liver, and despite reports of low circulating concentrations of all BPN metabolites in dogs administered intravenous BPN³¹, dog liver was as efficient as human liver for the conversion of BPN to BPN-3-β-D-G.¹⁷ An NADPH-generating system rather than NADPH was used to supply a reductive cofactor, and, while NADPH is not necessary for UGT enzymatic activity, it has been shown to enhance the activity of these enzymes.^{32–35} UDP-glucuronic acid was added in equimolar (or greater) amount to nBPN. The S9 fraction was sonicated immediately prior to the addition of the reaction components in order to create pores in the microsomal membranes.¹⁸ The progress of the reaction was monitored by LC/MS. An aliquot was removed from the total incubation, and the reaction stopped by adding three volumes of ice-cold acetonitrile. The supernatant was analyzed by selected ion monitoring on an 1100 MSD (Agilent) following the m/z 590 for the glucuronide and m/z 414 for the parent compound after LC separation. The conversion of nBPN to nBPN-3-β-D-G was not complete, even after an overnight incubation. In effort to increase the yield of the glucuronide, we repeated the reaction with UDP-glucuronic acid in 2-, 3-, and 5-fold molar excess and found that the latter resulted in the greatest conversion (36%). In every reaction, we observed a peak with a mass to charge ratio (m/z) of 576 at signal intensity equal to that of the nBPN-3-β-D-G (m/z 590) and nBPN (m/z 414). Since this is a 14 amu difference from the glucuronide (m/z 590), we hypothesized that this might correspond to a demethylated form of the glucuronide. Dealkylation by cytochrome P450 enzymes is a common step in drug metabolism. To test the hypothesis that this compound was a demethylated metabolite of nBPN-3-β-D-G, aminobenzotriazole (ABT), a non-specific cytochrome P450 inhibitor,³⁶ was added to the reaction. While the addition of ABT to the

reaction did not affect the presence or relative amount of m/z 576, it did increase the conversion of nBPN to nBPN-3- β -D-G, from 36 to 68% (figure 2). Having thus optimized the reaction conditions, a set of 30 1-ml reactions was initiated. Following incubation, extraction, and purification, approximately 10 mg of nBPN-3- β -D-G was obtained.

Both the biologically and chemically synthesized compound were characterized by HPLC and tandem mass spectrometry. Comparison to that of a purchased certified reference material showed that both the synthesized and reference compound had the same retention time (1.28 minutes), and a mix of the two clearly showed only one peak. Additionally, the fragmentation pattern of the synthesized was compared to the fragmentation pattern of the certified reference standard; both fragmented to give one major product ion with m/z of 414, corresponding to the mass of nBPN after the loss of the glucuronic acid (Figure 3).

In conclusion, nBPN-3- β -D-G was successfully prepared by two routes: a 4-step chemical synthesis using a silyl protecting group, and an enzyme-assisted synthesis using a metabolically active fraction of dog liver. While the enzyme-assisted synthesis had a greater yield per batch, the batch volume was limited, and multiple reactions were required for significant yield. The chemical synthesis resulted in more product per batch, albeit with a lesser overall yield. Preparative HPLC was required for final purification of nBPN-3- β -D-G prepared by either the chemical or biological synthetic route. Structure of the final product was confirmed by $^1\text{H-NMR}$, and purity was determined to be >95%.

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Figure 1. Structure of some of the byproducts of chemical synthesis

Dehydration product 8 formed during the coupling step when silver triflate was used; HBr elimination of 5 formed 9; $\Delta_{4,5}$ -glucuronides formed during the coupling and hydrolysis steps.

Figure 2. Addition of ABT to the enzyme-assisted reaction increased the conversion of nBPN to nBPN-3-β-D-G

HPLC analysis of the reaction mixture immediately preceding incubation showed one peak (RT = 1.56 min) corresponding to nBPN (Panel A). After overnight incubation, a second peak (RT = 1.28 min) corresponding to nBPN-3-β-D-G was observed (Panel B). Addition of 10 mM ABT to the reaction resulted in a greater conversion of nBPN to nBPN-3-β-D-G (Panel C).

Figure 3. A purchased nBPN-3-β-D-G standard and the synthesized nBPN-3-β-D-G have the same fragmentation pattern
nBPN-3-β-D-G precursor (m/z 590) fragments at the 3-ether linkage to give the product ion m/z 414 (bold arrows). Two less abundant product ions are m/z 396 and m/z 340 (small arrows). The purchased standard (A) and the synthesized product (B) had the same fragmentation pattern.

Scheme 1. Synthesis of norbuprenorphine-3-β-D-glucuronide (nBPN-3-β-D-G)

Reagents and conditions: (i) Et_3N , 1,4-dioxane; (ii) Ag_2CO_3 , quinoline; (iii) KOH , H_2O /THF; (iv) TBAF, THF Protection of the amine on nBPN with Teoc was followed by coupling Teoc-nBPN to a glucuronic acid by the donor acetobromo- α -D-glucuronic acid methyl ester (**5**) to give Teoc-nBPN-3-D-G (**6**). This step was followed by hydrolysis of acetyl groups with a base and then removal of the Teoc protecting group with fluoride, to yield nBPN-3-β-D-G (**1**).