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EFFICIENT SYNTHESIS OF 3-AMINODIGOXIGENIN AND 3-AMINODIGITOXIGENIN PROBES

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Abstract: Oxidation of digoxigenin and digitoxigenin to the 3-ketones followed by reductive amination produced a mixture of amine epimers. The inability to separate the epimeric mixtures of chemiluminescent digoxigenin probes derived by conjugation to the acridinium label prompted us to develop an HPLC method to separate the amines. Labeling of the pure amines resulted in good yields of the isomerically pure probes.

The plant family *Digitalis* has produced several biologically active steroidal glycosides.[1] The two major members of the family are digoxin and digitoxin, drugs still widely utilized for the treatment of various cardiac diseases.[2] Since these glycosides are toxic at higher concentrations, patient serum levels must be measured to maintain proper dosing.[3–6] While immunoassays are commonly used for this purpose, monitoring by immunoassay continues to be challenging, due to the cross-reactivities of the structurally similar but less pharmacologically active metabolites produced by stepwise deglycosidation.[7,8]

We required epimerically pure probes linked at the steroidal 3-position for immunoassay development which would contain the steroid backbone and an intact lactone ring.[9] Stereochemical integrity at the 3-position of these steroid derivatives is particularly important, as dramatically different pharmacological activities, for example, have been reported for different 3α and 3β derivatives of digoxigenin.[10] Since variable isomer ratios lead to inconsistent immunoassay performance, the availability of isomerically pure probes is critically important. Among the many labels utilized for probe preparation, fluorescent and biotinylated derivatives of digoxin and digitoxin and their metabolites have been previously described.[11–18] Of these, several probes were prepared using glycosylated substrates,[11–14] while the remaining derivatives were synthesized using epimeric mixtures of the 3-position steroidal compounds.[15–18] Although immunoassays employing chemiluminescently labeled antibodies have been noted,[19] the preparation of chemiluminescent digoxigenin or digitoxigenin probes, to the best of our knowledge, has not been previously described.

The pure 3-aminodigoxigenin and 3-aminodigitoxigenin epimers can be prepared from the separated 3-position alcohols by a multi-step sequence including 12-hydroxyl protection, tosylation, azide inversion, deprotection, and reduction.[20,21] Synthesis of new digoxigenin and digitoxigenin probes using these procedures, while potentially successful, would be rather laborious. We report here an efficient method for the preparation of the previously undescribed epimerically pure acridinium, biotin, and fluorescein probes of 3α- and 3β-aminodigoxigenin and 3α- and 3β-aminodigitoxigenin.

Oxidation of commercially available digitoxigenin with Jones reagent[22] smoothly produced ketone **4** (see below). Performing such an oxidation on digoxigenin, however, could result in oxidation of the 12-

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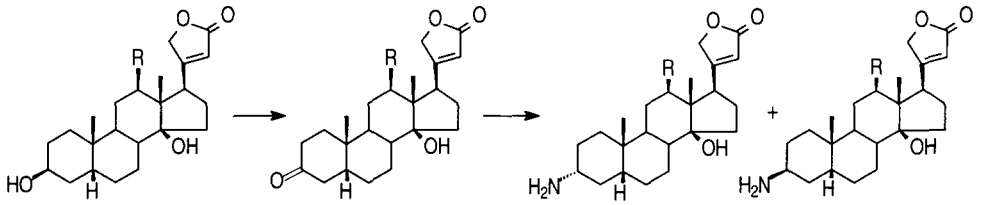
hydroxyl. Fortunately, catalytic oxidation (Pt, O_2) has been shown to selectively oxidize the 3-hydroxyl of digoxigenin in presence of the 12-hydroxyl without the need for protection.[23]

Reductive amination of the 3-ketosteroids with ammonium acetate proceeded selectively for **3** and **4** to yield epimeric mixtures of the 3α- and 3β-amines (**5a/5b** and **6a/6b**) without affecting lactone integrity, and for consistently high yields, care needs to be taken to rigorously exclude moisture by using freshly sublimed ammonium acetate and anhydrous methanol.[24]



1: R=OH **2:** R=H **3:** R=OH **4:** R=H **5a:** R=OH **6a:** R=H **5b:** R=OH **6b:** R=H

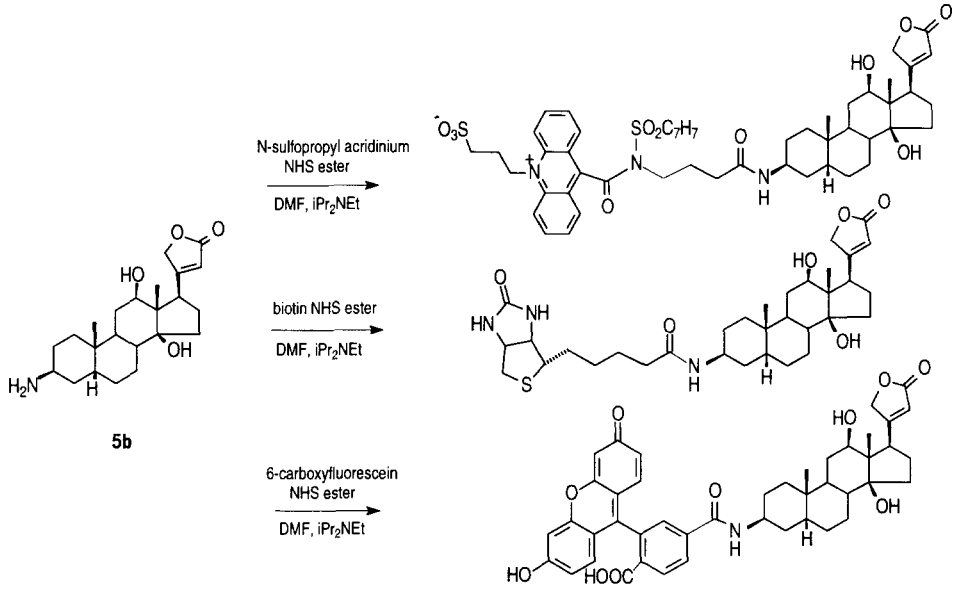
Since reaction of the epimeric 3-amine mixtures with the active esters of the labels produced good yields of mixtures of the probes, we first attempted to separate the epimeric chemiluminescent acridinium probe mixture by HPLC under a variety of conditions, but determined that the compounds were not practically separable in our hands. We thus focused on development of an efficient method for the separation of the 3-aminodigoxigenin epimers and the 3-aminodigitoxigenin epimers. We found that an effective separation of the amine epimers could be accomplished by preparative HPLC. Optimization of the method showed that separation of the epimers can be efficiently achieved using a C18 reversed-phase μBondapak column with acetonitrile/aqueous trifluoroacetic acid mixtures as eluents. Under these conditions, the chromatograms of both epimeric mixtures presented well-resolved peaks (**5b** and **5a**: 5.7 and 8.1 mins; **6b** and **6a**: 6.3 and 8.5 mins). Typically, β/α mixtures of 33:66 to 25:75 were observed for the digoxigenin and digitoxigenin amines, which displayed resonances at 3.19 ppm and 2.68 ppm for the 3-position protons of the 3β- and 3α-amines, respectively.[10,25] The presence of a polar 12-hydroxyl functionality in the digoxigenin series did not significantly alter the epimer ratio obtained for the epimeric 3-aminodigoxigenin products.

Reaction of the separated amines with the label *N*-hydroxysuccinimide (NHS) esters proceeded smoothly, giving good yields of the isomerically pure probes (illustrated below for **5b**) as lyophilized solids.[26] Analytical HPLC coinjection confirmed that the epimeric acridinium as well as biotin probes were not practically separable, while the fluorescein probes were potentially resolvable (α/β 3-aminodigoxigenin fluorescein probes: 7.0 min/8.2 min; α/β 3-aminodigitoxigenin fluorescein probes: 6.8 min/8.2 min).

The digoxigenin fluorescein probes were evaluated for binding with an antidigoxin antibody in a fluorescence polarization immunoassay (FPIA) format. While the 3α-aminodigoxigenin probe showed good binding and displacement, providing a calibration curve with a span of 96 mP (millipolarization units) for the clinically relevant digoxin concentration range (0-5 ng/mL), the 3β-aminodigoxigenin probe, having the same

stereochemistry as digoxigenin at the 3-position, showed no displacement. These data attest to the critical importance of the epimeric purity of the probes for assay performance and reproducibility.

In summary, we have prepared twelve novel epimerically pure probes, which include chemiluminescent, biotinylated, and fluorescent derivatives of 3α- and 3β-aminodigoxigenin and 3α- and 3β-aminodigitoxigenin. We believe these probes will find considerable utility in assays for digoxin and digitoxin.



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25. Digitoxigenone (100 mg, 0.27 mmol) was dissolved in anhydrous MeOH (56 mL), and freshly sublimed NH_4OAc (249 mg, 3.22 mmol) was added, followed by $NaBH_3CN$ (34 mg, 0.54 mmol). After 12 h, TLC indicated no starting material. The reaction was acidified to pH 2 with 1N HCl, basified to pH 9 with solid K_2CO_3, and extracted 4 x 20 mL with $CHCl_3$. The extracts were dried over Na_2SO_4, and concentrated to provide 98 mg of the amine mixture as a white foam. The mixture was dissolved in MeOH, and separated by reversed-phase HPLC (40 x 100 mm C^{18} μBondapak column, 25% CH_3CN/75% 0.05% aq. TFA, 40 ml/min, ~10 mg injections). The combined fractions of each epimer were concentrated, basified with K_2CO_3 to pH 9, extracted 4 x 20 mL with $CHCl_3$. The combined extracts were dried over Na_2SO_4, and concentrated to provide the epimeric amines as white solids. First peak: 3β-aminodigitoxigenin (**6b**, 21 mg, 21%): HPLC (25% CH_3CN/75% 0.05% aqueous TFA) T_R 6.3 min (>98%); ESMS $(M + H)^+$ at 374; ^1H NMR (CD_3OD) δ 5.90 (s, 1H), 5.03 (d, 1H, *J* = 18.2 Hz), 4.93 (d, 1H, *J* = 17.8 Hz, partially solvent obscured), 3.19 (br s, 1H), 2.83 (m, 1H), 2.18 (m, 2H), 1.94–1.20 (m, 19H), 0.98 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (CD_3OD) δ 178.6, 177.4, 117.9, 86.5, 75.4, 52.1, 51.0, 47.5, 42.6, 40.9, 37.2, 36.6, 33.7, 33.4, 30.5, 28.0, 27.9, 24.1, 22.5, 22.2, 16.3. 2nd peak: 3α-aminodigitoxigenin (**6a**, 52 mg, 52%): HPLC (25% CH_3CN/75% 0.05% aq. TFA) T_R 8.5 min (>98%); ESMS $(M + H)^+$ at 374; ^1H NMR (CD_3OD) δ 5.90 (s, 1H), 5.03 (d, 1H, *J* = 18.2 Hz), 4.95 (d, 1H, *J* = 17.8 Hz, partially solvent obscured), 2.83 (m, 1H), 2.68 (m, 1H), 2.18 (m, 2H), 1.90–1.04 (m, 19H), 0.95 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (CD_3OD) δ 178.6, 177.4, 117.9, 86.5, 75.4, 52.3, 52.1, 51.0, 43.6, 42.8, 40.9, 37.4, 37.1, 36.9, 36.0, 33.3, 31.2, 28.0, 23.9, 22.5, 22.1, 16.3.

26. For each probe, 25 mg (67 μmol of **6a/6b**, 64 μmol of **5a/5b**) of the amine epimer was dissolved in 500 μL anhydrous DMF. The NHS ester of each probe (67 μmol: 46 mg for N-sulfopropyl acridinium, 23 mg for biotin, or 32 mg for 6-carboxyfluorescein) was added, followed by *i*Pr_2NEt (12 μL, 67 mmol). After stirring for 18-40 h, the reactions were purified by reversed phase HPLC. Selected data (ACR = acridinium, BIO = biotin; CF = 6-carboxyfluorescein) is shown below.

5a-CF: 30 mg (61%): T_R 8.2 min (40% CH_3CN/60% 0.05% aq TFA); ESMS $(M + H)^+$ at 748.4; ^1H NMR (CD_3OD) δ 8.08 (d, 2H, *J* = 1.2 Hz), 7.69 (t, 1H, *J* = 1.2 Hz), 6.95 (dd, 2H, *J* = 8.9 Hz, 2.1 Hz), 6.65 (dd, 2H, *J* = 2.2 Hz, 0.8 Hz), 6.60 (dm, 2H, *J* = 8.9 Hz), 5.89 (s, 1H), 5.03 (d, 1H, *J* = 18.2 Hz), 4.95 (d, 1H, *J* = 17.8 Hz, partially solvent obscured), 3.92 (m, 1H), 3.38 (m, 1H, partially solvent obscured), 2.09–1.11 (m, 21H), 0.96 (s, 3H), 0.76 (s, 3H).

5b-CF: 29 mg (59%): T_R 6.8 min (40% CH_3CN/60% 0.05% aq TFA); ESMS $(M + H)^+$ at 748.4; ^1H NMR (CD_3OD) δ 8.09 (d, 1H, *J* = 8.0 Hz), 8.02 (dd, 1H, *J* = 8.0 Hz, 1.5 Hz), 7.65 (d, 1H, *J* = 1.2 Hz), 7.00 (d, 2H, *J* = 9.0 Hz, 2.1 Hz), 6.64 (d, 2H, *J* = 2.2 Hz), 6.60 (dd, 2H, *J* = 9.0 Hz, 2.0 Hz), 5.89 (s, 1H), 5.03 (d, 1H, *J* = 18.2 Hz), 4.95 (d, 1H, *J* = 17.8 Hz, partially solvent obscured), 4.24 (br s, 1H), 3.39 (m, 1H, partially solvent obscured), 2.19–1.11 (m, 21H), 0.96 (s, 3H), 0.78 (s, 3H).

6a-ACR: 32 mg (51%): T_R 4.2 min (50% CH_3CN/50% 0.05% aq TFA); ESMS $(M + H)^+$ at 941.5; ^1H NMR (CD_3CN) δ 8.84 (m, 2H), 8.39 (m, 2H), 8.14 (d, 2H, *J* = 8.4 Hz), 7.79 (t, 1H, *J* = 7.6 Hz), 7.74 (t, 1H, *J* = 7.5 Hz), 7.60 (d, 1H, *J* = 7.2 Hz), 7.10 (m, 2H), 5.81 (s, 1H), 5.62 (m, 2H), 4.86 (m, 2H), 4.35 (m, 2H), 3.70 (m, 1H), 3.46 (m, 1H), 3.09 (m, 2H), 2.27–1.13 (m, 21H), 0.92 (s, 3H), 0.82 (s, 3H).

6b-ACR: 39 mg (62%): T_R 4.3 min (50% CH_3CN/50% 0.05% aq TFA); ESMS $(M + H)^+$ at 941.5; ^1H NMR (CD_3CN) δ 8.85 (m, 2H), 8.40 (m, 2H), 8.14 (d, 2H, *J* = 7.8 Hz), 7.79 (t, 1H, *J* = 7.6 Hz), 7.74 (t, 1H, *J* = 7.3 Hz), 7.61 (d, 1H, *J* = 8.1 Hz), 7.10 (m, 2H), 5.83 (s, 1H), 5.63 (m, 2H), 4.89 (m, 2H), 4.24 (m, 1H), 4.08 (br s, 1H), 3.43 (m, 1H), 3.10 (m, 2H) 2.15–1.05 (m, 21H), 0.95 (s, 3H), 0.82 (s, 3H).

6a-BIO: 38 mg (94%): T_R 6.8 min (40% CH_3CN/60% 0.05% aq TFA); ESMS $(M - H)^-$ at 598.4; ^1H NMR ($CDCl_3$) δ 6.29 (br s, 1H), 5.88 (s, 1H), 5.55 (m, 2H), 4.98 (d, 1H, *J* = 18.3 Hz), 4.80 (d, 1H, *J* = 18.2 Hz), 4.59 (dd, 1H, *J* = 8.0, 4.8 Hz), 4.40 (dd, 1H, *J* = 7.8, 4.8 Hz), 3.70 (br s, 1H), 3.20 (m, 1H), 2.96 (dd, 1H, *J* = 12.9, 4.8 Hz), 2.79 (m, 2H), 2.11–1.12 (m 29H), 0.93 (s, 3H), 0.87 (s, 3H).

6b-BIO: 36 mg (89%): T_R 7.0 min (40% CH_3CN/60% 0.05% aq TFA); ESMS $(M - H)^-$ at 598.4; ^1H NMR ($CDCl_3$) δ 6.56 (br s, 1H), 5.96 (m, 2H), 5.88 (s, 1H), 4.99 (d, 1H, *J* = 18.5 Hz), 4.81 (d, 1H, *J* = 18.1 Hz), 4.59 (dd, 1H, *J* = 8.0, 4.4 Hz), 4.40 (dd, 1H, *J* = 7.8, 4.7 Hz), 4.19 (br s, 1H), 3.19 (m, 1H), 2.94 (dd, 1H, *J* = 13.1, 4.9 Hz), 2.79 (m, 2H), 2.27–1.12 (m 29H), 0.96 (s, 3H), 0.87 (s, 3H).