Journal of Labelled Compounds and Radiopharmaceuticals
J Label Compd Radiopharm 2007; **50**: 197–206.
Published online in Wiley InterScience
(www.interscience.wiley.com). DOI: 10.1002/jlcr.1260

JLCR

Research Article

Synthesis and deuterium labelling of the pure selective estrogen receptor modulator (SERM) acolbifene glucuronides

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Received 7 December 2006; Revised 12 January 2007; Accepted 14 January 2007

Abstract: Acolbifene (EM-652·HCl, SCH 57068·HCl), a highly potent and orally active selective estrogen receptor modulator (SERM), is at late stage clinical development for the treatment of estrogen-sensitive breast cancer. Acolbifene-7-glucuronide **1** (major) and acolbifene-4′-glucuronide **2** (minor) were identified as metabolites of acolbifene in the human. The two monoglucuronides and a diglucuronide **3** as well as the corresponding ^{2}H-labelled derivatives **4–6** were synthesised for use as preclinical and clinical standards for LC–MS/MS analysis. All glucuronides were prepared by the Schmidt glycosylation of monoprotected acolbifene with a glucuronyl imidate at −10°C to prevent epimerisation at the C-2 position. The two monoglucuronides **1** and **2** of acolbifene were separated by semi-preparative HPLC. Incorporation of three deuteriums was achieved by alkylation of chromanone **15** with $C^{2}H_{3}MgI$ followed by dehydration with $C^{2}H_{3}CO_{2}{}^{2}H/{}^{2}H_{2}O$. After chemical resolution and salt neutralisation, [$^{2}H_{3}$]acolbifene **19** was obtained with 99.4% enantiomeric purity and >98% isotopic purity. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: acolbifene (EM-652·HCl); acolbifene glucuronides; deuterium; SERM; metabolites

Introduction

Acolbifene (EM-652·HCl, SCH 57068·HCl), a highly potent and orally selective estrogen receptor modulator (SERM), is currently in advanced clinical trials for treatment of estrogen-dependent breast cancer.[1] Preclinical and clinical data indicate that acolbifene possesses characteristics superior to tamoxifen and raloxifene for breast and uterine cancer prevention and treatment as well as for hormone replacement therapy at menopause.[2–4] Metabolic studies in rats with ^{14}C-radiolabelled derivatives[5] of acolbifene and the dipivaloate prodrug EM-800 (SCH 57050) have shown that the known metabolites of acolbifene included acolbifene-7-glucuronide **1**, acolbifene-4′-glucuronide **2** and acolbifene-7,4′-diglucuronide **3** as illustrated in Figure 1.[6]

After oral administration of acolbifene to postmenopausal women, the main circulating metabolite detected in plasma was glucuronide **1** (78%) while trace amounts of the other regiosiomer **2** (3%) were also measured.[7] Reference standards of the three glucuronides **1–3** were required for assays of acolbifene glucuronides in preclinical and clinical trials. The corresponding stable ^{2}H-labelled derivatives (Figure 2) of these three glucuronides were also needed for use as LC/MS–MS internal standards. A preliminary disclosure of the syntheses of the three glucuronides **1–3** and their corresponding ^{2}H-labelled derivatives **4–6** has been recently reported[8]; further details of the syntheses are reported herein.

Results and discussion

Synthesis of the two acolbifene monoglucuronides **1–2** and diglucuronide **3** is outlined in Schemes 1 and 2. Previous reports on the preparation of aryl-*O*-*β*-glucuronides concerned traditional glycosylation with glucuronyl donors,[9] oxidation of glucopyranosides,[9,10] as well as enzymatic[9] and microbial[11] bioconversions. Initially,

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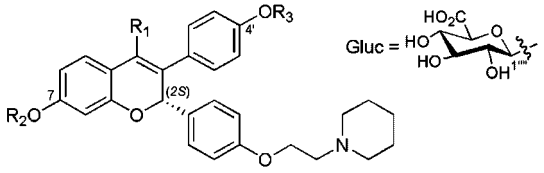
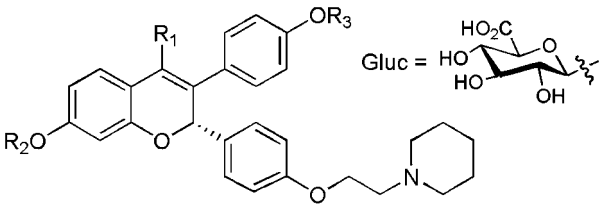


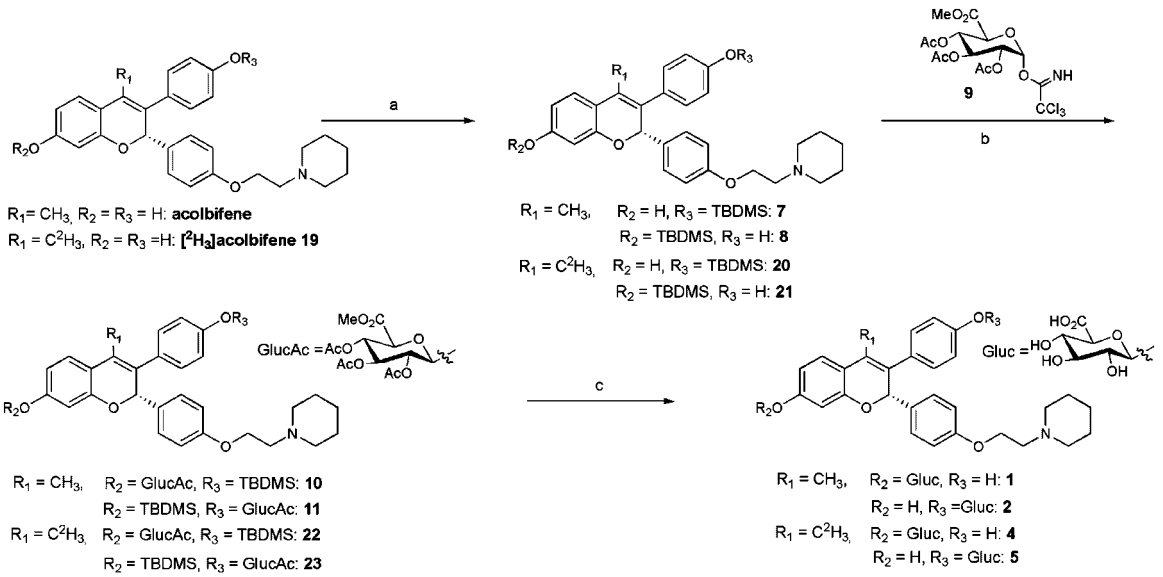
$R_1 = CH_3$; $R_2 = R_3 = H$: **acolbifene (EM-652·HCl)** and $R_2 = R_3$ = Piv: **EM-800**
R_2 = Gluc, R_3 = H: **acolbifene-7-β-glucuronide 1**
R_2 = H, R_3 = Gluc: **acolbifene-4'-β-glucuronide 2**
$R_2 = R_3$ = Gluc: **acolbifene-7,4'-β,β-diglucuronide 3**

Figure 1 Acolbifene, EM-800 and their glucuronidated derivatives **1**–**3**.



$R_1 = C^2H_3$; R_2 = Gluc, $R_3 = H$: **4**
$R_2 = H$, R_3 = Gluc: **5**
$R_2 = R_3$ = Gluc: **6**

Figure 2 Structures of [$^2H_3$]acolbifene glucuronides **4**–**6**.



R_1= CH_3, $R_2 = R_3 = H$: **acolbifene**
$R_1 = C^2H_3$, $R_2 = R_3$ =H: **[$^2H_3$]acolbifene 19**

$R_1 = CH_3$, $R_2 = H$, R_3 = TBDMS: **7**
R_2 = TBDMS, $R_3 = H$: **8**
$R_1 = C^2H_3$, $R_2 = H$, R_3 = TBDMS: **20**
R_2 = TBDMS, $R_3 = H$: **21**

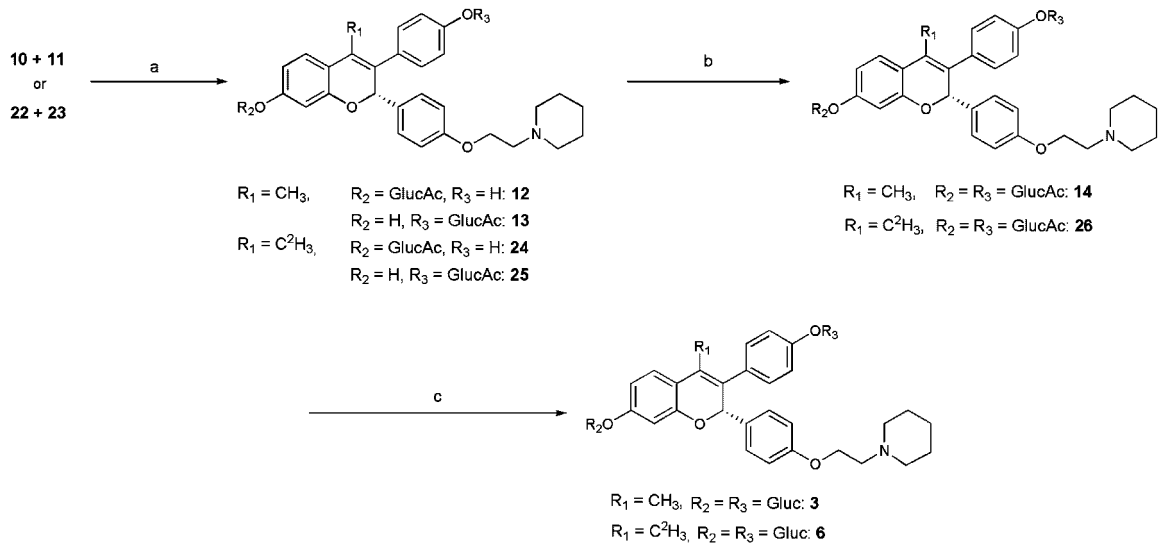
$R_1 = CH_3$, R_2 = GlucAc, R_3 = TBDMS: **10**
R_2 = TBDMS, R_3 = GlucAc: **11**
$R_1 = C^2H_3$, R_2 = GlucAc, R_3 = TBDMS: **22**
R_2 = TBDMS, R_3 = GlucAc: **23**

$R_1 = CH_3$, R_2 = Gluc, $R_3 = H$: **1**
$R_2 = H$, R_3 =Gluc: **2**
$R_1 = C^2H_3$, R_2 = Gluc, $R_3 = H$: **4**
$R_2 = H$, R_3 = Gluc: **5**

Scheme 1 Synthesis of acolbifene-7-glucuronide **1**, acolbifene-4′-glucuronide **2** and their corresponding ^2H-labelled derivatives **4**–**5** (as a mixture). Reagents and conditions: (a) TBDMSOTf (1.1 equiv.), 2,6-lutidine (4 equiv.), dichloromethane, −20°C, 1 h, 42%; (b) imidate **9** (1.2 equiv.), $BF_3 \cdot OEt_2$ (1.7 equiv.), dichloromethane, −10°C, 1 h, 59%; (c) 2 N LiOH (5 equiv.), dioxane, room temperature, 5 h, 70–85%.

we envisaged preparing glucuronides **1**–**3** from free acolbifene by classical Koenigs–Knorr glycosylation and its variants.[12] However, the reaction with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranosyluronate and Ag_2CO_3[12a] or $CdCO_3$[12b] catalysts led to complex mixtures. Similarly, no glucuronide was obtained on

DOI: 10.1002.jlcr



Scheme 2 Synthesis of acolbifene-7,4′-diglucuronide **3** and its corresponding ^{2}H-labelled derivative **6**. Reagents and conditions: (a) HF · Pyr, THF, room temperature, 12 h, 74%; (b) imidate **9** (1.25 equiv.), $BF_3 \cdot OEt_2$ (2 equiv.), dichloromethane, −10°C, 1 h, 52%; (c) 2 N LiOH (9 equiv.), 1:1 dioxane–water, room temperature, 5 h, 75%.

reaction of acolbifene with methyl 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosyluronate and $SnCl_4$.[13]

We suspected that the low solubility of acolbifene in the solvents used for these reactions was responsible for this problem. A solution to this issue has been the conversion of acolbifene into the more soluble monoTBDMS ethers.[14] Free acolbifene (100% enantiomeric excess (ee)) was treated with TBDMSOTf and 2,6-lutidine thus providing regioisomeric monosilylethers **7** and **8**, in 42% yield, with 55:45 regioselectivity, as determined by ^{1}H NMR. Glycosylation of this mixture, however, under the conditions mentioned above was again unsuccessful in our hands. We thus turned to a Schmidt glycosylation[15] recently used to prepare mono- and diglucuronides of indole-based SERMs.[16] Thus, monosilylethers **7** and **8** were treated with the readily available imidate[17] **9** in the presence of $BF_3 \cdot OEt_2$ at −10°C to give the fully protected β-glucuronates **10**–**11** as a 55:45 mixture in 59% yield. Exploratory test runs have shown that the temperature should be maintained at −10°C, even during neutralisation of the reaction mixture, in order to prevent epimerisation at position C-2 (<2%). Hydrolysis of **10**–**11** with 2 N LiOH in dioxane at room temperature yielded, after filtration on a HP-20SS resin, the free monoglucuronides **1** and **2** in 85% yield as a 55:45 mixture. Comparison of the HPLC profile of monoglucuronides **1**–**2** with that obtained from racemic (2*R*,2*S*)-acolbifene (EM-343) shows that the final material contains only small amounts (<2%) of monoglucuronides of (2*R*)-enantiomer (Figure 3).

Separation of monoglucuronides **1** and **2** was achieved by semi-preparative chromatography. First, analytical quantities (5 mg) of monoglucuronides **1** and **2** were obtained at high purity (>98%) using a Nova-Pak HR C18 column (40 × 100 mm). In order to obtain the quantities required for metabolic studies, the mixture was separated on a Supelco LC-8 DB column (10 × 250 mm) and the analytical conditions were optimised to increase the yield. After desalting and lyophilisation, glucuronides **1** and **2** were obtained in 41 and 35% yields as well as 94.6 and 93.5% purity, respectively. Each compound contained less than 2% of the other regioisomer.

The regiochemistry of each glucuronide was determined by ^{1}H NMR in comparison with acolbifene (HCl salt) and by 2D NMR experiments (COSY, HMQC, HMBC). Glucuronidation results in a low-field shift of the resonance for the protons flanking each of the newly formed sugars. Accordingly, the chemical shifts of H-8 and H-6 changed from 6.07 and 6.32 ppm in acolbifene to 6.32 and 6.57 ppm for metabolite **1**. In the case of metabolite **2**, the H-3′/5′ protons shifted from 6.70 ppm in acolbifene to 6.95 ppm in **2**. In addition, the relatively large coupling constant ($J = 7.4$ Hz) between anomeric H-1′′′′ proton and H-2′′′′ suggested that each of the glucuronides has the desired β-stereochemistry.

For the preparation of diglucuronide **3** (Scheme 2), neutral HF · Pyr was chosen for TBDMS-deprotection of glucuronates **10**–**11** in order to avoid the transesterification observed with TBAF. The resulting mixture of

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phenols **12** and **13** thus obtained in 74% yield was coupled with imidate **9**, as described above, thus giving the fully protected diglucuronate **14** in 52% yield. Finally, hydrolysis with 2 N LiOH in 1:1 dioxane–water gave, after purification, diglucuronide **3** as a pink solid in 75% yield (96.4% purity).

Having developed conditions for glucuronidation of acolbifene which preserve chirality at C-2, we then focused on the preparation of stable-labelled analogs having an appropriately high number of deuteriums (at least 3). An efficient and direct deuteration of phenols using $^{2}H_3PO_4 \cdot BF_3/^{2}H_2O$ has been reported on related polyphenolic systems (isoflavonoid).[18] However, in our case, chirality and chemical stability of acolbifene would likely be affected with this very acidic deuteration reagent.

We thus chose to introduce the labels on the aglycon part of the molecule by modification of our original synthesis of acolbifene.[19] We thought that the methyl group would be suitable for labelling because insertion of three deuteriums at this position would require only slight synthetic modifications. Furthermore, we could start directly with an advanced intermediate of the acolbifene commercial process synthesis, the diprotected *trans*-chromanone **15**.[20] The low cost of trideuterated methylating reagents with high enrichment was also considered.

As shown in Scheme 3, the deuterium atoms were incorporated by alkylation with freshly prepared $C^{2}H_3MgI$ (99.9 atom% ^{2}H). Dehydration and deprotection of alcohols **16** was achieved by heating in 19:1 $C^{2}H_3CO_2\,^{2}H$ (99.9 atom% ^{2}H)-$^{2}H_2O$ (100 atom%

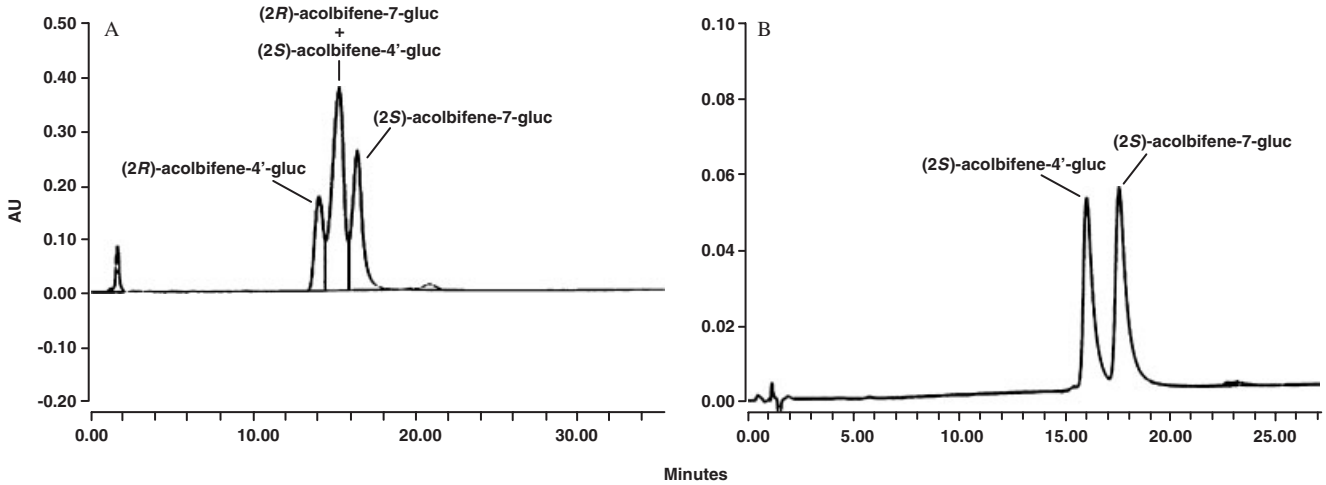
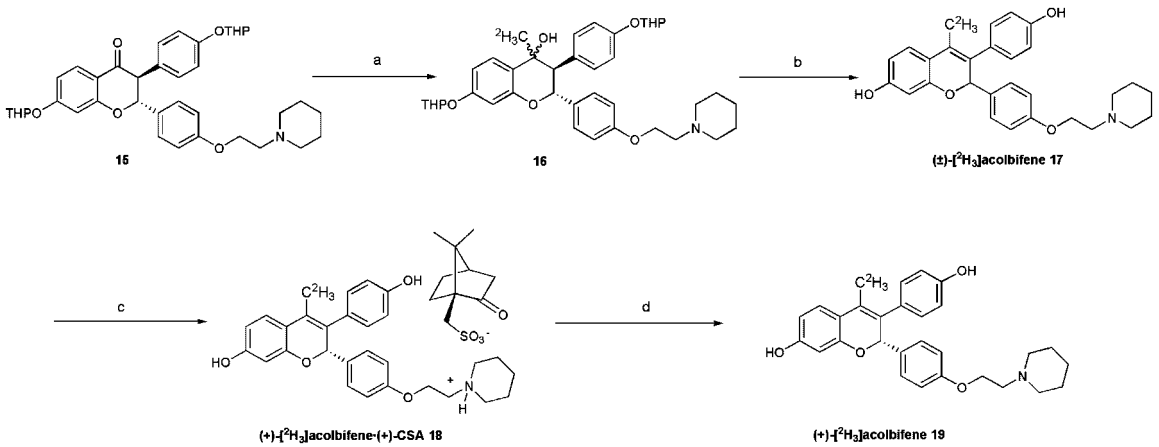


Figure 3 HPLC profiles of monoglucuronides (Nova-Pak C18 column). (A) racemic (2*S*,2*R*)-acolbifene monoglucuronides and (B) (2*S*)-acolbifene monoglucuronides **1**–**2**.



Scheme 3 Synthesis of [$^{2}H_3$]acolbifene **19**. Reagents and conditions: (a) $C^{2}H_3MgI$ (4 equiv.), THF, −40°C to room temperature, 5 h; (b) 19:1 $C^{2}H_3CO_2\,^{2}H$–$^{2}H_2O$, 90°C, 0.5 h, 82% from **15**; (c) (+)-CSA (1 equiv.), 20:1 acetone–water, 1 h; first recrystallisation in 95% EtOH, 2 days then second recrystallisation in 94:6 dichloromethane–DMF, 24% from **17**; (d) Na_2CO_3 (4 equiv.), EtOAc, 1 h, 100%.

DOI: 10.1002.jlcr

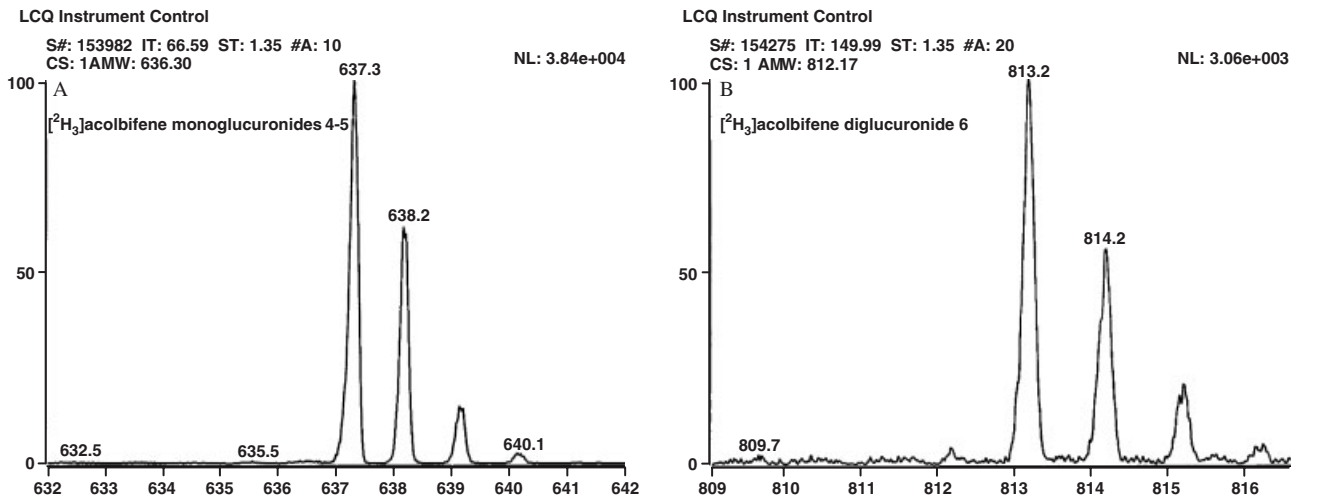


Figure 4 Full scan APCI mass spectra recorded in positive-ion mode. (A) [$^2H_3$]acolbifene monoglucuronides **4**–**5** and (B) [$^2H_3$]acolbifene diglucuronide **6**.

^2H) at 90°C for 30 min to give racemic [$^2H_3$]acolbifene **17** in 82% yield (from **15**). The APCI mass spectrum showed evidence of an uptake of three deuterium atoms: the molecular ion (M+H) was shifted from *m/z* 458 in acolbifene to *m/z* 461. Incorporation into the methyl was confirmed by the ^1H NMR spectrum where the singlet of the methyl protons (observed at 2.07 ppm in acolbifene) was absent. Isotopic purity of [$^2H_3$]acolbifene **17** was estimated at >98%. No d_1 and d_0 species were found in the mass spectrum. A fully deuterated solvent was necessary to minimize the amount of available protons in the equilibrium mixture. Thus, with 5% aqueous acetic acid, we observed partial $^2H/H$ exchanges that probably occur via acid catalysed *endo–exo* equilibrium. In this case, the APCI mass spectrum gave the following isotopic distribution: d_3 60%, d_2 25%, d_1 10% and d_0 5%. Chiral resolution of racemic **17** with (*S*)-(+)-camphorsulfonic acid gave [$^2H_3$]acolbifene·(+)-CSA **18** in 24% yield which, after neutralisation, provided quantitatively enantiomerically pure amine **19** (99.4% ee).[5,19,20] No significant loss of deuterium atoms was observed in the resolution process and amine **19** was obtained with >98% isotopic purity (APCI MS).

Conversion of [$^2H_3$]acolbifene **19** into its monoglucuronides **4**–**5** and diglucuronide **6** was achieved as described for unlabelled acolbifene (Schemes 1 and 2). After monoprotection as TBDMS ethers, the regioisomeric amines **20** and **21** were subjected to glycosylation with imidate **9**. Saponification of the resulting glucuronates **22** and **23** led, after purification, to a 55:45 mixture of ^2H-labelled monoglucuronides **4** and **5**, in 17% yield (from **19**). No attempt was made to separate the two monoglucuronides since they could be used directly as a mixture for MS quantifications. Finally, deprotection of the mixture **22** and **23**, followed by glycosylation and saponification gave ^2H-diglucuronide **6** in 29% yield. Examination of the APCI mass spectra of **4**–**5**, **6** (Figure 4) and their corresponding unlabelled metabolites **1**–**2**, **3** confirmed that each compound was >98% isotopically pure. Chemical purity was estimated at 96.4% for **4**–**5** and 93.7% for **6** (the major contaminants were probably didehydroglucuronides).

Conclusion

We have prepared the two acolbifene monoglucuronides **1** and **2** as well as diglucuronide **3**, and their corresponding ^2H-labelled derivatives **4**–**6**, by the Schmidt glycosylation. Only slight modifications of the original synthesis of acolbifene were necessary to accommodate the insertion of three deuterium atoms on the methyl group. It was advantageous to replace, in the dehydration step, the original CH_3CO_2H/H_2O mixture by the fully deuterated $C^2H_3CO_2{}^2H/{}^2H_2O$ mixture in order to minimize $^2H/H$ exchanges.

Experimental

General method

Trichloroacetimidate **9** was prepared in five steps from the commercially available D-glucuronolactone.[17] $C^2H_3CO_2{}^2H$ (99.9 atom% ^2H) and ^2H_2O (100 atom% ^2H) were purchased from CDN isotopes, and C^2H_3I

DOI: 10.1002.jlcr

(99.9 atom% ^{2}H) from Aldrich. All reactions were carried out in flame-dried glassware under a positive atmosphere of dry Ar. Column chromatography was carried out using a silica gel (230–400 mesh) (EM Science) and by reverse-phase using a LiChroprep® RP-18 resin (40–63 μm) (EM Science). Dianion® HP-20SS resin was obtained from Supelco and washed with MeOH and water before use. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker WH300 spectrometer or at 400 MHz on a Bruker Avance 400 spectrometer. APCI mass spectra were recorded on a LCQ (Thermo-Finnigan, San Jose, CA) with an APCI source (positive mode). High-resolution mass spectra were obtained at Le Laboratoire de Spectrométrie de Masse (Université de Sherbrooke) (EI/CI, 70 eV) and at Le Centre Régional de Spectrométrie de Masse (Université de Montréal) (ESI, positive mode). Chemical purities were determined by HPLC using a Nova-Pak C18 column (3.9 × 150 mm) eluted with 20 mM ammonium acetate in MeOH–water, flow rate 1.0 ml/min, UV detector at 240 nm.

(2S)-7-hydroxy-3-(4′-tert-butyldimethylsilyloxyphenyl)-4-methyl-2-(4″-(2‴–(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (7) and (2S)-7-tert-butyldimethylsilyloxy-3-(4′-hydroxyphenyl)-4-methyl-2-(4″-(2‴-(1-piperidino) ethoxy)phenyl)-2H-1-benzopyran (8). To a suspension of free acolbifene (10.3 g, 22.5 mmol) in dichloromethane (250 ml) was added 2,6-lutidine (10 ml, 90 mmol). The resulting red solution was cooled to −10°C (acetone–ice) and then TBDMSOTf (5.7 ml, 25 mmol) was added over 30 min. After removing the cooling bath, the mixture was stirred for 1 h while the temperature slowly rose to 5°C. It was then poured into water (200 ml). The organic phase was washed once with brine (50 ml), dried (Na_2SO_4), and concentrated. Flash chromatography of the residue (dichloromethane–EtOH 99:1) yielded amorphous monosilylated ethers **7** and **8** (5.5 g, 42%) as a 55:45 mixture. ^{1}H NMR ($CDCl_3$) δ 0.18 and 0.20 (s, 6H), 0.96 and 0.98 (s, 9H), 1.52 (m, 2H, CH_2), 1.74 (m, 4H, CH_2), 2.07 (s, 3H, Me), 2.67 (m, 4H, NCH_2), 2.97 (m, 2H, CH_2N), 4.02 and 4.09 (m, 2H, OCH_2), 5.81 and 5.82 (s, 1H, H-2 for **7** and **8**, respectively), 6.30 and 6.32 (d, $J = 2.4$ Hz, 1H, H-8 for **7** and **8**, respectively), 6.44 and 6.46 (dd, $J =$ 2.3 and 8.4 Hz, 1H, H-6 for **7** and **8**, respectively), 6.59–7.18 (m, 9H, Ar). HRSM (EI) calcd for $C_{35}H_{45}NO_4Si$: 571.3118. Found 571.3123.

(2S)-7-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-3-(4′-tert-butyldimethylsilyloxyphenyl)-4-methyl-2-(4″-(2‴-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (10) and (2S)-7-tert-butyldimethylsilyloxy-3-(4′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-phenyl)-4-methyl-2-(4″-(2‴-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (11). A mixture of monosilylated acolbifene **7** and **8** (8.00 g, 14.0 mmol) was azeotroped twice with dry benzene (100 ml) to ensure dryness and removal of residual ethanol. Freshly prepared trichloroacetimidate **9** (7.80 g, 16.8 mmol) was added to the oily residue followed by dry dichloromethane (50 ml). The solution was cooled with an acetone–ice bath (−10°C) and $BF_3 \cdot OEt_2$ (29 ml, 24 mmol) was slowly introduced. After stirring for 1 h, saturated $NaHCO_3$ (30 ml) was added and the resulting suspension stirred at −10°C until the yellow color turned red. Water (30 ml) was added and the aqueous phase was extracted with dichloromethane (2 × 50 ml), then the combined extracts were washed with brine and dried over Na_2SO_4. Flash chromatography of the residue with dichloromethane–EtOH (99.5:0.5) furnished protected monoglucuronates **10** and **11** (7.37 g, 59%) as a 55:45 amorphous mixture. ^{1}H NMR ($CDCl_3$) δ 0.18 and 0.20 (s, 6H), 0.96 and 0.98 (s, 9H), 1.45 (m, 2H, CH_2), 1.60 (m, 4H, CH_2), 2.05–2.06 (m, 9H, OAc), 2.07 and 2.08 (s, 3H, Me), 2.50 (m, 4H, NCH_2), 2.75 (m, 2H, CH_2N), 3.70 and 3.72 (s, 3H, CO_2Me), 4.05 (d, $J = 5.5$ Hz, 2H, CH_2O), 4.16 and 4.17 (d, $J = 9.5$ Hz, 1H, $CHCO_2Me$), 5.09 and 5.13 (d, $J = 7.2$ Hz, 1H, H-1‴′ for **10** and **11**, respectively), 5.23–5.37 (m, 3H, CHOAc), 5.82 and 5.86 (s, 1H, H-2 for **11** and **10**, respectively), 6.29 and 6.43 (d, $J = 2.4$ Hz, 1H, H-8 for **11** and **10**, respectively), 6.44 and 6.56 (dd, $J = 2.4$ and 8.7 Hz, 1H, H-6 for **11** and **10**, respectively), 6.72–7.23 (m, 9H, Ar). HRSM (EI) calcd for $C_{48}H_{61}NO_{13}Si$: 887.3912. Found 887.3917.

(2S)-7-(β-D-glucopyranosyluronic acid)-3-(4′-hydroxyphenyl)-4-methyl-2-(4″-(2‴-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (1) and (2S)-7-hydroxy-3-(4′-(β-D-glucopyranosyluronic acid)phenyl)-4-methyl-2-(4″-(2‴-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (2). To a solution of protected glucuronates **10** and **11** (7.00 g, 7.89 mmol) in dioxane (80 ml) was added 2 N LiOH (19.7 ml, 39.4 mmol) at room temperature. The mixture was stirred for 3 h and additional 2 N LiOH was added (2 ml), stirring being then continued for 2 h. Ten percent HCl (20 ml) was added (pH 7) followed by HP-20SS resin (4 g). After evaporation to dryness, the resulting pink powder was placed on a HP-20SS resin column. All the lithium salts were removed by a first elution with water. A second elution with water–MeOH (from 90:10 to 10:90) afforded a 55:45 mixture of monoglucuronides **1** and **2** (4.29 g, 85%). Separation of the regioisomers was performed on a Waters Delta Prep 4000 system equipped with a Supelco LC-8 DB column (10 × 250 mm, 5 μm). The mixture of monoglucuronides (2 mg) was dissolved in 1:1 75 mM ammonium formate–MeOH (1 ml) and injected on the column. The mobile

DOI: 10.1002.jlcr

phase was composed of (A) water–75 mM ammonium formate and (B) MeOH–75 mM ammonium formate. Elution was set as follows: 0–60 min 68% A, 60–61 min 61% A and 61–80 min 68% A (flow rate at 4 ml/min, UV detector at 240 nm). All fractions were analysed by HPLC, the 4′-glucuronide **2** eluting before the 7-glucuronide **1**. Based on the HPLC analysis, the fractions containing each glucuronide with >98% purity were combined and concentrated. Each glucuronide was separately desalted using a HP-20SS resin column and a water–MeOH gradient. This separation–desalting process was repeated until we obtained the quantities required for our metabolic studies. Thus, 450 mg of the regioisomeric mixture were separated leading to 185 mg of 7-glucuronide **1** (41% yield, 94.5% chemical purity) and 160 mg of 4′-glucuronide **2** (35% yield, 93.6% chemical purity) as yellow solids, m.p. >200°C. ^{1}H NMR (DMSO-d_6) δ (*acolbifene-7-glucuronide* **1**) 1.37 (m, 2H, CH_2), 1.51 (m, 4H, CH_2), 2.05 (s, 3H, Me), 2.58 (m, 4H, CH_2N), 2.77 (m, 2H, CH_2N), 3.19 (m, 1H, CHOH), 3.26 (m, 2H, CHOH), 3.69 (d, J = 9.54 Hz, 1H, $CHCO_2H$), 4.00 (m, 2H, CH_2O), 4.88 (d, J = 7.5 Hz, 1H, H-1′′′′), 5.11–5.37 (bs, 3H, OH), 5.97(s, 1H, H-2), 6.32 (d, J = 2.4 Hz, 1H, H-8), 6.57 (dd, J = 2.3 and 8.4 Hz, 1H, H-6), 6.72 (d, J = 8.7 Hz, 2H, H-3′), 6.77 (d, J = 8.7 Hz, 2H, H-3′′), 7.09 (d, J = 8.4 Hz, 2H, H-2′), 7.20 (d, J = 8.7 Hz, 2H, H-2′′), 7.24 (d, J = 8.7 Hz, 1H, H-5), 9.58 (bs, 1H, OH); (*acolbifene-4′-glucuronide* **2**) 1.37 (m, 2H, CH_2), 1.52 (m, 4H, CH_2), 2.01 (s, 3H, Me), 2.59 (m, 4H, CH_2N), 2.74 (m, 2H, CH_2N), 3.25 (m, 3H, CHOH), 3.70 (d, J = 9.2 Hz, 1H, $CHCO_2H$), 3.98 (m, 2H, CH_2O), 4.93 (d, J = 7.4 Hz, 1H, H-1′′′′), 5.2–5.37 (bs, 3H, OH), 5.95 (s, 1H, H-2), 6.09 (d, J = 2.3 Hz, 1H, H-8), 6.34 (dd, J = 2.3 and 8.4 Hz, 1H, H-6), 6.77 (d, J = 8.6 Hz, 2H, H-3′′), 6.95 (d, J = 8.6 Hz, 2H, H-3′), 7.14 (d, J = 8.5 Hz, 1H, H-5), 7.15 (d, J = 8.5 Hz, 2H, H-2′), 7.19 (d, J = 8.5 Hz, 2H, H-2′′), 9.60 (bs, 1H, OH). HRSM (ESI) (*glucuronide* **1**) calcd for $C_{35}H_{40}NO_{10}$ $[M+H]^+$: 634.2646. Found 634.2634; (*glucuronide* **2**) calcd for $C_{35}H_{40}NO_{10}$ $[M+H]^+$: 634.2646. Found 634.2657.

(2S)-7-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-3-(4′-hydroxyphenyl)-4-methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (12) and (2S)-7-hydroxy-3-(4′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)phenyl)-4-methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (13). To a solution of regioisomers **10** and **11** (310 mg, 0.35 mmol) in THF (5 ml) was added HF·Pyr (0.6 ml). After stirring overnight, saturated $NaHCO_3$ (25 ml) was added and the aqueous phase was extracted with dichloromethane (2 × 10 ml). The combined extracts were washed with brine and concentrated under vacuum. The residue was purified by flash chromatography (dichloromethane–MeOH 97:3) to give phenols **12** and **13** (201 mg, 74%) as a 55:45 mixture. ^{1}H NMR (CD_3OD) δ 1.46 (m, 2H, CH_2), 1.61 (m, 4H, CH_2), 1.97–2.03 (m, 9H, OAc), 2.05 (s, 3H, Me), 2.53 (m, 4H, CH_2N), 3.11 (d, J = 5.5 Hz, 2H, CH_2N), 3.65 and 3.66 (s, 3H, CO_2Me), 4.03 (d, J = 5.5 Hz, 2H, CH_2O), 4.48 and 4.49 (d, J = 9.9 Hz, 1H, $CHCO_2Me$), 5.18 (m, 2H, CHOAc), 5.34 and 5.37 (d, J = 7.8 Hz, 1H, H-1′′′′ for **12** and **13**, respectively), 5.45 (m, 1H, CHOAc), 5.80 and 5.86 (s, 1H, H-2 for **13** and **12**, respectively), 6.15 and 6.36 (d, J = 2.4 Hz, 1H, H-8 for **13** and **12**, respectively), 6.38 and 6.58 (dd, J = 2.3 and 8.4 Hz, 1H, H-6 for **13** and **12**, respectively), 6.72–7.23 (m, 9H, Ar). HRSM (EI) calcd for $C_{42}H_{47}NO_{13}$: 776.3235. Found 776.3243.

(2S)-7-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)-3-(4′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)phenyl)-4-methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (14). A mixture of phenols **12** and **13** (283 mg, 0.361 mmol) was coupled at −10°C with imidate **9** (211 mg, 0.452 mmol) in the presence of $BF_3 \cdot OEt_2$ (88 µl, 0.72 mmol) as described for preparation of **10** and **11**, to yield, after purification, amorphous diglucuronate **14** (207 mg, 52%). ^{1}H NMR (CD_3OD) δ 1.48 (m, 2H, CH_2), 1.62 (m, 4H, CH_2), 1.99–2.04 (6 s, 21H, OAc and Me), 2.56 (m, 4H, CH_2N), 2.76 (d, J = 5.5 Hz, 2H, CH_2N), 3.66 (s, 3H, CO_2Me), 3.68 (s, 3H, CO_2Me), 4.04 (d, J = 5.5 Hz, 2H, CH_2O), 4.49 (d, J = 9.9 Hz, 1H, $CHCO_2Me$), 4.50 (d, J = 10.1 Hz, 1H, $CHCO_2Me$), 5.13–5.23 (m, 4H, CHOAc), 5.38 (d, J = 7.8 Hz, 1H, H-1′′′′), 5.40 (d, J = 8.1 Hz, 1H, H-1′′′′), 5.42 (t, J = 9.9 Hz, 1H, CHOAc), 5.44 (t, J = 9.5 Hz, 1H, CHOAc), 5.87 (s, 1H, H-2), 6.36 (d, J = 2.4 Hz, 1H, H-8), 6.60 (dd, J = 2.3 and 8.7 Hz, 1H, H-6), 6.77 (d, J = 8.7 Hz, 2H, H-3′′), 6.97 (d, J = 8.7 Hz, 2H, H-3′), 7.13 (d, J = 8.7 Hz, 2H, H-2′), 7.20 (d, J = 8.7 Hz, 2H, H-2′′), 7.26 (d, J = 8.5 Hz, 1H, H-5). HRSM (EI) calcd for $C_{55}H_{63}NO_{22}$: 1093.1016. Found 1093.1021.

(2S)-7-(β-D-glucopyranosyluronic acid)-3-(4′-(β-D-glucopyranosyluronic acid)phenyl)-4-methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (3). Diglucuronate **14** (22 mg, 0.020 mmol) was hydrolysed with 2 N LiOH (90 µl, 0.18 mmol) in 1:1 dioxane-water (2 ml) to yield, after purification by reverse-phase chromatography (C18) with a water–MeOH gradient and drying, diglucuronide **3** (12 mg, 75%, 96.4% chemical purity) as a yellow solid, m.p. >200°C. ^{1}H NMR (DMSO-d_6) δ 1.34 (m, 2H, CH_2), 1.48 (m, 4H, CH_2), 2.02 (s, 3H, Me), 2.49 (m, 4H, CH_2N), 2.66 (t, J = 5.7 Hz, 2H, CH_2N), 3.41 (m, 6H, CHOH), 3.51 (d, J = 9.5 Hz, 2H, $CHCO_2H$), 3.98 (t, J = 5.7 Hz, 2H, CH_2O), 4.81

DOI: 10.1002.jlcr

(d, $J = 7.4$ Hz, 1H, H-1′′′′), 4.86 (d, $J = 7.0$ Hz, 1H, H-1′′′′), 5.04 (bs, 3H, OH), 5.24 (bs, 3H, OH), 5.97(s, 1H, H-2), 6.32 (d, $J = 2.4$ Hz, 1H, H-8), 6.57 (dd, $J = 2.3$ and 8.4 Hz, 1H, H-6), 6.76 (d, $J = 8.5$ Hz, 2H, H-3′′), 6.93 (d, $J = 8.5$ Hz, 2H, H-3′), 7.16 (d, $J = 8.4$ Hz, 2H, H-2′), 7.17 (d, $J = 8.5$ Hz, 2H, H-2′′), 7.23 (d, $J = 8.5$ Hz, 1H, H-5). HRSM (ESI) calcd for $C_{41}H_{48}NO_{16}$ $[M+H]^+$: 810.2967. Found 810.2959.

(2R,S)-7-hydroxy-3-(4′-hydroxyphenyl)-4-($^2H_3$)methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (17), (±)-($^2H_3$)acolbifene. To a cooled solution (−40°C) of *trans*-chromanone[20] **15** (3.10 g, 4.94 mmol) in dry THF (100 ml) was added dropwise a solution of C^2H_3MgI in ether (20 mmol), prepared from C^2H_3I (99.9 atom% ^2H, 24.8 mmol) and Mg (20.6 mmol). The cooling bath was removed and the green suspension was stirred at room temperature for 4 h, then quenched with saturated NH_4Cl (100 ml). The aqueous solution was extracted with EtOAc (2×50 ml). The combined organic phases were washed with brine (50 ml), dried (Na_2SO_4) and evaporated under reduced pressure affording alcohols **16** (3.16 g) as a foamy solid. This crude alcohol was dissolved in 19:1 $C^2H_3CO_2{}^2H$–^2H_2O (55 ml) and heated at 90°C for 30 min under a stream of argon. The deep red solution was concentrated under vacuum and the residue was treated with saturated Na_2CO_3 (100 ml) then stirred overnight with EtOAc (100 ml). After decantation, the aqueous phase was extracted with EtOAc (2×50 ml). The combined organic phase was washed with saturated Na_2CO_3 (50 ml) and brine (50 ml), and evaporated under reduced pressure. The crude red product was purified by flash chromatography (dichloromethane–EtOH 9:1) affording racemic (±)-[$^2H_3$]acolbifene **17** as a pink amorphous solid (1.89 g, 82%). ^1H NMR (CD_3OD) δ 1.47 (m, 2H, CH_2), 1.63 (m, 4H, CH_2), 2.54 (m, 4H, NCH_2), 2.75 (t, $J = 5.6$ Hz, 2H, CH_2N), 4.06 (t, $J = 5.6$ Hz, 2H, OCH_2), 5.80 (s, 1H, H-2), 6.15 (d, $J = 2.4$ Hz, 1H, H-8), 6.34 (dd, $J = 2.3$ and 8.9 Hz, 1H, H-6), 6.73 (d, $J = 8.4$ Hz, 2H, H-3′), 6.74 (d, $J = 8.4$ Hz, 2H, H-3′′), 7.00 (d, $J = 8.4$ Hz, 2H, H-2′), 7.13 (d, $J = 8.5$ Hz, 1H, H-5), 7.20 (d, $J = 8.8$ Hz, 2H, H-2′′). HRSM (EI) calcd for $C_{29}H_{28}{}^2H_3NO_4$: 460.2441. Found 460.2451.

(2S)-7-hydroxy-3-(4′-hydroxyphenyl)-4-($^2H_3$)methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (1S)-10-camphorsulfonic acid salt, (+)-($^2H_3$)acolbifene·(+)-CSA (18). To a solution of **17** (1.9 g, 4.12 mmol) in 20:1 acetone–water (5 ml) was added (1S)-(+)-10-camphorsulfonic acid (955 mg, 4.12 mmol). After 5 min, acetone (5 ml) was added and after 1 h the crystals were filtered, washed with acetone (5 ml) and dried to give racemic (±)-[$^2H_3$]acolbifene·(+)-CSA (2.51 g, 88%). This salt was dissolved in hot 95% EtOH (400 ml). After standing for 2 days at room temperature, the solid was collected and washed with 95% EtOH, then dried to afford (+)-[$^2H_3$]acolbifene·(+)-CSA **18** (1.11 g). One more recrystallisation from 94:6 dichloromethane–DMF (16 ml) gave optically pure salt **18** (0.78 g, 28%), $[\alpha]_D$ +136.2° (*c* 1.2, DMF). ^1H NMR spectrum is identical with previously reported data[5] for unlabelled salt without the signal for the methyl group.

(2S)-7-hydroxy-3-(4′-hydroxyphenyl)-4-($^2H_3$)methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (19), (+)-($^2H_3$)acolbifene. The labelled salt **18** (692 mg, 1.00 mmol) was suspended in EtOAc (10 ml). Na_2CO_3 (424 mg, 4.00 mmol) and water (10 ml) were added. The mixture was stirred at room temperature for 1 h and extracted with EtOAc (25 ml), washed with saturated $NaHCO_3$ (10 ml), brine (10 ml) and dried (Na_2SO_4). After concentration, the foamy residue was purified by flash chromatography (dichloromethane–EtOH 95:5) to yield (+)-[$^2H_3$]acolbifene **19** (495 mg, 100%, 99% chemical purity by HPLC) as a pink foam. A 99.4% enantiomeric purity was determined by chiral HPLC: Chiralpak AD column (4.6×250 mm) eluted with Hexanes-Reagent alcohol–Diethylamine 89.5:10:0.5, flow rate 1.0 ml/min, UV detector at 240 nm. ^1H NMR and HRMS of **19** are identical with that of racemic **17**.

Preparation of a mixture of (2S)-7-(β-D-glucopyranosyluronic acid)-3-(4′-hydroxyphenyl)-4-($^2H_3$)methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (4) and (2S)-7-hydroxy-3-(4′-(β-D-glucopyranosyluronic acid)phenyl)-4-($^2H_3$)methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (5). (+)-[$^2H_3$]Acolbifene **19** (460 mg, 1.00 mmol) was monosilylated as described for acolbifene to yield compounds **20** and **21** (246 mg, 42%) as an amorphous regioisomeric mixture. ^1H NMR spectrum is identical with that obtained in the preparation of compounds **7**–**8** without the signal at δ 2.07 ppm for the methyl group. HRSM (EI) calcd for $C_{35}H_{42}{}^2H_3NO_4Si$: 574.3306. Found 574.3314. Glycosylation of **20** and **21** as described for unlabelled material with trichloroacetimidate **9** and $BF_3 \cdot OEt_2$ catalyst furnished ^2H-glucuronates **22** and **23** (230 mg, 60%) as a regioisomeric mixture. ^1H NMR spectrum is identical with that of **10**–**11** without the signals at δ 2.07 and 2.08 ppm for the methyl group. HRSM (EI) calcd for $C_{48}H_{58}{}^2H_3NO_{13}Si$: 890.4100. Found 890.4089. Hydrolysis with 2 N LiOH in dioxane yielded, after purification by reverse-phase chromatography, evaporation and drying under vacuum, [$^2H_3$]monoglucuronides **4** and **5** (57 mg, 70%) as a pink solid. This labelled material was found to be a 55:45 mixture of **4** and **5** with >98% isotopic purity and

DOI: 10.1002.jlcr

96.4% chemical purity. ^{1}H NMR (DMSO-d_6) δ 1.38 (m, 2H, CH_2), 1.50 (m, 4H, CH_2), 2.56 (m, 4H, CH_2N), 2.76 (d, $J = 5.5$ Hz, 2H, CH_2N), 3.25 (m, 3H, CHOH), 3.73 (d, $J = 9.54$ Hz, 1H, $CHCO_2H$), 4.01 (m, 2H, CH_2O), 4.88 and 4.93 (d, $J = 7.4$ Hz, 1H, H-1′′′′ for **4** and **5**, respectively), 5.11–5.37 (bs, 3H, OH), 5.93 and 5.96 (s, 1H, H-2 for **5** and **4**, respectively), 6.07 and 6.31 (d, $J = 2.4$ Hz, 1H, H-8 for **5** and **4**, respectively), 6.34 and 6.56 (dd, $J = 2.3$ and 8.4 Hz, 1H, H-6 for **5** and **4**, respectively), 6.70–7.23 (m, 9H, Ar), 9.49 (bs, 1H, OH). HRSM (ESI) calcd for $C_{35}H_{37}{}^2H_3NO_{10}$ $[M+H]^+$: 637.2835. Found 637.2834.

Preparation of (2S)-7-(β-D-glucopyranosyluronic acid)-3-(4′-(β-D-glucopyranosyluronic acid)phenyl)-4-($^2H_3$) methyl-2-(4′′-(2′′′-(1-piperidino)ethoxy)phenyl)-2H-1-benzopyran (6). As described for preparation of diglucuronide **3**, a mixture of **22** and **23** (130 mg) was deprotected and then glucuronidated with imidate **9**. After saponification, [$^2H_3$]diglucuronide **6** was obtained (15 mg, 29% yield from **22** and **23**, >98% isotopic purity and 93.7% chemical purity) as a pink solid, m.p. >200°C. ^{1}H NMR spectra of the labelled intermediates **24**–**25** and **26** were identical with that of the corresponding unlabelled compounds without the signals for the methyl group. ^{1}H NMR (DMSO-d_6) δ (*diglucuronide* **6**) 1.37 (m, 2H, CH_2), 1.51 (m, 4H, CH_2), 2.60 (m, 4H, CH_2N), 2.77 (m, 2H, CH_2N), 3.69 (m, 6H, CHOH), 3.70 (m, 2H, $CHCO_2H$), 4.00 (m, 2H, CH_2O), 4.88 (d, $J = 6.3$ Hz, 1H, H-1′′′′), 4.93 (d, $J = 6.7$ Hz, 1H, H-1′′′′), 5.34 (bs, 6H, OH), 6.09 (s, 1H, H-2), 6.32 (bs, 1H, H-8), 6.58 (d, $J = 7.0$ Hz, 1H, H-6), 6.77 (d, $J = 7.0$ Hz, 2H, H-3′′), 6.95 (d, $J = 7.0$ Hz, 2H, H-3′), 7.09 (d, $J = 7.5$ Hz, 2H, H-2′), 7.11 (d, $J = 7.1$ Hz, 2H, H-2′′), 7.18 (d, $J = 7.0$ Hz, 1H, H-5). HRSM (ESI) calcd for $C_{41}H_{45}{}^2H_3NO_{16}$ $[M+H]^+$: 813.3156. Found 813.3151.

Acknowledgements

We thank Dr René Bérubé, Dr Kim High, Christine Boucher, Patrick Caron, Eric Gaudreau, Joëlle Pelletier, Gilles Provencher, Eric Tessier and Marie-Claude Trottier (NMR) from our laboratory for analytical support. We would also like to thank Schering-Plough Research Institute (NJ) for gift of chromanone **15** and Dr Philippe Couture for careful reading of the manuscript. This work was supported by Endorecherche.

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DOI: 10.1002.jlcr