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SHORT COMMUNICATION

TITLE PAGE

TITLE

NMR Characterization of an S-Linked Glucuronide Metabolite of the Potent, Novel, Nonsteroidal Progesterone Agonist Tanaproget

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RUNNING TITLE: Characterization of a Progesterone Agonist Glucuronide

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ABBREVIATIONS: NMR, nuclear magnetic resonance; 1D and 2D NMR, one and two dimensional NMR; gCOSY, gradient correlation spectroscopy; gHSQC, gradient heteronuclear multiple quantum coherence; gHMBC, gradient heteronuclear multiple bond correlation; NOESY, nuclear Overhauser spectroscopy; ROESY, rotational Overhauser spectroscopy; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry/mass spectrometry; HPLC, high-performance liquid chromatography; UDPGA, uridine diphosphoglucuronic acid; ppm, parts per million; DMSO-d_6, deuterated dimethyl sulfoxide.

ABSTRACT

Tanaproget is a first-in-class nonsteroidal progesterone receptor agonist which is being investigated for contraception indication. A major in vitro and in vivo metabolite of tanaproget formed in humans was initially characterized as a glucuronide of tanaproget. However, whether the glucuronide was linked to the nitrogen or sulfur of the benzoxazine-2-thione group in tanaproget could not be determined by LC/MS and LC/MS/MS analysis. In order to obtain additional structural details for this metabolite, additional quantities were generated from rat liver microsomal incubations and purified by HPLC for NMR analysis. The NMR data for the metabolite confirmed that the glucuronide was covalently bound to either the sulfur or the nitrogen of the benzoxazine-2-thione moiety. The lack of key through-bond (scalar) and through-space (dipolar) 1- and 2-D NMR couplings and correlations in the metabolite spectra (due primarily to low sample concentration) precluded an unambiguous structure elucidation. Subsequent synthesis of the S- and N-glucuronides of tanaproget from tanaproget facilitated the unambiguous regio- and stereochemical assignment of the metabolite by comparison of 1D NMR chemical shifts and scalar coupling constants, 2D NMR correlations, and HPLC and LC/MS characteristics between the synthetic compounds and the metabolite. From extensive comparison of the spectral and chromatographic data of the microsomally-derived metabolite and the synthetic compounds, the metabolite has been determined to be the S-(β)-D-glucuronide of tanaproget.

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Tanaproget is a potent, first-in-class nonsteroidal progesterone receptor agonist being investigated at Wyeth for use in contraception (Fensome et al., 2005; Zhang et al., 2005). Earlier studies indicated that a major conjugated metabolite (>10%), referred to here as **M1**, was formed in rat and human liver microsomal incubations, and initially characterized by LC/MS as a glucuronide of tanaproget. The glucuronic acid group was proposed to be either on the nitrogen or the sulfur of the benzoxazine-2-thione moiety of tanaproget, **1**, (Scheme 1). Oxidative metabolism represented a minor metabolic pathway of **1** in both in vitro and in vivo studies.

Analysis of the initial NMR data of **M1** from rat liver microsomes confirmed that the glucuronide was covalently bound to either the S- or the N- of the benzoxazine-2-thione moiety. However, due to an insufficient amount (~few μg) of the sample in solution, we could not record complete scalar and dipolar 1D and 2D NMR couplings and correlations for an unambiguous structure elucidation. The NMR analysis was especially challenging due to the lack of protons in the benzoxazine-2-thione moiety, particularly in the scenario of an S-glucuronide conjugate of tanaproget, since most heteronuclear correlations between the sugar and the parent molecule would be too long range to be observed.

Because of the challenges in preparing sufficient quantities of **M1**, the strategy turned away from liver microsome preparations to synthesis. Comparison of NMR, LC/MS, and HPLC data between synthetic compounds and **M1** isolated from microsomes facilitated the unambiguous regio- and stereochemical assignment of the metabolite.

The present study highlights the successful approach and powerful utility of extensive NMR analysis and the availability of synthetic metabolite standards in the unambiguous identification of this S-linked metabolite. The data are critical for the drug development of tanaproget, since it would allow for biological testing of its major metabolite.

MATERIALS and METHODS

Chemicals. Chemicals used in the model compound syntheses, and deuterated NMR solvents were from Aldrich (Milwaukee, WI). Ammonium acetate, magnesium chloride, and UDPGA were from Sigma (St. Louis, MO). Solvents for extractions and chromatography were HPLC grade or ACS Reagent Grade (Mallinckrodt Baker, Phillipsburg, NJ and EMD Chemicals, Gibbstown, NJ).

Synthesis of N- and S-Glucuronic Acid Derivatives of Tanaproget. A 0.5 mmol solution of tanaproget in anhydrous dimethyl formamide (5 mL) was added dropwise under a nitrogen atmosphere to a solution of 1.1 mmol sodium hydride in dimethyl formamide (25 mL) that was cooled to approximately –70 ^{o}C (Dry Ice). After stirring for 10 min, a 0.5 mmol solution of acetobromo-α-D-glucuronic acid methyl ester in dimethyl formamide (5 mL) was then added dropwise. The reaction solution was then warmed to room temperature, and stirred for a total of 8 h. The reaction solution was partitioned between water (100 mL) and ethyl acetate (100 mL). The aqueous layer was extracted again with ethyl acetate (100 mL). The combined organic layers were washed with saturated sodium chloride solution (100 mL), dried (magnesium sulfate), and solvent removed *in vacuo* to yield 0.350 g of crude reaction material. To a portion of this material (0.210 g) was added a solution of methanol/Hunig's base ((*iso*-Pr)$_2$NEt)/water (5 mL/2 mL/2 mL) and the solution was stirred for 8.5 h at room temperature. The reaction solution was then adjusted to pH 2.5 using HCl (conc., approximately 1.5 mL) and chromatographed by reversed-phase HPLC (YMC-Pack CN, 150 x 20 mm ID, S-5 μm particle size) using a gradient of 15 – 35% acetonitrile/10 mM (aqueous) ammonium acetate. From repeated injections, approximately 5 mg of **2**, the N-glucuronic acid derivative (3% isolated yield from tanaproget),

and 15 mg of **3**, the S-glucuronic acid derivative (10% isolated yield from tanaproget), were purified to > 98% purity based on NMR analysis.

Preparation of Tanaproget Glucuronide Conjugate from Rat Liver Microsomes for NMR. Liver microsomes from Sprague-Dawley rats were prepared in-house using a differential ultracentrifugation method (Lake, 1987) with slight modifications. Microsomal protein and cytochrome P450 content were determined by previously published methods (Bradford, 1976; Omura and Sato, 1964). The protein concentration and P450 content were 50.9 mg/mL and 0.42 nmol/mg protein, respectively. Incubations (100 mL) were performed with tanaproget (40 μM), UDPGA (5 mM), magnesium chloride (10 mM), and male rat liver microsomes (1.5 mg/mL), in 0.1 M potassium phosphate buffer, pH 7.4 at 37 °C. The samples were pre-incubated for 1 min at 37 °C, and the reactions were initiated by the addition of UDPGA. Incubation mixtures were placed on ice to stop the reaction after 3 h. Tanaproget was removed from the incubate by ether extraction (200 mL x 2). Tanaproget glucuronide, and remaining tanaproget were extracted using C18 cartridges and elution with methanol. The eluate was dried by rotary evaporation at room temperature. The residues were extracted with 50% acetonitrile in water (5 mL). Aliquots of supernatants were analyzed on a Waters 2690 HPLC system with a semi-preparative column. Separation was accomplished on a Luna column (C18, 250 x 10 mm ID, 5 μm, Phenomenex, Torrance, CA) and metabolites were detected by UV absorbance at 310 nm. The autosampler temperature was 6 ^{o}C, and the column was at room temperature. Ammonium acetate (10 mM, pH 4.5) and acetonitrile were used as mobile phase A and B, respectively. The following gradient (flow rate of 2 mL/min) was employed: 0-1 min 20% B, 10 min 40% B, 20 min 70% B, 25-28 min 95% B, 30 min 20% B. Fractions containing the glucuronide peak were collected and immediately frozen on Dry Ice. All glucuronide fractions were combined and acetonitrile was

removed by rotary evaporation. The glucuronide was extracted from the aqueous eluates by solid phase extraction using C18 cartridges. Water was used to wash residual buffer and 50% methanol in water was used to elute the glucuronide conjugate, **M1**. The methanol/water eluates were dried by rotary evaporation at room temperature followed by lyophilization.

LC/MS. LC/MS data for the synthetic compounds were acquired using a Waters Alliance 2695 HPLC coupled to a Waters ZQ mass spectrometer using an open-access LC/MS method previously described (Mallis et al., 2002). The LC/MS system used for **M1** analysis was a Waters Alliance model 2695 HPLC coupled to a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters). Separations were accomplished on a Phenomenex Luna C18(2) column (150 x 2 mm ID, 5 μm) with a Deltabond C18 guard column (10 x 2 mm) (Thermo Electron Corp., Bellefonte, PA). The flow rate was 0.3 mL/min. Mobile phase A was 10 mM ammonium acetate in water, pH 4.5, and mobile phase B was acetonitrile. The linear mobile phase gradient used was: 0-1 min 10% B, 10 min 15% B, 35 min 17.5% B, 36 min 25% B, 50 min 30% B, 55 min 50% B, 60-62 min 90% B, 65 min 10% B, 75 min 10% B. The mass spectrometer had an electrospray ionization interface and operated in both positive and negative ionization modes. Settings for the mass spectrometer were: electrospray ionization spray 2.5 KV, cone 50 V, desolvation gas flow 900-1000 L/h, source temperature 80 °C, desolvation gas temperature 250 °C.

NMR Spectroscopy. DMSO-d_6 was used for all NMR samples. NMR spectra were obtained on Bruker (Billerica, MA) instruments 300 MHz DPX and 600 MHz Avance, and Varian (Palo Alto, CA) instruments 400 and 500 MHz Inova. The bulk of the NMR work was done on the 500 MHz instrument equipped with a Varian 3 mm ^{1}H observe indirect detection probe. Chemical shifts (δ) are reported in ppm. ^{1}H chemical shifts are referenced to residual

protonated DMSO at δ2.49, and ^{13}C chemical shifts are referenced to internal DMSO-d_6 at δ39.5. Chemical shifts for ^{15}N are reported relative to liquid ammonia at δ0.0 and referenced to external formamide (δ112.0). General parameters for ^{1}H-NMR experiments were a 5000 Hz spectral width, 32K data points, 45° pulse width, 1 s relaxation delay and acquisition of 32 to > 1000 scans, depending on sample concentration. Line broadening (~0.5 Hz) or Gaussian processing routines were used to increase the signal-to-noise. General parameters for a ^{13}C-NMR experiment were a 25,000 Hz spectral width, 64K data points, 45° pulse width, 1 s relaxation delay and acquisition of at least 10,000 scans, with 2 Hz line broadening. All spectra were acquired at 25 ^{o}C. Several types of gradient 2D NMR experiments were utilized to determine ^{1}H-^{1}H and ^{1}H-^{13}C connectivities. These included gCOSY for the determination of three-bond ^{1}H-^{1}H connectivities, gHSQC and gHMBC for the determination of one-, two-, three-, and four-bond ^{1}H-^{13}C- connectivities, and a NOESY or ROESY for the determination of through-space connectivities.

HPLC. HPLC columns used to test retention times of **M1** vs. **2** and **3** were: Luna C8(2) (Polymeric) (150 x 3.0 mm, 5μ, Phenomenex); Everest Monomeric C18 (250 x 4.6 mm, 5μ, Grace Vydac, Hesperia, CA); YMC-Pack CN, (150 x 4.6 mm, 5μ, Waters); XTerra RP C18-Hybrid, (150 x 4.6 mm, 5μ, Waters); YMC-Pack Phenyl, (150 x 4.6 mm, 5μ, Waters).

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RESULTS and DISCUSSION

Both the N-glucuronide, **2**, and the S-glucuronide, **3**, forms of tanaproget were synthesized starting from tanaproget and acetobromo-α-D-glucuronic acid methyl ester (Scheme 1). The purity was excellent (> 98%) and a sufficient amount of each (at least 5 mg) was obtained, enabling complete NMR assignments of each. Table 1 summarizes the NMR results for **2** and **3**.

The protonated and deprotonated LC/MS spectra (not shown) of the S-glucuronide **3** gave an $[M+H]^+$ ion at 474 and an $[M-H]^-$ ion at m/z 472, respectively, (as was also the case for **2**, spectra not shown), indicating glucuronidation of tanaproget. Also observed for **3** in the positive and negative ionization mode, respectively, are the ions m/z 298 and m/z 296, assigned as tanaproget, that is, the loss of glucuronic acid. In addition, a fragment at m/z 175, assigned as glucuronic acid, was also detected in the negative ionization spectrum.

Metabolite **M1** LC/MS spectra (not shown) gave protonated and deprotonated molecular ions $[M+H]^+$ and $[M-H]^-$, at m/z 474 and 472, respectively, which indicated a molecular weight of 473. This was 176 Da larger than tanaproget. Loss of 176 Da from m/z 474 in the positive ionization mass spectrum and from m/z 472 in the negative ionization mass spectrum generated the fragment ions at m/z 298 and 296, respectively, which are assigned as tanaproget. A glucuronic acid ion fragment was observed at m/z 175 in negative ionization mode. These data were consistent with glucuronic acid conjugation of tanaproget.

A ^{1}H NMR spectrum of **M1** is shown in Figure 1A and a compilation of the results from NMR experiments on **M1** is given in Table 1. Despite several attempts at using freshly isolated **M1** samples, the purity and concentration of the samples were typically low (< 70% pure and an estimated total amount of < 20 μg of **M1** in solution) which hampered acquisition of complete

2D NMR datasets. We note that during the process of the microsomal incubation and subsequent isolation steps, the glucuronide solution tended to readily undergo oxidative transformation to the carbamate analog of tanaproget (replacement of the sulfur with oxygen), rendering it difficult to obtain the glucuronide conjugate in a stable form. Nevertheless, sufficient chemical shift, coupling, and 2D NMR correlations were obtained to make nearly complete ^{1}H and ^{13}C assignments of **M1**. However, only one key ^{1}H-^{13}C heteronuclear correlation was observed in the gHMBC NMR spectrum of **M1**, that from H-1' to C-6. This same crosspeak was expected however, whether the metabolite was an N-or an S-glucuronide (i.e. 3-bond coupling in either case).

Key NMR chemical shifts and correlations in synthetic compounds **2** and **3**, which located the site of glucuronidation in tanaproget, are drawn in Figure 2. The 1D ^{1}H NMR spectrum of **2** is shown in Figure 1B. Note that the carbon chemical shift of the benzoxazine-2-thione carbon (C-6) of **2** is 188.0 ppm; this compared with the thiocarbonyl carbon of the benzoxazine-2-thione group observed in the parent molecule, tanaproget, at 182.8 ppm. There are four important 3-bond correlations observed in the HMBC spectrum (not shown) of **2**, drawn as arrows in Figure 2. The chemical shift of the proton (H-10) that is in a position *peri*- to the N-glucuronide has moved downfield to 7.80 ppm, compared to 7.13 ppm in the parent molecule, indicating the proximity of the glucuronic acid group (and possibly the carboxylic acid moiety) to this proton. ^{1}H-^{15}N gHMBC experiments were run on all compounds studied, but signals from the key nitrogen N-5 were not observed for any compound except **1** (δ144.8), which had an HMBC crosspeak to H-10. Signals from N-15 were only observed in **1** (δ155.0) and in **2** (δ155.4), and in each compound had HMBC correlations to H-17, H-18, and H-19.

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The ^{1}H NMR spectrum for **3** is shown in Figure 1C. The carbon chemical shift of the derivatized benzoxazine-2-thione carbon (C-6) of **3** is observed at 161.3 ppm, quite upfield shifted from the observed thiocarbonyl carbon chemical shift (182.8 ppm) of the benzoxazine-2-thione group observed in tanaproget. Three important two- and three-bond observed correlations for **3** are indicated in Figure 2. These data confirm that the benzoxazine-2-thione (N(C=S)O) of tanaproget did not rearrange to a thionocarbamate (N(C=O)S) group before glucuronidation. Key ^{1}H and ^{13}C NMR chemical shift data reported above for the S-glucuronide **3** were similar to those as reported in the literature for two other S-β-(D)-glucuronides (Martin et al., 2003; Ethell et al., 2003).

HPLC comparisons were made to confirm whether the major synthetic glucuronide of tanaproget, S-glucuronide **3,** was identical to **M1**, now proposed to be the S-glucuronide from the NMR and mass spectral results described above. Five different types of reverse phase HPLC columns, as described in Materials and Methods, were each used under two different mobile phase gradient conditions, as follows. One mobile phase was a gradient of 10 mM ammonium acetate in water/acetonitrile (90/10 to 10/90 in 20 minutes), and the other mobile phase was a gradient of 10 mM formic acid in water/acetonitrile (90/10 to 10/90 in 20 minutes). These ten HPLC conditions covered a wide range of selectivity as evidenced by the change of elution order of the major and minor components in the samples. To compare and match the retention time of the major peaks in the two samples, spiking experiments were performed. The synthetic glucuronide **3** that was determined to be S-glucuronide was found to have identical retention times as metabolite **M1** under all ten HPLC conditions.

Literature reports of glucuronide conjugation through an S-linkage are rare (Ethell et al., 2003; Jeffcoat et al., 1980; Martin et al., 2003; Nakaoka et al., 1989; Smith et al., 1992), with

little data available on their structural characterization or the UDP-glucuronosyltransferases (UGTs) catalyzing their formation. N- and O-glucuronides are more commonly observed (Green and Tephly, 1998; Hawes, 1998; Kuehl and Murphy, 2003; Tricker, 2003). We recently reported that UGT1A9 and 2B7 were the major isoforms involved in the formation of tanaproget S-glucuronide (Elmarakby et al., 2005).

Direct NMR structure elucidation of the metabolite **M1** generated from rat liver microsomal incubation was challenging because of the lack of protons proximal to the glucuronic acid group attached to the benzoxazine-2-thione moiety of tanaproget which, therefore, precluded homonuclear correlations to the attached sugar group. The lack of NOEs from the sugar to the remainder of the molecule constituted only negative proof that **M1** was an S-glucuronide and not an N-glucuronide. Further, chemical shift arguments for the benzoxazine-2-thione carbonyl and the glucuronic acid group were also insufficient evidence for an unambiguous structure proof. Synthetic compounds turned out to be a more straightforward path to structure elucidation, since they could be made in relatively pure, concentrated amounts.

In summary, from extensive chromatographic and spectral comparison of the microsomally derived metabolite **M1** and the synthetic compounds **2** and **3**, the glucuronide metabolite of tanaproget has been determined to be the S-β-(D)-glucuronide conjugate.

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LEGENDS FOR FIGURES

Fig. 1. 500 MHz ^{1}H NMR spectra of (A) S-glucuronide **M1** isolated from rat liver microsomes, (B) synthetic N-glucuronide **2**, (C) synthetic S-glucuronide **3**. * Indicates broad impurity peaks. All samples are in DMSO-d_6.

Fig. 2. Comparison of key ^{1}H-^{13}C NMR correlations (curved arrows) for the N-glucuronide, **2**, and the S-glucuronide, **3**, of tanaproget. Relevant chemical shift values for tanaproget, **1**, are: C-6 (δ182.8), H-10 (δ7.13).

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TABLE 1

*NMR Results for **M1** and the Synthetic N-Glucuronide **2** and the S-Glucuronide **3** of Tanaproget*

Position	$\delta^{13}C$			$\delta^{1}H$, ^{1}H Multiplicity [a] / J in Hz [a]			$^{1}H - ^{13}C$ HMBC (^{1}H to ^{13}C)
	2	**3**	**M1**	**2**	**3**	**M1**	**M1**
2	82.1	81.5	82.4	–	–	–	
3	132.0	131.2	127.4	–	–	–	
4	131.5	137.8	138.7	–	–	–	
6	188.0	161.3	161.6	–	–	–	
7	123.1	123.4	125.0	7.50, d / 1.7	7.40, d / 2.0	7.40, s	C-2, C-4, C-9, C-14
8	127.7	128.3	132.6	–	–	–	
9	128.1	128.9	130.0	7.57, dd/1.7, 8.5	7.41, dd/2.0,7.9	7.39, dd/ ~9, 2.4	C-4, C-7, C-14
10	118.1	123.4	124.0	7.80, d/8.5	7.11, d/7.9	7.09, d / 8.0	C-3, C-8, C-9
11	25.3[b]	27.2	28.2	1.63, s	1.70, s	1.62, s	C-2, C-3
12	25.5[b]	27.8	28.2	1.83, s	1.59, s	1.62, s	C-2, C-3
14	138.6	139.5	140.7	–	–	–	
16	104.8	104.7	105.8	–	–	–	
17	119.2	119.6	120.3	7.05, d / 4	7.03, d / 4	7.05, d / 3.6	C-14, C-16, C-18

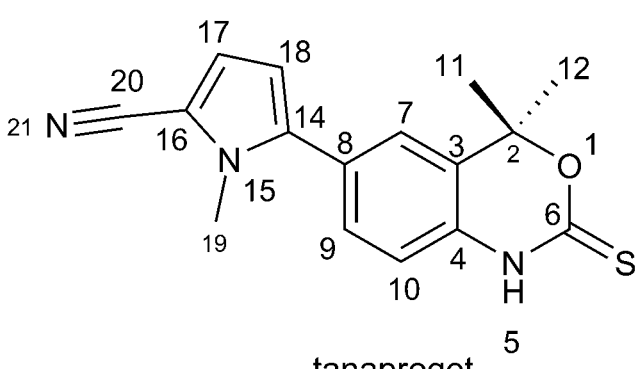
18	109.8	109.7	110.5	6.43, d / 4	6.38, d / 4	6.36, d / 4.0	C-14, C-16, C-17
19	33.6	33.8	34.9	3.75, s	3.73, s	3.69, s	C-14, C-16
20	113.7	114.2		–	–	–	
1’	90.2	85.0	85.6	6.31, d / 9.5	5.11, d / 10.2	5.10, d / 10.3	C-6
2’	68.6	72.1	70.4	3.70, m	3.11, t / 9.4	3.14, m	
3’	78.0	78.3	71.3[c]	3.31, m	3.22, t / 9.3	3.24,[c] t / 8.7	
4’	71.9	71.7	70.6[c]	3.30, m	3.11, t / 9.4	3.29[c]	
5’	76.8	78.4	74.6	3.39, m	3.31, d / 10.0	3.37, d / 10.0	COOH
COOH	171.2	171.3	172.7	–	–	–	3.29[c]

[a] ^{1}H multiplicities are reported as s = singlet, d = doublet, dd = doublet of doublet, and m = multiplet. ^{1}H – ^{1}H J coupling constants are stated in Hz.

[b] ^{13}C signals δ25.3 and δ 25.5 could not be individually assigned to C-11 vs. C-12 for **2**.

[c] Unambiguous assignments to H3’ vs. H4’ could not be made for **M1**. δ3.24 / 71.3 (t, J=8.7 Hz)) is observed in gHSQC, 1D and 2D TOCSY (total correlation spectroscopy) spectra, and δ3.29 / 70.6 is observed in an HMBC spectrum.

Scheme 1



tanaproget

1

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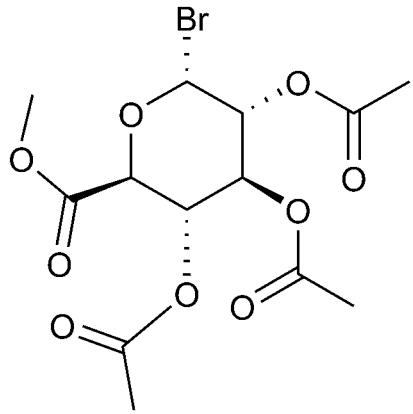
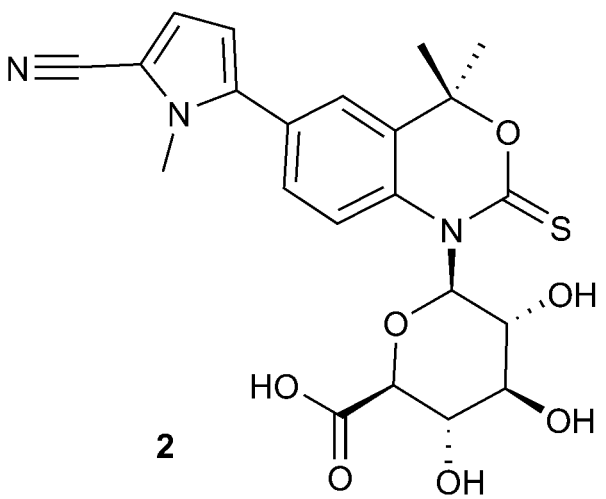


1) NaH, DMF, -70 °C, - - → rt

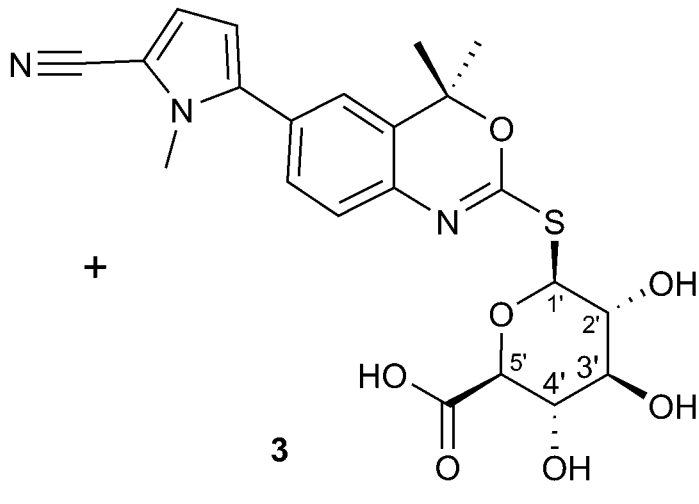
2) $(isoPr)_2NEt$ / MeOH / H_2O (2:5:2), rt

3) HPLC: CN (ACN / 10 mM (aq.) NH_4OAc)



2

+



3

Figure 1

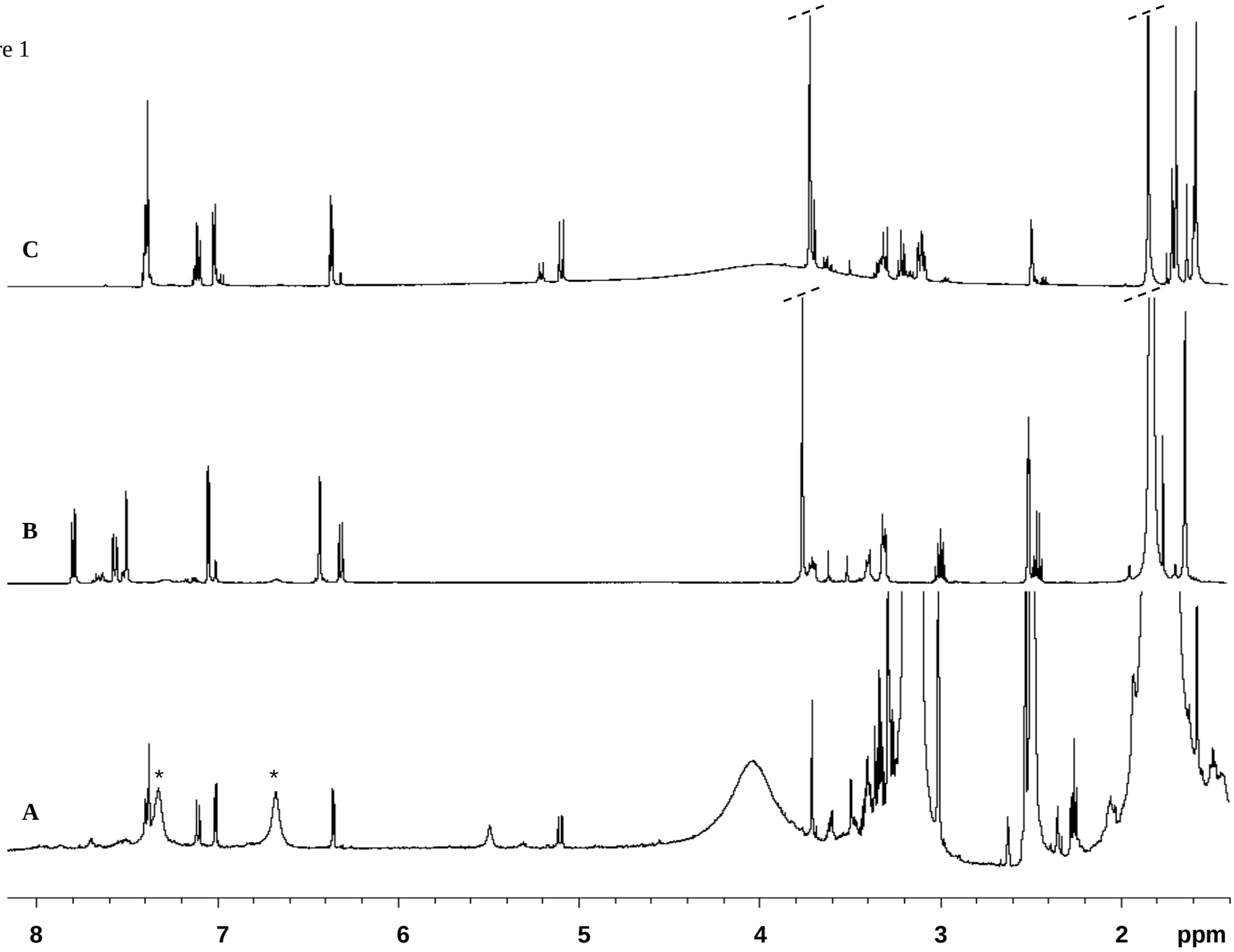
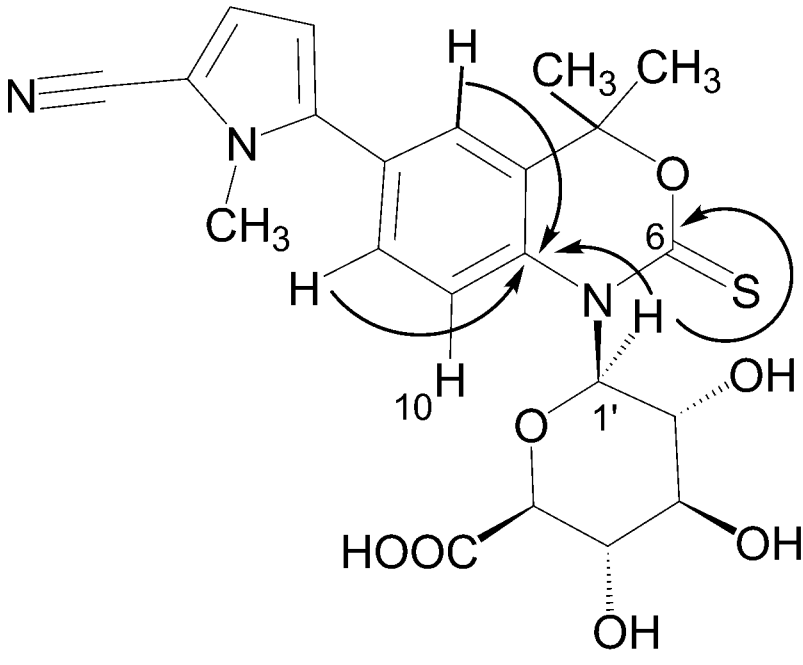
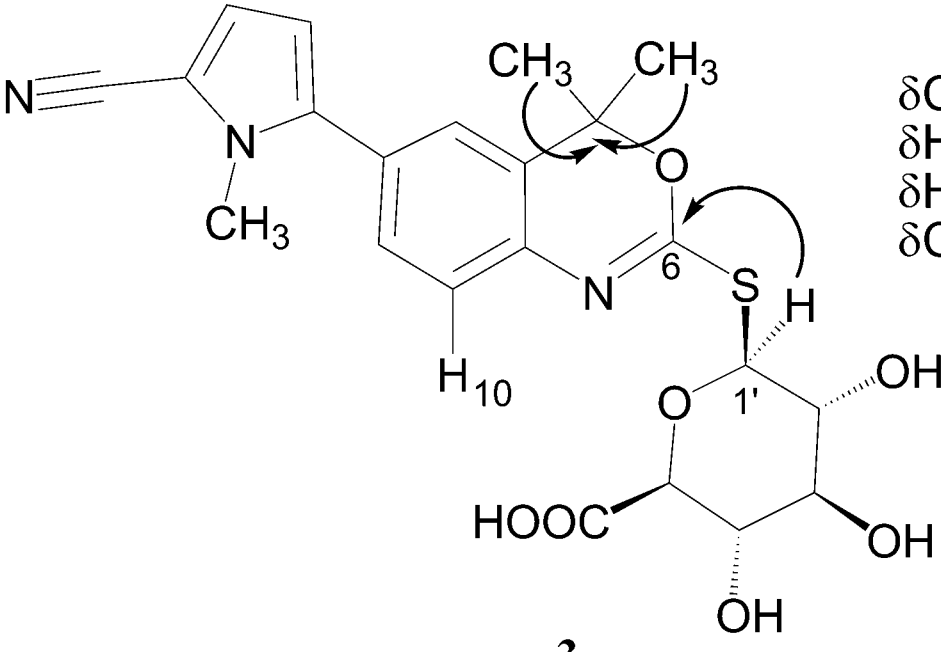


Figure 2



δC-6 = 188.0
δH-10 = 7.80
δH-1' = 6.31
δC-1' = 90.2

2



δC-6 = 161.3
δH-10 = 7.11
δH-1' = 5.11
δC-1' = 85.0

3