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Microbial process for preparation of glucuronides of raloxifene

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Raloxifene, also known as Evista®, has recently been approved for the prevention of osteoporosis. Three glucuronidated compounds: raloxifene-6-glucuronide, raloxifene-27-glucuronide, and raloxifene-6,27-diglucuronide are known metabolites of raloxifene in man. Reference standards of the three glucuronides were needed for clinical trials. Although chemical routes exist to make the two mono-glucuronides, these routes were unable to provide material to meet the needs of clinical trial standards. No chemical route existed to synthesize the di-glucuronide. A bioconversion process using the microorganism *Streptomyces* sp NRRL 21489 was identified and scaled up. The biotranformation products were prepared in a tank fermentation, purified, and characterized by UV, LC/MS and NMR spectroscopy.

Keywords: raloxifene; glucuronidation; *Streptomyces* sp NRRL 21489

Introduction

Osteoporosis occurs as a result of an imbalance between normal resorption of bone and bone formation. Estrogen is critical in maintaining this balance [6,8]. Although estrogen therapy in postmenopausal women decreases bone resorption, estrogen treatments are linked with an increase in uterine cancer and, possibly, breast cancer [1,11]. Raloxifene, 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy) benzoly] benzo[b]thiophene, has recently been approved for the prevention of osteoporosis. *In vivo* studies indicate that this benzothiophene exhibits estrogen agonist effects on bone or the ability to inhibit bone resorption [2]. However, this study also indicated that raloxifene exhibits no estrogen agonist activity in breast and uterine tissues.

Raloxifene is converted extensively to glucuronidated forms after oral administration. Three metabolites of raloxifene (Figure 1, I) in man are glucuronidated compounds: raloxifene-6-glucuronide (Figure 1, II), raloxifene-27-glucuronide (Figure 1, III), and raloxifene-6, 27-diglucuronide (Figure 1, IV). Reference standards of the three glucuronides were needed for clinical trials. Although a chemical method exists for making the two mono-glucuronides, it was unable to provide yields to meet the amounts required for clinical trial standards [7]. No route to synthesize the di-glucuronide had been explored.

Examination of microbial bioconversion of raloxifene to produce its glucuronidated analogues was a logical step. Microorganisms have been reported to conduct biochemical reactions similar to mammalian metabolism [10]. Over 30 organisms isolated from soil have been cited either to hydroxylate aromatic rings or dealkylate amines or ethers. Other organisms have been used to achieve steroid hydroxylation and ester or amide hydrolysis. Glucuronidation, however, is not as well documented. A literature search revealed that only four organisms were reported to add glucuronic acid to either natural products or synthetic organic compounds. The organisms are *Streptomyces* sp ATCC 55043, *S. peucetius* var *vinaceus* NRRL 15344, *S. peucetius* var *castaneus* NRRL 15345, and a blocked mutant of *Actinomadura roseoviolacae* [3,4,9].

This article describes a novel process for the preparation of glucuronides from raloxifene utilizing the microorganism *Streptomyces* sp NRRL 21489. The process for glucuronidation of raloxifene was previously disclosed in a US patent for the preparation of certain benzo[B]thiophene glucuronides [2].

Materials and methods

Shake flask bioconversion

The initial bioconversion studies with *Streptomyces* sp NRRL 21489 (ATCC 55043) were conducted using shake flask cultures. Frozen vegetative preparations were maintained in 10% glycerol/5% lactose solution stored at –70°C for use as working stocks. To prepare inoculum for the bioconversion phase, one frozen vegetative preparation (2.0% v/v) was used to inoculate a 250-ml wide-mouth Erlenmeyer flask containing 50 ml vegetative medium. The composition of the medium was (per liter): glucose, 5.0 g; soluble starch, 10.0 g; yeast extract, 2.5 g; N-Z amine Type A, 2.5 g; reagent grade $CaCO_3$, 0.5 g; KCl, 0.2 mg; $MgSO_4 \cdot 7\ H_2O$, 0.2 mg; $FeSO_4 \cdot 7\ H_2O$, 0.004 mg. The pH was adjusted to 7.0–7.5 before autoclaving the medium. Flasks were incubated at 30°C for 17 h on a rotary shaker orbiting in a 2-inch circle at 250 rpm. This vegetative growth was used to inoculate bioconversion medium (2% v/v). Bioconversion medium consisted of (per liter): glucose, 25.0 g; soybean flour, 15.0 g; NZ amine Type A, 1.0 g; black strap molasses, 3.0 g; reagent grade $CaCO_3$, 2.5 g; KCl, 0.2 mg; $MgSO_4 \cdot 7\ H_2O$, 0.2 mg; $FeSO_4 \cdot 7\ H_2O$, 0.004 mg. The pH was adjusted to 7.2–7.5 before the medium was autoclaved. After inoculation of the bioconversion medium, the substrate was added aseptically to obtain a final concentration of 0.5 mg ml^{-1}. The flasks were then incubated at 30°C for 48–96 h. Methanol extracts of

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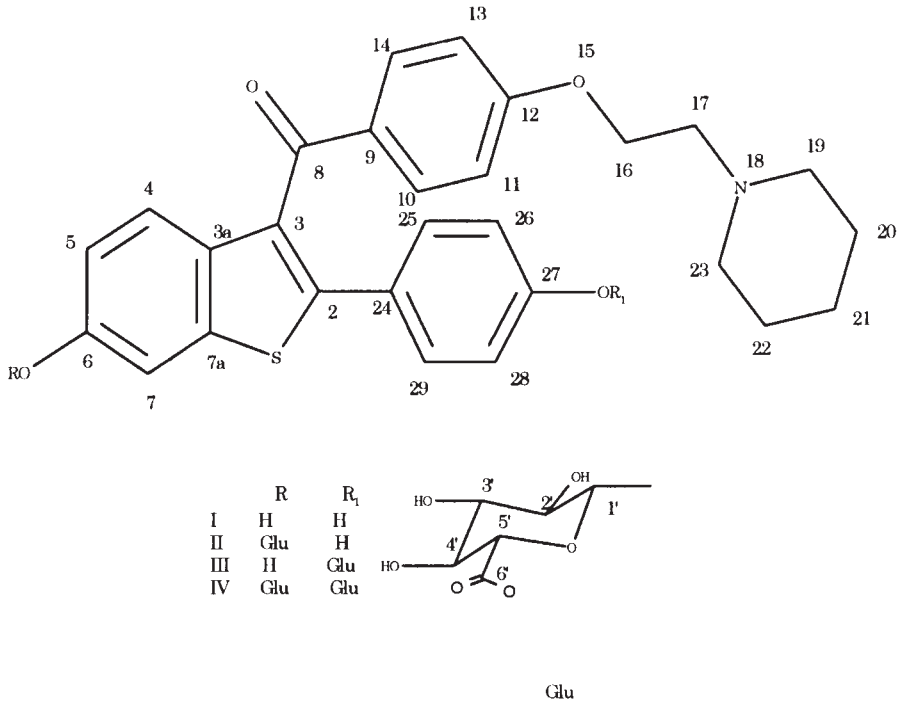

Figure 1 Raloxifene and its glucuronidated derivatives.

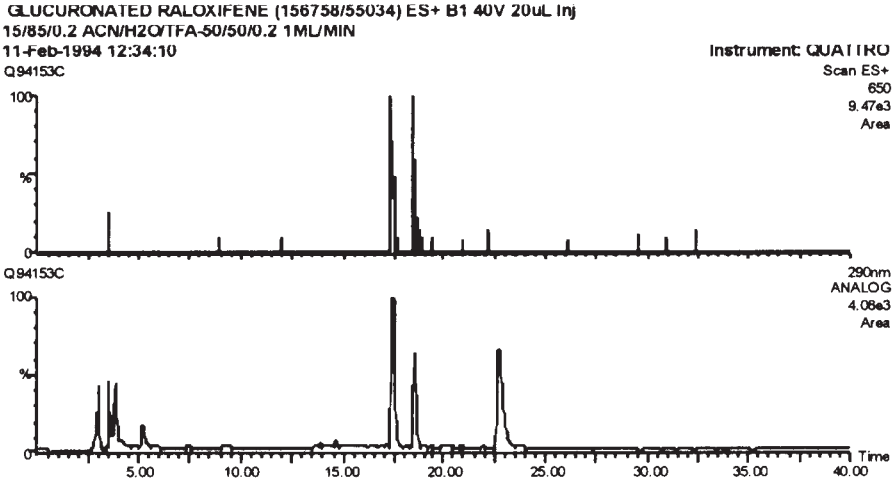

Figure 2 Chromatographic data collected by simultaneous LC-UV and LC-MS. The top trace is the extracted ion chromatogram for m/z 650 and the bottom trace is the UV chromatogram. The coincidence of the two peaks in the middle of the run clearly identifies the components possessing the mass expected for the mono-glucuronates.

the whole broth were examined by LC/MS for the presence of biotransformation products.

HPLC and LC/MS analysis of bioconversion

Bioconversion was monitored by HPLC utilizing a gradient elution system at 1.0 ml min^{-1} on a Waters RCM 8×10 column containing a NovaPak C18 cartridge with detection at 290 nm. The gradient system was as follows: 0.2% aqueous trifluoroacetic acid/acetonitrile (85/15) held for 5 min, then to 0.2% aqueous trifluoroacetic acid/acetonitrile (50/50) in 20 min, held for 10 min, then decreased to 0.2% aqueous trifluoroacetic acid/acetonitrile (85/15) in 3 min.

All LC/MS analyses were carried out using a Micromass (Beverly, MA, USA) Quattro 1 triple quadrupole mass spectrometer. The ionization method used was pneumatically assisted electrospray in the positive ion mode. The LC equipment was comprised of a Waters (Milford, MA, USA) HPLC system consisting of a 610 pump and solvent delivery system, a 910 UV photodiode array (PDA) detector and a 710 autosampler. The gradient program and solvent compositions were as described above. The 1 ml min^{-1} eluent flow was passed to a Supelco (Bellefonte, PA, USA) splitter T, one side of which was connected to an SGE (Austin, TX, USA) MCVT-1 micro needle valve providing a variable back-pressure on one arm of the T connection. This was adjusted so that only approximately 30 μl min^{-1}

was passed to the mass spectrometer interface. The relatively high level of TFA in the mobile phase causes a loss of sensitivity [5]. However, by optimizing source parameters and increasing the amount of the sample, acceptable spectra could be obtained.

Tank fermentation

To conduct preparative scale bioconversions of raloxifene, *Streptomyces* sp NRRL 21489 was cultured in two 150-L fermentors with a working volume of 100 L per tank. The inoculum for each fermentor was prepared by inoculating 50 ml of vegetative medium in a 250-ml Erlenmeyer flask with frozen stock (2.0% v/v) of *Streptomyces* sp. This first stage seed culture was incubated at 30°C for 48 h on a rotary shaker (250 rpm). A second stage was prepared by inoculating vegetative medium with first stage growth (2.5% v/v) and incubating it at 30°C for 48 h. Second stage culture (2.0% v/v) was used to inoculate each 150-L tank. Substrate was added at 0 h to obtain an initial concentration of 0.5 mg ml^{-1}. A second addition of substrate (50 g) was added to the bioconversion at 16–22 h. Glucose feeding (25 g L^{-1} day^{-1}) began at 22–23 h. pH was controlled at 7.0 with 14.5 M ammonium hydroxide. Temperature was maintained at 30°C. Agitation was started at 150 rpm then increased to 425 rpm from 12–17 h post-inoculation. The dissolved oxygen level was controlled by the addition of sterile air at an initial rate of 0.5 standard cubic feet per minute (scfm), then increasing the rate to 3.5 scfm from 12–17 h post-inoculation. Raloxifene bioconversion tanks were harvested after 66 h.

Purification of glucuronides

The whole fermentation broth was treated with an equal volume of methanol and stirred for about 30 min. The resulting slurry was filtered through a ceramic filter unit. The enriched extract contained the glucuronides. This extract was diluted with an equal volume of water and loaded onto a 10-L HP20 ss column at the rate of 1 L min^{-1}. The column was eluted with a linear gradient of 0.5% aqueous ammonium acetate, pH 9.0/methanol (50:50) to 0.5% aqueous ammonium acetate, pH 9.0/methanol (70:30) at a flow rate of 1 L min^{-1}. Fractions were collected and analyzed on HPLC for content and purity. Fractions containing the mono-glucuronides were recycled over an HP20 ss column to increase the purity. The fractions containing the di-glucuronide were concentrated under vacuum to remove the methanol and then the aqueous solution was passed over a 5×50 cm column of HP20 ss resin at 50 ml min^{-1}. The column was eluted with a gradient of 0.5% aqueous ammonium acetate (pH 9.0)/acetonitrile. Fractions containing material of interest were combined, concentrated, and lyophilized. This material was then dissolved in 20 ml of water and passed over a 7×40 cm column of HW40C resin eluting with water. Fractions containing material of interest were combined, concentrated and lyophilized. Purified material was obtained by utilizing a reversed phase C-18 column eluted with a gradient of 0.5% aqueous ammonium acetate (pH 9.0)/acetonitrile (90/10 to 70/30, v/v). Desalting was achieved using a reversed phase C-18 HPLC and a methanol/water gradient.

Results and discussion

The bioconversion of raloxifene by the microorganism *Streptomyces* sp NRRL 21489 to glucuronides was monitored by HPLC. The initial HPLC chromatogram of the raloxifene bioconversion revealed only two compounds that co-chromatographed with the chemically made mono-glucuronides. A UV profile scan of the fermentation broth indicated the presence of a third glucuronidated compound. This material later was identified as the di-glucuronide derivative of raloxifene.

Positive ion electrospray usually produces protonated pseudo-molecular ions [M+H] with very little deposition of internal energy so that fragmentation is minimized. Typically, the power of the technique lies in the routine assignment of molecular weight; operating conditions can be adjusted to produce spectra containing a pseudomolecular ion and very little else. LC/MS data confirmed the presence of the two mono-glucuronides in the raloxifene bioconversion. The molecular weight of mono-glucuronated raloxifene is 649 (nominal mass) and the extracted ion chromatogram in Figure 2 reveals that the compounds eluting at 17.4 and 18.5 min produce the expected [M+H] ion of *m/z* 650. In contrast, the control sample yielded no peaks in the extracted ion chromatogram in this region.

Large-scale bioconversions were run to generate sufficient material needed for the clinical standards. Because the need for the glucuronides was immediate, little time for process development was available. Flask conditions were utilized in the fermentors with minimal adjustments. Since cell mass appeared to be a critical issue for maximum conversion, a two-stage vegetative inoculum was grown to ensure an inoculum of maximum cell density. Two 150-L fermentors were run. Two 50-g additions of raloxifene (0.210 mol) were added to each tank. HPLC data on the methanol extraction of the pooled 17-h fermentations as well as isolated yields are in Table 1. At the time little information was known regarding the physical and chemical properties of the glucuronides. This lack of understanding affected the purification and resulted in low isolated yields.

The ^1H NMR assignments for raloxifene were made using spin patterns and homonuclear decoupling. Identification of each of the glucuronides was completed by com-

Table 1 Raloxifene glucuronidation

Purification step	Raloxifene-6-glucuronide	Raloxifene-27-glucuronide	Raloxifene-6,27-glucuronide
Methanol extract[a] (115 L)	37.1 g (0.057 mol)	23.5 g (0.036 mol)	ND[b]
HP-20 ss column fraction pools	34.1 g (0.052 mol)	23.5 g (0.036 mol)	ND[b]
Isolated yield purity	8.3 g 99.5% (0.013 mol)	4.0 g 98.7% (0.006 mol)	0.199 g (0.0002 mol)

[a]Cell-free methanol extraction of bioconversion broth.
[b]ND, Not detected.

Table 2 The ^{1}H NMR data for raloxifene, I, and its mono-glucuronides, II, and III (in DMSO-d_6) and the diglucuronide, IV (in D_2O)

Position	I[a]	II	III	IV
4	7.29	7.33	7.42	7.32
5	6.91	7.06	6.89	7.05
7	7.41	7.73	7.37	7.64
10/14	7.71	7.65	7.63	7.47
11/13	6.98	6.91	6.85	6.65
16	4.47	4.11	4.18/4.12	4.14
17	3.45	2.74	2.92	3.38
19/23	3.47/2.98	2.52	2.73	3.41/2.87
20/22	1.78	1.50	1.60	1.83/1.68
21	1.67/1.31	1.38	1.43	1.76/1.41
25/29	7.19	7.20	7.22	7.05
26/28	6.73	6.69	6.89	6.79
1′/1″[b]		5.10	5.00	5.09/4.84
2′/2″		3.29	3.19	3.75/3.62
3′/3″		3.34	3.26	3.75/3.65
4′/4″		3.32	3.26	3.75/3.69
5′/5″		3.83	3.66	3.95/3.86

[a]HCl salt.
[b]The assignments are not specifically made for each proton of the di-glucuronide.

paring the ^{1}H NMR spectra to that of raloxifene (Table 2, I) and through different nuclear overhauser effect (NOE) or rotating overhauser effect spectroscopy (ROESY) measurements. A diagnostic NOE is observed between the anomeric proton and the adjacent aromatics. As an example, raloxifene-6-glucuronide (Table 2, II) shows a NOE between H-1′ and H-5 and H-7 while raloxifene-27-glucuronide (Table 2, III) shows a NOE between H-1′ and H-26/28. Raloxifene-6,27-diglucuronide (Table 2, IV) was assigned by comparison to raloxifene and the mono-glucuronides. The ^{1}H NMR data are shown in Table 2.

The work presented here identified a microbial bioconversion process for the glucuronidation of raloxifene. At present only one organism, *Streptomyces* sp NRRL 21489, has been identified as conducting this reaction. Application of LC/MS technology provided confirmation of the presence of the glucuronides without the cost and time of isolating the material for confirmation via physical chemistry. Although the bioconversion and isolated yields were not quantitative, this microbial route provided sufficient material to meet the amount of material needed for clinical trial standards for raloxifene. The route also provided the di-glucuronide of raloxifene for which no chemical route has been described.

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