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Research Article

Synthesis of [^{14}C]- and [^{35}S]-labelled PI-88 for pharmacokinetic and tissue distribution studies

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Summary

PI-88, uniformly labelled with [^{14}C] was prepared by incorporating D-[^{14}C]glucose into the fermentation of *Pichia (Hansenula) holstii* NRRL Y-2448 under controlled conditions to produce [^{14}C]-labelled extracellular phosphomannan. Subsequent acid catalyzed hydrolysis gave the [^{14}C]-labelled oligosaccharide phosphate fraction which was sulfonated with excess sulfur trioxide pyridine complex to give [^{14}C]PI-88. [^{35}S]-labelled PI-88 was similarly prepared by the sulfonation of unlabelled oligosaccharide phosphate fraction with [^{35}S] sulfur trioxide pyridine complex. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: PI-88; ^{14}C; ^{35}S; *Pichia* (*Hansenula*) *holstii*; phosphomannan

Introduction

The novel sulfonated oligosaccharide agent known as PI-88[1] (**1**) is a promising inhibitor of tumour growth and metastasis and is currently

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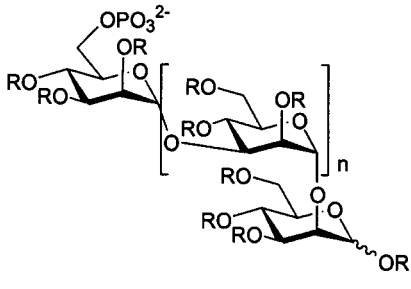
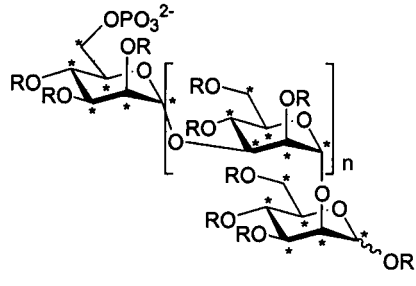
undergoing Phase II clinical trials in oncology patients. PI-88 inhibits tumour growth by blocking heparan sulfate binding of growth factors that induce angiogenesis.[1] PI-88 also inhibits metastasis by inhibiting the enzyme heparanase,[2] blocking the breakdown of the extracellular matrix, thus preventing the spread of tumour cells. In addition to its anti-cancer activities, PI-88 shows promise as a potential anticoagulant/antithrombotic agent with a novel mode of action.[3,4]

A suitable, sensitive and direct assay for PI-88 in biological fluids is not currently available to provide useful pharmacokinetic data. The use of the activated partial thromboplastin time (APTT) has thus been used as a surrogate marker of PI-88 concentration in plasma samples.[5] This assay relies on the fact that PI-88 causes an increase in APTT, i.e., the time it takes for plasma to clot following activation of the intrinsic coagulation pathway. In order to obtain more detailed information about the disposition of PI-88 *in vivo*, radiolabelled PI-88 was prepared for use in pharmacokinetic and tissue distribution studies in rats and monkeys.





1 R = SO_3^- Na^+ or H
2 R = H
5 R = [^{35}S]SO_3^- Na^+ or SO_3^- Na^+ or H

*All carbons labelled with ^{14}C
3 R = H
4 R = SO_3^- Na^+ or H

n = 0 - 4

Results and discussion

PI-88 (**1**) is prepared[6] by the chemical sulfonation of the oligosaccharide phosphate fraction (OPF, **2**) obtained by mild acid catalyzed hydrolysis of the extracellular phosphomannan produced by the yeast *Pichia (Hansenula) holstii* NRRL Y-2448.[7,8] The OPF has been extensively studied[9–11] and recent analysis by capillary electrophoresis (CE) has shown it to be made up of predominantly penta- and tetrasaccharide

phosphates (**2**, $n = 3$, 2), along with minor amounts of di-, tri- and hexasaccharide phosphates (**2**, $n = 0$, 1, 4) and a tetrasaccharyl amine.[11] The large-scale preparation of the OPF[10] and its sulfonation[6] to produce PI-88 have recently been reported.

Uniformly [^{14}C]-labelled PI-88 was prepared by incorporating D-[^{14}C]glocuose into the fermentation of *P. holstii* to produce [^{14}C]-labelled phosphomannan.[7] Unfortunately, this critical step resulted in a disappointingly low yield and low incorporation of radiolabel (specific activity 1.45 μCi/mg), probably due to the difficulty in maintaining suitable glucose levels to prevent the consumption by the yeast of the phosphomannan as a carbon source. The [^{14}C]phosphomannan was subjected to mild acid catalyzed hydrolysis. The resulting [^{14}C]OPF (**3**) and unphosphorylated oligosaccharides were separated from the high molecular weight core polysaccharide by ultrafiltration (NMWCO 5000). Ion-exchange chromatography on DEAE-Spherilose then furnished pure (**3**). Sulfonation with excess sulfur trioxide pyridine complex, conversion to the sodium salt, and desalting and purification by size exclusion chromatography on a column of Bio-Gel P-2 then gave [^{14}C]PI-88 (**4**). The identity of the final product was confirmed by CE[6] and the chemical and radiochemical purities were determined by HPLC to be 99.0 and 98.7%, respectively. The yields in the hydrolysis and sulfonation steps were satisfactory and the chemical and radiochemical purity were excellent. However, the specific activity of the final product (0.0885 μCi/mg) was suitable only for preliminary pharmacokinetic studies.

In order to obtain a radiolabelled product of higher specific activity, the use of ^{35}S was examined. Sperling *et al.*[12] have reported that the sulfonation of heparin (ATIII-binding) pentasaccharide analogues with [^{35}S]sulfur trioxide pyridine complex gives products of high specific activity, depending on the quality of the reagent. [^{35}S]Sulfur trioxide pyridine complex (1 mCi/mg) was thus reacted in *N*,*N*-dimethylformamide (DMF) with a large excess of unlabelled OPF at room temperature for 3 days. Excess unlabelled sulfur trioxide pyridine complex was then added to sulfonate the remaining unreacted hydroxyl groups. Conversion to the sodium salt and chromatographic purification on Bio-Gel P-2 gave [^{35}S]PI-88 (**5**) in good yield and high specific activity (43.05 μCi/mg). Similarly to Sperling *et al.*,[12] it was also found that the specific activity of the product was not as high when the [^{35}S]sulfur trioxide pyridine complex used was of inferior quality (i.e., colored and not crystalline). The identity of the product was confirmed by CE and

the chemical and radiochemical purities were determined by HPLC to be 94.0 and 97.0%, respectively. The major impurities were inorganic salts (from incomplete desalting), which could be easily removed by further chromatography on the Bio-Gel P-2 column. However, the product was of sufficient purity for the required pharmacokinetic studies.[13]

Experimental

Material and methods

[^{35}S]Sulfur trioxide pyridine complex and D-[^{14}C]glucose were purchased from Amersham Pharmacia Biotech and NEN, respectively. Sulfur trioxide pyridine complex and TLC plates (Kieselgel 60 F_{254} aluminum-backed sheets) were purchased from Merck. Tryptone and yeast extract were purchased from Oxoid, D-glucose from APS Chemicals Ltd, ethanol, KH_2PO_4, salts and trace minerals from BDH, DEAE-Spherilose from ISCO, and Bio-Gel P-2 from BioRad. HPLC was performed on a Waters Alliance 2690 Separations Module, or a Waters 6000A or GBC LC 1150 HPLC pump (with a Rheodyne 7125 manual injector fitted with a 20 µl loop) using a Tosohaas TSK-Gel G2500PWXL 6 µ (300 × 7.8 mm) size exclusion column (flow rate: 1 ml/min, mobile phase: 0.05 M NaCl). Detection was with a Shimadzu RID-10A or a Waters 2410 refractive index detector. Capillary electrophoresis was performed on a Beckman P/ACE 5000 System as previously described.[6] Scintillation counting was performed on an LKB Wallac 1217 Rackbeta or a Wallac 1450 MicroBeta Trilux liquid scintillation counter. Aliquots (10 µl) were mixed with scintillant (1 ml Packard Ultima Gold™ LSC-cocktail or Perkin-Elmer OptiPhase 'SuperMix') and counted for 1 min. All water used was purified in house to USP Purified Water standard.

Growth media

Growth medium A contained yeast extract (1 g), tryptone (1 g), KH_2PO_4 (5 g), D-glucose (40 g), salts solution (5 ml), trace minerals solution (1 ml) and Dow Corning Medical Anti-foam C (1 ml) per liter. Growth medium B contained yeast extract (0.2 g), tryptone (0.2 g), KH_2PO_4 (5 g), D-glucose (3.5 g), salts solution (5 ml), trace minerals solution (1 ml) and Medical Anti-foam C (1 ml) per liter.

[^{14}C]Phosphomannan

P. holstii NRRL Y-2448 was grown for 48 h at 28°C in 100 ml of growth medium A (shaking incubator at 160 rpm). Ten milliliters of this inoculum culture was transferred to a 100 ml shake flask and grown for 24 h at 28°C. The flask culture was then centrifuged (15 min × 7000 *g*). The supernatant was discarded and the pellet was resuspended in 100 ml of growth medium B. This was then incubated at 28°C for 0.5 h and then D-[^{14}C]glucose (5 ml, 1 mCi/ml in 9:1 EtOH–water, 5.0 mCi) was added. The culture was incubated for a further 15.5 h, by which time the glucose concentration was ~0 g/l (Diastix™ glucose strips, Bayer). The culture was centrifuged (10°C, 30 min × 11000 *g*). To the supernatant were added 3 M KCl (1 ml) and absolute EtOH (100 ml). After standing for 3 h at room temperature the phosphomannan precipitate was isolated by centrifugation (10°C, 40 min × 13000 *g*). The residue was dissolved in water (20 ml) and concentrated by rotary evaporation to yield 14 mg of [^{14}C]phosphomannan with a specific activity of 2.92 μCi/mg. More product was recovered from the supernatant as follows. The supernatant was concentrated to dryness and the residue was dissolved in water (20 ml). Salts and low molecular weight species were then removed by repeated ultrafiltration through a 3000 NMWCO membrane (Amicon Centriprep-3). The retentate was then concentrated to yield a further 37 mg of [^{14}C]phosphomannan with a specific activity of 0.91 μCi/mg. The total yield was 51 mg with a specific activity of 1.45 μCi/mg.

*[^{14}C]Oligosaccharide phosphate fraction (OPF, **3**)*

To a mixture of [^{14}C]phosphomannan (51 mg, 74 μCi) and unlabelled phosphomannan[†] (62 mg) was added a solution of KCl (40 mg) in water (2.5 ml). The pH was adjusted to ~2 by addition of 1 M HCl (~60 μl) and the mixture was heated at reflux for 6 h. The pH was monitored and maintained at 1.5–2 by further addition of 1 M HCl as required. TLC analysis (7 : 3 EtOH–1 M NH_4OAc) showed three components: high molecular weight core polysaccharide ($R_F = 0.1$), OPF ($R_F = 0.3$) and non-phosphorylated oligosaccharides ($R_F = 0.6$). After standing overnight at room temperature, the mixture was adjusted to pH 9 by addition of 1 M NaOH and then transferred to a water-rinsed Millipore Ultrafree®-15 centrifugal concentrator (NMWCO 5000) and

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centrifuged (50 min × 2000 *g*). The permeate, which contained OPF and unphosphorylated products but no core polysaccharide (TLC), was collected whilst the retentate was re-diluted with water and re-centrifuged. This process was continued until TLC analysis showed that no more OPF was left in the retentate. The combined permeate (25 ml) was loaded onto a column of DEAE-Spherilose (60 ml) equilibrated with 0.01 M NH_4HCO_3. The column was washed with three column volumes of 0.01 M NH_4HCO_3 to remove any traces of non-phosphorylated material and then the pure product was eluted off the column with 0.25 M NH_4HCO_3. The fractions containing product were pooled and lyophilized to give [^{14}C]OPF (**3**) as a white, amorphous solid. The yield was 73 mg with a specific activity of 0.19 μCi/mg. The product was identical to an authentic non-radioactive sample by TLC analysis.[10]

*[^{14}C]PI-88 (**4**)*

[^{14}C]OPF (**3**, 73 mg, 77 μmol) was suspended in DMF (4 ml) at 60°C. Sulfur trioxide pyridine complex (1.05 g, 6.58 mmol, 5 eq/OH) was added and the mixture was stirred at 60°C for 6 h and then left to stand overnight at room temperature, by which time the product had separated out as an oil. The DMF was decanted off and the residue was washed with EtOH (3 × 2 ml). More product was recovered by precipitation of the combined DMF and EtOH washings with EtOH (40 ml), which was then isolated by centrifugation (10 min × 6000 *g*). The combined product residues were dissolved in water (2 ml), the pH was adjusted to 9 with 1 M NaOH and the solution was then concentrated. The crude product was purified on a column of Bio-Gel P-2 (1.5 × 100 cm, equilibrated with 0.01 M NH_4HCO_3). The fractions containing product were pooled and lyophilized to give [^{14}C]PI-88 (**4**) as a white, amorphous solid which was ≥99.0% pure by HPLC. The identity of the product was confirmed by CE.[6] The yield was 52 mg with a specific activity of 0.0885 μCi/mg. The radiochemical purity was determined to be 98.7% by HPLC.

*[^{35}S]PI-88 (**5**)*

The OPF (**2**)[10] (13 mg, 14 μmol) was added to a solution of [^{35}S]sulfur trioxide pyridine complex (2 mg, 12 μmol, 2 mCi) in DMF (2 ml). Unlabelled sulfur trioxide pyridine complex (39 mg, 245 μmol, 1 eq/OH)

was then added and the mixture was stirred at room temperature for 3 days. More sulfur trioxide pyridine complex (364 mg, 2.29 mmol, 9.6 eq/OH) and DMF (0.5 ml) were added and the mixture was stirred at room temperature for a further 3 days. The DMF was removed via pipette and the residue was washed with EtOH (3×1 ml). The EtOH washings and more EtOH (30 ml) were added to the DMF. The resulting precipitate was isolated by centrifugation (10 min $\times$ 6000 *g*), dissolved in water (1 ml) and added to the residue. More water (2 ml) was added and the pH of the solution was then adjusted to 9.5 with 1 M NaOH. The solution was then concentrated, redissolved in water (2 ml) and reconcentrated ($\times 2$). The residue was purified by chromatography on a column of Bio-Gel P-2 (1×100 cm) equilibrated with 0.1 M NH_4HCO_3. The fractions containing carbohydrate (determined by spotting onto a TLC plate and charring with 10% H_2SO_4) were pooled and lyophilised to give [^{35}S]PI-88 (**5**) as a white, amorphous solid (37 mg). The identity of the product was confirmed by CE.[6] The specific activity was 43.05 μCi/mg. The chemical and radiochemical purity were determined by HPLC to be 94 and 97.0% respectively.

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