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Synthesis and PC3 androgen-independent prostate cells antiproliferative effect of fagaronine derivatives

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Abstract

Fagaronine derivatives syntheses were optimized and their effect on PC3 androgen-independent prostate cell line was evaluated. An assessment of the lipophilicity of the benzo[*c*]phenanthridine derivatives was achieved at pH 7.4 and et 6.7 by determining log D.

Keywords: *Fagaronine, benzo[*c*]phenanthridine, PC3 androgen-independent cell line, log D*

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Introduction

Prostate cancer (PC) is the second most prevalent malignancy and the second leading cause of cancer-related deaths among men in many western countries [1]. Its treatment will represent one of the main important goals in the next decade. Although the treatment of PCs by radical prostatectomy, radiotherapy and antiandrogen therapy has a high curability rate in patients diagnosed with localized and androgen-dependent PCs, the progression to the hormone-refractory prostate cancer (HRPC) forms is associated with disease relapse and poor patient survival [2].

Benzo[*c*]phenanthridines (BZP) are very attractive synthetic targets because of their widespread occurrence in plants and their broad range of biological activities [3–4]. Many synthetic approaches to natural benzo[c]phenanthridinic alkaloids have been reported, but relatively few studies of structurally original synthetic quaternary BZP have been described [5–6]. Fagaronine **1**, the natural compound exhibiting the most promising biological properties in that series, was first isolated from *Zanthoxylum fagara* (L.) [7] and synthesised by different groups [8]. This lead molecule demonstrated a narrow range of antitumoral activity. Its interaction with topoisomerase I and II was also ascertained [9–10]. A recent study reported the impact of the 12-alkoxy substitution on the *in vitro* antileukaemic activity of N-methyl-12-alkoxybenzo[c]phenanthridinium salts [11]. Ethoxidine **2** was the lead derivative in our BZP series and could also be considered as the 12-ethoxy O-methylfagaronine. The patterns of DNA cleavage by topo I showed linear enhancement of CPT-dependent sites of fagaronine, whereas ethoxidine suppressed both top I-specific and Camptothecine-dependent sites [12]. New antitumoral 12-alkoxybenzo[c]phenanthridinium derivatives were prepared and their strong interaction with DNA and important topoisomerase I inhibition were demonstrated [13].

In this paper, we report a facile multi-step synthesis of fagaronine and 12-ethoxy derivatives. The activity of these compounds on PC3 androgen-independent

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prostate cells is assessed. The question of the cellular permeability of Bzp has been asked but never answered. In order to address this question with regards to pharmacological results, the lipophilic-behaviour dependence of new compounds was studied. This was performed by measuring the HPLC distribution coefficient (log D) [14] at tumoral and physiological tissues pHs, respectively 6.7 and 7.4. As a matter of fact, cellular pH is crucial for biological functions such as proliferation, invasion and metastasis, drug resistance and apoptosis. Moreover, hypoxic conditions are often observed during the development of solid tumours and lead to intracellular and extracellular acidosis [15].

Materials and methods

Chemistry

NMR spectra were recorded on a Jeol GX 270 MHz and a Bruker 500 Mhz instruments and the chemical shifts (δ-scale) are expressed in parts per million (ppm) value downfield from tetramethylsilane. Chemical mass spectra were recorded on a Jeol JMS 700. TLC was performed on a DC Alufolien Kieselgel 60 F_{254} (VWR). Column chromatography was performed using Silica-gel 60 (0,040–0,063 mm, E. Merck). Organic solutions were concentrated in a rotary evaporator under reduced pressure at a bath temperature below 35°C.

*3-(4-mesyloxy-3-methoxyphenyl)-6,7-dimethoxy-4-vinyl-1,2,3,4-tetrahydroisoquinolin-4-ol (**5a**)*. To a solution of aminoketone **4** (6.25 mmol) in 25 mL of dry THF in a cold bath (−10°C) was added vinylmagnesium bromide (15 mmol). The reaction mixture was stirred for 4 h and a saturated aqueous solution of ammonium chloride was added until the precipitate disappeared. Product was then extracted by 3 × 10 mL of dichloromethane and the organic layer was dried over sodium sulfate and evaporated. The crude material is rapidly purified on silica gel with dichloromethane/methanol (97/3) to lead to **5a** (81% yield).

5a ^{1}H NMR ($CDCl_3$): δ = 2.27 (s, 3H, NCH_3), 3.19 (s, 3H, OSO_2CH_3) 3.40 (s, 1H, CH), 3.59 (d, *J* = 15.0 Hz, 1H, C*H*$_2$N), 3.78–4.05 (m, 3 × OCH_3 + C*H*$_2$N), 5.14 (d, *J* = 11.0 Hz, 1H, C*H*$_2$ vinyl), 5.19 (d, *J* = 17.5 Hz, 1H, C*H*$_2$ vinyl), 5.82 (dd, *J* = 11.0 Hz, 17.0 Hz, 1H, CH vinyl), 6.55 (s, 1H, arom), 6.82 (d, *J* = 8.0 Hz, 1H, arom), 6.87 (s, 1H, arom), 7.14 (s, 1H, arom), 7.23 (s, 1H, arom). ^{13}C NMR ($CDCl_3$): δ = 38.7 (OSO_2CH_3), 44.0 (NCH_3), 55.7 (OCH_3), 55.8 (2 × OCH_3), 58.06 (CH_2N), 74.0 (COH), 75.1 (CHN), 107.9 (CH arom), 110.3 (CH arom), 113.0 (CH arom), 113.2 (CH arom), 113.6 (CH_2 vinyl), 123.7 (CH vinyl), 125.9 (C arom), 127.8 (C arom), 130.1 (C arom), 134.6 (C arom), 141.2 (CH arom), 142.7 (C arom), 147.1 (C arom), 148.5 (C arom).

*3-(4-Hydroxy-3-methoxyphenyl)-6,7-dimethoxy-4-vinyl-1,2,3,4-tetrahydroisoquinolin-4-ol (**5b**)*. To a solution of aminoketone **4** (6.25 mmol) in 25 mL of dry THF in a cold bath (−10°C) was added vinylmagnesium bromide (31.3 mmol). The reaction mixture was stirred for 4 h and a saturated aqueous solution of ammonium chloride was added until precipitate disappeared. The product was then extracted by 3 × 10 mL of dichloromethane and the organic layer was dried over sodium sulfate and evaporated. The crude material was rapidly purified on silica gel with dichloromethane/methanol (97/3) to lead to **5b** (respectively 97%). **5b** was used without further purification.

5b ^{1}H NMR ($CDCl_3$): δ 2.09 (s, 3H, NCH_3), 3.31 (s, 1H, C*H*NCH_3), 3.41 (d, *J* = 15.0 Hz, 1H, C*H*$_2$$NCH_3$), 3.50 (d, *J* = 15.0 Hz, 1H, C*H*$_2$$NCH_3$), 3.69 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 5.04 (d, *J* = 11.0 Hz, 1H, C*H*$_2$CH), 5.15 (d, *J* = 17.1 Hz, 1H, C*H*$_2$CH), 5.71–5.86 (m, 1H, CH_2C*H*), 6.47 (s, 1H, arom), 6.63 (d, *J* = 8.0 Hz, 1H, arom), 6.73 (dd, *J* = 2.5 and 8.0 Hz, 1H, arom), 6.88 (s, 1H, arom), 7.09 (s, 1H, arom). ^{13}C NMR ($CDCl_3$): δ 43.5 (NCH_3), 55.4 (OCH_3), 55.5 (2 × OCH_3), 55.8 (OCH_3), 57.7 (CH_2N), 73.9 (COH), 74.9 (CHN), 107.8 (CH arom), 110.4 (CH arom), 112.9 (CH arom), 113.0 (CH arom), 113.7 (CH_2 vinyl), 123.4 (CH vinyl), 125.7 (C arom), 127.5 (C arom), 130.4 (C arom), 141.1 (CH arom), 144.6 (C arom), 145.7 (C arom), 147.3 (C arom), 148.0 (C arom).

*3-(4-Isopropoxy-3-methoxyphenyl)-6,7-dimethoxy-4-vinyl-1,2,3,4-tetrahydroisoquinolin-4-ol (**6**)*. To a solution of **5a** (1.03 mmol) in dry DMF (15 mL) was added potassium carbonate (2.06 mmol). The mixture was stirred for 20 min and isopropylbromide (2.06 mmol) is added. The mixture was heated at 80°C for 3 h. After the reaction mixture was cooled to RT, 20 mL of water were added and **6** was extracted by 3 × 10 mL of dichloromethane. The oil residue was then purified by silica gel with dichloromethane/methanol (98/2) to lead to compound **6** (yield 55%).

^{1}H NMR ($CDCl_3$): δ = 1.37 (d, *J* = 6 Hz, 6H, (CH(C*H*$_3$)$_2$), 2.21 (s, 3H, NCH_3), 3.46 (s, 1H, CHPh), 3.58 (d, *J* = 15.0 Hz, 1H, C*H*$_2$N), 3.743.81 (m, 4H, OCH_3 + C*H*$_2$N), 3.84 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.52 (sept, *J* = 6 Hz, 1H, $OCHMe_2$), 5.10 (dd, *J* = 10.0 Hz, 1.0 Hz, 1H, C*H*$_2$ vinyl), 5.15 (dd, *J* = 18.0 Hz, 1.0 Hz, 1H, C*H*$_2$ vinyl), 5.875.98 (m, 1H, CH vinyl), 6.55 (s, 1H, arom), 6.70 (dd, *J* = 1.0 Hz, 8.5 Hz, 1H, arom), 6.79 (d, *J* = 8.5 Hz,

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1H, arom), 6.906.92 (m, 2H, arom). ^{13}C NMR ($CDCl_3$): δ = 22.0 ($CH(CH_3)_2$), 22.1 ($CH(CH_3)_2$), 43.7 (NCH_3), 55.8 (OCH_3), 55.8 (OCH_3), 55.9 (OCH_3), 57.4 (CH_2), 71.0 ($CH(CH_3)_2$), 74.4 (COH), 74.8 (CHN), 108.0 (CH arom), 110.5 (CH vinyl), 114.0 (CH_2 vinyl), 114.2 (CH arom), 114.3 (CH arom), 122.9 (CH arom), 126.2 (C arom), 128.4 (C arom), 130.7 (C arom), 141.8 (CH arom), 146.7 (C arom), 147.8 (C arom), 148.5 (C arom), 149.5 (C arom).

2-Isopropoxy-3,8,9-trimethoxy-5-methyl-4b,5,6,12-tetrahydrobenzo[c]phenanthridine (7). To a solution of **6** (1.09 mmol) in dry nitromethane (10 mL) at −55°C, was quickly added boron trifluoride-diethyl ether complex (10.9 mmol). The mixture was left under stirring until it reached RT and a saturated aqueous solution of sodium hydrogen carbonate (10 mL) was added. The crude product was extracted by 3 × 10 mL of dichloromethane and the organic layers were dried over anhydrous sodium sulfate and evaporated. The oil was then purified by silica gel with dichloromethane/methanol (95/5) to lead to compound 7 (yield 61%).

^1H NMR ($CDCl_3$): δ = 1.40 (s, *J* = 6.2 Hz, 6H, $CH(CH_3)_2$), 2.39 (s, 3H, NCH_3), 3.60 (t, *J* = 4.0 Hz, 1H, CH_2), 3.67 (t, *J* = 4.0 Hz, 1H, CH_2), 3.83 (s, 1H, CH_2N), 3.86 (s, 1H, CH_2N), 3.91 (s, 3H, OCH_3), 3.96 (s, 7H, 2 × OCH_3 + CHN), 4.58 (sept, *J* = 6.2 Hz, 1H, $CH(CH_3)_2$), 5.11 (t, *J* = 4.0 Hz, 1H, $CHCH_2$), 6.64 (s, 1H, arom), 6.73 (s, 1H, arom), 6.75 (s, 1H, arom), 7.12 (s, 1H, arom). ^{13}C NMR ($CDCl_3$): δ = 22.0 ($CH(CH_3)_2$), 22.1 ($CH(CH_3)_2$), 29.6 ($CHCH_2Ph$), 40.8 (NCH_3), 49.6 (CH_2N), 55.7 (OCH_3), 56.2 (OCH_3), 56.5 (OCH_3), 63.4 ($CH(CH_3)_2$), 71.4 (NCH), 105.1 (CH arom), 109.2 (OCH_3), 113.2 (OCH_3), 114.3 (OCH_3), 121.5 ($CHCH_2$), 148.3 (C arom), 148.5 (C arom), 149.2 (C arom), 149.5 (C arom), 149.7 (C arom), 149.9 (C arom), 150.0 (C arom), 150.2 (C arom), 150.3 (C arom), 150.4 (C arom).

2-Isopropoxy-3,8,9-trimethoxy-5-methylbenzo[c]phenanthridinium iodide (8). To a solution of tetrahydrobenzo[*c*]phenanthridine **7** (0.41 mmol) in absolute ethanol (1.7 mL) was added iodine (4.1 mmol). The solution was then refluxed for 2 h and an aqueous saturated solution of sodium sulfite was added until the brown colour disappeared. The precipitate was then filtered and washed with acetone to lead to quaternary benzo[*c*]phenanthridine **8** (yield 92%).

^1H NMR (DMSO-d_6): δ = 1.43 (s, 6H, $CH(CH_3)_2$), 3.74 (s, 1H, $CH(CH_3)_2$), 4.06 (s, 6H, 2 × OCH_3), 4.23 (s, 3H, OCH_3), 4.97 (s, 3H, N^+CH_3), 7.82 (s, 1H, arom), 7.89 (s, 1H, arom), 8.15 (s, 1H, arom), 8.35 (s, 2H, arom), 8.88 (s, 1H, arom), 9.87 (s, 1H, arom). ^{13}C NMR (DMSO-d_6): δ = 21.6 ($CH(CH_3)_2$), 51.4 (N^+CH_3), 55.8 (OCH_3), 56.1 (OCH_3), 57.2 (OCH_3), 70.2 ($CH(CH_3)_2$), 102.9 (CH arom), 107.8 (CH arom), 108.4 (CH arom), 110.0 (CH arom), 117.9 (C arom), 118.6 (CH arom), 119.2 (C arom), 123.5 (C arom), 129.5 (CH arom), 130.8 (C arom), 131.6 (C arom), 131.9 (C arom), 148.4 (C arom), 149.3 (C arom), 150.4 (C arom), 151.1 (C arom), 157.9 (C arom).

Fagaronine chloride (1). In a plastic bottle, were introduced **8** (0.53 mmol) and 5 mL of hydrofluoric acid. The bottle was sealed and the mixture stirred overnight. The acid was then evaporated and a saturated aqueous solution of sodium chloride was added. The mixture was stirred for 2 h and the precipitate filtered off to lead to fagaronine **1** (yield 92%).

^1H NMR (DMSO-d_6): δ = 4.05 (s, 3H, OCH_3), 4.09 (s, 3H, OCH_3), 4.23 (s, 3H, OCH_3), 4.99 (s, 3H, N^+CH_3), 7.57 (s, 1H, arom), 7.89 (s, 1H, arom), 8.17 (s, 1H, arom), 8.22 (d, *J* = 9.1 Hz, 1H, arom), 8.37 (s, 1H, arom), 8.84 (d, *J* = 9.1 Hz, 1H, arom), 9.86 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ = 51.4 (N^+CH3), 55.9 (OCH_3), 56.2 (OCH_3), 57.2 (OCH_3), 103.0 (CH arom), 108.2 (CH arom), 108.6 (CH arom), 111.8 (CH arom),117.5 (C arom), 118.6 (CH arom), 119.3 (C arom), 123.3 (C arom), 129.3 (CH arom), 131.1 (C arom), 132.1 (C arom), 132.2 (C arom), 148.7 (C arom), 149.0(C arom), 150.4 (C arom), 151.3 (C arom), 158.1 (C arom).

12-Ethoxy-3,8,9-trimethoxy-5-methyl-2-octanoyloxy-5,6-dihydrobenzo[c]phenanthridine (11). To a solution of **10** (130 mg, 0.33 mol) in dry dichloromethane (4.5 mL) was added triethylamine (0.230 mL, 1.65 mmol). Octanoyl chloride (0.282 mL, 1.65 mmol) was then added to the mixture. The reaction was monitored by TLC (dichloromethane/methanol: 98/2).The reaction was quenched with water and the organic layer was separated and washed 3 times with water (5 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to lead to compound **11** (135 mg, 94%).

^1H NMR ($CDCl_3$): δ = 0.90 (t, *J* = 5.5 Hz, 3H, $OCO(CH_2)_6CH_3$), 1.30–1.,60 (m, 10H, 5 × CH_2), 1.65 (t, *J* = 7.0 Hz, 3H, OCH_2CH_3), 2.53 (s, 3H, NCH_3), 2.80 (t, *J* = 8.0 Hz, 2H, $OCOCH_2$), 4.02 (s, 2H, CH_2N), 4.14 (s, 3H, OCH_3), 4.18 (s, 3H, OCH_3), 4.38 (s, 3H, OCH_3), 4.53 (q, 2H, *J* = 7.0 Hz, OCH_2CH_3), 7.09 (s, 1H, arom.), 7.42 (s, 1H, arom.), 7.56 (s, 1H, arom.), 7.61 (s, 1H, arom.), 7.82 (s, 1H, arom.).

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*12-Ethoxy-3,8,9-trimethoxy-5-methyl-2-octanoyl-oxybenzo[c]phénanthridinium iodide (**12**).* **11** (135 mg, 0.26 mmol) and iodine (674 mg, 2.6 mmol) were dissolved into absolute ethanol (3 mL). The solution was refluxed for 2 h then cooled to room temperature. A saturated sodium sulfite aqueous solution was then added until disappearance of brown color. The precipitate was then filtered off and dried in dessicator to lead to the benzo[*c*]phenanthridinium iodide salt **12** (141 mg, 84%).

^{1}H NMR (DMSO-d$_6$): δ = 0.94 (t, *J* = 5.5 Hz, 3H, OCO(CH$_2$)$_6$C*H$_3$*), 1.30–1.60 (m, 10H, 5 × CH$_2$), 1.70 (t, *J* = 7.0 Hz, 3H, OCH$_2$C*H$_3$*), 2.72 (t, *J* = 8.0 Hz, 2H, OCOCH$_2$), 4.11 (s, 3H, OCH$_3$), 4.17 (s, 3H, OCH$_3$), 4.31 (s, 3H, OCH$_3$), 4.52 (q, 2H, *J* = 7.0 Hz, OC*H$_2$*CH3), 5.08 (s, 3H, N$^+$CH$_3$), 7.49 (s, 1H, arom.), 7.80 (s, 1H, arom.), 7.98 (s, 1H, arom.), 8.01 (s, 1H, arom.), 8.22 (s, 1H, arom.), 9.73 (s, 1H, CHN$^+$). ^{13}C NMR (DMSO-d$_6$): δ = 14.1 (CH$_3$), 14.6 (CH$_3$), 22.6 (CH$_2$), 25.0 (CH$_2$), 29.0 (CH$_2$), 29.1 (CH$_2$), 31.7 (CH$_2$), 34.0 (CH$_2$), 52.3 (N$^+$CH3), 57.0 (OCH$_3$), 57.3 (OCH$_3$), 57.7 (OCH$_3$), 65.2 (OCH$_2$), 95.6 (CH arom.), 102.0 (CH arom.), 108.1 (CH arom.), 109.3 (CH arom.), 117.3 (CH arom.), 120.5 (C arom.), 123.2 (CH arom.), 124.0 (C arom.), 127.4 (C arom.), 127.5 (C arom.), 131.4 (C arom.), 141.9 (C arom.), 147.8 (C arom.), 151.5 (C arom.), 152.5 (C arom.), 155.7 (C arom.), 158.4 (C arom.), 171,6 (CO).

Partition Coefficients: log D (pH 7.4 or pH 6.7)

In this study log D (pH 7.4 or 6.7) was assessed by the micro-HPLC method [14]. The determinations were performed with a chromatographic apparatus (Spectra Series, San Jose, USA) equipped with a model P1000XR pump and a model SCM 1000 vacuum membrane degasser, a model UV 150 ultraviolet detector (λ = 278 nm) and a ChromJet data module integrator (ThermoFinnigan, San Jose, USA). A reversed phase column was used: a Waters XTerra™MS C$_{18}$ (3.9 × 150 mm; 5 μm particle size) with a mobile phase consisting of acetonitrile – potassium dihydrogen phosphate (0.066 M) adjusted to pH = 3.5 with orthophosphoric acid (30:70, v/v (**1**), 40:60, v/v (**2** and **3**) and 65:35, v/v (**12**)).

The compounds were partitioned between n-octanol (HPLC grade) and phosphate buffer (pH = 6.7 or 7.4). Octanol was presatured with buffer, and conversely. An amount of 1 mg of each compound was dissolved in an adequate volume of methanol in order to achieve 1 mg/mL stock solutions. Then an appropriate aliquot of these methanolic solutions was dissolved in buffer to obtain final concentration of 100 μg/mL. Under the above-described chromatographic conditions, 20 μL of this aqueous phase was injected into the chromatograph, leading to the determination of a peak area before partitioning (W_0).

In screw-capped tubes, 1000 μL of the aqueous phase (V_{aq}) was then added to 50 μL of n-octanol (V_{oct}). The mixture was shaken by mechanical rotation during 30 min. Then, the centrifugation was achieved at 3000 rpm in 15 min. A 20 μL amount of the lower phase was injected into the chromatograph column. This led to the determination of a peak area after partitioning (W_1). The log D was determined by the formula: $\log D = \log [(W_0 - W_1)V_{aq}/W_1V_{oct}]$.

Pharmacology

Survival assays. Cells were plated at a density of $5 \cdot 10^3$ cells in 190 ll culture medium in each well of 96-well microplates (Nunclon; Nunc, Roskilde, Denmark) and were allowed to adhere for 16 h before treatment with the benzo[*c*]phenanthridine (Bzp). A stock solution of Bzp was prepared in sterile 0.15 M NaCl (Eurobio, Les Ulis, France) and kept at −20°C until use. Then 10 μl of a 20x Bzp solution in 0.15 M NaCl was added to the cultures. A 48-h continuous drug exposure protocol was sed. The antiproliferative effect of Bzp was assessed by both the resazurin reduction test (RRT) and determination of DNA cellular content after cell lysis according to Debiton et al. [16]. Each experiment was done at least 3 times.

Resazurin reduction test (RRT). The RRT was carried out as follows. Briefly, plates were rinsed with 200 μL PBS (Gibco) at 37°C using an automatic microplate washer (Cell Wash; Labsystems, Helsinki, Finland) emptied by overturning on absorbent towelling. Then 150 μL of a 25 μg/ml solution of resazurin in MEM without phenol red was added to each well using an automatic microvolume dispenser (Multidrop 384; Labsystems). Plates were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO$_2$. Fluorescence was then measured on an automated 96-well plate reader (Fluoroskan Ascent FL; Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Under the conditions used, fluorescence was proportional to the number of living cells in the well. The IC$_{50}$, defined as the drug concentration required to inhibit cell proliferation by 50%, was calculated from the curve of concentration-dependent survival percentage, defined as fluorescence in experimental wells compared with fluorescence in control wells, after subtraction of blank values. After reading, cells were prepared for cellular DNA quantitation with Hoechst dye 33342. They were rinsed with PBS, resazurin solution was then eliminated using an automatic micro plate washer and plates were stored at 80°C until the Hoechst assay.

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Hoechst dye 33342 assay (Ho 33342) [17]. On the day of assay, plates were thawed at room temperature for 10 min. A volume of 100 μL 0.01% (m/v) SDS solution in sterile distilled water was then distributed into each well with an automatic dispenser, and the plates were incubated for 1 h at room temperature and frozen again at −80°C for 1 h. After thawing (approximately 15 min), 100 μL per well of Hoechst dye 33342 solution (Sigma) at 30 μg/mL in a hyper saline buffer (10 mM Tris HCl, pH 7.4, 1 μM EDTA and 2 M NaCl) was added to each well. The plates were incubated in this solution for 1 h protected from light at room temperature on a plate shaker. Fluorescence was then measured at 360/460 nm on a microplate fluorescence reader. Under the used conditions, fluorescence was proportional to the amount of biomass, and the IC50 was calculated as above.

Results and discussion

Chemistry

3-Oxoisoquinoline **4** was previously obtained via anhydrous fluorhydric acid catalysed intramolecular cyclisation, in a quantitative yield and used without further chromatographic purification [13]. The ketone **4** in the presence of vinylmagnesium bromide, led to the vinylic precursors **5a** or **5b** in very high yields (Scheme 1). Structures of compounds **5a,b** were easily confirmed by the presence of three non-ambiguous signals relative to vinylic protons in ^{1}H NMR spectra (between 5.04 an 5.86 ppm). It is noticeable that the mesyloxy protective group was eliminated when an excess of Grignard reagent was used. Using a slight excess of this reagent allowed to isolate the mesyloxy product **5a**. Intramolecular cyclisation of the phenolic compound **5b** was attempted either in protic or Lewis acid media in different solvents. The very low yields observed could be explained by the presence of the phenolic acidic proton, unfavourable to the reaction. Knowing that an ether function was a necessary condition for a successful cyclisation, the isopropoxy group was chosen and introduced, as a protective group, on the isoquinoline skeleton **5b** using classical *O*-alkylation methods to yield **6** (Scheme 1). On another hand, as expected, the reaction did not occur with the mesyl protected compound **5a**. We then found out that the optimal acid conditions consisted in using methanesulphonic acid in a polar aprotic solvent (nitromethane) yielding the desired benzo[*c*]phenanthridine precursor **7** in 94–96% yield, as a single product. Bearing in mind this solvent effect, the synthesis of fully aromatized Bzp skeleton was then undertaken in nitromethane using BF_3-Et_2O with precursor **7** (Scheme 1). Then, this derivative was oxidised by iodine in absolute ethanol to lead to quaternary Bzp **8**. Fagaronine **1** was then obtained from the iodide **8** when regenerating the phenolic function by the use of anhydrous fluorhydric acid. This strong acid enabled both the isopropoxy group hydrolysis and the exchange of the iodide to the fluoride counter-ion. After this salt was stirred in saturated sodium chloride aqueous solution, the fagaronine chloride **9** precipitated out of the solution. This pathway allowed the isolation of fagaronine chloride from **4** in a 6 steps in a good overall yield (27%).

In order to synthesize a new Bzp lipophilic prodrug, we used a versatile phenolic dihydrobenzo[*c*]phenanthridinic intermediate **10**. This molecule was readily obtained by a sodium borohydride reduction of the iminium bond of a BZP mesylated intermediate [13]. Oxidation of **10** using iodine in ethanolic solution led to ethoxyfagaronine **3** [13]. On another occasion, esterification of **10** was conducted using octanoyl chloride in basic dichloromethane solution to yield **11**. Oxidation of the dihydroester **11** afforded **12** in a good overall yield (Scheme 2).

Pharmacology

The four BZP compounds **1**–**3**, **12** were tested on PC3 prostatic adenocarcinoma tumour cell line and their effect assessed using two complementary methods: the RRT and Hoechst assay. The RRT measured the residual metabolic activity of cultures, and the Hoechst assay, the DNA content in each well and, hence, the corresponding biomass. Statistical analysis revealed that ethoxidine **2** was the most efficient compound in that series, with biological activity in the μmolar range (Table I). Fagaronine **1** was less potent while ethoxyfagaronine **3** antitumoral activity values were average. Surprisingly, the prodrug **12** potency was quite at the same level compared to **3**.

Lipophilicity

The preliminary pharmacological results could be discussed in terms of physicochemical behaviour through the partitioning theory which involved the influence of the lipophilicity of studied compounds.

For compounds **1**, **2**, **3** and **12**, HPLC determination of the distribution coefficient (log$D$) was achieved at two distinct pHs (6.7 considered close to the probable pH of tumoral tissues [15] and 7.4 assumed to be the physiological pH). In Table II, it can be seen that there was only a slight difference between the log D values measured at pH 6.7 and those determined at pH 7.4. Three of the studied compounds (**1**, **2** and **3**) were found to be hydrophilic with log D between −0.15 and 0.38 (pH = 6.7) and log D between −0.29 and 0.38 (pH = 7.4), respectively. Compounds **1** and **3** both have an hydroxy group on position 2, with an additional

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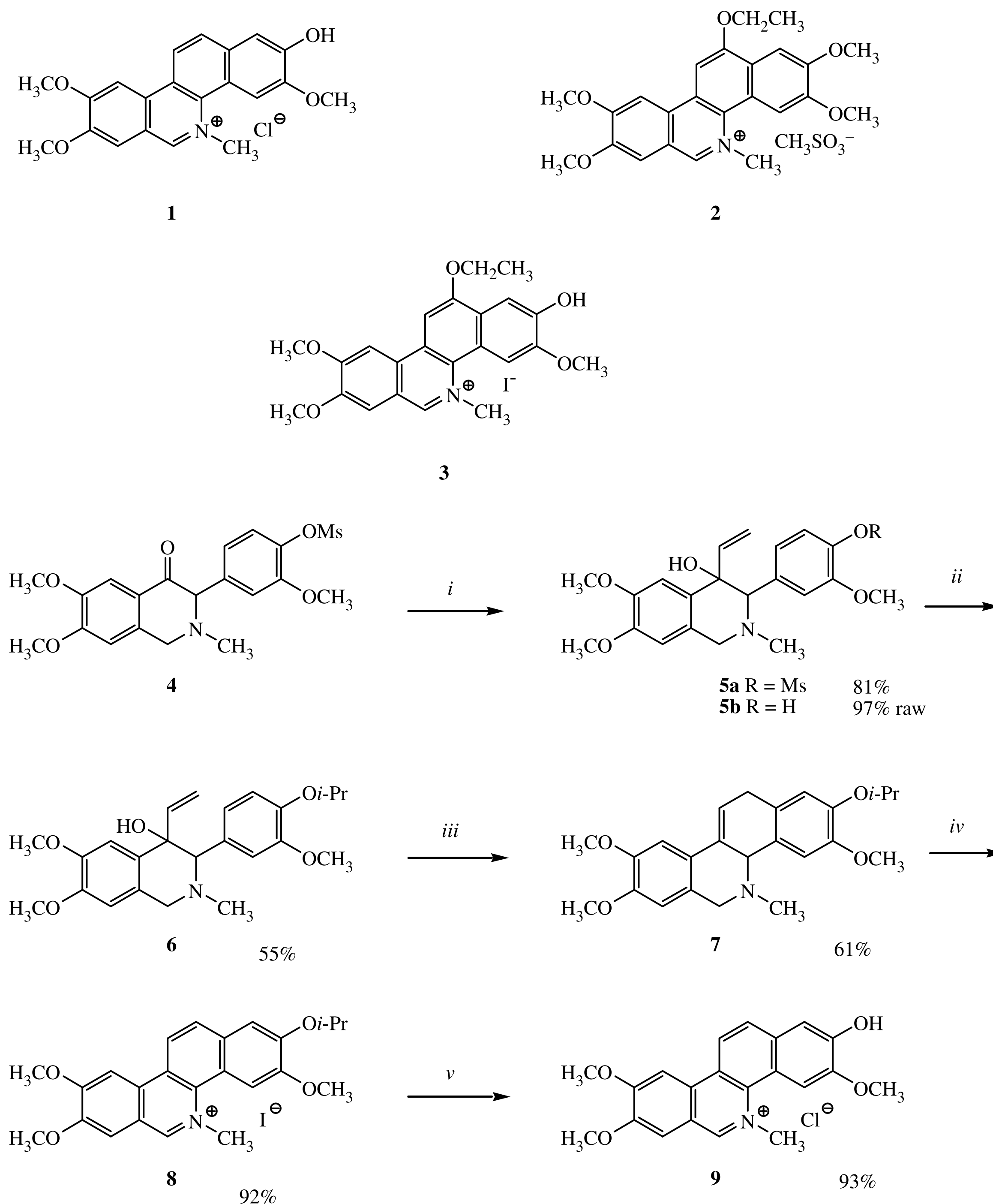

Scheme 1. *i.* vinylMgBr, THF, -10°C *ii.* i-PrBr, DMF, K_2CO_3 *iii.* Et_2OBF_3, CH_3NO_2, -55°C *iv.* I_2, EtOH, 80°C *v.* HF, NaCl aq 10%.

bulkier ethoxy group on position 12 for **3**. Compound **2** bears this ethoxy group on the same position but without the hydrogen bond donor function on position 2. Our results indicates that these structural modifications very slightly affects the lipophilic behaviour at studied pHs.

On the other hand (Table II), log D values of compound **12** highlighted a more lipophilic profile at studied pHs. As an example, by comparison with the reference drug **1** a near 5000 fold difference was observed at pH 6.7 and 200 fold at pH 7.4. Compound **12** was synthesized to be used as a prodrug with an octanoyloxy ester side chain. Whereas the introduction of an ethoxy substituent in position 12 is non significant, the C-2 octanoyloxy chain introduction produced a large increase of the

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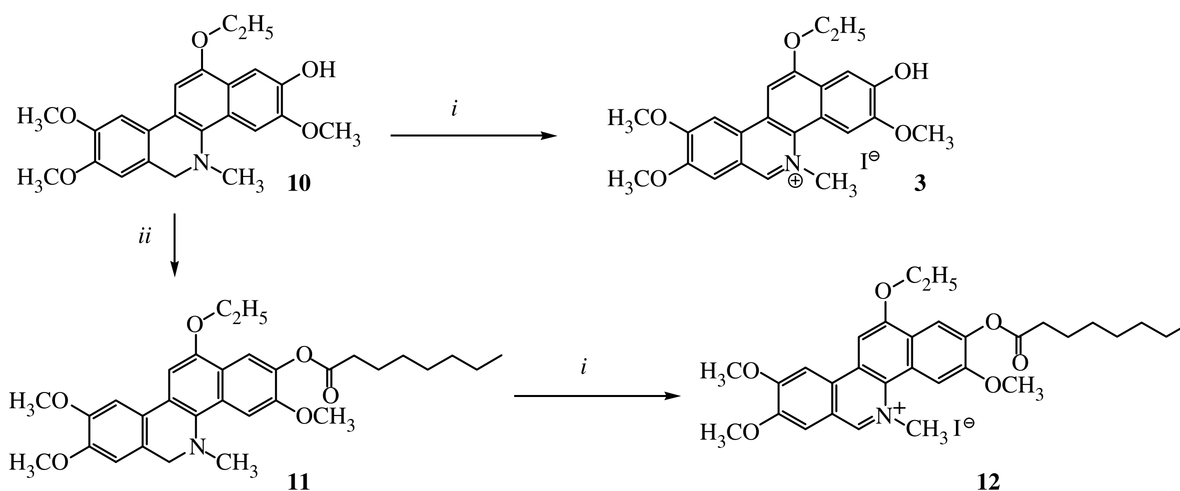

Scheme 2. *i.* I_2, EtOH, 80°C *ii.* $C_7H_{15}COCl$, Et_3N, CH_2Cl_2.

lipophilic character of **12** compared to compound **1**. Nevertheless, this measured increase of lipohilicity is not related to an increase of pharmacological activity**.** On another hand, only slight variations affected the log *D* values measured at pH 6.7 and at pH 7.4. Moreover, when the pH decreased from 7.4 to 6.7, an opposite lipophilicity behaviour between **1** and **2**, **3**, **12** was noticed. This feature, in relation with the biological results, could explain the relative drop of activity observed with **1**.

In conclusion, we described in this paper an improved access to fagaronine and the synthesis of the first prodrug of ethoxyfagaronine. We showed that the studied BZP derivatives were all active against a PC3 androgen-independent prostate cell line in the micromolar range. Among these molecules, ethoxidine **2** was the most active derivative.

Although up to now only a very limited number of compounds have been studied, the following preliminary conclusions could be put forward. Introduction of an ethoxy group at carbon 12 of **1**, leading to **3,** induces but a slight increase of the activity. Blockade of the phenol group of **3**, affording **2**, on the other hand, induces a clear-cut increase of the level of activity: **2** is about five fold as potent as **1**. Blockade of the same function by esterification (**12**, Table II) hardly modifies the activity level of **3**; this could be linked to a fast hydrolysis of the ester, before entering the tumoral cell or the fact that such a high lipophilicity exert a detrimental effect on membrane transport. Work is now in progress considering these two hypothesis.

Iminium function plays a major role in that series; the nitrogen charged iminium function is definitively a part of the mechanism of action and is in pH-dependent equilibrium with the more lipophilic pseudobase form (Scheme 3) [18]. On the other hand, it represents a major drawback when it comes to the question of transport through biological membrane. To overcome this problem, Clement et al recently described the synthesis and the antitumoral activity of 6-aminobenzo[*c*]phenanthridine derivatives, also bearing a phenyl group on C-11. These molecules were shown to be very potent agents against various tumour cell lines [19]. Compared to our BZP series, the iminium function, characterized by its positively charged nitrogen, has been replaced by an amidine group, potentially bearing a positive charge. Such a structural modification could be applied to our BZP series to potentially enhance antitumoral activities. These

Table I. Bzp Survival tests assays: resazurin reduction test and Hoechst dye 33342 assay.

	PC3 cells **IC50 (μM)**			
Compound	**1**	**2**	**3**	**12**
Resazurin test	11.6	2.0	7.8	7.0
CMF	21.5	5.0	11.7	8.0

Table II. Measured values of drug lipophilicity (log D) at two different pHs (pH 6.7 and pH 7.4).

	log $D_{6.7}$	log $D_{7.4}$
1	− 0.15	0.38
2	0.38	0.07
3	0.01	−0.29
12	3.54	2.68

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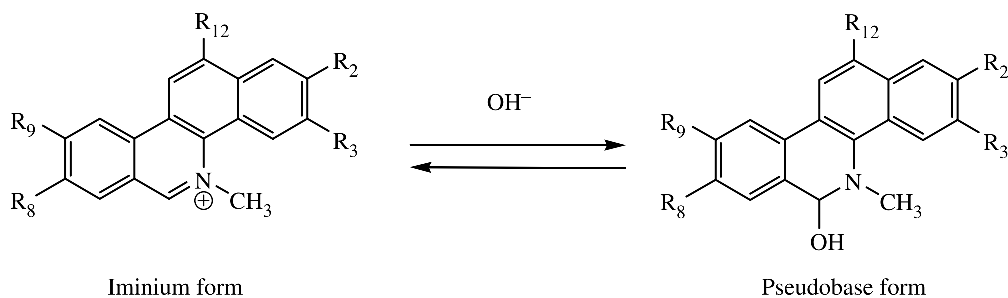

Scheme 3. iminium and pseudobase equilibrium in the BZP series.

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modifications may help to design new structures for the discovery of more efficient BZP.

References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. CA Cancer J Clin 2006;56:106–130.
[2] Gronberg H. Lancet 2003;361:859–864.
[3] Suffness M, Cordell GA In: Brossi A, editor. "The Alkaloids". 25 New York: Academic; 1985. p 178–187.
[4] Simanek V In: Brossi A, editor. In The Alkaloids. Orlando, FL: Academic Press; 1986; 26: 229–230.
[5] Simeon S, Rios JL, Villar A. Pharmazie 1989;44:593–597.
[6] MacKay SP, Meth-Cohn O, Waigh RD. Adv. Heterocyclic Chem. 1997;67:345–388.
[7] Messmer WM, Tin-Wa M, Fong HHS, Bevelle C, Farnsworth NR, Abraham DJ, Trojanek J. J Pharm Sci 1972;61: 1858–1859.
[8] Ishikawa T, Ishii H. Heterocycles 1999;50:627–639.
[9] Ianoul A, Fleury F, Duval O, Waigh R, Jardillier JC, Alix AJP, Nabiev I. J Phys Chem 1999;103:2008–2013.
[10] Wang LK, Johnson RK, Hecht SM. Chem Res Toxicol 1993;6:813–818.
[11] Mackay SP, Comoe L, Desoize, Duval O, Jardillier JC, Waigh RD. Anti-Cancer Drug Design 1998;13(7):797–813.
[12] Fleury F, Sukhanova A, Ianoul A, Devy J, Kudelina I, Duval O, Alix AJP, Jardillier JC, Nabiev I. J Biol Chem 2000;275: 3501–3509.
[13] Lynch MA, Duval O, Sukhanova A, Devy J, MacKay SP, Waigh RD, Nabiev I. Bioorgan & Med Chem Lett 2001; 11:2643–2646.
[14] Pehourcq F, Thomas J, Jarry C. J Liq Chromatogr Relat Technol 2000;23:443–453.
[15] Hiroto I, Takayuki T, Hiroshi I, Hidetaka U, Yoichiro Y, Mizuho T, Tomoko I, Tadashi M, Takeshi Y, Minoru N, Kimitoshi K. Cancer Treat Rev 2003;29:541–549.
[16] O'Brien J, Wilson I, Orton T, Pognan F. Eur J Biochem 2000;267:5421–5426.
[17] Rago R, Mitchen J, Wilding G. Anal Biochem 1990;191: 31–34.
[18] Kerry MA, Duval O, Waigh RD, MacKay SP. J Pharm Pharmacol 1998;50:1307–1315.
[19] Kock I, Heber D, Weide M, Wolschendorf U, Clement B. J Med Chem 2005;48:2772–2777.

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