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Synthesis and Antiviral Effect against *Herpes Simplex* Type 1 of 12-substituted Benzo[*c*]phenanthridinium Salts

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The synthesis of benzo[*c*]phenanthridine alkaloid derivatives is described. *In vitro* antiviral activity against *herpes simplex* type 1 (HSV1) has been investigated. Contrary to the natural product fagaronine, which did not have any activity in the HSV1 antiviral tests, four 12-alkoxy derivatives showed good activity demonstrating the importance of the 12-substitution in the structure-activity relationships.

Keywords: Benzo[*c*]phenanthridines; Fagaronine; 12-alkoxy derivatives; Antiviral activity; HSV1

INTRODUCTION

Quaternary benzo[*c*]phenanthridines (BZPs) are naturally occurring alkaloids with various biological activities.[1] Among these structures, nitidine **1** and fagaronine **2** have gained much attention for their anticancer and antiviral activities. For example, fagaronine chloride demonstrated potent activity in the HIV-1 RT system and was adopted as a positive-control substance.[2] Three other synthetic 12-substituted benzo[*c*]phenanthridines have shown anti-HIV activity.[3] The *herpesviridae* include viruses that infect a variety of different animal species. Eight distinct human herpes viruses have been identified and are subdivided into alpha, beta and gamma subfamilies. HSV-1 is a member of the alpha subfamily and is commonly associated with cold sores. However, active viral replication can lead to more serious cytopathy in the immunocompromised.[4]

The nuclear enzyme topoisomerase II is a potential cellular target of drugs to treat *herpes simplex* virus infection since biochemical studies show that the enzyme is a functional host factor for HSV replication.[5–6] The natural antitumor benzo[*c*]-phenanthridines, fagaronine and nitidine, have both been shown to be inhibitors of topoisomerases I and II[7–8] and as part of our work on the search for new pharmacological applications in this series, we now report on the HSV1 antiviral activity of synthetic BZP derivatives **3**, **4**, **5**, **16** (Figure 1 and Scheme 1).

MATERIALS AND METHODS

Chemistry

Instrumentation

Melting points were determined on an Electrothermal 8100 melting point apparatus and are uncorrected. ^{1}H, ^{13}C NMR spectra experiments were recorded on a Jeol GSX WB 270 MHz [270 MHz (^{1}H) and 67.5 MHz (^{13}C)] instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Bruker FT IR Vector 22 using potassium bromide disc for solids or neat liquid films for liquids. HREIMS (70 eV) were recorded on a Varian MAT 311 spectrometer.

Reagents and Chemicals

Organic solvents were purified when necessary according to literature methods or purchased from Aldrich Chimie (St Quentin-Fallavier, France). All solutions were dried over anhydrous magnesium

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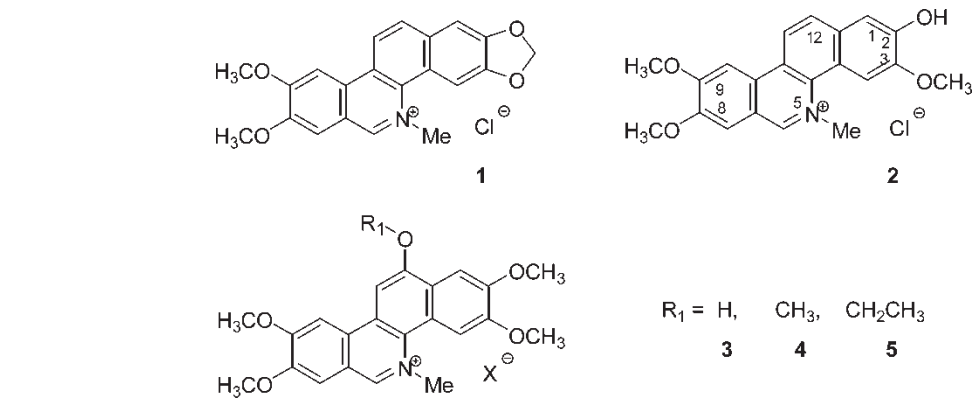
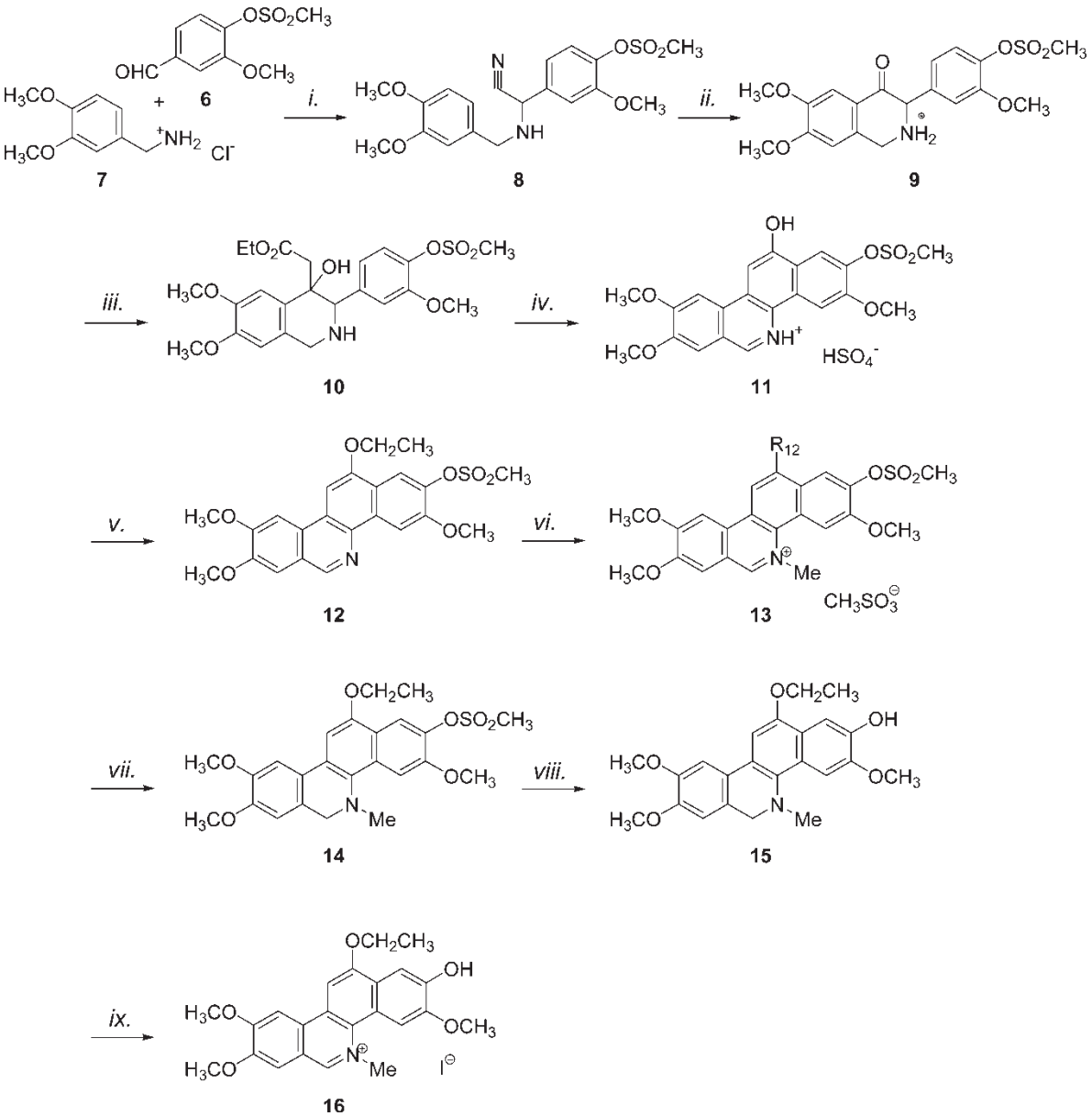


FIGURE 1



SCHEME 1 i. KCN, H_2O/C_2H_5OH, RT; ii. HF, brine, 15°C; iii. $BrCH_2CO_2Et$, Zn, dioxane/DMM; iv. H_2SO_4; v. K_2CO_3, acetone, $(C_2H_5O)_2SO_2$; vi. $CH_3SO_3CH_3$, 180°C; vii. $NaBH_4$, CH_3OH; viii. KOH (5 M), CH_3OH; ix. I_2, C_2H_5OH.

sulfate and evaporated on a Buchi rotatory evaporator. The column chromatography solvents employed were distilled and solvent mixtures were reported as volume to volume ratios. Starting materials were purchased from Aldrich Chimie (St Quentin-Fallavier, France) or Acros (Noisy-le-Grand, France). Literature procedures were used for the preparation of **2**, **3**, **4** and **5**.[9,10]

Synthesis of 12-ethoxy-fagaronine

2-(3,4-Dimethoxybenzylamino)-2-(3-methoxy-4-mesyloxyphenyl)acetonitrile (**8**)

A mixture of 3,4-dimethoxybenzylamine hydrochloride salt **7** (5.95 g, 2.94×10^{-2} mol) in 55 mL of water and 3-methoxy-4-mesyloxybenzaldehyde **6** (6.76 g, 2.94×10^{-2} mol) in 55 mL of ethanol was vigorously stirred. To the reaction mixture was added dropwise an aqueous potassium cyanide solution (2.90 g, 4.41×10^{-2} mol, 15 mL) and the mixture was vigorously stirred for 48 h. The ethanol was removed under reduced pressure and the resulting aqueous layer was extracted with 3×15 mL of dichloromethane. The combined organic layers were dried over sodium sulfate, and the solvent evaporated to yield 10.87 g of the acetonitrile **8**. Yield: 91%. M.p. = 84, 5°C. IR(cm^{-1}): 3312, 2235. ^{1}H NMR($CDCl_3$): δ (ppm): 1.92 (1H, s coalescent, —NH—), 3.20 (3H, s, $—OSO_2CH_3$), 3.89 (3H, s, $—OCH_3$), 3.90 (3H, s, $—OCH_3$), 3.93 (3H, s, $—OCH_3$), 4.03 (1H, d, $J = 12$ Hz, AB system $—CH_2—$), 4.73 (1H, s, —CHCN—), 6.85 (1H, d, $J = 8$ Hz, Ar), 6.93 (2H, s, Ar), 7.17 (1H, s, Ar), 7.17 (1H, dd, $J = 3$ and 10 Hz, Ar), 7.33 (1H, d, $J = 8$ Hz, Ar); ^{13}C NMR($CDCl_3$): δ (ppm): 38.38 ($—OSO_2CH_3$), 50.96 ($—CH_2—$), 52.73 (—CHCN—) 55.79 ($—OCH_3$), 55.86 ($—OCH_3$), 56.05 ($—OCH_3$), 111.07 (CH Ar), 111.39 (CH Ar), 111.75 (CH Ar), 118.22 (CH Ar), 119.72 (CH Ar), 120.53 (CH Ar), 124.67 (—CN), 111.07 (CH Ar), 130.14 (C Ar), 134.94 (C Ar), 138.38 (C Ar), 148.44 (C Ar), 148.99 (C Ar), 151.59 (C Ar).

3-(4-Mesyloxy-3-methoxyphenyl)-6,7-dimethoxy-1,2-dihydroisoquinolin-4(3H)-one, Hydrochloride (**9**)

The aminonitrile **8** (3.27 g, 8.05 mmol) was poured into a plastic bottle at 0°C. 15 mL of hydrogen fluoride was added and the bottle sealed. Magnetic stirring was maintained for 48 h. The bottle was opened and the hydrogen fluoride was allowed to evaporate. A saturated aqueous solution of sodium chloride (30 mL) was added and the mixture was stirred for 24 h. The reaction mixture was then filtered to obtain the isoquinolinium salt **9** in quantitative yield. M.p.: 101–103°C. IR(cm^{-1}): 3049, 2921, 2600, 1695, 1599, 1517. ^{1}H NMR(d_6-DMSO): δ (ppm): 3.16 (3H, s, $—SO_2CH_3$), 3.72 (2H, d, $J = 2.5$ Hz, $—CH_2—$), 3.85 (9H, s, $—OCH_3$), 4.18 (1H, s, —CHCO—), 6.78–7.03 (4H, m, Ar), 7.23 (1H, s, Ar); ^{13}C NMR(d_6-DMSO): δ (ppm): 38.56 ($—OSO_2CH_3$), 43.85 ($—CH_2—$), 55.59 ($—OCH_3$), 56.11 ($—OCH_3$), 56.19 ($—OCH_3$), 63.87 (—CH), 107.64 (CH Ar), 109.19 (CH Ar), 114.89 (CH Ar), 122,27 (C Ar), 122.61 (CH Ar), 123.61 (CH Ar), 132.46 (C Ar), 133.48 (C Ar), 137.93 (C Ar), 148.63 (C Ar), 151.18 (C Ar), 154.34 (C Ar), 188.33 (CO).

Ethyl 3-(4-mesyloxy-3-methoxyphenyl)-4-hydroxy-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-4-acetate (**10**)

A mixture of zinc (7.68 g, 0.117 mol), dimethoxymethane (24 mL) and trimethylsilyl chloride (1.5 mL, 1.18×10^{-2} mol) was stirred under an anhydrous atmosphere for 30 min. The reaction mixture was then refluxed and a solution of ethyl bromoacetate (12 mL, 0.108 mol) in dimethoxymethane (24 mL) was added dropwise cautiously to maintain reflux. The reaction mixture was stirred for 4 h. A solution of the dried hydrochloride **9** (7.70 g, 1.28×10^{-2} mol) in dry dioxan (20 mL) was added slowly to the organozinc solution (43 mL, 76.8×10^{-2} mol) into a Schlenk tube under anhydrous atmosphere. The reaction mixture was stirred overnight at room temperature. An aqueous saturated solution of ammonium chloride was then added until the precipitate disappeared. The organic layer was separated and the aqueous layer was extracted with dichloromethane (3×10 mL). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The ethyl ester **10** (5.21 g, 82%) was precipitated by addition of petroleum ether. M.p.: 186°C. IR(cm^{-1}): 3220, 1730. ^{1}H NMR($CDCl_3$): δ (ppm): 1.15 (3H, t, $J = 7$ Hz, $—CH_3$), 2.69 (1H, d, $J = 14$ Hz, AB system $—CH_2—$), 3.10 (1H, $J = 14$ Hz, AB system $—CH_2—$), 3.18 (3H, s, $—OSO_2CH_3$), 3.85 (3H, s, $—OCH_3$), 3.87 (3H, s, $—OCH_3$), 3.92 (3H, s, $—OCH_3$), 4.00 (2H, q, $J = 7$ Hz, $—CH_2—CH_3$), 4.03 (1H, d, $J = 14$ Hz, AB system $—CH_2—$), 4.19 (1H, d, $J = 14$ Hz, AB system $—CH_2—$), 4.49 (1H, s, —CH), 6.53 (1H, s, Ar), 7.02 (1H, s, Ar), 7.05 (1H, dd, $J = 3$ and 7.5Hz, Ar), 7.21 (1H, d, $J = 3$ Hz, Ar), 7.27 (1H, d, $J = 7.5$ Hz, Ar); ^{13}C NMR($CDCl_3$): δ (ppm): 14.05 ($—CH_2CH_3$), 38.30 ($—OCH_3$), 41.23 ($—CH_2—$), 48.81 ($—CH_2—$), 52.01 (C), 55.88 ($—OCH_3$), 55.97 ($—OCH_3$), 60.56 ($—CH_2$), 64.94 ($—SO_2CH_3$), 65.95 (—CH), 108.60 (CH Ar), 108.94 (CH Ar), 113.79 (CH Ar), 121.93 (CH Ar), 123.93 (CH Ar), 127.39 (C Ar), 130.57 (C Ar), 137.75 (C Ar), 139.89 (C Ar), 147.79 (C Ar), 148.60 (C Ar), 151.03 (C Ar), 170.83 (—CO). HRMS (FAB+): $[M + H]^+$ = 354.1705 (theoret. 354.1703).

12-Hydroxy-2-mesyloxy-3,8,9-trimethoxybenzo[*c*]-phenanthridinium Hydrogensulfate (**11**)

The ester **10** (4.17 g, 2.83×10^{-3} mol) was dissolved in concentrated sulfuric acid (20 mL) in a round bottom flask fitted with a stopper using

magnetic stirring to give a deep red solution. When the red colour disappeared (about 4 h), the reaction mixture was poured into ice. The yellow precipitate was filtered under vacuum and washed with cold water (3 × 20 mL) to obtain the hydrogensulfate **11** (2.02 g, 46%). M.p.: 243°C. IR(cm^{-1}): 1605, 1514, 1365, 1183. ^{1}H NMR(d_6-DMSO): δ (ppm): 3.91 (3H, s, —OSO_2CH_3), 4.42 (3H, s, —OCH_3), 4.54 (6H, s, —OCH_3), 8.22 (2H, s, Ar), 8.29 (1H, s, Ar), 8.57 (1H, s, Ar), 8.97 (1H, s, Ar), 9.74 (1H, s, Ar), 11.72 (1H, s, —NH^+). HRMS (FAB+): $M^+ = 430.0945$ (theoret. 430.0960).

12-Ethoxy-2-mesyloxy-3,8,9-trimethoxybenzo[*c*]-phenantridine (**12**)

A mixture of the hydrogensulfate **11** (638 mg, 1.21×10^{-3} mol), potassium carbonate (668 mg, 4.84×10^{-3} mol) and diethyl sulfate (0.23 mL, 1.81×10^{-3} mol) in dry acetone (15 mL) was refluxed overnight. Water was added (30 mL) and acetone removed under vacuum. The aqueous layer was extracted by dichloromethane (3 × 10 mL). The combined organic layers were dried over sodium sulfate and evaporated. The yellow solid was washed with diethyl ether and filtered to give the ester **12** (371 mg, 78%). M.p.: 258°C. IR (cm^{-1}): 1354, 1148. ^{1}H NMR($CDCl_3$): δ (ppm)?: 1.65 (3H, t, $J = 5.5$ Hz,-CH_2CH_3), 3.31 (3H, s, —OSO_2CH_3), 4.11 (3H, s, —OCH_3), 4.17 (6H, s, —OCH_3), 4.36 (2H, q, $J = 5.5$Hz, —CH_2CH_3), 7.36 (2H, s, Ar), 7.68 (1H, s, Ar), 8.29 (1H, s, Ar), 8.79 (1H, s, Ar), 9.10 (1H, s, Ar); ^{13}C NMR($CDCl_3$): δ; (ppm): 14.82 (—CH_2CH_3), 38.53 (—OSO_2CH_3), 56.09 (—OCH_3), 56.15 (—OCH_3), 56.22 (—OCH_3), 63.97 (—OCH_2—), 95.34 (CH Ar), 101.52 (CH Ar), 105.98 (CH Ar), 107.05 (CH Ar), 117.66 (CH Ar), 120.88 (C Ar), 122.35 (C Ar), 122.82 (C Ar), 127.85 (C Ar), 133.04 (C Ar), 135.26 (C Ar), 138.62 (C Ar), 120.88 (C Ar), 147.07 (CH Ar), 149.96 (C Ar), 150.98 (C Ar), 152.48 (C Ar), 153.07 (C Ar). HRMS (FAB+): $[M + H]^+ = 458.1272$ (theoret. 458.1273).

12-Ethoxy-2-mesyloxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridinium Mesylate (**13**)

Methyl methanesulfonate (402 mg, 7.09×10^{-4} mol), diisopropylethylamine (0.42 mL, 2.41×10^{-3} mol) and **12** (1.5 mL, 17.7×10^{-3} mol) were heated to 170°C during 20 min. The reaction mixture was cooled to RT and a yellow precipitate appeared. The precipitate was filtered off and washed with dry acetone to give mesylate **13** (294 mg, 73%). M.p.: 193°C. IR (cm^{-1}): 1370, 1163. ^{1}H NMR(d_6-DMSO): δ; (ppm): 1.70 (3H, t, $J = 7$ Hz, —CH_2CH_3), 2.99 (3H, s, $SO_3CH_3^-$), 3.38 (3H, s, —OSO_2CH_3), 4.13 (3H, s, —OCH_3), 4.14 (3H, s, —OCH_3), 4.28 (3H, s, —OCH_3), 4.49 (2H, q, $J = 7$ Hz, —CH_2CH_3), 5.00 (3H, s, —NCH_3), 7.49 (1H, s, Ar), 7.74 (1H, s, Ar), 7.78 (1H, s, Ar), 8.06 (1H, s, Ar), 8.49 (1H, s, Ar), 9.49 (1H, s, Ar); ^{13}C NMR(d_6-DMSO): δ; (ppm): 14.40 (—CH_2CH_3), 51.34 (—CH_2CH_3), 56.26 (—OCH_3), 56.74 (—OCH_3), 57.38 (—OCH_3), 65.29 (—N^+CH_3), 97.04 (CH Ar), 103.43 (CH Ar), 108.83 (CH Ar), 110.11 (CH Ar), 117.06 (C Ar), 119.00 (C Ar), 121.83 (C Ar), 124.49 (C Ar), 126.70 (C Ar), 127.30 (C Ar), 130.49 (C Ar), 138.83 (C Ar), 149.04 (C Ar), 150.66 (C Ar), 151.9 (C Ar), 154.33 (C Ar), 157.69 (C Ar). HRMS (FAB+): $M^+ = 472.1461$ (theoret. 472.1430).

12-Ethoxy-2-mesyloxy-3,8,9-trimethoxy-5-methyl-5,6-dihydrobenzo[*c*]phenantridine (**14**)

Compound **13** (4.0 g, 7.05 mmol) was dissolved in methanol (100 mL). Sodium borohydride (293 mg, 7.75 mmol) was added cautiously with magnetic stirring. The methanol was evaporated, water was then added (20 mL) and the ester **14** was extracted with dichloromethane (3 × 10 mL). The combined organic layers were dried over sodium sulfate and the solvent evaporated under vacuum to give the ester **14** (2.8 g, 84%). M.p.: 204°C. IR (cm^{-1}): 1375, 1182. ^{1}H NMR ($CDCl_3$): δ; (ppm): 1.57 (3H, t, $J = 7$ Hz, —CH_2CH_3), 2.54 (3H, s, —NCH_3), 3.27 (3H, s, —OSO_2CH_3), 3.96 (3H, s, —OCH_3), 4.02 (3H, s, —OCH_3), 4.06 (3H, s, —OCH_3), 4.12 (2H, s, —CH_2-), 4.27 (2H, q, $J = 7$ Hz, —CH_2CH_3), 6,82 (1H, s, Ar), 7.02 (1H, s, Ar), 7.25 (1H, s, Ar), 7.73 (1H, s, Ar), 8.17 (1H, s, Ar); ^{13}C NMR($CDCl_3$): δ; (ppm): 14.77 (—CH_3), 38.27 (—OSO_2CH_3), 40.55 (—OCH_3), 54.84 (—CH_2—), 55.86 (—OCH_3), 56.21 (—OCH_3), 63.91 (—OCH_2—), 99.12 (CH Ar), 104.15 (CH Ar), 106.55 (CH Ar), 110.00 (CH Ar), 117.72 (CH Ar), 120.22 (C Ar), 124.50 (C Ar), 124.94 (C Ar), 126.38 (C Ar), 129.88 (C Ar), 134.77 (C Ar), 137.58 (C Ar), 148.24 (C Ar), 149.02 (C Ar), 150.63 (C Ar), 151.80 (C Ar). HRMS (FAB+): $[M + H]^+ = 474.1557$ (theoret. 474.1586).

12-Ethoxy-2-hydroxy-3,8,9-trimethoxy-5-methyl-5,6-dihydro-benzo[*c*]phenanthridine (**15**)

Compound **14** (2.8 g, 7.1 mmol) and potassium hydroxide (5.2 g, 92 mmol) were refluxed in a mixture of ethanol (3.6 mL) and water (1.6 mL). After 6 h, the reaction mixture was neutralized with aqueous HCl (10%) and extracted with dichloromethane (3 × 10 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated under vacuum to yield the phenol **15** (2.4 g, 85%). Mp = 190–193°C. IR(cm^{-1}): 3450, 1610. ^{1}H NMR ($CDCl_3$): δ; (ppm): 1.56 (3H, t, $J = 7$ Hz, —CH_2CH_3), 2.55 (3H, s, —NCH_3), 3.95 (3H, s, —OCH_3), 4.04 (3H, s, —OCH_3), 4.07 (3H, s, —OCH_3), 4.12 (2H, s, —CH_2—), 4.26 (2H, q, $J = 7$ Hz, —CH_2CH_3), 6.81 (1H, s, Ar), 7.00 (1H, s, Ar), 7.24 (1H, s, Ar), 7.61 (1H, s, Ar), 7.71 (1H, s, Ar); ^{13}C NMR($CDCl_3$): δ; (ppm): 14.96 (—CH_2CH_3),40.75 (—CH_2CH_3), 55.12 (—OCH_3), 55.91 (—NCH_3), 55.99 (—OCH_3), 56.30 (—OCH_3), 64.10 (—CH_2—), 99.32 (CH Ar), 102.18 (CH Ar), 105.21 (CH Ar), 106.46 (CH Ar), 110.22 (CH Ar), 121.93 (C Ar), 123.36 (C Ar), 124.52 (C Ar), 125.32 (C Ar), 125.59 (C Ar), 135.61 (C Ar), 145.30 (C Ar),

148.11 (C Ar), 148.42 (C Ar), 148.63 (C Ar), 151.43 (C Ar). HRMS (FAB+): $M^+ = 394.1669$ (theoret. 394.1654).

12-Ethoxy-2-hydroxy-3,8,9-trimethoxy-5-methyl-benzo[c]phenanthridinium Iodide (**16**)

A mixture of **15** (2.4 g, 6.0 mmol), absolute ethanol (10 mL) and iodine (15 g, 60 mmol) was refluxed for 2.5 h. The addition of a saturated aqueous sodium thiosulfate solution gave first a pale yellow solution and then a precipitate. The solid was filtered off to yield the iodide **16** (1.7 g, 55%). M.p.: 264-280°C. IR (cm^{-1}): 3420, 1625. ^{1}H NMR(d_6-DMSO): δ; (ppm): 1.58 (3H, t, $J = 7$ Hz, $-CH_2CH_3$), 4.01 (3H, s, $-OCH_3$), 4.06 (3H, s, $-OCH_3$), 4.22 (3H, s, $-OCH_3$), 4.52 (2H, q, $J = 7$ Hz, $-CH_2CH_3$), 4.89 (1H, s, $-NCH_3$), 7.77 (1H, s, Ar), 7.80 (1H, s, Ar), 8.04 (1H, s, Ar), 9.64 (1H, s, Ar), 10.51 (1H, s, Ar); ^{13}C NMR(d_6-DMSO): δ; (ppm): 14.52 ($-CH_2CH_3$), 51.34 ($-CH_2CH_3$), 55.94 ($-OCH_3$), 56.17 ($-OCH_3$), 57.19 ($-OCH_3$), 64.77 ($-NCH_3^+$), 96.07 (CH Ar), 102.85 (CH Ar), 106.07 (CH Ar), 108.40 (CH Ar), 108.47 (CH Ar), 118.63 (C Ar), 119.34 (C Ar), 123.57 (C Ar), 124.90 (C Ar), 127.65 (C Ar), 130.90 (C Ar), 147.79 (C Ar), 148.81 (C Ar), 148.86 (C Ar), 151.24 (C Ar), 154.07 (C Ar), 157.46 (C Ar). HRMS (FAB+): $M^+ = 394.1644$ (theoret. 394.1654).

Biology

Cells and Virus

Eagle's Minimal Essential Medium (MEM) supplemented with 10% newborn calf serum was used for growth and maintenance of Vero cell cultures (African green monkey kidney). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. *Herpes simplex* Virus type 1 (HSV-1) strain KOS was grown in Vero cells with MEM supplemented with 10% newborn calf serum and then titrated by the Reed and Muench method.[11] The titre expressed in 50% tissue culture infectious dose ($TCID_{50}$) was $2.10^{6.5}$ $TCID_{50}$/mL.

Test Compounds

Stock solutions (10 mM) of alkaloids **2**, **3**, **4**, **5**, **16** and fagaronine were prepared in 50% aqueous DMSO and distributed in 0.5 mL fractions which were stored at + 4°C. For the experiments, aliquots were diluted with MEM to obtain the indicated concentrations (analogous dilutions of DMSO did not interfere with the assays -data not shown).

Cytotoxicity of Compounds

To assess the effect of alkaloids on uninfected Vero cells, increasing concentrations of compounds were added to confluent 1-day-old monolayers of Vero cells grown in microtitre tissue culture plates (96 wells). After incubation for 72 h at 37°C, cytotoxicity was determined by microscopic examination of cell morphology and the viability of cells was determined by the MTT colorimetric method in treated and untreated cultures.[12] The concentration of compound at which the cell viability was reduced to 50%, as compared with the control, was the 50% cytotoxic concentration (CC_{50}).

Effect of Compounds on the Viral Cytopathic Effect (CPE)

A range of 6 non-cytotoxic concentrations of alkaloids from 0.03 to 1 mM was prepared in MEM and added to confluent 1-day-old monolayers of Vero cells grown in microtitre tissue culture plates (96 wells) just before inoculation with HSV-1 at two multiplicity of infection (MOI): 0.3, 0.03, $TCID_{50}$ per cell. Toxicity, cell and viral controls were run simultaneously. The assay of each drug was carried out in triplicate. Cells were incubated at 37°C during 72 h corresponding to four cycles of replication. Then, the monolayers of cells were observed under a phase-contrast microscope. The level of cytopathic inhibition was evaluated as follows:++++ = 100% viral inhibition;+++ = 75%;++ = 50%; + = 25%; - = 0%. The viability of Vero cells was measured by the MTT colorimetric method. The inhibition of the CPE was calculated as follows: % inhibition = [Optical Density (OD) assay-OD viral control]/(OD toxicity control-OD viral control) × 100. The 50% efficacy dose (ED_{50}) was calculated as the concentration of compound reducing to 50% the CPE.

Effect of Compounds on Virus Yield

Monolayers of Vero cells were treated with the alkaloids, and inoculated by virus as described above. After examination of cells under a phase-contrast microscope, the plates were frozen and thawed three times. The contents of the wells were harvested, clarified by low-speed centrifugation, and virus titrations were performed on the supernatant fluids by the Reed and Muench method. The antiviral activity of the compound was determined as the reduction factor (log_{10}) of the viral titre by comparison with untreated-infected controls.

RESULTS AND DISCUSSION

Chemistry

Fagaronine **2**, 12-hydroxy, 12-methoxy and 12-ethoxy-2,3,8,9-tetramethoxybenzo[*c*]phenanthridinium salts **3**, **4**, **5** (Figure 1) were synthesized according to previously described procedures.[9,10]

The 12-Ethoxy-2-hydroxy-3,8,9-benzo[*c*]phenanthridine (12-ethoxyfagaronine) was a novel structure in this series. This molecule took into account

the substitution of the OH group of fagaronine and the 12-ethoxy group of the 12-ethoxy-2,3,8,9-tetramethoxybenzo[*c*]phenanthridine which both already have shown good biological activities.[1,3]

Harsh acidic conditions were required for the formation of the B and C rings during the synthesis of 12-alkoxyBZPs.[9] In a previous study, **2** was prepared through de-ethylation of a phenolic ether protecting group using concentrated sulfuric acid.[9] The major drawbacks in this type of deprotection are controlling the reaction time and the need for laborious purification steps to separate related polar by-products. To avoid this, we chose the methanesulfonate phenolic protection of vanillin in the early steps of our synthesis, which was easily carried out using methanesulfonyl chloride and triethylamine in dichloromethane to give **6** (Scheme 1). The amino nitrile **8** was obtained under Strecker reaction conditions in 86% yield. A nearly quantitative hydrogen fluoride cyclisation of **8** at room temperature, led to the ketone **9**. The Reformatskii reaction, which introduced the two-carbon synthon required for the ensuing cyclisation, yielded the amine **10**. Sulfuric acidic cyclisation led to the BZP **11,** which was thereafter *O*-ethylated in the 12-position using diethylsulfate and sodium carbonate in dried acetone giving **12**, and then N-methylated in the presence of methyl methanesulfonate at 180°C to yield the 12-ethoxy-2-mesyloxy-3,8,9-trimethoxybenzo[*c*] phenanthridinium salt **13**. The target compound, 12-ethoxyfagaronine **16**, was obtained following the reduction of the iminium bond in **13** with sodium borohydride in methanol to give the tertiary amine **14**, sulfonyl ester hydrolysis of **14** using 5 M potassium hydroxide to yield the 2-hydroxyBZP **15** and oxidation of **15** with iodine in ethanol to **16**. The overall yield of this multistep synthesis was 20% (Scheme 1).

TABLE I Cytotoxicity (Vero cells) and anti-HSV-1 (MOI 0.03) activity of fagaronine **2** and the alkaloids **3**, **4**, **5** and **16**

	Cytotoxicity (CC_{50}; mM)	Antiviral activity (ED_{50}; mM)	Selectivity index (CC_{50}/ED_{50})
2	>1	inactive	–
3	0.03	0.03	1
4	>1	0.18	>5.5
5	>1	0.28	>3.5
16	0.33	0.22	1.5

Biological Results

Antiviral Assays

Because of the poor solubility of these benzophenanthridine alkaloids in diluted DMSO, a 1 mM concentration was the maximal concentration usable for assays. Cytotoxicity results (CC_{50}) on Vero cells and anti-HSV-1 activity (ED_{50}) at a 0.03 multiplicity of infection (MOI) are shown in Table I. The cytotoxicity of compounds **4**, **5**, **16** and fagaronine **2** on Vero cells was weak ($CC_{50} > 0.3$ mM), but 12-hydroxylation resulted in enhanced cytotoxicity (CC_{50} of **3** = 0.03 mM). Fagaronine, described as an inhibitor of AMV and HIV reverse transcriptases,[2] was found inactive on the DNA-virus HSV-1. Analogs **3** and **16** exhibited anti-HSV-1 activities at toxic doses while **4** and **5**, which do not bear any hydroxyl group, appeared to be active with selectivity indexes >3.5. Although in the 10^{-4} M range, their anti-HSV-1 activity was quantified for 0.3 and 0.03 MOI using the MTT assay and measurement of the viral yield reduction (Table II).

TABLE II Vero cells viability and protection from HSV-1 infection with compounds **4** and **5**

			Antiviral effect					
			MOI[c] 0.3			MOI 0.03		
			CPE Inhibition			CPE Inhibition		
	CT[a] (mM)	Cytotoxicity[b] (%)	microscopy[d]	MTT[e] (%)	YR[f] (log_{10})	microscopy	MTT (%)	YR (log_{10})
4	1	40	++++	98	4	++++	97	4
	0.5	40	+++	79	4	++++	90	4
	0.25	33	++	51	2	+++	70	3
	0.12	28	+	31	1.5	+	31	1.5
	0.06	0	−	17	1.5	−	30	1
5	1	48	++++	93	4	++++	92	4
	0.5	44	++++	92	2.5	++++	97	3.5
	0.25	31	+	20	0.5	++	44	2
	0.12	0	+	10	0.5	++	34	2
	0.06	0	−	6	0.5	+	24	1

[a] CT: Concentrations tested (mM). [b] Cytotoxicity: Percent of cell controls. [c] MOI: Multiplicity of Infection. [d] Microscopy: CPE inhibition evaluated by microscopy. [e] MTT: Percentage of inhibition was calculated as follows:

$$\frac{(DO_{sample} - DO_{virus\ control})}{(DO_{drug\ control} - DO_{virus\ control})} \times 100$$

[a] YR: Yield reduction. Reduction in titre (log_{10} $TCID_{50}$/mL) compared with that of virus control.

Complete viral inhibition was obtained for **3** and **4** with both MOI but corresponding concentrations were also toxic for almost 50% of Vero cells. However, a 30% CPE inhibition with a 2log and 1log viral titre reduction was obtained with non toxic concentrations on Vero cells for compound **5** (0.12 mM) and compound **4** (0.06 mM) respectively.

In conclusion, BZPs which have a 12-alkoxy-substitution have demonstrated good anti-HSV-1 activity in the usual tests. It is noticeable that the less toxic and the most active compounds do not bear any hydroxyl groups. The charged BZP compounds are probably subject to several dynamics within the mammalian systems that limit their absorption and bioavailability. In order to improve cell penetration by the drugs, further work toward the search for an increase in lipophilicity will be carried out in that series. Moreover, other studies are in progress to ascertain the role of the topoisomerases in the antiviral activity of our products.

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