

DRUG SYNTHESIS

DIFFERENTIAL INHIBITORY EFFECT OF NEWLY SYNTHESIZED
PYRIDINE-2-ONE DERIVATIVES ON THE CERCARIAL SERINE PROTEASE
ACTIVITY OF THE PARASITE SCHISTOSOMA MANSONIMAHMOUD BAHGAT^{1,2} AND NAGY KHALIFA¹¹Therapeutic Chemistry Department, National Research Center,
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Abstract: Herein, we report on the synthesis of 4 novel pyridine derivatives (P1, 2, 3 and 4) with differential serine protease inhibitory effects utilizing pyridine-2 (1H)-ones as starting materials. When these 4 compounds were tried to inhibit cercarial serine protease, the major penetration enzyme of the parasite *Schistosoma mansoni*, P1 and P3 exerted the most potent inhibitory effects as demonstrated by abolishing 90% and 97%, respectively, of the enzyme activity, whereas, P4 showed moderate inhibitory effect (abolished 60% of the enzyme activity) and P2 exerted the lowest inhibitory effect (only 10%). The biochemical characteristics of this parasite enzyme were previously reported by using specific substrate and inhibitors for such class of enzymes (13). Kinetics of the cercarial serine protease inhibition by the pyridine derivatives that exerted the most potent inhibition, P1 and P3, was further studied with respect to Michaelis-Menten constants (K_m), maximum velocity (V_{max}) and inhibitory coefficients (K_i). The results showed that serial dilutions of both P1 and P3 abolished the enzyme activity at different concentrations of the substrate Boc-Val-Leu-Gly-Arg-PNA as demonstrated by drop in V_{max} . The K_i values for P1 and P3 were calculated 0.17 and 0.12 mM, respectively.

Keywords: Pyridin-2-ones, *Schistosoma mansoni*, cercarial serine protease

Several pyrimidine derivatives have been reported to have serine protease inhibitory effect. Alkyl [sulfonyl(oxy)] uracils 1-2, dihydrouracil derivatives were found to be efficient, time-dependent inhibitors of leukocyte elastase and found to form acyl-enzymes that exhibited variable hydrolytic stability which appeared to be dependent on the nature of the R1 group (believed to be accommodated at the primary specificity site, S1 (1)). When some hexahydroimidazo[1,2-c]pyrimidine derivatives were tested for their anti-inflammatory effects on mouse-paw edema, they exerted a dose-dependent inhibition of paw swelling with significant reduction of leukocyte elastase activity (2). Several pyrimidine derivatives were recently reported for their selective inhibitory effect on factor Xa, a known serine protease (3). Continuous oral and intravenous infusion of nifedipine, a calcium channel blocker pyrimidine derivative, in patients undergoing cardiopulmonary bypass significantly lowered levels of granulocyte elastase by forming alpha-proteinase

inhibitor complex (4). Replacement of the Val-Pro unit of the peptidic chymase, chymotrypsin-like serine protease, inhibitor Val-Pro-Phe-CF₃ with a (5-amino-6-oxo-2-phenyl-1,6-dihydro-1-pyrimidinyl)acetyl moiety and studying structure-activity relationship revealed that phenyl substitution at the 2-position of the pyrimidinone ring results in the formation of a superior protease inhibitor ($K_i = 0.0506$ mmol) than the parent peptidic inhibitor (5, 6).

Only 2 reports were found to be concerned with the peptidase inhibitory effects of tetralin derivatives. The first demonstrated that among several derivatives tested for their inhibitory effect on the clotting activity of thrombin, a known serine protease, the most potent inhibitor was 1-[N_α-(4,6-dimethoxynaphthalene-2-sulfonyl)-arginyl]-4-methylpiperidine, with an IC₅₀ of 7.5×10^{-8} M, also, arginine amide derivatives of 4-methyl- or 4-ethylpiperidine with tetralin or an oxygen-containing heterocyclic compound as an N_α-substituent showed inhibition with IC₅₀ value lower than 10^{-5} M (7).

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The second report demonstrated that 3-{3-[(S)-2-(1,2,3,4-tetrahydronaphthyl)acetyl]-L-thiopropyl}-thiazolidine exhibited approximately 20-fold (IC_{50} = 2.3 nM) increase in potency to abolish prolyl endopeptidase activity compared with 1-[1-(4-phenylbutanoyl)-L-prolyl]-pyrrolidine or any of its formyl or cyano derivatives, while, 1-{1-(2-indanylacetyl)-L-prolyl}prolinal, 1-{1-[(S)-2-(1,2,3,4-tetrahydronaphthyl)acetyl]-L-prolyl}prolinal, 1-{3-[(S)-2-(1,2,3,4-tetrahydronaphthyl)acetyl]-L-thiopropyl}prolinal, (S)-2-cyano-1-{2-[(S)-2-(1,2,3,4-tetrahydronaphthyl)acetyl]-L-prolyl}pyrrolidine, and (S)-2-cyano-1-{3-[(S)-2-(1,2,3,4-tetrahydronaphthyl)acetyl]-L-thiopropyl}pyrrolidine showed approximately 2-fold (IC_{50} congruent to 0.5 nM) increase in potency compared with 1-[1-(benzyloxy-carbonyl)-L-prolyl]prolinal (Z-Pro-prolinal) (8).

The initial step in human infection by schistosome parasites is penetration of host skin by a potent serine protease, cercarial elastase (CE), secreted from the preacetabular glands of the invading larvae, cercariae (9-12). In previous work (13), we characterized the kinetics and substrate specificity of this enzyme in terms of Michaelis-Menten constant (K_m), maximum reaction velocity (V_{max}), inhibitory coefficients (K_i) of several standard serine protease inhibitors and potencies of these inhibitors were confirmed by direct biochemical assays. Moreover, in a very recent study carried out by our group we described a potent inhibitory effect of N'-(4-methylbenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonohydrazide on the cercarial serine protease and when this compound was formulated in jojoba oil and used to paint mice tails before infection with *S. mansoni* cercariae, it caused a significant reduction (93%; $p < 0.05$) in the worm burden when compared to control mice (14).

Based on the above mentioned evidences that several pyrimidine derivatives and tetralins have serine protease inhibitory effects and also based on our own previous work where CE was characterized as a serine protease, in the present report we describe the differential inhibitory effects of several newly synthesized pyridine derivatives on CE activity hoping to discover new serine protease inhibitors.

EXPERIMENTAL

The melting points were recorded in open glass capillaries. Elemental analysis was carried out at the Microanalytical Unit, Cairo University, Giza, Egypt and were within $\pm 0.4\%$ of the theoretical values. The IR spectra (KBr discs) were recorded on a Perkin Elmer 1430 spectrophotometer. The 1H NMR and ^{13}C NMR

spectra were measured in DMSO- d_6 using a Japanese Jeol-EX apparatus including TMS as internal standard and all the chemical shifts were recorded in ppm relative to TMS. The value of the applied frequencies for running 1H and ^{13}C NMR spectra was 270 MHz. The mass spectra were run with a Finnegan SSQ GC/MS spectrometer using EI (70 eV) and Fast Atom Bombardment (FAB) mass spectra on a Kratos MS 50 Rf. The reactions were followed by TLC (silica gel, aluminium sheets 60 F254, Merck). Merck silica gel (0.040-0.063 mm) was used for column chromatography.

4-(Aryl)-1,2-dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridine-3-carbonitrile (1a,b)

General Procedure

A mixture of 6-acetyltetralin (0.01 mole), aryl aldehyde namely, 2,4-dichlorobenzaldehyde or 2-chloro-6-fluorobenzaldehyde (0.01 mole), ethyl cyanoacetate (0.01 mole) and ammonium acetate (0.06 mole) in ethanol (30 mL) was heated under reflux for 3 h. The obtained precipitate was filtered off, washed with water and crystallized to give (1a,b).

4-(2,4-Dichlorophenyl)-1,2-dihydro-6-(1',2',3',4'-tetrahydronaphthalen-6-yl)-2-oxopyridine-3-carbonitrile (1a; P1)

Yield 83%; m. p. 291-293°C (AcOH); IR (cm^{-1}): 3276 (NH), 2219 (CN), 1671 (CO); 1H NMR (DMSO- d_6 , ppm): 1.7, 2.73 (m, m, 8H, 4CH₂ of tetralin), 6.71 (s, 1H, CH of pyridine ring), 7.18-7.60 (m, 6H, Ar-H), 12.31 (s, 1H, NH exchangeable); MS, m/z 395 (M⁺). Anal. calcd. for C₂₂H₁₆Cl₂N₂O (395.28): C, 66.85; H, 4.08; N, 7.09. Found: C, 66.73; H, 3.97; N, 6.90.

4-(2-Chloro-6-fluorophenyl)-1,2-dihydro-6-(1',2',3',4'-tetrahydronaphthalen-6-yl)-2-oxopyridine-3-carbonitrile (1b)

Yield 71%; m. p. > 300°C (AcOH); IR (cm^{-1}): 3269 (NH), 2221 (CN), 1677 (CO); 1H NMR (DMSO- d_6 , ppm) 1.71, 2.73 (s, s, 8H, 4CH₂ of tetralin), 6.73 (s, 1H, CH), 7.10-7.59 (m, 6H, Ar-H), 12.01 (s, 1H, NH exchangeable); MS, m/z 378 (M⁺). Anal. calcd. for C₂₂H₁₆ClFNO (378.83): C, 69.75; H, 4.26; N, 7.39. Found: C, 69.71; H, 4.15; N, 7.14.

1-(2,3,4,6-Tetra-O-acetyl-β-D-gluco and D-galactopyranosyl)-3-cyano-pyridine-2-ones (3a-d)

General Procedure

To a solution of 2 (1H)-pyridinones 1a,b (0.01 mol) in aqueous potassium hydroxide (0.01 mol) a solution of 2,3,4,6-tetra-O-acetyl-α-D-gluco or galactopyranosyl bromide 2 (0.011 mol) in acetone (30 mL) was added. The reaction mixture was stirred at

room temperature until judged complete by TLC (8-12 h), then was evaporated under reduced pressure at 40°C and the residue was washed with distilled water to remove the formed potassium bromide. The product was dried and purified by column chromatography on silica gel (eluent: ethyl acetate/ petroleum ether 30%).

4-(2,4-Dichlorophenyl)-1,2-dihydro-1-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridine-3-carbonitrile (3a; P2)

Yield 63%; 183-185°C; IR (cm⁻¹): 2220 (CN), 1753 (CO ester), 1670 (CO pyridone); ¹H NMR (DMSO-d₆, ppm): 1.70, 2.73 (s, s, 8H, tetralin), 1.89-2.10 (4s, 12H, 4CH₃CO), 4.17 (m, 2H, H-6', 6''), 4.76 (m, 1H, H-5), 5.24 (m, 2H, H-4' and H-3'), 5.63 (m, 1H, H-2), 6.70 (d, *J* = 8.35 Hz, 1H, H-1'), 6.79 (s, 1H, pyridine H-5), 7.12-7.63 (m, 6H, Ar-H); MS: *m/z* 725 (M⁺). Anal. calcd. for C₃₆H₃₄Cl₂N₂O₁₀ (725.57): C, 59.59; H, 4.72; N, 3.86. Found: C, 59.47; H, 4.59; N, 3.71.

4-(2-Chloro-6-fluorophenyl)-1,2-dihydro-1-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyrimidin-3-carbonitrile (3b)

Yield 61%; 197-199°C; IR (cm⁻¹): 2221 (CN), 1750 (CO ester), 1676 (CO pyridone); ¹H NMR (DMSO-d₆, ppm): 1.71, 2.75 (s, s, 8H, 4CH₂ of tetralin), 1.79-2.10 (4s, 12H, CH₃CO), 4.20 (m, 2H, H-6', 6''), 4.67 (m, 1H, H-5'), 5.21 (m, 2H, H-4' and H-3'), 5.69 (m, 1H, H-2'), 6.73 (d, *J* = 8.38 Hz, 1H, H-1'), 6.85 (s, 1H, pyridine H-5), 7.10-7.59 (m, 6H, Ar-H); MS, *m/z* 709 (M⁺). Anal. calcd. For C₃₆H₃₄ClFN₂O₁₀ (709.11): C, 60.98; H, 4.83; N, 3.95. Found: C, 60.83; H, 4.79; N, 3.87.

4-(2,4-Dichlorophenyl)-1,2-dihydro-1-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxo-pyrimidin-3-carbonitrile (3c)

Yield 72%; 164-166°C; IR (cm⁻¹): 2220 (CN), 1760 (CO ester), 1670 (CO pyridone); ¹H NMR (DMSO-d₆, ppm): 1.70, 2.73 (s, s, 8H, tetralin), 1.86-2.12 (4s, 12H, 4 x CH₃CO), 4.23 (m, 2H, H-6', 6''), 4.63 (m, 1H, H-5'), 5.44 (m, 2H, H-4' and H-3'), 5.60 (m, 1H, H-2'), 6.61 (d, *J* = 8.68 Hz, 1H, H-1'), 6.81 (s, 1H, pyridine H-5), 7.10-7.61 (m, 6H, Ar-H); MS: *m/z* 725 (M⁺). Anal. calcd. for C₃₆H₃₄Cl₂N₂O₁₀ (725.57): C, 59.59; H, 4.72; N, 3.86. Found: C, 59.40; H, 4.61; N, 3.73.

4-(2-Chloro-6-fluorophenyl)-1,2-dihydro-1-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl)-

6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyrimidin-3-carbonitrile (3d)

Yield 69%; 206-208°C; IR (cm⁻¹): 2218 (CN), 1774 (CO ester), 1648 (CO pyridone); ¹H NMR (DMSO-d₆, ppm) 1.71, 2.73 (s, s, 8H, tetralin), 1.94-2.16 (4s, 12H, 4 x CH₃CO), 4.16 (m, 2H, H-6', 6''), 4.64 (m, 1H, H-5'), 5.41 (m, 2H, H-4' and H-3'), 5.60 (m, 1H, H-2'), 6.58 (d, *J* = 8.70 Hz, 1H, H-1'), 6.85 (s, 1H, pyridine H-5), 7.10-7.59 (m, 5H, Ar-H); MS: *m/z* 709 (M⁺). Anal. calcd. for C₃₆H₃₄ClFN₂O₁₀ (709.11): C, 60.98; H, 4.83; N, 3.95. Found: C, 60.79; H, 4.70; N, 3.79.

1-(β-D-glucopyranosyl and D-galactopyranosyl)-3-cyanopyridine-2-ones (4a-d)

General Procedure

A solution of an individual nucleoside 3a-d (0.01 mol) in anhydrous methanol (10 mL) was added at 0°C to a saturated solution of ammonia in anhydrous methanol (25 mL) and the mixture was stirred at 0°C until judged complete by TLC (16-20 h). The mixture was evaporated under reduced pressure at 40°C giving a solid residue which was crystallized from methanol to afford an analytically pure compounds 4a-d.

4-(2,4-Dichlorophenyl)-1,2-dihydro-1-(β-D-glucopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyrimidin-3-carbonitrile (4a)

83%; 263-265°C; IR (cm⁻¹): 3610-3200 (OH), 2221 (CN), 1635 (CO pyridone); ¹H NMR (DMSO-d₆, ppm): 1.70, 2.73 (s, s, 8H, tetralin), 3.24-3.71 (m, 6H, H-6', 6'', H-5', H-4', H-3', H-2'), 4.63 (t, 1H, 2'-OH), 5.17 (d, 1H, 3'-OH), 5.26 (s, 1H, 4'-OH), 5.53 (d, 1H, 6'-OH), 6.17 (d, *J* = 8.70 Hz, 1H, H-1'), 6.79 (s, 1H, pyridine H-5), 7.10-7.61 (m, 5H, Ar-H); MS: *m/z* 557 (M⁺). Anal. Calcd. for C₂₈H₂₆Cl₂N₂O₆ (557.42): C, 60.33; H, 4.70; N, 5.03. Found: C, 60.27; H, 4.73; N, 5.11.

4-(2-Chloro-6-fluorophenyl)-1,2-dihydro-1-(β-D-glucopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyrimidin-3-carbonitrile (4b)

84%; 296-298°C; IR (cm⁻¹): 3630-3170 (OH), 2218 (CN), 1620 (CO pyridone); ¹H NMR (DMSO-d₆, ppm) 1.70, 2.73 (s, s, 8H, tetralin), 3.21-3.77 (m, 6H, H-6', 6'', H-5', H-4', H-3', H-2'), 4.65 (t, 1H, 2'-OH), 5.16 (d, 1H, 3'-OH), 5.22 (s, 1H, 4'-OH), 5.44 (d, 1H, 6'-OH), 6.18 (d, *J* = 8.75 Hz, 1H, H-1'), 6.75 (s, 1H, pyridine H-5), 7.12-7.59 (m, 5H, Ar-H); MS: *m/z* 540 (M⁺). Anal. Calcd. for C₂₈H₂₆ClFN₂O₆ (540.97): C, 62.17; H, 4.84; N, 5.18. Found: C, 62.23; H, 4.71; N, 5.29.

4-(2,4-Dichlorophenyl)-1,2-dihydro-1-(β-D-glac-

topyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridin-3-carbonitrile (4c)

82%; 285-287°C; IR (cm⁻¹): 3660-3200 (OH), 2220 (CN), 1625 (CO pyridone); ¹H NMR (DMSO-d₆, ppm) 1.71, 2.73 (s, s, 8H, 4 CH₂, tetralin), 3.19-3.64 (m, 6H, H-6', 6'', H-5', H-4', H-3', H-2'), 4.58 (t, 1H, 2'-OH), 5.09 (d, 1H, 3'-OH), 5.19 (s, 1H, 4'-OH), 5.48 (d, 1H, 6'-OH), 6.16 (d, *J* = 8.77 Hz, 1H, H-1'), 6.80 (s, 1H, pyridine H-5), 7.10-7.59 (m, 5H, Ar-H); MS: *m/z* 557 (M⁺). Anal. Calcd. For C₂₈H₂₆Cl₂N₂O₆ (557.42): C, 60.33; H, 4.70; N, 5.03. Found: C, 60.42; H, 4.51; N, 4.97

4-(2-Chloro-6-fluorophenyl)-1,2-dihydro-1-(β-D-galactopyranosyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridin-3-carbonitrile (4d)

81%; 290-292°C; IR (cm⁻¹): 3600-3200 (OH), 2220 (CN), 1620 (CO pyridone); ¹H NMR (DMSO-d₆, ppm) 1.71, 2.75 (s, s, 8H, 4 CH₂, tetralin), 3.20-3.65 (m, 6H, H-6', 6'', H-5', H-4', H-3', H-2'), 4.62 (t, 1H, 2'-OH), 5.10 (d, 1H, 3'-OH), 5.19 (s, 1H, 4'-OH), 5.49 (d, 1H, 6'-OH), 6.16 (d, *J* = 8.77 Hz, 1H, H-1'), 6.75 (s, 1H, pyridine H-5), 7.10-7.60 (m, 5H, Ar-H); MS: *m/z* 540 (M⁺). Anal. Calcd. for C₂₈H₂₆ClFN₂O₆ (540.97): C, 62.17; H, 4.84; N, 5.18. Found: C, 62.25; H, 4.73; N, 5.03.

1-Acetyl-4-(2,4-dichlorophenyl)-1,2-dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridin-3-carbonitrile (5; P3)

(0.01 mole) of compound 1 was refluxed with 30 mL of acetic anhydride for 3 h. The cooled mixture was poured into ice-cold water and the solid separated was crystallized from acetic acid to give the N-acetyl derivative **5** in 91% yield; m.p. 160-161°C; IR (cm⁻¹): 2223 (CN), 1710 (CO acetyl), 1677 (CO pyridone); ¹H NMR (DMSO-d₆, ppm): 1.70, 2.73 (s, s, 8H, tetralin), 2.40 (s, 3H, CH₃), 6.79 (s, 1H, pyridine H-5), 7.12-7.61 (m, 5H, Ar-H); MS: *m/z* 437 (M⁺). Anal. Calcd. for C₂₄H₁₈Cl₂N₂O₂ (437.23): C, 65.91; H, 4.15; N, 6.41. Found: C, 65.70; H, 4.03; N, 6.33.

7-(2,4-Dichlorophenyl)-5-(1,2,3,4-tetrahydronaphthalen-6-yl)-3-methyl-[1,2,4]triazolo[3,4-a]pyridin-8-carbonitrile (6, P4)

Compound **5** (0.01 mole) was refluxed for 4 h with 3 mL of hydrazine hydrate and 30 mL of absolute ethyl alcohol. The product obtained after cooling was crystallized from methanol to give the triazole derivative **6** with 73% yield; m.p. 269-270°C; IR (cm⁻¹): 2221 (CN), 1653 (C=N); ¹H NMR (DMSO-d₆, ppm): 1.70, 2.73 (s, s, 8H, tetralin), 2.30 (s, 3H, CH₃), 7.10-7.62 (m, 5H, Ar-H), 7.90 (s, 1H,

pyridin H-5); MS: *m/z* 433 (M⁺). Anal. Calcd. for C₂₄H₁₈Cl₂N₄ (433.33): C, 66.52; H, 4.19; N, 12.93. Found: C, 66.39; H, 4.07; N, 12.79. Note that **1a**, **3a**, **5** and **6** were designated **P1**, **P2**, **P3** and **P4** where P states for pyridine derivative.

Preparation of *S. mansoni* cercarial secretions (13)

Infected *Biomphalaria alexandrina* snails, the intermediate host of *S. mansoni*, were placed in deionized water containing beakers. After 15 min the water containing the snails' faeces was replaced by fresh water and the snails were exposed to light for 1 h.

The cercariae-containing water (500–1,000 cercariae/mL) was slowly decanted into a new beaker. A total of 10,000 actively swimming cercariae in water were allowed to sediment on ice and the water was discarded and the cercarial pellet was recovered. The cercarial pellet was washed 3 times by resuspension in water followed by sedimentation on ice to remove any remaining traces from snail materials. After the last wash, the cercariae were resuspended in 10 mL of water and checked for their viability then poured onto a Petri dish which was previously painted with linoleic acid (0.9 g/mL; Sigma, St. Louis, Mo., USA) and air dried. The plates were kept at 37°C for 30 min. After this incubation the water containing secretions and dead cercariae was collected in 15 mL Falcon tubes and the dead cercariae were sedimented on ice. The water containing the cercarial secretions (CSs) was collected and spun down at 3,000 g for 2 min to ensure that the secretions were completely free from cercarial debris. The protein concentration of the CSs was measured using the BCA protein determination kit (Pierce, Ill., USA).

Assay for cercarial serine protease activity (13)

Serine protease activity in *S. mansoni* CSs was assessed using the specific substrate L-1195 (Boc-Val-Leu-Gly-Arg-PNA; Bachem Biochemica, Heidelberg, Germany) (15). The enzymatic hydrolysis of the covalent bond between the arginine and the p-nitroanilide group results in release of p-nitroaniline (yellow-colored). The color intensity was recorded by measuring the absorption at 405 nm. A stock solution of the substrate (10 mg/mL) was prepared in dimethylsulfoxide (DMSO) and then diluted to the desired final concentration with the substrate buffer (30 mM TRIS-HCl, 60 mM NaCl, 0.05% NaN₃, pH 10.5). Specific protease activity is expressed in nmol of p-nitroaniline/min/μg protein. The enzymatic assay was carried out at an alkaline pH optimum as previously described for the cercarial serine protease by ourselves (13)

and all incubations were done at 37°C and optical densities were measured at 405 nm.

Inhibition of CE activity by 4 pyridine derivatives

Possible inhibitory effects of the prepared pyridine derivatives (**1a**, **3a**, **5** and **6**) on the cercarial serine protease activity were studied. Stock solution of each derivative was prepared in DMSO/ethanol (1/1). For performing inhibition assays, 50 μ L CSs were incubated for 10 min with 5 μ L of each compound appropriately diluted to give the desired final concentration. Control assays contained 50 μ L CSs, 5 μ L of DMSO/ethanol (1/1) and the substrate and were carried out as described above.

Enzyme kinetics (13)

For calculating the Michaelis-Menten constants (K_m), maximum velocity (V_{max}) and inhibitory coefficients (K_i), serial dilutions of the substrate L-1195 were prepared ranging from 1 to 0.0625 mM. In addition, serial dilutions of the pyridine derivatives **1a** (**P1**) and **5** (**P3**) were prepared ranging from 0.25 to 0.03125 mM. CSs (50 μ L; 20 mg protein/mL) were mixed with each of the pyridine derivatives (5 μ L at each of the prepared concentration), incubated for 10 min at room temperature and then mixed with the serially diluted substrate. Control assays contained DMSO/ethanol (the solvents used to dissolve the pyridine derivatives; 1/1) (5 μ L; instead of the deazapyrimidines) and were carried out as described above. All plotted results for the cercarial serine protease activity were means of duplicate measurements.

RESULTS AND DISCUSSION

Chemistry

We report here on the synthesis of novel pyridine derivatives with differential serine protease inhibitory effects utilizing pyridine-2 (1H)-ones **1** as starting materials. Compounds **1a** (**P1**), **1b** were prepared by the reaction of α,β -unsaturated nitriles with 1-acetyltetralin in boiling ethanol containing ammonium acetate. Compounds **1a,b** reacted with 2,3,4,6-tetra-O-acetyl- α -D-gluco and galactopyranosyl bromides (**2**) in the presence of aqueous potassium hydroxide to give the corresponding N-glycosides **3a** (**P2**), **b** and N-galactosides **3c,d**, respectively. The structures of the reaction products **3** (**P2**) were established and confirmed on the basis of their elemental analysis and spectral data (MS, IR, 1 H NMR, 13 C NMR).

Compound **3c** served as an example for the series. The analytical data for compound **3c** revealed

a molecular formula $C_{36}H_{34}Cl_2N_2O_{10}$ (m/z 725.57). The 1 H NMR spectrum showed the anomeric proton as a doublet at 6.51 ppm with spin-spin coupling constant equal to 9.6 Hz which corresponds to the diaxial orientation of H-1' and H-2' protons indicating the presence of only β -configuration. The other six protons of the galactopyranosyl ring resonate at δ 4.12-5.47 ppm region. The remaining four acetoxy groups appear as four singlets at δ 1.93-2.17 ppm region. The 13 C NMR spectrum was characterized by a signal at δ 93.1 ppm corresponding to C-1 atom of the β -D-galactopyranose. Four signals appeared at δ 168.9-169.7 ppm due to the four acetoxy carbonyl carbon atoms of the sugar moiety, with four additional signals at δ 20.31-25.2 ppm attributed to the acetoxy methyl carbons.

Another five signals at 61.3, 67.1, 67.9, 70.6 and 71.1 ppm were assigned to C-6', C-4', C-2', C-3' and C-5' of galactose, respectively. On the other hand, the signal of the carbonyl carbon atom of pyridone appeared at δ 161.9 ppm and the nitrile carbon atom at 116.3 ppm. The preparation of the crystalline 1-(β -D-glycopyranosyl)-3-cyanopyridin-2-one derivatives **4a-d** was achieved by removing the blocking acetyl groups on treatment with methanolic ammonia at 0°C.

The structures of compounds **4a-d** were established on the basis of elemental analysis and spectral data. Thus, the analytical data for **4b** revealed a

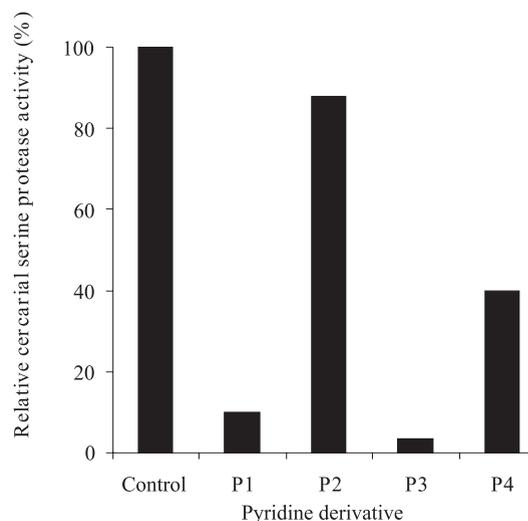
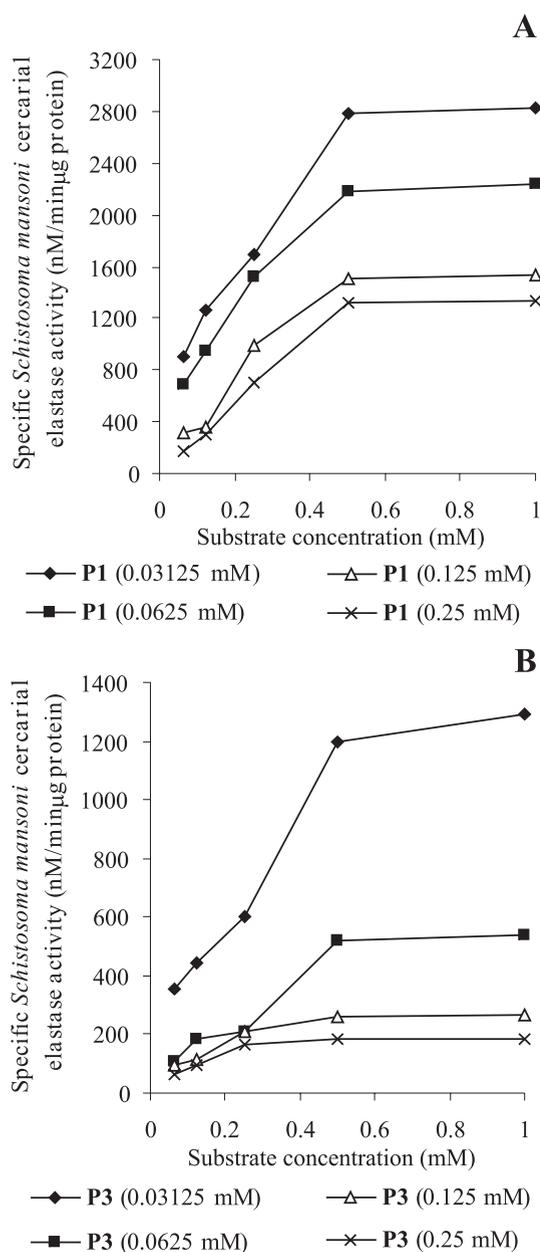


Figure 1. Preliminary inhibition assay of *Schistosoma mansoni* cercarial serine protease activity using 0.5 mM of each of the 4 tested pyridines derivatives **1a** (**P1**), **3a** (**P2**), **5** (**P3**) and **6** (**P4**). **P1** and **P3** exerted the most potent inhibitory effects as demonstrated by abolishing 90% and 97% of the enzyme activity, respectively. While **P4** showed moderate inhibitory effect (abolished 60% of the enzyme activity), **P2** exerted the lowest inhibitory effect (only 10%).



P1 = 1a; P3 = 5

Figure 2. Changes in Michaelis-Menten constants (K_m), maximum enzymatic reaction velocity (V_{max}) upon inhibiting the cercarial serine protease activity using serial dilutions of the pyridine derivatives **P1** (A) and **P3** (B). K_m was calculated as the substrate concentration at which the velocity of the enzymatic reaction is half-maximum, while, V_{max} was the maximum enzymatic activity recorded in nmol/min/μg protein. Activity was measured by hydrolysis of the serine protease specific chromogenic substrate Boc-Val-Leu-Gly-Arg-PNA at the indicated concentrations of the inhibitors **P1** and **P3**. Cercarial secretions (50 μL; 20 mg protein/mL) were mixed with each inhibitor concentration (5 μL at appropriate concentration), incubated for 10 min at room temperature and then mixed with the serially diluted substrate followed by incubation at 37°C and optical densities were measured at 405 nm.

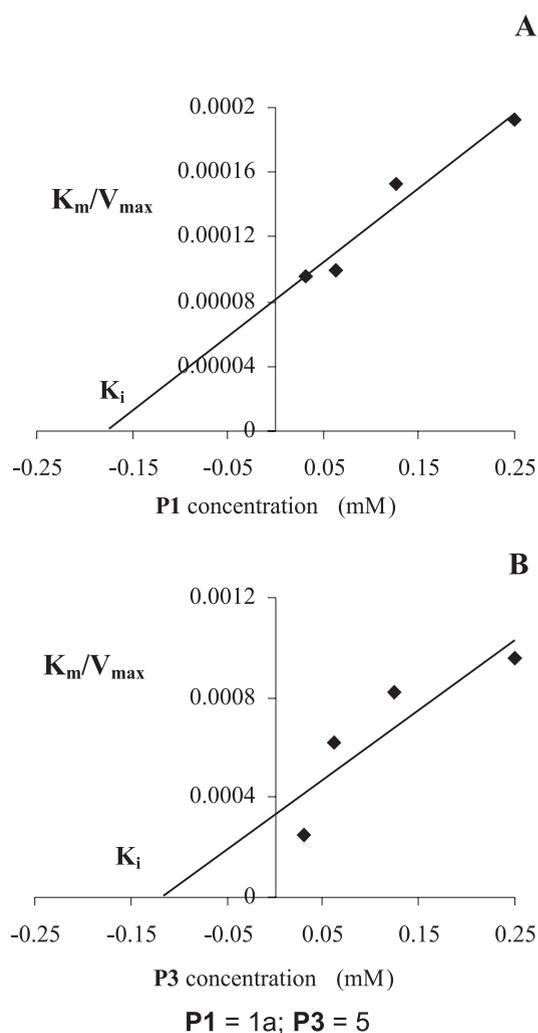
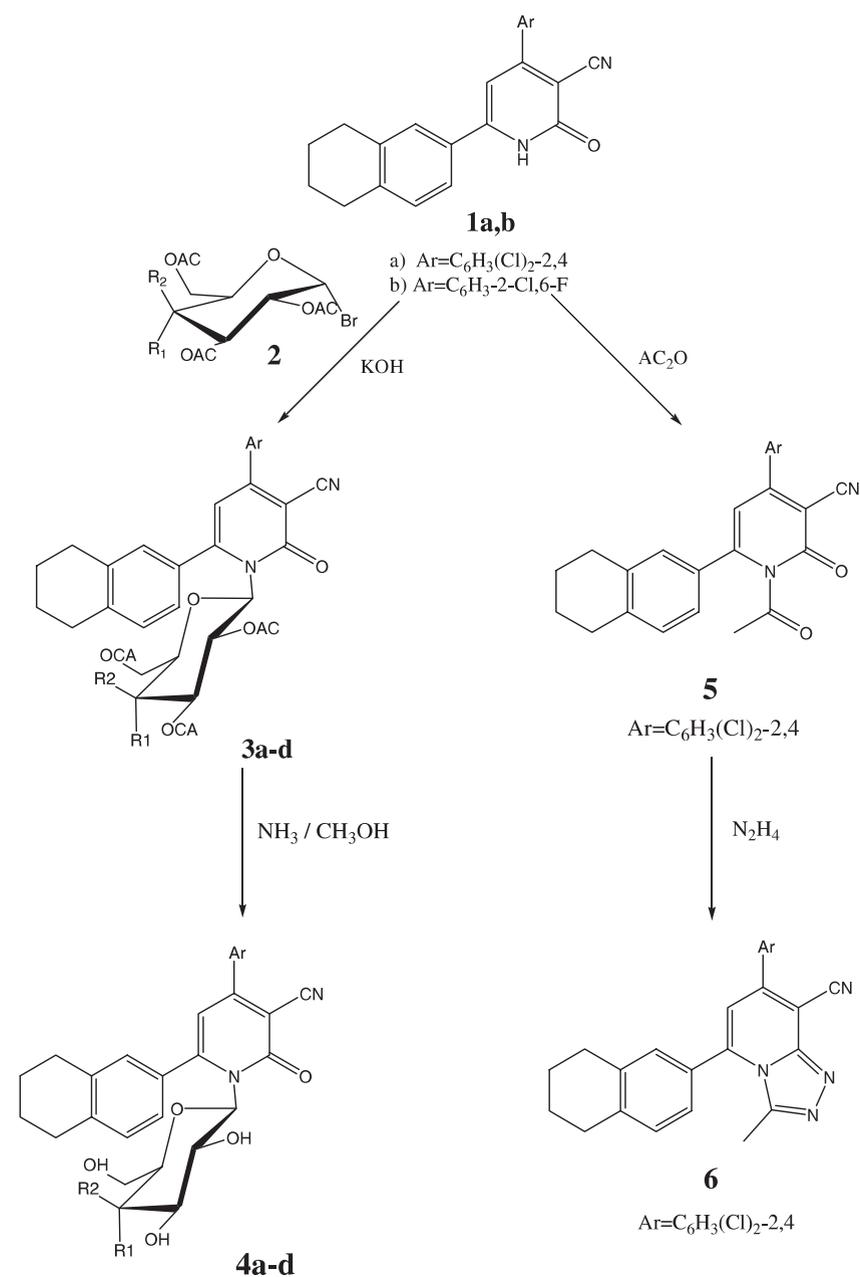


Figure 3. Dixon plots for *Schistosoma mansoni* cercarial serine protease using the pyridine derivatives **P1** and **P3**. The ratio K_m/V_{max} is plotted against the concentrations of each of the 2 pyridine derivatives and the K_i values were graphically determined as the intercept with the abscissa and were 0.17 and 0.12 mM for **P1** and **P3**, respectively.

molecular formula $C_{28}H_{26}ClFN_2O$ (m/z 540). The 1H NMR spectra showed the anomeric proton as a doublet at 6.09 indicating the presence of only the β -configuration.

The other six protons of the glucose ring appear as a multiplet at 3.27-3.76 ppm, while the four hydroxyl groups resonate at δ 4.60-5.57 (exchangeable by D_2O). The ^{13}C NMR spectra were characterized by a signal at δ 96.7 ppm corresponding to the C-1' atom of β -D-glucopyranose. Another five signals at 61.1, 69.7, 72.2, 76.7 and 78.1 ppm were assigned as C-6', C-4', C-2', C-3' and C-5' of the glucose moiety, respectively.



Comp.	Ar	R ¹	R ²	Comp.	Ar	R ¹	R ²
3a	2,4-Dichlorophenyl	OAc	H	4a	2,4-Dichlorophenyl	OH	H
3b	2-Chloro-6-fluorophenyl	OAc	H	4b	2-Chloro-6-fluorophenyl	OH	H
3c	2,4-Dichlorophenyl	H	OAc	4c	2,4-Dichlorophenyl	H	OH
3d	2-Chloro-6-fluorophenyl	H	OAc	4d	2-Chloro-6-fluorophenyl	H	OH

Scheme 1.

The carbonyl carbon atom of the pyridone appears at 162.1 ppm and the nitrile carbon atom at 115.5 ppm. Reaction of compounds **1a,b** with acetic anhydride leads to the formation of the N-acetyl derivative **5 (P3)**, which on treatment with hydrazine hydrate in ethanol gave the triazole derivative **6 (P4)**.

Note that **1a, 3a, 5** and **6** were designated **P1, P2, P3** and **P4** where **P** states for pyridine derivative.

Biochemical studies

Preliminary inhibition experiments of the cercarial serine protease activity using the prepared pyridine derivatives at final concentration 0.5 mM were carried out (Fig. 1). Results showed that compound **1a (P1)** and **5 (P3)** exerted the most potent inhibitory effects as demonstrated by abolishing 90% and 97% of the cercarial serine protease activity, respectively. While compound **6** showed moderate inhibitory effect (abolished 60% of the enzyme activity), compound **3a** exerted the lowest inhibitory effect (only 10%). Kinetics of the inhibition of the serine protease activity in CSs of *S. mansoni* by the pyridine derivatives that exerted the most potent inhibition, **P1** and **P3**, was further studied with respect to V_{max} , K_m and K_i . The V_{max} and K_m values were determined in four independent experiments by plotting the enzymatic activity (nMol/min/ μ g protein) against the substrate concentration (mM) at 4 different concentrations of each of **P1** and **P3** (Fig. 2 A and B). Our results clearly showed that serial dilutions of both **P1** and **P3** abolished the cercarial serine protease activity at different concentrations of the serine proteases specific substrate Boc-Val-Leu-Gly-Arg-PNA as demonstrated by drop in the maximum reaction velocity (V_{max} ; Fig. 2A and B). The K_i values for our newly described protease inhibitors were deduced from Dixon plot (13) which expresses K_m/V_{max} as a function of the inhibitor concentrations. From these plots, K_i values for **P1** and **P3** were calculated as 0.17 and 0.12 mM, respectively, as the negative value of the intercept with the x-axis (Fig. 3A and B). Both values were comparable to the inhibitory coefficient of the standard serine protease inhibitor phenylmethylsulfonylfluoride (PMSF) against the *S. mansoni* cercarial serine protease calculated by ourselves in a previous report (13).

The protease inhibitory effects for our newly described pyridine derivatives are generally not surprising in the light of previous reports where other pyrimidine derivatives could block several serine proteases activities like leukocyte, serine protease, elastase (1, 4), blood clotting factor Xa (3) and chy-

mase (5, 6). In addition, our results are particularly supporting the very few reports that described serine protease inhibitory effect of tetralins on thrombin and prolyl endopeptidase, respectively (7, 8).

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