SYNTHESIS AND ANTIPROLIFERATIVE ACTIVITY IN VITRO
OF NEW 2-, 3- or 4-SUBSTITUTED PYRIDO[2′,3′:3,4]
PYRAZOLO[1,5-a]PYRIDINES

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Abstract: The synthesis of several new 2-, 3- or 4-substituted pyrido[2′,3′:3,4]pyrazolo[1,5-a]pyrimidines is described. The obtained compounds were tested for their antiproliferative activity in vitro. Two of them: 3-chloro-2-methylpyrido[2′,3′:3,4]pyrazolo[1,5-a]pyrimidin-4-one [X] and 2,3-cyclopentylpyrido [2′,3′:3,4]pyrazolo[1,5-a]pyrimidin-4-one [XII] revealed weak cytotoxic activity against the cells of human bladder cancer cell lines: LoVo, MCF-7, MES-SA and HCTV29T. The structures of the products II - XII were established on the basis of elemental analysis and spectral data (IR, H NMR and MS).

Keywords: pyrido[2′,3′:3,4]pyrazolo[1,5-a]pyrimidine derivatives, synthesis, antiproliferative activity in vitro

In the early papers (1, 2) the syntheses of 3-substituted aminopyrazolo[3,4-b]pyridines: 3-chloro-acetyl- and 3-(2-bromopropionylamino)pyrazole [3,4-b]pyridine and 4-phenyl-2-(3,4,5-trimethoxy -β-styryl)pyrido[2′,3′:3,4]pyrazolo[1,5-a] pyrimidine derivatives which showed antiproliferative activity in vitro against cancer cells were described.

Numerous derivatives of tricyclic heteroaromatic systems containing the biologically active moiety of pyrazolo[3,4-b]pyridine (3) display cyto-static (4), anxiolytic (5), antimalarial (6), bacteristatic (7), and hypotensive (8) actions. Pyrazolepyrimidines have attracted much attention because of their important biological activities (9). A broad range of pharmacological activity of this class of compounds prompted us to investigate new derivatives of a little-known tricyclic system, pyrido[2′,3′:3,4]pyrazolo[1,5-a]pyrimidine. Only few syntheses of this fused heterocyclic ring system are described in the literature (10,11) and some of pyridopyrazolepyrimidines were evaluated for their fungistatic activity (12). The aim of our present work was the synthesis of new pyrazolo[3,4-b]pyridines and pyrido[2′,3′:3,4]pyrazolo[1,5-a] pyrimidines, as well as their examination for antiproliferative activity in vitro against the cells of human cancer cell lines.

EXPERIMENTAL

Chemistry

Melting points (uncorrected) were measured in a Boethius melting point apparatus. Elemental analyses were performed on a Perkin Elmer 2400 analyzer and satisfactory results within ±0,4% of calculated values were obtained for the new compounds. The IR spectra (in KBr) were recorded with an IR 75 spectrophotometer (Unicam SP-1000) and 1H NMR spectra on a Tesla BS 587 (80 MHz) apparatus. The positions of the resonances were referred to the residual solvent signals (DMSO-d6, δ 2,5 ppm). The mass spectra were determined on a GCMS-LKB 2091 spectrometer at an ionisation energy of 15 or 70 eV. The course of reaction and the purity of products were checked by TLC on Merck aluminium foil with silica gel F254.

Reaction 3-aminopyrazolo[3,4-b]pyridine [I] with ethyl acetoacetate:
Method A

2-Methylpyrido[2′,3′:3,4]pyrazolo[1,5-a]pyrimidin-4-one [II]

Ethyl 3-(pyrazolo[3,4-b]pyridylamino)-crotonate [III]

A mixture containing 1.34 g (0.01 mole) of 3-aminopyrazolo[3,4-b]pyridine [I] (13) 1.5 g (0.02 mole) of ammonium acetate and 1.3 g (0.01 mole) of ethyl acetoacetate was stirred and heated in an oil bath at 100 – 110°C for 2 h. After cooling, the reaction mixture was triturated with warm water (3 x 100 mL). The separated solid was filtered, dried and refluxed in 100 mL of benzene for 15 min. The insoluble in benzene residue was filtered, washed with benzene and crystallized from DMF to give product II, which was identical with compound
obtained during the thermal cyclization by Kocevar and Stanovnik (11).

Yield of II: 1.5 g (75%), yellow crystals, mp. 310°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3420, 3000 (CH, NH, OH), 1640 (NCOO), 1600, 1590, 1460 (C=C, C≡N). Anal.: C₁₂H₁₄N₄O₂ (246.27). MS (70 eV): m/z (%) = 200 (100) M⁺.

The combined benzene filtrates were evaporated. After cooling, filtering and recrystallization from benzene product III was obtained.

Yield of III: 0.32 g (13%), yellow crystals, mp. 152.5- 3°C (benzene). IR (KBr) ν [cm⁻¹] = 3080, (CH, NH), 1660, 1650 (CO), 1600, 1555, 1450 (C=C, C≡N). ¹H NMR (CDCl₃) δ [ppm] = 1.27 (t, 3H, CH₃), 2.22 (s, 3H, CH₃), 4.17 (q, 2H, CH₂), 4.85 (s, 1H, CH=), 7.20 (dd, 1H, H-5), 8.14 (dd, 1H, H-5), 8.55 (dd, 1H, H-6), 10.8 (s, 1H, NH), 13.24 (s, br, NH). Anal.: C₉H₁₀N₄O₂ (218.22). MS (70 eV): m/z (%) = 246 (100) M⁺.

Method B
To a solution of 3-aminopyrazole[3,4-b]pyridine [I] (1.34 g, 0.01 mole) in 50 mL of ethanol was added 1.3 g (0.01 mole) of ethyl acetoacetate and 1 mL of glacial acetic acid. The reaction mixture was refluxed for 8 h. The solvent was removed and the residue was filtered. The separated solid was dried and refluxed in 100 mL of benzene for 15 min. The insoluble in benzene residue was filtered. After cooling, filtering and recrystallization from benzene product III was obtained (yield 1.85 g, 75.2 %).

Method C
A solution of 0.61 g (2.5 mmole) of ethyl 3-pyrazole[3,4-b]pyridylamino)-crotonate [III] in 10 mL of glacial acetic acid was refluxed for 8 h. The reaction mixture was cooled and the product was filtered off and recrystallized from DMF. Yield of compound II 0.36 g (72 %).

Method D
N-3-(Pyrazole[3,4-b]pyridyl)-acetoacetamide [IV]
The solution of ethyl acetoacetate (1.3 g, 0.01 mole) in 40 mL of o-xylene was added to a solution of 3-aminopyrazole[3,4-b]pyridine [I] (1.34 g, 0.01 mole) in 100 mL of dry o-xylene. The reaction mixture was refluxed for 15 min. in a flask equipped with a Dean-Stark apparatus. After cooling, the crude product was filtered, washed with hexane and recrystallized from ethanol.

Yield of compound IV 1.83 g (83.9 %), colorless crystals mp. 214 – 5°C (ethanol). IR (KBr) ν [cm⁻¹] = 3080, (CH, NH), 1660, 1650 (NCOO), 1740 (COCH₃), 1600, 1555, 1450 (C=C, C≡N), 1H NMR (DMSO-d₆) δ [ppm] = 2.52 (s, 3H, CH₃), 3.72 (s, 2H, CH₃), 7.32 (dd, 1H, H-5), 8.12 (dd, 1H, H-5), 8.46 (m, 2H, H-6), 10.79 (s, 1H, NH), 13.23 (s, br, NH). Anal.: C₁₀H₁₀N₄O₂ (218.22). MS (70 eV): m/z (%) = 218 (100) M⁺.

Method E
4-Methylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-2-one [VI]
A solution of 1.09 g (5 mmole) of N-3-(pyrazole[3,4-b]pyridyl)-acetoacetamide [IV] in 10 mL of glacial acetic acid was refluxed for 15 min. The reaction mixture was cooled and the product was filtered off and recrystallized from hot water.

Yield of compound VI 0.75 g, (75%), yellow crystals, mp. 335°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3420, 3000 (CH, NH, OH), 1640 (CO), 1650, 1560 (NCOO), 1600, 1510, 1420 (C=C, C≡N). Anal.: C₁₀H₁₀N₄O₂ (218.22). MS (70 eV): m/z (%) = 200 (100) M⁺.

Reaction of 3-aminopyrazole[3,4-b]pyridine [I] with ethyl benzoylacetate
Method D
N-3-(Pyrazole[3,4-b]pyridyl)-benzoylacetamide [V]
The solution of ethyl benzoylacetate (0.99 g, 5 mmole) in 10 mL of dry o-xylene was added to a solution of 3-aminopyrazole[3,4-b]pyridine [I] (0.67 g, 5 mmole) in 50 mL of dry o-xylene. The reaction mixture was refluxed for 3 h. in a flask equipped with a Dean-Stark apparatus. After cooling, the crude product was filtered, washed with hexane and recrystallized from ethanol.

Yield of compound V 0.76 g (54.28 %), colorless crystals, mp. 204 – 5°C (ethanol). IR (KBr) ν [cm⁻¹] = 3080, (CH, NH), 1700 (COPh), 1670, 1560 (NCHO), 1600, 1555, 1450 (C=C, C≡N). ¹H NMR (DMSO-d₆) δ [ppm] = 4.27 (s, 2H, CH₃), 7.22 (dd, 1H, H-5), 8.12 – 8.58 (m, 8H, H-4 + Ph), 10.91 (s, 1H, NH), 13.24 (s, br, NH). Anal.: C₁₀H₁₀N₄O₂ (280.29). MS (70 eV): m/z (%) = 280 (100) M⁺.

Method E
4-Phenylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-2-one [VII]
A solution of 0.70 g (2.5 mmole) of N-3-(pyrazolyl[3,4-b]pyridyl)-benzoylacetamide [V] in 10 mL of acetic acid was refluxed for 15 min. The reaction
mixture was cooled and the product was filtered off and recrystallized from acetic acid.

Yield of compound VII 0.5 g (76.92 %), yellow crystals, mp. 310°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3030, 3000 (CH, NH, OH), 1650, 1560 (NHCO), 1600, 1590, 1460 (C=C, C=N). Anal.: C₁₅H₁₀N₄O (262.27). MS (70 eV): m/z (%) = 262 (100) M⁺.

Method F
2-Phenylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [VIII]
A mixture containing 1.34 g (0.01 mole) of 3-aminopyrazole[3,4-b]pyridine[1,5-a]pyrimidin-4-one [VIII] was prepared. Method F was used for this synthesis.

Compounds IX - XII were synthesized using a similar procedure as that for compound VIII.

2-Methoxymethylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [IX]
Yield of compound IX 1.72 g (74.78 %), yellow crystals, mp. 303-5°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3030, 3000 (CH, NH), 1650 (CO), 1590, 1510, 1400 (C=C, C=N). Anal.: C₁₇H₁₉N₅O₂ (270.23). MS (70 eV): m/z (%) = 230 (100) M⁺.

3-Chloro-2-methylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [X]
Yield of compound X 1.75 g (74.78 %), yellow crystals, mp. 350-5°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3050, (CH, NH), 1640, (NCO), 1600, 1510, 1440 (C=C, C=N), 830 (CCl). Anal.: C₁₃H₁₀ClN₄O (234.64). MS (70 eV): m/z (%) = 234 (86) M⁺.

2-Chloromethylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [XI]
Yield of compound XI 1.73 g (73.93 %), yellow crystals, mp. 345-350°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3050, (CH, NH), 1640, (CO), 1600, 1510, 1440 (C=C, C=N), 830 (CCl). Anal.: C₁₃H₁₀ClN₄O (234.64). MS (70 eV): m/z (%) = 234 (86) M⁺.

2,3-Cyclopentylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [XII]
Yield of compound XII 1.68 g (74.33 %), yellow crystals, mp. 300-5°C (dec.) (DMF). IR (KBr) ν [cm⁻¹] = 3200, 2980 (CH, NH), 1640, (NCO), 1600, 1500, 1450 (C=C, C=N). Anal.: C₁₅H₁₀N₄O (226.24). MS (70 eV): m/z (%) = 226 (100) M⁺.

Biology
Antiproliferative activity in vitro
Test solutions of the compounds (1 mg/mL) were prepared in 100 µL of DMSO + 900 µL of culture medium. After that, the compounds were diluted in culture medium (described below) to reach final concentrations 100, 10, 1 and 0.1 µg/mL.

Cell lines
Cells of the following human cancer lines were used: MES-SA (uterine carcinoma), SW707 or LoVo (colon adenocarcinoma), MCF-7 (breast carcinoma). All lines were obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.) and cultured in the Cell Culture Collection of Department of Tumor Immunology, Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Human uroepithelial cell line HCV29T established in Fübig Institute (Copenhagen, Denmark), was obtained from Dr. J. Kieler in 1982. The established in vitro murine Lewis lung cancer (LLC) cell line was also applied.

Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 10⁴ cells per well. The cells were cultured in the opti-MEM medium supplemented with 2 mM glutamine (Gibco, Grand Island, U.S.A.). The cell cultures were maintained at 37°C in humid atmosphere saturated with 5% CO₂.

SRB (Sulforodamine B) assay
The cytotoxic assays were performed after 72-hour exposure of the cultured cells to varying concentrations (from 0.1 to 100 µg/mL) of the tested agents. The SRB method was used as described by Skehan et al. (14). The optical densities of the samples were measured on a Multiskan RC photometer (Labsystems, Helsinki, Finland) at 570 nm. The results were calculated as an ID₅₀ (inhibitory dose 50 %) – the dose of compound which inhibits proliferation rate of the tumor cells by 50% as compared to control untreated cells. Each compound in every concentration was tested in triplicates per
experiment. Every experiment was repeated 3 times.

RESULTS AND DISCUSSION

Chemistry


In the condensation reaction of 3-aminopyrazole[3,4-b]pyridine [I] (12) with selected β-ketoesters: ethyl acetoacetate, ethyl benzoylacetate, methyl 4-methoxyacetoacetate, ethyl 2-chloroacetoacetate, ethyl 4-chloroacetoacetate and ethyl cyclopentanone-2-carboxylate in o-xylene, acetic acid or in the presence of ammonium acetate the respective 3-substituted aminopyrazole[3,4-b]pyridines [III – V] and new 2-, 3- or 4-substituted pyrido[2’3’;3,4]pyrazolo[1,5-a]pyrimidine derivatives [II, VI – XII] were obtained.

By heating of amine I with an equimolar quantity of ethyl acetoacetate in the presence of two equivalents of ammonium acetate at 110°C a mixture was obtained containing two compounds, namely: 2-methylpyrido[2’3’;3,4]pyrazolo[1,5-a]pyrimidin-4-one [II], which was identical with compound obtained during the thermal cyclization by Kočevar and Stanovnik (13) and ethyl N-3-(pyrazole[3,4-b]pyridylamino)-crotonate [III], in relation 5 : 1. Moreover, in the reaction of amine I with ethyl acetoacetate in boiling ethanol in the presence of glacial acetic acid in the presence of ammonium acetate the respective 3-substituted aminopyrazole[3,4-b]pyridines [III – V] and new 2-, 3- or 4-substituted pyrido[2’3’;3,4]pyrazolo[1,5-a]pyrimidine derivatives [II, VI – XII] were obtained.


Reagents: A: ethyl acetoacetate/AcONH4/fused; B: ethyl acetoacetate/EtOH/AcOH; C: AcOH/reflux; D: ethyl acetoacetate or ethyl 4-chloroacetoacetate, ethyl cyclopentanone-2-carboxylate/AcONH4/fused.
the following double coupled signals appear: triplet at $\delta$ 1.27 ppm and quartet at $\delta$ 4.17 ppm representing $-\text{CH}_2\text{CH}_3$ of the ester group. A three-proton singlet at $\delta$ 2.22 ppm indicates the presence of the methyl group, singlet at $\delta$ 4.85 ppm belongs to the methine group. A broad singlet at $\delta$ 10.8 ppm represents the NH in the position 3 and a broad singlet at 13 ppm corresponds with the proton linked to the pyrazole ring. Heating the aminocrotonate III in a glacial acetic acid under reflux yielded the cyclized compound II.

During heating of amine I with ethyl acetoacetate in boiling o-xylene using a Dean – Stark apparatus, only N-3-(pyrazolo[3,4-b]pyridyl)-acetoacetamide [IV] of molecular weight 246 was isolated. The absorbance bands at $\nu$ 1740, 1660 and 1550 cm$^{-1}$ in the IR spectrum indicated the presence of ketone and amide groups. In the $^1\text{H}$ NMR spectrum, three-proton singlet appeared at $\delta$ 2.52 ppm representing the methyl group and double-proton singlets at $\delta$ 3.72 ppm for two protons of the methylene group. Then, attempts have been made to cyclize amide IV in boiling acetic acid to 4-methyl derivative of pyrazolepyridopyrimidine VI.

Similarly, amide V was prepared from amine I and ethyl benzoyletacetate in boiling o-xylene and subsequently cyclized to 4-phenylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-2-one [VII]. In the $^1\text{H}$ NMR spectrum of the compound V apart from the signals of aromatic protons, there was a double-proton singlet at $\delta$ 4.27 ppm for two protons of the methylene group and two broad signals for NHCO and NH of pyrazole ring. The absorption bands at $\nu$ 1670 and 1560 cm$^{-1}$ in the IR spectrum indicated the presence of NHCO group in the pyrimidine ring.

Amine I was fused at 130°C with ethyl benzoyletacetate in the presence ammonium acetate to afford only one product (of molecular weight 262) namely: 2-phenylpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [VIII]. The IR spectrum of compound VIII contains an absorption band at $\nu$ 1640 cm$^{-1}$ which is characteristic for NCO in the 6-member lactam. The low frequency absorption of the group C=O is caused by the advantage of mesomeric effect of the next nitrogen atom and also by coupling with the double bond C=C.

Then, amine I was subjected to cyclocondensation reaction, under the same reaction conditions, with of selected $\beta$-ketoesters: methyl 4-methoxyacetooctetate, ethyl 2-chloroacetooctetate, ethyl 4-chloroacetooctetate and ethyl cyclopentanone-2-carboxylate to afford cyclic products: pyrido[2í,3í:3,4]pyrazolo[1,5-a]pyrimidines IX – XII. The structure of all new compounds was confirmed by elemental analysis and their MS and IR spectra.

### Biology

**Antiproliferative activity in vitro**

The compounds: 3-chloro-2-methylepyrido [2',3:3,4]pyrazole[1,5-a]pyrimidin-4-one [X] and 2,3-cyclopentolpyrido[2',3':3,4]pyrazole[1,5-a]pyrimidin-4-one [XII] were tested for their antiproliferative activity in vitro against the cells of human cancer lines (LoVo, MCF-7, MES-SA and HCV29T).

Both compounds revealed very weak cytotoxic activity, with ID$_{50}$ values (9.2 – 98.5 $\mu$g/mL, respectively), which doesn’t fulfil activity demands (Table 1). All other compounds tested did not reveal any cytotoxic activity.

### REFERENCES


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