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The Synthesis of 3,5,6,7-Tetrasubstituted Isoxazolo[4,5-B]Pyridines and an Evaluation of Their *In Vitro* Antiproliferative Activity

Synteza 3,5,6,7-tetrapodstawionych izoksazolo[4,5-b]pirydyn i badanie ich aktywności antyproliferacyjnej

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Derivatives of isoxazolopyridines exhibit diverse biological activity. One method of synthesizing isoxazolo[4,5-b]pyridines is Friedländer condensation.
Objectives. To establish the conditions necessary for conventional and microwave synthesis of new 3,5,6,7-tetrasubstituted isoxazolo[4,5-b]pyridines and their antiproliferative activity.
Material and Methods. The substrates in the synthesis of new isoxazolo[4,5-b]pyridines were 4-amino-5-benzoylisoxazole-3-carboxamide and selected carbonyl compounds containing a reactive α-methylene group. Reactions were carried out using classical methods in the presence of catalysts $ZnCl_2$ or In $(OTf)_3$, and in a microwave reactor in the presence of $ZnCl_2$ under solvent-free conditions. Selected compounds were tested *in vitro* on eight tumor cell lines to assess their antiproliferative activity.
Results and Discussion. A series of new derivatives of 3,5,6,7-tetrasubstituted isoxazolo [4,5-b]pyridines was obtained from Friedländer condensation of 4-amino-5-benzoyloisoxazolo-3-carboxamide with selected carbonyl compounds with an active methylene group. The compounds were obtained by conventional and microwave methods, in the presence of catalysts $ZnCl_2$ or In $(OTf)_3$. The structures of the products were determined on the basis of elemental analysis and infrared (IR), Nuclear Magnetic Resonance (^{1}H NMR) and Mass Spectrometry (MS) data. Selected compounds were tested *in vitro* on eight tumor cell lines in the direction of antiproliferative activity.
Conclusions. Only the use of conventional heating in a thermostated oil bath in the presence of catalysts $ZnCl_2$, or In $(OTF)_3$ or microwave irradiation in the presence $ZnCl_2$ in the solvent-free conditions allowed good yields of the new derivatives of poly-substituted isoxazolo[4,5-b]pyridines to be obtained. Among the compounds tested *in vitro* only 6-benzoyl-5, 7-difenyloisoxazolo[4,5-b]pyridine showed antyproliferative activity at a concentration of 3.9 μg/ml (**Adv Clin Exp Med 2012, 21, 5, 563–571**).

Key words: isoxazolopyridines, microwave synthesis, antitproliferative activity.

Streszczenie

Wprowadzenie. Pochodne układu izoksazolopirydyny wykazują różnorodną aktywność biologiczną. Jedną z metod syntezy pochodnych izoksazolo[4,5-b]pirydyny jest kondensacja Friedländera.
Cel pracy. Opracowanie warunków syntezy konwencjonalnej i mikrofalowej nowych pochodnych izoksazolo[4,5-b]pirydyny o aktywności antyproliferacyjnej.
Materiał i metody. Substratami w syntezie nowych pochodnych izoksazolopirydyny były: amid kwasu 4-amino-5-benzoiloizoksazolo-3-karboksylowego i wybrane związki karbonylowe mające aktywną grupę metylenową. Reakcje były prowadzone metodami klasycznymi wobec $ZnCl_2$ lub $In(OTf)_3$ jako katalizatorów oraz w reaktorze mikrofalowym w obecności $ZnCl_2$ w warunkach bez rozpuszczalnika. Wybrane związki były testowane *in vitro* na ośmiu liniach nowotworowych w kierunku aktywności antyproliferacyjnej.

Wyniki. W wyniku reakcji kondensacji Friedländera amidu kwasu 4-amino-5-benzoiloizoksazolo-3-karboksylowego z wybranymi związkami karbonylowymi posiadającymi aktywną grupę metylenową otrzymano serię nowych 3,5,6,7-tetrapodstawionych pochodnych izoksazolo[4,5-b]pirydyny. Związki były otrzymane 2 metodami: konwencjonalną w obecności katalizatorów $ZnCl_2$ lub $In(OTf)_3$ oraz mikrofalową wobec $ZnCl_2$. Strukturę otrzymanych nowych pochodnych określono na podstawie analizy elementarnej i widm spektralnych IR, ^1H NMR i MS. Wybrane związki były testowane *in vitro* w kierunku aktywności antyproliferacyjnej.
Wnioski. Tylko zastosowanie konwencjonalnego ogrzewania na termostatowanej łaźni olejowej w obecności katalizatorów: $ZnCl_2$ lub $In(OTf)_3$ lub pod wpływem promieniowania mikrofalowego wobec $ZnCl_2$ w warunkach bez rozpuszczalnika pozwoliło otrzymać nowe polipodstawione pochodne izoksazolo[4,5-b]pirydyny z dobrymi wydajnościami. Spośród testowanych *in vitro* związków tylko 6-benzoilo-5,7-difenyloizoksazolo[4,5-b]pirydyna wykazała aktywność antyproliferacyjną w stężeniu 3,9 μg/ml (**Adv Clin Exp Med 2012, 21, 5, 563–571**).

Słowa kluczowe: izoksazolopirydyny, synteza mikrofalowa, aktywność antyproliferacyjna.

As a continuation of the authors' research [1–3] into the synthesis of bicyclic heteroaromatic systems containing the biologically active moiety of isoxazole, several new derivatives of substituted isoxazolo[4,5-b]pyridine were synthesized. Previously obtained derivatives of 5-alkyl-6,7-diphenyl-4,5,6,7-tetrahydroisoxazolo[4,5-d] pyrimidine-3-carboxamides revealed antidepressive activity [1, 2] and 3-chloroacetylo-, 3-(2-bromopropionyloamino)isoxazolo[5,4-b]pyridine [3] and 3-benzoylisoxazolo[4,3-d]pyrimidine-3-formamidine were found to have antiproliferative activity *in vitro* against cancer cells lines [4].

The derivatives of isoxazolopyridine have interesting biological properties. Isoxazolo[5,4-b] pyridines show antibacterial [5], antiviral [6], muscle relaxant and anticonvulsant [7] and analgesic [8]. Derivatives of isoxazolo[4,3-b]pyridines (THIP and THOPO) have been investigated in clinical studies and show anxiolytic activity [9–11]. Many techniques of Friedländer condensation have previously been reported for the synthesis of a variety of substituted quinoline derivatives [12–14], imidazo[4,5-b]pyridines [15] and pyrazolo[3,4-b] pyridines[16]. Friedländer condensation is one of the simplest and most efficient methods of synthesis. It entails the condensation of o-aminoaryl ketones or o-aminoaryl aldehydes with a carbonyl compound containing a reactive α-methylene moiety. Synthesis is carried out in polar solvents in the presence $ZnCl_2$ [17], a base [18] or Brønsted acids [19, 20] and Lewis acids [21].

Up until now, only two methods of synthesis of isoxazolo[4,5-b]pyridine derivatives have been described. The first method is Friedlander annulation in the presence of pyridine as a solvent and base catalyst [22]. The second is the condensation 3,5-dimethyl-4-nitro-isoxazole with β-dicarbonyl compounds in a one-step reaction [23, 24].

It was interesting to investigate the $ZnCl_2$ and $In(OTf)_3$ (triflate) as Lewis acid catalysts or using microwave irradiation in the synthesis of new derivatives. $ZnCl_2$ and $In(OTf)_3$ have been used as potential mild Lewis acid catalysts for many organic transformations [25, 26]; and microwave-assisted solvent-free synthesis of quinolones, azolopyrimidines and other heterocyclic derivatives has been studied in recent years [27–29].

The present work is devoted to both conventional and microwave synthesis of novel derivatives of isoxazolo[4,5-b]pyridines and preliminary biological studies investigating their antiproliferative activity *in vitro*.

Material and Methods

Chemistry

Melting points were determined with a Boethius apparatus and are uncorrected. Elemental analyses were performed on a Perkin Elmer 2400 analyzer (Waltham, MA, USA) and the results are within ±0.4% of the theoretical values obtained for the new compounds. Infrared (IR) spectra were recorded with a Specord M80 spectrophotometer (Zeiss/Analytic Jena, Germany) for KBr pellets. Hydrogen-1 NMR (^1H NMR) spectra were recorded with a Bruker Avance ARX-300 instrument (Bruker Analytic, Karlsruhe, Germany) using DMSO-d_6 or $CDCl_3$ as internal standards. Chemical shifts are reported in ppm from the internal tetramethylsilane reference. Mass spectra (MS) were recorded on a Finningan Mat 95 GC-MS (Finningan, Bremen, Germany) with an ionization energy of 70 eV. The progress of the reaction and the purity of the compounds were monitored using thin layer chromatography (TLC) on analytical silica gel plates (Merck F_{254}, Darmstadt, Germany). Microwave-assisted synthesis was performed in a laboratory microwave RM 800PC reactor (Plazmatronika, Wrocław, Poland). Water was purified using an Aquadem SDF-Ion exchanger system (TKA, Thermo Scientific). All chemicals and reagents for the synthesis were obtained from Alfa Aesar (Karlsuhe, Germany), Lancaster Synthesis (Morecambe, England) and Chempur (Piekary Śląskie, Poland).

Conventional Conditions

Method A (in solution): 6-Acetyl--5-methyl-7-phenylisoxazolo[4,5-b] pyridine-3-carboxa-mide (4)

Acetyloaceton (0.025 mole) was added to a solution 0.01 mole of 4-amino 5-benzoylisoxazole-3-carboxamide (1) in 100 ml glacial acetic. The reaction mixture was refluxed for 24 hours. The solvent was distilled under vacuum and the residue was mixed with 100 ml of water. The resulting precipitate solid was filtered, dried and recrystallized from ethanol to give 4 as colorless crystals (mp. 225–226°C, yield 58%).

IR (KBr) ν = 3390, 3180 (NH, CH), 1710 (CO), 1690, 1680 ($CONH_2$) [cm^{-1}], ^1H NMR ($CDCl_3$) δ = 2.14 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 7.52-7.61 (m, 5H, Ph), 8.37 (br s, 2H, $CONH_2$) (ppm), MS: (70 ev, electron impact) m/z 295 (molecular ion). Anal. Calcd. for $C_{16}H_{13}N_3O_3$ (295.30): C 65.08, H 4.44, N 14.23; Found: C 64.80, H 4.31, N 14.08.

Method B (solvent-free)

The compound 4-amino-5-benzoylisoxazole-3-carboxamide (**1**) (0.01 mole), selected methylene compound (0.015 mole) and anhydrous $ZnCl_2$ (0.01 mole) or $In(OTf)_3$ were mixed thoroughly in a mortar. The reaction mixture was then transferred to a round-bottomed flask and heated, while stirring, at 125-130° for 8-16 hours in an oil bath. After cooling to room temperature, 50 ml of ethyl ether was added to the reaction mixture and stirred for 1 hour. The obtained precipitate was filtered off. Then 100 ml of water was added to the solid and stirred for 1 hour. The precipitate formed was filtered and washed with cold water, dried and recrystallized.

Microwave Conditions

Method C

A mixture of 4-amino-5-benzoyl-isoxazole-3-carboxamide (1) (0.01mole), a selected methylene compound (0.015 mole) and 2.4 g of anhydrous $ZnCl_2$ were mixed thoroughly in a mortar. The reaction mixture was heated while being stirred in the microwave reactor in an aluminum bath at 60-65°C for 15 minutes (3 × 5 min with 1-minute breaks, at microwave power P = 240 W). After cooling to room temperature, 100 ml of ethyl ether was added to the reaction mixture and stirring was continued for 1 hour. The precipitate was filtered. To the resulting precipitate 100 ml of distilled water was added and then stirred for 30 minutes. The precipitate was filtered, washed with distilled water, dried and recrystallized.

Compound 2 was also obtained by procedure B. In the presence of $ZnCl_2$ the yield was 75%; in the presence of $In(OTf)_3$, the yield was 76%. By the microwave procedure C, the yield was 80%.

5,7-Diphenylisoxazolo[4,5-b]pyridine--3-carboxamide (2)

This compound was obtained by condensing compound 1 with acetophenone. Using procedure B in the presence of $ZnCl_2$ the yield was 77%; in the presence of $In(OTf)_3$ the yield was 76%. Using the microwave method (C) the yield was 80% as colorless mp 219–220°C (from ethanol). IR (KBr) ν : 3300, 3100 (CH, NH), 1680, 1580 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 7.50–7.75 (m, 10H, phenyl protons), 8.30 (br s, 2H, $CONH_2$), 8.5 (s, 1H, CH)ppm. MS: m/z = 315 (molecular ion). Anal. Calcd. for $C_{19}H_{13}N_3O_2$ (315.33): C, 72.37; H, 4.16; N, 13.33. Found: C, 72.27; H, 4.19; N, 13.18.

5-(4-Bromophenyl)-7--phenylisoxazolo[4,5-b]pyridine-3--carboxamide (3)

This compound was obtained by condensing compound 1 with 4-bromoacetophenone. Using procedure B in the presence of $ZnCl_2$ the yield was 68%; in the presence of $In(OTf)_3$ the yield was 67%. Using the microwave method (C) the yield was 78% as colorless mp 179-180°C (from ethanol). IR (KBr) ν = 3200, 3100 (CH, NH), 1680, 1580 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 7.376–7.75 (m, 9H, phenyl protons), 8.36 (br s, 2H, $CONH_2$), 8.5 (s, 1H, CH) ppm, MS: m/z = 394 (molecular ion). Anal. Calcd. for $C_{19}H_{12}N_3BrO_2$ (394.23): C, 57.89; H, 3.07; N, 10.66. Found: C, 57.95; H, 3.27; N, 10.46.

6-Benzoyl-5-methyl-7--phenylisoxazolo[4,5-b]pyridine-3--carboxamide (5)

This compound was obtained by condensing compound 1 with benzoylacetone. Using procedure B in the presence of $ZnCl_2$ the yield was 68%; in the presence of $In(OTf)_3$ the yield was 70%. Using the microwave method (C) the yield was 85% as colorless IR (KBr) ν = 3380, 3180 (NH, CH), 1705 (CO), 1680, 1610 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 2.48 (s, 3H, CH_3), 7.36–7.70 (m, 10H, phenyl protons), 8.41 (br s, 2H, NH_2) ppm, MS: m/z = 357 (molecular ion). Anal. Calcd. for $C_{21}H_{15}N_3O_3$ (357.37): C, 70.58; H, 4.23; N, 11.76. Found: C, 70.32; H, 4.11; N, 11.96

6-Benzoyl-5,7-diphenylisoxazolo [4,5-b]pyridine-3-carboxamide (6) [30]

This compound was obtained by condensing compound 1 with dibenzoylmethane. Using pro-

cedure B in the presence of $ZnCl_2$ the yield was 71%; in the presence of In $(OTf)_3$ the yield was 70%. Using the microwave method (C) the yield was 85% as colorless mp 213–215°C (from ethanol).IR (KBr) ν: 3300, 3100 (NH, CH), 1720 (CO), 1680, 1580 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.25–7.59 (m, 15H, phenyl protons), 8.40 (br s, 2H, NH_2); MS: m/z = 419 (molecular ion). Anal. Calcd. for $C_{26}H_{17}N_3O_3$ (419.44): C, 74.45; H, 4.09; N, 11.02. Found: C, 74.10; H, 4.19; N, 10.01.

6-Acetyl-7-phenyl-5-trifluoromethylisoxazolo [4,5-b]pyridine-3--carboxamide (7)

This compound was obtained by condensing compound 1 with 1,1,1-trifluoroacetylacetone. Using procedure B in the presence of $ZnCl_2$ the yield was 68%; in the presence of In $(OTf)_3$ the yield was 72%. Using the microwave method (C) the yield was 86% as colorless mp 176–179°C (from ethanol). IR (KBr) ν = 3360, 3080 (CH, NH), 1700 (CO), 1680, 1620 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 2.65 (s, 3H, CH_3), 7.36–7.75 (m, 5H, phenyl protons), 8.46 (br s, 2H, $CONH_2$) ppm, MS: m/z = 349 (molecular ion). Anal. Calcd. for $C_{16}H_{10}N_3F_3O_3$ (349.27): C, 55.02; H, 2.89; N, 12.03. Found: C, 55.17; H, 2.01; N, 12.34.

6-Acetyl-5-chlorodifluoromethyl-7--phenylisoxazolo[4,5-b]pyridine--3-carboxamide (8)

This compound was obtained by condensing compound 1 with 1-chloro-1,1-difluoroacetylacetone. Using procedure B in the presence of $ZnCl_2$ the yield was 75%; in the presence of In $(OTf)_3$ the yield was 76%. By the microwave method (C) the yield was 80% as colorless mp 176–177°C (from ethanol). IR (KBr) ν = 3300, 3000 (CH, NH), 1700 (CO), 1680, 1620 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 2.65 (s, 3H, CH_3), 7.36–7.75 (m, 5H, phenyl protons), 8.46 (br s, 2H, $CONH_2$) ppm, MS: m/z = 365 (molecular ion). Anal. Calcd. for $C_{16}H_{10}N_3F_2ClO_3$ (365.72): C, 55.55; H, 2.76; N, 11.49. Found: C, 55,51; H, 2.65; N, 11.45.

7-Phenyl-6-phenylcarbamoyl-5--methylisoxazolo [4,5-b]pyridine-3--carboxamide (9)

This compound was obtained by condensing compound 1 with acetylacetanilide. Using procedure B in the presence of $ZnCl_2$ the yield was 67%; in the presence of In $(OTf)_3$ the yield was 76%. Using the microwave method (C) the yield was 80% as colorless mp 144–145°C (from ethanol). IR (KBr): 3300, 3100 (CH, NH), 1680, 1580 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 2.75(s, 3H, CH_3), 7.26–7.75 (m, 10H, phenyl protons), 8.24 (br s, 2H, $CONH_2$), 10.5(s, 1H, NH) ppm, MS: m/z = 372 (molecular ion). Anal. Calcd. for $C_{21}H_{16}N_4O_3$ (372.38): C, 67. 73; H, 4.33; N, 15.05. Found: C, 67.40; H, 4.50; N, 15.21.

Ethyl-7-phenyl-5-methylisoxazolo [4,5-b]pyridine-6-carboxylate (10)

This compound was obtained by condensing compound 1 with ethyl acetoacetate. Using procedure B in the presence of $ZnCl_2$ the yield was 67%; in the presence of In $(OTf)_3$ the yield was 70%. Using the microwave method (C) the yield was 85% as colorless mp 148–149°C (from ethanol). IR (KBr) ν = 3390, 3000 (CH, NH), 1730 (COOR), 1690, 1570 ($CONH_2$), 1210 (COOR) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 1.05 (t, J=7.2 Hz, 3H, CH_3), 2.78 (s, 3H, CH_3), 4.20 (q, J=7.2 Hz, 2H, CH_2), 7.51–7.61 (m, 5H, phenyl protons), 8.39 (br, s, 2H, $CONH_2$) ppm; MS: m/z = 325 (molecular ion). Anal. Calcd. for $C_{17}H_{15}N_3O_4$ (325.32): C, 62.76; H, 4.65; N, 12.92. Found: C, 62.97; H, 4.66; N, 13.08.

Ethyl 5,7-diphenylisoxazolo[4,5-b] pyridine-6-carboxylate (11)

This compound was obtained by condensing of compound 1 with ethyl benzoylacetate. Using procedure B in the presence of $ZnCl_2$ the yield was 65%; in the presence of In $(OTf)_3$ the yield was 66%. Using the microwave method (C) the yield was 80% as colorless mp 163–164°C (from ethanol), mp 161–164°C. IR (KBr) ν : 3290, 3000 (CH, NH), 1730 (COOR), 1680, 1590 ($CONH_2$), 1210 (COOR) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 0.96 (t, J = 7.2 Hz, 3H, CH_3), 4.0 (q, J = 7.2Hz, 2H, CH_2), 7.45–7.65 (m, 10H, phenyl protons), 8.36 (br s, 2H, $CONH_2$) ppm. MS: m/z = 387 (molecular ion). Anal. Calcd. for $C_{22}H_{17}N_3O_4$ (387.39): C, 68.21; H, 4.42; N, 10.85. Found: C, 68.47; H, 4.48; N, 10.52.

Ethyl-5-chloromethyleno-7--phenylisoxazolo [4,5-b]pyridine-6--carboxylate (12)

This compound was obtained by condensing compound 1 with ethyl 2-chloroacetoacetate. Using procedure B in the presence of $ZnCl_2$ the yield was 63%; in the presence of In $(OTf)_3$ the yield was 66%. Using the microwave method (C) the yield was 70% as colorless, mp 280–282°C (from methoxyethanol). IR (KBr) ν = 3280, 3100 (CH, NH), 1760 (COOR), 1680, 1560 ($CONH_2$), 1445, 1260 (CH_2Cl) cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.05

(t, J = 7.2Hz, 3H, CH_3), 4.18 (q, J = 7.2Hz, 2H, CH_2), 4.51 (s, 2H, CH_2), 7.35–7.61 (m, 5H, phenyl protons), 8.37 (br. s, 2H, $CONH_2$) ppm. Anal. Calcd. for $C_{17}H_{14}N_3ClO_4$ (359.77): C, 56.76; H, 3.92; N, 11.68. Found: C, 56.42; H, 3.41; N, 11.56.

5-Amino-6-cyano-7-phenylisoxazolo [4,5-b]pyridine-3-carboxamide (13)[a]

This compound was obtained by condensing compound 1 with malononitrile. Using procedure B in the presence of $ZnCl_2$ the yield was 63%; in the presence of In $(OTf)_3$ the yield was 66%. Using the microwave method (C) the yield was 70% as colorless, mp 280–282°C (from methoxyethanol). IR (KBr) ν = 3280, 3100 (CH, NH), 2200 (CN), 1680, 1560 ($CONH_2$) cm^{-1}; ^1H NMR (DMSO-d_6): δ = 6.50 (br s, 2H, NH_2), 7.27–7.68 (m, 5H, phenyl protons), 8.35 (br s, 2H, $CONH_2$) ppm. Anal. Calcd. for $C_{14}H_9N_5O_2$ (279.26): C, 60.21; H, 3.25; N, 25.08. Found: C, 60.05; H, 3.41; N, 25.38.

Biology Antiproliferative Assay *in vitro*

The Compounds. The compounds 2–13 were examined in an *in vitro* screening assay. Test solutions of the compounds (1 mg/ml) were prepared *ex tempore* for each test by dissolving them in 100 μl of DMSO + 900 μl of culture medium. After that, the compounds were diluted in the culture medium (described below) to obtain final concentrations of 100, 10, 1 and 0.1 μg/ml.

The Cell Lines. Cells of the following human cancer lines were used: MES-SA (uterine carcinoma), HCV 29T (transitional epithelial cells), KB (human nasopharynx carcinoma), SW707 or LoVo (colon adenocarcinoma), MCF-7 (breast carcinoma), A549 (lung adenocarcinoma), LLC (lung cancer). All the lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and cultured in the Cell Culture Collection of the Department of Tumor Immunology at the Institute of Immunology and Experimental Therapy in Wroclaw, Poland. Human uroepithelial cell line HCV29T, established at the Fibiger Institute (Copenhagen, Denmark) was obtained from Dr. J. Kieler in 1982. The established *in vitro* murine Lewis lung cancer (LLC) cell line was also used.

Twenty-four hours before the addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at a density of 10^4 cells per well. The cells were cultured in the opti-MEM medium supplemented with 2mM glutamine (Gibco, Warsaw, Poland), streptomycin (50 μg/ml), penicillin (50U/ml) (both antibiotics from Polfa, Tarchomin, Poland) and 5% fetal calf serum (Gibco, Grand Island, USA). The cell cultures were maintained at 37°C in humid atmosphere saturated with 5% CO_2.

The SulphorodamineB (SRB) 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. [31]. The optical densities of the samples were measured on a Multiskan RC photometer (Labsystems, Helsinki, Finland) at 70 nm. The results were calculated as an inhibitory dose 50% (ID_{50}) – the dose of compound which inhibits proliferation rate of the tumor cells by 50% as compared to untreated control cells. Each compound was tested in triplicate in every concentration for each experiment. Every experiment was repeated three times.

Results and Discussion

Chemistry

The synthesis of new isoxazolo[4,5-b]pyridine derivatives 2–13 is presented in Fig. 1. 4-Amino-5-benzoylisoxazole-3-carboxamide (1) [20] was subjected to Friedländer condensation with selected carbonyl compounds containing a reactive α-methylene group, such as α-methylene ketones (acetophenone, p-bromoacetophenone), β-diketones (acetylacetone, benzoylacetone, dibenzoylomethane, 1,1,1-trifluoroacetylacetone, 1-chloro-1,1-difluoro-acetylacetone and acetylacetanilide) and β-keto esters (ethyl acetoacetate, ethyl benzoylacetate and ethyl 2-chloroacetoacetate) or malononitrile. The reactions were carried out in boiling acetic acid solution, or at higher temperatures in the presence of anhydrous $ZnCl_2$ or $In(OTf)_3$ as catalysts, or under solvent-free conditions. For comparison, these reactions were carried out using Microwave-Assisted Organic Synthesis (MAOS) in the presence of the same catalysts. In a typical case, a molar equivalent of substrates 4-amino-5-benzoylisoxazole-3-carboxamide, α-metylene compounds and catalyst were mixed and then irradiated in a microwave reactor at 240 W for several minutes as required to complete the reaction (determined by TLC).

Only heating the 4-amino-5-benzoylisoxazolo-3-carboxamide (1) with acetylacetone in refluxing acetic acid resulted in a good yield of 6-acetyl-5-methyl-7-phenylisoxazolo[4,5-b]pyridine-3-carboxamide (2) (method A). In the ^1H NMR spectrum of this compound, in addition to the signals of aromatic protons, three-proton singlets at δ = 2.14 ppm and δ = 2.60 ppm are found, representing two methyl groups. The broad signal at δ = 8.37 ppm corresponds to the protons of the amide group.

However, when o-amino ketone 1 was heated with selected α-methylene ketones, β-diketones and β-keto esters or malononitrile in boiling acetic acid, trace amounts of products 2–13 were obtained. In all cases, the starting compound 1 was isolated from the reaction mixture almost quantitatively. However, good yields of all the desired products (2–13) were obtained by heating o-amino ketone 1 with the aforementioned carbonyl compounds containing a reactive methylene group, in the presence of catalysts – $ZnCl_2$ or $In(OTf)_3$ – under solvent-free conditions. These reactions were carried out in a conventional manner, in a thermostated oil bath at 125–130°C for 8–16 hours and under microwave irradiation at 60–65°C for 15 minutes with same catalysts, under solvent-free conditions, The results are summarized in Table 1. The structures of compounds 2–13 were confirmed on the basis of spectral data and elemental analysis.

In the condensation reaction of 4-amino-5-benzoylisoxazole-3-carboxamide (1) with selected α-methylene ketones such as acetophenone, p-bromoacetophenone in the presence of catalysts $ZnCl_2$ or $In(OTf)_3$ under conventional heating (method B) or under microwave irradiation (method C), the respective substituted 7-phenylisoxazolo[4,5-b] pyridine-3-carboxamides 2–3 were obtained in good yields. The absorption bands at ν 1680 and 1580 cm^1 in the IR spectrum indicate the presence of amide groups. In the ^1H NMR spectrum of the compounds, multiplets appeared at 7.50–7.75 ppm, representing aromatic protons. The broad signals at δ 8.30 ppm correspond to protons of the amide groups.

Similary, good yields of 7-phenylisoxazolo[4,5-b]pyridine-3-carboxamides derivatives 4-7 were isolated as a result of condensing amide 1 with selected β-diketones (acetyloacetone, benzoylacetone, α-acetylacetanilide, dibenzoylmethane, 1,1,1-trifluoroacetylacetone and 1-chloro-1,1-difluoroacetylacetone), carried out by the investigated methods. The ^1H NMR spectra of compounds 4-7, apart from the signals of aromatic protons, showed a singlet at δ ~ 2.48–2.60 ppm, for two protons of the group and two broad signals for $CONH_2$. The IR spectrum of these products contains the absorption band of the carbonyl group at ~ 1700 cm^{-1} and the frequency amide I and II occurs at 1680 and 1610 cm^{-1}.

O-amino ketone 1 was then subject to Friedländer condensation under the same reaction conditions, with selected β-keto esters: ethyl acetoacetate, ethyl benzoylacetate and ethyl 2-chloroacetoacetate, to obtain cyclic product derivatives 8–12. The structures of compounds 8–12 were confirmed on the basis of spectral data and elemental analysis. For example, the IR spectrum contained strong absorption bands for the $CONH_2$ group at 1690 and 1570 cm^{-1}. The ^1H NMR spectrum showed a triplet and quartet at δ = 1.05 and δ = 4.20 ppm respectively for ethoxy protons, and a singlet at δ = 2.78 ppm for methyl protons. Aromatic protons appeared in the δ ~ 7.5–7.61 ppm range, and one broad singlet at δ=8.39 ppm corresponding to the protons of the $CONH_2$ group. Conventional heating of compound 1 with equimolar amounts of

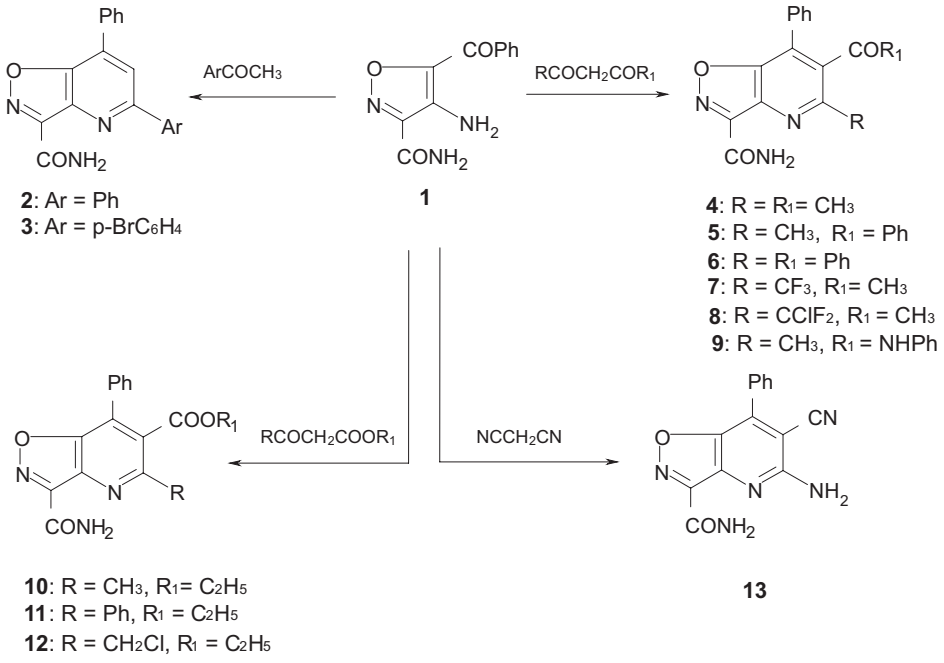

Fig. 1. Synthesis of poly-substituted isoxazolo[4,5-b]pyridines 2–13

Ryc. 1. Synteza polipodstawionych pochodnych izoksazolo[4,5-b]pirydyny 2–13

Table 1. A comparison of synthesis yields of poly-substituted isoxazolo[4,5-b]pyridine derivatives 2–13

Tabela 1. Porównanie wydajności syntez polipodstawionych pochodnych izoksazolo[4,5-b]pirydyn 2–13

No	Carbonyl compound	Product	Yields (%) / time $ZnCl_2$, In(OTf)3 (Conventional)	Yields (%)/ time $ZnCl_2$ (Microwave)
1	acetylacetone	2	75%, 76% / 10 h	79% / 10 min
2	acetophenone	3	77%, 76% / 10 h	80% / 10 min
3	p-Bromoacetophenone	4	68%, 67% / 10 h	78% / 10 min
4	benzoyloacetone	5	68%, 70% / 12 h	85% / 12 min
5	dibenzoylomethane	6	71%, 70% / 10 h	85% / 10 min
6	1,1,1-trifluoroacetylacetone	7	68%, 72% / 10 h	86% / 10 min
7	1-chloro-1,1-difluoroacetyl-acetone	8	75%, 76% / 10 h	80% / 10 min
6	acetyloacetanilide	9	67%, 76% / 12 h	80% / 12 min
7	ethyl acetoacetate	10	67%, 70% / 10 h	85% / 10 min
8	ethyl benzoylacetate	11	65%, 66% / 10 h	80% / 10 min
9	ethyl 2-chloroacetoacetate	12	63%, 66% / 10 h	70% / 10 min
7	malononitrile	13[a]	63%, 66% / 9 h	70% / 10 min

[a] Compound 13 was synthesized by Gewald and Bellmann in the presence of pyridine [22].

malononitrile in the presence of $ZnCl_2$ (method B) or $In(OTf)_3$ (method C) as catalysts, or under microwave irradiation with these catalysts (method C) led respectively to 65% and 70% yields of 5-amino-6-cyano-7-phenylisoxazolo[4,5-b]pyridine-3-carboxamide (13). These results were identical to those reported by Gewald and Bellmann [22] but they were obtained using another method. The IR spectrum of compound 13 showed a strong absorption band at 2200 cm^{-1}, which is characteristic for the CN group, and two absorption bands at 3100–3280 cm^{-1} for the NH_2 group.

Biology

Antiproliferative activity *in vitro*

The results of the experiments, expressed as ID_{50} (inhibitory dose 50%) values determined for given cancer cell lines for two compounds (5 and 6) are summarized in Table 2. The activity criterion adopted for the new compounds in the *in vitro* screening tests was an ID_{50} level not exceeding 4μg/cm^3 [32]. Only 6-benzoyl-5,7-diphenylisoxazolo[4,5-b]pyridine-3-carboxamide (6) fulfills this criterion. Compounds 5 and 6 revealed the highest cytotoxic effect *in vitro* against colon cancer cell lines; their activity was close to the international activity criterion.

Table 2. The *in vitro* cytotoxic activity (ID_{50} in μg/cm^3) of the tested compounds against various tumor cell lines

Tabela 2. Aktywność cytotoksyczna *in vitro* (ID_{50} w μg/cm^3) testowanych związków przeciw różnym liniom komórek nowotworowych

Cell line	Compound/ID50 in μg/ml	
	5	6
MES-SA	32.3 ± 1.0	–
HCV29T	50.9 ± 1.0	127.2 ± 1.30
KB	–	85.2 ± 1.25
SW707	–	3.9 ± 1.20
LoVo	9.2 ± 1.90	–
MCF-7	17.0 ± 2.21	7.2 ± 1.70
A549	–	41.8 ± 1.70
LLC	39.4 ± 1.1	–

Summarizing, the authors have described a novel application of $ZnCl_2$ and $In(OTf)_3$ as catalysts for the synthesis of new isoxazolo[4,5-b]pyridines using conventional heating and microwave irradiation under solvent-free conditions. Only the condensation reaction of compound 1 with acetylactone, conducted in a solvent, produced the desired product. In the absence of catalysts the reactions did not proceed even after a long reaction

time (48 h). Compared with conventional heating, the microwave method produced only slightly higher yields of the desired products, but in a much shorter time. It can therefore be concluded that the Friedländer condensation of 4-amino-5-benzoyl-isoxazole-3-carboxamide (1) with carbonyl compounds containing active α-methylene groups, conducted using both traditional and microwave techniques in the presence of catalysts, may be a convenient method for the synthesis of new isoxazolo[4,5-b]pyridine derivatives.

Among the compounds tested only 6-benzoyl-5,7-diphenylisoxazolo[4,5-b]pyridine-3-carboxamide (6) fulfills the international activity criterion. Compounds 5 and 6 showed the highest cytotoxic effect *in vitro* against colon cancer cell lines and their activity was close to the international criterion. These two compounds could be selected for further advanced *in vitro* studies using a larger panel of human cancer lines of different tissue origin, and *in vivo* using an experimental mouse tumor model.

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