Title: NOVEL DERIVATIVES OF A 3,4-DIHYDROISOQUINOLINE-3-CARBOXYLIC ACID HAVING ANTI-CANCER PROPERTIES, A METHOD FOR THEIR SYNTHESIS, PHARMACEUTICAL COMPOSITIONS COMPRISING SAID DERIVATIVES, AND THEIR USE

Abstract: The object of the invention is 3,4-dihydroisoquinoline derivative of structure represented by formula I, wherein R1s selected from the group consisting of H, COOEt, COOH, COOBn; R2 is selected from the group consisting of H, COOEt, COOH, COOBn; R3s selected from the group consisting of OH, OBn; R4s selected from the group consisting of OH, OBn; and isomers, salts, hydrates and solvates thereof, as well as the method of preparation thereof; pharmaceutical composition comprising the above mentioned derivative and use thereof.

Formula I

![Formula I Image]
Novel derivatives of a 3,4-dihydroisoquinoline-3-carboxylic acid having anti-cancer properties, a method for their synthesis, pharmaceutical compositions comprising said derivatives, and their use

The present invention relates to new 3,4-dihydroisoquinoline derivatives, the method of preparation thereof, pharmaceutical compositions comprising 3,4-dihydroisoquinoline derivatives and to the uses of said derivatives and said compositions.

The National Cancer Registry (Krajowy Rejestr Nowotworow) in Poland in 2010 received information about 70,024 initial notifications of malignant cancer in men and 70,540 in women (over 140 thousand cancer notifications in total). The estimation of the number of cases in 2010, taking the completeness of the register into account, indicates that the number of new cases was approximately 155 thousand. For every 100 thousand of the Polish population there are 365 cases of malignant cancers. In 2010 the number of deaths caused by malignant cancer among men was around 52 thousand, and among women around 41 thousand. For every 100 thousand of the Polish population there are 132 thereby induced deaths. In men the predominating cancers are: lung (21 percent), prostate (13 percent), colorectal (11 percent), bladder (7 percent) and stomach cancer (5 percent). The remaining 41 percent are other cancers. In case of women the major problem is: breast (23 percent), colorectal (10 percent), lung (9 percent), endometrial (7 percent), ovarian (5 percent) and cervical cancer (4 percent). The remaining cancers make up 42 percent of the cases. The authors predict that in the coming years the incidence structure will not change: the most prevalent cancers in men will remain: lung, prostate and colorectal cancer, while in women breast, colorectal, lung and endometrial cancer [Wojciechowska U., Didkowska J., Zatonski W.: Nowotwory zosliwe w Polsce w 2010 r.].

The limitations in cancer chemotherapy arise mainly from the fact that the cytostatic agents used carry very severe side effects and many cancer cells exhibit acquired or congenital resistance to the administered drugs. The cause of insensitivity to chemotherapy is believed to be the numerous changes occurring on the cellular and genetic level. Therefore it is necessary to search for new, less toxic therapeutic agents and more effective therapies [Sliwinska-Hill U., Trocha J.: Najnowsze Terapie Przeciwnowotworowe, Post. Farm. 2011, 14-19].


The aim of the present invention was to provide new active compounds exhibiting anti-proliferative activity against human cancer cell lines as well as leucyl aminopeptidase inhibitory properties, which could be used as therapeutic agents or pharmaceutical preparations in cancer treatment. A further aim of the invention is to provide a convenient method of chemical synthesis of such compounds.

Unexpectedly, this objective has been achieved with new 3,4-dihydroisoquinoline derivatives, exhibiting the above-mentioned properties.

Hence, the object of the invention is a 3,4-dihydroisoquinoline derivative of structure represented by formula I:

![Formula I](image)

wherein

- $R_1$ is selected from the group consisting of H, COOEt, COOH, COOBn
- $R_2$ is selected from the group consisting of H, COOEt, COOH, COOBn
- $R_3$ is selected from the group consisting of OH, OBn
- $R_4$ is selected from the group consisting of OH, H, OBn
- $R_5$ is selected from the group consisting of OH, OBn, wherein Bn is benzyl and Et is ethyl;

and isomers, salts, hydrates and solvates thereof.

Preferably, the compound according to the present invention is selected from such as

- 6,8-dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,8-dibenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,7,8-tribenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid dibenzyl ester
- 6,7,8-trihydroxy-3,4- dihydroisoquinoline-3-carboxylic acid

and isomers, salts, hydrates and solvates thereof.

The object of the invention is also a method of preparation of a 3,4-dihydroisoquinoline derivative of structure represented by formula I:
wherein the substituents are as defined above, characterized by the fact that it involves steps, wherein 
a) the compound of formula II 

wherein 
R₃ is OBn 
R₄ is selected from the group consisting of H or OBn 
R₅ is OBn, and the remaining substituents are as defined above 
is reacted with diethyl or dibenzyl formamide malonate, yielding a compound of formula III 

wherein 
R₃ is OBn 
R₄ is selected from the group consisting of H or OBn 
R₅ is OBn, X is selected from the group consisting of Et or Bn; and the remaining substituents are as defined above 
b) the compound of formula III is subjected to the Bischler-Napieralski cyclization reaction yielding a compound of formula IV
wherein

$R_3$ is OBn

$R_4$ is selected from the group consisting of H or OBn

$R_5$ is OBn

$X$ is selected from the group consisting of Et or Bn;

and the remaining substituents are as defined above;

and optionally

c) the compound of formula IV is subjected to removal of benzyl groups, yielding a compound of formula I
wherein

\( R_1 \) is selected from the group consisting of \( H, \text{ COOH} \)

\( R_2 \) is selected from the group consisting of \( H, \text{ COOH} \)

\( R_3 \) is \( \text{OH} \)

\( R_4 \) is selected from the group consisting of \( \text{OH}, \text{ H} \)

\( R_5 \) is \( \text{OH} \).

Preferably, the method is characterized by the fact that in step c) and c1) boron tribromide is used.

The further object of the invention is a 3,4-dihydroisoquinoline derivative of structure represented by formula I:

![Formula I](image)

wherein the substituents are as defined above, as well as isomers, salts, hydrates and solvates thereof, for use as a medicament, in particular for use in the prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.

The invention also relates to a pharmaceutical composition, characterized by the fact that it comprises a compound according to the present invention as an active agent.

The object of the invention is also the above-mentioned pharmaceutical composition for use as a medicament, in particular for use in the prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.

The composition according to the present invention is characterized by the fact that it is in liquid form, for example for oral, intranasal, rectal, intravaginal, intragastric administration in the form of a solution, syrup or elixir; and for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (as via injection) in the form of a sterile solution or emulsion, or in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.

The further object of the invention is the use of the compound according to the present invention for use in the manufacture of a medicament for use in the prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia, preferably the use, wherein the compound according to the invention is used for the manufacture of a
medicament in liquid form, such as a solution, syrup, elixir, sterile solution or emulsion, or in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.

The new 3,4-dihydroisoquinoline derivatives according to the present invention exhibit anti-proliferative activity against human cancer cell lines as well as leucyl aminopeptidase inhibitory properties. Therefore they may be used as therapeutic agents or pharmaceutical preparations in the treatment of cancer, as well as leucyl aminopeptidase inhibitors.

Due to the above properties, the compounds according to the invention may be used as therapeutic agents, in the form of pharmaceutical preparations, in particular in the treatment of cancer. The compounds according to the present invention exhibit a substantially strong activity against prostate cancer, colorectal cancer and leukemia. Therefore, they may be applied in a treatment for these cancer types.

The 3,4-dihydroisoquinoline-3-carboxylic acid derivatives were obtained in a several-step synthesis, where the key steps were the alkylation reaction with the use of benzyl or ethyl formamide malonate and the Bischler-Napieralski cyclization (Bischler, A.; Napieralski, B.: A new method for the synthesis of isoquinolines Chem. Ber. 1893, 26, 1903-1908). The use of boron tribromide in the last step allowed for the removal of all protecting groups and shortening the synthesis. The products were purified with standard methods.

The chemical synthesis of the compounds according to the invention, as well as their physicochemical properties and biological assays are provided in the examples.

Examples

Example 1

Preparation of 6,8-dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester

\[ \text{Diethyl formamide malonate (20.8 g, 100 mmol) was added to the solution of 3,5-dibenzyl oxybenzyl chloride (34 g, 100 mmol) in acetone (500 ml), followed by K}_2\text{CO}_3 (186 g, 1.333 mol) and KI (49.9 g, 301 mmol). The reaction was conducted under anhydrous conditions, with vigorous stirring and heating (bath temperature 60 °C) under reflux condenser for 35 hours. After this time the mixture was cooled and filtered} \]
through Celite. Subsequently, the solvent was evaporated and the product crystallized from hexane. 47.5 g of the product (94%) was obtained.

$^1$H NMR (CDCl$_3$) δ: 1.27 (t, J=7.1 Hz, 6H); 3.56 (s, 2H); 4.14-4.32 (m, 4H); 5.0 (s, 4H); 6.24 (d, J=2.2 Hz, 2H); 6.53 (t, J=2.3Hz, 1H); 6.56 (bs, 1H); 7.28-7.44 (m, 10H); 7.92 (d, J=0.8Hz, 1H).

$^{13}$C NMR (CDCl$_3$) δ: 13.9; 38.0; 62.8; 66.5; 69.9; 101.1; 109.2; 127.3; 127.9; 128.6; 136.8; 136.9; 159.7; 159.8; 166.9.

HR MS ESI calculated for C$_{36}$H$_{41}$N$_{6}$O$_{6}$ (M+H) 506.2173. Measured 506.2164.

1,2,3,4-Dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester 3

POCl$_3$ (0.45 ml) was added to the solution of 3,5-dibenzyloxybenzyl-2-(formylamide) propanedioic acid diethyl ester (0.8 g, 1.58 mmol) in acetonitrile (40 ml). The reaction was conducted under argon atmosphere, with stirring and heating (bath temperature 75°C) under reflux condenser for 48 hours. After this time the solvent was evaporated on a rotary evaporator and a mixture of NaHCO$_3$ (20 ml) and CH$_2$Cl$_2$ (20 ml) was added. The mixture was extracted with CH$_2$Cl$_2$ (3 x 15 ml). The organic layers were combined and dried with anhydrous MgSO$_4$. After separation from the drying agent the solvent was evaporated. The product was crystallized from a mixture of AcOEt : hexane, (1:1). 0.64 g (83%) was obtained.

$^1$H NMR (CDCl$_3$) δ: 1.24 (t, J=7.1 Hz, 6H); 3.3 (s, 2H); 4.13-4.32 (m, 4H); 5.0 (s, 2H); 5.1 (s, 2H); 6.41 (d, J=1.6 Hz, 1H); 6.44 (d, J=2.0Hz, 1H); 7.29-7.46 (m, 10H); 8.86 (d, J=0.5Hz, 1H).

$^{13}$C NMR (CDCl$_3$) δ: 13.9; 31.5; 62.1; 69.8; 70.2; 99.1; 105.8; 111.4; 127.0-128.7; 136.1; 136.2; 136.9; 157.4; 158.1; 162.8; 169.3.

HR MS ESI calculated for C$_{36}$H$_{43}$O$_{10}$ (M+H) 488.2068. Measured 488.2060.

1,3,4-Dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester 4

BB$_3$ (0.05 ml, 0.54 mmol) was added dropwise to the solution of 6,8-dibenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester 3 (120 mg, 0.25 mmol) in CH$_2$Cl$_2$ (20 ml) at -70°C. The reaction was conducted under argon atmosphere at -70°C for 0.5 hour. After this time H$_2$O (20 ml) was added to the mixture and the whole mixture was stirred for 0.5 hour. Next, the mixture was washed 3 times with CH$_2$Cl$_2$. The obtained product was purified on Biogel P-2, eluting with 0.1 % TFA solution in H$_2$O. 63 mg (83%) of the product was obtained.

$^1$H NMR (D$_2$O) δ: 1.26 (t, J=7.1Hz, 6H); 3.65 (s, 2H); 4.34 (q, J=7.1Hz, 4H); 6.32 (d, J=2.1Hz, 1H); 6.48 (dt, J=2.1Hz, 1.0Hz, 1H); 8.9 (d, J=0.8Hz, 1H).

$^{13}$C NMR (D$_2$O) δ: 12.9; 31.3; 65.1; 66.1; 101.3; 105.2; 109.9; 138.0; 158.7; 165.3; 165.9; 170.1.

HR MS ESI calculated for (M+H) C$_{16}$H$_{18}$N$_{6}$O$_{6}$ 308.1 129. Measured 308.1 120.
Example 2

Preparation of 6,7,8-trihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid

[Diagram]

2.1. 3,4,5-tribenzyloxybenzyl-2-(formylamide) propanedioic acid dibenzyl ester 6

Dibenzyl formamide malonate (7.4 g; 22.02 mmol) was added to the solution of 3,4,5-tribenzyloxybenzyl chloride 5 (10 g, 22.02 mmol) in acetone (100 ml), followed by K₂CO₃ (41 g, 293 mmol) and KI (10 g, 22.02 mmol). The reaction was conducted under anhydrous conditions, with vigorous stirring and heating (bath temperature 60 °C) under reflux condenser for 24 hours. After this time the mixture was cooled and filtered through Celite. Next, the solvent was evaporated and the product was crystallized from hexane. 13.87 g of the product (86%) was obtained.

¹H NMR (CDCl₃) δ: 3.54 (s, 2H); 4.96 (s, 4H); 5.04 (s, 4H); 5.10 (s, 2H); 6.20 (s, 2H); 6.37 (bs, 1H); 7.16-7.46 (m, 25H); 7.72 (d, J=1.2Hz, 1H).

¹³C NMR (CDCl₃) δ: 38.0; 66.7; 68.1; 70.9; 75.0; 109.8; 127.1-128.6; 129.9; 134.5; 137.1; 137.7; 152.4; 159.8; 166.5.

HR MS ESI calculated for C₄₆H₄₀NO₇ (M+H) 718.2799. Measured 718.2793.

2.2. 6,7,8-trihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid dibenzyl ester 7

POCl₃ (2.52 ml) was added to the solution of 3,4,5-tribenzyloxybenzyl-2-(formylamide) propanedioic acid dibenzyl ester (6.48 g, 8.81 mmol) in acetonitrile (70 ml). The reaction was conducted under argon atmosphere, with stirring and heating (bath temperature 75 °C) under reflux condenser for 24 hours. After this time the solvent was evaporated on a rotary evaporator and a mixture of NaHC0₃ (20 ml) and CH₂Cl₂ (20 ml) was added. The mixture was extracted with CH₂Cl₂ (3 x 15 ml). The organic layers were combined and dried with anhydrous MgSO₄. After separation from the drying agent the solvent was evaporated. The product was crystallized from a mixture of AcOEt : hexane, (1:3). 5.6 g (89%) was obtained.

¹H NMR (CDCl₃) δ: 3.27 (s, 2H); 5.01 (s, 2H); 5.03 (s, 2H); 5.08 (s, 2H); 5.10 and 5.19 (AB, J=1.2Hz, 4H); 6.53 (s, 1H); 7.17-7.44 (m, 25H); 8.73 (s, 1H).

¹³C NMR (CDCl₃) δ: 29.7; 31.1; 67.6; 70.4; 70.9; 75.6; 108.3; 115.5; 127.4-128.7; 130.1; 135.2; 136.0; 136.6; 137.1; 140.3; 151.7; 156.3; 158.2; 168.9.

HR MS ESI calculated for C₄₆H₄₀NO₇ (M+H) 718.2799. Measured 718.2793.
2.3. 6,7,8-trihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid 8

BBR₃ (1.75 ml, 17.97 mmol) was added dropwise to the solution of 6,7,8-tribenzylxoxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid dibenzyl ester (2.15 g, 3.0 mmol) in CH₂Cl₂ (60 ml) at -20 °C. The reaction was conducted under argon atmosphere at -20 °C for 0.5 hour. After this time a solution of 10% HCl (20 ml) was added to the mixture and the whole mixture was stirred for 0.5 hour. Next, the mixture was washed 3 times with CH₂Cl₂. The product was crystallized from H₂O. 0.6 g of the product (90%) was obtained.

¹H NMR (D₂O) δ: 3.00 and 3.10 (ABX, J= 0.7Hz, 8.6Hz, 17.2Hz, 2H); 4.59 (ddd, J=8.6Hz, 7.3Hz, 1.2Hz, 2H); 6.21 (s, 1H); 8.6 (s, 1H).

¹³C NMR (D₂O) δ: 27.3; 53.2; 105.8; 108.7; 129.8; 130.5; 151.6; 156.8; 159.5; 171.0.

HR MS ESI calculated for (M+H) C₁₀H₁₀NO₅ 224.0559. Measured: 224.0594.

**Example 3**


**Reagents:**

1. Enzyme: pig kidney microsomal leucyl aminopeptidase, Type IV-S (Sigma-Aldrich) 3.5 mg of protein/ml, 28 U/mg of protein; diluted with 0.02M Tris·HCl + 0.5 mM CaCl₂ buffer pH=8.5 until activity 0.01 225U achieved

2. Substrate: L-leucine p-nitroanilide: 4.2 mg / 269 μl DMSO; solution diluted 1:9 with 0.02 M Tris·HCl buffer, pH = 8.5 + 0.5 mM CaCl₂

3. 0.02 M Tris·HCl buffer, pH = 8.5 + 0.5 mM CaCl₂

4. DMSO, inhibitor solution in DMSO

**Assay procedure:**

5 μl of leucyl aminopeptidase (0.245 U), 70 μl of Tris, 5 μl of inhibitor solution in different concentrations (in DMSO) were added to the wells of a microtitration plate, followed by pre-incubation for 30 minutes at 37 °C. Next, 20μl of diluted L-leucine p-nitroanilide solution was added, whereas the reaction mixture was incubated for 20 minutes at 37 °C, with measurement of absorbance changes for wavelength 405 nm (Fluorostar, Omega, BMG Labtech). The control sample for the enzyme contained analogous components, while instead of the inhibitor solution DMSO (5 μl) was added.

For each inhibitor concentration the percentage inhibition was calculated. The inhibitor concentration required for 50% of enzyme activity inhibition (IC₅₀) was calculated based on linear regression data between the percentage inhibition and the concentration for a given inhibitor.

IC₅₀ for compound 3 is 0.028 mM, for 4 it is 1.35 mM; for 8 it is 3.8 mM.

**Example 4**

a) colorectal cancer LoVo,
b) colorectal cancer LoVo/DX (with multidrug resistance),
c) breast cancer MCF-7,
d) prostate cancer LNCaP,
e) bladder cancer HCV29T,
f) kidney cancer A498,
g) lung cancer A549

4.2. Anti-proliferative activity evaluation using the MTT test [Marcinkowska et al.: J. Steroid Biochem. Mol. 76: 71-78, 1998] against human cancer cell lines with diverse tissue origins and determination of the IC_{50} concentration:
a) promyelocytic leukemia HL-60,
b) promyelocytic leukemia HL-60/MX2 (with multidrug resistance)

4.3. Cytotoxic activity evaluation against normal cell lines with diverse tissue origins using the SRB test:
a) normal mouse fibroblasts (BALB/3T3),
b) normal mammary gland cells (MCF-10),
c) normal human epithelium (HLMEC)

Preparations

The stock solution of the tested compound with concentration of 20 mg/ml was prepared day before the analysis by dissolving the preparation in DMSO, followed by storage in -20°C. After 24 hours the preparation was thawed and diluted with the culture medium. The compounds were tested in concentrations of 100, 10, 1, 0.1, 0.01 µg/ml.

Cell lines

The following human cancer cell lines were used in the study: HL-60 (promyelocytic leukemia), HL-60/MX2 (promyelocytic leukemia, line resistant to mitoxantrone), MCF7 (mammary gland cancer), LoVo (colorectal cancer), LoVo/Dx (colorectal cancer with multidrug resistance), HCV29T (bladder cancer), A498 (kidney cancer), A549 (lung cancer) and LNCaP (prostate cancer), as well as normal cell lines: BALB3T3 (normal mouse fibroblasts), MCF-10 (normal mammary gland cells) and HLMEC (normal human endothelium).

The lines are in the cell line bank of The Polish Academy of Sciences Institute of Immunology and Experimental Therapy (Instytut Immunologii i Terapii Doswiadczalnej PAN) (obtained from the ATCC collection). The composition of culture media is summarized in Table 1. All media contained antibiotics: 100 µg/ml streptomycin and 100 U/ml penicillin. The cells were cultured in humidified atmosphere 5% CO₂ at 37°C.
<table>
<thead>
<tr>
<th>No.</th>
<th>Cell line</th>
<th>Medium</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB3T3</td>
<td>Dulbecco</td>
<td>10% FBS, L-glutamine [2 mM]</td>
</tr>
<tr>
<td>2</td>
<td>MCF-10A</td>
<td>Ham’s F12 Nutrient Mix with glutamine</td>
<td>5% HS (Gibco), Insulin [0.01 mg/ml], EGF [20 ng/ml], hydrocortisone [0.5 μg/ml], cholera toxin [0.05 μg/ml]</td>
</tr>
<tr>
<td>3</td>
<td>HLMEC</td>
<td>RPMI-1640</td>
<td>10% FBS (HyClone), L-glutamine [2 mM]</td>
</tr>
<tr>
<td>4</td>
<td>HL60,</td>
<td>RPMI-1640</td>
<td>10% FBS, L-glutamine [4 mM], sodium pyruvate [1 mM], glucose [4.5 g/l]</td>
</tr>
<tr>
<td></td>
<td>HL60MX2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MCF7</td>
<td>Eagle</td>
<td>10% FBS, L-glutamine [2 mM], amino acids, insulin [0.01 mg/ml]</td>
</tr>
<tr>
<td>6</td>
<td>Lovo</td>
<td>OptiMEM +RPMI(1:1)</td>
<td>5% FBS (HyClone), L-glutamine [2 mM], sodium pyruvate [1 mM]</td>
</tr>
<tr>
<td>7</td>
<td>Lovo/Dx</td>
<td>OptiMEM +RPMI(1:1)</td>
<td>5% FBS (HyClone), L-glutamine [2 mM], sodium pyruvate [1 mM], doxorubicin [10 µg/100 ml]</td>
</tr>
<tr>
<td>8</td>
<td>HCV29T</td>
<td>OptiMEM +RPMI(1:1)</td>
<td>5% FBS(HyClone), L-glutamine [2 mM]</td>
</tr>
<tr>
<td>9</td>
<td>A498</td>
<td>OptiMEM +RPMI(1:1) + G.MAX</td>
<td>5% FBS (HyClone), sodium pyruvate [1 mM]</td>
</tr>
</tbody>
</table>
Cytotoxicity assays

The cells in the phase of logarithmic growth were seeded on a 96-well plate (Sarsted) in quantity of 1 x 10^4 cells/well, and then incubated at 37°C, 5% CO₂ for 24 hours. Subsequently, the tested compound was added in different concentrations and the incubation was carried out. The assays measured the inhibition of target cells proliferation in a 72-hour culture in vitro. For each assay the samples containing predetermined preparation concentrations were applied in triplicates. The experiments were repeated 3-5 times.

The MTT method

The method involves a measurement of cell number increase inhibition utilizing the redox activity of mitochondria. The determination of biological activity with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is based on the assumption that reduction of the dye occurs only in viable cells. As a result of mitochondrial dehydrogenase activity the water-soluble tetrazolium salt (thiazoly blue formazan - MTT) is converted to water-insoluble formazan crystals. The quantity of the formed formazan crystals is proportional to the number of cells.

3-4 hours before the end of cell incubation 20 μl of MTT solution (5 mg/ml) was added to each well. Next, 80 μl of dissolving mixture (with the following composition : 225 ml DMF (POCh), 67.5 g SDS (Sigma-Aldrich), 275 ml distilled H₂O) was added. After 24 hours, formazan crystals were dissolved and OD of the samples was measured for wavelength 570 nm (Multiskan PC photometer, Labsystem, Helsinki).

The SRB method

Sulphorhodamine B (SRB) is an anionic dye which binds to essential amino acids of cellular proteins. The determination of cytotoxic activity in the assay is based on the measurement of the amount of cellular proteins.

After the incubation is finished the attached cells are fixed with cold 50% TCA (4°C) at 4°C for 1 hour. Next, each well is washed with water and air dried, repeating this step 5 times.

0.4% SRB solution (Sigma-Aldrich) dissolved in 1% acetic acid is added consecutively to each well and the staining is carried out for 30 minutes. The unbound dye is removed and the cells are washed 4 times with 1% acetic acid. Next, the plate is air dried for about 5 minutes.

The bound dye is dissolved by adding 10 mM Tris-base to each well and then OD is determined for wavelength 540 nm (Multiscan RC photometer, Labsystes, Helsinki).
Results

The results of the assays in the form of IC50 (the concentration causing proliferation inhibition of 50% of a cancer cells population) determined for the tested compounds and cisplatin are summarized in Table 2 [Geran RI et al.: Cancer Chemotherapy Reports, 3, 2 (part3) : 59-61 , 1972].

DMSO, which is a solvent for the compound, caused proliferation inhibition only in the highest concentration: MCF-7 - 3.1%, Balb3T3 - 23%, Lovo - 62%, Lovo/Dx - 0%, HCV29T - 17%, A549 - 8%, A498 - 29% and LNCaP - 37% respectively. Since the IC50 values of the tested compound are lower than 100 µg/ml this did not influence the arithmetically calculated IC50 value.

Table 2

Anti-proliferative activity expressed as IC50 value for a compound against cancer cells and cytotoxic activity against normal cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound 3 IC50 (µg/ml)</th>
<th>Compound 8 IC50 (µg/ml)</th>
<th>Cisplatin IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/3T3 (normal mouse fibroblasts)</td>
<td>17.36 +/- 7.9</td>
<td>52.5 +/- 22.3</td>
<td>2.4 +/- 0.3</td>
</tr>
<tr>
<td>MCF-10 (normal mammary gland cells)</td>
<td>15.3</td>
<td>18.9 +/- 11.4</td>
<td>0.7</td>
</tr>
<tr>
<td>HLMEC (normal human epithelium)</td>
<td>3.3 +/- 0.3</td>
<td>33.4 +/- 5.0</td>
<td>2.6 +/- 0.3</td>
</tr>
<tr>
<td>HL-60 (promyelocytic leukemia)</td>
<td>0.12</td>
<td>27.7 +/- 4.3</td>
<td>0.4 +/- 0.1</td>
</tr>
<tr>
<td>HL-60 MX2 (promyelocytic leukemia with multidrug resistance)</td>
<td>1.3</td>
<td>33.2 +/- 2.7</td>
<td>0.32 +/- 0.02</td>
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<td>MCF-7 (breast cancer)</td>
<td>4.7 +/- 0.8</td>
<td>18.9 +/- 11.4</td>
<td>3.6 +/- 0.6</td>
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<td>Lovo (colorectal cancer)</td>
<td>3.1 +/- 0.3</td>
<td>33.6 +/- 9.5</td>
<td>2.5 +/- 1.3</td>
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<td>Lovo/Dx (colorectal cancer with multidrug resistance)</td>
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<td>30.1 +/- 2.3</td>
<td>3.0 +/- 0.7</td>
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<td>LNCaP (prostate cancer)</td>
<td>1.6 +/- 0.3</td>
<td>30.9 +/- 5.7</td>
<td>6.2 +/- 1.3</td>
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<td>HCV29T (bladder cancer)</td>
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<td>28.7 +/- 7.2</td>
<td>2.7 +/- 0.6</td>
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<td>A549 (lung cancer)</td>
<td>6.4 +/- 2.3</td>
<td>44.3 +/- 6.7</td>
<td>3.2 +/- 1.0</td>
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<td>A498 (kidney cancer)</td>
<td>6.5 +/- 1.5</td>
<td>40.0 +/- 18.4</td>
<td>2.8 +/- 0.6</td>
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</table>
1. 3,4-dihydroisoquinoline derivative of structure represented by formula I:

![Formula I](image)

wherein

- \( R_1 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_2 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_3 \) is selected from the group consisting of OH, OBn
- \( R_4 \) is selected from the group consisting of OH, H, OBn

and isomers, salts, hydrates and solvates thereof.

2. The compound according to claim 1, selected from such as:

- 6,8-dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,8-dibenzylxoy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,7,8-tribenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid dibenzyl ester
- 6,7,8-trihydroxy-3,4- dihydroisoquinoline-3-carboxylic acid

and isomers, salts, hydrates and solvates thereof.

3. A method of preparation of 3,4-dihydroisoquinoline derivative of structure represented by formula I:

![Formula I](image)

wherein the substituents are as defined above,

**characterized in that** it involves steps, wherein

a) the compound of formula II
wherein

$R_3$ is OBn

$R_4$ is selected from the group consisting of H or OBn

$R_5$ is OBn,

and the remaining substituents are as defined above

is reacted with diethyl or dibenzyl formamide malonate, yielding a compound of formula II

$$\text{Formula II}$$

wherein

$R_3$ is OBn

$R_4$ is selected from the group consisting of H or OBn

$R_5$ is OBn,

$X$ is selected from the group consisting of Et or Bn;

and the remaining substituents are as defined above

b) the compound of formula II is subjected to the Bischler-Napieralski cyclization reaction yielding a compound of formula IV

$$\text{Formula IV}$$

wherein

$R_3$ is OBn

$R_4$ is selected from the group consisting of H or OBn

$R_5$ is OBn
X is selected from the group consisting of Et or Bn;
and the remaining substituents are as defined above;
and optionally

c) the compound of formula IV is subjected to removal of benzyl groups, yielding a compound of formula I

![Chemical Structure](image)

Formula I

wherein

- $R_1$ is selected from the group consisting of COOEt, COOBn
- $R_2$ is selected from the group consisting of COOEt, COOBn
- $R_3$ is OH
- $R_4$ is selected from the group consisting of OH, H
- $R_5$ is OH

or

1) the compound of formula IV is subjected to removal of benzyl groups under acidic conditions, yielding a compound of formula I

![Chemical Structure](image)

Formula I

wherein

- $R_1$ is selected from the group consisting of H, COOH
- $R_2$ is selected from the group consisting of H, COOH
- $R_3$ is OH
- $R_4$ is selected from the group consisting of OH, H
- $R_5$ is OH.

4. The method according to claim 3, characterized in that in step c) and c1) boron tribromide is used.

5. The compound according to claim 1 for use as a medicament.
6. The compound according to claim 1 for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.

7. A pharmaceutical composition characterized in that it comprises the compound according to claim 1 as an active agent.

8. The composition according to claim 7 for use as a medicament.

9. The composition according to claim 7 or 8 for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.

10. The composition according to any of claims 7-9, characterized in that it is in liquid form, for example for oral, intranasal, rectal, intravaginal, intragastric administration in the form of a solution, syrup or elixir; and for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (as via injection) in the form of a sterile solution or emulsion, as well as in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.

11. Use of the compound according to claim 1 in the manufacture of a medicament for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.

12. The use according to claim 11, wherein the compound according to claim 1 is used in the manufacture of a medicament in liquid form, such as a solution, syrup, elixir, sterile solution or emulsion, or in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.
1. 3,4-dihydroisoquinoline derivative of structure represented by formula I:

\[
\text{Formula I}
\]

wherein

- \( R_1 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_2 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_3 \) is selected from the group consisting of OH, OBn
- \( R_4 \) is selected from the group consisting of OH, H, OBn
- \( R_5 \) is selected from the group consisting of OH, OBn,

with the proviso that if each of \( R_1, R_2 \) and \( R_4 \) is H then at least one of \( R_3 \) and \( R_5 \) is OH, and isomers, salts, hydrates and solvates thereof.

2. The compound according to claim 1, selected from:

- 6,8-dihydroxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,8-dibenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid diethyl ester
- 6,7,8-tribenzyloxy-3,4-dihydroisoquinoline-3,3-dicarboxylic acid dibenzyl ester
- 6,7,8-trihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid

and isomers, salts, hydrates and solvates thereof.

3. A method of preparation of 3,4-dihydroisoquinoline derivative of structure represented by formula I:

\[
\text{Formula I}
\]

wherein

- \( R_1 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_2 \) is selected from the group consisting of H, COOEt, COOH, COOBn
- \( R_3 \) is selected from the group consisting of OH, OBn

**AMENDED SHEET (ARTICLE 19)**
R₄ is selected from the group consisting of OH, H, OBn
R₅ is selected from the group consisting of OH, OBn,

characterized in that it involves steps, wherein

a) the compound of formula II

\[
\begin{array}{c}
\text{R}3 \quad \text{Cl} \\
\text{R}4 \\
\text{R}5
\end{array}
\]

Formula II

wherein

R₃ is OBn
R₄ is selected from the group consisting of H or OBn
R₅ is OBn,

and the remaining substituents are as defined above

is reacted with diethyl or dibenzyl formamide malonate, yielding a compound of formula III

\[
\begin{array}{c}
\text{R}3 \\
\text{R}4 \\
\text{R}5 \\
\text{COOX} \\
\text{HN} \\
\text{CHO}
\end{array}
\]

Formula III;

wherein

R₃ is OBn
R₄ is selected from the group consisting of H or OBn
R₅ is OBn
X is selected from the group consisting of Et or Bn;

and the remaining substituents are as defined above

b) the compound of formula III is subjected to the Bischler-Napieralski cyclization reaction yielding a compound of formula IV

\[
\begin{array}{c}
\text{R}3 \\
\text{R}4 \\
\text{R}5 \\
\text{COOX} \\
\text{COOX}
\end{array}
\]

Formula IV

AMENDED SHEET (ARTICLE 19)
wherein

\( R_3 \) is OBn

\( R_4 \) is selected from the group consisting of H or OBn

\( R_5 \) is OBn

\( X \) is selected from the group consisting of Et or Bn;

and the remaining substituents are as defined above;

and optionally

c) the compound of formula IV is subjected to removal of benzyl groups, yielding a compound of formula I

\[
\text{Formula I}
\]

wherein

\( R_1 \) is selected from the group consisting of COOEt, COOBn

\( R_2 \) is selected from the group consisting of COOEt, COOBn

\( R_3 \) is OH

\( R_4 \) is selected from the group consisting of OH, H

\( R_5 \) is OH

or

c1) the compound of formula IV is subjected to removal of benzyl groups under acidic conditions, yielding a compound of formula I

\[
\text{Formula I}
\]

wherein

\( R_1 \) is selected from the group consisting of H, COOH

\( R_2 \) is selected from the group consisting of H, COOH

\( R_3 \) is OH
4. The method according to claim 3, characterized in that step c) and c1) boron tribromide is used.
5. The compound according to claim 1 for use as a medicament.
6. The compound according to claim 1 for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.
7. A pharmaceutical composition characterized in that it comprises the compound according to claim 1 as an active agent.
8. The composition according to claim 7 for use as a medicament.
9. The composition according to claim 7 or 8 for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.
10. The composition according to any of claims 7-9, characterized in that it is in liquid form, for example for oral, intranasal, rectal, intravaginal, intragastric administration in the form of a solution, syrup or elixir; and for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (as via injection) in the form of a sterile solution or emulsion, as well as in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.
11. Use of the compound according to claim 1 in the manufacture of a medicament for use in prevention, treatment and amelioration of symptoms of conditions associated with excessive activity of leucyl aminopeptidase and the conditions associated with excessive cell proliferation, such as cancer, in particular prostate cancer, colorectal cancer or leukemia.
12. The use according to claim 11, wherein the compound according to claim 1 is used in the manufacture of a medicament in liquid form, such as a solution, syrup, elixir, sterile solution or emulsion, or in solid form, such as a tablet, pill, capsule, granule, coated tablet and powder.
STATEMENT UNDER ARTICLE 19 (1)

Applicant submit herewith an Amendment under Article 19 in which the differences between the claims as filed and the claims as amended are as follows:

Claim 1 - in line 9 the expression "with the proviso that if each of R₁, R₂ and R₄ is H then at least one of R₃ and R₅ is OH," is added in order to disclaim the compound disclosed in document D₁ and to render novelty of the subject matter of claim 1.

Claim 2 - in line 1 the expression "such as" is deleted for the sake of clarity.

Claim 3 - in line 3 instead of the expression "wherein the substituents are as defined above", which is deleted, definitions of the substituents are added for the sake of clarity as follow:

"wherein

R₁ is selected from the group consisting of H, COOEt, COOH, COOBn
R₂ is selected from the group consisting of H, COOEt, COOH, COOBn
R₃ is selected from the group consisting of OH, OBn
R₄ is selected from the group consisting of OH, H, OBn
R₅ is selected from the group consisting of OH, OBn,"

Claims 4 to 12 - unchanged.

The amendments are fully supported by the application as filed and no new matter has been introduced.

The present amendments have no impact on the description of the International Application.
## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

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<th>A61K31/47</th>
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**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
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<td>X</td>
<td>JAEBONG JANG ET AL: &quot;Asymmetric c formal synthesis of schul zeines A and C&quot;, ORGANIC &amp; BIOMOLECULAR CHEMISTRY, vol. 10, no. 27, 1 January 2012 (2012-01-01), page 5202, XP055147868, ISSN: 1477-0520, DOI: 10.1039/c2ob25772f compound 7 in Scheme 1 on page 5203</td>
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**Further documents are listed in the continuation of Box C.**

**See patent family annex.**

- **A** document defining the general state of the art which is not considered to be of particular relevance
- **E** earlier application or patent but published on or after the international filing date
- **L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **O** document referring to an oral disclosure, use, exhibition or other means
- **P** document published prior to the international filing date but later than the priority date claimed
- **T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- **X** document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- **Y** document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- **S** document member of the same patent family

**Date of the actual completion of the international search**

23 October 2014

**Date of mailing of the international search report**

04/11/2014

**Name and mailing address of the ISA**

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax. (+31-70) 340-3016

**Authorized officer**

Guspanova, Jana

Form PCT/ISA/210 (second sheet) (April 2005)
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<td>Y</td>
<td>F. N. ALVAREZ ET AL: &quot;6,7-Di hydroxy-3,4-Di hyrodi soquinol i ne: A Novel Inhibi tor of Nuclear Factor-kappaB and in vitro Invasion on Muri ne Mammary Cancer Cells&quot;, CHEMOTHERAPY, vol. 55, 30 April 2009 (2009-04-30), pages 175-182, XP009180893, cited in the application on compound 7 on page 176; figure 3</td>
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<td>5 March 1992 (1992-03-05) Tabl e on page 11; page 1, paragraph 1; claims 1,9,12-16; examples 1-8,21 ,22</td>
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<td>US 2010/267767 AI (NARAYANAN RAMESH [US] ET AL) 21 October 2010 (2010-10-21) page 2, paragraph 10 page 5, paragraph 71 - page 6, paragraph 77 compounds of exampl es; page 52, paragraph 464; exampl es 31-35</td>
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<td>WO 9203419</td>
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