Synthesis and Antiproliferative Activity *in Vitro* of Novel 1,5-Benzodiazepines. Part II[1)]

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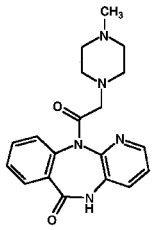
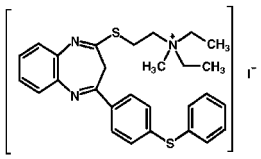
Summary

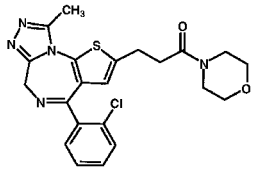
The reaction of 2,2,4-trimethyl-1*H*-2,3-dihydro-1,5-benzodiazepine (**1**) with cinnamoyl chloride leading to the formation of 1-cinnamoyl derivative **2** is described. Two novel benzodiazepines, 2,2,4-trimethyl-1*H*-2,3,4,5-tetrahydro-1,5-benzodiazepine (**3**) and 1-cinnamoyl-2,2,4-trimethyl-1*H*-2,3,4,5-tetrahydro-1,5-benzodiazepine (**4**), were synthesized by the reduction of **1** and **2** using $NaBH_4$ in i-PrOH and two other derivatives **5** and **6** were obtained by reaction of **4** with equimolar and dimolar quantity of cinnamoyl chloride, respectively. The structures of **1–6** were confirmed by analytical and spectral data (IR, ^1H NMR, and MS). 7-Carboxy-2,2,4-trimethyl-1*H*-2,3-dihydro-1,5-benzodiazepine (**7**) was synthesized and its crystals were subjected to X-ray analysis. Benzodiazepines **1**–**6** were evaluated for antiproliferative activity *in vitro*. Among the compounds tested, **4–6** exhibited cytotoxic activity against human cancer cell lines, namely SW707 (colon cancer), MCF-7 (breast cancer), A549 (lung cancer), and HCV29T (bladder cancer).



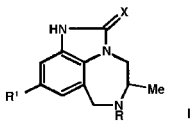
Pirenzepine



Tibenzonium Iodide



Apafant



TIBO

Introduction

Substituted or tricyclic derivatives of benzodiazepines belong to the most abundant and still growing group of the anxiolytic drugs. In recent years the synthesis and biological studies of benzodiazepines derivatives or diazepines condensed with heterocyclic rings have confirmed their diverse pharmacological properties.

The tricyclic derivative pirenzepine [1] acts selectively as a muscarinic receptor (M1) antagonist. Derivatives described in a patent [2] and in a paper [3] show similar properties.

Tibenzonium iodide [4,5] is an example of benzodiazepine with antibacterial activity against *Streptococus pyogenes* A88 and also virucidal. Thienotriazolodiazepine derivative applied as Apafant acts as the platelet activating factor (PAF) inhibitor [5], while benzodiazepines also possess an antiaggregating activity towards the blood platelets [6–9]. Novel antithrombotic drugs have been recently obtained by placing the essential functional groups of the thrombin antagonist MD-805 into the benzodiazepine nucleus [10]. TIBO has been found to be the most active inhibitor of the HIV-1 virus, comparable to AZT (Zidovudine) [11]. Benzodiazepines, also non-nucleoside inhibitors of HIV-1 reverse transcriptase, have been described in several papers and patents [12–17].

Benzodiazepines with analgesic and/or antiinflammatory activities [18], antinflammatory ulcer inhibitors [19], salidiuretic activity [20] and oxytocin antagonists [21], antidiabetic [22], antileukemic [23], cholecystokinin A (CCK-A) receptor antagonistic activity [24], and orally active CCK-A agonists [25] have been synthesized.

Benzodiazepine and diazepine derivatives condensed with heterocyclic or heteroaromatic structure have been found and identified in various plants and fungi, commonly as alkaloids. This class of natural compounds shows diverse biological activity encouraging to syntheses of their structural analogs of similar pharmacological activity but of lower toxicity.

Auranthine was discovered as an alkaloid metabolite of *Penicillinum aurotiogriseum* [26]. Benzomalvines were isolated from broth culture of *Penicillium sp.* Benzomalvin A is anhibitor of substance P [27].

Pentostatin [5], adenosine deaminase inhibitor, was isolated from *Streptomyces antibioticus* and structurally identified by Woo [28].

[1)] Part I: W. Nawrocka, B. Sztuba, M. Rutkowska, J. Barczynska, A. Opolski, J. Wietrzyk, *Acta Polon. Pharm.*, **1998,** *55*, 397–402.

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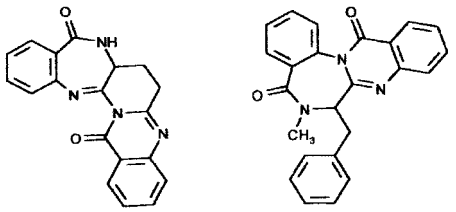
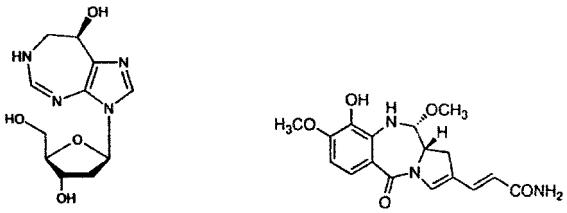


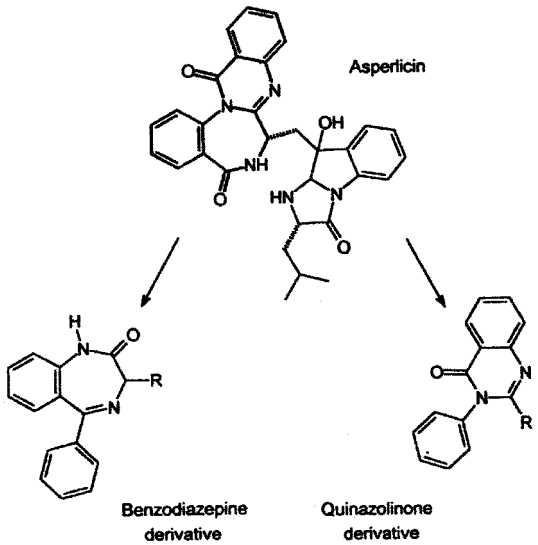
Auranthine Benzomalvine

A group of natural compounds called pyrrolobenzodiazepines (PDBs) shows interesting cytotoxicity profiles but their clinical use has been limited because of various toxicity problems, e.g. anthramycin, which reveals broad antitumor activity *in vivo* against a variety of transplanted tumors, is unfortunately highly cardiotoxic [29–31].



Pentostatin Anthramycin

Benzodiazepinic and quinazolinic compounds derived from Asperlicin, an alkaloid of *Asperillus alliacens* [32], have been reported as selective cholecystokinin (CCK) subtype B ligands [33–38].



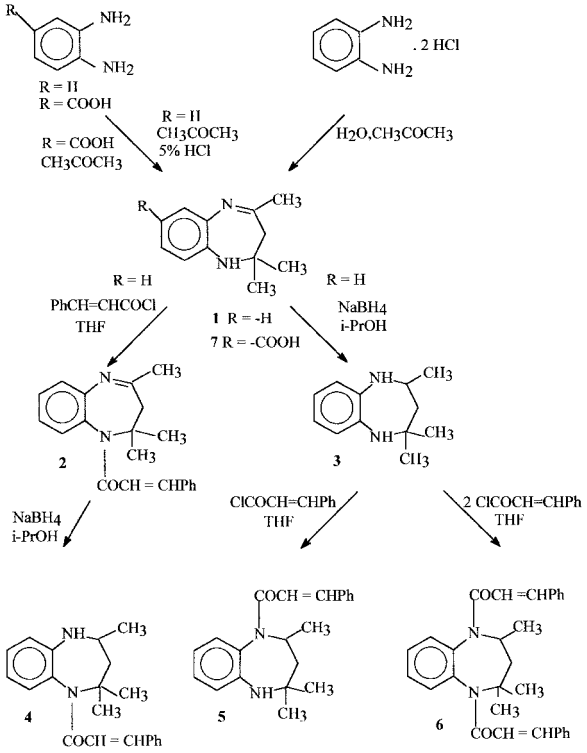
Our previous studies regarding cinnamoyl derivatives showed that some of them, namely 3-amino-2(1*H*)-tioxo-4(3H)-quinazolinone [39] and 2-aminobenzimidazole [40,41], were more active immunotropically than referential Levamisole or Cyclosporine A and were not toxic. It seemed interesting to synthesize some cinnamoyl derivatives of 1,5-benzodiazepines and examine them for their antiproliferative activity *in vitro* against tumor cells.

Results and Discussion

Chemistry

2,2,4-Trimethyl-1*H*-2,3-dihydro-1,5-benzodiazepine (**1**) (Scheme 1) was obtained as described previously[42,43]. Acetone in hydrochloric acid milieu underwent enolization forming mesityl oxide which subsequently reacted with *o*-phenylenediamine and benzodiazepine **1** was produced. Benzodiazepine **1** can be also obtained in the reaction of 1,2-phenylenediamine dihydrochloride with acetone. Using 3,4-diaminobenzoic acid in the reaction with acetone only, the formation of 2,2,4-trimethyl-7-carboxy-1*H*-2,3-dihydro-1,5-benzodiazepine (**7**) was found. The structure of compound **7** was confirmed by X-ray crystal structure analysis.

In the next step benzodiazepine **1** was subjected to an acylation by cinnamoyl chloride while heating in anhydrous THF in the presence of triethylamine. The acylation of N-1 nitrogen atom was unfavourable due to the steric hindrance present at the 2-position of diazepine ring, i.e. two methyl substituents. Chemical structure of 1-cinnamoyl-2,2,4-trimethyl-1*H*-2,3-dihydro-1,5-benzodiazepine (**2**) was confirmed by elemental analysis and IR, ^{1}H NMR, and MS spectra. Its MS spectrum displays base ion *m/z* = 131 corresponding to cinnamoyl group, while in the ^{1}H NMR spectrum two signals characteristic for two one-proton doublets belonging to vinylene (CH=CH) protons are present at δ = 6.98 and 7.56 ppm, respectively. Their coupling constant *J* = 15.78 Hz indicates that these protons are located in *trans* configuration. However, there is no signal corresponding to the proton of the NH group which was recorded for the compound **1** at δ = 4.70 ppm.



Scheme 1

Table 1. Selected bond lengths (Å) and torsion angles (°).

Compound (mol.)					
5(1)		**5**(2)		**7**	
O(11)-C(113)	1.240(2)	O(21)-C(213)	1.239(2)	O(1)-C(7)	1.3231(15)
N(11)-C(113)	1.358(2)	N(21)-C(213)	1.360(2)	O(2)-C(7)	1.2208(16)
N(11)-C(11)	1.431(2)	N(21)-C(21)	1.432(2)	N(1)-C(8)	1.2796(16)
N(11)-C(17)	1.478(2)	N(21)-C(27)	1.475(2)	N(1)-C(3)	1.4174(15)
N(12)-C(12)	1.386(2)	N(22)-C(22)	1.375(2)	N(2)-C(4)	1.3674(16)
N(12)-C(19)	1.490(2)	N(22)-C(29)	1.474(2)	N(2)-C(10)	1.4621(17)
C(11)-C(12)	1.402(2)	C(21)-C(22)	1.408(2)	C(3)-C(4)	1.4165(17)
C(17)-C(18)	1.522(2)	C(27)-C(28)	1.519(2)	C(8)-C(9)	1.4990(18)
C(18)-C(19)	1.530(2)	C(28)-C(29)	1.530(2)	C(9)-C(10)	1.5396(18)

5			
C(11)-N(11)-C(17)-C(18)	–43.6(1)	C(21)-N(21)-C(27)-C(28)	–40.0(1)
N(11)-C(17)-C(18)-C(19)	–47.8(1)	N(21)-C(27)-C(28)-C(29)	–49.0(1)
C(17)-C(18)-C(19)-N(12)	72.1(1)	C(27)-C(28)-C(29)-N(22)	77.4(1)
C(11)-C(12)-N(12)-C(19)	–54.9(1)	C(21)-C(22)-N(22)-C(29)	–37.8(1)
C(12)-N(12)-C(19)-C(18)	3.6(1)	C(22)-N(22)-C(29)-C(28)	–9.9(1)
C(12)-C(11)-N(11)-C(17)	72.0(1)	C(22)-C(21)-N(21)-C(27)	69.6(1)

7	
C(3)-N(1)-C(8)-C(9)	0.0(1)
N(1)-C(8)-C(9)-C(10)	–68.2(1)
C(8)-C(9)-C(10)-N(2)	71.3(1)
C(3)-C(4)-N(2)-C(10)	–26.8(1)
C(4)-N(2)-C(10)-C(9)	–13.2(1)
C(4)-C(3)-N(1)-C(8)	35.7(1)

Table 2. Lengths (Å) and angles (°) of the hydrogen bonds for compounds **5** and **7**.

D-H...A [symmetry code]		D-H	H...A	D...A	∠D-H-A
Compound **5**					
N(12)-H(8)...O(21)	[–*x*+2/3, *y*+1/2, –*z*+1/2]	0.91(2)	2.06(2)	2.957(2)	170(2)
N(22)-H(32)...O(11)	[*x*+1/2, –*y*+1/2, *z*+1/2]	0.89(2)	2.09(2)	2.972(2)	169(2)
Compound **7**					
O(1)-H(1)...N(1)	[–*x*+3/2, –*y*, z –1/2]	1.00(3)	1.62(3)	2.616(2)	173(2)
N(2)-H(7)...O(2)	[–*x*+1, *y*+1/2, –*z*+1/2]	0.88(2)	2.32(2)	3.186(2)	168(2)
C(5)-H(3)...O(2)	[–*x*+1, *y*+1/2, –*z*+1/2]	0.99(2)	2.52(2)	3.410(2)	150(2)

On reduction with sodium borohydride ($NaBH_4$) compound **2** gave 1-cinnamoyl-2,2,4-trimethyl-1*H*-2,3,4,5-tetrahydro-1,5-benzodiazepine (**4**). The product obtained was identified by its ^{1}H NMR spectrum in which proton signals appeared at δ = 4.75 ppm and at δ = 3.15 ppm assigned to the NH-group at 5-position and the methine group at 4-position, respectively. Protons of methyl group at 4-position are present as a doublet at δ = 1.23 ppm, J = 6.27 Hz, while the methylene protons appear as a multiplet at δ = 1.75 ppm.

Similar reduction occurred in the case of benzodiazepine **1** leading to the formation of 2,2,4-trimethyl-1*H*-2,3,4,5-tetrahydro-1,5-benzodiazepine (**3**). The ^{1}H NMR spectrum recorded for this compound contains protons signals of two NH groups at δ = 4.24 and 4.48 ppm. Methyl group protons at 4-position resonate at δ = 1.13 ppm, J = 6.33 Hz, methine and methylene protons appear as multiplets at δ = 3.11 and 1.48 ppm, respectively.

In the next stage of synthesis, benzodiazepine **3** was used in the reaction with equimolar or dimolar quantity of cinnamoyl chloride. Selective substitution of benzodiazepine **3** in 5-position was found in the first case when the reaction occurred at room temperature, in the second case the product of 1,5 substitution was obtained using boiling THF. 5-Cinnamoyl- (**5**) and 1,5-dicinnamoyl-2,2,4-trimethyl-1*H*-2,3,4,5-tetrahydro-1.5-benzodiazepine (**6**) were isolated and their purity and structures were confirmed by TLC, elemental analysis and IR, ^{1}H NMR, and MS spectra.

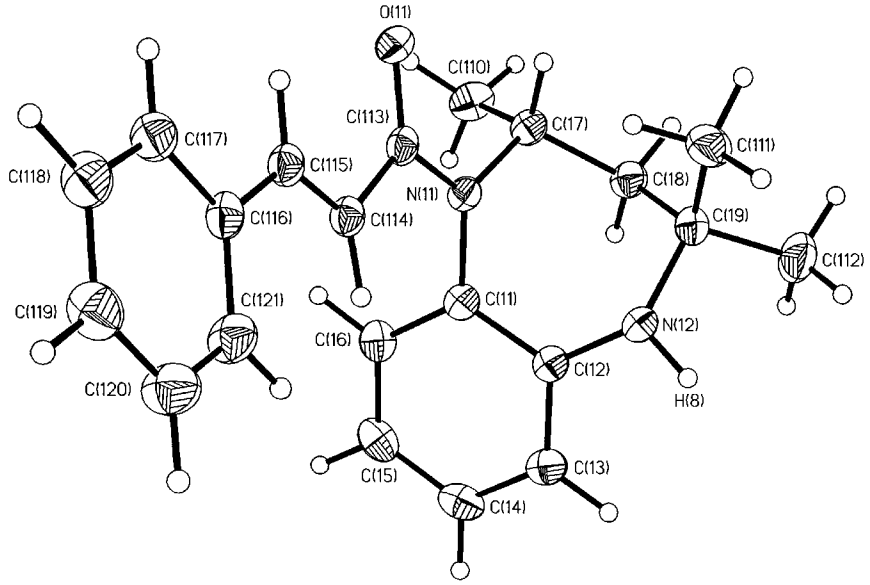


Figure 1. Numbering of atoms and schematic structure of compound **5**.

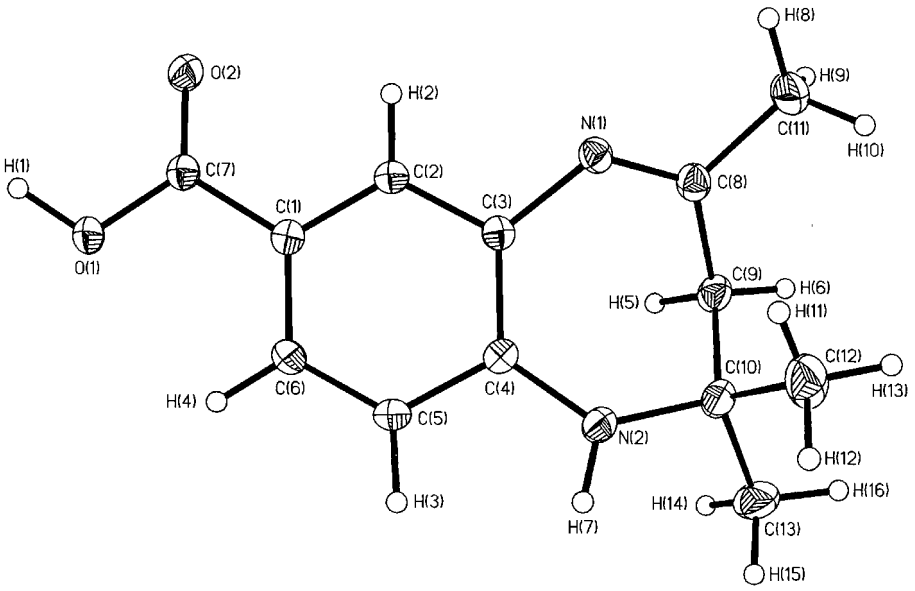


Figure 2. Numbering of atoms and schematic structure of compound **7**.

Crystallographic Part

Molecular structure and atoms labelling system are demonstrated in Figures 1 and 2. In **5** two non-equivalent molecules in the basic crystal unit were found. Table 1 presents selected bond distances and torsion angles. The presence of a double bond between the N(1) and C(8) atoms in **7** is a reason for essential conformational diferences between the two diazepine rings. The C(3)-N(1)-C(8)-C(9) torsion angle equals 0.0(2) in **7** whereas in both symmetry independent molecules of **5** the respective angles equal –43.6(2) and –40.0(2)°. There are also some differences between both symmetry independent molecules. The most important are between the C(11)-C(12)-N(12)-C(19) and C(21)-C(22)-N(22)-C(29) torsion angles which equal –54.9(2) and –37.8(2)°, respectively, and the C(12)-N(12)-C(19)-C(18) and C(22)-N(22)-C(29)-C(28) torsion angles equal 3.6(2)- and –9.9(2)°. The interplanar angles calculated for aromatic ring of both symmetry independent molecules of **7** are 88.1(2) and 67.8(2)°. The molecular packing in studied crystals is stabilized by a system of intermolecular hydrogen bonds (Table 2).

Pharmacology

Antiproliferative Activity in Vitro

The results of cytotoxic activity *in vitro* are expressed as ID_{50} – the dose of compound (in μg/ml) that inhibits a proliferation rate of the tumor cells by 50% as compared to control untreated cells. Compound **1** served as reference.

Compounds **4–6** exhibited cytotoxic activity against the cells of all 4 target cell lines applied (Table 3). Compounds **1–3** did not show any cytotoxic activity (ID_{50}>100 μg/ml).

The compounds **1** and **2** are 1*H*-2,3-dihydro-1,5-benzodiazepine derivatives while compounds **3–6** have the structure of 1*H*-2,3,4,5-tetrahydro-1,5-benzodiazepine. Antiproliferative activity was exhibited by compounds **4–6** with saturated benzodiazepine ring containing in their structure cinnamoyl substituents at the 1-position or the 5- and the 1,5-position.

Three selected compounds, namely **4–6** seem to be good candidates for further studies *in vitro* using a broad panel of human and murine tumor cell lines with the aim to select one compound for eventual studies *in vivo*.

Table 3. Antiproliferative activity of the compounds coded **4–6** against the cells of human cancer cell lines.

Compounds	Cell line/ID_{50} ± SD [μg/ml]			
	SW707	A549	MCF-7	HCV29T
4	35 ± 1.0	34 ± 1.0	29 ± 1.0	37 ± 1.0
5	35 ± 1.0	37 ± 1.0	34 ± 1.0	35 ± 1.0
6	44 ± 2.0	54 ± 1.0	42 ± 1.0	49 ± 1.0

Experimental

Chemistry

Melting points (uncorrected) were measured with a Boethius melting point apparatus Analyses were performed on a Perkin Elmer 2400 analyser and satisfactory results within ± 0.4% calculated values were obtained for the new compounds. IR spectra (in KBr) were recorded with an IR 75 spectrophotometer, ^{1}H NMR spectra – on a Bruker AMX 300 using DMSO-d_6 as a solvent and TMS as an internal standard. Mass spectra were determined on a GCMS-LK 82091 spectrometer at the ionisation energy 15 or 70 eV. The course of reaction and the purity of products were checked by TLC (Kieselgel G, Merck) in diethyl ether:ethanol = 5:1 for eluation.

*2,2,4-Trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**1**)*

Method A. The compounds **1** was prepared exactly as described previously [42,43].

Method B. A mixture of 1,2-phenyldiamine dihydrochloride (0.01 mol) dissolved in water (20 ml) and acetone (50 ml) was stirred at room temperature for 7 h (TLC). After neutralisation with 25% NH_4OH the precipitate was filtered off and crystallized.

Yield 1.57 g (83%), white precipitate from benzene, mp 132–133 °C.– IR (KBr): ν = 3328 cm^{-1} (NH), 3032 (CH), 2964 (CH_3), 1632 (C=N, ring), 1594 (NH), 1460 ($C(CH_3)_2$), 960, 770 (CH aromatic).– ^{1}H NMR: δ = 1.23(s, 6H), 2.15(s, 2H), 2.22(s, 3H), 4,70(s, 1H), 6.77–7.94(m, 4H).– Anal.: $C_{12}H_{16}N_2$ (188.27).– MS (70 eV): *m/z* (%) = 189 (7), 188 (66), 174 (14), 173 (100), 133 (64), 132 (89), 131 (13), 65 (10), 57 (13), 39 (11).

*Reaction of 2,2,4-Trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**1**) and 2,2,4-Trimethyl-1H-2,3,4,5-tetrahydro-1,5-benzodiazepine (**3**) with Cinnamoyl Chloride **2, 5, 6***

The compounds **2**, **5, 6** were prepared exactly as described previously[39–41].

*1-Cinnamoyl-2,2,4-trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**2**)*

Yield 2.5 g (79%), white solid from ethanol, mp 170–172 °C.– IR (KBr): ν = 3072 cm^{-1} (CH), 2976, 2950 (CH_3), 2930 (CH_2), 1694 (COCH=CH), 1660 (NCO, C=N), 1620 (CH=CH-Ph), 1604 (C=N, ring), 1480 (CH_2), 1384, 1364 ($C(CH_3)_2$), 1220, 1210 ($C(CH_3)_2$), 976, 760 (CH, aromatic), 980 (CH=CH trans).– ^{1}H NMR: δ = 1.27 (s, 6H), 2.09 (s, 3H), 2,79 (s, 2H), 6.81 (m, 1H), 6.98 (d, *J* = 15.78 Hz, 1H), 7.05 (m, 2H), 7.45 (m, 5H), 7.56 (d, *J*=15.78 Hz, 1H), 7.64 (m, 1H).– Anal.: $C_{21}H_{22}N_2O$ (318.42).– MS (70 eV): *m/z* (%) = 318 (12), 303 (20), 267 (11), 132 (14), 131 (100), 103 (31), 77 (16), 39 (12).

*5-Cinnamoyl-2,2,4-trimethyl-1H-2,3,4,5-tetrahydro-1,5-benzodiazepine (**5**)*

Yield 1.52 g (47%), white crystal from ethanol, analyzed by X-ray method, mp 133–135 °C.– IR (KBr): ν = 3340 cm^{-1} (=CH), 3336 (NH), 3020, 2956, 2844 (CH), 1658 (COCO=CH), 1650 (CON, O=C), 1630 (CH=CHPh), 1594 (NH), 1448 (CH_2), 1350 (NH), 990 (CH=CH trans), 764 (CH, aromatic).– ^{1}H NMR: δ = 1.04 (d, *J* = 6.21 Hz, 3H), 1,16 (s, 6H), 1.39 (m, 1H), 1,62 (m, 1H), 4,71 (m, 1H), 5.05 (s, br, 1H), 5.97(d, *J* = 15.63 Hz, 1H), 6.81 (m, 3H), 7.21 (m, 6H), 7.45 (d, *J* = 15.66 Hz, 1H).– Anal.: $C_{21}H_{24}N_2O$ (322.43).– MS (70 eV): *m/z* (%) = 322 (2), 321 (15), 320 (74), 305 (24), 190 (40), 189 (78), 175 (36), 133 (100), 132 (11), 131 (75), 92 (18), 77 (17), 39 (6).

*1,5-Dicinnamoyl-2,2,4-trimethyl-1H-2,3,4,5-tetrahydro-1.5-benzodiazepine (**6**)*

Yield 1.83 g (41%), white precipitate from ethanol, mp 213–215 °C.– IR (KBr): ν = 3016 cm^{-1} (CH), 2972, 2956 (CH_3), 2844 (CH_2), 1680 (NCO), 1648 (COCH=CH), 1632 (CH=CHPh), 1460 (CH_2), 900 (CH=CH trans), 744, 738, 724 (aromatic).– Anal. $C_{30}H_{30}N_2O_2$ (450.58).– MS (70 eV): *m/z* (%) = 450 (30), 435 (13), 321(6), 320 (27), 190 (65), 189 (33), 175 (77), 133 (100), 129(31), 39(13).

Table 4. Crystal data and structure refinement.

Compound	**5**	**7**
Empirical formula	$C_{21}H_{24}N_2O$	$C_{13}H_{16}N_2O_2$
Formula weight	320.42	232.28
T [K]	100(1)	100(1)
λ [Å]	0.71073	0.71073
Crystal system	Monoclinic	Orthorhombic
Space group	$P2_1/n$	$P2_12_12_1$
a [Å]	16.081(3)	9.770(2)
b [Å]	12.699(3)	9.896(2)
c [Å]	17.742(4)	12.610(3)
β [°]	95.81(3)	–
V [Å^3]	3604.5(14)	1219.2(5)
Z	8	4
D_c [Mg·m]$^{-3}$	1.181	1.265
μ[mm^{-1}]	0.073	0.087
F(000)	1376	496
Crystal size [mm]	0.15 × 0.15 × 0.20	0.15 × 0.20 × 0.20
Diffractometer	Kuma KM4CCD	Kuma KM4CCD
θ range for data collection [°]	3.21 – 27.00	3.83 – 28.76
h,k,l ranges	→20→19,–16→16,–20→22	–12→11,–12→13,–16→16
Reflections collected	23920	8744
Independent reflections (R_{int})	7757 (0.0341)	2924 (0.0222)
Data/parameters	6551 / 625	2818 / 219
Goodness-of-fit (F^2)	1.105	1.033
Final R_1/wR_2 indices ($I>2\sigma_I$)	0.0539 / 0.01051	0.0327 / 0.0757
Final R_1/wR_2 indices (all data)	0.0675 / 0.1114	0.0345 / 0.0771
Extinction coefficient	–	0.011(2)
Flack parameter	–	–0.2(9)
Largest diff. peak/hole /e· Å^{-3}	0.209 / –0.197	0.208 / –0.149

*Reduction of 2,2,4-Trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**1**) and 1-Cinnamoyl-2,2,4-trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**2**) with $NaBH_4$, **3, 4***

Three portions of $NaBH_4$ (each containing 0.01 mol) were added to boiling mixture of **1** or **2** in 50 ml of isopropanol during heating for 3–4 h (TLC). Then the alcohol was refluxed under reduced pressure and 200 ml of water was added to residue. Undissolved residue was filtered off, washed with water to neutral reaction. After drying precipitates were crystallized.

*2,2,4-Trimethyl-1H-2,3,4,5-tetrahydro-1,5-benzodiazepine (**3**)*

Yield 1.42 g (74%), white precipitate from water, mp 90–91 °C.– IR (KBr): ν = 3344 cm^{-1}, 3320 (NH), 2956 (CH_3), 2856 (CH_2), 1598 (NH), 1476 (CH_2), 1376 ($C(CH_3)_2$), 1342 (NH), 974, 800, 748 (CH, aromatic).– ^{1}H NMR:δ = 0.98 (s, 3H), 1.13 (d, *J* = 6.33 Hz, 3H), 1.22 (s, 3H), 1.48 (m, 2H), 3.11 (m, 1H), 4.24 (s, 1H), 4.48 (s, 1H), 6.55 (m, 2H), 6.72 (m, 2H).– Anal.: $C_{12}H_{18}N_2$ (190.29).– MS (70 eV): *m/z* (%) = 191 (3), 190 (16), 126 (10), 114 (10), 112(11), 83 (18), 81 (14), 73 (17), 72 (100), 69 (35), 60 (34), 59 (99), 55 (79), 45 (19), 39 (8).

*1-Cinnamoyl-2,2,4-trimethyl-1H-2,3,4,5-tetrahydro-1,5-benzodiazepine (**4**)*

Yield 1.70 g (53%), white precipitate from ethanol, mp 124–127 °C.– IR (KBr): ν = 3340 cm^{-1} (NH), 3020 (CH), 2968, 2952 (CH_3), 2920 (CH_2), 1662 (CO-CH=CH), 1654 (CON, C=C), 1630 (CH=CHPh), 1568 (NH), 1452 (CH_2), 1354 (NH), 984 (CH=CH trans), 766 (CH aromat.) – ^{1}H NMR: δ = 1.19 (s, 3H), 1.23 (d, *J* = 6.27 Hz, 3H), 1.43 (s, 3H), 1.75 (m, 2H), 3.15 (m, 1H), 4.75 (br, 1H), 5.99 (d, *J* = 15.63 Hz, 1H), 6.88 (m, 2H), 7.14 (m, 3H), 7.29 (m, 3H), 7.45 (d, *J* = 15,63 Hz, 1H), 7.53 (d, *J* = 7.77 Hz, 1H).– Anal.: $C_{21}H_{24}N_2O$ (320.43).– MS (70 eV): *m/z* (%) = 321 (13), 320 (76), 305 (21), 190 (32), 175 (30), 133 (100), 131 (76), 65 (7), 39 (4).

*7-Carboxy-2,2,4-trimethyl-1H-2,3-dihydro-1,5-benzodiazepine (**7**)*

3,4-Diaminobenzoic acid was heated in acetone and left for crystallisation. The colorless crystals, mp 207–208 °C, were analyzed by X-ray method.– IR (KBr): ν = 3352 cm^{-1} (OH), 2960 (CH_3), 2832 (CH_2), 1668 (CO), 1630 (NH, C=N ring), 1356 ($C(CH_3)_2$), 954, 760 (aromat.).– ^{1}H NMR: δ = 1.24 (s, 3H), 2.21 (s, 3H), 2.35 (s, 2H), 5.91 (s, 1H), 6.78 (d, *J* = 8.43 Hz, 1H), 7.44 (d, *J* = 8.40 Hz, 1H), 7.55 (s, 1H), 12.22 (s, br, 1H).–Anal.: $C_{13}H_{16}N_2O_2$ (232.28).– MS (70 eV): *m/z* (%) = 233 (6), 232 (48), 218 (15), 217 (100), 117 (47), 176 (77), 92 (6), 77 (6), 65 (5), 39 (13).

Crystallography

Crystal data of **5** and **7** are given in Table 4, together with refinement details. All measurements on both crystals were performed on a Kuma KM4CCD κ-axis diffractometer with graphite-monochromated MoKα radiation. Crystals were positioned at 65 mm from the KM4CCD camera. 612 frames were measured at 0.75° intervals with a counting time of 20 s. The data were corrected for Lorentz and polarization effects. No absorption correction was applied. Data reduction and analysis were carried out with the Kuma Diffraction (Wroclaw) programs. The structure was solved by direct methods (program SHELXS97 [45]) and refined by the full-matrix least-squares method on all F^2 data using the SHELXL97 [46] programs. Non-hydrogen atoms were refined with anisotropic thermal parameters; hydrogen atoms were included from Δρ maps and were refined with isotropic thermal parameters. The selected bond lengths and bond angles are listed in Table 1.

The numbering of atoms and schematic structure of the title compounds are presented in Figure 1 and Figure 2.

Antiproliferative Assay In Vitro

Compounds

The compounds coded **1–6** were examined in *in vitro* screening assay. Test solutions of the compounds tested (1 mg/ml) were prepared *ex tempore* by dissolving the substance in 100 µl of DMSO completed with 900 µl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium (described below) to reach the final concentrations of 100, 10, 1, and 0.1 µg/ml. The solvent (DMSO) in the highest concentration used in test did not reveal any cytotoxic activity.

Cells

The following established *in vitro* human cancer cell lines were applied: SW707 (rectal adenocarcinoma), A549 (non-small cell lung carcinoma) and MCF-7 (breast cancer). All lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and are maintained in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Human uroepithelial cell line HCV29T established in the Fibiger Institute, Copenhagen, Denmark, was obtained from Dr. J. Kieler in 1982 and maintained at the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, U.S.A.) 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 (50 U/ml) (both antibiotics from Polfa, Tarchomin, Poland) and 5% fetal calf serum (Gibco, Grand Island, U.S.A.). The cell cultures were maintained at 37 °C in humid atmosphere saturated with 5% CO_2.

SRB Assay

The details of this technique were described by Skehan et al. [44]. The cytotoxicity assay was performed after 72-hour exposure of the cultured cells to varying concentrations (from 0.1 to 100 µg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 minutes. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10mM unbuffered Tris base (POCh, Gliwice, Poland) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland). Each compound in given concentration was tested in triplicates in each experiment, which was repeated 3–5 times.

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