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Synthesis and *in vitro* Cytotoxicity Studies of Novel Triazolo[4,3-*b*]pyridazinones

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New triazolo[4,3-*b*]pyridazinones were synthesized and evaluated for their potential *in vitro* cytotoxic antitumor properties. The compounds were prepared by 1,3-dipolar cycloaddition of pyridazin-3-ones with *N*-aryl-*C*-ethoxycarbonylnitrile imines, generated *in situ* from ethylhydrazono-α-bromoglyoxylates. The peri- and regioselectivity of the reaction were ascertained by ^{1}H and ^{13}C NMR spectroscopy of the cycloadducts.

Keywords: Triazolo[4,3-*b*]pyridazinones, Cycloaddition, Cytotoxicity, Hep cells

INTRODUCTION

Heterocyclic annelated pyridazines attract considerable attention, which mainly arises from the large variety of interesting pharmacological activities observed with pyridazine derivatives [1-6]. Triazolopyridazine derivatives are frequently used in biological research as adenine receptor ligands [7-9]. In our ongoing research program aiming at new pyridazino-fused ring systems [10], we report herein the synthesis of the triazolo[4,3-b]pyridazinones **3-9**, which were obtained by 1,3-dipolar cycloaddition. Evaluation of their *in vitro* antitumor activities is also reported.

EXPERIMENTAL

Melting points were determined using a Büchi-Tottoli apparatus. IR spectra were recorded on a Perkin-Elmer 577 spectrometer using KBr disks, only noteworthy IR absorptions are listed (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and solution (unless otherwise specified) with TMS as an internal reference using Bruker AC 300 (^{1}H) or 75 MHz (^{13}C) instruments. Chemical shifts are given in ppm downfield from TMS. Multiplicities of ^{13}C NMR resources were assigned by distortionless enhancement by polarization transfer (DEPT) experiments. Elemental analysis data were taken on a Perkin-Elmer 240C instrument. Column chromatography was carried out on SiO_2 (silica gel 60 Merck 0.063-0.200 mm). TLC was carried out on SiO_2 (silica gel 60, F 254 Merck 0.063-0.200 mm) and the spots located with UV light. Commercial reagents were used without further purification. Compounds **2e** and **2f** were prepared according to the method described in the literature [11].

General Procedure for the Preparation of Triazolo[4,3-*b*]pyridazinones 3-9

To a solution of pyridazin-3(2H)-one **2e-f** (5 mmol) and ethylhydrazono-α-bromoglyoxylate **1a-d** (5 mmol) in THF (50 ml), triethylamine (2 ml, 9 mmol) dissolved in THF (5 ml)

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was added dropwise. The mixture was stirred for 18 h at room temperature. After evaporation of the solvent, the residue was purified by column chromatography on silica gel using hexane-ethyl acetate 80:20 as eluent.

1-(4-Chloro-phenyl)-8a-methyl-6-oxo-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (3). Yield: 60%; m.p.: 230-232 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.32 (t, *J* = 7.0 Hz, 3H, CH_3), 1.72 (s, 3H, CH_3), 2.08 (m, 2H, CH_2), 2.53 (m, 2H, CH_2), 4.30 (q, *J* = 7.0 Hz, 2H, CH_2O), 7.27 (d, *J* = 7.8 Hz, 2H, ArH), 7.37 (d, *J* = 7.8 Hz, 2H, ArH), 9.02 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.4 (CH_3), 28.8 (CH_3), 29.3 (CH_2), 32.4 (CH_2), 62.1 (CH_2O), 85.9 (C-8a), 118.3 and 129.5 (4CH), 159.1 (CONH), 179.8 (CO); IR (KBr, cm^{-1}): 1677 (CONH), 1720 (CO ester), 3020 (NH). Anal.: Calcd. for $C_{15}H_{17}ClN_4O_3$: C, 53.50; H, 5.09; N, 16.64. Found: C, 53.54; H, 5.11; N, 16.61.

8a-Methyl-1-(4-nitro-phenyl)-6-oxo-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (4). Yield: 54%; m.p.: 218-220 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.34 (t, *J* = 7.0 Hz, 3H, CH_3), 1.76 (s, 3H, CH_3), 2.18 (m, 2H, CH_2), 2.82 (m, 2H, CH_2), 4.31 (q, *J* = 7.0 Hz, 2H, CH_2O), 7.67 (d, *J* = 9.2 Hz, 2H, ArH), 8.05 (d, *J* = 9.2 Hz, 2H, ArH), 9.12 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.3 (CH_3), 28.2 (CH_3), 28.6 (CH_2), 31.2 (CH_2), 62.4 (CH_2O), 85.4 (C-8a), 116.7 and 124.5 (4CH), 160.0 (CONH), 178.4 (CO); IR (KBr, cm^{-1}): 1520, 1300 (NO_2), 1667 (CONH), 1720 (CO ester), 3060 (NH). Anal.: Calcd. for $C_{15}H_{17}N_5O_5$: C, 52.89; H, 5.83; N, 19.27. Found: C, 52.92; H, 5.82; N, 19.25.

8a-Methyl-6-oxo-1-*p*-tolyl-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (5). Yield: 56%; m.p.: 160-162 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.36 (t, *J* = 7.2 Hz, 3H, CH_3), 1.84 (s, 3H, CH_3), 2.26 (m, 2H, CH_2), 2.32 (s, 3H, CH_3), 2.64 (m, 2H, CH_2), 4.35 (q, *J* = 7.2 Hz, 2H, CH_2O), 7.12 (d, *J* = 7.5 Hz, 2H, ArH), 7.17 (d, *J* = 7.5 Hz, 2H, ArH), 8.23 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.2 (CH_3), 20.7 (CH_3), 29.0 (CH_2), 29.3 (CH_3), 30.9 (CH_2), 62.1 (CH_2O), 87.4 (C-8a), 118.8 and 129.8 (4CH), 133.6, 138.8, 141.2 (3C), 158.3 (CONH), 175.1 (CO); IR (KBr, cm^{-1}): 1670 (CONH), 1710 (CO ester), 3080 (NH). Anal.: Calcd. for $C_{16}H_{20}N_4O_3$: C, 60.75; H, 6.37; N, 17.71. Found: C, 60.70; H, 6.35; N, 17.68.

1-(4-Methoxy-phenyl)-8a-methyl-6-oxo-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (6). Yield: 61%; m.p.: 106-108 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.38 (t, *J* = 7.2 Hz, 3H, CH_3), 1.83 (s, 3H, CH_3), 2.28 (m, 2H, CH_2), 2.45 (m, 2H, CH_2), 3.81 (s, 3H, CH_3O), 4.35 (q, *J* = 7.2 Hz, 2H, CH_2O), 6.90 (d, *J* = 6.9 Hz, 2H, ArH), 7.22 (d, *J* = 6.9 Hz, 2H, ArH), 7.93 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.2 (CH_3), 28.9 (CH_2), 29.2 (CH_3), 32.5 (CH_2), 55.5 (CH_3O), 62.0 (CH_2O), 88.0 (C-8a), 114.5 and 121.8 (4CH), 134.8, 141.5, 157.3 (3C), 158.5 (CONH), 174.9 (CO); IR (KBr, cm^{-1}): 1666 (CONH), 1725 (CO ester), 3100 (NH). Anal.: Calcd. for $C_{16}H_{20}N_4O_4$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.78; H, 6.05; N, 16.84.

1-(4-Chloro-phenyl)-8a-phenyl-6-oxo-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (7). Yield: 68%; m.p.: 156-158 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.38 (t, *J* = 7.2 Hz, 3H, CH_3), 2.42 (m, 2H, CH_2), 2.85 (m, 2H, CH_2), 4.38 (q, *J* = 7.2 Hz, 2H, CH_2O), 6.98 (d, *J* = 7.7 Hz, 2H, ArH), 7.16 (d, *J* = 7.7 Hz, 2H, ArH), 7.46 (m, 3H, ArH), 7.54 (m, 2H, ArH), 7.56 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.2 (CH_3), 28.6 (CH_2), 28.9 (CH_2), 62.4 (CH_2O), 88.5 (C-8a), 118.3, 126.5, 129.0, 129.2, 129.4 (9CH), 130.8, 135.4, 140.5, 144.1 (4C), 160.5 (CONH), 175.4 (CO); IR (KBr, cm^{-1}): 1670 (CONH), 1730 (CO ester), 3120 (NH). Anal.: Calcd. for $C_{20}H_{19}ClN_4O_3$: C, 60.23; H, 4.80; N, 14.05. Found: C, 60.20; H, 4.78; N, 14.02.

1-(4-Nitro-phenyl)-8a-phenyl-6-oxo-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-*b*]pyridazine-3-carboxylic acid ethyl ester (8). Yield: 54%; m.p.: 228-230 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.37 (t, *J* = 7.2 Hz, 3H, CH_3), 2.45 (m, 2H, CH_2), 2.88 (m, 2H, CH_2), 4.35 (q, *J* = 7.2 Hz, 2H, CH_2O), 7.13 (d, *J* = 9.0 Hz, 2H, ArH), 7.49 (m, 3H, ArH), 7.56 (m, 2H, ArH), 7.76 (d, *J* = 9.0 Hz, 2H, ArH), 8.01 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.1 (CH_3), 27.4 (CH_2), 28.1 (CH_2), 62.5 (CH_2O), 89.0 (C-8a), 118.3, 126.5, 129.2, 129.4, 134.5 (9CH), 130.6, 135.4, 140.5, 144.2 (4C), 161.0 (CONH), 175.8 (CO); IR (KBr, cm^{-1}): 1675 (CONH), 1720 (CO ester), 3080 (NH). Anal.: Calcd. for $C_{20}H_{19}N_5O_5$: C, 58.68; H, 4.68; N, 17.11. Found: C, 58.54; H, 4.68; N, 17.10.

6-Oxo-8a-phenyl-1-*p*-tolyl-1,5,6,7,8,8a-hexahydro-[1,2,4]triazolo[4,3-b]pyridazine-3-carboxylic acid ethyl ester (9). Yield: 64%; m.p.: 159-161 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.40 (t, *J* = 7.2 Hz, 3H, CH_3), 2.28 (s, 3H, CH_3), 2.78 (m, 2H, CH_2), 2.98 (m, 2H, CH_2), 4.39 (q, *J* = 7.2 Hz, 2H, CH_2O), 6.94 (d, *J* = 8.1 Hz, 2H, ArH), 7.03 (d, *J* = 8.1 Hz, 2H,

ArH), 7.45 (m, 3H, ArH), 7.55 (m, 2H, ArH), 7.62 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 14.2 (CH_3), 20.7 (CH_3), 28.8 (CH_2), 28.9 (CH_2), 62.2 (CH_2O), 89.6 (C-8a), 118.0, 126.7, 129.2, 129.3, 129.7 (9CH), 132.9, 138.3, 140.2, 141.4 (4C), 158.2 (CONH), 174.9 (CO); IR (KBr, cm^{-1}): 1665 (CONH), 1725 (CO ester), 3090 (NH). Anal.: Calcd. for $C_{21}H_{22}N_4O_3$: C, 66.65; H, 5.86; N, 14.80. Found: C, 66.70; H, 5.85; N, 14.82.

Cell Culture

The human laryngeal carcinoma cell line (Hep) was kindly provided by Prof. G. Lemaire, Institute of Biochemistry, University of Paris XI, France. This cell line is routinely cultured in an humidified incubator at 37 °C with 5% CO_2 in complete medium [Dulbecco's modified eagles medium (DMEM)] supplemented with 5% foetal calf serum (Gibco BRL, France), 1% penicillin-streptomycin-neomycin, 0.2% sodium bicarbonate (Sigma Chemical Co., France).

Cytotoxic Activity Assay

This test was performed using the crystal violet assay as previously described [12-13]. Briefly, cells were trypsinized and 7.5×10^4 cells per ml were incubated in flat-bottomed 96-well microtiter plates in 100 µl of complete medium. Several dilutions of these products were then added in a final volume of 100 µl of complete medium. After 48 h incubation at 37 °C and 5% CO_2, medium was removed and replaced by 100 µl of 0.5% crystal violet solution. After 10 min-incubation at room temperature, the plates were washed and viable crystal-violet-stained cells were lysed using SDS solution (1%). Optical density was then measured in each well at a wavelength of 540 nm using a Multiscan plate reader (Labsystem, Finland). Using this colorimetric procedure, product-mediated cell lysis and adriamycin (used as a positive control) cytotoxic effect were measured as compared to the viability of untreated cells according to the following calculation: % of cell killing = 100 × (1-OD/OD_0), where OD_0 and OD are the optical densities obtained from negative control cells and product (or adriamycin) treated cells, respectively. Test compounds were compared using the IC_{50} value, which corresponds to the concentration of an individual compound required to lyse 50% of the cells.

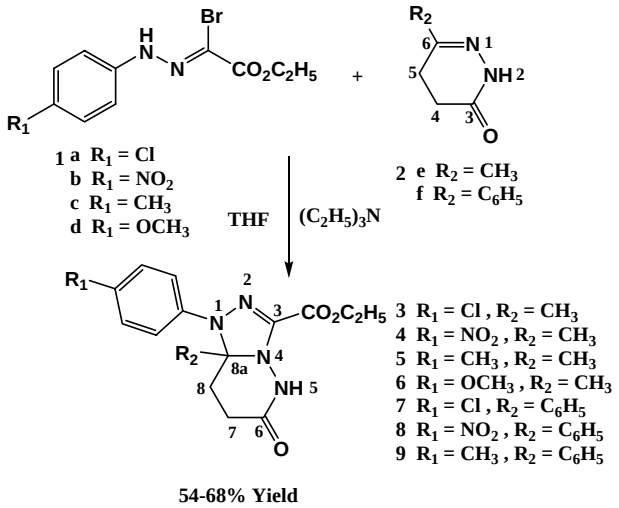
Statistical Analysis

Values are recorded as means ± SD. Statistical differences between the compound effects were assessed using the one-way analysis of variance (ANOVA). P values less than 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Chemistry

1,3-Dipolar cycloaddition reactions are among the best and more general methods for the construction of five-membered rings in a convergent and stereocontrolled manner [14]. Pyridazinones **2e-f** have two potential dipolarophilic sites: the C=N double bond and the C=O carbonyl double bond. The reaction of compounds **2e-f** with *N*-aryl-*C*-ethoxycarbonylnitrile imines, generated *in situ* from ethylhydrazono-α-bromoglyoxylates **1a-d** [15] and triethylamine, was performed in tetrahydrofuran (THF) at room temperature. In all cases, only one type of triazolopyridazinone (**3-9**) was obtained in moderate to good yields (Scheme 1). No adduct resulting from a condensation on the double bond C=O was detected under identical conditions. The reaction was exclusively periselective.



Scheme 1

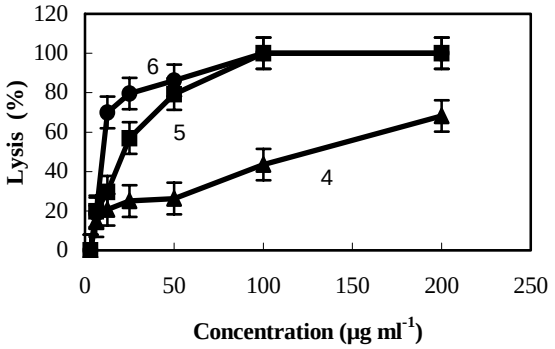


Fig. 1. *In vitro* cytotoxic effect of the compounds **4**, **5** and **6** against Hep cell line.

Table 1. *In vitro* Antitumor Activity of Triazolo [4,3-*b*]pyridazinone Derivatives *vs*. Adriamycin

Compound	IC_{50} (µg ml^{-1})
3	NT
4	119
5	20.76
6	9.2
8	NT
ADR	1.2

NT: Not Tested.

The structural assignments of the triazolo[4,3-*b*]pyridazinones **3-9** are based on a full characterization by 300 MHz ^{1}H NMR and 75 MHz ^{13}C NMR spectra. The ^{13}C NMR spectra of cycloadducts **3-9,** in particular, exhibit a signal at 85.9-89.6 ppm due to the **C-8a** carbon atom and a signal at 158.2-161.0 ppm assigned to the double bond C=O, hence confirming the addition of the dipole to the C=N double bond. The direction of the addition was inferred from the ^{13}C NMR spectra: the **C-8a** signal at 85.9-89.6 ppm, slightly deshielded, rules out any other direction of the addition on the double bond C=N; otherwise the **C-8a** signal would appear upfield (expected value between 50 and 60 ppm) [16-17]. The reaction is thus regioselective.

Pharmacological Results

The new triazolo[4,3-b]pyridazinones **3-9** have been synthesized with the aim to evaluate their cytotoxic activity against tumor cells.

Cells (105/ml) were treated with increasing concentrations of compounds **4-6**, followed by a 48 h incubation. Cell lysis data are the mean ±SD of three independent experiments.

In vitro evaluation of the cytotoxic effect of compounds **5** and **6** (Fig. 1, Table 1) shows that both of these products are cytotoxic to the Hep cell line in a dose-dependent manner, with IC_{50} values of 20.76 µg ml^{-1} and 9.2 µg ml^{-1}, respectively. It is noteworthy that the cytotoxic effect of these products is weaker than that induced by adriamycin (IC_{50}:1.2 µg ml^{-1}). Statistical analysis shows that **6** is more cytotoxic than compound **5** ($P < 0.05$).

In conclusion, we have described a straightforward synthesis of new triazolo[4,3-b]pyridazinones and studied their antiproliferative activities. Compound **6** exhibits significant cytotoxicity against the Hep cell line.

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