

Synthesis and Pharmacological Activities of Fused Pyrimidinones

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Abstract: A series of substituted 6-pyrazol-3-yl-pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-5-ones **8** were prepared *via* reaction of 5-pyrazol-3-yl-2-thioxo-2,3-dihydro-1*H*-pyrido[2,3-*d*]pyrimidin-4-one **3** or its methylthio derivatives **4** with hydrazonoyl chlorides **5**. Both conventional thermal and microwave irradiation techniques were used for synthesis of the target products **8**. A comparative study of these techniques in presence of a basic catalyst was performed. The mechanism of the studied reactions was discussed. Also, the products **8** were screened for their biological activities.

Key words: Hydrazonoyl chloride • 2-thioxo-2,3-dihydro-1*H*-pyrido [2,3-*d*] pyrimidin-4-one • Microwave irradiation technique • Antitumor • Anti-HCV • Anti-SSPE • Antioxidant activities

INTRODUCTION

Hydrazonoyl halides are used as precursors for synthesis of various heterocyclic systems [1-7], [1, 2, 4] Triazolo [4, 3-*a*] pyrimidines are pharmacological scaffold that represent a wide range of biological activities such as antitumor [8], human A3 adenosine receptor [9], antibacterial [10], CNS depressant [11] antiallergy [12] and anti-inflammatory [13]. In the field of coordination chemistry, [1, 2, 4]triazolo [4,3-*a*]pyrimidine can be used as bridging ligand [14]. On the other hand, pyridopyrimidines, another class of heterocyclic compounds, can be used as active modulators of cannabinoid-1 receptor (CB1R) [15]. Fusion of [1, 2, 4] triazole ring to pyridopyrimidines tends to exert novel biological activities. In addition, pyrazole derivatives use as the core structure in many drug substances, covering wide range of pharmacological applications [16-20]. This encouraged us to use hydrazonoyl halide and pyrazole ring to obtain another new heteropolycyclic derivatives including pyrazole and pyrido [2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-5-ones moieties in one molecular frame. This novel heterocyclic systems are expected to sustain interesting biological activities.

Experimental

General: All melting points were determined on an electrothermal Gallenkamp apparatus and they are uncorrected. Solvents were generally distilled and dried by standard literature procedures prior to use. The IR spectra were measured on a Pye-Unicam SP300 Infrared

Spectrophotometers in potassium bromide discs. The ¹H NMR spectra were recorded on a Varian Mercury VXR-300 spectrometer (300 MHz) and the chemical shifts were related to that of the solvent DMSO-*d*₆. The mass spectra were recorded on a GCMS-Q1000-EX Shimadzu and GCMS 5988-A HP spectrometers, the ionizing voltage was 70 eV. Elemental analyses were carried out by the Microanalytical Center of Cairo University, Giza, Egypt. Microwave experiments were carried out using CEM Discover Labmate microwave apparatus (300 W with Chem. Driver Software). Compounds **1**, **2** and hydrazonoyl chloride **5** were prepared as previously described [21-29].

Synthesis of 5-(4-cyano-1, 5-diphenylpyrazol-3-yl)-2-thioxo-2, 3-dihydro-1*H*-pyrido [2, 3-*d*] pyrimidin-4-one (**3**):

A mixture of 3-[3-(dimethylamino)-2-propenoyl]-4-cyano-1, 5-diphenyl-1*H*-pyrazole (**1**) (3.42 g, 10 mmol) and 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**2**) (1.43 g, 10 mmol) in acetic acid (30 mL) was refluxed for 6 hours. The reaction mixture was cooled and diluted with ethanol and the solid product was collected by filtration and recrystallized from dioxane to give **3**, as yellow crystals (3.79 g, 90%), mp 238°C; IR (KBr) $\nu = 3481, 3200$ (2NH), 2233 (CN), 1690 (C=O) cm^{-1} ; ¹H NMR (DMSO-*d*₆) 7.45-7.52 (m, 10H, ArH), 7.94 (d, $J = 4.5$ Hz, 1H, pyridine-H), 8.44 (d, $J = 4.5$ Hz, 1H, pyridine-H), 12.64 (s, 1H, NH), 13.40 (s, 1H, NH) ppm; MS, *m/z* (%) 423 (*M*⁺+1, 11), 422 (*M*⁺, 17), 381 (17), 147 (20), 115 (20), 77 (15), 63 (100). Anal. Calcd. for C₂₃H₁₄N₆OS (422.47): C, 65.39; H, 3.34; N, 19.89. Found: C, 65.32; H, 3.52; N, 19.71%.

Synthesis of 2-methylsulfanyl-5-(4-cyano-1,5-diphenylpyrazol-3-yl)-3H-pyrido[2,3-d]pyrimidin-4-one (4):

To a stirred solution of 5-(4-cyano-1,5-diphenylpyrazol-3-yl)-2-thioxo-2,3-dihydro-1H-pyrido[2,3-d]pyrimidin-4-one (**3**) (0.422 g, 1 mmol) in dimethylformamide (10 mL) was added anhydrous potassium carbonate (0.207 g, 1.5 mmol) and methyl iodide (0.142 g, 1 mmol). The reaction mixture was stirred overnight at room temperature then poured into ice-water. The solid formed was filtered, washed with water, dried and recrystallized from dioxane to give compound **4** as yellow solid, mp > 300°C; IR (KBr) ν = 3405 (NH), 1684 (CO), cm^{-1} ; $^1\text{H NMR}$ (DMSO- d_6) δ = 2.6 (s, 3H, CH₃), 7.10-8.00 (m, 10H, Ar-H), 8.10 (d, J = 4.5 Hz, 1H, pyridine-H), 8.24 (d, J = 4.5 Hz, 1H, pyridine-H), 11.22 (s, 1H, NH) ppm. *Anal.* Calcd. for C₂₄H₁₆N₆OS (436.11): C, 66.04; H, 3.69; N, 19.25. Found: C, 66.37; H, 3.38; N, 19.04%.

Synthesis of pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one derivatives (8a-j)

Method A: To a mixture of equimolar amounts of **3** and the appropriate hydrazoneyl chlorides **5a-j** (1 mmol of each) in dioxane (15 mL) was added triethylamine (0.14 mL, 1 mmol). The reaction mixture was refluxed till all of the starting materials have disappeared and hydrogen sulfide gas ceased to evolve (5 h, monitored by TLC). The solvent was evaporated and the residue was triturated with methanol. The solid that formed was filtered and recrystallized from dioxane to give compounds **8a-j**.

Method B: To a mixture of equimolar amounts of **4** and the appropriate hydrazoneyl chlorides **5a, e, i** (1 mmol of each) in dioxane (15 mL) was added triethylamine (0.14 mL, 1 mmol). The reaction mixture was refluxed till all methanethiol gas ceased to evolve (5 h, monitored by TLC). The solvent was evaporated and the residue was treated with methanol. The solid that formed was filtered and recrystallized from dioxane to give products identical in all respects (mp, mixed mp and IR) with **8a, e, i**.

Method C: To a solution of **3** and the appropriate hydrazoneyl chlorides **5a, e, i** (1 mmol of each) in dioxane (15 mL) was added triethylamine (0.14 mL, 1 mmol) at room temperature. The reaction mixture was irradiated in a pressurized microwave (17.2 Bar, 140°C) for 10 min. at a power of 300 W. After cooling, dil. HCl was added till pH became acidic and the solid product was collected and recrystallized from dioxane to give products **8a, e, i**.

Method D: To a solution of **3** (0.13 g, 1 mmol) and the appropriate hydrazoneyl chlorides **5a, e, i** (1 mmol of

each) in dioxane (15 ml) was added chitosan (0.1 g) at room temperature. The reaction mixture was irradiated under constant pressure (17.2 Bar, 140°C) for 10 min. at a power of 300 W. The hot solution was filtered to remove chitosan. After cooling, dil. HCl was added till pH became acidic and the solid product was collected and recrystallized from dioxane to give products **8a, e, i**.

Method E: A mixture of 3-[(3-dimethylamino)-2-propenoyl]-1,5-diphenyl-1H-pyrazole-4-carbonitrile (**1**) (3.42 g, 10 mmol) and 7-amino-3-ethoxycarbonyl-1-phenyl[1,2,4]triazolo[4,3-a]pyrimidin-5(1H)-one (**10**) [30] (10 mmol) in acetic acid (30 mL) was refluxed for 6 hours. The reaction mixture was cooled and diluted with ethanol and the solid product was collected by filtration and recrystallized from dioxane to give **8e**. The physical constants together with the spectral data of products **8a-j** are listed below:

3-Acetyl-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-phenyl-1,5-dihydro-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (8a): Yellow solid, mp 120°C; IR (KBr) ν = 2231 (CN), 1709, 1690 (2CO), cm^{-1} ; $^1\text{H NMR}$ (DMSO- d_6) δ = 2.79 (s, 3H, COCH₃), 7.36-7.69 (m, 15H, ArH), 8.08 (d, J = 4.5 Hz, 1H, pyridine-H), 8.22 (d, J = 4.5 Hz, 1H, pyridine-H) ppm; MS, m/z (%) 549 (M^+ +1, 15), 548 (M^+ , 40), 533 (3), 505 (22), 91 (21), 77 (100). *Anal.* Calcd. for C₃₂H₂₀N₈O₂ (548.57): C, 70.07; H, 3.67; N, 20.43%. Found: C, 70.03; H, 3.54; N, 20.61%.

3-Acetyl-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-methylphenyl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (8b): Orange solid, mp 200°C; IR (KBr) ν = 2232 (CN), 1699, 1685 (2CO), cm^{-1} ; $^1\text{H NMR}$ (DMSO- d_6) δ = 2.41 (s, 3H, Ar-CH₃), 2.77 (s, 3H, COCH₃), 7.05-7.47 (m, 14H, Ar-H), 8.06 (d, J = 5 Hz, 1H, pyridine-H), 8.72 (d, J = 5 Hz, 1H, pyridine-H), ppm; MS, m/z (%) 563 (M^+ +1, 15), 562 (M^+ , 55), 519 (25), 493 (19), 104 (14), 91 (23), 77 (100); *Anal.* Calcd. for C₃₃H₂₂N₈O₂ (562.60): C, 70.45; H, 3.94; N, 19.92. Found: C, 70.26; H, 4.29; N, 20.21%.

3-Acetyl-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-chlorophenyl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (8c): Yellow crystals, mp 220°C; IR (KBr) ν = 2232 (CN), 1707, 1660 (2CO), cm^{-1} ; $^1\text{H NMR}$ (DMSO- d_6) δ = 2.80 (s, 3H, COCH₃), 7.28-7.47 (m, 10H, Ar-H), 7.74 (d, J = 8 Hz, 2H, ArH), 8.12 (d, J = 4.5 Hz, 1H, pyridine-H), 8.28 (d, J = 8 Hz, 2H, ArH), 8.78 (d, J = 4.5 Hz, 1H, pyridine-H), ppm; MS, m/z (%) 585 (M^+ +2, 5), 583 (M^+ , 16), 581 (6), 541 (7), 539 (16), 422 (17),

180 (20), 125 (23), 111 (25), 84 (100), 77 (79); *Anal.* Calcd. for $C_{32}H_{19}ClN_9O_2$ (583.01): C, 65.93; H, 3.28; N, 19.22. Found: C, 65.66; H, 3.03; N, 19.34%.

3-Acetyl-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-nitrophenyl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (8d): Brown solid, mp 180°C; IR (KBr) $\nu = 2232$ (CN), 1705, 1665 (2CO), cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 2.62$ (s, 3H, COCH₃), 7.50-7.97 (m, 14H, Ar-H), 8.44 (d, $J = 4.6$ Hz, 1H, pyridine-H), 8.52 (d, $J = 4.6$ Hz, 1H, pyridine-H), ppm; MS, m/z (%) 593 (M^+ , 2), 423 (27), 422 (100), 405 (14), 77 (86). *Anal.* Calcd. for $C_{32}H_{19}N_9O_4$ (593.57): C, 64.75; H, 3.23; N, 21.24. Found: C, 64.61; H, 3.20; N, 21.35%.

Ethyl 5-oxo-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-phenyl-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8e): Yellow crystals, mp 220°C; IR (KBr) $\nu = 2233$ (CN), 1725, 1705 (2CO) cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 1.42$ (t, $J = 7$ Hz, 3H, CH₃), 4.58 (q, $J = 7$ Hz, 2H, CH₂), 7.48-7.66 (m, 15H, Ar-H), 8.07 (d, $J = 5$ Hz, 1H, pyridine-H), 8.74 (d, $J = 5$ Hz, 1H, pyridine-H) ppm; MS, m/z (%) 579 ($M^+ + 1$, 16), 578 (M^+ , 48), 533 (5), 505 (24), 91 (22), 77 (100). *Anal.* Calcd. for $C_{33}H_{22}N_8O_3$ (578.60): C, 68.51; H, 3.83; N, 19.37. Found: C, 68.35; H, 3.49; N, 19.29%.

Ethyl 5-oxo-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-methylphenyl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8f): Yellow crystals, mp 280°C; IR (KBr) $\nu = 2233$ (CN), 1748, 1711 (2CO), cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 1.20$ (t, $J = 7$ Hz, 3H, CH₃), 2.41 (s, 3H, Ar-CH₃), 4.56 (q, $J = 7$ Hz, 2H, CH₂), 7.44-7.52 (m, 14H, Ar-H), 8.03 (d, $J = 4.5$ Hz, 1H, pyridine-H), 8.73 (d, $J = 4.5$ Hz, 1H, pyridine-H), ppm; MS, m/z (%) 593 ($M^+ + 1$, 33), 592 (M^+ , 84), 591 (22), 547 (15), 519 (38), 105 (51), 91 (75), 77 (100). *Anal.* Calcd. for $C_{34}H_{24}N_8O_3$ (592.62): C, 68.91; H, 4.08; N, 18.91. Found: C, 68.75; H, 4.27; N, 18.81%.

Ethyl 5-oxo-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-chlorophenyl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8g): Yellow solid, mp 260°C; IR (KBr) $\nu = 2233$ (CN), 1747, 1713 (2CO) cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 1.43$ (t, $J = 7$ Hz, 3H, CH₃), 4.56 (q, $J = 7$ Hz, 2H, CH₂), 7.34-7.75 (m, 10H, Ar-H), 7.71 (d, $J = 8$ Hz, 2H, Ar-H), 8.08 (d, $J = 5$ Hz, 1H, pyridine-H), 8.21 (d, $J = 8$ Hz, 2H, Ar-H), 8.74 (d, $J = 5$ Hz, 1H, pyridine-H) ppm; MS, m/z (%) 615 ($M^+ + 2$, 6), 613 (M^+ , 18), 612 (54), 569 (9), 539 (25), 334 (13), 180 (12), 111 (22), 77 (100). *Anal.* Calcd. for $C_{33}H_{21}ClN_8O_3$ (613.04): C, 64.66; H, 3.45; N, 18.28. Found: C, 64.43; H, 3.27; N, 18.07%.

Ethyl 5-oxo-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-nitrophenyl)-1,5-dihydro-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (8h): Yellow crystals, mp 160°C; IR (KBr) $\nu = 2233$ (CN), 1735, 1715 (2C=O) (2C=O) cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 1.23$ (t, $J = 7$ Hz, 3H, CH₃), 3.07 (q, $J = 7$ Hz, 2H, CH₂), 7.48 (m, 14H, Ar-H), 8.01 (d, $J = 5$ Hz, 1H, pyridine-H), 8.73 (d, $J = 5$ Hz, 1H, pyridine-H) ppm; MS, m/z (%) 623 (M^+ , 5.9), 550 (10.3), 422 (13.2), 180 (17.6), 119 (29.4), 77 (100). *Anal.* Calcd. for $C_{33}H_{21}N_9O_5$ (623.59): C, 63.56; H, 3.39; N, 20.22. Found: C, 63.41; H, 3.60; N, 20.00%.

N3,1-Diphenyl-5-oxo-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1,5-dihydro-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxamide (8i): Yellow crystals, mp 200°C; IR (KBr) $\nu = 3388$ (NH), 2233 (CN), 1698, 1658 (2C=O) cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 7.02$ -8.35 (m, 20H, Ar-H), 8.44 (d, $J = 5$ Hz, 1H, pyridine-H), 8.75 (d, $J = 5$ Hz, 1H, pyridine-H), 11.69 (s, 1H, NH) ppm; MS, m/z (%) 626 ($M^+ + 1$, 10), 625 (M^+ , 10), 457 (7), 318 (31), 148 (15), 119 (47), 91 (57), 89 (22), 77 (53), 64 (100). *Anal.* Calcd. for $C_{37}H_{23}N_9O_2$ (625.65): C, 71.03; H, 3.71; N, 20.15. Found: C, 71.13; H, 3.43; N, 20.32%.

5-Oxo-N3-phenyl-6-(4-cyano-1,5-diphenylpyrazol-3-yl)-1-(4-methylphenyl)-1,5-dihydro-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxamide (8j): Yellow crystals, mp 190°C; IR (KBr) $\nu = 3358$ (NH), 2230 (CN), 1690, 1666 (2CO) cm^{-1} ; 1H NMR (DMSO- d_6) $\delta = 2.27$ (s, 3H, Ar-CH₃), 7.14-7.97 (m, 19H, Ar-H), 8.11 (d, $J = 4.5$ Hz, 1H, pyridine-H), 8.44 (d, $J = 4.5$ Hz, 1H, pyridine-H), 10.14 (s, 1H, NH) ppm; MS, m/z (%) 639 (M^+ , 3), 422 (33), 201 (13), 135 (13), 119 (33), 106 (25), 91 (52), 77 (100). *Anal.* Calcd. for $C_{38}H_{25}N_9O_2$ (639.68): C, 71.35; H, 3.94; N, 19.71. Found: C, 71.22; H, 3.90; N, 19.53%.

Determination of Minimum Inhibitory Concentration (MIC), of Ribavirin and Different Tested Compounds in HCV Replicon Cells was Performed as Follow: Briefly, 1×10^4 replicon cells per well were plated in 96-well plates. On the following day, replicon cells were incubated at 37°C for the indicated period of time with antiviral agents were serially diluted in DMEM plus 2% FBS and 0.5% dimethyl sulfoxide (DMSO). Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA) and the copy number of HCV-RNA was determined using a quantitative RT-PCR (QRT-PCR) assay. Each datum point represents the average of five replicates in cell culture. The cytotoxicity of tested

compounds was measured under the same experimental settings using a tetrazolium (MTS)-based cell viability assay (Promega, Madison, WI). For the cytotoxicity assay with human hepatocyte cell lines, 1×10^4 parental Huh-7 cells per well or 4×10^5 HepG2 cells per well were used.

MIC of the Tested Compounds in Hamster Brains for Antiviral Chemotherapy for Subacute Sclerosing Panencephalitis (SSPE): Under ether anesthesia, 50 μ l of ribavirin or tested compound solutions at dosages of 5, 10 and 20 mg/kg/day was injected for 10 days intracranially to a depth of 2 mm by using a 27-gauge needle and was placed within the subarachnoid space. On 1, 2, 3, 5, 7, 10, 12, 15 and 20 days after the initial injection, four hamsters from each group were sacrificed. The brains were aseptically removed, washed twice with phosphate-buffered saline (PBS), homogenized and suspended in PBS. The suspension was centrifuged at 1600 for 10 min. The supernatant was collected, ethanol was added to remove proteins and the mixture was heated at 90°C to evaporate ethanol. The protein-free samples were used to evaluate the MIC in brain tissue by HPLC and bioassay.

Radical Scavenging Assays:

DPPH Radical Scavenging Assay: The antioxidant activities of the tested compounds and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH[•] free radical [31]. Weighed quantities of the ten compounds were dissolved in distilled DMSO and used. Solution of ascorbic acid used as standard for this study was prepared in distilled H₂O. All these solutions were serially diluted with respective solvents to get lower dilutions.

Each compound (10 μ l) or standard (from 0.0 μ M/ml to 100 μ M/ml) was added to 90 μ l of DPPH[•] in methanol solution (100 μ M) in a 96-well microtitre plate. After incubation in the dark at 37°C for 30 min, the decrease in absorbance of each solution was measured at 515 nm using ELISA microplate reader (Bio Rad Laboratories Inc., California, USA, Model 550). Absorbance of blank sample containing the same amount of DMSO and DPPH[•] solution was done prepared and measured as well. The experiment was carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. Radical scavenging activity was calculated by the following formula: % Reduction of

absorbance = $[(A_B - A_A) / A_B] \times 100$, where: A_B indicates absorbance of blank sample and A_A indicates absorbance of tested extract solution ($t = 30$ min).

Reaction with Reactive Nitrogen Species:

Synthesis of Peroxynitrite: Peroxynitrite (ONOO⁻) was synthesized as previously described by Beckman *et al.* [32]. Briefly, an acidic solution (0.6 M HCl) of H₂O₂ (0.7 M) was mixed with KNO₂ (0.6 M) on ice for one second and the reaction was quenched with ice-cold NaOH (1.2 M). Residual H₂O₂ was removed by mixing with granular MnO₂ prewashed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (-20°C) and the top layer of the solution was collected for the experiment. Concentrations of stock ONOO⁻ were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm⁻¹ M⁻¹. Concentrations of 200-250 mM were usually obtained. Once thawed, ONOO⁻ solutions were kept in ice for no longer than 30 min before use.

Reaction of Compounds with Peroxynitrite: The ability of tested compounds to inhibit peroxynitrite-induced tyrosine nitration was investigated via reaction of equimolar concentrations (100 μ M) of tyrosine and peroxynitrite in the presence of increasing concentrations of each tested compound (0 - 300 μ M) in 100 mM phosphate buffer, pH 7.4, at 37°C for 15 min. Snap-freezing reaction mixtures prior to HPLC analysis successfully terminated the reactions. The formation of 3-nitrotyrosine (3-NT) was monitored by HPLC analyzed with photodiode array detection (see below). 3-NT formed was characterized and quantified by use of an authentic standard (elution time and unique spectral characteristics).

HPLC Analysis: Reaction mixtures were analyzed using reverse-phase HPLC analysis performed on an Agilent 1100 system with a Zorbax ODS C18 column (150 \times 4.6 mm i.d., 4 μ m) and a guard column (15 \times 4.6 mm i.d., 4 μ m). Mobile phase A consisted of methanol/water/ 5 N HCl (5: 94.90.1 v/v/v) and mobile phase B of acetonitrile/water/5 N HCl (50: 49.9: 0.1 v: v: v). The following gradient system was used (min/% acetonitrile): 0: 0, 5: 0, 40: 50, 60: 100, 65: 100 and 65.1: 100 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm for 3-NT measurements with spectra of products obtained over the 220-600 nm range.

Antitumor Activity of Novel Topoisomerase II Inhibitors:

DNA topoisomerase II has been shown to be an important therapeutic target in cancer chemotherapy. Herein, we describe studies on the antitumor activity of novel topoisomerase II inhibitors. The tested compounds inhibited topoisomerase II activity at 10 times lower concentration than etoposide in relaxation assay and cells. Murine Colon 38 cancer, murine leukemia P388 and human lung cancer LX-1 were obtained from the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan). Human lung cancer PC-1 and human gastric cancer MKN-1, MKN-7, MKN-28 and MKN-74 were obtained from Immuno Biology Laboratory (Gunma, Japan). Human lung cancer A549 and human colon cancer WiDr were obtained from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). Human gastric cancer HGC-27 and GT3TKB were obtained from Riken Cell Bank (Ibaraki, Japan). The other cell lines were purchased from American Type Culture Collection (Rockville, MD).

Growth-Inhibitory Effect: Exponentially growing solid tumor cells in 0.1 ml of medium were seeded in 96-well plates on day 0. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the cell plates. After incubation at 37°C for 72 h, the cell number was estimated by sulforhodamine B (SRB) assay. The cell number on day 1 was also measured separately. GI50 values are the drug concentrations causing a 50% reduction in the net protein increase in control cells. The antitumor spectrum of ER-37328 was compared with those of other drugs by means of the NCI COMPARE analysis procedure. Exponentially growing P388 cells were seeded in 96-well plates on day 0. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the wells. After incubation at 37°C for 72 h, the cell number was determined by MTT assay. IC₅₀ values were calculated as the drug concentrations that reduced the number of cells to 50% of the control number.

In vivo Efficacy Study: Female C57BL/6 mice (6 weeks of age; Charles River, Atsugi, Japan) were housed in barrier facilities on a 12 h light/dark cycle, with food and water ad libitum. About 30 mg of Colon 38 tumor tissues was inoculated s.c. into the flank of animals. Mice were randomized and separated into control and treatment (n = 5 each) groups when the tumor volume reached approximately 300 mm³ on day 1. The tested compound

was dissolved in 5% glucose. Etoposide was diluted in saline and doxorubicin was dissolved in saline. The tested compound was administered on day 1 (single dose) by injection into the tail vein and Etoposide and doxorubicin were given at the maximum tolerated dose on the reported administration schedule. Control animals received 5% glucose. Tumor volume and body weight were measured on the days indicated in the figures. Tumor volume was calculated according to the following equation: tumor volume (mm³) = (length - width²)/2.

Relaxation Assay: Topoisomerase II was purified from P388 cells. One unit of the enzyme was defined as the activity to relax completely 0.125 µg of supercoiled pBR-322 DNA at 30°C for 1 h. For the assay, 0.125 µg of supercoiled pBR-322 DNA (Takara Shuzo Co., Ltd, Tokyo, Japan) was relaxed with one unit of topoisomerase II in 20 µl of the assay buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.5 µM EDTA, 1 mM ATP and 30 µg/ml BSA] in the presence or absence of an inhibitor at 30°C for 1 h. Samples were subjected to electrophoresis in 0.7% agarose gels with TBE buffer [89 mM Tris, 89 mM boric acid and 2 mM EDTA (pH 8.0)]. The DNA was stained with ethidium bromide and photographed under UV light.

Growth-Inhibitory Effect: The growth-inhibitory effects of the tested compounds **8** on 21 human solid tumor cell lines (8 lung, 7 colon and 6 gastric cancer cell lines) were determined. The tested compounds inhibited the growth of these cell lines with an average GI50 given in Table 1 and relative potency to etoposide were determined. The correlation coefficients with etoposide, doxorubicin, SN-38 (topoisomerase I inhibitor) and cisplatin, calculated according to the NCI COMPARE analysis procedure using the tested compound as the benchmark, are given in Table 3b, respectively. That is, the pattern of the growth-inhibitory effect of the tested compounds was similar to that of etoposide, but less similar to that of doxorubicin.

In vivo Efficacy Study: The *in vivo* activity of the tested compounds, etoposide, against murine colon cancer Colon38 was tested (Table 3). Treatment of Colon 38 implanted into mice with ER-37328 induced a clear dose-dependent inhibition of tumor growth. Moreover, marked tumor regression was observed at 3 mg/kg. No body weight was observed at 3 mg/kg.

Table 1: Formation of **8a**, **8e** and **8i** using Microwave and Conventional Heating Procedures

Compound No.	Microwave irradiation			Thermal heating		
	Time (min)	Yield (%) (Chitosan)	Yield (%) (TEA)	Time (min)	Yield (%) (Chitosan)	Yield (%) (TEA)
8a	10	90	86	5	84	78
8e	10	92	87	5	80	75
8i	10	89	82	5	80	78

Table 2a: MIC of Ribaverin and the ten tested compounds against HCV and SSPE

Tested Compound	MIC µg/ml	
	HCV	Subacute Sclerosing Panencephalitis (SSPE)
Ribaverin	16.15	77.89
8a	0.541	4.723
8b	0.610	4.811
8c	0.747	5.089
8d	0.778	5.098
8e	0.664	4.873
8f	0.695	4.988
8g	0.444	4.711
8h	0.716	5.011
8i	0.623	4.812
8j	0.593	4.800

Table 2b: Decrease of DPPH absorbance (%) by tested compounds

Compound No.	Decrease of DPPH absorbance % mean ± SD (n = 3)
Ascorbic acid (standard)	79.042±2.928
8a	92.22±0.083
8b	91.19±0.034
8c	89.77±0.065
8d	89.19±0.057
8e	89.99±0.067
8f	89.89±0.045
8g	92.30±0.037
8h	89.88±0.045
8i	90.00±0.054
8j	91.99±0.032

Table 2c: IC₅₀ values of the compounds tested for the inhibition of ONOO⁻ mediated 3-nitrotyrosine formation

Compound No.	IC ₅₀ (µM) mean ± SD (n = 3)
Trolox	58.430±5.9000
8a	49.211±0.0454
8b	49.457±0.0464
8c	50.049±0.0466
8d	51.004±0.0466
8e	49.466±0.0422
8f	49.678±0.0434
8g	49.160±0.0466
8h	50.000±0.0456
8i	49.555±0.0433
8j	49.321±0.0435

Table 3a: The Average GI₅₀ and relative potencies of the newly synthesized compounds to Etoposide

Compound No	Average GI ₅₀	Relative Potency to Etoposide
8a	0.081	37.03703704
8b	0.088	34.09090909
8c	0.103	29.12621359
8d	0.120	25.00
8e	0.091	32.96703297
8f	0.093	32.25806452
8g	0.080	37.5
8h	0.099	30.3030303
8i	0.090	33.33333333
8j	0.084	35.71428571

Table 3b: The correlation coefficients of the newly synthesized compounds with topoisomerase I inhibitors

Compound No	Etoposide	Doxorubicin	SN-38	Cisplatin
8a	3.73	3.350	3.22	2.94
8b	3.55	3.330	3.13	2.92
8c	2.91	2.822	2.56	2.34
8d	2.52	2.311	2.11	2.00
8e	3.32	3.220	3.05	2.89
8f	3.23	3.190	3.01	2.82
8g	3.75	3.530	3.32	2.96
8h	3.12	3.110	3.00	2.81
8i	3.46	3.290	3.12	2.91
8j	3.62	3.340	3.21	2.93

Table 3c: The relative potencies of the newly synthesized compounds to Etoposide

Compound No	Relative potency to Etoposide
8a	37.00
8b	34.00
8c	29.10
8d	25.00
8e	32.90
8f	32.10
8g	37.40
8h	30.20
8i	33.30
8j	35.70

Table 3d: The relative potencies of the newly synthesized compounds to Etoposide

Compound No	Relative potency to Etoposide
8a	36.00
8b	34.00
8c	29.00
8d	25.00
8e	32.80
8f	32.00
8g	36.40
8h	30.00
8i	33.10
8j	35.10

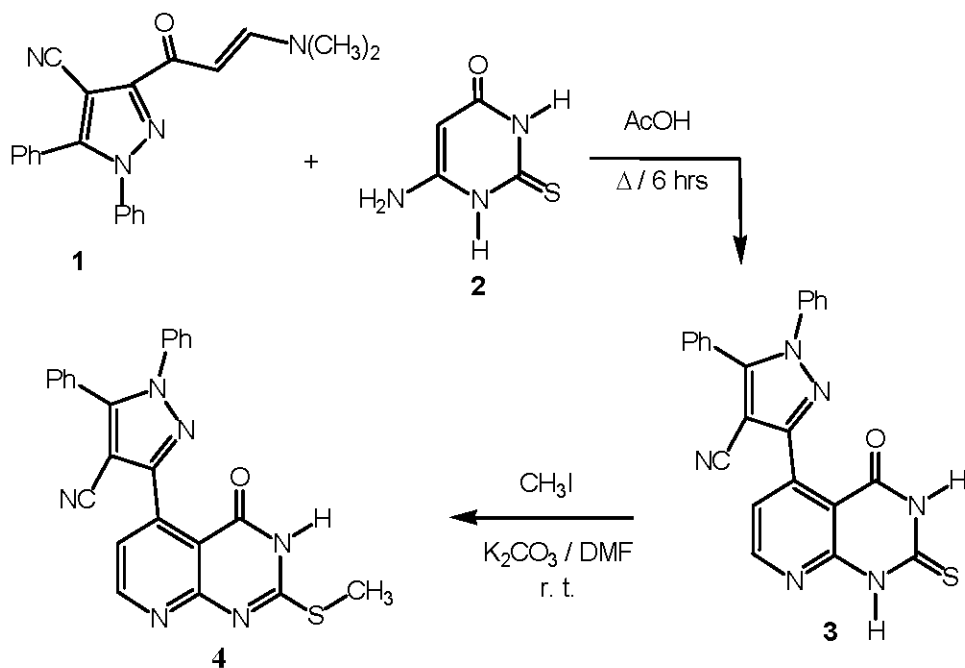
Inhibition of Topoisomerase II Activity: The effects of the tested compounds (Table 3b) and etoposide on topoisomerase II activity were analyzed by means of relaxation assay. The tested compounds and topoiside inhibited topoisomerase II activity and the relative potency of each compound were determined.

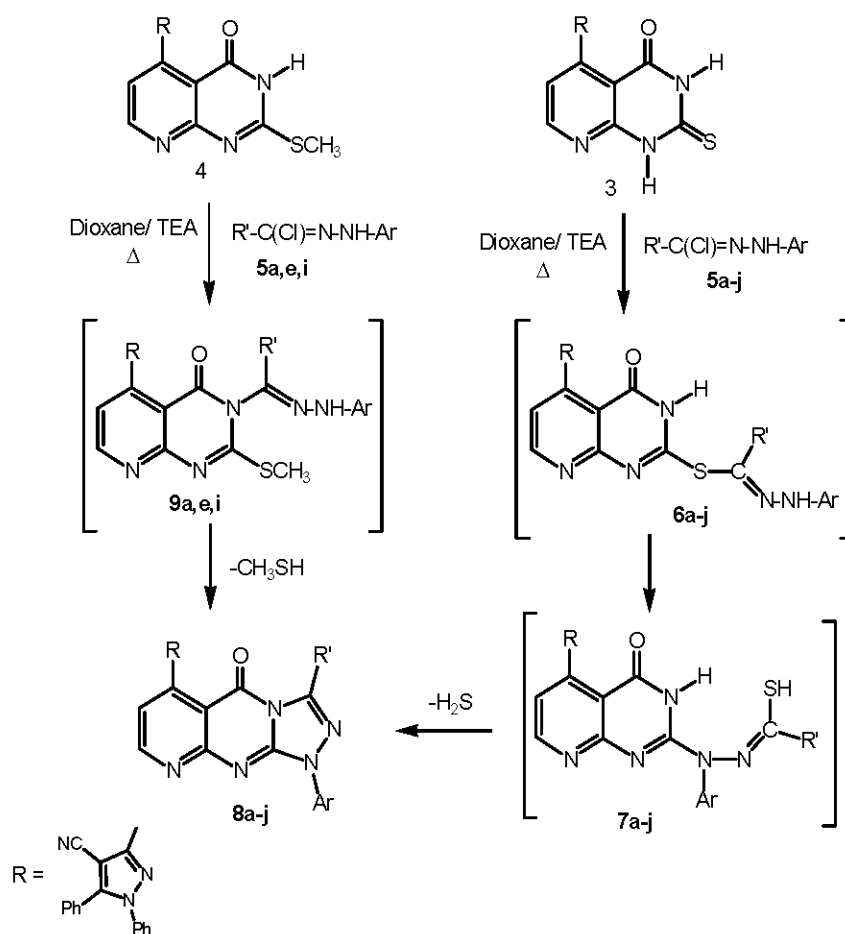
RESULTS AND DISCUSSION

The required new starting reactant **3**, namely 5-substituted-2-thioxo-2, 3-dihydro-1*H*-pyrido [2,3-*d*] pyrimidin-4-one was prepared by reaction of 3-[(3-dimethylamino)-2-propenoyl]-4- cyano-1, 5-diphenyl-pyrazole **1** and 6-amino-2-thioxo-2, 3-dihydro-pyrimidin-4(1*H*)-one **2** in boiling acetic acid. In addition, unreported

compound, 2-methylthio derivative **4** was prepared by reaction of **3** with methyl iodide in dimethylformamide and in the presence of anhydrous potassium carbonate at room temperature (Scheme 1).

The reaction of **3** with hydrazoneyl chlorides **5a-j** was carried out in dioxane in the presence of triethylamine under reflux until all the hydrogen sulfide gas ceased to evolve (**Scheme 2**). The reaction gave in each case after working up, only one isolable product as evidenced by TLC monitoring of the crude product. The structures of the compounds **8** were established on the basis of spectral and analytical data (see Experimental). For example, the IR spectra of the products **8** revealed in each case an additional absorption band at 1748-1690 cm^{-1} due to carbonyl groups. ^1H NMR spectra exhibited the absence of the 2NH groups in compound **3** and instead revealed the presence of the signal due to the protons of acetyl, ester and anilide substituents at position 3. The microanalyses and mass spectral data are in consistent with the expected molecular weight of the products **8**. The assignment of the proposed mechanism and the formation of **8** were further manifested by alternate synthesis. Thus, treatment of 2-methylthio derivative **4** with hydrazoneyl chlorides **5a,e,i** in dioxane in the presence of triethylamine under reflux led to evolution of methanethiol and the formation of products that proved to be identical in all respects (mp., mixed mp. and IR) with compounds **8** (**Scheme 2**).

Scheme 1: Synthesis of thione **3** and methylthio derivative **4**



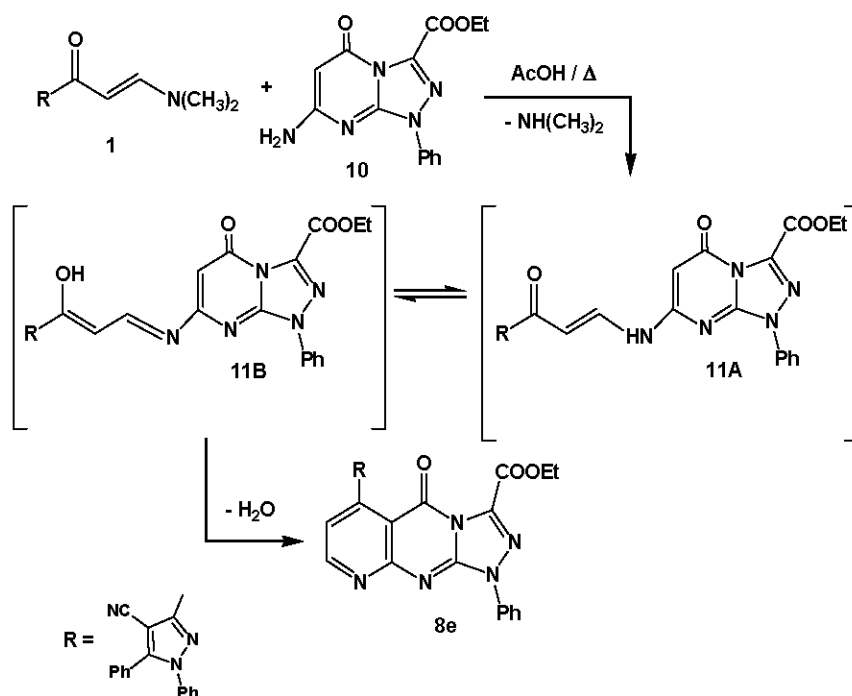
Compd. no.	R'	Ar	Compd. no.	R'	Ar	Compd. no.	R'	Ar
5a, 8a	CH ₃ CO	C ₆ H ₅	5e, 8e	CO ₂ Et	C ₆ H ₅	5i, 8i	PhNHCO	C ₆ H ₅
5b, 8b	CH ₃ CO	4-CH ₃ C ₆ H ₄	5f, 8f	CO ₂ Et	4-CH ₃ C ₆ H ₄	5j, 8j	PhNHCO	4-CH ₃ C ₆ H ₄
5c, 8c	CH ₃ CO	4-ClC ₆ H ₄	5g, 8g	CO ₂ Et	4-ClC ₆ H ₄			
5d, 8d	CH ₃ CO	4-NO ₂ C ₆ H ₄	5h, 8h	CO ₂ Et	4-NO ₂ C ₆ H ₄			

Scheme 2: Synthesis of compound **8a-j**

The mechanism explained for the reaction of **4** with **5** may be proceed *via* formation of amidrazones **9** which in turn, underwent cyclization with elimination of methanethiol to give **8a,e,i** as end products (**Scheme 2**), according to Smiles rearrangement mechanism [33, 34]. To substantiate further the assigned structure **8** for the isolated products, we examined alternate synthesis of these products. Thus, reaction of 7-amino-3-ethoxycarbonyl-1-phenyl [1, 2, 4] triazolo [4, 3-*a*] pyrimidin-5(1*H*)-one (**10**) [30] with enaminone **1** in acetic acid under reflux gave product identical in all respects to that isolated product **8e** (**Scheme 3**).

Finally, we used microwave irradiation technique as an alternative method for the syntheses of products **8a, 8e** and **8i** using the same reaction sequence outlined in Scheme 1. It is noteworthy to mention that the catalyst used in this method is either triethylamine or chitosan which is a very efficient and environmentally benign heterogeneous basic catalyst. The reaction results (time, % yield) were compared with that of the traditional thermal procedure using triethylamine as basic catalyst (Table 1).

As shown from Table 1, the use of microwave irradiation substantially reduced the reaction times from hours scale to minutes scale and appreciably increased



Scheme 3: Alternative method for synthesis of compound **8e**

the yields. Also, chitosan can be used as ecofriendly basic catalyst for preparation of desired products **8a, e, i** in high yield under thermal heating or microwave irradiation.

Pharmacological Screening: The newly synthesized compounds have structural similarity to many antioxidants and topoisomerase II inhibitors so they were tested for the following activities.

Hepatitis C Virus (HCV) NS3-4A Protease Inhibitory Activities in both HCV Replicon Cells and in Hamster Brains: The results showed that all the tested compounds are more active than ribavirin in the following ascending order **8d, 8c, 8h, 8f, 8e, 8i, 8b, 8j, 8a** and **8g** (Table 2a).

DPPH Radical Scavenging Activity: It is worth to mentions that all the tested compounds are more active than ascorbic acid in the following ascending order **8d, 8c, 8h, 8f, 8e, 8i, 8b, 8j, 8a** and **8g** (Table 2b).

Inhibition of Peroxynitrite-Induced Tyrosine Nitration: It is worth to mention that all the tested compounds are more active than trolox in the following ascending order **8d, 8c, 8h, 8f, 8e, 8i, 8b, 8j, 8a** and **8g** (Table 2c).

In vivo Efficacy Study: The *in vivo* activity of the tested compounds and etoposide, against murine Colon 38 cancer was tested (Table 3a). Treatment of Colon 38 implanted into mice with ER-37328 induced a clear dose-dependent inhibition of tumor growth. Moreover, marked tumor regression was observed at 3 mg/kg. No body weight was observed at 3 mg/kg. It is worth to mention that all the tested compounds showed potent antitumor activities and the order of activity in ascending order is **8d, 8c, 8h, 8f, 8e, 8i, 8b, 8j, 8a** and **8g**.

In a progress to determine the mechanism of action of the antitumor activities, the correlation coefficients with (topoisomerase I inhibitor) and cisplatin, were calculated according to the NCI COMPARE analysis procedure using the tested compound as the benchmark. These calculations culminated on the newly synthesized compounds that act as topoisomerase inhibitors (Table 3b).

To confirm this mechanistic pathway of action the following assays are done.

To confirm this mechanistic pathway of action the following assays are done:

Inhibition of Topoisomerase II Activity: The effects of the tested compounds and etoposide on topoisomerase II activity were analyzed by means of a relaxation assay.

The tested compounds and topoisomerase II activity and the relative potency of each compound was determined and confirmed in the following ascending order **8d**, **8c**, **8h**, **8f**, **8e**, **8i**, **8b**, **8j**, **8a** and **8g** as in Table 3c and Table 3d.

All the previously mentioned measurements revealed that all the tested compound are more active than etoposide and act similar to its mechanism of action as topoisomerase I inhibitors.

CONCLUSION

In conclusion, the aim of the present investigation was to synthesize novel series of pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-5-ones incorporating substituted pyrazole moiety. The structures of all the newly synthesized compounds were established on the basis of spectral analysis. The newly synthesized compounds seem to be interesting for biological activity studies. These compounds were investigated for anti-HCV, antioxidant and antitumor (as topoisomerase I inhibitors) activities. All the tested compounds are highly effective at very low concentrations compared to the reference drug in all biological activity studies.

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