Synthesis of new pentaheterocyclic ring systems as anti-androgene, anti-HCV and anti-H1N1 agents

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Abstract

A new series of pentaheterocycles, namely, benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]pyrimidine-7,13(3*H*)-diones **10** was prepared *via* the reaction of hydrazonoyl chlorides **7** with 2,4-dihydro-3-thioxo-benzo[5',6']pyrano[3',4':5,6]pyrido[2,3-*d*]pyrimidine-1,7-dione **4** in presence of chitosan as ecofriendly catalyst. The structure of the newly synthesized compounds were established on the basis of spectral data (Mass, IR, ^{1}H and ^{13}C NMR) and elemental analyses.

Keywords: Hydrazonoyl halides, 4-hydroxycoumarine, chitosan, antiandrogene, anti HCV, anti H1N1

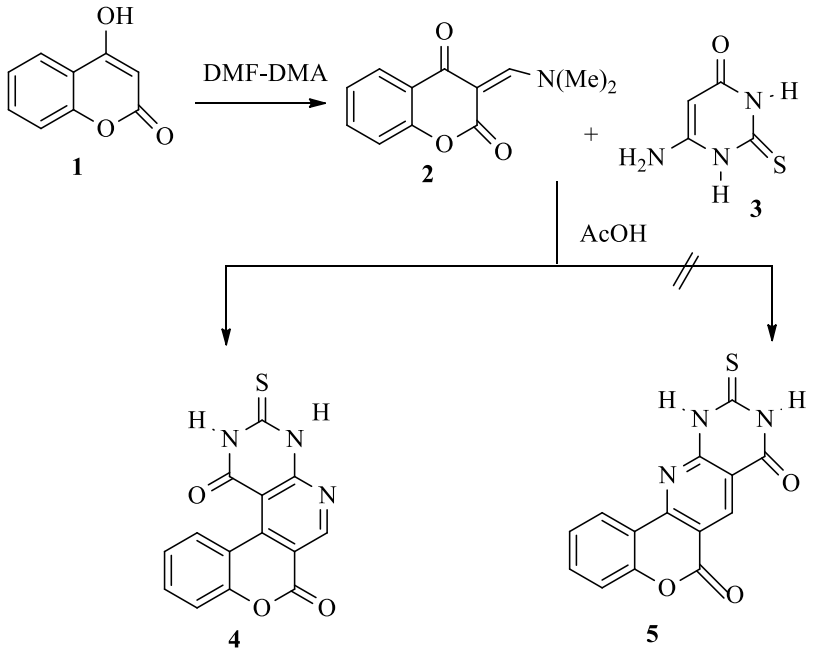
Introduction

Hydrazonoyl halides have been widely employed in the synthesis of heterocyclic derivatives.[1-5] Coumarine is an important structural moiety present in a variety of natural and synthetic products that possess significant biological activities,[6] such as, anticoagulants, antifungal, antineoplastic, antibacterial, spasmolytic or cytotoxic activity.[7-9] In addition, chitosan, the naturally occurring polysaccharide, can be used as heterogeneous phase transfer catalyst in heterocyclic synthesis,[10] as well as transition metal support for the preparation of heterogeneous catalysts.[11] In addition, chitosan is a copolymer containing both glucoseamine units and acetylglucoseamine units. The presence of amino groups is responsible for the basic nature of chitosan. All the above findings encouraged us to synthesize a new pentaheterocyclic ring system, namely, benzo[5',6']pyrano [4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]pyrimidine-7,13(3*H*)-diones **10** *via* the reaction of 2,4-dihydro-3-thioxo-

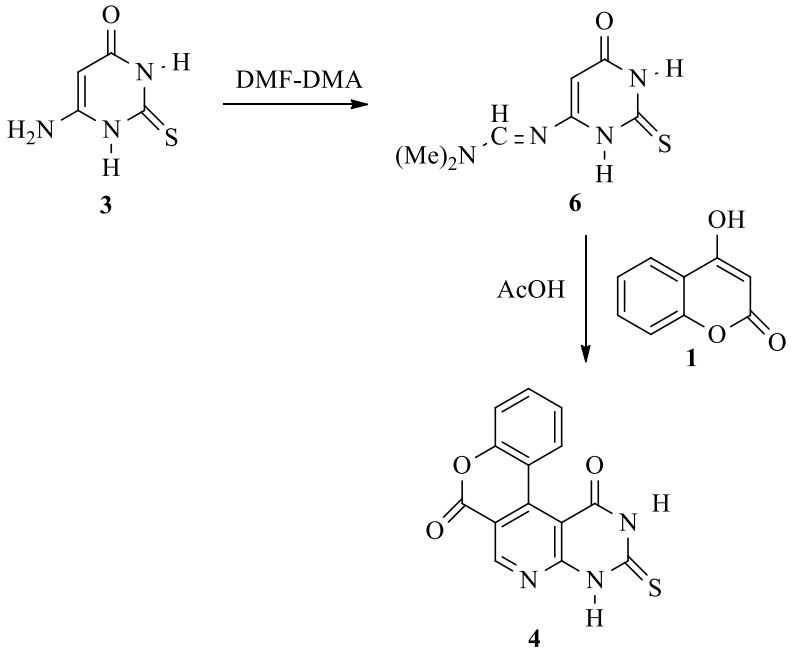
benzo[5',6']pyrano[3',4':5,6]pyrido[2,3-*d*]pyrimidine-1,7-dione **4** with hydrazonoyl halides **7** in presence of chitosan as ecofriendly basic catalyst instead of the traditional toxic catalyst, and study their biological activity.

Results and Discussion

Reaction of 3-(*N*,*N*-dimethylaminomethylene)-chromane-2,4-dione (**2**) with 6-amino-2-thioxopyrimidin-4(3*H*)-one (**3**) in glacial acetic acid under reflux gave a new tetra-heterocyclic ring system **4** or **5** (Scheme 1). Mass, IR spectra and elemental analysis data of the isolated product is consistent with each of the isomeric structures **4** and **5** (Scheme 1). However, ^{1}H NMR spectral data was found to be consistent with structure **4.** This is because, ^{1}H NMR spectrum revealed singlet signal at δ 8.78 ppm assigned for pyridine-H at position 2 not pyridine-H at position 4.[12] Furthermore, alternative synthesis of compound **4** was accomplished *via* condensation of 6-amino-2-thioxopyrimidin-4(3*H*)-one (**3**) with dimethylformamide-dimethylacetal (DMF-DMA) to give compound **6**,[13] followed by treatment of product **6** with 4-hydroxy-coumarine (**1**) (Scheme 2). In addition, we published recently[14] that the reaction of heterocyclic amine with enaminone proceeded firstly *via* nucleophilic attack of the amino group of the heterocyclic amine to the double bond of the enaminone with elimination of dimethylamine rather than condensation of amino group with the ketonic group with elimination of water molecule.[15-17] Based on these findings, structure **5** was discarded and the isolated product from the studied reaction was assigned structure **4** (Scheme 1).

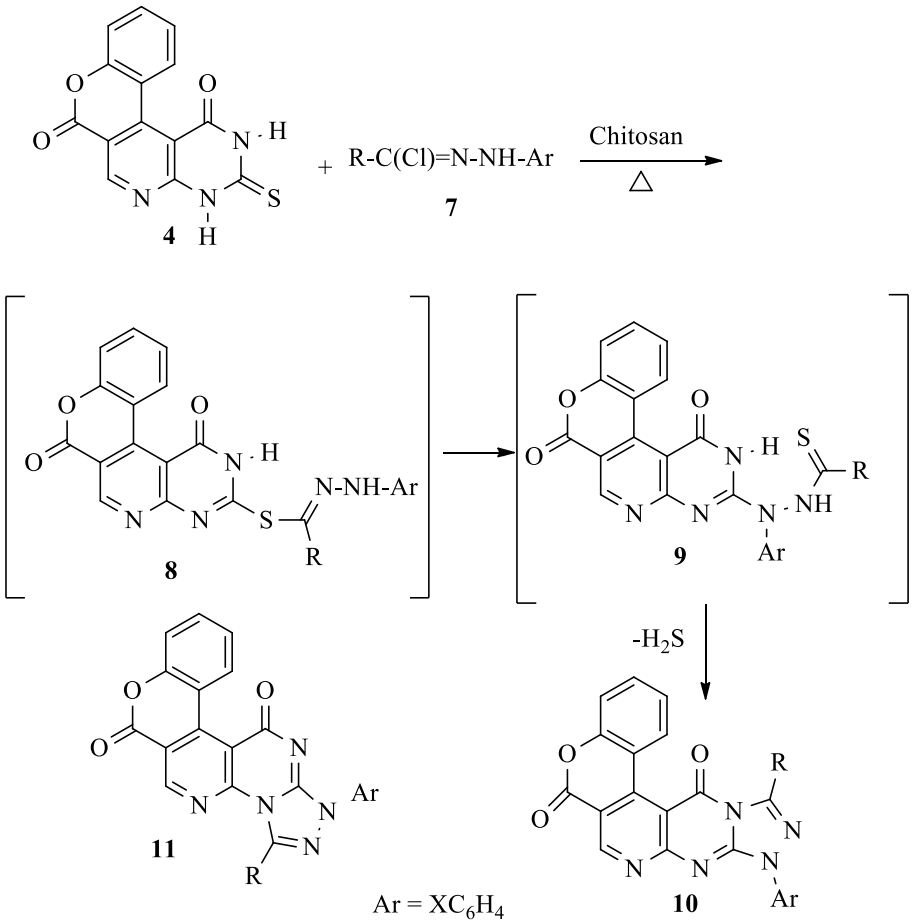


Scheme 1. Synthesis of compound **4.**



Scheme 2. Alternative synthesis of compound **4**.

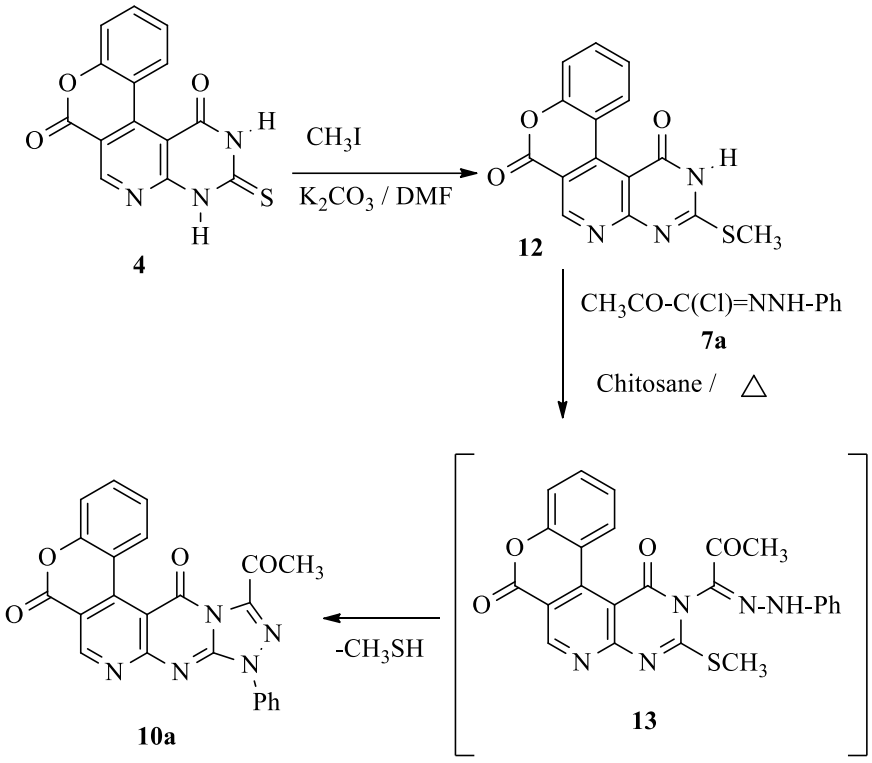
In continuation of our studies in the utility of hydrazonoyl halides in synthesis of poly-heterocyclic ring systems,[5] we investigated the reaction of hydrazonoyl halides **7** with heterocyclic thione **4.** Reaction of **4** with **7a-o** in dioxane in presence of chitosan as a base catalyst under reflux gave a single product in each case consistent with structure **10** or **11** (Scheme 3). An immediate distinction between these two structures was reached by comparison of the ^{13}C NMR spectra with those of similar annulated pyrimidinones. Literature reports[18-21] have shown that the chemical shift for the carbonyl carbon in 4-pyrimidinone derivatives is markedly affected by the nature of the adjacent nitrogen (N3) (pyrrole type in structure **10** and pyridine type as in structure **11**). For example, the ^{13}C NMR spectra of **10a** and **10e** taken as typical examples of the series prepared, revealed the signals of the carbonyl carbon of the pyrimidinone ring residue at δ 163.05 and 164.27 ppm, respectively. Such chemical shift values are similar to those of annulated pyrimidines with N3 pyrrole type rather than those of N3 pyridine type. On the basis of this similarity, the isolated products were assigned structure **10** and the isomeric structure **11** was excluded.



R / X : a, CH_3CO / H; b, CH_3CO / 4-CH_3; c, CH_3CO / 4-Cl; d, CH_3CO / 4-NO_2; e, EtOCO / H; f, EtOCO / 4-CH_3; g, EtOCO / 4-Cl; h, EtOCO / 4-NO_2; i, EtOCO / 3-CH_3; j, EtOCO / 3-Cl; k, PhNHCO / H; l, PhNHCO / 3-CH_3; m, PhNHCO / 4-Cl; n, PhNHCO / 4-MO_2; o,  CO / H

Scheme 3. Synthesis of compounds **10a-o**.

Also, compound **10a** was synthesized *via* the methylthio-derivative **12** (prepared by the reaction of **4** with methyl iodide in dimethylformamide in presence of anhydrous potassium carbonate) with hydrazonoyl chloride **7a** under the same reaction conditions through the intermediate **13** with concurrent elimination of methanethiol. The product **10a** found to be identical in all respects with the product produced from the reaction of **4** with **7a** (Scheme 4).



Scheme 4. Alternative synthesis of compound **10a**.

Pharmacology

Antiandrogenic activity in female rats. Neuman and Elger described[22] the method for testing the antiandrogenic activity in ovariectomized female rats. The protection of the antiandrogen Cyperoterone against the trophic effect of testosterone on uterine and prenuptial growth was equally studied in intact as well as castrated female rats.

Table 1. Antiandrogenic activity

Compound No.	ED_{50} mg/kg	LD_{50} mg/kg	LD_{90} mg/kg
10a	0.12±0.000011	123.22±0.022	287.23±1.21
10g	0.21±0.000012	321.09±0.034	324.45±1.23
10h	0.29±0.000013	213.69±0.032	333.67±3.44
10b	0.34±0.000014	214.63±0.34	432.67±4.34
4	0.56±0.000015	224.47±0.23	543.56±2.32
10e	0.65±0.000016	324.23±0.21	689.67±5.36
Cyproterone	1.7 ± 0.0031	518 ± 0.016	800 ± 0.012

Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

All tested compounds showed potent antiandrogenic activities and more potent than Cyperoterone in descending activity order **10a**, **10g**, **10h**, **10b**, **4**, **10e** (Table 1).

Hepatitis C virus (HCV) NS3-4A protease inhibitory activities in HCV replicon cells and EC_{50} of the tested compounds in Hamster Brains for antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE). Determination of minimum inhibitory concentration (EC_{50} CC_{50}), of ribavirin and different tested compounds in HCV replicon cells and EC_{50} CC_{50} of the tested compounds in Hamster Brains for antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE) were lead to the results depicted in Table 2.

Table 2. EC_{50},CC_{50} of Ribavirin and the ten tested compounds against HCV and SSPE

Tested Compounds	HCV			Subacute sclerosing panencephalitis (SSPE)
	EC_{50} (μg mL^{-1})[a]	CC_{50} (μg mL^{-1})[a]	SI	EC_{50} (μg/mL)
4	0.00295±0.000011	2.733±0.033	926.4407	0.017±0.00034
10a	0.00325±0.000023	3.8454±0.021	1183.2	0.019±0.00045
10g	0.00376±0.000045	4.1235±0.033	1096.676	0.021±0.0033
10h	0.00397±0.000034	5.84±0.0023	1471.033	0.022±0.000044
10b	0.00416±0.000022	6.83365±0.023	1642.704	0.027±0.00043
10e	0.00445±0.000067	7.53±0.023	1692.135	0.045±0.00047
10l	0.00494±0.000043	8.22±0.0057	1663.968	0.067±0.00044
10f	0.00513±0.000022	8.455±0.043	1648.148	0.089±0.00043
10c	0.00562±0.000033	9.356±0.045	1664.769	0.090±0.00033
10m	0.00612±0.000023	9.513332±0.021	1554.466	0.091±0.0003
Ribavirin	16.1500±0.000023			77.890±0. 0033

[a]Average and average ± SE, n = 12, for EC_{50} and CC_{50}, resp. Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

The order of activity in ascending order is **10m, 10c, 10f, 10l**, **10e, 10b, 10h, 10g, 10a,** and **4**

The mechanism of action is NS3-4A protease inhibitor in HCV replicon cells.

Anti-H1N1 activity of the newly synthesized compounds. A viral focus reduction assay was used to characterize the in vitro anti-influenza activity of the tested compounds. Human influenza A (H1N1) virus particles were used to infect Madin-Darby canine kidney NBL-2 (MDCK) cells. The tested compounds showed clear dose-dependent inhibition of H1N1 virus infection. The 50% inhibition concentration (IC_{50}) of the tested compounds for H1N1 and the 100% inhibition of H1N1 infection were achieved and tabulated as follow (Table 3).

The order of activity in ascending order were **10m, 10c, 10f, 10l**, **10e, 10b, 10h, 10g, 10a,** and **4**. The mechanism of action thought to be *via* inhibition of RNA synthesis.

Table 3. Inhibition of H1N1Infection

Tested compound	EC_{50} (μg mL^{-1})[a]	CC_{50} (μg mL^{-1})[a]	SI
Oseltamivir	0.1		
Amantadine	4.1		2283.636
4	0.0055±0.000002	12.56±0.0034	3028.814
10a	0.0059±0.000003	17.87±0.0032	3085.075
10g	0.0067±0.000004	20.67±0.0045	5008.696
10h	0.0069±0.000006	34.56±0.0076	5397.403
10b	0.0077±0.000007	41.56±0.0046	5934.177
10e	0.0079±0.000006	46.88±0.0087	6186.42
10l	0.0081±0.000007	50.11±0.0085	6360.976
10f	0.0082±0.000008	52.16±0.0075	5962.222
10c	0.0092±0.000009	53.66±0.0078	5803.191
10m	0.0094±0.000009	54.55±0.0054	2283.636

[a]Average and average ± SE, n = 12, for EC_{50} and CC_{50}, resp. Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

Conclusions

The new pentaheterocyclic compounds, namely, benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*] triazolo[4,3-*a*]pyrimidine-7,13(3*H*)-diones **10** were prepared via one-pot reaction of hydrazonoyl chlorides **7** with thione compound **4** using chitosan as ecofriendly base catalyst. The newly synthesized compounds showed promising activities against HCV, H1N1, and can also be used as antiandrogenic agents.

Experimental Section

General. All melting points were determined on an electrothermal Gallenkamp apparatus and 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 instrument in potassium bromide discs. The ^{1}H and ^{13}C-NMR spectra were recorded on a Varian Mercury VXR-300 spectrometer (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C NMR) 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. Hydrazonoyl halides **7a-g** and enaminone **2** were prepared following literature methods.[23-28]

2,4-Dihydro-3-thioxo-benzo[5',6']pyrano[3',4':5,6]pyrido[2,3-*d*]pyrimidine-1,7-dione (4)

Method A. A mixture of enaminone (**2**) (2.17 g, 10 mmol) and 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**3**) (1.43 g, 10 mmol) in acetic acid (30 mL) was refluxed for 6 hours. The reaction mixture was cooled, filtered off and recrystallized from ethanol to give compound **4** as yellow crystals, mp > 300 ºC; IR (KBr) υ = 3430, 3120 (2 NH), 1720, 1686 (2 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 7.47-8.51 (m, 4H, Ar-H), 8.78 (s, 1H, pyridine-H), 12.84 (s, 1H, NH), 13.44 (s, 1H, NH) ppm; MS, *m/z* (%) 297 (M^+, 20), 269 (18), 240 (13), 212 (15), 124 (36), 100 (34), 86 (25), 76 (58). *Anal*. Calcd. for $C_{14}H_7N_3O_3S$ (297.29): C,56.56; H,2.37; N,14.13. Found: C, 56.32; H, 2.20, N, 14.07%.

Method B. A mixture of compound **6** (1.98 g, 10 mmol) and 4-hydroxycoumarine (**1**) (1.62 g, 10 mmol) in acetic acid (30 mL) was refluxed for 10 hours (monitored by TLC). The reaction mixture was cooled, filtered off and recrystallized from ethanol to give product identical in all respects (mp, mixed mp, IR and ^{1}H NMR) with **4**.

3-Methylthio-benzo[5',6']pyrano[3',4':5,6]pyrido[2,3-d]pyrimidine-1,7(2*H*)-dione (12). To a stirred solution of thione (**4**) (1.5 g, 5 mmol) in dimethylformamide (20 mL) was added anhydrous potassium carbonate (1.14 g, 7.5 mmol), and methyl iodide (0.71 g, 5 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 ethanol / dioxan mixture to give compound **12** as yellow solid, mp 240 ºC; IR (KBr) υ = 3431 (NH), 1724,1685 (2 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 3.17 (s, 3H, CH_3), 7.43 -8.16 (m, 4H, Ar-H), 8.78 (s, 1H, pyridine-H), 12.86 (s, 1H, NH) ppm.; MS, *m/z* (%) 311 (M^+, 3), 262 (3), 240 (4), 238 (3.4), 196 (6.6), 127 (100) 73 (26); *Anal*. Calcd. for $C_{15}H_9N_3O_3S$ (311.32): C,57.87; H,2.91; N, 13.50. Found: C,57.62; H,2.84; N, 13.70 %.

Synthesis of compounds (10a-o)

Method A. To a mixture of equimolar amounts of **4** and the appropriate hydrazonoyl chlorides **7** (2 mmol of each) in dioxane (20 mL) was added chitosan (0.2 g). The reaction mixture was refluxed till all of the starting materials have disappeared and hydrogen sulfide gas ceased to evolve (10 h, monitored by TLC). 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 **10**

Method B. To a mixture of equimolar amounts of **12** and the hydrazonoyl chloride **7a** (2 mmol) in dioxane (20 mL) was added chitosan (0.2 g). The reaction mixture was refluxed till all methanethiol gas ceased to evolve (20 h, monitored by TLC). 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 product identical in all respects (mp, mixed mp, IR and ^{1}H NMR) with **10a**.

1-Acetyl-3-phenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]pyrimidine-7,13-(3*H*)-diones (10a). Brown crystals, mp 218-220 ºC; (ethanol/dioxane); IR (KBr) υ = 1727, 1682, 1640 (3 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.49 (s, 3H, $COCH_3$), 7.10 – 8.16 (m, 9H, Ar-H), 8.96 (s, 1H, pyridine-H) ppm; ^{13}C NMR (DMSO-d_6) δ = 18.24, 116.21, 119.05, 121.0, 125.31, 127.08, 127.94, 128.01, 129.0, 129.58, 134.17, 139.24, 139.58, 149.11, 152.50, 154.24, 159.12, 163.1, 165.27, 169.21, 198.80. MS, *m/z* (%) 423 (M^+, 8), 380 (8), 306 (9), 262 (17), 242 (11), 190 (11), 91 (20), 77 (100). *Anal*. Calcd. for $C_{23}H_{13}N_5O_4$ (423.38): C,65.25; H,3.09; N,16.54. Found: C, 65.04; H, 3.25; N, 16.38 %.

1-Acetyl-3-(4-methylphenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones (10b). Brown crystals, mp 200 ºC; (ethanol/dioxane); IR (KBr) υ = 1734, 1635, 1625 (3 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.41 (s, 3H, $COCH_3$), 2.79 (s, 3H, CH_3), 7.18 – 8.14 (m, 4H, Ar-H), 8.38 (d, *J* = 7 *Hz*, 2H, Ar-H), 8.52 (d, *J* = 7 *Hz*, 2H, Ar-H), 8.78 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 439 (M^++2, 12), 437 (M^+, 28), 395 (34), 367 (21), 263 (12), 209 (16), 170 (10), 104 (58), 90 (100), 77 (72) . *Anal*. Calcd. for $C_{24}H_{15}N_5O_4$ (437.41): C,65.90; H,3.46; N,16.01. Found: C, 65.84, H, 3.21; N, 15.93 %.

1-Acetyl-3-(4-chlorophenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones (10c). Yellow crystals, mp 145 ºC; (dioxane); IR (KBr) υ = 1731, 1681, 1637 (3 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.66 (s, 3H, $COCH_3$), 7.39 (d, 2H, Ar-H), 7.49 – 7.90 (m, 4H, Ar-H), 8.34 (d, 2H, Ar-H), 8.80 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 457 (M^+, 6), 263 (7), 125 (15), 104 (7), 89 (6), 84 (25), 76 (24) 63 (100). *Anal*. Calcd. for $C_{23}H_{12}ClN_5O_4$ (457.83): C,60.34; H,2.64; N,15.30. Found: C, 60.21; H, 2.45; N,15.08 %.

1-Acetyl-3-(4-nitrophenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones (10d). Brown crystals, mp 210 ºC; (ethanol/dioxane); IR (KBr) υ = 1724,1690,1625 (3 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.67 (s, 3H, $COCH_3$), 7.45 -8.39 (m, 8H, Ar-H), 8.77 (s, 1H, pyridine-H), ppm; MS, *m/z* (%) 468 (M^+, 8), 411 (16), 384 (19), 297 (75) 237 (20), 210 (32), 126 (50), 104 (685), 91 (52), 85 (46), 76 (100). *Anal*. Calcd. for $C_{23}H_{12}N_6O_6$ (468.38): C, 58.98; H, 2.58; N, 17.94. Found: C, 58.72; H, 2.45; N, 17.64 %.

1-Ethoxycarbonyl-3-phenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones (10e).Yellow crystals, mp 270 ºC; (ethanol/dioxane); IR (KBr) υ = 1744, 1685, 1670 (3 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 1.43 (t, *J = 7 Hz*, 3H, CH_3), 4.59 (q, *J = 7 Hz*, 2H, CH_2), 7.45 - 8.56 (m, 9H, Ar-H), 9.18 (s, 1H, pyridine-H) ppm; ^{13}C NMR (DMSO-d_6) δ = 14.16, 60.21, 116.01, 118.94, 122.05, 128.32, 128.19, 130.24, 131.28, 131.99, 132.45, 135.36, 136.29, 142.18, 145.96, 150.0, 152.51, 156.34, 159.24, 160.24, 164.0, 170.12. MS, *m/z* (%) 453 (M^+,9), 380 (80), 355 (15), 326 (11),104 (11), 91 (26), 76 (100). *Anal*. Calcd. for $C_{24}H_{15}N_5O_5$ (453.41): C,63..58; H, 3.33; N, 15.45. Found: C, 63.41; H, 3.19; N, 15.28%.

1-Ethoxycarbonyl-3-(4-methylphenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo [4,3-*a*]pyrimidine-7,13 (3*H*)-diones (10f). Yellow crystals, mp 248-250 ºC; (ethanol); IR (KBr) υ = 1745, 1672, 1637 (3 CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 1.45 (t, *J* = 7 *Hz*, 3H, CH_3), 2.42 (s,

3H, Ar-CH_3), 4.56 (q, *J* = 7 *Hz*, 2H, CH_2), 7.45 – 8.54 (m, 8H, Ar-H), 9.15 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 467 (M^+, 4), 394 (10), 264 (9), 91 (14), 77 (12), 60 (100). *Anal*. Calcd. for $C_{25}H_{17}N_5O_5$ (467.44): C,64.24; H,3.67; N,14.98. Found: C, 64.04; H, 3.46; N,14.74 %.

3-(4-Chlorophenyl)-1-ethoxycarbonyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo [4,3-*a*]pyrimidine-7,13(3*H*)-diones (10g). Yellow crystals, mp 247-250 ºC; (ethanol); IR (KBr) υ = 1730, 1721, 1634 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 1.14 (t, *J* = *7Hz*, 3H, CH_3), 4.21 (q, *J* = 7 *Hz*, 2H, CH_2), 7.40 - 7.94 (m, 8H, Ar-H), 8.86 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 487 (M^+, 8), 458 (5), 209 (11), 151 (24), 111 (52), 86 (41), 77 (100). *Anal*. Calcd. for $C_{24}H_{14}ClN_5O_5$ (487.86): C,59.09; H,2..89; N,14.36. Found: C, 58.94; H, 2.95; N, 14.22%.

1-Ethoxycarbonyl-3-(4-nitrophenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]pyrimidine-7,13(3*H*)-diones (10h). Red crystals, mp 135-140 ºC; (ethanol/dioxane); IR (KBr) υ = 1736, 1654, 1621 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 1.19 (t, *J* = 7 *Hz*, 3H, CH_3), 4.41 (q, *J* = 7 *Hz*, 2H, CH_2), 7.37 - 8.55 (m, 8H, Ar-H), 8.77 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 499 (M^++1, 1), 498 (M^+, 6), 154 (3), 108 (11), 90 (16), 63 (100). *Anal*. Calcd. for $C_{24}H_{14}N_6O_7$ (498.42): C, 57.84; H, 2.83; N, 16.86. Found: C, 57.71; H, 2.66; N,16.60 %.

1-Ethoxycarbonyl-3-(3-methylphenyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo-[4,3-*a*]-pyrimidin-7,13(3*H*)-diones (10i). Pale brown crystals, mp 185-186 ºC; (ethanol/dioxane); IR (KBr) υ = 1737, 1725, 1625 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 1.34 (t, *J* = 7 *Hz*, 3H, CH_3), 2.20 (s, 3H, Ar-CH_3), 4.39 (q, *J* = 7 *Hz*, 2H, CH_2), 7.41 - 8.0 (m, 8H, Ar-H), 8.78 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 467 (M^+, 63), 395 (100), 105 (38), 77 (64). *Anal*. Calcd. for $C_{25}H_{17}N_5O_5$ (467.44): C,64.24; H,3.67; N,14.98. Found: C, 64.10; H, 3.54; N,14.79 %.

3-(3-Chlorophenyl)-1-ethoxycarbonyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo [4,3-*a*]pyrimidin-7,13(3*H*)-diones (10j). Yellow solid, mp 210-212 ºC; (dioxane); IR (KBr) υ = 1734, 1695, 1606 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 1.37 (t, *J* = *7Hz*, 3H, CH_3), 4.40 (q, *J* = 7 *Hz*, 2H, CH_2), 7.38 – 8.39 (m, 8H, Ar-H), 8.79 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 487 (M^+, 10), 111 (45), 95 (51), 84 (50), 78 (12). *Anal*. Calcd. for $C_{24}H_{14}ClN_5O_5$ (487.86): C, 59.09; H, 2.89; N, 14.36. Found: C, 58.94; H, 2.95; N, 14.22%.

***N*,3-Diphenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]pyrimidine-7,13(3*H*)-diones-1-carboxamide (10k).** Yellow crystals, mp 150 ºC; (ethanol); IR (KBr) υ = 3377 (NH), 1697, 1666, 1633 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.35 - 8.75 (m, 14H, Ar-H), 8.72 (s, 1H, pyridine-H), 11.69 (s, 1H, NH) ppm; MS, *m/z* (%) 500 (M^+, 2), 237 (2), 185 (3), 118 (21), 76 (100). *Anal*. Calcd. for $C_{28}H_{16}N_6O_4$ (500.46): C, 67.20; H, 3.22; N,16.79. Found: C, 67.05; H, 3.01; N,16.65 %.

3-(4-Methylphenyl)-*N*-phenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*] pyrimidine-7,13(3*H*)-diones-1-carboxamide (10l). Brown crystals, mp 235 ºC; (ethanol/dioxane); IR (KBr) υ = 3391 (NH), 1725, 1686, 1626 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.27 (s, 3H, Ar-CH_3), 7.14-7.77 (m, 13H, Ar-H), 8.80 (s, 1H, pyridine-H), 11.73 (s, 1H, NH) ppm; MS, *m/z* (%) 514 (M^+, 5), 445 (3), 361 (4), 145 (13), 90 (100), 76 (60). *Anal*. Calcd. for $C_{29}H_{18}N_6O_4$ (514.49): C,67.70; H,3.53; N,16.33. Found: C, 67.52; H, 3.40; N,16.08 %.

3-(4-Chlorophenyl)-*N*-phenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*] pyrimidine-7,13(3*H*)-diones-1-carboxamide (10m). Pale green crystals, mp 270 ºC; (ethanol/dioxane); IR (KBr) υ = 3398 (NH), 1725, 1686, 1626 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.37 - 8.85 (m, 13H, Ar-H), 8.79 (s, 1H, Pyridine-H), 11.72 (s, 1H, NH) ppm; MS, *m/z* (%) 536 (M^++2, 0.5), 535 (M^++1, 1), 534 (M^+, 2), 118 (100), 111 (7), 91 (73), 77 (18). *Anal*. Calcd. for $C_{28}H_{15}ClN_6O_4$ (534.91): C,62.87; H,2.83; N,15.71. Found: C, 62.74; H, 2.66; N, 15.79 %.

3-(4-Nitrophenyl)-*N*-phenyl-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones 1-carboxamide (10n). Brown crystals, mp 290 ºC; (ethanol / dioxane); IR (KBr) υ = 3371 (NH), 1700, 1666, 1620 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.36 – 8.38 (m, 13H, Ar-H), 8.78 (s, 1H, pyridine-H), 12.86 (s, 1H, NH) ppm; MS, *m/z* (%) 545 (M^+, 10), 518 (16), 260 (12), 210 (16), 157 (20), 118 (76), 104 (40), 92 (45), 77 (78), 62 (100). *Anal*. Calcd. for $C_{28}H_{15}N_7O_6$ (545.46): C,61.65; H,2.77; N,17.98. Found: C, 61.52; H, 2.49; N,17.78%.

3-Phenyl-1-(2-thienylcarbonyl)-benzo[5',6']pyrano[4',3':4,5]pyrido[2,3-*d*]triazolo[4,3-*a*]-pyrimidine-7,13(3*H*)-diones (10o). Yellow solid, mp > 340 ºC; (ethanol/dioxane); IR (KBr) υ = 1736, 1686, 1634 (3 CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ = 7.50 – 8.43 (m, 12H, Ar-H), 8.80 (s, 1H, pyridine-H) ppm; MS, *m/z* (%) 491 (M^+, 1), 297 (75), 181 (14), 104 (60), 90 (44), 76 (100). *Anal*. Calcd. for $C_{26}H_{13}N_5O_4S$ (491.49): C,63.54; H,2.67; N,14.25. Found: C, 63.28; H, 2.54; N,14.02 %.

Pharmacology

Animals: Female Sprague–Dawley rats were obtained from Animal House Laboratory, Sanofi-Aventis, France, and acclimatized for one week in the animal facility that has a 12 h light/dark cycle with the temperature controlled at 21–238C. The animals were housed individually in stainless steel cages in temperature-controlled and humidity-monitored quarters. Test animals were provided with a continuous access to tap water.

Procedure for antiandrogenic activity in female rats

Female Sprague–Dawley rats weighting 40–45 g were overotomized as described above. After one week, animals were divided into 38 groups (12 animals) of which 36 received new compounds, a group received control, and a group received Cyperoterone. The treatment was continued over a period of 12 days with daily subcutaneous injection of 0.3 mg testosterone propionate and the same dose of each individual new tested compound. The control received only testosterone propionate. On the 13th day, the animals were sacrificed, and the uteri and perpetual glands were weighed. The increase in female accessory sexual organs due to testosterone treatment was dose-despondently reduced by antiandrogen.[29,30] Dose–response curves were established for various doses of the antiandrogen at a given dose of testosterone propionate or for various doses of testosterone propionate at a given dose of the antiandrogen.

From the dose–response curve, the relative antiandrogenic potency of the newly synthesized compounds was calculated and compared to that of Cyperoterone as a reference drug.

Determination of acute toxicity (*LD*$_{50}$ and *LD*$_{90}$). Female Sprague–Dawley albino rats were used to determine intraperitoneal *LD*$_{50}$ and *LD*$_{90}$ of the tested compounds. Prior to the determination of *LD*$_{50}$ and *LD*$_{90}$ values, a range finding screen was conducted using 20 rats treated with each tested compound at dose ranging from 3 to 2000 mg/kg/dose level. Based on the mortality observed within 14 days, the doses used for the *LD*$_{50}$ and *LD*$_{90}$ determination were 3, 10, 30, 100, 300, 1000, and 2000 mg/kg of compound administered by intraperitoneal injection as a 10% solution in dimethyl sulfoxide (DMSO). Control animals received intraperitoneal injections of DMSO.

For each concentration and control, 10 female rats were injected with the tested compounds for viability twice daily for two weeks. From the mortality data of all tested animals, the intraperitoneal LD50 and LD90 values for each agent were determined according to Austen and Brocklehurst.[31]

Procedure hepatitis C virus (HCV) NS3-4A protease inhibitory activities in HCV replicon cells and EC$_{50}$,CC$_{50}$of the tested compounds in hamster brains for antiviral chemotherapy for subacute sclerosing panencephalitis (SSPE)

Determination of CC_{50} **and** SI of different tested compounds in HCV replicon cells was performed as follow. Briefly, 1 X 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 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 RTPCR (QRT-PCR) assay.

Each datum point represents the average of five replicates in cell culture. The cytotoxicity of tested compound 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 X10^4 parental Huh-7cells per well or 4X10^4 HepG2 cells per well were used.

EC_{50},CC_{50} of the tested compounds in Hamster Brains for antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE). Under ether anesthesia, 50 mL 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. At 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 3g for 10 min. The supernatant was collected, ethanol was added to remove proteins, and the mixture was heated at 90 ºC to evaporate the ethanol. The protein-free samples were used to evaluate the **EC$_{50}$,CC$_{50}$** in brain tissue by HPLC and bioassay.

Procedure For Anti-H1N1 activity of the newly synthesized compounds

The virus for this study was Influenza A (H1N1) virus strain A/PR/8/34 (ATCC, Manassas, VA; ATCC No. VR-1469). The tested synthesized compounds were dissolved in a minimal volume of EtOH (USP grade) prior to dilution in DMEM (pH 7.4).

Approximately 100 focus-forming units (FFU) of influenza virus were incubated with dilutions of the tested synthesized compounds solution in DMEM for 1 h at room temperature and then allowed to infect confluent MDCK cells for 1 h at room temperature. After infection, cells were fixed with Formalde-fresh then permeabilized with EtOH (USP). The FFU's were visualized using goat anti-influenza A virus IgG polyclonal antibody, rabbit Anti-Goat IgG (H&L) horseradish peroxidase conjugated affinity purified antibody (Chemicon,Temecula, CA) and AEC chromogen substrate (Dako, Carpinteria,CA).

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