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Synthesis and Antiproliferative Activity of [1,2,4]Triazino[4,3-*a*]indoles

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Abstract. *A series of [1,2,4]triazino[4,3-a]indoles was prepared in good yield by reacting 2-diazo-3-ethoxycarbonylindole with methylene active compounds. Derivatives of the title ring system were tested against a panel of 60 human tumor cell lines, and showed inhibitory activity against a wide range of cancer cell lines at micromolar concentration.*

As part of our ongoing program directed towards the design, synthesis and evaluation of novel compounds of biological interest containing the pyrrole and the indole moieties, we have reported the synthesis of 2-diazopyrroles (1) and 2-diazoindoles (2) which are versatile key intermediates for the synthesis of biologically active compounds. In fact we synthesized 2-triazenopyrroles and 2-triazenoindoles by reaction of the corresponding diazo compounds with secondary amines. Such compounds can be considered deaza analogues of dacarbazine, a triazenoimidazole derivative used in therapy as the single most active drug available for the treatment of malignant melanoma (3). Biological screenings of both classes of derivatives gave quite interesting results, especially those of the pyrrole series. In fact one of them displayed cytotoxicity against leukemia, lymphoma and carcinoma cell lines at micromolar level (3.9-21.2) (4), while those of the indole series were less active with GI_{50} in the range 23-99 μM (5).

By reacting 2-diazopyrroles with aryl or alkyl isocyanates, we also obtained several derivatives of the new ring system pyrrolo[2,1-*d*][1,2,3,5]tetrazine (6), a deaza analogue of the antitumor drug temozolomide, today on the market under the trade name TEMODAL® and used against malignant melanoma, mycosis fungoides and brain tumors (7). The synthesised pyrrolotetrazinones, tested *in vitro* against a panel of 60 human cancer cell lines, showed potent antiproliferative activity in the low micromolar or submicromolar range, some of them reaching GI_{50} up to nanomolar concentrations (8).

Furthermore, upon reaction with the sodium salts of β-diketones, β-carbonitriles, β-dinitriles and β-ketoesters, 2-diazopyrroles afforded derivatives of the new ring system pyrrolo[2,1-*c*][1,2,4] triazine that inhibited the growth of a wide range of human cancer cell lines at micromolar concentration (9). The title heterocyclic system contains the 1,2,4-triazine moiety, to which a wide range of biological properties such as antibacterial, antibiotic, antiviral and antitumor have been ascribed (10). In particular, pyrazolo[5,1-*c*][1,2,4]triazine derivatives exhibited antineoplastic activity against the mouse sarcoma 180 and methylcolanthrene-induced rat sarcoma (11).

Encouraged by these results and considering the great versatility of 2-diazoindoles, we thought it was interesting to verify whether the condensation of the 1,2,4-triazine moiety with the indole system increases the biological activity.

In this paper, we report a simple synthesis of the [1,2,4]triazino[4,3-*a*]indole system based on the reaction of 2-diazoindole of type 1, obtained by diazotization of 2-aminoindoles according to a method recently reported, (2) with methylene active compounds. The reactions were carried out in absolute ethanol at room temperature for 24 hours, with the sodium salts of type 2a-d prepared *in situ* with sodium ethoxide in absolute ethanol. The primary coupling products 3a-d, which are analogous with similar compounds in the azole series (12) and exist in the hydrazo form, were not isolated as they spontaneously cyclize to the desired [1,2,4]triazino[4,3-*a*]indoles in good yields (72-80%) (Figure 1). The structure of [1,2,4]triazino[4,3-*a*]indoles 4a-d was confirmed by IR and NMR data as well as elemental analysis. An evaluation of the spectroscopic data led to the

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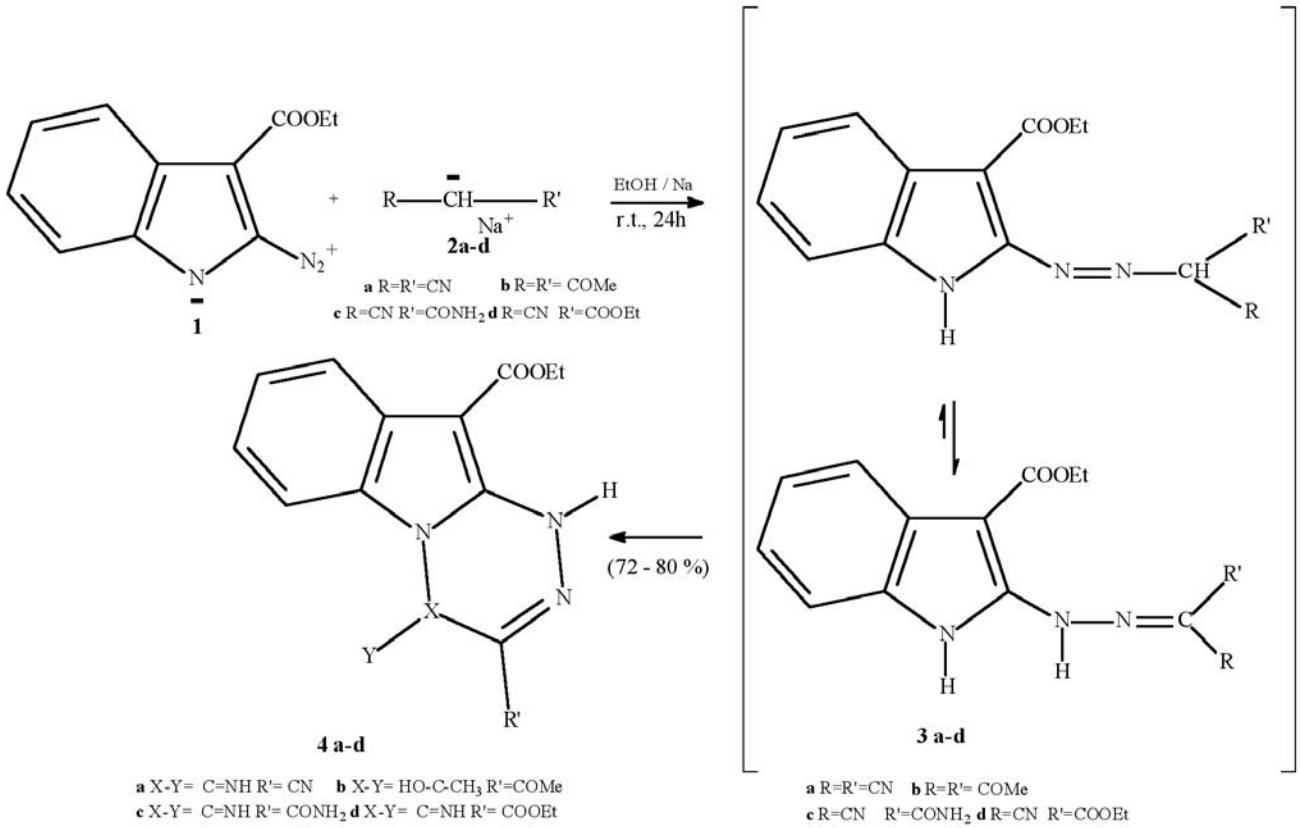

Figure 1. *Synthetic pathway for derivatives 4.*

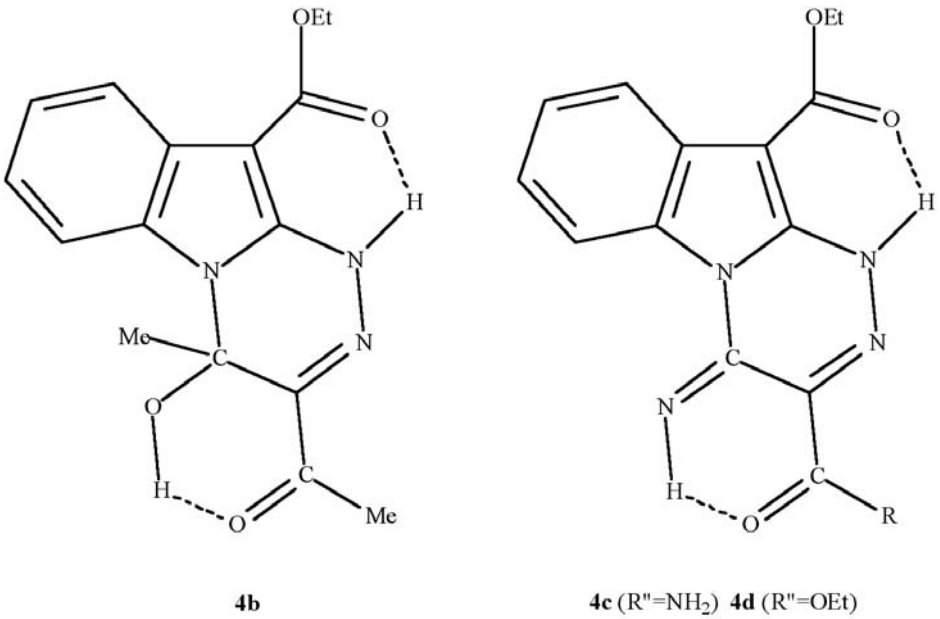

Figure 2. *Hydrogen bondings in compounds 4.*

conclusion that compounds 4a,c,d exist in the imino form. Such a form is stabilized by the hydrogen bonding between the hydrogen on the N-1 and the ethoxycarbonyl functionality on position 10 of the triazinoindole ring system. In the case of compounds 4c and 4d, an additional hydrogen bonding of the imino hydrogen with the adjacent carboxamido and ethoxycarbonyl moieties respectively, further stabilizes the compounds in such a form (Figure 2). On the other hand, in the azolo[1,2,4]triazine series, it has already been observed that hydrogen bonding determined the tautomer populations of the bicyclic systems (13).

In the case of compound 4b, the presence of the two hydrogen bonds, analogous to those present in compounds 4c and 4d, stabilizes the hydrate form which can be isolated, giving rise to a full aromatic system.

Materials and Methods

Chemistry. All melting points were taken on a Buchi-Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer; ^{1}H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz, respectively, in DMSO-d_6 (TMS as internal reference), using a Bruker AC-E series 200 MHz spectrometer. Column chromatography was performed with a BIOTAGE FLASH40i chromatography module (prepacked polyethylene cartridge system). For all new compounds, elemental analyses were within ± 0.4% of theoretical values.

Preparation of 2-diazo-3-ethoxycarbonylindole (1). According to the procedure described previously (2), to a stirred solution of 2-amino-3-ethoxycarbonylindole (10 mmol) in acetic acid (80%, 10 mL) at 0˚C, a solution of sodium nitrite (10 mmol) in the minimum amount of water (2 mL) was added under a nitrogen atmosphere and in the dark. The reaction mixture was stirred for 3 hours and neutralised with an aqueous solution of sodium carbonate (10%), keeping the temperature at 0˚C. The brown solid separated was filtered, dried in the dessicator under vacuum and in the dark. The crude product was quickly washed with cyclohexane to give one which furnished an IR spectrum identical to an authentic sample.

General method for the preparation of [1,2,4]triazino[4,3-a]indoles 4a-d. To a solution of active methylene compounds 2a-d (2 mmol) in anhydrous ethanol (50 mL), a solution of sodium ethoxide (2 mL, 1N) in anhydrous ethanol (10 mL) was added dropwise. The reaction mixture was stirred at 0˚C for 30 minutes then a solution of 2-diazo-3-ethoxycarbonylindole 1 (2 mmol) in anhydrous ethanol was added dropwise with stirring at 0˚C. The mixture was stirred at room temperature for an additional 24 hours, after which the solvent was removed under reduced pressure, the residue taken up with water, filtered and air dried. The crude [1,2,4]triazino[4,3-*a*]indoles were purified by chromatography using dichloromethane: ethyl acetate (9:1) as eluant, and recrystallized from ethanol.

Ethyl 3-cyano-4-imino-1,4-dihydro[1,2,4]triazino[4,3-a]indole-10-carboxylate (4a), (yield 80%), mp >320˚C; ir: 3296 (NH), 3209 (NH), 2229 (CN), 1675 (CO) cm^{-1}; ^{1}H nmr (ppm): 1.39 (3H, t, J=6.8 Hz, CH_3), 4.41 (2H, q, J=6.8 Hz, CH_2), 7.37 (1H, t, J=7.5 Hz, H-7), 7.48 (1H, t, J=7.5 Hz, H-8), 8.10 (1H, d, J=7.5 Hz, H-6), 8.73 (1H, d, J=7.5 Hz, H-9), 8.80 (1H, vbs, C=NH), 11.50 (1H, vbs, NH); ^{13}C nmr (ppm): 14.1 (q), 59.8 (t), 90.3 (s), 113.9 (s), 116.5 (d), 119.7 (d), 123.0 (d), 123.4 (s), 125.4 (d), 126.1 (s), 129.3 (s), 138.3 (s), 143.9 (s), 163.1 (s).

Ethyl 3-acetyl-4-hydroxy-4-methyl-1,4-dihydro[1,2,4]triazino[4,3-a]indole-10-carboxylate (4b), (yield 72%), mp 174-176˚C; ir: 3449 (OH), 3284 (NH), 1671 (CO), 1614 (CO) cm^{-1}; ^{1}H nmr (ppm): 1.27 (3H, t, J=7.0 Hz, CH_3), 2.02 (3H, s, CH_3), 2.34 (3H, s, CH_3), 4.26 (2H, q, J=7.0 Hz, CH_2), 7.05 (1H, t, J=7.8 Hz, H-7), 7.12 (1H, t, J=7.8 Hz, H-8), 7.37 (1H, s, OH), 7.69 (1H, d, J=7.8 Hz, H-6), 7.83 (1H, d, J=7.8 Hz, H-9), 11.70 (1H, s, NH);^{13}C nmr (ppm): 14.7 (q), 25.1 (q), 26.6 (q), 59.0 (t), 78.8 (s), 85.0 (s), 113.6 (d), 119.7 (d), 121.2 (d), 122.4 (d), 126.0 (s), 130.4 (s), 137.1 (s), 140.1 (s), 163.9 (s), 194.7 (s).

Ethyl 3-(aminocarbonyl)-4-imino-1,4-dihydro[1,2,4]triazino[4,3-a]indole-10-carboxylate (4c), (yield 80%), mp >320˚C; ir: 3477 (NH), 3257-3165 (NH and NH_2), 1676 (CO), 1607 (CO) cm^{-1}; ^{1}H nmr (ppm): 1.51 (3H, t, J=7.7 Hz, CH_3), 4.51 (2H, q, J=7.7 Hz, CH_2), 7.45 (1H, t, J=7.7 Hz, H-7), 7.58 (1H, t, J=7.7 Hz, H-8), 7.90 (1H, bs, NH), 8.05 (1H, bs, NH), 8.25 (1H, d, J=7.7 Hz, H-6), 8.90 (1H, d, J=7.7 Hz, H-9), 10.70 (1H, vbs, C=NH), 12.01 (1H, vbs, NH); ^{13}C nmr (ppm): 14.1 (q), 59.0 (t), 116.3 (d), 119.1 (d), 121.7 (d), 124.1 (s), 124.7 (d), 126.4 (s), 128.9 (s), 139.9 (s), 144.8 (2xs), 163.0 (s), 166.0 (s).

Diethyl 4-imino-1,4-dihydro[1,2,4]triazino[4,3-a]indole-3,10-dicarboxylate (4d), (yield 75%), mp >320˚C; ir: 3341 (NH), 3301 (NH), 1697 (CO), 1674 (CO) cm^{-1}; ^{1}H nmr (ppm): 1.31 (3H, t, J=6.5 Hz, CH_3), 1.34 (3H, t, J=7.4 Hz, CH_3), 4.31 (2H, q, J=6.5 Hz, CH_2), 4.38 (2H, q, J=7.4 Hz, CH_2), 7.30 (1H, t, J=8.3 Hz, H-7), 7.42 (1H, t, J=8.3 Hz, H-8), 8.08 (1H, d, J=8.3 Hz, H-6), 8.87 (1H, d, J=8.3 Hz, H-9), 9.40 (1H, vbs, C=NH), 10.00 (1H, vbs, NH);^{13}C nmr (ppm): 14.0 (q), 14.6 (q), 59.4 (t), 61.2 (t), 116.8 (s), 117.2 (d), 119.3 (d), 122.2 (d), 124.2 (s), 124.8 (d), 126.4 (s), 130.2 (s), 145.7 (2 x s), 163.0 (s), 163.8 (s).

Results and Discussion

The triazinoindoles 4c and 4d were selected by NCI and their cytotoxicity was evaluated in *in vitro* disease-oriented antitumor screenings (14) against a panel of 60 human tumor cell lines derived from leukemia, non-small lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. The test compounds were evaluated using five concentrations at 10-fold dilutions, the highest being 10^{-4} M and the others 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. The results obtained are shown in Table I, taking into consideration the growth inhibitory power (GI_{50}).

Most of the susceptible tumors were generally inhibited at micromolar concentration, expecially for the triazine 4d bearing an ethoxycarbonyl functionality in position 10, which generally resulted more active than 4c bearing a carbamoyl group in the same position. Compound 4d gave the best result in the leukemia and melanoma subpanels, with GI_{50} values in the low micromolar range, 1.12-6.91 and

Table I. *Inhibition of in vitro tumor cell growth by triazinoindoles 4c,d*

Cell lines	GI_{50} (μM)[a,b] 4c	4d	Cell lines	GI_{50} (μM)[a,b] 4c	4d
Leukemia			Melanoma		
CCRF-CEM	>100	2.22	LOX IMVI	28.3	5.96
HL-60(TB)	>100	2.15	MALME-3M	70.9	2.10
K-562	>100	1.12	M14	>100	5.97
MOLT-4	>100	4.40	SK-MEL-2	20.5	5.15
RPMI-8226	>100	1.54	SK-MEL-28	>100	5.85
SR	>100	6.91	SK-MEL-5	74.3	5.53
Non-small cell lung			UACC-257	99.7	2.09
A549/ATCC	>100	10.5	UACC-62	19.9	2.42
EKVX	18.0	25.8	Ovarian cancer		
HOP-62	17.3	68.8	IGROV1	1.33	1.12
NCI-H23	16.7	5.94	OVCAR-3	>100	5.20
NCI-H322M	62.4	14.5	OVCAR-4	>100	>100
NCI-H460	50.3	8.13	OVCAR-5	16.1	5.16
NCI-H522	28.8	6.21	OVCAR-8	13.9	6.03
Colon cancer			SK-OV-3	21.9	67.0
COLO 205	>100	10.2	Renal cancer		
HCC-2998	>100	34.0	786-0	21.0	4.85
HCT-116	31.1	29.3	A498	6.07	7.19
HCT-15	79.0	7.66	ACHN	32.8	7.49
HT29	>100	22.7	CAKI-1	16.1	>100
KM12	>100	4.43	RXF 393	>100	6.66
SW-620	>100	8.26	SN12C	11.3	7.90
CNS cancer			TK-10	74.3	7.87
SF-268	16.0	19.3	UO-31	59.9	5.35
SF-295	3.31	26.9	Breast cancer		
SF-539	1.62	5.66	MCF7	17.6	13.1
SNB-19	1.93	4.93	NCI/ADR-RES	35.3	3.62
SNB-75	1.17	11.0	MDA-MB231/ATCC	18.7	19.8
U251	1.52	5.41	HS 578T	4.36	7.16
Prostate cancer			MDA-MB-435	4.36	5.04
PC-3	59.9	53.5	MDA-N	>100	4.35
DU-145	>100	37.4	BT-549	22.1	13.7
			T-47D	43.0	30.0

[a]Data obtained from NCI's *in vitro* disease-oriented tumor cells screen.
[b]GI_{50} is the molar concentration causing 50% growth inhibition of tumor cells. Compounds with GI_{50} >100 μM are considered inactive.

2.09-5.97, respectively. The most sensitive cell lines were K-562 (1.12 μM) and RPMI-8226 (1.54 μM) of the former subpanel; UACC-257 (2.09 μM) and MALME-3M (2.10 μM) belong to the latter subpanel. Moreover, in some ovarian cancer cell lines, IGROV1, OVCAR-3, OVCAR-5, OVCAR-8 and CNS cancer cell lines, SF539, SNB19 and U251, good values of GI_{50} were observed: 1.12-6.03 μM and 4.93-5.66 μM, respectively. Compound 4c showed remarkable inhibitory activity only in the case of the CNS subpanel, with the exception of the SF268 cell line, showing GI_{50} in the range 1.17-3.31 μM, and the ovarian cancer cell line IGROV1 with GI_{50} 1.33 μM. In the other cell lines compound 4c, with some exceptions when the potency was at the same level as compound 4d, was one magnitude order less active and, in the case of the leukemia and in most of the colon cancer cell lines, not active at all.

In order to discern the mechanism of action of pyrrolotriazines, we performed COMPARE computations (15) on the NCI screening data, using [1,2,4]triazino[4,3-*a*]indoles 4c,d as seed compounds. Both had a Pearson Correlation Coefficient <0.5 (data not shown) against the "Standard Agents" database, suggesting that they probably act with a different mechanism from those of the Standard Agents and make this class of compounds worthy of considerable attention. It is our intention to undertake studies directed at elucidating their biochemical mechanism of action.

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References

1 Cirrincione G, Almerico AM, Diana P, Grimaudo S, Barraja P, Mingoia F, Dattolo G and Aiello E: 2-Diazopyrroles: synthesis and antileukemic activity. Il Farmaco *51*: 275-277, 1996.

2 Barraja P, Diana P, Lauria A, Almerico AM, Dattolo G and Cirrincione G: 2-Diazo-2H-indoles. Helv Chim Acta *84*: 2212-2219, 2001.

3 a) Lucas US and Huang AT: Clinical management of melanoma. *In*: Development in Oncology; Siegler HF ed; Martinus Nijhoff: The Hague *5*: 382, 1982; b) Viviani S, Bonadonna G, Santoro A, Bonfante V, Zanin M, Devizzi L, Soncini F and Valagussa P: Alternating *versus* hybrid MOPP and ABVD combinations in advanced Hodgkin's disease: ten year results. J Clin Oncol *14*: 1421-1430, 1996.

4 Diana P, Barraja P, Lauria A, Almerico AM, Cirrincione G, Loi AG, Congeddu E, Musiu C, Putzolu M and La Colla P: 2-Triazenopyrroles: synthesis and biological activity. Eur J Med Chem *34*: 353-360, 1999.

5 Barraja P, Diana P, Lauria A, Montalbano A, Almerico AM, Dattolo G and Cirrincione G: 2-Triazenoindoles: synthesis and biological activity. Anticancer Res *22*: 837-840, 2002.

6 Diana P, Barraja P, Lauria A, Almerico AM, Dattolo G and Cirrincione G: Pyrrolo[2,1-*d*][1,2,3,5]tetrazinones, a new class of azolotetrazines related to the antitumor drug temozolomide. Synthesis: 2082-2086, 1999.

7 a) Newlands ES, Stevens MFG, Wedge SR, Wheelhouse RT and Brock C: Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rep *23*: 35-61, 1997; b) Bleehen NM, Newlands ES, Lee SM, Thatcher N, Selby P, Calvert AH, Rustin GJS, Brampton M and Stevens MFG: Cancer research campaign phase II trial of temozolomide in metabolic melanoma. J Clin Oncol *13*: 910-914, 1995; c) O'Reilly SM, Newlands ES, Glaser MG, Brampton M, Rice-Edwards JM, Illinworth RD, Richards PG, Kennard C, Colquhoun IR, Lewis P and Stevens MFG: Temozolomide: a new oral cytotoxic chemotherapeutic agent with promising activity against primary brain tumors. Eur J Cancer *29A*: 940-943, 1993; d) Newlands ES, Blackledge GRP, Slack JA, Rustin GJS, Smith DB, Stuart NSA, Quarterman CP, Hoffman R, Stevens MFG, Brampton MH and Gibson AC: Phase I trial of temozolomide (CCRG81045M&B39831: NSC362856). Br J Cancer *65*: 287-291, 1992.

8 Diana P, Barraja P, Lauria A, Almerico AM, Dattolo G and Cirrincione G: Pyrrolo[2,1-*d*][1,2,3,5]tetrazine-4-(3H)-ones, a new class of azolotetrazines with potent antitumor activity. Bioorg Med Chem Lett *11*: 2371-2380, 2003.

9 Diana P, Barraja P, Lauria A, Almerico AM, Montalbano A, Dattolo G and Cirrincione G: Pyrrolo[2,1-*c*][1,2,4]triazines from 2-diazopyrroles: synthesis and antiproliferative activity. Eur J Med Chem *37*: 267-272, 2002.

10 a) Neunhoeffer H: 1,2,4-Triazines and their benzoderivatives. *In*: Comprehensive Heterocyclic Chemistry; Katritzky AR ed; Pergamon Press Oxford *3*: 385-456, 1984; b) Neunhoeffer H: 1,2,3-Triazines. *In*: Chemistry of Heterocyclic Compounds; Neunhoeffer H and Wiley PF eds *33*: 189-574, 1978.

11 Partridge MW and Stevens MFG: Pyrazolo-as-triazines.I. J Chem Soc (C): 1127-1131, 1966.

12 Cirrincione G, Almerico AM, Aiello E and Dattolo G: Diazoazoles. Adv Heterocycl Chem *48*: 65-175, 1990.

13 Baig GU and Stevens MFG: Triazine and related products. Part 22. Synthesis and reactions of imidazo[5,1-*c*][1,2,4]triazines. J Chem Soc Perkin I: 1424-32, 1981.

14 a) Grever MR Sherpatz SA and Chabner BA: The National Cancer Institute: cancer drug discovery and development program. Semin Oncol *19*: 622-638, 1992; b) Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langely J, Cronise P, Vaigro-Woiff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd MR: Feasibility of a high-flux anticancer drug screen using a diverse panel cultured human tunor cell lines. J Natl Cancer Inst *83*: 757-766, 1991; c) Boyd NR, Paull KD and Rubinstein LV: Data display and analysis strategies for the new NCI disease-oriented *in-vitro* anti-tumor drug screen. *In*: Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development; Valeriote FA, Corbett TH and Baker LH eds., Kluwer Academic Publ., Norwell, Mass., 11-34, 1992.

15 Paull K, Shoemaker R, Hodes L, Monks A, Scudiero D, Rubistein L, Plowman J and Boyd MR: Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. J Natl Cancer Inst *81*: 1088-1092, 1989.

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