

# Synthesis and Anticancer Evaluation of Some New Unsymmetrical 3,5-Diaryl-4H-1,2,4-Triazole Derivatives

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A series of 4-arylidenamino-4H-1,2,4-triazole derivatives (**3-11**) were synthesized from the treatment of 4-amino-4H-1,2,4-triazoles (**2**) with certain aldehydes. Compounds **3-11** were reduced with NaBH<sub>4</sub> to afford the corresponding 4-arylmethylenamino-4H-1,2,4-triazoles (**12-20**). Compounds **1-10** and **12-19** were characterized by elemental analyses and <sup>1</sup>H NMR, <sup>13</sup>C NMR, IR and UV spectral data. Compounds **11** and **20** were characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, IR and mass spectral data. Compounds **14**, **16**, **17**, and **18** were tested for anticancer activities. Compound **17**, chosen for its higher anticancer activity in the preliminary tests with the cancer cell lines of MCF7, NCI-H460, and SF-268, exhibited remarkable anticancer potential in screening tests with 60 human cancer cell lines.

**Key Words:** 4H-1,2,4-Triazoles, 4-Amino-4H-1,2,4-triazoles, 4-Arylidenamino-4H-1,2,4-triazoles, 4-Arylmethylenamino-4H-1,2,4-triazoles, Synthesis, Anticancer activity.

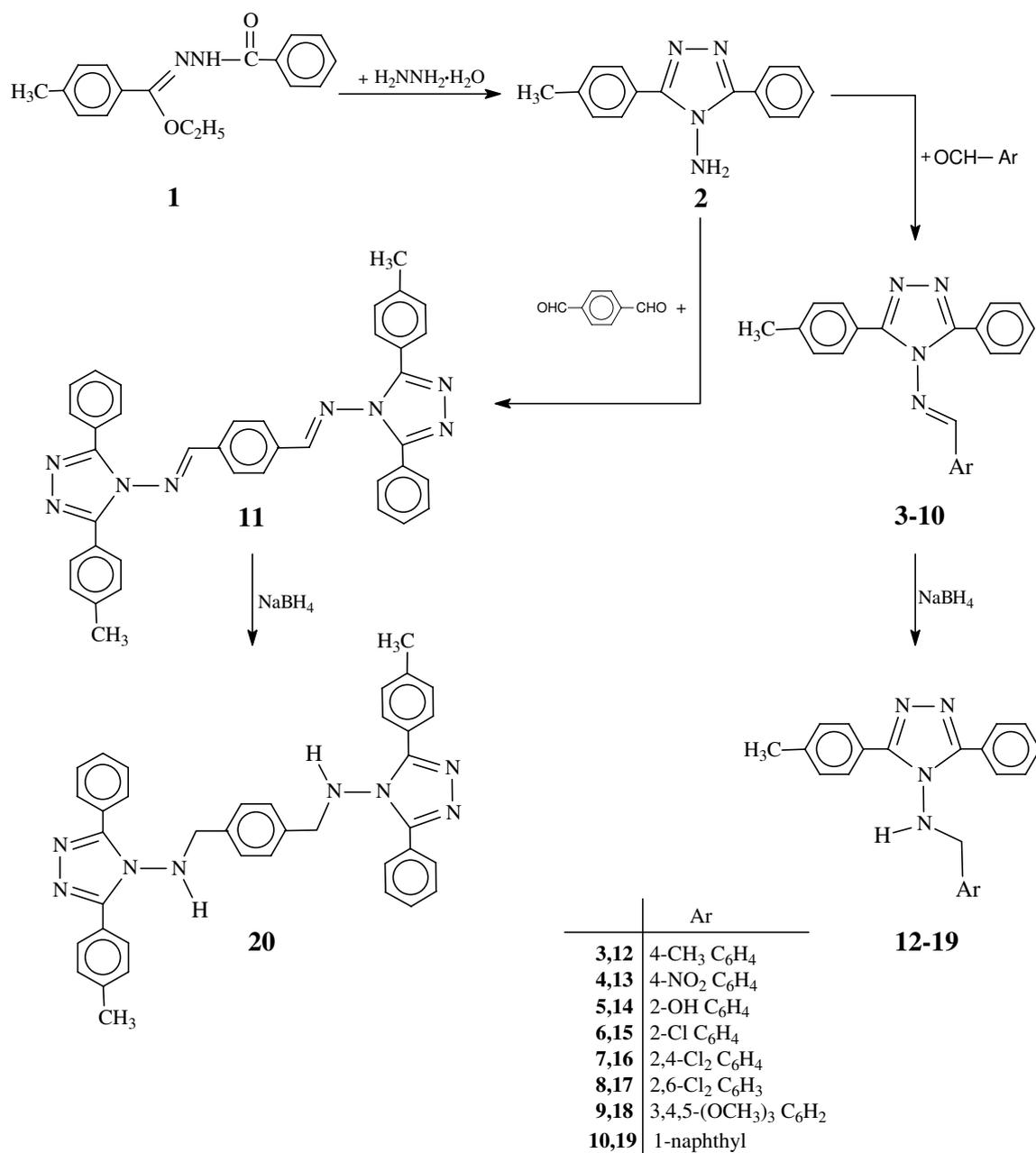
## Introduction

In recent years, various 1,2,4-triazoles and 4,5-dihydro-1H-1,2,4-triazol-5-ones have been found to be associated with diverse pharmacological activities such as anticonvulsant, antifungal, anticancer, anti-inflammatory and antibacterial<sup>1-13</sup>. Several articles have been devoted to the syntheses and biological activities of some 4-arylidenamino-4,5-dihydro-1H-1,2,4-triazol-5-ones<sup>8-13</sup>. Due to the structural resemblance to 1,2,4-triazoles and 4,5-dihydro-1H-1,2,4-triazole-5-ones, the synthesis of 4-arylidenamino-4H-1,2,4-triazoles may be important for their potential biological activity<sup>14,15</sup>.

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The syntheses of 4-arylidenamino-4H-1,2,4-triazole (**3-11**) derivatives were accomplished in the current study according to the reaction scheme outlined in Figure 1. First, 3-phenyl-5-p-tolyl-4-amino-4H-1,2,4-triazole (**2**) was treated with selected aldehydes. Compound **2** was obtained from the reaction of ethyl p-methylbenzoate benzoylhydrazone (**1**) with hydrazine by using the published method (Figure 1)<sup>15</sup>. Generally the imine group in such compounds as **3-11** is expected to be reduced<sup>16-19</sup>. However, attempts to reduce the imines in **3-11** may also lead to reduction of the hetero ring. For this reason, the reduction of only the imino group of compounds **3-11** without affecting the hetero ring was also aimed in the study. Thus, a general and convenient method was employed for the synthesis of 4-arylmethylenamino-4H-1,2,4-triazoles **12-20** in good yields by the use of NaBH<sub>4</sub> as a selective reducing agent (Figure 1).



**Figure 1.** The reaction scheme for the complete syntheses of compounds **1** through **20**.

## Results and Discussion

Many symmetrically substituted 4-amino-3,5-diaryl-4H-1,2,4-triazoles have been prepared by several methods<sup>24–33</sup> and only a few unsymmetrically substituted 4-amino-3,5-diaryl-4H-1,2,4-triazoles have been reported in the literature<sup>15</sup>. The first 2 compounds in the reaction scheme (Figure 1), ethyl p-methylbenzoate benzoylhydrazone (**1**) and 4-amino-3-phenyl-5-p-tolyl-4H-1,2,4-triazole (**2**), were synthesized according to the literature method<sup>15</sup>. Schiff bases **3-11** were prepared by the condensation of 4-amino-3-phenyl-5-p-tolyl-4H-1,2,4-triazole (**2**) with certain aldehydes in acetic acid. In the reduction of compounds **3-11**, the formation of different products was possible due to the possibility of the reduction of the hetero ring. However, the reduction was performed on the imino group of compounds **3-11** without affecting the hetero ring by using NaBH<sub>4</sub> as the selective reducing agent<sup>10,11,18</sup>.

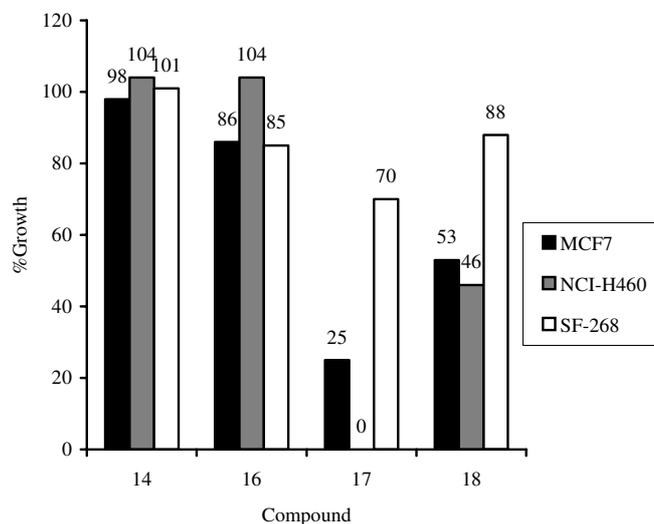
The IR spectra of Schiff bases **3-11** showed characteristic absorption bands between 1570 and 1600 cm<sup>-1</sup> ( $\nu_{C=N}$ ). The <sup>1</sup>H NMR characteristic signals of compounds **3-11** were observed at  $\delta$  8.58-8.78 ppm (s, 1H, N=CH). The <sup>13</sup>C NMR signals for the -N=CH- group of compounds **3-11** were recorded at 156-160 ppm. Arylmethylamino derivatives **12-20** showed IR absorption bands around 3200-3300 cm<sup>-1</sup> ( $\nu_{NH}$ ). The <sup>1</sup>H NMR characteristic signals of **12-20** were observed as a triplet at  $\delta$  5.30-6.63 ppm (t, 1H, NH) and as a doublet at  $\delta$  3.63-4.15 ppm (d, 2H, CH<sub>2</sub>). The signal of the reduced carbon atom in -NH-CH<sub>2</sub>- of the compounds **12-20** was recorded at around 50 ppm in the <sup>13</sup>C NMR spectra.

## Pharmacology

The tumor growth inhibition properties of the 4 reduced compounds **14**, **16**, **17**, and **18** with the NCI codes NSC 731084, NSC 731082, NSC 731083, and NSC 731085, selected among compounds **3-19** by the National Cancer Institute (NCI), USA, were screened on 3 human tumor cell lines, breast cancer (MCF7), non-small cell lung cancer (NCI-H460), and CNS cancer (SF-268) at the NIH, Bethesda, Maryland, USA, under the drug discovery program of the NCI in a primary one-dose anticancer assay (Figure 2). Each cell line was inoculated on a pre-incubated microtiter plate. The test agents were added at a single concentration (10<sup>-4</sup> M) and the culture was incubated for 48 h. End-point determinations were made with Alamar Blue<sup>20</sup>. Results for each test agent are reported as the percent growth of the tested cells when compared with the untreated control cells. Only the compounds exhibiting 32% growth inhibition or higher activity are considered for further anticancer testing by the NCI. Compound **17**, which reduced the growth of 2 cell lines to 25% and 0% (negative numbers would indicate cell killing) (Figure 2), was passed on for evaluation in the full panel of 60 cancer cell lines.

Compound **17** was tested by the NCI with 60 human tumor cell lines over a 5-log dose range (five 10-fold dilutions). Following incubation for 48 h, the cell viability and growth was estimated with sulforhodamine B (SRB) protein assay. The inhibitory activity is expressed as the  $\mu$ M concentration of the agent providing 50% inhibition of the growth of the tumor cells, denoted as GI<sub>50</sub> in the Table. The GI<sub>50</sub> values of **17** ranged between 7.25 (HOP-92, HCT 116) and 26.6 (NCI-H226  $\mu$ M), mostly below 15  $\mu$ M. Its growth inhibition activity was low only for the NCI-H522 (lung cancer) cell line (GI<sub>50</sub> 50  $\mu$ M). Although compound **17** was not referred to the Biological Evaluation Committee for further testing to be a drug candidate, with 2 chloro atoms at the 2,6 position of the benzylamino moiety, it adds to the understanding of the anticancer potential of compounds with specific functional groups within a series. When the 4 compounds

were compared based on their performance in the 3 human tumor cell line test, it is evident that the tumor antiproliferative activity increases by changing the positions of chloro groups from 2,4 to 2,6 on benzylamino moiety. The fact that not only the position but also the type of functional group plays an important role in biological activity is evident from various % Growth values of the 4 test compounds.



**Figure 2.** Three cell line antitumor screening data for selected compounds **14**, **16**, **17** and **18** at  $10^{-4}$  M concentration.

The results of the 2 test methods reveal that the use of the 3 cell lines MCF7, NCI-H460, and SF-268, as an indication of total anticancer potential, is a good, cost-effective selection, since the  $GI_{50}$  values for these 3 cell lines (10.9, 11.8, and 9.89  $\mu$ M, respectively) are close to the lower end of the results range (between 7.25 and  $>50$   $\mu$ M). However, some discrepancy exists between the results of the 2 test methods as % Growth values for MCF7, NCI-H460, and SF-268 with compound **17** were 25, 0, and 70, respectively, which indicates some difference between cell types, while the  $GI_{50}$  values for these cell lines were 10.9, 11.8, and 9.89  $\mu$ M, respectively, which show a closer cancer antiproliferative activity. In conclusion, there appears a need for the synthesis of series of various homologous unsymmetrical 3,5-diaryl-4H-1,2,4-triazole derivatives having various substituent groups at various positions of the benzylamino ring, and possibly on the other 2 aryl groups for a better evaluation of such compounds in terms of anticancer activity.

## Experimental

Melting points were determined on a Büchi oil-heated melting point apparatus and are uncorrected.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra ( $\delta$ , ppm) were recorded on a Varian-Mercury 200 MHz spectrometer using tetramethylsilane as the internal reference. IR spectra ( $\lambda$ ,  $\text{cm}^{-1}$ ) were obtained with a Perkin-Elmer 1600 FTIR spectrometer in KBr pellets. The UV absorption measurements were carried out in  $1.10^{-4}$ - $1.10^{-6}$ M ethanolic solutions and the spectra were recorded between 200 and 400 nm with a Shimadzu-1201 spectrophotometer using 10 mm quartz cells. Combustion analyses were performed on a Carlo Erba 1106 elemental analyzer. The mass spectra were recorded on a MicroMass Quattro LC-MS/MS spectrometer. The necessary chemicals were purchased from Merck and Fluka. The starting compounds **1** and **2** were synthesized by the methods reported earlier<sup>15</sup>.

**Table.** Sixty human tumor cell line anticancer screening data for compound **17**.

Cancer Type	Cell	GI <sub>50</sub> <sup>a</sup> (μM)
Leukemia	HL-60 (TB)	14.3
	K-562	10.3
	MOLT-4	11.1
	RPMI-8226	8.79
	SR	10.9
Non-Small Cell Lung Cancer	A549/ATCC	13.6
	EKVX	13.8
	HOP-62	17.4
	HOP-92	7.25
	NCI-H226	26.6
	NCI-H23	11.2
	NCI-H322M	9.94
	NCI-H460	11.8
	NCI-H522	6.50
	NCI-H527	11.5
Colon Cancer	COLO 205	24.9
	HCT-116	7.25
	HCT-15	10.5
	HT29	15
	KM12	8.69
CNS Cancer	SW-620	16.7
	SF-268	9.89
	SF-295	9.51
	SF-539	10.3
	SNB-19	16.6
	SNB-75	17.1
	U251	9.54
Melanoma	LOX IMVI	8.59
	M14	9.1
	SK-MEL-2	19.6
	SK-MEL-28	14.4
	SK-MEL-5	6.18
	UACC-257	10.7
	UACC-62	7.94
	UACC-122	10.7
Ovarian Cancer	IGROV1	18.7
	OVCAR-3	8.12
	OVCAR-4	16
	OVCAR-5	16.9
	OVCAR-8	24.6
	SK-OV-3	18.4
Renal Cancer	786-0	16.5
	A498	6.59
	ACHN	9.27
	CAKI-1	10.5
	RXF 393	7.18
	SN12C	18.5
	TK-10	10.9
	UO-31	17.1
	UO-511	17.1
Prostate Cancer	PC-3	12.6
	DU-145	9.98
Breast Cancer	MCF7	10.9
	NCI/ADR-RES	8.57
	MDA-MB-231/ATCC	16
	HS 578T	11.4
	MDA-MB-435	9.15
	BT-549	9.27

<sup>a</sup>GI<sub>50</sub> represents the μM concentration of the test compound providing 50% inhibition of tumor cell growth.

**Synthesis of compound 1:** A solution of benzhydrazide (0.01 mol) in 25 mL of absolute ethanol was added to a solution of ethyl imido-p-methylbenzoate hydrochloride (0.01 mol) in 25 mL of absolute ethanol. The mixture was stirred for 6 h at 0-5 °C and subsequently for 2 h at room temperature. The reaction mixture was poured into a beaker containing 40 mL of cold water and 10 g of ice. The precipitate formed was washed with 50 mL of ice-water and then dried. The product was recrystallized from benzene-petroleum ether (40-60 °C) (1:2) to give pure compound **1**. Yield 85%, m.p. 78-79 °C, IR (KBr)  $\text{cm}^{-1}$ : 3169 ( $\nu_{\text{NH}}$ ), 1616 ( $\nu_{\text{C=N}}$ ), 1650 ( $\nu_{\text{C=O}}$ ), 824, 793, 698 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.44 (t, 3H,  $\text{CH}_3$ ), 2.24 (s, 3H,  $\text{CH}_3$ ), 4.30 (q, 2H,  $\text{CH}_2$ ), 9.86 (s, 1H, NH), 7.18-8.10 (m, 9H, Ar-H), UV [ $\lambda_{\text{max, nm}}$  ( $\epsilon \times 10^{-3}$ ): 282 (19.2), 221 (18.7), 205 (22.7), Anal. Calcd. for ( $\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_2$ ): C: 72.31, H: 6.43, N: 9.93. Found: C: 72.85, H: 5.94, N: 9.95.

**Synthesis of compound 2:** Compound **1** (0.005 mol) was added to a solution of hydrazine hydrate (0.01 mol) in 50 mL of 1-propanol and the mixture was refluxed for 24 h. On cooling, a precipitate was formed. This product was filtered and, after drying, was washed with 20 mL of benzene. The insoluble part in benzene was recrystallized from 1-propanol to afford pure compound **2**. Yield 85%, m.p. 283-284 °C, IR (KBr)  $\text{cm}^{-1}$ : 3345, 3250 ( $\nu_{\text{NH}_2}$ ), 1619 ( $\nu_{\text{C=N}}$ ), 824, 768, 696 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  2.38 (s, 1H,  $\text{CH}_3$ ), 6.30 (s, 2H,  $\text{NH}_2$ ), Ar-H: [7.30 (d, 2H), 7.50 (m, 3H), 7.98 (d, 2H), 8.10 (m, 2H, Ar-H)],  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  154.15 (triazole  $\text{C}_3$ ), 154.02 (triazole  $\text{C}_5$ ), Ar-C: [139.11, 129.42, 128.94 (2C), 128.36 (2C), 128.18 (2C), 128.09 (2C), 127.10, 124.31], 20.87 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max, nm}}$  ( $\epsilon \times 10^{-3}$ ): 256 (19.9), 210 (16.0), Anal. Calcd. for ( $\text{C}_{15}\text{H}_{14}\text{N}_4$ ): C: 71.96, H: 5.64, N: 22.39. Found: C: 72.11, H: 5.23, N: 22.66.

**General method for the synthesis of compounds 3-10:** The corresponding aldehyde (0.005 mol) was added to a solution of compound **2** (0.005 mol) in 20 mL of glacial acetic acid and the mixture was refluxed for 4 h. After cooling, the mixture was poured into a beaker containing 100 mL of ice-water. The precipitate formed was filtered. After drying in vacuo, the product was recrystallized from an appropriate solvent to give the desired compound.

**3-Phenyl-5-p-tolyl-4-(4-methylbenzylidenamino)-4H-1,2,4-triazole (3):** Recrystallized from ethanol-water (1:1) to yield 90%, m.p. 178-179 °C, IR (KBr)  $\text{cm}^{-1}$ : 1600, 1592 ( $\nu_{\text{C=N}}$ ), 821, 769, 695 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  2.32 (s, 3H,  $\text{CH}_3$ ) 2.38 (s, 3H,  $\text{CH}_3$ ), Ar-H: [7.34 (m, 4H), 7.50 (m, 3H), 7.72 (m, 4H), 7.84 (m, 2H)], 8.58 (s, 1H, CH),  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  170.57 (N=CH), 149.68 (triazole  $\text{C}_3$ ), 149.50 (triazole  $\text{C}_5$ ), Ar-C: [143.56, 139.00, 129.43 (2C), 129.22, 128.91 (2C), 128.54 (2C), 128.34 (2C), 128.23, 127.66 (2C), 127.60 (2C), 126.00, 123.09], 20.13 ( $\text{CH}_3$ ), 20.06 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max, nm}}$  ( $\epsilon \times 10^{-3}$ ): 264 (33.9), 209 (31.6), Anal. Calcd. for ( $\text{C}_{23}\text{H}_{20}\text{N}_4$ ): C: 78.38, H: 5.72, N:15.90, Found: C: 78.11, H: 5.95, N: 15.94.

**3-Phenyl-5-p-tolyl-4-(4-nitrobenzylidenamino)-4H-1,2,4-triazole (4):** Recrystallized from ethanol to yield 76%, m.p. 209-210 °C, IR (KBr)  $\text{cm}^{-1}$ : 1617, 1591 ( $\nu_{\text{C=N}}$ ), 824, 777, 693 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  2.32 (s, 3H,  $\text{CH}_3$ ), Ar-H: [7.32 (d, 2H), 7.52 (m, 3H), 7.72 (d, 2H), 7.83 (m, 2H), 8.06 (d, 2H), 8.36 (d, 2H)], 8.78 (s, 1H, CH),  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  168.05 (N=CH), 150.11 (triazole  $\text{C}_3$ ), 149.93 (triazole  $\text{C}_5$ ), Ar-C: [149.87, 139.68, 136.70, 130.04 (2C), 129.88, 129.43 (2C), 128.87 (2C), 128.26 (2C), 128.17 (2C), 126.17, 124.32 (2C), 123.43], 20.83 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max, nm}}$  ( $\epsilon \times 10^{-3}$ ): 264 (29.7), 210 (27.1), Anal. Calcd. for ( $\text{C}_{22}\text{H}_{17}\text{N}_5\text{O}_2$ ): C: 68.92, H: 4.47, N: 18.27, Found: C: 69.87, H: 3.85, N: 18.29.

**3-Phenyl-5-p-tolyl-4-(2-hydroxybenzylidenamino)-4H-1,2,4-triazole (5):** Recrystallized from

ethanol to yield 85%, m.p. 195-196 °C, IR (KBr)  $\text{cm}^{-1}$ : 3031 ( $\nu_{\text{OH}}$ ), 1604, 1576 ( $\nu_{\text{C=N}}$ ), 822, 768, 758, 694 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.34 (s, 3H,  $\text{CH}_3$ ), Ar-H: [6.95 (m, 2H), 7.28 (m, 2H), 7.47 (m, 4H), 7.72 (d, 2H), 7.88 (m, 3H)], 8.70 (s, 1H, CH), 10.43 (s, 1H, OH),  $^{13}\text{C}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  165.75 (N=CH), 149.34 (triazole  $\text{C}_3$ ), 149.16 (triazole  $\text{C}_5$ ), Ar-C: [157.79, 138.62, 134.23, 128.88, 128.56 (2C), 127.99 (2C), 127.40 (2C), 127.33 (2C), 126.80, 125.73, 122.81, 118.95, 116.78, 115.86], 20.05 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max}}$ , nm ( $\epsilon \times 10^{-3}$ )]: 261 (30.0), 214 (37.7). Anal. Calcd. for ( $\text{C}_{22}\text{H}_{18}\text{N}_4\text{O}$ ): C: 74.56, H: 5.12, N: 15.81, Found: C: 74.05, H: 5.86, N: 15.58.

**3-Phenyl-5-p-tolyl-4-(2-chlorobenzylidenamino)-4H-1,2,4-triazole (6):** Recrystallized from ethanol-water (1:1) to yield 82%, m.p. 150-151 °C, IR (KBr)  $\text{cm}^{-1}$ : 1607, 1590 ( $\nu_{\text{C=N}}$ ), 820, 771, 724, 693 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.38 (s, 3H,  $\text{CH}_3$ ), Ar-H : [7.38 (d, 2H), 7.58 (m, 6H), 7.76 (d, 2H), 7.82 (m, 2H), 8.12 (m, 1H)], 8.78 (s, 1H, CH),  $^{13}\text{C}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  165.70 (N=CH), 150.20 (triazole  $\text{C}_3$ ), 149.96 (triazole  $\text{C}_5$ ), Ar-C: [139.59, 139.53, 134.95, 134.43, 130.04, 129.80, 129.38 (2C), 128.85 (2C), 128.51 (2C), 128.39 (2C), 128.21, 128.04, 126.49, 123.57], 20.15 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max}}$ , nm ( $\epsilon \times 10^{-3}$ )]: 256 (25.4), 214(22.3). Anal. Calcd. for ( $\text{C}_{22}\text{H}_{17}\text{N}_4\text{Cl}$ ): C: 70.87, H: 4.60, N: 15.03, Found: C: 70.27, H: 4.86, N: 15.43.

**3-Phenyl-5-p-tolyl-4-(2,4-dichlorobenzylidenamino)-4H-1,2,4-triazole (7):** Recrystallized from ethanol to yield 85%, m.p. 139-140 °C, IR (KBr)  $\text{cm}^{-1}$ : 1602, 1582 ( $\nu_{\text{C=N}}$ ), 870, 828, 804, 771, 694 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.38 (s, 3H,  $\text{CH}_3$ ), Ar-H: [7.34 (d, 2H), 8.10 (d, 2H), 7.50-7.85 (m, 8H)], 8.72 (s, 1H, CH),  $^{13}\text{C}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  164.85 (N=CH), 150.19 (triazole  $\text{C}_3$ ), 149.94 (triazole  $\text{C}_5$ ), Ar-C: [139.63, 138.37, 135.76, 129.83, 129.73, 129.39 (2C), 128.88 (2C), 128.52 (2C), 128.47 (2C), 128.41, 128.37, 127.82, 126.43, 123.51], 20.93 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max}}$ , nm ( $\epsilon \times 10^{-3}$ )]: 260 (26.7), 209 (30.0), Anal. Calcd. for ( $\text{C}_{22}\text{H}_{16}\text{N}_4\text{Cl}_2$ ): C: 64.88, H: 3.96, N: 13.76, Found: C: 64.53, H: 4.05, N: 13.98.

**3-Phenyl-5-p-tolyl-4-(2,6-dichlorobenzylidenamino)-4H-1,2,4-triazole (8):** Recrystallized from ethanol to yield 86%, m.p. 167-168 °C, IR (KBr)  $\text{cm}^{-1}$ : 1603, 1577 ( $\nu_{\text{C=N}}$ ), 826, 783, 769, 724, 691 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.38 (s, 3H,  $\text{CH}_3$ ), Ar-H: [7.34 (d, 3H), 7.58 (m, 5H), 7.80 (m, 4H), 8.76 (s, 1H, CH)],  $^{13}\text{C}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  165.78 (N=CH), 150.25 (triazole  $\text{C}_3$ ), 150.18 (triazole  $\text{C}_5$ ), Ar-C: [139.75, 134.78 (2C), 133.65, 129.89, 129.68 (2C), 129.26 (2C), 128.95 (2C), 128.83 (2C), 128.50 (2C), 127.11, 126.26, 123.21], 20.95 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max}}$ , nm ( $\epsilon \times 10^{-3}$ )]: 255 (27.3), 213 (29.9), Anal. Calcd. for ( $\text{C}_{22}\text{H}_{16}\text{N}_4\text{Cl}_2$ ): C: 64.88, H: 3.96, N: 13.76, Found: C: 64.06, H: 4.63, N: 13.98.

**3-Phenyl-5-p-tolyl-4-(3,4,5-trimethoxybenzylidenamino)-4H-1,2,4-triazole (9):** Recrystallized from ethanol to yield 86%, m.p. 195-196 °C, IR (KBr)  $\text{cm}^{-1}$ : 1610, 1575 ( $\nu_{\text{C=N}}$ ), 852, 825, 765, 694 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.38 (s, 3H,  $\text{CH}_3$ ), 3.82 (s, 6H,  $2\text{OCH}_3$ ), 3.75 (s, 3H,  $\text{OCH}_3$ ), Ar-H: [7.18 (s, 2H), 7.30 (d, 2H), 7.50 (m, 3H), 7.75 (d, 2H), 7.87 (m, 2H)], 8.58 (s, 1H, CH),  $^{13}\text{C}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  170.65 (N=CH), 149.95 (triazole  $\text{C}_3$ ), 149.79 (triazole  $\text{C}_5$ ), Ar-C: [153.10 (2C), 141.74, 139.34, 129.55, 129.26 (2C), 128.68 (2C), 127.92 (2C), 127.86 (2C), 126.44, 126.18, 123.26, 106.17 (2C)], 60.05 (C,  $\text{OCH}_3$ ), 55.98 (2C,  $2\text{OCH}_3$ ), 20.80 ( $\text{CH}_3$ ), UV [ $\lambda_{\text{max}}$ , nm ( $\epsilon \times 10^{-3}$ )]: 327 (6.24), 260 (24.8), 210 (27.9), Anal. Calcd. for ( $\text{C}_{25}\text{H}_{24}\text{N}_4\text{O}_3$ ): C: 70.08, H: 5.65, N: 13.08, Found: C: 69.85, H: 5.72, N: 13.15.

**3-Phenyl-5-p-tolyl-4-(naphthylidenamino)-4H-1,2,4-triazole (10):** Recrystallized from ethanol to yield 80%, m.p. 165-166 °C, IR (KBr)  $\text{cm}^{-1}$ : 1589, 1572 ( $\nu_{\text{C=N}}$ ), 819, 778, 696 ( $\nu_{\text{arm.ring}}$ ),  $^1\text{H}$  NMR (DMSO- $\text{d}_6$ )  $\delta$  2.30 (s, 3H,  $\text{CH}_3$ ), Ar-H: [7.30 (d, 2H), 7.50 (d, 3H), 7.65 (m, 3H), 7.80 (d, 2H), 7.92 (m,

2H), 8.08 (d, 2H), 8.22 (d, 1H), 8.38 (m, 1H)], 9.28 (s, 1H, CH), <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ 170.65 (N=CH), 150.24 (triazole C<sub>3</sub>), 150.08 (triazole C<sub>5</sub>), Ar-C: [139.50, 133.83, 133.27, 130.52, 130.28, 129.70, 129.38 (2C), 128.94, 128.81 (2C), 128.37 (2C), 128.29 (2C), 128.14, 126.98, 126.75, 126.63, 125.55, 123.70, 123.59], 20.98 (CH<sub>3</sub>), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 320 (8.0), 250 (28.8), 222 (31.0), Anal. Calcd. for (C<sub>26</sub>H<sub>20</sub>N<sub>4</sub>): C: 80.39, H: 5.19, N: 14.42, Found: C: 80.73, H: 5.06, N: 14.21.

**N,N'-bis (3-Phenyl-5-p-tolyl-4H-1,2,4-triazolyl)-1,4-xylenediimine (11):** Recrystallized from acetic acid to yield 83%, m.p. 304-305 °C, IR (KBr) cm<sup>-1</sup>: 1596 ( $\nu_{C=N}$ ), 826, 776, 697 ( $\nu_{arm.ring}$ ), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 2.50 (s, 6H, 2CH<sub>3</sub>), Ar-H: [7.33 (d, 4H), 7.50 (s, 6H), 7.71 (d, 4H), 7.80 (m, 4H), 7.93 (s, 4H)], 8.68 (s, 2H, CH), LC-MS/MS for C<sub>38</sub>H<sub>30</sub>N<sub>8</sub>(Molecular weight: 598.71 g/mol), m/z: 235.86 (96%) [3-Phenyl-5-p-tolyl-4H-1,2,4-triazole]<sup>+</sup>, 599.23 (23%) [M+1]<sup>+</sup>, 621.22 (20%) [M+Na]<sup>+</sup>.

**General method for the synthesis of compounds 12-20:** The corresponding compound (3-11) (0.005 mol) was dissolved in 50 mL of dried methanol, and NaBH<sub>4</sub>(0.005 mol) was added in small portions to this solution. The mixture was refluxed for 20 min and then allowed to cool. After evaporation at 25-30 °C under reduced pressure, the solid residue was washed with cold water. After drying in vacuo, the solid product was recrystallized from an appropriate solvent to afford the desired compound.

**3-Phenyl-5-p-tolyl-4-(4-methylbenzylamino)-4H-1,2,4-triazole (12):** Recrystallized from ethyl acetate to yield 95%, m.p. 179-180 °C, IR (KBr) cm<sup>-1</sup>: 3301 ( $\nu_{NH}$ ), 1617 ( $\nu_{C=N}$ ), 824, 807, 771, 694 ( $\nu_{arm.ring}$ ), <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.28 (s, 3H, CH<sub>3</sub>), 2.45 (s, 3H, CH<sub>3</sub>), 3.71 (d, 2H, CH<sub>2</sub>), 5.30 (t, 1H, NH), Ar-H: [6.72 (d, 2H), 6.96 (d, 2H), 7.32 (d, 2H), 7.50 (m, 2H), 7.84 (d, 4H), 7.89 (m, 1H)], <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 153.94 (triazole C<sub>3</sub>), 153.22 (triazole C<sub>5</sub>), Ar-C: [140.34, 138.11, 131.10, 130.03, 129.54 (2C), 129.23 (2C), 128.91 (2C), 128.73 (2C), 128.14 (2C), 127.96 (2C), 126.82, 123.87], 56.18 (CH<sub>2</sub>), 21.08 (CH<sub>3</sub>), 21.49 (CH<sub>3</sub>), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 261 (23.7), 212 (25.0), Anal. Calcd. for (C<sub>23</sub>H<sub>22</sub>N<sub>4</sub>): C: 77.94, H: 6.26, N: 15.81, Found: C: 78.07, H: 6.15, N: 15.78.

**3-Phenyl-5-p-tolyl-4-(4-nitrobenzylamino)-4H-1,2,4-triazole (13):** Recrystallized from ethanol to yield 88%, m.p. 210-211 °C, IR (KBr) cm<sup>-1</sup>: 3260 ( $\nu_{NH}$ ), 1606 ( $\nu_{C=N}$ ), 853, 822, 772, 692 ( $\nu_{arm.ring}$ ), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 2.45 (s, 3H, CH<sub>3</sub>), 3.82 (d, 2H, CH<sub>2</sub>), 5.85 (t, 1H, NH), Ar-H: [6.92 (d, 2H), 7.30 (d, 2H), 7.48 (m, 3H), 7.81 (m, 6H)], <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ 152.08 (triazole C<sub>3</sub>), 151.98 (triazole C<sub>5</sub>), Ar-C: [147.65, 141.96, 140.78, 130.29, 129.87 (2C), 129.69 (2C), 128.85 (2C), 128.19 (2C), 127.98 (2C), 126.59, 123.59, 123.43 (2C)], 55.23 (CH<sub>2</sub>), 21.53 (CH<sub>3</sub>), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 258 (29.3), 210 (24.6). Anal. Calcd. for (C<sub>22</sub>H<sub>19</sub>N<sub>5</sub>O<sub>2</sub>): C: 68.56, H: 4.97, N: 18.17, Found: C: 68.65, H: 4.83, N: 18.27.

**3-Phenyl-5-p-tolyl-4-(2-hydroxybenzylamino)-4H-1,2,4-triazole (14):** Recrystallized from ethanol to yield 83%, m.p. 233-234 °C, IR (KBr) cm<sup>-1</sup>: 3340 ( $\nu_{NH}$ ), 3049 ( $\nu_{OH}$ ), 1598 ( $\nu_{C=N}$ ), 818, 775, 759, 692 ( $\nu_{arm.ring}$ ), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 2.42 (s, 3H, CH<sub>3</sub>), 3.84 (d, 2H, CH<sub>2</sub>), 6.63 (t, 1H, NH), Ar-H: [6.86 (m, 2H), 7.04 (m, 2H), 7.35 (d, 2H), 7.53 (m, 3H), 8.03 (m, 4H)], 9.56 (s, 1H, OH), <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ 153.58 (triazole C<sub>3</sub> and triazole C<sub>5</sub>), Ar-C: [155.63, 139.29, 130.12, 129.59, 129.07 (2C), 128.75, 128.40 (2C), 127.97 (2C), 127.82 (2C), 127.23, 124.38, 121.64, 118.52, 114.82], 49.05 (CH<sub>2</sub>), 20.95 (CH<sub>3</sub>), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 261 (22.0), 209 (27.3). Anal. Calcd. for (C<sub>22</sub>H<sub>20</sub>N<sub>4</sub>O): C: 74.14, H: 5.66, N: 15.72, Found: C: 74.63, H: 5.27, N: 15.65.

**3-Phenyl-5-p-tolyl-4-(2-chlorobenzylamino)-4H-1,2,4-triazole (15):** Recrystallized from ethyl acetate to yield 90%, m.p. 182-183 °C, IR (KBr) cm<sup>-1</sup>: 3257 ( $\nu_{NH}$ ), 1617 ( $\nu_{C=N}$ ), 821, 770, 755, 692

( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.44 (s, 3H,  $\text{CH}_3$ ), 3.85 (d, 2H,  $\text{CH}_2$ ), 5.78 (t, 1H, NH), Ar-H: [6.70 (m, 1H), 7.04 (m, 4H), 7.26 (d, 2H), 7.47 (m, 2H), 7.78 (m, 4H)],  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  153.44 (triazole  $\text{C}_3$  and triazole  $\text{C}_5$ ), Ar-C: [140.24, 134.41, 132.01, 131.25, 129.94, 129.83, 129.52 (2C), 129.48, 128.73 (2C), 128.05 (2C), 127.90 (2C), 126.90, 126.48, 123.58], 53.95, 21.46, UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 260 (29.0), 209 (34.3), Anal. Calcd. for ( $\text{C}_{22}\text{H}_{19}\text{N}_4\text{Cl}$ ): C: 70.49, H: 5.11, N: 14.95, Found: C: 70.69, H: 5.04, N: 14.81.

**3-Phenyl-5-p-tolyl-4-(2,4-dichlorobenzylamino)-4H-1,2,4-triazole (16):** Recrystallized from ethyl acetate to yield 92%, m.p. 192-193 °C, IR (KBr)  $\text{cm}^{-1}$ : 3242 ( $\nu_{NH}$ ), 1598 ( $\nu_{C=N}$ ), 867, 818, 805, 765, 686 ( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.45 (s, 3H,  $\text{CH}_3$ ), 3.82 (d, 2H,  $\text{CH}_2$ ), 5.85 (t, 1H, NH), Ar-H: [6.55 (m, 1H), 6.95 (m, 2H), 7.30 (d, 2H), 7.45 (m, 3H), 7.75 (m, 4H)],  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  153.56 (triazole  $\text{C}_3$  and triazole  $\text{C}_5$ ), Ar-C: [140.33, 135.07, 131.92, 130.67, 129.98, 129.48 (2C), 129.45, 129.30, 128.68 (2C), 127.96 (2C), 127.82 (2C), 127.07, 126.37, 123.48], 53.15 ( $\text{CH}_2$ ), 21.46 ( $\text{CH}_3$ ), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 260 (22.6), 213 (24.6). Anal. Calcd. for ( $\text{C}_{22}\text{H}_{18}\text{N}_4\text{Cl}_2$ ): C: 64.56, H: 4.43, N: 13.69, Found: C: 64.75, H: 4.41, N: 13.58.

**3-Phenyl-5-p-tolyl-4-(2,6-dichlorobenzylamino)-4H-1,2,4-triazole (17):** Recrystallized from ethyl acetate to yield 94%, m.p. 209-210 C, IR (KBr)  $\text{cm}^{-1}$ : 3277 ( $\nu_{NH}$ ), 1618 ( $\nu_{C=N}$ ), 827, 777, 764, 729, 691 ( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.42 (s, 3H,  $\text{CH}_3$ ), 4.10 (d, 2H,  $\text{CH}_2$ ), 5.98 (t, 1H, NH), Ar-H: [6.94 (s, 2H), 7.24 (d, 3H), 7.42 (m, 3H), 7.72 (m, 4H)],  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  153.59 (triazole  $\text{C}_3$ ), 153.50 (triazole  $\text{C}_5$ ), Ar-C: [139.94, 136.15 (2C), 130.55, 129.90, 129.67, 129.36(2C), 128.57 (2C), 128.01 (2C), 127.94 (2C), 127.37 (2C), 126.45, 123.56], 51.75 ( $\text{CH}_2$ ), 21.45 ( $\text{CH}_3$ ), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 261 (23.1), 210 (30.6). Anal. Calcd. for ( $\text{C}_{22}\text{H}_{18}\text{N}_4\text{Cl}_2$ ): C: 64.56, H: 4.43, N: 13.69, Found: C: 64.48, H: 4.22, N: 14.27.

**3-Phenyl-5-p-tolyl-4-(3,4,5-trimethoxybenzylamino)-4H-1,2,4-triazole (18):** Recrystallized from ethyl acetate to yield 96%, m.p. 144-145 C, IR (KBr)  $\text{cm}^{-1}$ : 3229 ( $\nu_{NH}$ ), 1592 ( $\nu_{C=N}$ ), 836, 822, 772, 693 ( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  2.41 (s, 3H,  $\text{CH}_3$ ), 3.63 (d, 2H,  $\text{CH}_2$ ), 3.60 (s, 6H, 2 $\text{OCH}_3$ ), 3.78 (s, 3H,  $\text{OCH}_3$ ), 5.82 (t, 1H, NH), Ar-H: [5.90 (s, 2H), 7.28 (m, 2H), 7.45 (m, 3H), 7.82 (m, 4H)],  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  152.30 (triazole  $\text{C}_3$ ), 152.15 (triazole  $\text{C}_5$ ), Ar-C: [151.32 (2C), 138.62, 135.98, 128.37, 128.22, 127.96 (2C), 127.13 (2C), 126.53 (2C), 126.36 (2C), 125.36, 122.45, 104.23 (2C)], 59.10 ( $\text{OCH}_3$ ), 54.51 ( $\text{CH}_2$ ), 54.13 (2 $\text{OCH}_3$ ), 19.87 ( $\text{CH}_3$ ), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 261 (22.6), 211 (36.5), Anal. Calcd. for ( $\text{C}_{25}\text{H}_{26}\text{N}_4\text{O}_3$ ): C: 69.75, H: 6.09, N: 13.01, Found: C: 69.87, H: 6.17, N: 12.87.

**3-Phenyl-5-p-tolyl-4-(naphthylmethylamino)-4H-1,2,4-triazole (19):** Recrystallized from ethyl acetate to yield 92%, m.p. 218-219 °C, IR (KBr)  $\text{cm}^{-1}$ : 3220 ( $\nu_{NH}$ ), 1617 ( $\nu_{C=N}$ ), 820, 775, 688 ( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.45 (s, 3H,  $\text{CH}_3$ ), 4.15 (d, 2H,  $\text{CH}_2$ ), 5.45 (t, 1H, NH), Ar-H: [7.28 (m, 5H), 7.42 (m, 5H), 7.72 (m, 3H), 7.86 (m, 3H)],  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  153.93 (triazole  $\text{C}_3$ ), 153.73 (triazole  $\text{C}_5$ ), Ar-C: [140.31, 133.61, 131.57, 130.06, 129.94, 129.56 (2C), 129.43, 128.79 (2C), 128.50, 128.21 (2C), 128.15, 128.04 (2C), 126.57, 126.28, 125.71, 125.13, 123.59, 123.30], 54.31 ( $\text{CH}_2$ ), 21.52 ( $\text{CH}_3$ ), UV [ $\lambda_{max}$ , nm ( $\epsilon \times 10^{-3}$ ): 262 (25.2), 223 (46.8). Anal. Calcd. for ( $\text{C}_{26}\text{H}_{22}\text{N}_4$ ): C: 79.97, H: 5.68, N: 14.35, Found: C: 79.58, H: 5.75, N: 14.67.

**N,N'-bis (3-Phenyl-5-p-tolyl-4H-1,2,4-triazolyl)-1,4-xylenediamine (20):** Recrystallized from ethanol to yield 92%, m.p. 324-325 °C, IR (KBr)  $\text{cm}^{-1}$ : 3305 ( $\nu_{NH}$ ), 1616 ( $\nu_{C=N}$ ), 818, 769, 730, 693 ( $\nu_{arm.ring}$ ),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  2.50 (s, 6H, 2 $\text{CH}_3$ ), 3.64 (d, 4H, 2 $\text{CH}_2$ ), 7.09 (t, 2H, 2NH), Ar-H: [6.54 (s, 4H), 7.38 (m, 4H), 7.54 (s, 6H), 7.86 (m, 8H)],  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$  153.55 (2C, triazole  $\text{C}_3$  and tria-

zole C<sub>5</sub>), Ar-C: [139.36 (2C), 134.57 (2C), 129.60 (2C), 129.08 (4C), 128.38 (4C), 128.13 (4C), 127.93 (4C), 127.73 (4C), 127.13 (2C), 124.30 (2C)], 53.33 (2CH<sub>2</sub>), 20.91 (2CH<sub>3</sub>), LC-MS/MS for C<sub>38</sub>H<sub>34</sub>N<sub>8</sub> (Molecular weight: 602.74 g/mol), m/z: 235.98 (15%) [3-Phenyl-5-p-tolyl-4H-1,2,4-triazole]<sup>+</sup>, 603.14 (100%) [M+1]<sup>+</sup>.

## Pharmacology

### Three Cancer Cell Line Anticancer Prescreening

The 3-cell lines', breast cancer (MCF7), non-small cell lung cancer (NCI-H460) and CNS cancer (SF-268), 1-dose anticancer activity prescreening is performed by the Developmental Therapeutics Program of the NCI, USA, for it identifies a large proportion of the compounds that would be inactive in multi-dose 60-cell line screening. The cell lines were grown in the same manner as for the 60-cell line screen. The cells were plated with densities of 5000 cells/well (MCF7), 1000 cells/well (NCI-H460), and 7500 cells/well (SF-268) to allow for varying doubling time of the cell lines. Each plate contained all 3-cell lines, a series of dilutions of standard agents, total kill wells and appropriate controls. Plates were incubated under standard conditions for 24 h prior to addition of the test compounds.

The test compounds (**14**, **16-18**), selected by the NCI, were dissolved in dimethyl sulfoxide (DMSO) at 400 times the desired maximum test concentration (maximum final DMSO concentration of 0.25%) and stored frozen. Compounds were then diluted with complete media with 0.1% gentamicin sulfate (5  $\mu$ L of test sample in 100% DMSO was added to 565  $\mu$ L of complete medium). An aliquot (20  $\mu$ L) from this solution was then dispensed into test wells containing cell suspension (50  $\mu$ L) to yield a test concentration of 10<sup>-4</sup> M.

Two standard drugs, meaning that their activities against the cell lines are well documented, were tested against each cell line: NSC 19893 (5-FU) and NSC 123127 (Adriamycin). The end-point determinations were carried out with Alamar Blue<sup>20</sup>. After compound addition, plates were incubated under standard conditions for 48 h, Alamar Blue (10  $\mu$ L/well) was added and the plates were incubated for an additional 4 h. Fluorescence was measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth is expressed as the ratio of fluorescence of the test well to the average fluorescence of the control wells x 100. Compound **17**, which inhibited the growth of NCI-H460 to 26% (Figure 2), was forwarded for testing in the 60-cell line assay.

### Sixty Human Cancer Cell Line Anticancer Screening

The 60-cell line cancer screening tests were performed by Developmental Therapeutics Program of the NCI, USA, according to the literature<sup>21-23</sup>. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing fetal bovine serum (5%) and L-glutamine (2 mM). For the screening experiment, cells were inoculated into 96-well microtiter plates in 100  $\mu$ L at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity for 24 h prior to addition of the test compound (**17**).

After 24 h, 2 plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of compound addition (Tz). The test compound was

dissolved in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of test compound addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing gentamicin (50 µg/mL). Additional four 10-fold or 1/2 log serial dilutions were made to provide a total of 5 compound concentrations plus a control. Aliquots (100 µL) of these different compound dilutions were added to the appropriate microtiter wells already containing the medium (100 µL), resulting in the required final compound concentrations.

Following test compound addition, the plates were incubated for an additional 48 h at 37 °C, 5% CO<sub>2</sub>, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of cold TCA (50 µL, 50%, w/v) (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed 5 times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µL, 0.4%, w/v) in acetic acid (1%) was added to each well, and the plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing 5 times with acetic acid (1%) and the plates were air dried. Bound stain was subsequently solubilized with trizma base (10 mM), and the absorbance was read at 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding TCA (50 µL, 80%) (final concentration, 16% TCA). Using the 7 absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of the compound at the 5 concentration levels (Ti)], the percentage growth was calculated at each compound concentrations level. Percentage growth inhibition was calculated as:

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations for which } Ti < Tz.$$

Growth inhibition of 50% (GI<sub>50</sub>) was calculated from  $[(Ti-Tz)/(C-Tz)] \times 100 = 50$ , which was the compound concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the compound incubation. Values were calculated for growth inhibition if the level of activity was reached; however, if the effect was not reached the value for that parameter was expressed as greater than the maximum concentration tested.

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