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Anticancer Evaluation of 1,5-Disubstituted Tetrazoles using Ugi-Azide Four-Component Reactions (UA-4CRs)

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ABSTRACT

Azide isocyanide-based multicomponent reactions allow the formation of relatively complex molecules through a one-pot synthesis. The proposed reactions have been coupled of four classes of compounds including 3-phenoxybenzaldehyde, various aromatic amines, TMS-N_3 and tertiary butylisocyanide, which is known as Ugi-azide four-component reactions (UA-4CRs). It generated a diverse class of 1,5-disubstituted tetrazoles which are an important drug-like scaffold known for their ability to mimic the carcinogenic conformers used in medicinal chemistry. This work presents a concise, novel, general strategy to access a surplus of new heterocyclic scaffolds through the Ugi-azide reaction. Frequency in anticancer drug design can be partly attributed to their being extremely common in nature and there are multiple metabolic pathways and cellular processes within cancer pathology that can be susceptible to heterocycles-based drugs. The anticancer screening of derived molecules were carried out using one dose response study using NCI-60 cell-lines and found most active in breast cancer cell-lines.

KEYWORDS

Tetrazoles, Anticancer screening, Ugi coupling reaction.

INTRODUCTION

The Ugi reaction is an easily performed one-pot reaction that is applicable to the synthesis of many distinct types of organic compounds [1]. Some of the products represent important classes of synthetic targets, while others are useful as intermediates for the preparation of a variety of nitrogen compounds [2]. The classical Ugi MCR is comprised of four components, an aldehyde (or ketones), amine, isocyanide and carboxylic acid, which on mixing generate the peptidic-like structure. As such, it is probably the premiere isocyanide based multicomponent reaction and subsequent chemical manipulation of the flexible product has received immense interest in the medicinal chemistry community providing access to arrays of highly diverse small molecules [3-5].

Nitrogen rich tetrazoles are a class of nitrogen rich heterocyclic compounds. The development of tetrazole chemistry has been largely associated with wide scale of applications of these compounds in medicine, biochemistry, agriculture, photography, as well as corrosion inhibitors [6]. To determine effective mimics of the *cis*-amide bond (a protein secondary

structures), the tetrazole ring and more specifically the 1,5-disubstituted tetrazole, has proven to be a valuable bioisostere, reported by Creighton *et al.* [7]. The biological significance of related ring systems has grown in recent years with a number of tetrazole analogs reported to exhibit biological activity toward the cannabinoid-1 receptor (CB1) [8], fatty acid amide hydrolase [9], melanin-concentrating hormone receptor 1 [10] and to act as orally effective human growth hormone secretagogues [11]. The development of concise routes to novel 1,5-disubstituted tetrazole chemical space has to generate active molecule partners or probes for new or established chemotherapy.

Moreover, originally reported in 1961 [12], the $TMSN_3$-modified Ugi reaction, denoted the Ugi-azide reaction, offers a concise chemical route to 1,5-disubstituted tetrazoles which is initiated with simple replacement of the carboxylic acid with $TMSN_3$, delivering 1,5-disubstituted tetrazoles [13]. Through use of a variety of assorted reagents and systematically exploring different ring closing possibilities of the Ugi-azide product, unique scaffolds such as keto piperazine-tetrazoles, azepine-tetrazoles, benzodiazepine-tetrazoles and quinoxaline-tetrazoles have been successfully generated [14-16].

As part of our continuing efforts in using consecutive multi-component reactions to obtain novel molecules in a reduced number of steps [17], herein we describe a concise and efficient strategy for the synthesis of 1,5-disubstituted tetrazoles using Ugi-azide reactions in only single step procedure. During the course of our work, some of the selected molecules by NIH (National Institute of Health) were shown considerable potency against NCI-60 cell-lines.

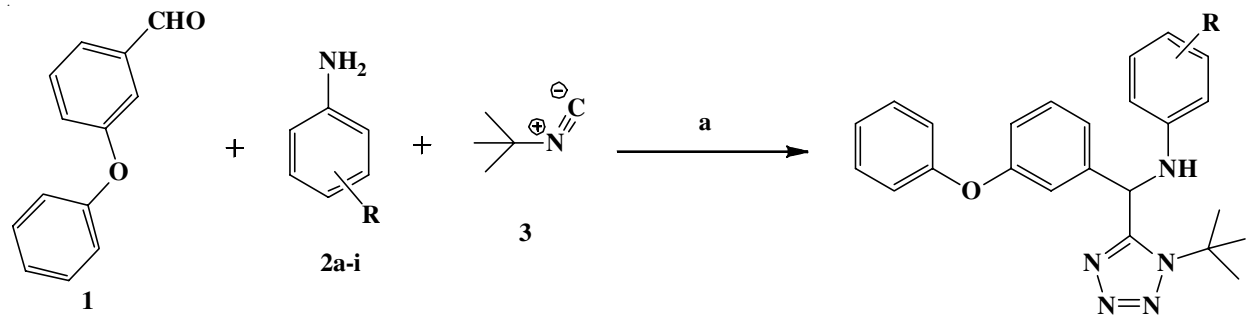
EXPERIMENTAL

All the chemicals and reagents were received from Sigma-Aldrich and Merck. Silica gel plate G60 F254 (Merck) was used in thin layer chromatography to monitor the completion of the reaction. Visualization was made under UV light (254 and 365 nm). Infrared spectra of the compounds were recorded on IR Affinity-1S spectrophotometer (Shimadzu). ^1H (400 MHz) and ^{13}C (101.1 MHz) NMR spectra were recorded on a Bruker AVANCE II spectrometer in DMSO-d_6. Mass spectrometer GCMS-QP 2010 (Shimadzu) was used to resolute the mass spectra of compounds and rotary evaporator was used for drying the compounds. Melting point was measured by open capillary method.

Synthesis of *N*-((1-*tert*-butyl-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)methyl)-substituted benzenamine (4a-i): To a methanol containing round bottom flask, phenoxy benzaldehyde (0.0050 mol) and substituted aromatic amines (0.0050 mol) were added and stirred for 1 h at room temperature to generate a reactive intermediate. After 1 h stirring *tert*-butyl isocyanide (0.0075 mol) and trimethylsilyl azide ($TMSN_3$) (0.0085 mol) were added. The reaction mixture was stirred for 12 h at room temperature. After completion of reaction, the reaction mixture was poured into ice-cold water and stirred for 1 h to isolate free product. The separated product was filtered and washed with cold water. The isolated product was dried for next 12 h at room temperature. For the purification purpose, column chromatography was performed by using Silica gel (60-120 mesh) as a stationary phase and ethyl acetate:hexane (10: 90) as a mobile phase (**4a-i**) **(Scheme-I)**.

Preparation of single crystals of *N*-((1-(*tert*-butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)methyl)-3-nitroaniline (4a): Synthesized compound **4a** (0.250 g) purified by column chromatography was taken in chloroform:methanol (1:1) and heated up to 50-60 °C for 10-15 min till it dissolved completely. Activated charcoal was added and further heated up to 50-60 °C for 5 min. The hot solution was filtered through Wattmann 41 filter paper followed by using hyflow (celite) bed under high *vacuo*. The solution was allowed to cool gradually and kept in a stoppered conical flask. The crystals have grown due to thin layer evaporation.

Procedure for the preparation of single crystals of *N*-((1-(*tert*-butyl)-1*H*-tetrazol-5-yl)(3-phenoxy phenyl)methyl)-4-methylaniline (4b): Synthesized compound **4b** (0.250 g) purified by column chromatography was taken in chloroform: methanol:DMF (5: 4: 1) and heated up to 50-60 °C for 10-15 min till it dissolved completely. Activated charcoal was added and further heated up to 50-60 °C for 5 min. The hot solution was filtered through Wattmann 41 filter paper followed by using hyflow (celite) bed under high *vacuo*. The solution was allowed to cool gradually and kept in a stoppered conical flask. The crystals have grown due to thin layer evaporation.



R= 4a= 3-NO_2, 4b= 4-CH_3, 4c= 4-C_2H_5, 4d= 4-OH, 4e= 4-F, 4f= 4-OCH_3, 4g= 4-OC_2H_5, 4h= 3,4-Di OH, 4i= 4-Cl

Reaction condition: (a) Methanol: Dimethylformamide (8:2), $TMSN_3$, RT, 12 h

Scheme-I: Synthetic route for the synthesis of 1,5-disubstituted tetrazole using Ugi multicomponent reactions (**4a-i**)

Analytical data and physical data

***N*-((1-(*tert*-Butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)-methyl)-3-nitroaniline (4a):** Yield: 82 %; m.p.: 256 °C; IR (KBr, ν_{max}, cm^{-1}): 3303.13 (N-H *str.*), 3087.03 (aromatic ring C-H *str.*), 2987.64 (aliphatic C-H asym.), 2935.25 (aliphatic C-H sym.), 1939.95 (C-H bonding overtone), 1586.46 (C=N *str.*), 1530.17, 1486.99, 1457.37 (aromatic ring skeleton), 1346.90 (C-N *str.*), 792.93; 897.61 (*m*-substituted ring); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.711 (s, 9H), 6.429-6.450 (d, 1H), 6.937-6.979 (q, 3H), 7.102-7.164 (m, 2H), 7.231-7.275 (q, 2H), 7.333-7.454 (m, 5H), 7.486-7.517 (q, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 29.21, 51.44, 62.18, 106.89, 111.60, 118.27, 118.31, 118.67, 119.52, 123.33, 123.38, 129.95, 130.09, 130.15, 140.19, 147.54, 148.64, 154.46, 156.41, 156.47.

***N*-((1-(*tert*-Butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)-methyl)-4-methylaniline (4b):** Yield: 75 %; m.p.: 210 °C; (KBr, ν_{max}, cm^{-1}): 3348.37 (N-H *str.*), 3024.84 (aromatic ring C-H *str.*), 2956.76 (aliphatic C-H asym.), 2925.32 (aliphatic C-H sym.), 1584.21 (C=N *str.*), 1541.38, 1487.24, 1456.34 (aromatic ring skeleton), 1373.22 (C-N *str.*), 841.20 (*p*-substituted ring), 699.18 (*m*-substituted ring); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.708 (s, 9H), 2.086-2.124 (d, 3H), 6.190-6.214 (d, 1H), 6.610-6.631 (d, 2H), 6.865-6.956 (m, 6H), 7.097-7.134 (t, 1H), 7.252-7.273 (t, 1H), 7.333-7.373 (t, 4H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 20.03, 62.04, 113.36, 117.80, 118.17, 118.77, 123.26, 123.36, 125.76, 129.34, 129.76, 129.94, 141.39, 144.00, 155.00, 156.17, 156.56.

***N*-((1-(*tert*-Butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)-methyl)-4-ethylaniline (4c):** Yield: 72 %; m.p.: 240 °C; (KBr, ν_{max}, cm^{-1}): 3335.15 (N-H *str.*), 3023.34 (aromatic ring C-H stretch.), 2950.70 (aliphatic C-H asym.), 2919.30 (aliphatic C-H sym.), 1540.98, 1485.15, 1455.15 (aromatic ring skeleton), 1583.85 (C=N *str.*), 1372.30 (C-N *str.*), 840.93 (*p*-substituted ring), 698.85 (*m*-substituted ring); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.703 (s, 9H), 2.48-2.51 (q, 2H), 1.29-1.31 (t, 3H), 6.180-6.205 (d, 1H), 6.590-6.618 (d, 2H), 6.840-6.950 (m, 6H), 7.065-7.115 (t, 1H), 7.230-7.268 (t, 1H), 7.330-7.364 (t, 4H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 13.20, 28.15, 28.20, 52.98, 57.95, 115.15, 117.30, 118.03, 119.16, 121.98, 123.25, 129.08, 130.03, 132.45, 133.85, 137.94, 142.95, 143.48, 156.40, 156.63.

4-(((1-(*tert*-Butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)-methyl)amino)phenol (4d): Yield: 65 %; m.p.: 198 °C; (KBr, ν_{max}, cm^{-1}): 3650.65 (aromatic ring O-H *str.*), 3302.52 (N-H *str.*), 2986.50 (aliphatic C-H asym.), 2934.40 (aliphatic C-H sym.), 1585.66 (C=N *str.*), 1530.05, 1486.75, 1456.98, (aromatic ring skeleton), 1345.85 (C-N *str.*), 792.05; 897.25 (*m*-substituted ring); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.703 (s, 9H), 6.415-6.440 (d, 1H), 6.920-6.965 (q, 3H), 7.101-7.155 (m, 2H), 7.214-7.199 (q, 2H), 7.309-7.535 (m, 5H), 7.475-7.508 (q, 2H), 8.05 (s,1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 29.15, 50.46, 61.98, 106.08, 111.50, 117.98, 118.30, 118.45, 119.45, 123.15, 123.36, 129.40, 130.03, 130.09, 140.14, 147.45, 148.08, 154.40, 155.98, 156.50.

***N*-((1-(*tert*-Butyl)-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)-methyl)-4-fluoroaniline (4e):** Yield: 58 %; m.p.: 223 °C; (KBr, ν_{max}, cm^{-1}): 3302.33 (N-H *str.*), 2986.45 (aliphatic C-H asym.), 1258.87 (aromatic ring C-F *str.*), 2934.66 (aliphatic C-H sym.), 1585.50 (C=N *str.*), 1530.05, 1486.85, 1456.98 (aromatic ring skeleton), 1346.84 (C-N *str.*), 792.54; 897.36 (*m*-substituted ring); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.698 (s, 9H), 6.415-6.429 (d, 1H), 6.915-6.965 (q, 3H), 7.101-7.145 (m, 2H), 7.218-7.260 (q, 2H), 7.325-7.449 (m, 5H), 7.495-7.510 (q, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 29.05, 50.99, 61.85, 105.99, 111.45, 118.15, 118.29, 118.55, 119.45, 122.99, 123.05, 129.40, 129.85, 129.98, 140.10, 147.45, 148.38, 154.40, 155.98, 155.40.

Anticancer screening protocol: NCI-60 cell-lines were used for evaluation of *in vitro* anticancer activity of synthesized tetrazoles at National Institute of Health (NIH) using nine different cancer cell panels including leukemia, non-small cell lung cancer, colon cancer, central nervous system (CNS) cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. The screening was a two-stage process; beginning with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. Data analysis is available by the "COMPARE program" and it was reported as the single dose screen. The data is reported as a mean graph of the percent growth of treated cells and were similar in appearance to mean graphs from the 5-dose assay (if data allowed from single dose study). Drug activity was determined by the DTP (Developmental Therapeutics Program) human cancer cell line screen and reported the values in terms of GI_{50} (Growth Inhibition of 50 % of the cells) values. No control drug was used to identify a good anticancer agent by NCI as per protocol used in NIH [18].

The cytotoxicity of the tested compounds **4a-i** were determined on 16 different human cancer cell lines on cell viability measured at 24 h after exposure. As per the protocol by NCI, computational studies were carried out to identify the probable active scaffolds out of screened molecules. Only when promising results are obtained are the *in vitro* studies performed [19].

RESULTS AND DISCUSSION

The target molecules for this study are shown in **Scheme-I**. The scope of this reaction was studied by using various amines, as it is seen in reaction **Scheme-I**, Ugi-azide based coupling reaction was performed by using phenoxy aldehyde (**1**), *tert*-butylisocynide, trimethylsilyl azide ($TMSN_3$) and various forms of aromatic amines to afford *N*-((1-*tert*-butyl-1*H*-tetrazol-5-yl)(3-phenoxyphenyl)methyl)-substituted benzenamine (**4a-i**). The core structure 1,5-tetrazoles based various adducts were produced in good yield. The obtained results from this reaction were preeminent compared to recent reported multi-component reactions in terms of the reaction yield, catalyst, solvent and reaction time. Moreover, the present methodology, compared to other reported procedures, has several advantages, for example, easy work-up and eco-friendly feature.

Spectroscopic confirmations: The structural assignment for **4a-i** was established on the basis of consistent single crystal XRD study of representative molecules **(4a** and **4b)** and various spectral data. The IR spectrum showed no aldehydic absorption at ~1720 cm^{-1}, but absorption bands at ~3310 and ~1590 cm^{-1} which were assigned to -NH and C=N functions. Moreover, the ^{1}H NMR spectrum revealed the absence of aldehydic

protons and the presence of signals for asymmetric protons at δ 6.2 ppm in doublet splitting pattern and aromatic protons in their expected positions confirms the formation of final adducts. The ^{13}C NMR of synthesized compounds were exactly fit in to the theoretical value of specified group *i.e.* the chiral carbon shown confirmative peak at ~55 δ ppm, tetrazole ring carbon (C-5) showed peak at ~143 δ ppm, which confirms the predicted route of synthesis. The mass spectrum showed fragment ions irrespective of molecular ion peak due to the bulky molecule. A sharp fragment peak was observed by cleavage from C-NH bond at *m/z* = 307 and also a peak by cleavage *tert*-butyl group from tetrazole motifs at *m/z* = 387 for the molecule **4a** and the same pattern observed in rest of the synthesized molecules.

X-ray diffraction study: A single crystal was carried out of two representative molecules to confirms the formation of desired adduct. The compounds **4a** and **4b** were characterized by single crystal XRD for structure elucidation which gave exact result as we were designing. Data Collection of yellow blocks crystal of compound **4a** ($C_{24}H_{24}N_6O_3$) having approximate dimensions of 0.390 mm × 0.370 mm × 0.160 mm and a colourless prism crystal of compound **4b** ($C_{25}H_{27}N_5O$) having approximate dimensions of 0.740 mm × 0.550 mm × 0.100 mm were mounted on a glass fiber. All measurements were made on a Rigaku SCX mini diffractometer using graphite monochromated Mo-Kα radiation (Fig. 1).

Crystal data and experimental parameters used for the intensity data collection are summarized in Table-1.

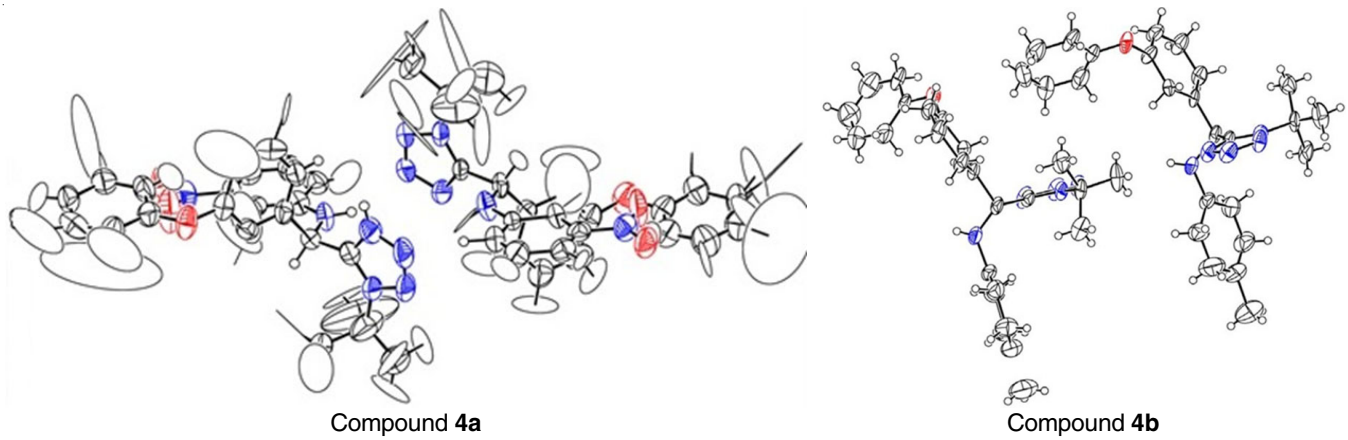


Fig. 1. Oak ridge thermal ellipsoid plot (ORTEP) of the compounds **4a** and **4b** molecule at 50 % probability

TABLE-1
CRYSTAL DATA OF MOLECULES **4a** AND **4b**

Compound ID	**4a**	**4b**
CCDC deposition number	1813428	1811962
Empirical Formula	$C_{24}H_{24}N_6O_3$	$C_{25}H_{27}N_5O$
Formula Weight	444.49	413.52
Crystal Colour, Habit	Yellow, block	Colourless, Prism
Crystal Dimensions	0.390 × 0.370 × 0.160 mm	0.740 × 0.550 × 0.100 mm
Crystal System	Monoclinic	Triclinic
Lattice Type	Primitive	Primitive
Lattice Parameters	a = 8.070(1) Å; b = 10.815(2) Å c = 26.880(4) Å; V = 2321.6(6) Å^3	a = 7.2(7) Å; b = 17(2) Å c = 26(3) Å; V = 3006(455) Å^3
Space Group	P21 (#4)	P-1 (#2)
Z value	4	6
Dcalc	1.272 g/cm^3	1.371 g/cm^3
F000	936.00	1320.00
? (MoKα)	0.871 cm^{-1}	0.868 cm^{-1}
Diffractometer	SCX mini	SCX mini
Radiation	MoKα (α = 0.71075 Å) (graphite monochromated	MoKα (α = 0.71075 Å) graphite monochromated
Temperature	20.0 °C	20.0 °C
Detector aperture	75 mm (diameter)	75 mm (diameter)
θ oscillation range	-120.0 - 60.0°	-120.0 - 60.0°
Exposure rate	10.0 s/°	10.0 s/°
2θ max	55.0°	51.7°
No. of reflections measured	Total: 23187; Unique: 10492 (Rint = 0.0640)	Total: 21658; Unique: 10264
Corrections	Lorentz-polarization absorption (trans. factors: 0.516 - 0.986)	Lorentz-polarization absorption (trans. factors: 0.223 - 0.991)
Reflection/Parameter Ratio	10.23	18.36
Residuals: R1 (I>2.00s(I))	4.0154	0.1793
Residuals: R (All reflections)	44.8675	0.2637
Residuals: wR2 (All reflections)	0.8432	0.4765
Goodness of Fit Indicator	12.240	1.125

Anticancer screening: The single dose response studies of selected molecules were shown in Table-2. The cytotoxicity of the tested compounds (**4a-i**) were determined on 16 different human cancer cell lines on cell viability measured at 24 h after exposure. As per the protocol by NCI, computational studies were carried out to identify the probable active scaffolds out of screened molecules. Only when promising results are obtained are the *in vitro* studies performed.

TABLE-2
SINGLE DOSE RESPONSE STUDY
(ANTICANCER ACTIVITY) OF COMPOUNDS **4a-i**

Sample code	GI_{50} value (μM/mL)	Cell lines	Cancer panels
CP-101	59.83	T-47D	Breast
	66.97	MOLT-4	Leukemia
	67.77	UACC-62	Melanoma
	69.76	NCI-H522	Non-small cell lung
	70.10	K-562	Leukemia
	77.83	UO-31	Renal
CP-102	50.64	T-47D	Breast
	54.27	UO-31	Renal
	65.78	NCI-H522	Non-small cell lung
	66.52	HCT-116	Colon
	67.83	MOLT-4	Leukemia
	68.55	K-562	Leukemia
	69.03	RPMI-8226	Leukemia
	72.31	UACC-62	Melanoma
	74.28	UACC-257	Melanoma
	75.23	PC-3	Prostate
	77.91	SR	Leukemia

Data from Table-2 revealed that the $–NO_2$ and $–CH_3$ group containing ugi adducts showed promising response against in T-47D and UO-31 cell lines (< 60 %). Compounds **4a** and **4b** were showed maximum potency in breast cancer panels against T-47D cell-lines with GI_{50} values 59.83 and 50.64, respectively. Furthermore, compound **4b** was showed promising response in renal cancer panel (GI_{50} = 54.27).

Compounds **4a** and **4b** were displaying comparable *in vitro* cytotoxic activity with varying GI_{50} value in leukemia, melanoma, non-small cell lung and colon cancer. However, the remaining 1,5-tetrazole derivatives were not selected for the cancer study, which meant that modification should be import with adding potent functionalities. In above discussion, it was generalized that out of the 9 synthesized molecules only a few compounds were revealed to possess antitumor activities but we could correlate the tendency of ugi-azide compounds and research them further following the results obtained.

Conclusion

In conclusion, we evaluated 2 synthesized compounds out of 15 analogous against NCI-60 cell-lines. It was observed that compounds (**4a** and **4b**) were given comparative GI_{50} values against T47-D (GI_{50} = 50.64 μM/mL and 59.83 μM/mL in compounds **4b** and **4a**, respectively) cell lines in breast cancer panel. The mean value in compound **4b** was 90.28 and the same for compound **4a** was 92.54 which was much higher than the experimental value of standard sample *i.e.* 5-flouro uracil (mean value, 17.98). On the synthetic side, the approach developed herein allows the synthesis of a wide range of 1,5-disubstituted tetrazoles in only single step. The procedure offers several advantages, such as high atom-economy, a simple synthetic procedure with an easy work-up and ready access to highly functionalized compounds in a low number of steps. In addition, the obtained compounds may allow further modification reactions to generate lead scaffolds in the field of medicinal chemistry.

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