Synthesis, Cytotoxic Activity, and Thermal Studies of Novel *N*-[(1,3-Diphenylpyrazol-4-yl)methyl] α-Amino Acids

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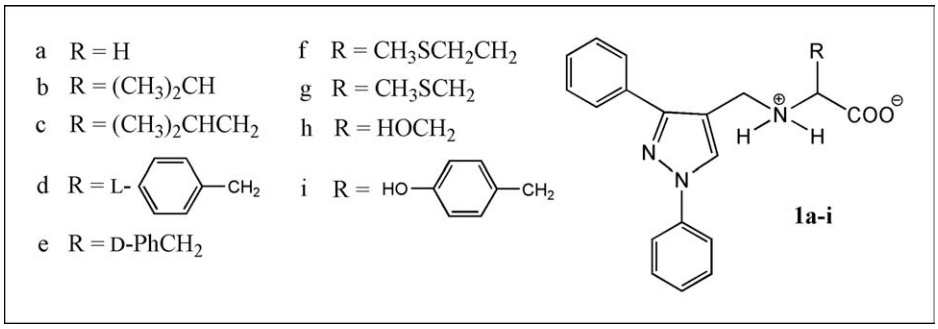

New *N*-[(1,3-diphenylpyrazol-4-yl)methyl]α-amino acids (**1a–i**) have been synthesized and tested *in vitro* for their antiproliferative activity against human myelogenous leukemia K562, colon adenocarcinoma HT-29, cervix carcinoma HeLa, and normal fetal lung fibroblasts, MRC-5. Compounds derived from both phenylalanine enantiomer precursors appeared to be the most active against myelogenous leukemia K562 cell lines with a high cytotoxic potential.

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INTRODUCTION

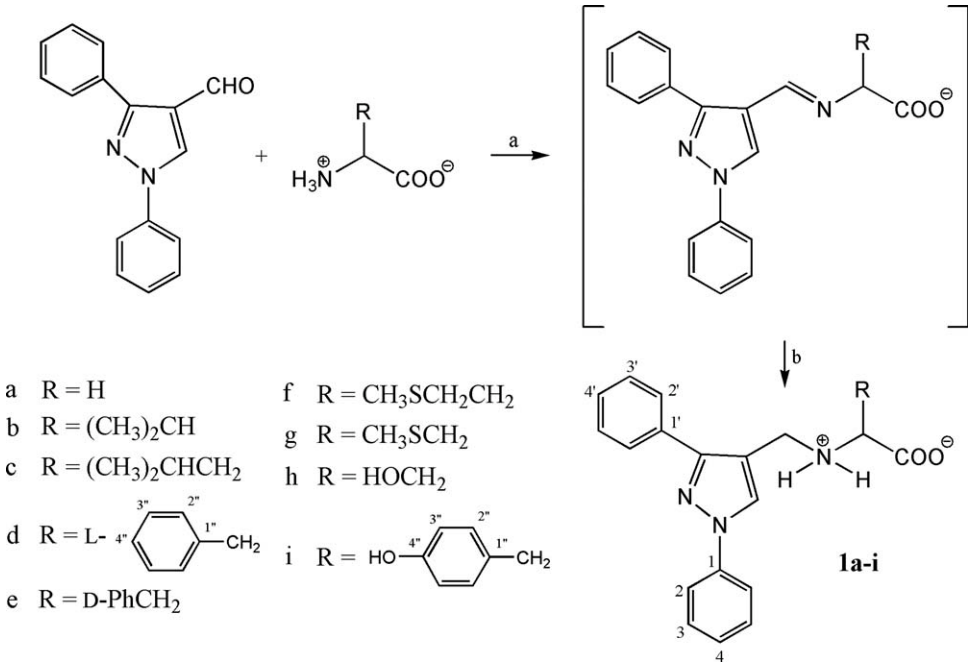
Arylpyrazole derivatives play an important role in biologically active compounds and therefore represent an interesting template for medicinal chemistry. These compounds displayed diverse biological properties such as antiparasitic [1], antifungal [2], antibacterial [3], and antidiabetic [4]. Moreover, arylpyrazole derivatives have shown several biological activities as seen in cyclooxygenase-2 [5], p38 MAP kinase [6], and nonnucleoside HIV-1 reverse transcriptase inhibitors [7]. In the research for antitumor agents, arylpyrazole derivatives exhibited promising antiproliferative properties against several kinds of human tumor cell lines [8–10].

The cytotoxic drugs, such as doxorubicin, 5-fluorouracil, and camptothecins, can damage DNA or affect cellular metabolic pathways causing indirect block of the cell cycle. Unfortunately, these agents produce an irreversible damage to both normal and tumor cells, resulting in a significant toxicity and side effects [11–13]. In this respect, it is desirable to synthesize new highly specific antitumor agents with comparable efficacy and reduced toxicity than the currently available drugs. In view of these observations and as a continuation of our interest toward synthesis and biological activity of arylpyrazole derivatives [14,15], some new *N*-[(1,3-diphenylpyrazol-4-yl)methyl] α-amino acids were prepared with the aim to have promising antiproliferative properties against several kinds of human tumor cell lines.

RESULTS AND DISCUSSION

In this work, condensation of the optically active L- and D-amino acids (except **1a**) with 1,3-diphenylpyrazole-4-carboxaldehyde in the presence of NaOH led to the formation of Schiff base intermediates. As α-amino acids are sparingly soluble in alcoholic solvents, the Schiff base formation takes long time, and the reaction gives lower yields even under reflux conditions. Thus, the condensation reaction was accelerated using solventless method [16] combining aldehyde, α-amino acids, and NaOH in a porcelain mortar with pestle and aggregating the dry solid mixture until a white powder was formed. The condensation was completed by an additional heating at reflux for 2 h in dry methanol (Scheme 1).

Scheme 1. Reagents and conditions: (a) NaOH, continuous aggregating at rt, then MeOH, reflux, 2 h; (b) $NaBH_4$, 0–5°C, then rt, 12 h, AcOH.



The stability of the formed Schiff base products depends on several factors such as amino acid side chain polarity [17], the metal, pH, solvent, and temperature [18]. The problem with Schiff base instability can be overcome by their reduction to give a more flexible amine and not constrained compounds. In the light of these facts, we have performed out the reduction with an excess of $NaBH_4$ without isolation of Schiff base to afford the novel *N*-[(1,3-diphenylpyrazol-4-yl)methyl] α-amino acids.

The structures of the new compounds were confirmed using different spectral data (IR, ^1H, and ^{13}C NMR) and elemental analysis. The IR spectra revealed the presence of NH_2^+ stretching frequencies between 2650 and 2300 cm^{-1} in the form of broad band with multiple peaks on the low frequency wing, which continue until about 2200 cm^{-1}, confirming their zwitterionic nature. The ν_{as} (COO) was related to the strong absorption band appearing in the spectra between 1619 and 1601 cm^{-1}, whereas the symmetric carboxylate stretches ν_s (COO) correspond to the medium–strong peaks about 1410 cm^{-1} [19]. The other characteristic very strong absorption bands in the IR spectra were those attributed to the pyrazole ring: ν (C=C) and ν (C=N) between 1601 and 1547 cm^{-1} as well as δ (C=C) about 1505 cm^{-1} [20].

The ^1H NMR spectra of all compounds exhibited a characteristic AB system except **1a** and ABX system for **1d**, **1e**, **1g**, **1h**, and **1i**. The ^1H NMR spectra revealed the presence of characteristic singlet peak attributed to pyrazole ring proton appeared between 7.97 and 8.67 depending on the applied solvent system. The complete assignment of all reported signals (^1H and ^{13}C) in the experimental part was carried out by means of 1D and 2D homo- and heteronuclear correlated NMR spectroscopy.

The newly synthesized compounds **1a–i** were evaluated *in vitro* for their antiproliferative activity against human myelogenous leukemia K562, colon adenocarcinoma HT-29, cervix carcinoma HeLa, and normal fetal lung fibroblasts, MRC-5 using sulforhodamine B (SRB) assay [21] and doxorubicin (Dox) as reference drug. The results are presented in Table 1.

The malignant cell line K562 was the most sensitive, where the pronounced cytotoxicity was achieved by seven of nine tested samples. The compounds with benzyl group **1d** and **1e** showed the most potent cytotoxic activity against human myelogenous leukemia K562 cell lines. The replacement of the benzyl group in **1d** by a hydrophobic isobutyl group leading to **1c** would retain potential hydrophobic bonding while creating spatial property differences between the benzyl and isobutyl group. The comparison of the cytotoxicity of **1d** or **1e** with **1c** indicates that **1d** and **1e** are more active in inhibition of these cells. When the side chain was replaced with an isopropyl group **1b**, a complete inactivity against K562 was observed. The moderate activity was achieved when an additional heteroatom was

Table 1

In vitro cytotoxicity of compounds **1a–i**.

Compounds	IC_{50} (μM)[a]			
	K562	HeLa	HT29	MRC-5
1a	8.89	>100	>100	>100
1b	>100	9.21	>100	>100
1c	4.17	>100	72.31	>100
1d	1.02	14.43	8.31	>100
1e	0.97	11.97	6.45	>100
1f	6.37	>100	>100	>100
1g	>100	7.64	>100	>100
1h	4.69	96.34	>100	>100
1i	3.25	>100	67.32	>100
Dox	0.36	1.17	0.51	0.32

[a] IC_{50} is the concentration of compound required to inhibit the cell growth by 50% compared to an untreated control.

incorporated into the side chain **1f**. These differences in cytotoxicity could be related to their conformation and size of the substitutes. The bulky isopropyl group **1b** and voluminous sulfur atom **1g** closely to aminocarboxylate skeleton induce the decrease of cytotoxic activity, probably preventing the formation of intermolecular interaction with cell receptors. Comparison of the cytotoxicity of compounds derived from different enantiomers of phenylalanine indicates that D-phenylalanine demonstrated similar activity with respect to L-phenylalanine analog. We could also suggest that **1d** and **1e** are the most cytotoxic compounds because of their planarity and hydrophobic properties. These results indicate that spatial effects and side chain length should be an important factor in the design of future molecules. It is interesting to note that the compounds **1h** and **1i** having polar hydroxyl groups proved to be less active than **1d** and **1e**, suggesting that electronic character could be diminished in comparison with hydrophobic requirements of amino acid part of molecule. Compounds **1g** and **1e** were also active against HeLa and HT29 cell lines, respectively, but their activity was significantly lower compared with doxorubicin. On the other hand, all compounds were devoid of any cytotoxicity against the normal fetal lung fibroblasts MRC-5. Generally, the hydrophobic character and steric effects in amino acid moiety appear to be the main factors for the growth suppressing potential against K562 cell lines. Further modification of compounds **1d** and **1e**, especially the transformation of carboxylate group and identification of its molecular target, is under investigation.

Thermal analysis. Thermal analysis of pharmaceutical compounds is a very reliable method for purity control, and it is a necessary part of the characterization of new compounds with potential bioactivity [22,23]. As amino acid–water–protein interface interactions are very important in biological systems [24]; the thermal properties of these new amino acid derivatives may be of special interest.

In the series of nine new compounds, two of them are pure N-substituted amino acids, whereas the others crystallize with different number of water molecules.

Thermal measurements reveal that the stability of N-substituted amino acids **1a** and **1b** is rather high in both atmospheres (in N_2 200 and 205°C, respectively). Most probably as a consequence of some sensitivity toward oxidation, in air the thermal stability of the compounds is somewhat less. The decomposition mechanism in inert and oxidative atmosphere also differs (see Fig. 1).

The dehydration pattern of the hydrates does not depend on the atmosphere. The dehydrated compounds **1c**, **1g**, **1h**, and **1i** are stable to above 200°C, with the previous remark: their stability in air is less with about 5°C. Compounds **1d**, **1e**, and **1f** decompose further after dehydration, without giving a stable anhydrous intermediate.

The decomposition of all samples was accompanied by melting, observed visually. The decomposition mechanism of the first decomposition step of dry compounds is proposed only on the basis of the mass loss. The fragmentation begins with that of the side chain, *e.g.*, the mass loss to the minimum in DTG curve of **1a** amounts 14.5% in N_2 that agrees well with the loss of CO_2 molecule (calcd. 14.32%). The valine **1b** derivative most probably decomposes by the loss of the methyl groups. However, it is pointless to give a decomposition mechanism in lack of coupled measurements. It is more important to have a closer look to the dehydration process of the hydrates from both the theoretical and practical point of view. The mass loss with the corresponding temperature of the dehydration is given in Table 2 together with the decomposition temperature of the anhydrous compound.

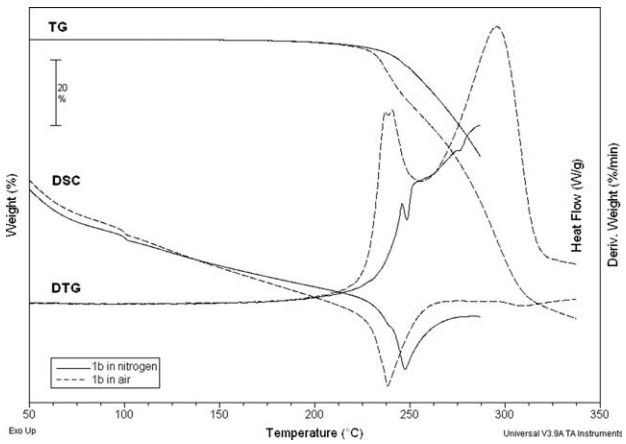


Figure 1. Thermal decomposition curves of **1b** in air and nitrogen.

Table 2

Mass loss of the dehydration, the dehydration temperature, and the onset temperature of the decomposition of the anhydrous compound in N_2.

Compound	Dehydration, Δm (%)		$t_{DEHYD.}$ (°C)	$t_{DECOMP.}$ (°C)	Comments
	Exp.	Calcd.			
1c	4.2	4.72	<105	215	Stable anhydrous compound
1d, 1e	7.3	8.31	<130	–	Stepwise dehydration, continuous decomposition
1f	10.4	10.17	<135	–	Stepwise dehydration, continuous decomposition
1g	1.3	4.67	<50	203	Crystal water
1h	2.2	5.34	<150	225	Structural water
1i	0.5	4.36	<30	235	Loosely bond crystal water

As the decomposition of the compounds **1d**, **1e**, and **1f** is continuous to obtain better separated decomposition steps, quasi-isothermal [25] (SWI) measurement was carried out. Figure 2 illustrates SWI curve of the compound **1f**. The five water molecules evaporate to 150°C (exp. 15.8%, calcd. 16.95%) in five clearly distinguished steps, not one by one, but through a complex process governed by macroscopic properties of the sample (*e.g.*, the rate of the diffusion) as well as by their different bonding energy. The dehydration of this compound is most probable followed by the evaporation of CH_3COOH molecule. The difference in experimentally determined mass loss and the theoretical one is most probable because of the evaporation of the crystal water at room temperature. The dehydration of **1d** and **1e** derivatives shows similar complexity of the dehydration. Derivative **1h**, according to elemental analysis data, contains both crystal and structural water, belonging to more than one molecule. By TA only the structural water is detected, because in air the compound lost its crystal water completely. Compound **1i** has some crystal water left, while most of it has evaporated during the storage time.

Taking into account the importance of the interactions in the relation of amino acid–water–protein, the cytotoxicity of the compounds might be related to H-bond-forming capability in the molecules. This, indirectly, may be investigated using TA data of hydrates. However, the correlation is not straightforward. TA data may refer only to the preferences of an amino acid to bind water molecules and the strength of the bond between them [26].

CONCLUSIONS

In summary, a new class of *N*-[(1,3-diphenylpyrazol-4-yl)methyl] α-amino acids were synthesized, and their antiproliferative activity against human myelogenous leukemia K562, colon adenocarcinoma HT-29, cervix carcinoma HeLa, and normal fetal lung fibroblasts, MRC-5 was evaluated. The nature, spatial effects and the distance of bulky substitutes from aminocarboxylate part of molecule appeared to play an important role in antiproliferative activity against different malignant cell lines. The dehydration temperature is related to the water bond energy and might be connected with the active sites of some bioactive molecules.

EXPERIMENTAL

All chemicals were obtained from commercial sources (Aldrich) and used as supplied. 1,3-Diphenylpyrazole-4-carboxaldehyde was synthesized by Vilsmeier-Haack reaction [27].

Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001, and are uncorrected. Optical rotations were measured on a Rudolph Research Analytical automatic polarimeter Autopol IV. Elemental (C, H, N, and S) analysis of the samples was carried out by standard micromethods in the Center for Instrumental Analysis, Faculty of Chemistry, Belgrade. IR spectra were recorded on a Perkin Elmer Spectrum One FTIR spectrometer with a KBr disc. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 (200 MHz) and a Bruker Avance III spectrometer operating at 500 MHz. As a consequence of the poor solubility in DMSO-d_6, the NMR spectra were recorded in pyridine-d_5/D_2O and NaOD/D_2O solvent system for **1h** and **1i**. Thermal measurements were conducted using SDT Q600 TA Instruments'

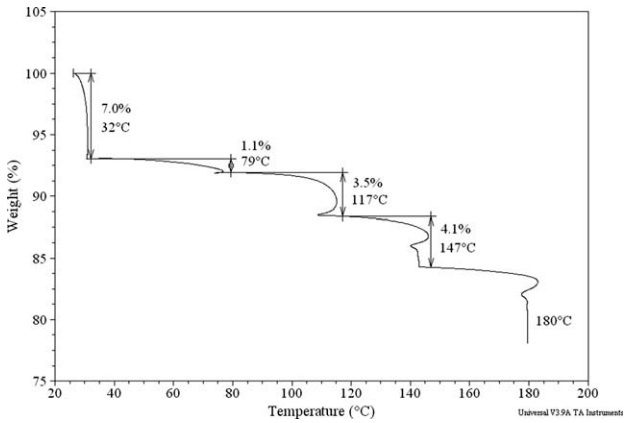


Figure 2. SWI decomposition curve of **1g**, illustrating the complexness of its dehydration.

thermal analyzer with about 2 mg sample masses and a heating rate of 20°C/min in air and nitrogen atmospheres. The sample holder and the reference cells were made of alumina.

General procedure for the preparation of 1a–i. 1,3-Diphenylpyrazole-4-carboxaldehyde (0.62 g, 2.5 mmol), α-amino acid (2.75 mmol), and NaOH (0.11 g, 2.75 mmol, for **1i** 0.22g, 5.50 mmol) were milled using a porcelain mortar and pestle to obtain a homogenous white powder until the release of water was observed. This mixture was transferred into 50 cm^3 of dry methanol and heated to reflux for 2 h. After cooling in an ice bath, sodium borohydride (0.11 g, 3 mmol) was added in several portions with stirring. The solution was stirred for additional 2 h at room temperature, then diluted with 50 cm^3 of deionized water, and left for 12 h, after which time the white precipitate had formed by addition of glacial acetic acid. The crude product was purified by dissolving in 1*M* NaOH and precipitation with glacial acetic acid. Recrystallized compound was collected by filtration, washed with plenty of water, and dried over anhydrous $CaCl_2$.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]glycine (1a). White powder; yield: 0.56 g (73%); mp 190–191°C (Dec.); IR (KBr, cm^{-1}): 3063 ν (C—H)$_{Ar}$, 2960 and 2820 ν (C—H)$_{Al}$, 2650–2400 ν (NH_2^+), 1626 ν_{as} (COO^-), 1601 ν (C=C)$_{Ar}$, 1547 ν (C=N)$_{Ar}$, 1502 δ (C=C)$_{Ar}$, 1453 δ (C=N)$_{Ar}$, 1412 ν_s (COO^-), 1066 δ (C—H)$_{ip}$, 758 δ (C—H)$_{oop}$; ^{1}H NMR (200 MHz, pyridine-d_5/D_2O, 9/1 v/v): 4.19 (s, 2H, CH_2—COO), 4.74 (s, 2H, Pz—CH_2), 7.40–7.71 (m, 6H, 1H at C-4, 1H at C-4′, 2H at C-3/5, and 2H at C-3′/5′), 7.97 (d, 2H at C-2/6, J = 8.00 Hz), 8.00 (d, 2H at C-2′/6′, J = 8.00 Hz), 8.94 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 41.87 (Pz—CH_2), 50.13 (*C*H_2—COO), 113.09 (C-4, Pz), 119.15 (C-2/6), 127.31 (C-4), 128.66 (C-2′/6′), 129.08 (C-4′), 129.46 (C-3′/5′), 130.02 (C-3/5), 130.40 (C-5, Pz), 132.42 (C-1′), 139.72 (C-3, Pz), 152.50 (C-1), 170.83 (COO); Anal. Calcd. for $C_{18}H_{17}N_3O_2$ (307.35 g/mol): C, 70.34; H, 5.58; N, 13.67; Found: C, 70.12; H, 5.62; N, 13.55.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-valine (1b). White powder; yield: 0.72 g (82%); mp 198°C (Dec.); $[\alpha]_D^{20}$ = +12.77 ($c = 1.096 \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3059 ν (C—H)$_{Ar}$, 2965 and 2876 ν (C—H)$_{Al}$, 2600–2400 ν (NH_2^+), 1614 ν_{as} (COO^-), 1600 ν (C=C)$_{Ar}$, 1550 ν (C=N)$_{Ar}$, 1504 δ (C=C)$_{Ar}$, 1451 δ (C=N)$_{Ar}$, 1411 ν_s (COO^-), 1069 δ (C—H)$_{ip}$, 756 δ (C—H)$_{oop}$; ^{1}H NMR (200 MHz, pyridine-d_5/D_2O, 9/1 v/v): 1.16 (d, 3H, J = 6.62 Hz, CH_3), 1.19 (d, 3H, J = 6.56 Hz, CH_3), 2.31 (m, 1H, $(CH_3)_2$C*H*), 3.55 (d, 1H, J = 5.46 Hz, CH—COO), δ_A = 4.31 and δ_B = 4.01 (AB system, 2H, J_{AB} = 13.50 Hz, Pz—CH_2), 7.33–7.62 (m, 6H, 1H at C-4, 1H at C-4′, 2H at C-3/5, and 2H at C-3′/5′), 8.02 (d, 2H at C-2/6, J = 8.00 Hz,), 8.36 (d, 2H at C-2′/6′, J = 8.00 Hz), 8.55 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 18.81 (CH_3), 19.95 (CH_3), 31.78 ($(CH_3)_2$C*H*), 43.25 (Pz—CH_2), 67.49 (*C*H—COO), 118.80 (C-2/6), 120.14 (C-4, Pz), 126.46 (C-4), 128.33 (C-4′), 128.56 (C-2′/6′), 128.98 (C-3′/5′), 129.14 (C-5, Pz), 129.85 (C-3/5), 134.13 (C-1′), 140.46 (C-3, Pz), 151.93 (C-1), 177.01 (COO); Anal. Calcd. for $C_{21}H_{23}N_3O_2$ (349.43 g/mol): C, 72.18; H, 6.63; N, 12.03; Found: C, 72.02; H, 6.68; N, 11.86.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-leucine monohydrate (1c). White powder; yield: 0.82 g (86%); mp 184°C (Dec.); $[\alpha]_D^{20}$ = −6.09 ($c = 1.313 \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3065 ν (C—H)$_{Ar}$, 2956 and 2869 ν (C—H)$_{Al}$, 2550–2300 ν (NH_2^+), 1612 ν_{as} (COO^-), 1600 ν (C=C)$_{Ar}$, 1560 ν (C=N)$_{Ar}$, 1503 δ (C=C)$_{Ar}$, 1454 δ (C=N)$_{Ar}$, 1412 ν_s (COO^-), 1076 δ (C—H)$_{ip}$, 755 δ (C—H)$_{oop}$; ^{1}H NMR (200 MHz, pyridine-d_5/D_2O, 9/1 v/v): 0.92 (d, 3H, J = 6.72 Hz, CH_3), 0.96 (d, 3H, J = 6.74 Hz, CH_3), 1.89 (m, 2H, CH—CH_2—CH), 2.16 (m, 1H, $(CH_3)_2$C*H*), 3.87 and 3.90 (2d, 1H, J = 6.36 Hz and J = 6.60 Hz, NH—C*H*—COO), δ_A = 4.42 and δ_B = 4.15 (AB system, 2H, J_{AB} = 13.50 Hz, Pz—CH_2), 7.32–7.61 (m, 6H, 1H at C-4, 1H at C-4′, 2H at C-3/5, and 2H at C-3′/5′), 8.03 (d, 2H at C-2/6, J = 8.00 Hz), 8.36 (d, 2H at C-2′/6′, J = 8.00 Hz), 8.67 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 21.17 (CH_3), 21.94 (CH_3), 24.14 ((CH_3)$_2$*C*H), 41.38 (CH—*C*H_2—CH), 41.62 (Pz—*C*H_2), 59.50 (*C*H—COO), 117.67 (C-2/6), 118.20 (C-4, Pz), 125.36 (C-4), 127.23 (C-4′), 127.43 (C-2′/6′), 127.67 (C-3′/5′), 128.22 (C-5, Pz), 128.71 (C-3/5), 132.66 (C-1′), 139.28 (C-3, Pz), 150.78 (C-1), 176.47 (COO); Anal. Calcd. for $C_{22}H_{27}N_3O_3$ (381.48 g/mol): C, 69.27; H, 7.13; N, 11.02; Found: C, 68.99; H, 7.21; N, 10.89.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-phenylalanine dihydrate (1d). White powder; yield: 0.95 g (88%); mp 185–186°C (Dec.); $[\alpha]_D^{20}$ = −1.82 ($c = 1.100 \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3062 ν (C—H)$_{Ar}$, 2972 and 2853 ν (C—H)$_{Al}$, 2620–2380 ν (NH_2^+), 1619 ν_{as} (COO^-), 1599 ν (C=C)$_{Ar}$, 1553 ν (C=N)$_{Ar}$, 1503 δ (C=C)$_{Ar}$, 1453 δ (C=N)$_{Ar}$, 1412 ν_s (COO^-), 1072 δ (C—H)$_{ip}$, 754 δ (C—H)$_{oop}$; ^{1}H NMR (200 MHz, pyridine-d_5/D_2O, 9/1 v/v): δ_A = 3.42, δ_B = 3.21, and δ_X = 4.04 (ABX system, 3H, J_{AB} = 13.59 Hz, J_{AX} = 5.15 Hz, J_{BX} = 8.26 Hz, C*H*—CH_2), δ_A = 4.29 and δ_B = 3.99 (AB system, 2H, J_{AB} = 13.50 Hz, Pz—CH_2), 7.28–7.62 (m, 11H, Ar—H), 7.93 (d, 2H at C-2/6, J = 8.00 Hz), 8.20 (d, 2H at C-2′/6′, J = 8.00 Hz), 8.29 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 40.10 (Ph—CH_2), 42.88 (Pz—CH_2), 63.30 (*C*H—COO), 118.77 (C-2/6), 120.66 (C-4, Pz), 126.36 (C-4″), 126.78 (C-4), 128.19 (C-4′), 128.50 (C-2′/6′), 128.69 (C-3″/5″), 128.75 (C-5, Pz), 128.96 (C-3′/5′), 129.78 (C-3/5), 130.05 (C-2″/6″), 134.18 (C-1′), 139.35 (C-1″), 140.48 (C-3, Pz), 151.76 (C-1), 176.98 (COO); Anal. Calcd. for $C_{25}H_{27}N_3O_4$ (433.51 g/mol): C, 69.27; H, 6.28; N, 9.69; Found: C, 69.51; H, 6.31; N, 9.74.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-D-phenylalanine dihydrate (1e). Yield: 0.93 g (86%); $[\alpha]_D^{20}$ = +1.82 ($c = 1.100 \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v).

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-methionine acetate pentahydrate (1f). White powder; yield: 1.18 g (89%); mp 185–186°C (Dec.); $[\alpha]_D^{20}$ = −6.94 ($c = 1.296 \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3291 ν (O—H, broad), 3060 ν (C—H)$_{Ar}$, 2916 and 2853 ν (C—H)$_{Al}$, 2600–2440 ν (NH_2^+), 1681 ν (C=O), 1607 ν_{as} (COO^-), 1598 ν (C=C)$_{Ar}$, 1558 ν (C=N)$_{Ar}$, 1504 δ (C=C)$_{Ar}$, 1452 δ (C=N)$_{Ar}$, 1412 ν_s (COO^-), 1067 δ (C—H)$_{ip}$, 756 δ (C—H)$_{oop}$; ^{1}H NMR (200 MHz, pyridine-d_5/D_2O, 9/1 v/v): 2.04 (s, 3H, CH_3—S), 2.43 (s, 3H, CH_3—COO), 2.53 (m, 2H, CH—CH_2), 3.00 (t, 2H, J = 6.09 Hz, CH_2—S); 4.13 (t, 1H, J = 5.60 Hz, C*H*—CH_2), δ_A = 4.58 and δ_B = 4.35 (AB system, 2H, J_{AB} = 13.50 Hz, Pz—CH_2), 7.36–7.70 (m, 6H, 1H at C-4, 1H at C-4′, 2H at C-3/5, and 2H at C-3′/5′), 8.03 (d, 2H at C-2/6, J = 8.00 Hz), 8.21 (d, 2H at C-2′/6′, J = 7.52 Hz); 8.66 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 14.92 (CH_3—S), 23.38 (*C*H_3—COO), 30.91 (S—CH_2), 32.70 (S—CH_2—*C*H_2), 42.28 (Pz—CH_2), 62.92 (*C*H—COO), 117.35 (C-4, Pz), 118.95 (C-2/6), 126.74 (C-4), 128.59 (C-2′/6′), 128.64 (C-4′), 129.18

(C-3′/5′), 129.90 (C-3/5), 130.05 (C-5. Pz), 133.35 (C-1′), 140.11 (C-3, Pz), 152.10 (C-1), 177.02 (COO), 177.39 (CH_3—*C*OO); Anal. Calcd. for $C_{23}H_{37}N_3O_9S$ (531.63 g/mol): C, 51.96; H, 7.02; N, 7.90; S, 6.03; Found: C, 51.86; H, 6.94; N, 7.82; S, 5.93.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-S-methyl-L-cysteine monohydrate (1g). White powder; yield: 0.88 g (92%); mp 187°C (Dec.); $[\alpha]_D^{20} = -11.04$ ($c = 1.177 \cdot \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3051 ν (C—H)$_{Ar}$, 2923 and 2853 ν (C—H)$_{Al}$, 2601–2388 ν (NH_2^+), 1628 ν_{as} (COO^-), 1599 ν (C=C)$_{Ar}$, 1549 ν (C=N)$_{Ar}$, 1504 δ (C=C)$_{Ar}$, 1451 δ (C=N)$_{Ar}$, 1413 ν_s (COO^-), 1067 δ (C—H)$_{ip}$, 757 δ (C—H)$_{oop}$; ^{1}H NMR (500 MHz, pyridine-d_5/D_2O, 9/1 v/v): 2.20 (s, 3H, CH_3—S), $\delta_A = 3.25$, $\delta_B = 3.16$, and $\delta_X = 4.01$ (ABX system, 3H, $J_{AB} = 13.50$ Hz, $J_{AX} = 5.73$ Hz, $J_{BX} = 7.27$ Hz, C*H*—CH_2), $\delta_A = 4.34$ and $\delta_B = 4.14$ (AB system, 2H, $J_{AB} = 13.50$ Hz, Pz—CH_2), 7.29 (t, 1H, $J = 7.50$ Hz, C-4), 7.44 (t, 1H, $J = 7.50$ Hz, C-4′), 7.50 (t, 2H, $J = 7.50$ Hz, C-3/5), 7.57 (t, 2H, $J = 7.50$ Hz, C-3′/5′), 8.01 (d, 2H, $J = 8.00$ Hz, C-2/6), 8.35 (d, 2H, $J = 8.00$ Hz, C-2′/6′), 8.53 (s, 1H, Pz); ^{13}C NMR (pyridine-d_5/D_2O, 9/1 v/v): 16.35 (CH_3—S), 38.08 (S—CH_2), 43.02 (Pz—CH_2), 61.60 (CH), 118.93 (C-2/6), 120.69 (C-4, Pz), 126.52 (C-4), 128.41 (C-4′), 128.74 (C-2′/6′), 128.98 (C-5, Pz), 129.12 (C-3′/5′), 129.95 (C-3/5), 134.43 (C-1′), 140.75 (C-3, Pz), 151.96 (C-1), 175.98 (COO); Anal. Calcd. for $C_{20}H_{23}N_3O_3S$ (385.48 g/mol): C, 62.32; H, 6.01; N, 10.90; S, 8.32; Found: C, 62.49; H, 5.99; N, 10.92; S, 8.35.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-serine monohydrate (1h). White powder; yield: 0.71 g (80%); mp 190–191°C (Dec.); $[\alpha]_D^{20} = +3.96$ ($c = 1.263 \cdot \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3445 ν (O—H, broad), 3055 ν (C—H)$_{Ar}$, 2961 and 2853 ν (C—H)$_{Al}$, 2600–2390 ν (NH_2^+), 1628 ν_{as} (COO^-), 1600 ν (C=C)$_{Ar}$, 1547 ν (C=N)$_{Ar}$, 1504 δ (C=C)$_{Ar}$, 1452 δ (C=N)$_{Ar}$, 1417 ν_s (COO^-), 1074 δ (C—H)$_{ip}$, 752 δ (C—H)$_{oop}$; ^{1}H NMR (500 MHz, NaOD/D_2O): $\delta_A = 3.71$, $\delta_B = 3.65$, and $\delta_X = 3.17$ (ABX system, 3H, $J_{AB} = 11.50$ Hz, $J_{AX} = 4.66$ Hz, $J_{BX} = 6.09$ Hz, CH—CH_2), $\delta_A = 3.37$ and $\delta_B = 3.62$ (AB system, 2H, $J_{AB} = 13.50$ Hz, Pz—CH_2), 7.35 (t, 1H, $J = 7.50$ Hz, C-4), 7.44–7.52 (m, 5H, 2H at C-3/5, 1H at C-4′, and 2H at C-3′/5′), 7.58 (d, 2H at C-2/6, $J = 8.00$ Hz), 7.60 (d, 2H at C-2′/6′, $J = 8.00$ Hz), 8.05 (s, 1H, Pz); ^{13}C NMR (NaOD/D_2O): 43.88 (Pz—*C*H_2); 65.67 (CH_2—OH), 67.22 (CH), 121.65 (C-4, Pz), 122.09 (C-2/6), 129.75 (C-4), 130.69 (C-2′/6′), 131.32 (C-4′), 131.58 (C-3′/5′), 132.13 (C-5, Pz), 132.33 (C-3/5), 134.68 (C-1′), 141.78 (C-3, Pz), 154.57 (C-1), 181.84 (COO); Anal. Calcd. for $C_{19}H_{21}N_3O_4$ (355.39 g/mol): C, 64.21; H, 5.96; N, 11.82; S, 8.32; Found: C, 64.40; H, 5.94; N, 11.81.

N-[(1,3-Diphenylpyrazol-4-yl)methyl]-L-tyrosine monohydrate (1i). White powder; yield: 0.85 g (79%); mp 190–191°C (Dec.); $[\alpha]_D^{20} = -6.00$ ($c = 1.166 \cdot \times 10^{-3}$ g/cm^3, pyridine/H_2O, 9/1 v/v); IR (KBr, cm^{-1}): 3418 ν (O—H, broad), 3061 ν (C—H)$_{Ar}$, 2957 and 2812 ν (C—H)$_{Al}$, 2622–2400 ν (NH_2^+), 1611 ν_{as} (COO^-), 1597 ν (C=C)$_{Ar}$, 1559 ν (C=N)$_{Ar}$, 1504 δ (C=C)$_{Ar}$, 1451 δ (C=N)$_{Ar}$, 1410 ν_s (COO^-), 1072 δ (C—H)$_{ip}$, 757 δ (C—H)$_{oop}$; ^{1}H NMR (500 MHz, NaOD/D_2O): $\delta_A = 2.80$, $\delta_B = 2.63$, and $\delta_X = 3.28$ (ABX system, 3H, $J_{AB} = 13.75$ Hz, $J_{AX} = 5.67$ Hz, $J_{BX} = 7.83$ Hz, CH—CH_2), $\delta_A = 3.75$ and $\delta_B = 3.54$ (AB system, 2H, $J_{AB} = 13.50$ Hz, Pz—CH_2), 6.54 (d, 2H at 3″, $J = 8.00$ Hz), 6.91 (d, 2H at 2″, $J = 8.50$ Hz), 7.37 (t, 1H at C-4, $J = 7.50$ Hz), 7.49 (m, 7H, 2H at C-3/5, 1H at C-4′, 2H at C-3′/5′, and 2H at C-2/6), 7.61 (d, 2H at C-2′/6′, $J = 8.00$ Hz), 7.97 (s, 1H, Pz); ^{13}C NMR (500 MHz, NaOD/D_2O): 40.84 (Ph—CH_2), 43.62 (Pz—CH_2), 67.60 (CH), 121.38 (3″), 121.64 (C-4, Pz), 122.24 (C-2/6), 126.28 (1″), 129.81 (C-4), 130.72 (C-2′/6′), 131.35 (C-4′), 131.63 (C-3′/5′), 132.15 (C-5, Pz), 132.39 (C-3/5), 133.03 (2″), 134.60 (C-1′), 141.83 (C-3, Pz), 154.77 (C-1), 167.28 (4″), 184.13 (COO); Anal. Calcd. for $C_{25}H_{25}N_3O_4$ (431.48 g/mol): C, 69.59; H, 5.84; N, 9.74; Found: C, 69.69; H, 5.82; N, 9.74.

Biological evaluation. Three human tumor cell lines and one human nontumor cell line were used in this study: K562 (chronic myelogenous leukemia), HeLa (epitheloid carcinoma of cervix), HT-29 (colon adenocarcinoma), and MRC5 (lung fetal fibroblasts). The cells were grown in RPMI 1640 (K562 cells) or Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose (HeLa, HT-29, and MRC5 cells). Media were supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/mL of penicillin and 100 μg/mL of streptomycin (ICN Galenika). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37°C in the 100% humidity atmosphere and 5% of CO_2. Only viable cells were used in the assay. Viability was determined by dye exclusion assay with Trypan blue.

Cytotoxicity was evaluated by colorimetric SRB assay as described by Skehan et al. [21]. Briefly, single cell suspension was plated into 96-well microtiter plates (Costar, flat bottom): 1×10^4 of K562 and 5×10^3 of HeLa, HT29, and MRC5 cells, per 180 mL of medium. Plates were preincubated for 24 h at 37°C, 5% CO_2. Tested substances at concentration ranging from 10^{-8} to $10^{-4}M$ were added to all wells except to the control ones. After incubation period (48 h/37°C/5% CO_2), SRB assay was carried out as follows: 50 μL of 80% trichloroacetic acide (TCA) was added to all wells; an hour later, plates were washed with distillate water, and 75 μL of 0.4% SRB was added to all wells; half an hour later, plates were washed with citric acid (1%) and dried at room temperature. Finally, 200 μL of 10 mmol TRIS (pH = 10.5) basis was added to all wells. Absorbance was measured on the microplate reader (Multiscan MCC340, Labsystems) at 540/690 nm. The wells without cells, containing compete medium only, acted as blank.

Cytotoxicity was calculated according to the formula:

$$(1 - A_{TEST}/A_{CONTROL}) \times 100$$

and expressed as a percent of cytotoxicity (CI %).

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