New Carriers for Representative Peptides and Peptide Drugs

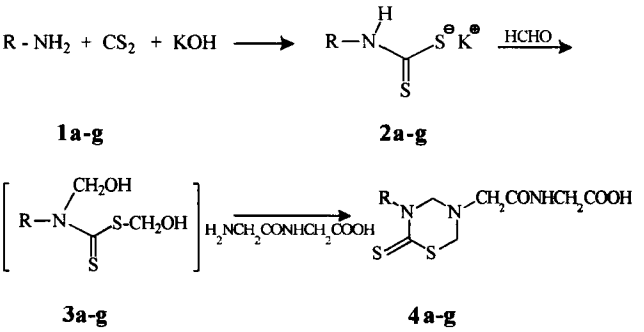
Tarek Aboul-Fadl and Abdel-Nasser El-Shorbagi*

Faculty of Pharmacy, University of Assiut, Assiut-71526, Egypt

Key Words: *Tetrahydro-2H-1,3,5-thiadiazine-2-thione; glycylglycine; prodrugs; lipophilicity; hydrolysis kinetics*

Summary

3,5-Disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) derivatives; **4a–g** were prepared and found to be a promising prodrug approach for peptide drugs. The pH profile for their degradation in aqueous buffer solutions was determined using HPLC technique and accounted for, in terms of specific base-catalyzed reactions. All of the compounds however, showed high acid-stability. Enzymatic (human serum) hydrolysis of the different derivatives offered an advantageous range of $t_{1/2}$'s, the property that permits controlling onset and duration of actions of drugs.



Scheme 1. In formulae: a) R= CH_3; b) R = CH_3CH_2; c) R = $CH_3CH_3CH_2$; d) R = nC_4H_9; e) R = *cyclo*-C_6H_{11}; f) R = $C_6H_5CH_2$; g) R = $C_6H_5CH_2CH_2$.

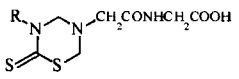
Introduction

Development of peptide drugs is presently a major area in drug research and, in recent years, several biologically active peptides, including those consisting of two or three amino acids, have been discovered. Application of peptides as clinically useful drugs is, however, seriously hampered due to substantial delivery problems. Most peptides are rapidly metabolized by proteolysis at most routes of administration, they are in general nonlipophilic compounds showing poor biomembrane penetration characteristics, and they possess short biological half-lives due to rapid metabolism[1–7].

A possible approach to solve these delivery problems, specially in the case of small peptides, may be derivatization of the bioactive peptides to produce prodrugs or transport forms which possess enhanced physicochemical properties in comparison with the parent compounds with regard to delivery and metabolic stability[8]. Bioreversible derivatization may protect small peptides against degradation by peptidase present at the mucosal absorption barrier and render hydrophilic peptides more lipophilic and hence facilitate their absorption. To be useful, however, the derivatives should be cleaved enzymatically in blood following their absorption, with release of the parent bioactive peptide[9–16].

As a part of the studies in progress in our laboratories to develop various types of bioreversible derivatives for functional or chemical entities occurring in amino acids and peptides[17,18], the present work outlines the incorporation of glycylglycine in a tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) structure as a model for a bioreversible derivatives of peptides capable of releasing these peptides through chemical or enzymatic hydrolysis.

Table 1: Physicochemical data of the synthesized THTT derivatives (**4a–g**).



Compd. No.	R	Yield (%)	M.p. °C	Formula	Elemental analysis [a]	R_M [b]	Clog P [c]
4a	CH_3	57	160	$C_8H_{13}N_3O_3S_2$	C,H,N,S	–0.158	–1.782
4b	C_2H_5	65	165	$C_9H_{15}N_3O_3S_2$	C,H,N,S	–0.105	–1.253
4c	C_3H_7	78	179	$C_{10}H_{17}N_3O_3S_2$	C,H,N,S	–0.052	–0.724
4d	C_4H_9	80	173	$C_{11}H_{19}N_3O_3S_2$	C,H,N,S	–0.017	–0.195
4e	cyclo-C_6H_{11}	77	197-9	$C_{13}H_{21}N_3O_3S_2$	C,H,N,S	0.02	0.159
4f	$C_6H_5CH_2$	86	175	$C_{14}H_{17}N_3O_3S_2$	C,H,N,S	–0.105	–0.142
4g	$C_6H_5C_2H_4$	88	169	$C_{15}H_{19}N_3O_3S_2$	C,H,N,S	–0.052	0.165

[a] Elemental analyses were satisfactory within ±0.5% of the calculated values.

[b] R_M value of glycylglycine is –0.75 under the same experimental conditions.

[c] Clog *P* value for glycylglycine is –3.728 and the reported log *P* value is –2.920 [23]

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Results and Discussion

Chemistry

Primary amines **1a–g** were allowed to react with carbon disulfide, in presence of KOH to form their corresponding potassium dithiocarbamate derivatives **2a–g.** Addition of formalin to the appropriate **2a–g** resulted in the formation of compounds **3a–g** (*in situ*). **3a–g** were then added portionwise to the aqueous solution of glycylglycine in the presence of phosphate buffer (pH = 7.8) to form the designated THTT derivatives **4a–g** (Scheme 1, Table 1). The structures of the prepared compounds were verified on the basis of elemental analyses and spectroscopic methods. In their IR spectra, **4a–g** showed combination stretching absorption of the carboxylic OH and the amidic NH in the range 3500–3150 cm^{-1} (OH and NH free and hydrogen bonded), the aliphatic C-H stretching at 3100–2850 cm^{-1}, stretching of the carbonyl groups at 1660–1680 (for the amidic C=O) and at 1705–1715 cm^{-1} (for the carboxylic C=O), and finally stretching of the thiocarbonyl group (C=S) at 1150–1155 cm^{-1}. In their ^{1}H-NMR spectra, the synthesized derivatives showed the C-4 and the C-6 methylenes of the tetrahydrothiadiazine-2-thione ring as one singlet integrating for four protons in derivatives **4a–e**, but separated as two singlets each of two protons in **4f,g**. The amide CONH proton appeared as a triplet due to coupling with the adjacent methylene which appeared as doublet integrating two protons. There is no interaction between the C-2 and C-4 protons with each other, but there is a small restricted rotation of the substituents (R) at N-3 of the ring system around the sigma bond evidenced by slightly non-typical ethylenic coupling in **4b,c** and **4g** of N-3-β-CH_2- and N-3-α-CH_2- as shown by ^{1}H-NMR (Table 2).

Lipophilicity

As expected, the synthesized derivatives possess greatly increased lipophilicity relative to the parent peptide glycylglycine indicated from their estimated R_M values[19–22], Table 1.

A fairly linear relation is observed between the estimated lipophilicity of the synthesized derivatives expressed by the R_M values and the logarithms of the partition coefficient (log *P*) ($r = 0.783$, $n = 7$). The latter computed with a routine method called calculated log *P* (Clog P) contained in a PC-software package (MacLogP 2.0, BioByte Corp., CA, USA). A representation of the molecular structure where hydrogens are omitted, or "suppressed" (SMILES notation), is entered into the program, which computes the log *P* based on the fragment method developed by ref.[23]. The regression coefficient of the relation is significantly improved when considering the parent compound in the equation ($r = 0.923$, $n = 8$). This improvement in lipophilicity by these THTT derivatives relative to glycylglycine may render them more capable of penetrating various biomembranes [24], thus may enhance its bioavailability to the requisite sites of action.

Kinetic Measurements

The kinetics of degradation of the THTT derivatives **4a–g** were studied in aqueous buffer solution at 37°C over the pH range 1.2–9 (μ = 0.5). At constant pH and temperature,

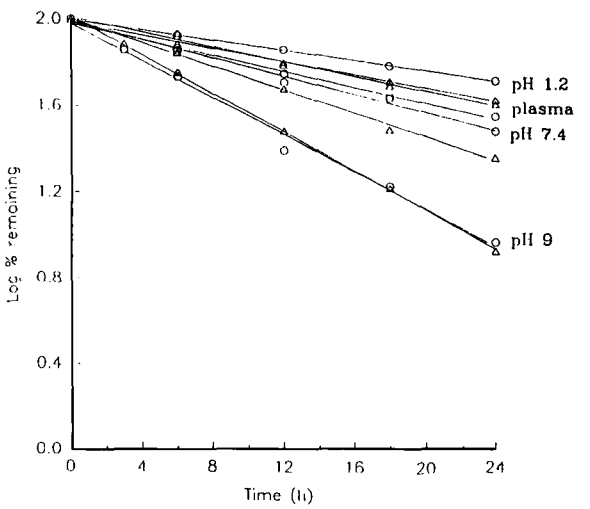


Fig. 1: Apparent first-order kinetics plot of the degradation of **4d** (Δ) and **4g** (○) in aqueous buffer solution and in 80% human plasma at 37°C.

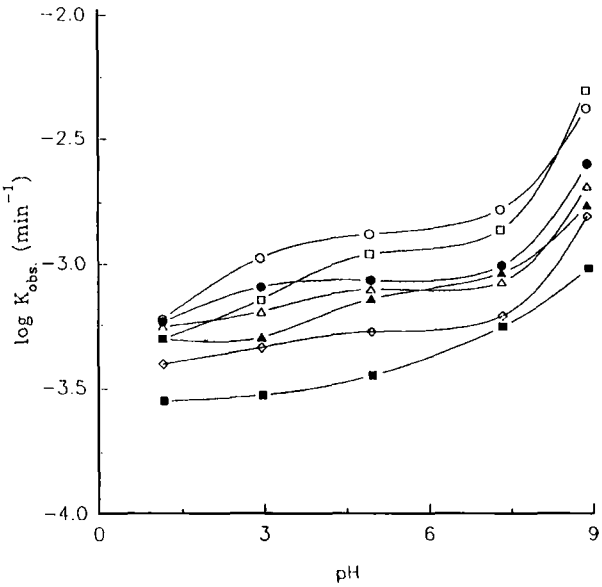


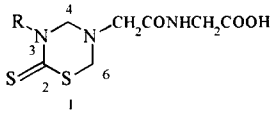
Fig. 2: pH rate profiles for the degradaton of the synthesized compounds **4a** (○), **4b** (●), **4c** (Δ), **4d** (▲), **4e** (❑), **4f** (■), and **4g** (✧) in aqueous buffer solutions at 37°C.

disappearance of the derivatives displayed strict first-order kinetics over several half-lives, Table 3, and all reactions proceeded to completion. Some typical first-order plots are shown in Fig. 1.

Influence of pH on the rate of hydrolysis is shown in Fig. 2, by plotting the logarithms of observed pseudo-first-order rate constants (log $k_{obs.}$) against pH. The observed pH-rate relationship indicates that the overall hydrolysis can be described in terms of base-catalyzed reactions.

Rate data obtained for the various derivatives, Table 3, show that the stability is maximal at pH 1.2 simulated to gastric fluid (SGF) contrary to that observed at alkaline pH 9. Stability is seen to be affected by substituents at N-3 of THTT moiety. N-3 aralkyl substituents decrease the reaction rates. In case of N-3 alkyl substituents, methyl group increased the

Table 2: ^{1}H NMR spectral data of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione derivatives.



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No.	R	Chemical shifts (δ values) ppm, in DMSO-d6, *J* = *Hz*.						
		4-CH_2 or	4-CH_2 & 6-CH_2	6-CH_2	N^5-CH_2-CO	CONH	CONHCH_2CO	N^3-R
4a	H_3C		4.68 (4H, bs)		3.72 (2H, s)	8.45 (1H, t, *J* = 6.5	4.00 (2H, d, *J* = 7.0	3.58 (3H, s, CH3)
4b	H_5C_2		4.71 (4H, bs)		3.63 (2H, s)	8.45 (1H, t, *J* = 6.5	3.95 (2H, d, *J* = 7.0	1.32 (3H, t, *J*= 7.6, CH_2CH_3), 4.20 (2H, q, *J* = 7.5, CH_2CH_3)
4c	*n*-H_7C_3		4.65 (4H, bs)		3.61 (2H, s)	8.42 (1H, t, *J* = 6.4	3.95 (2H, d, *J* = 7.0	0.92 (3H, t, *J*= 7.5, propyl CH_3), 1.75 (2H, m, N^3-$CH_2CH_2CH_3$), 3.95 (2H, t, *J* = 7.5, N^3-$CH_2CH_2CH_3$, combined with the $CONHCH_2CO$
4d	*n*-H_9C_4		4.59 (4H, bs)		3.54 (2H, s)	8.44 (1H, t, *J*= 6.3	4.00 (2H, d, *J* = 7.0	0.90 (3H, t, *J*= 7.3, butyl CH_3), 1.10-1.90 (4H, br.m, N^3-$CH_2CH_2CH_2CH_3$), 3.96 (2H, t, *J* = 7.6, N^3-$CH_2CH_2CH_2CH_3$).
4e	*cyclo*-$H_{11}C_6$	4.60 (2H, s)		4.56 (2H, s)	3.50 (2H, s)	8.38 (1H, t, *J* = 6.5	3.89 (2H, d, *J* = 7.3	0.90-2.05 (10H, br.m, the cyclohexyl five methylene groups), 5.62 (1H, br.m, N^3-CH methine of cyclohexyl)
4f	$C_6H_5CH_2$-	4.71 (2H, s)		4.67 (2H, s)	3.49 (2H, s)	8.30 (1H, t, *J* = 6.4	3.86 (2H, d, *J* = 7.2	5.42 (2H, s, N^3-$CH_2C_6H_5$), 7.49 (5H, s, benzyl C_6H_5)
4g	$C_6H_5(CH_2)_2$		4.60 (4H, s)		3.52 (2H, s)	8.36 (1H, t, *J* = 6.4	3.86 (2H, d, *J* = 7.2	3.01 (2H, t, *J* = 8.0, N^3-CH_2), 4.16 (2H, t, *J* = 7.9, N^3-CH_2), 7.43 (5H, s, phenethyl C_6H_5)

degradation rate, whereas chain elongation (ethyl, propyl, butyl) was accompanied by retardation of the reaction rate. Cycloalkyl substituent, on the other hand, revealed a varied pattern in the investigated buffer solutions.

Liberation of glycylglycine from these derivatives was confirmed using HPLC by matching the retention time of the reaction products in different pH's with that of the authentic peptide (glycylglycine) at 218 nm .

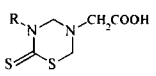
The rates of degradation of THTT derivatives were determined in 80% human plasma at 37 °C in order to obtain information on susceptibility of these derivatives to enzymatic catalysis. Strict first-order kinetic reactions were observed for the tested compounds under the investigation conditions, representative example is demonstrated in Fig. 1. As shown in Table 3, degradation rates are varied in presence of plasma compared to a buffer solution of the same pH (7.4) for the investigated derivatives. Here again, stability is seen to be affected by substituents at N-3 of THTT moiety. Compounds having N-3 aralkyl substituents hydrolyzed rapidely in plasma than in buffer pH 7.4, e.g. a two-fold change in the half-life of **4f** (R= benzyl) in plasma relative to physiological buffer solution, pH 7.4. In case of N-3 alkyl and cycloalkyl substituents, the compounds hydrolyzed more slowely in plasma (cf. **4c**) than at buffer of pH 7.4, e.g. a 2.7-fold change in half-life of **4e** (R = cyclohexyl) in plasma than buffer of

Table 3: Rate data for the hydrolysis of various synthesized THTT in aqueous buffer solutions and in 80% human plasma (pH 7.4) at 37 °C.

Compd. No.	$t_{1/2}$ (h)			
	pH			plasma
	1.2	7.4	9	
4a	19.7	7.0	2.8	8.9
4b	20.2	12.0	4.6	18.2
4c	21.1	13.9	5.7	9.6
4d	23.4	12.8	6.8	15.1
4e	23.6	8.5	2.4	22.8
4f	41.1	21.2	12.2	11.8
4g	29.5	19.2	7.5	15.8

pH 7.4. Release of the model peptide; glycylglycine was also detected by hydrolysis in plasma. The rates of liberation of glycylglycine in human plasma compared with buffer pH 7.4 appeared slower in most N-3 alkyl and aralkyl containing compounds. Analogous results were obtained with similar drug delivery systems[25–28], attributed to binding of a pro-

Table 4: The variables obtained by modeling and molecular mechanics of the optimized structures.



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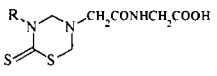

4a-g

No	$t_{1/2}$ (h)	mmx k cal.	str	tor	vdw	dm Debye	Inc. hf	nps Å^2	npu	pol
I	0.68	50.34	0.83	38.18	7.69	6.81	–52.27	114.60	0.00	75.80
II	1.80	17.06	1.11	2.05	9.09	6.82	–93.47	132.30	0.00	78.20
III	2.40	17.75	1.21	2.05	9.57	6.81	–99.20	146.40	0.00	78.20
IV	2.40	18.35	1.29	2.04	9.99	6.81	–105.01	168.20	0.00	76.60
V	0.93	20.64	1.59	3.48	10.90	6.93	–107.64	190.00	0.00	79.30
VI	0.68	23.48	1.13	8.03	9.34	6.99	–32.04	132.50	63.60	73.70
VII	0.69	22.86	1.09	8.06	9.78	6.93	–39.08	160.00	51.90	75.80
4a	8.90	3.77	0.97	1.62	8.18	5.06	–142.25	143.80	0.00	100.40
4b	18.20	6.40	1.23	1.46	9.51	5.00	–147.54	161.20	0.00	102.40
4c	9.60	7.08	1.33	1.45	9.98	4.99	–153.28	175.30	0.00	103.00
4d	15.10	7.69	1.43	1.44	10.37	4.95	–159.08	196.60	0.00	103.60
4e	22.80	12.64	1.55	5.82	10.13	4.80	–159.04	212.80	0.00	110.50
4f	11.80	12.99	1.17	7.52	9.85	5.14	–85.94	160.60	62.70	103.00
4g	15.80	14.14	1.22	7.43	11.12	5.11	–91.21	177.80	63.20	101.80

mmx: energy, str: strain, tor: torsion, Inc. hf: incremental heat of formation, vdw: Van der Waals forces, dm: dipole moment, nps: surface area (Å^2) of non-polar saturated part of the molecule, npu: surface area (Å^2) of non-polar unsaturated part, pol: surface area (Å^2) of polar part.

Table 5: The correlation matrix of $t_{1/2}$ with each variable obtained by modeling and molecular mechanics of the optimized structures.

variable	t1/2	mmx	str	tor	vdw	dm	Inc. hf	nps	npu	pol
t1/2	1.000									
mmx	0.590	1.000								
str	0.407	–00.451	1.000							
tor	–0.268	0.889	–0.556	1.000						
vdw	0.336	–0.417	0.808	–0.531	1.000					
dm	–0.917	0.674	–0.259	0.283	–0.210	1.000				
Inc. hf	–0.701	0.717	–0.550	0.520	–0.236	0.742	1.000			
nps	0.638	–0.529	0.895	–0.485	0.794	–0.527	–0.628	1.000		
npu	–0.039	0.078	–0.219	0.087	0.250	0.044	0.628	–0.114	1.000	
pol	0.936	–0.678	0.349	–0.308	0.279	–0.991	–0.756	0.600	–0.049	1.000

Critical value (1-tail, .05) = ±0.459. Critical value (2-tail, .05) = ±0.531. N = 14.

drug to plasma proteins, resulting in partial inhibition of hydrolytic mechanism. Aralkyl substituents at N-3 provided compouds that hydrolyze rapidely in plasma than in buffer pH 7.4. To find out responsbilty of enzymes for hydrolysis two compounds, **4e** and **4f**, were selected to be tested in enzyme-deactivated plasma (heated at 80 °C, for 5 min)[29]. It was found that for **4e** no significant change in its half-life in plasma and in enzyme-deactivated plasma ($t_{1/2}$ = 22.8 and 21.5 h, respectively), thus, there is no rule for the plasma enzymes in degradation process. **4f,** On the other hand, rapidly hydrolyzed in plasma ($t_{1/2}$ =11.8 h) than in enzyme-deactivated plasma ($t_{1/2}$ =21.0 h), the latter is matchable with that of pH 7.4. Such an observation confirms the rule of enzymes in the degradation process.

About the amide linkage, the HPLC results revealed that amide bond of tested peptide model is not affected by chemical or enzymatic catalysis, however, degradation of the THTT moiety *via* ring cleavage at N-5 of the structure takes place with liberation of compound of interest as previously reported [17,18].

Correlation of the Rates of Degradation in Plasma with the Molecular Kinetics

Correlations of the enzymatic susceptibility of THTT derivatives, expressed by $t_{1/2}$'s in 80% human plasma at 37 °C, and the variables obtained by molecular modeling and molecular mechanics[30–33] for these derivatives, listed in Table 4, were estimated. These may serve as sufficient, if not essential, conditions for *in vivo* reversion of the parent peptide. Table 5 shows the correlation matrix of the interrelations between all variables. Some of these variables, such as polar area (pol) and dipole moment (dm), exhibited strong correlations with the enzymatic degradation; however, the others revealed a varied interrelations. The variables that govern the enzymatic susceptibility of the tested derivatives can be arranged in the following order : pol > dm > Inc. hf > others.

Statistical treatment of different combinations of the orthogonal variables were also investigated using multiple regression analysis. No significant improvement was observed in the regression coefficient compared with those obtained with single variables. Such results indicate that the different N-3 functional groups affecting the polar surface area or the dipole moment of the molecule should enhance its enzymatic susceptibility.

Conclusion

It can be concluded from the obtained data, apparently for the first time, that the THTT derivatives may be useful as drug delivery system (DDS) for small peptides. This modification in the peptide structures is readily bioreversible, the parent peptide model being formed either by spontaneous hydrolysis at physiological or slightly alkaline pH's, as demonstrated for the synthesized derivatives, or by enzymes such as those in plasma which do not attack the peptide amide bond. The results and the correlations obtained with THTT derivatives revealed that such derivatives fulfill the requirement of the prodrug approach in that they are almost quantitatively cleaved to the parent peptide in plasma. The results indicate that as regards prodrug formation it is possible to vary the stability of the derivatives by selecting different substituents at N-3 of the THTT moiety. Furthermore, such derivatization may be useful to provide orally administerable peptides as revealed by stability studies in simulated gastric fluid (SGF).

Experimental

General

Precoated silica gel 60 F-254 plates (Merck) were used for thin layer chromatography; spots were detected by ultraviolet light and/or staining with iodine vapor. Melting points were determined on an electrothermal melting point apparatus [Stuart Scientific, England], and were uncorrected. ^{1}H NMR spectra were determined on an EM-60 Varian spectrometer in DMSO-d_6, using TMS as internal standard and the chemical shifts were given in δ ppm. IR spectra were recorded (KBr discs) on a Shimadzu-408 spectrophotometer. Elemental analyses (C, H, N, and S) were performed at the Department of Chemistry, Faculty of Science, Assiut University. HPLC system consisting of a pump [Knauer HPLC pump 64, Germany], a variable-wavelength detector [Knauer], a reversed-phase HPLC column [stainless steel (25 × 0.5 cm i.d.) C-18 Eurospher 80], a Shimadzu C-R 6A chromatopac recording integrator, and a 20-µl injection loop was used. Mobile phase systems of acetonitrile, water and 1% phosphoric acid (85%) were used and the ratio of acetonitrile:water was adjusted in order to give a retention time of 3.5–5 min. The column effluent was monitored at 258 nm and the flow rate was 1 ml/min. Glycylglycine was purchased from Wako pure chemical industries [Tokyo, Japan]. All of the other chemicals were of commercial grade except the HPLC solvents and the buffer reagents (analytical grade).

General Procedure for Synthesis of 3,5-Disubstituted tetrahydro-2H-1,3,5-thiadiazine-2-thione ***4a–g***

Carbon disulfide (60 mmol) was added portionwise to a stirred mixture of the appropriate alkyl-, cycloalkyl or aralkylamine; **1a–g** (10 mmol) and potassium hydroxide (20%, 10 mmol) in ethanol (10 ml). The stirring was continued for 3 h at ambient temperature. To the reaction mixture, which contains the dithiocarbamates **2a–g**, formaldehyde solution (35%, 22 mmol), was added and the stirring was continued for further 1 h. The resulting clear solution of **3a–g** was added portion-wise during 15 min to a stirred solution of glycylglycine (10 mmol) in phosphate buffer (pH 7.8, 20 ml). After stirring for 4 h at ambient temperature, the reaction mixture was acidified with dilute hydrochloric acid (5%, ~ 15–18 ml) to pH 2. Methylene chloride (100 ml) was added and the stirring was continued for further 30 min. The formed precipitate was collected by filtration, washed with 0.5% hydrochloric acid and dried, however, the organic phase was separated, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude solid collected was crystallized from ethanol to afford **4a–g**. Yields, melting points, physical and spectral data are given in Tables 1 and 2.

Determination of R_M Values of the THTT Derivatives

Silica gel TLC plates [20 × 20] were soaked for 5 h. in acetone containing 3% *n*-octanol, then left to dry overnight. From the methanolic solution (1 mg/ml) of each compound, three spots (each of 5 µl) were loaded at 1.5 cm intervals. The compounds were allowed to develop by ascending technique in a chromatographic tank under condition of equilibrium using a mobile phase of aqueous buffer phosphate solution and acetone (9:1) containing 3% *n*-octanol. The plates were dried and the developed spots were localized under UV lamp and/ or staining with iodine vapor. The R_f values were determined for each compound as the average of three readings, and the corresponding R_M values were calculated using the following formula: $R_M = \log (1/R_f - 1)$. Data are given in Table 1.

Kinetic Measurements

Degradation rates of the THTT derivatives **4a–g** in aqueous buffer solutions of pH 1.2 (simulated gastric fluid without enzyme), pH 3.0 (phosphate buffer), pH 5.0 (acetate buffer), pH 7.4 (isotonic phosphate buffer), and pH 9.0 (glycine/NaOH buffer), were determined at 37 °C. Ionic strength of the prepared buffer solutions was adjusted with NaCl to µ = 0.5.

Reactions were initiated by adding 25 µl of the stock methanolic solution of the derivatives (1 mg/ml) to 2.5 ml of preheated buffer solutions in screw-capped test tubes. At appropriate intervals samples were taken and chromatographed. Pseudo-first-order rate constants for the degradation were obtained from the slopes of linear plots of the logarithm of residual derivative against time as the average of three experiments for each compound.

Degradation of these derivatives was also studied at 37 °C in isotonic buffer of pH 7.4 containing 80% human plasma. At appropriate times' samples of 50 µl were withdrawn and mixed with 50 µl of acetonitrile for deproteinization and centrifuged at 10^4 rpm for 5 min. 20 µl of the clear supernatant was analyzed by HPLC as described above. The resulting data are given in Table 3.

Molecular Modeling and Molecular Mechanics

The powerful and fast DFP [30] procedure allowed to optimize the various molecules in their ground states without restrictions at full self-consistent field SCF [31]. MINDO/3; an improved version of the MINDO semiempirical SCF-MO method [31–33] allows recording the geometries of the fully minimized electroneutral closed shell disubstituted THTT derivatives. The energy (kcal/mole) of minimization (mmx), strain (str), torsion (tor), incremental heat of formation (Inc. hf), Van der Waals forces (vdw), the dipole moment (dm in Debye units), and the surface areas (Å^2) such as non-polar saturated (nps), non-polar unsaturated (npu), and polar (pol) parts for the synthesized compounds (**4a–g**) and the previously reported **I–VII,**

designated as **4a–g**, respectively, a–g are the same of the corresponding glycylglycine derivatives[18], are all counted in Table **4**.

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