Site-Specific Acid–Base Properties of Tenoxicam

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The *Hammett* approach, as a new deductive tool, was introduced to characterize the otherwise inaccessible minor protonation pathway of tenoxicam (**1**), the non-steroidal anti-inflammatory drug. A total of eight compounds, constituting a systematic series of side chain-substituted analogues of tenoxicam and piroxicam (**2**), were synthesized and studied in terms of acid–base properties and *Hammett* constants to identify the ideal replacement of the unprotonated pyridin-2-yl group, a key moiety in both molecules. *Hammett* constants of the phenyl substituents have been found to be in a linear correlation with the experimental log *K* values of the enolate sites, the basic moiety of the extended conjugated system in this family of piroxicam derivatives. Then, a similar correlation was observed for the analogous tenoxicam derivatives. After identifying the 2-aza *Hammett* constant of the pyridin-2-yl group and the corresponding log *K* value, the site-specific acid–base properties of tenoxicam could be quantitated. This novel method is assessed to be a fine-tuning tool to find the ideal substituent by using analogue-based deductive method to obtain site-specific constants of the minor protonation/deprotonation pathway in drugs and biomolecules. The tenoxicam microconstant values indicate that the enolate moiety is of extremely low basicity (reflected by the $\log k^{O} = 3.70$ and $\log k^{O}_{N} = 1.09$ values), which can, however, be interpreted in terms of the peculiar ring system and the overwhelming electron-withdrawing effects of the adjacent heteroatoms. A diagram depicting the pH-dependent distribution of **1** microspecies is also presented.

Introduction. – The protonation state of the non-steroidal anti-inflammatory drugs (NSAIDs) highly influences their pharmacokinetic processes and the concomitant bioavailability. It is also related to undesired side effects such as gastrointestinal or cardiovascular toxicity [1–3].

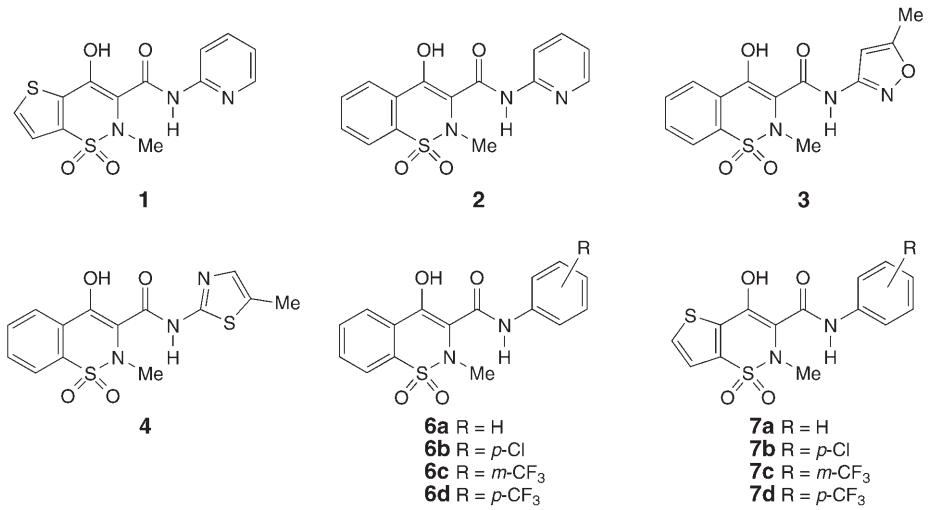
Oxicams, an important group of NSAIDs, act by blocking the formation of prostaglandins through the inhibition of COX isoenzymes [4]. Concerning molecular properties, oxicams are certainly examples of spectroscopic and structural peculiarities due to the various heteroatom sites suitable for coordination, protonation, H-bonding interactions, and tautomeric and conformational changes. To understand their mechanism of action at the molecular level, and, also, to design more effective derivatives, it is necessary to know their site-specific acid–base behavior in solution.

The site-specific protonation constants (microconstants and the less frequently applicable group constants) are physico-chemical parameters of crucial importance to describe the interactions of ampholyte drugs and biomolecules in the body [5][6]. Macroconstants, the most frequently used parameters in acid–base equilibria, describe only the overall acid–base properties of the chemical entity of two or more ionizing groups. In principle, however, they cannot be assigned to individual functional groups.

Thus, the protonation microconstants are essential parameters to characterize protonation equilibria at the molecular and submolecular levels [7][8]. Their significance is even greater in biological systems where the minor protonation isomer can well be the reactive one in highly specific (patho)biochemical processes [6][9].

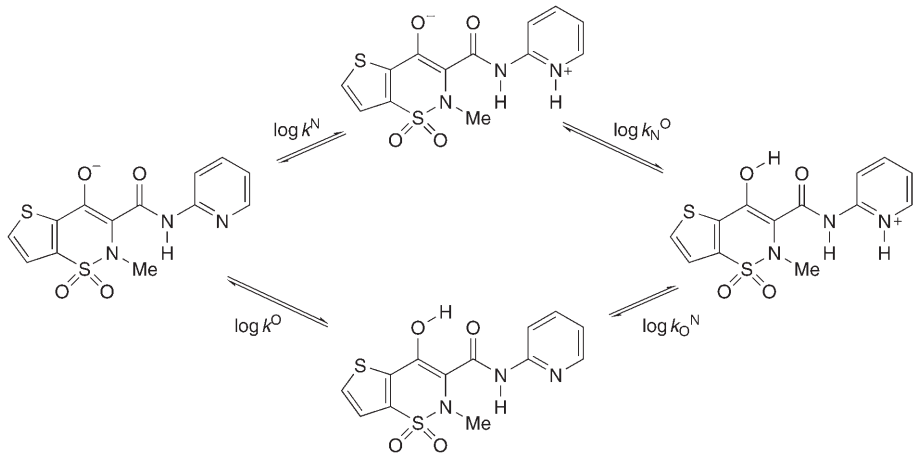
Two fundamental approaches exist to determine microconstants: combined spectroscopic/pH-metric direct methods and deductive methods by using synthetic model compounds. The former is widespread, and it frequently includes UV/VIS or NMR techniques (see refs. cit. in [6]; for some recent articles, see [10–13]). However, it requires some basic criteria in the molecule to be met. It is not applicable if acid–base properties of the functional groups are very different, or the protonation state of one of the proton-binding sites cannot be monitored selectively by spectroscopic methods, or the molecule is too complicated for spectral resolution. In these cases, the deductive method can be effective. It uses an auxiliary ligand which is usually a close derivative of the parent compound, but it contains a reduced number of functional groups. Macroconstant of the auxiliary molecule can then be handled as the appropriate microconstant of the main compound. Common such replacements for $-$COOH are $-$COOMe [14][15] and $-CONH_2$ [16].

Earlier, we resolved the piroxicam (**2**) protonation at the microscopic level [15]. Here, we report the microspeciation of tenoxicam (=*4-hydroxy-2-methyl*-N-*(pyridin-2-yl)-2*H-*thieno[2,3*-e*][1,2]thiazine-3-carboxamide 1,1 dioxide*; **1**), which differs from **2** in a fused five-membered thiophene heterocycle, replacing the fused benzene ring of **2**. Compound **1** has some advantages in therapy. It has a longer half-life and one-a-day advantage over **2**. Thus, it has achieved a significant role in the treatment of rheumatic diseases over the last decades [17][18].



Compound **1** has a bifunctional amphoteric structure: the enolic acid and the pyridin-2-yl moieties represent the acidic and basic sites, respectively. Accordingly, four individual microspecies exist in protic solvents (*e.g.*, in simple aqueous solutions and in tissue fluids) namely anionic, zwitterionic, uncharged, and cationic ones (*Scheme 1*).

Scheme 1. *Microspeciation Scheme of* **1**



The fused thienothiazine system and the conjugated enol-carboxamide moieties change significantly upon protonation/deprotonation, as shown by the strongly pH-dependent UV and NMR spectra.

The protonation properties of **1** have been studied at various levels [19][20]. *Bernhard* and *Zimmermann* [19] determined the macroscopic protonation properties of some oxicams by UV-titrations. They reported $\log K_1 = 5.34 \pm 0.06$ and $\log K_2 = 1.07 \pm 0.11$ for **1**. *Tsai et al.* [20] made estimations about the site-specific protonation constants by using the following considerations. They assumed that the average of the protonation macroconstants of isoxicam (**3**) and meloxicam (**4**) can approximate the microconstant of the anionic-uncharged protonation equilibrium of **2**. They also assumed that replacement of the pyridin-2-yl group in **2** with a 5-methylisoxazol-3-yl ring in **3** ($\log K = 3.93$) and 5-methylthiazol-2-yl ring in **4** ($\log K = 4.08$) means the elimination of the basic pyridin-2-yl moiety without significant influence on the acidity of the enolic OH group. They introduced the average of the macroconstants of **3** and **4** ($\log K \approx 4$) to calculate the microconstants of **2**. Applying the microconstants, they have also calculated the interactivity parameter that quantitates how protonation at one site reduces the basicity of the other site. Finally, the interactivity parameter of **2** was combined with the macroconstants of **1**, which allowed the approximation of the microconstants of **1** [20].

Microconstants of the minor protonation pathway obtained by the above method for piroxicam (**2**), however, differ by 0.6 log k units from the more recent corresponding values obtained by the well-established *O*-methyl deductive method [15], while macroconstants and microconstants of the major protonation pathway are in agreement within 0.04 log units.

Our study aimed, therefore, at the possible most accurate and reliable characterization of **1** at the microscopic level. This goal necessitated a new approach, since the *O*-methyl-derivative formation for **1** has been proven infeasible, as we have recently

shown [21] due to fast hydrolytic decomposition of *O*-methyl-tenoxicam in aqueous media. For this purpose, we synthesized auxiliary compounds with no ionizing group other than the enolic OH, but with pyridin-2-yl-mimicking, electron-withdrawing character in the side chain. The set of new compounds provided a possibility to assign *Hammett* constants to certain ring replacements after determining calibration lines between acidity and ring-substituent constants. By means of this novel method, the microspeciation of **1** could be carried out, based on experimental data of model molecules in this family, and the related observations in their pK_a values and *Hammett* constants.

Results and Discussion. – Preliminary experiments demonstrated that characteristic differences occur in positions of UV spectra, and also, in the protonation constants of tenoxicam analogues, depending on the quality and position of the substituent in the phenyl moiety. Substituent effects play a significant role in a variety of physical and chemical properties. For example, they influence the reactivity of the substrates, the rates of nucleophilic substitutions [22], positions of UV absorption bands [23], NMR chemical shifts [24], and the conformations of molecules [15]. The nature and position of substituents also affect the protonation constants (log *K* values) [25].

Thus the choice of the right substituent in deductive modelling is of crucial importance. Since the introduction of *Hammett* constants (σ) [26], these parameters have been eminent means for quantifying substituent effects. The *Hammett* constant, by definition, is the difference between the logarithmic protonation constants of the unsubstituted and substituted benzoic acids, taking into account the type and position of the substituent [27–29]. The slope of the correlation line between log *K* values and *Hammett* constants for benzoic acid is 1.00. For other molecules, the slope may differ from unity, depending on the sensitivity of the basic site, and various steric and inductive effects in the molecule.

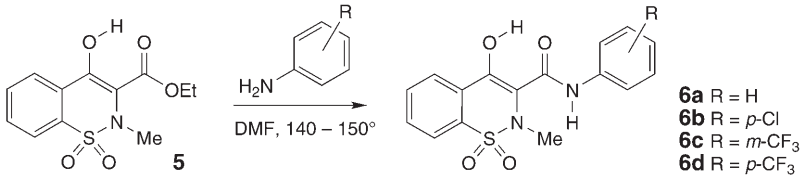
Electron-withdrawing and steric effects of an adjacent substituent are the main factors that influence the acid–base properties of such a molecule. For oxicams, the conjugated enolic carboxamide structure and the relatively long distance between the enolic and the pyridin-2-yl moieties, the electronic effects highly influence the extent of acidity. Thus, they can be characterized in terms of *Hammett* constants. As a consequence, acidity–*Hammett* constant correlation relationship can be established.

Since the most reliable value for log k^O, one of the microconstants in the minor protonation pathway of **2** is 4.60 [15], an ideally substituted phenyl moiety would also set log $K = 4.60$. We assumed that such a molecule does not exactly exist, but close derivatives can be prepared.

Our synthetic efforts to produce piroxicam derivatives **6a**–**6d** (*Scheme 2*) resulted in compounds with log *K* values in the range of 4.40–4.81, and the corresponding *Hammett* constants between 0.00 and 0.53 (*Table 1*). A close correlation ($R^2 = 0.990$) has been found between the log *K* values and the *Hammett* constants (*Fig. 1*), reinforcing the common origin of these two parameters.

By substitution of the value 4.60 into the linear-regression equation, an ideal *Hammett* constant of 0.29 could be marked. This represents the substituent on the phenyl group that would exert exactly the same influence on the enolic site as the pyridin-2-yl moiety. The results are in good correlation with the work of *Katritzky et al.*

Scheme 2



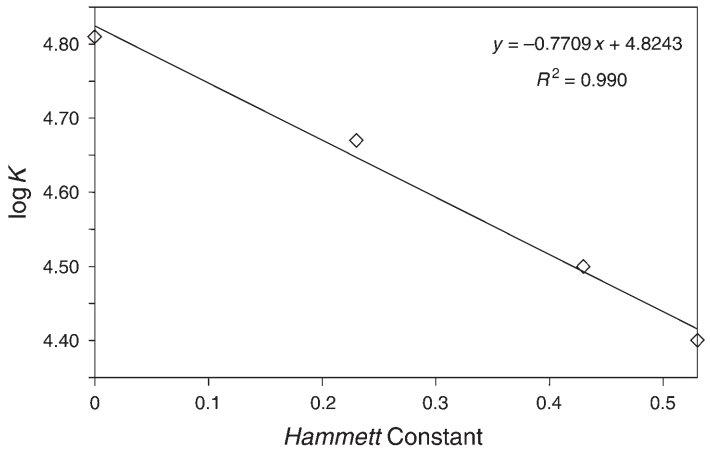


Fig. 1. *Correlation between protonation constants and* Hammett *constants of* **6a**–**6d**

Table 1. *Protonation Constants of* **6a**–**6d** *and* **7a**–**7d** *as a Function of Substituents and* Hammett *Constants*

Substituent	*Hammett* constant	log *K* of **6a**–**6d**[a])	log *K* of **7a**–**7d**[a])
H	0.00	4.81	3.89
p-Cl	0.23	4.67[b])	3.77
m-CF_3	0.43	4.50[b])	3.59
p-CF_3	0.53	4.40[b])	3.53

[a]) Standard deviation is within 0.01–0.03. [b]) Using *Yasuda–Shedlovsky* extrapolation.

[30], who assessed a 2-aza σ_o value of 0.268 of pyridin-2-yl group, based on IR intensity measurements.

An analogous set of tenoxicam derivatives, **7a**–**7d**, were prepared and characterized in our laboratory (details in *Exper. Part*). The correlation between the protonation constant of **7a**–**7d** and the *Hammett* constants of the substituents of the phenyl group (*Table 1*) gave also a linear fit with $R^2 > 0.98$ (*Fig. 2*). Due to the high structural similarity between **2** and **1**, especially in the region where inductive, electron-withdrawing effects take place, a moiety with a *Hammett* value of 0.29 can well be assumed to mark the log k^O constant of **1** in an analogous linear regression diagram.

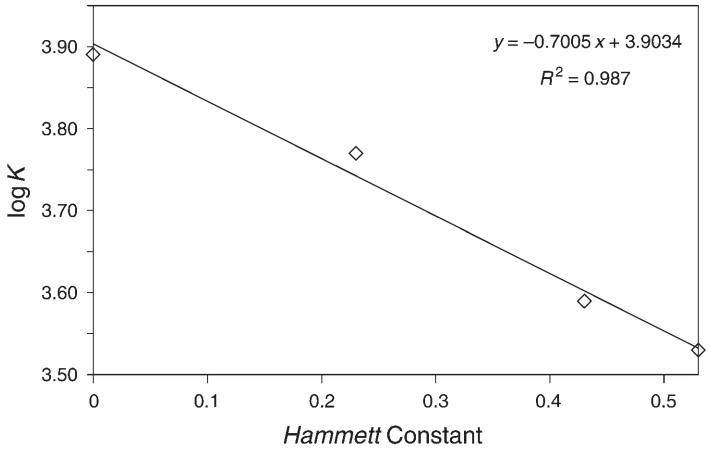


Fig. 2. *Correlation between protonation constants and* Hammett *constants of* **7a**–**7d**

Applying the *Hammett* constant of 0.29, a protonation constant of 3.70 was pointed out for the ideal phenyl analogue thus for the log k^O of the parent compound. All the microconstants could be calculated, using the macroconstants values (log $K_1 = 5.34 \pm 0.06$, log $K_2 = 1.07$) [19] and the following relationships between macro- and microconstants:

$$K_1 = k^N + k^O \tag{1}$$

$$K_1 K_2 = k^N k^O_N = k^O k^N_O \tag{2}$$

In *Table 2*, all the protonation microconstants of **1** are listed.

Table 2. *Summary of the Microconstants of* **2** *and* **1**

Microconstant	**2**		**1**	
	Tsai et al. [20]	*Takács-Novák et al.* [15]	*Tsai et al.* [20]	This work
log k^N	5.44	5.40	5.34[a])	5.32
log k^O	*ca.* 4[b])	4.60	3.17[a])	3.70[c])
log k^O_N	1.88	1.92	1.07[a])	1.09
log k^N_O	3.32	2.72[d])	3.24[a])	2.71

[a]) Calculated assuming same interactivity parameters of **2** and **1**. [b]) Average of log *K* values of **3** and **4**. [c]) Determined by the novel deductive method (estimated standard deviation is 0.03). [d]) Determined through *O*-methylpiroxicam.

The microconstants show that the two oxicam derivatives have nearly identical interactivity parameters ($\log k^O_{Pir} - \log k^O_{NPir} = 2.68$, $\log k^O_{Ten} - \log k^O_{NTen} = 2.61$), an obvious consequence of the structural homology.

Using these microconstant values, the pH-dependent distribution of the four microspecies could be calculated for tenoxicam (*Fig. 3*). The zwitterionic species

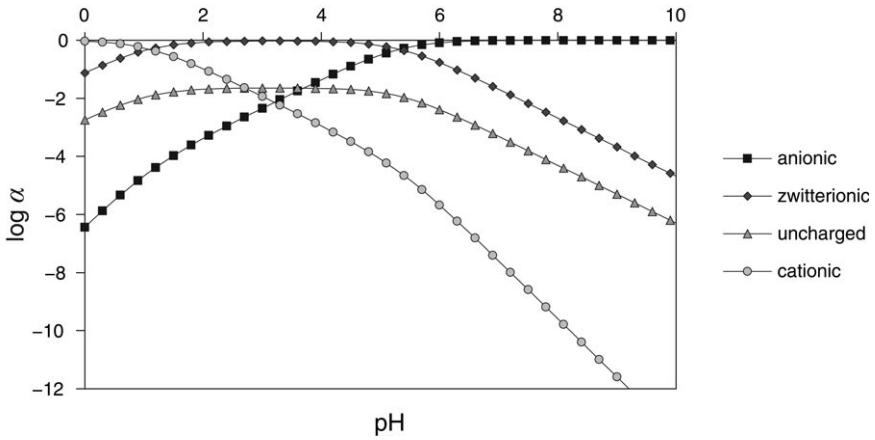


Fig. 3. *Logarithmic distribution curves for microspecies of* **1**

dominates over the uncharged form by a factor of 42. In fact, the major protonation route goes through the zwitterionic form in 97.7%. It can also be seen that the whole protonation takes place in the acidic region.

Conclusions. – This study of oxicams demonstrates that extended *Hammett* σ parameters can be used to account for substituent effects of pyridin-2-yl heterocycles in determination of protonation microconstants. In this novel deductive method, *Hammett* constants, harnessing molecular analogies, have been used to elucidate protonation microconstants in **1**.

The close correlation between log *K* values and *Hammett* constants for both **1** and **2** derivatives is a promising finding to determine hardly accessible macro- and micro-constants for compounds in structurally related molecular families. The precise microconstants provide the possibility to design more reliable COX – enzyme binding operational model.

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Experimental Part

General. Materials. Tenoxicam (**1**) was a generous gift from *Mihály Kata*, Department of Pharmaceutical Technology, University of Szeged. Saccharin sodium was obtained from *Hungaropharma* (H-Budapest). All other reagents and solvents for the synthetic work were purchased from *Sigma* (D-Steinheim) and were used as received. Of the chemicals used for pH electrode calibration and log *K* determination, KCl (anal. grade), KOH, and HCl were from *Sigma*, and MeOH (HPLC grade) from *Merck* (D-Darmstadt). KOH and HCl were standardized by titration against primary standards KH-phthalate and (amino)tris(hydroxymethyl)methane, resp. (*Sigma*). Bidistilled H_2O was used throughout this study. M.p.: in open cap. tubes on *Stuart Scientific SMP* melting-point apparatus. UV/VIS Spectra: *Jasco V-550* spectrophotometer at r.t.; λ_{max} in nm, ε in $dm^3\ mol^{-1}\ cm^{-1}$. ^{1}H-NMR Spectra: *Varian Unity Inova 600 MHz* spectrometer in (D_6)DMSO at r.t.; δ in ppm rel. TMS, *J* in Hz.

*Ethyl 4-Hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-Dioxide* (**5**). It was prepared from the readily accessible saccharin sodium in three steps as described in [31 – 33]. Saccharin sodium

(61.5 g, 0.30 mol) was dissolved in 240 ml of DMF and reacted with ethyl 2-chloroacetate (equiv. quantity) at 100° for 3 h according to [31]. The soln. was poured onto ice-water (4 × excess in volume), that resulted in 57.7 g (72%) of the ethyl (3-oxo-1,2-benzoisothiazol-2(3*H*)-yl)acetate 1,1-dioxide) as a white precipitate. The product (20 g, 0.074 mol) was dissolved in 3 equiv. instead of 2 [32] of freshly prepared EtONa soln. (5.12 g of Na in 80 ml abs. EtOH). The mixture was kept at 55° for 1 h so that a *Gabriel–Colman* rearrangement could occur. HCl (9 ml, of 9%) was added during continuous stirring and not allowing the temp. to rise above 5°. The whole mixture was kept in a refrigerator overnight. The white precipitates were filtered off to yield 13.6 g (68%) of ethyl 4-hydroxy-2*H*-benzo[1,2]thiazine-3-carboxylate 1,1-dioxide. According to [33], 10 g (0.037 mol) of the former product was combined to 8 ml of MeI, 40 ml of H_2O, 140 ml of EtOH, and 40 ml of 1N NaOH. After standing at r.t. for 18 h, the yellow precipitation was filtered, washed with H_2O, and dried to yielding 7.5 g (72%) of **5**. M.p. 161–163°. UV/VIS (MeOH): 322 (14000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 1.33 (*t*, *J* = 7.1, *Me*CH_2); 2.82 (*s*, MeN); 4.36 (*q*, *J* = 7.1, MeCH_2); 7.84 (*t*, *J* = 7.4, H–C(6)); 7.86 (*t*, *J* = 7.4, H–C(7)); 7.88 (*d*, *J* = 7.1, H–C(5)); 8.04 (*d*, *J* = 7.1, H–C(8)).

Piroxicam Analogues Suitable for Deductive Characterization (**6a**–**6d**). Compound **5** (0.28 g, 0.001 mol) was suspended in DMF (3 ml), excesses (3 ×) of substituted anilines were added, and the mixtures were heated under N_2 for 12 h at 140–150° (oil bath). The mixtures were cooled and then poured into 15 ml of HCl soln. containing mol-equivalent quantity of acid. The precipitous mixtures were placed into refrigerator and kept there for 16 h, then filtered, and the precipitates were washed 3 times with H_2O and cold i-PrOH.

4-Hydroxy-2-methyl-N-*phenyl-2*H-*1,2-benzothiazine-3-carboxamide 1,1-Dioxide* (**6a**). White crystalline solid. Yield: 57%. M.p. 216–217°. UV/VIS (MeOH): 334 (17000), 240 (14000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 2.87 (*s*, MeN); 7.19 (*m*, H–C(4′)); 7.39 (*t*, *J* = 7.1, H–C(3′), H–C(5′)); 7.68–7.76 (*m*, H–C(6), H–C(7)); 7.85–7.96 (*m*, H–C(5), H–C(2′), H–C(6′)); 8.04 (*d*, *J* = 7.4, H–C(8)); 10.30 (*s*, OH).

N-*(4′-Chlorphenyl)-4-Hydroxy-2-methyl-2*H-*1,2-benzothiazine-3-carboxamide 1,1-Dioxide* (**6b**). White crystalline solid. Yield: 64%. M.p. 230–232°. UV (MeOH): 334 (23000), 249 (19000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 2.86 (*s*, MeN); 7.45 (*d*, *J* = 7.8, H–C(3′), H–C(5′)); 7.79 (*d*, *J* = 7.8, H–C(2′), H–C(6′)); 7.86–7.95 (*m*, H–C(5), H–C(6), H–C(7)); 8.04 (*d*, *J* = 7.4, H–C(8)); 10.42 (*s*, OH).

4-Hydroxy-2-methyl-N-*[3-(trifluoromethyl)phenyl]-2*H-*1,2-benzothiazine-3-carboxamide 1,1-Dioxide* (**6c**). White crystalline solid. Yield: 60%. M.p. 199–201°. UV (MeOH): 334 (24000), 249 (18000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 2.88 (*s*, MeN); 7.54 (*d*, *J* = 8.0, H–C(4′)); 7.64 (*t*, *J* = 8.0, H–C(5′)); 7.88–7.95 (*m*, H–C(5), H–C(6), H–C(7)); 8.06 (*d*, *J* = 7.6, H–C(8)); 8.12 (*d*, *J* = 8.0, H–C(6′)); 8.19 (*s*, H–C(2′)); 10.57 (*s*, OH).

4-Hydroxy-2-methyl-N-*[4-(trifluoromethyl)phenyl]-2*H-*1,2-benzothiazine-3-carboxamide 1,1-Dioxide* (**6d**). White crystalline solid. Yield: 56%. M.p. 237–239°. UV (MeOH): 334 (20000), 253 (16000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 2.88 (*s*, MeN); 7.77 (*d*, *J* = 8.6, H–C(3′), H–C(5′)); 7.88–7.95 (*m*, H–C(5), H–C(6), H–C(7)); 8.02 (*d*, *J* = 8.6, H–C(2′), H–C(6′)); 10.60 (*s*, OH).

Synthesis of Tenoxicam Analogues **7a**–**7d**. The substituted benzene derivatives of tenoxicam were prepared as described in [21]. Detailed description of the procedures and products for phenyl, 3-(trifluoromethyl)phenyl, and 4-(trifluoromethyl)phenyl derivatives are provided therein.

N-*(4-Chlorophenyl)-4-hydroxy-2-methyl-2*H-*thieno[2,3-*e*][1,2]thiazine-3-carboxamide 1,1-Dioxide* (**7c**). Yellow crystals. Yield: 60%. M.p. 192–194°. UV (MeOH): 386 (19000), 296 (14000), 270 (15000). ^{1}H-NMR (600 MHz, (D_6)DMSO): 2.99 (*s*, MeN); 7.23 (*d*, *J* = 7.8, H–C(3′), H–C(5′)); 7.41 (*d*, *J* = 7.8, H–C(2′), H–C(6′)); 7.39 (*d*, *J* = 5.2, H–C(7)); 7.86 (*d*, *J* = 5.2, H–C(8)); 10.55 (*s*, OH).

Spectrophotometric log K *Determination*. The pH-metric titrations were performed at 25.0 ± 0.1° and at constant ionic strength (0.15 mM KCl) on a *GLpKa* automatic titrator (*Sirius Analytical Instruments Ltd.*, Forest Row, UK). The four-parameter technique (*Four Plus*™ method) was used for electrode calibration in both aqueous medium and MeOH/H_2O mixtures [34][35]. The *Refinement Pro* software (*Sirius*) was used to record and evaluate the titration curves. The log *K* values of samples were measured spectrophotometrically using the *D-PAS*™ UV spectrometer attachment for *GLpKa* [36]. In the spectrophotometric method, multi-wavelength UV absorbance of the sample soln. is monitored

throughout the titration using the fiber-optic probe *in-situ*. This method allows significantly smaller sample concentrations for log *K* measurements (10^{-5} M or below).

UV-pH Titration in Aqueous Medium. For all compounds, a stock sample soln. of *ca.* 1.0-mM concentration was prepared in MeOH. Aliquots (100 µl or 500 µl) of the stock solns. were then transferred into a sample vial containing 15 ml of 0.15M KCl soln. to achieve the required sample concentration. Sample concentrations of 6–30 µM were employed. In each experiment, the pH of the sample soln. of 15-ml volumes was adjusted, using 0.5M KOH, to *ca.* 8 and then titrated with 0.5M HCl to a suitably low value (*ca.* 2). Spectral data were recorded in the region of 200–700 nm after each pH measurement. About 20 pH readings and absorption spectra were collected from each titration. The titrations were carried out at constant ionic strength ($I = 0.15$M KCl) and temp. ($T = 25.0 \pm 0.5°$) under N_2. A minimum of three parallel measurements were carried out, and the log *K* values were calculated with RefinementPro™ software.

UV-pH Titration in MeOH/H_2O Mixtures and Extrapolation of Sample log K′ *Values Measured in MeOH/H_2O Mixtures to Aqueous log* K *Values*. The cosolvent dissociation constants of H_2O-insoluble compounds **6b**, **6c**, and **6d** were determined in various MeOH/H_2O mixtures between 16–44 wt-%. The same titration protocol was performed as described above. Each sample was measured at least in four different MeOH/H_2O mixtures. The measured log *K′* values in MeOH/H_2O solns. were extrapolated to aq. conditions according to the *Yasuda–Shedlovsky* procedure [37][38], which is the most widely used method among cosolvent techniques [39][40].

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