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CEPHEM SULFONES AS INACTIVATORS OF HUMAN LEUKOCYTE ELASTASE. II.[1] KETO-ENOL TAUTOMERISM IN CEPHEM-4-KETONES.

Marco Alpegiani, Pierluigi Bissolino, Daniela Borghi, Riccardo Corigli, Stefano Del Nero, Ettore Perrone, Giuseppe Razzano & Vincenzo Rizzo*

Farmitalia Carlo Erba R&D, 20014 Nerviano (MI), Italy

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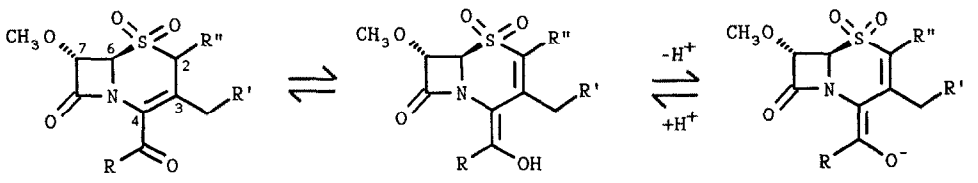
Abstract: Keto-enol tautomerism has been investigated in cephem-4-ketones, a new class of human leukocyte elastase inhibitors. Efficient enzyme inhibition was found compatible with ionization of the compounds as enolates, which confers solubility and hydrolytic stability at physiological pH.

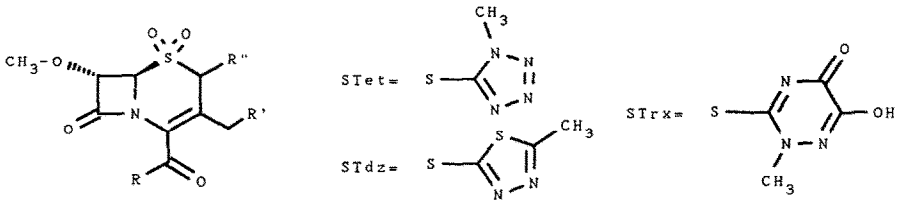
The chemical preparation of 1,1-dioxo-cephem-4-ketones of general structure I_a has been recently reported by our group[2] as part of an extensive investigation on the inhibitory properties of β-lactam compounds towards human leukocyte elastase (HLE). In previous studies, derivatization of the carboxylic function at the C-4 position of the cephem nucleus as esters,[3-5] amides[4,6] or thiolesters[1] was found mandatory for HLE inhibition. This result, in striking contrast with structural requirements for the antibiotic function of cephalosporins, was considered[3,4] as an indication that HLE (an endopeptidase) dislikes substrates or inhibitors bearing a negative charge close to the cleavable amide bond. Cephem-4-ketones pose an interesting problem in this regard, as they may exist in the tautomeric enol form I_b, and be converted to the negatively charged enolate I_c after proton abstraction. An investigation of these equilibria was therefore necessary before undertaking a systematic structure-activity analysis in the novel class of compounds.



I_a I_b I_c

The predominance of tautomeric form I_a for the undissociated molecule, both in the solid state and in solution, is substantiated by spectral data. Compounds **1-8** with a *tert*-butyl group next to the carbonyl function (Table 1) all have a C=O stretching at 1690-1705 cm^{-1}, well distinct from that of the β-lactam C=O (1790-1810 cm^{-1}). This absorption shifts to slightly lower frequency (1670-1680 cm^{-1}), as expected, for the corresponding phenyl ketones **9-13**.

Table 1. Structure, pK_a, chemical half-lives in buffer ($t_{1/2}$)[a] and HLE inhibition second order rate constant (k_{on})[b] of cephem-4-ketones.



Compound	R	R'	R"	pK_a	$t_{1/2}$ ± SD [hours]	(k_{on} ± SD) × 10^3 [$M^{-1}s^{-1}$]
1	tBu	H	H	ND	130 ± 5	0.09 ± 0.03
2	tBu	STet	H	11.6	24 ± 1	19 ± 2
3	tBu	STdz	H	11.9	37 ± 2	32 ± 5
4	tBu	H	STrx	8.9	16.3 ± 0.4	54 ± 4
5	tBu	H	STet	7.9	14.8 ± 0.4	100 ± 10
6	tBu	H	STdz	7.5	26 ± 1	42 ± 1
7	tBu	STet	STet	6.0	24 ± 1	26 ± 2
8	tBu	STdz	STdz	5.8	46 ± 3	21 ± 1
9	Φ	H	H	9 6	33 ± 1	0.13 ± 0.01
10	Φ	STet	H	8.4	8.6 ± 0.3	18 ± 7
11	Φ	H	STrx	5.7	86 ± 4	0.93 ± 0.12
12	Φ	H	STet	4.8	120 ± 6	0.2 ± 0.1
13	Φ	STet	STet	3.5	850 ± 150	2.2 ± 0.1

a) Chemical half-lives at 37°C, in the presence of 0.1 M phosphate buffer, pH 7.4. 2% MeCN was added as a solubilizing vehicle. With initial concentration c= 0.5 mM, $t_{1/2}$ values were obtained from plots of log(c) against time, by monitoring the decrease of c with an HPLC assay.[1]

b) Second order inhibition rate constants, k_{on}, were determined from plots of pseudo-first order inhibition rate constants against inhibitor concentration. Enzyme activity was monitored at 37°C, in the presence of 0.055 M phosphate buffer, pH 7.4, with the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-NMC. Reference 1 should be consulted for more details.

The UV spectra (MeCN or $CHCl_3$) of all of the compounds show a maximum centered at 252-273 nm (ε 8,500 ca. for **1-8** and 14,000-16,000 for **9-13**), which is typical of the Δ^3-cephem chromophore, independently of oxidation at sulfur.[7] The keto form I_a is also evident in the ^{1}H-NMR spectra ($CDCl_3$), where resonances from the C-2 methylene proton(s) appear either as an AB pattern in the range 3.5-4.4 ppm (2-unsubstituted compounds **1-3,9,10**) or as a sharp singlet, only marginally influenced by variations in concentration and temperature, at 4.98-6.08 ppm (2-substituted compounds **4-8,11-13**).

When a base of adequate strength (DBN for **1-3,9,10**, triethylamine for the others) was added to solutions of the compounds in organic solvents, impressive modifications of the IR, UV and NMR spectra were recorded, suggestive of formation of the enolate anion I_c; all of these spectroscopic changes were completely reversed upon addition of trifluoracetic acid. Thus, in the IR spectra ($CHCl_3$) the ketone carbonyl stretching at 1680-1700 cm^{-1} disappeared altogether, and the β-lactam band shifted to much lower frequencies (1765 cm^{-1}). The UV maximum at 252-273 nm (MeCN) was replaced by a new

absorption band (λ_{max} 380-401 nm, ϵ 10,000-15,000), conferring an intense yellow color to the solutions; for the most acidic compounds (13 and, to a minimal extent, 7,8,11,12), traces of the enolate were detectable in the absence of base. In the ^{1}H-NMR spectra, the H-2 singlet (2-substituted compounds) disappeared, and the two-proton ABq (2-unsubstituted compounds) was replaced by a broad, one-proton band at around 5 ppm. The H-6, H-7 coupling constant in all of the products became significantly smaller ($J \leq 1.5$ Hz), which is a feature we have observed in other sulfones blocked in the Δ^2-cephem structure.[8,9] Similarly, in the ^{13}C-NMR spectra the C-2 resonance was most affected: when a $CDCl_3$ solution of 12 was treated with triethylamine, this resonance shifted from 66.8 ppm down to 79.9 ppm. Interestingly, there was no indication of the presence of substantial amounts of the undissociated enol form I_b for any compound,[10] even under kinetic protonation conditions.

As a consequence of enolate formation under alkaline conditions, the absorption spectra of compounds in aqueous solutions are strongly influenced by the pH (Figure 1). This light absorption change has been exploited for pK_a determination, and the results are collected in Table 1. As expected, the relative acidity of the compounds is dictated by the type of substitution. Substitution at C-2 with a sulfur atom bound to electron-withdrawing heterocycles lowers the pK_a by more than 3 units, while substitution with the same moieties at the exocyclic methyl carbon (C-3') is much less effective at stabilizing the enolate ion. Conjugation with a phenyl group as in compounds 9-13 further increases acidity. The apparent pK_a of the phenyl ketones is about 3 units lower than that of the corresponding *tert*-butyl ketones, and is in the range of carboxylic acids.

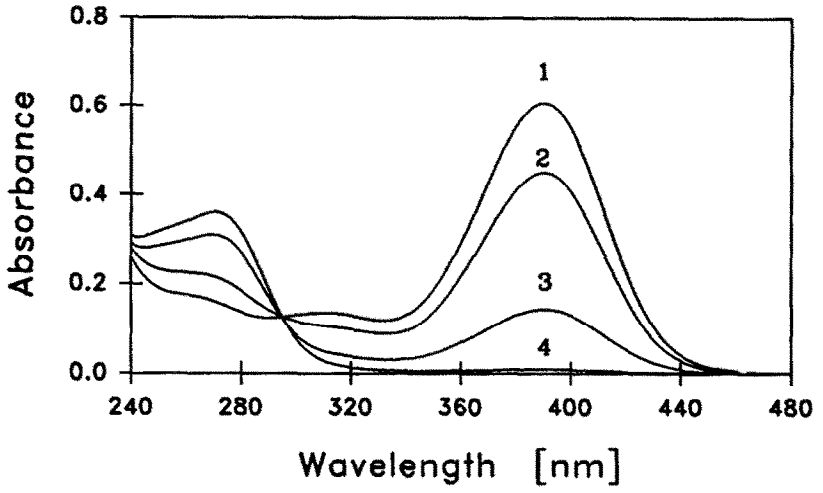


Figure 1. Absorption spectra of a 34 μM aqueous solution of 7 at various pH values: 1, pH 8.56; 2, pH 6.53; 3, pH 5.53 ; 4, pH 4.20. The isosbestic point at 295 nm indicates that only one prototropic equilibrium is present.

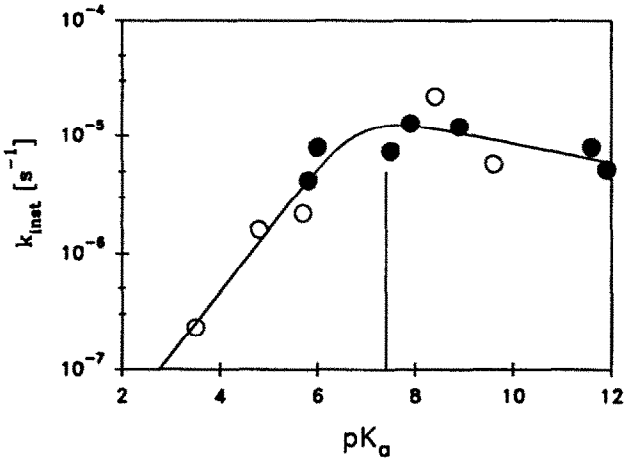


Figure 2. Correlation between the pseudo-first-order rate constant, $k_{inst} = 0.693/t_{1/2}$, for chemical hydrolysis at pH 7.4 and 37°C, and pK_a for cephem phenyl-ketones (compounds **9-13**, empty circles) and *tert*-butyl-ketones (compounds **2-8**, filled circles). Compound **1** is not included, as the corresponding pK_a value could not be determined. The interpolating line is a result of data fitting to a bilinear model:[12] $\log_{10}k_{inst} = 0.083\ \log_{10}K_a - 0.625\ \log_{10}(6.8\ 10^6K_a + 1) - 4.23$. The vertical line corresponds to $pK_a = 7.4$, the pH value of the chemical stability assay.

The pK_a values provide a mean for estimating the relative amount of the enolate form present under the conditions (pH 7.4, 37°C) of two assays routinely performed on the compounds, i.e. hydrolytic stability and efficiency of HLE inhibition, whose results are also reported in Table 1. Stability towards hydrolysis (chemical half-life) is an important parameter to monitor, as potent inhibitors usually have highly activated β-lactam bonds, and are therefore chemically unstable.[1] Activation of the cephem nucleus by inserting substituents at the C-2 or C-3′ position of the prototypic ketones **1** and **9** was expected to be accompanied by a decrease in their hydrolytic stability. This is the case, indeed, for compounds **2** and **3**, and for others, whose pK_a is not compatible with a substantial degree of enolate formation at pH 7.4. In the phenyl ketone series, in particular (compounds **10-13**), there is a strict inverse correlation between pK_a and chemical half-life. This increase in stability for the acidic compounds can be accounted for on the basis that mesomeric delocalization of the nitrogen lone pair out of the β-lactam ring, considered to be an important contribute to the reactivity of the Δ^3-cephem nucleus,[11] cannot be assisted in the enolate anion structure. In general, the correlation between the log of hydrolytic rate constants and pK_a (see Figure 2) is well depicted by a bilinear model: an empirical treatment introduced to manage cut-off or

breaks in structure-activity relationships.[12] Significantly, the break occurs at $pK_a \cong 7$, which is near the pH value used for stability investigation and thus at the border between predominance of keto or enolate form under the assay conditions. In the low pK_a branch stability increases (k_{inst} decreases) with lowering pK_a and thus paralleling enolate form stabilization. As already mentioned, in the high pK_a branch, where keto-forms predominate, stability decreases with decreasing pK_a as a result of increasing electron withdrawing ability of the R', R'' substituents. The limited range of overlap for the two data series requires a word of caution on possible generalization of the model, which remains however a useful empirical guideline for data interpretation.

The efficiency of HLE inhibition is reported in Table 1 as the second order rate constant, k_{on}, for the loss of enzyme activity. Suitable activation with an electron withdrawing group at C-3' is a well known requirement for high k_{on} values in cephem sulfone derivatives, and the new family of 4-ketones makes no exception (compare 2,3 vs. 1 and 10 vs. 9). Interestingly, in the sub-group of the *tert*-butyl ketones even higher k_{on} values were attained by compounds substituted at C-2 (4-6), in support of the electronic equivalence of the C-2 and C-3' position at β-lactam activation. In the case of phenyl ketones (11-13) this trend was not observed; values of k_{on} drop 10-100 fold for these highly acidic C-2 substituted compounds with respect to the C-3' substituted analogue 10. Such a drop in inhibitory potency might have been anticipated by considering the relative inertness of the β-lactam ring in the enolate form, and the presence of the negative charge close to the scissile bond. However, other factors influence recognition by the enzyme. In fact, enolate form in a *tert*-butyl ketone is perfectly tolerated by the enzyme; compound **8** (pK_a 5.8), that is mostly in the charged form I_c at pH 7.4, is equally potent as an HLE inhibitor as compound **2** (pK_a 11.6).

In conclusion, we have shown that it is possible to combine in one structure excellent HLE-inhibitory potency, high hydrolytic stability,[13] and adequate solubility in water[14]. These properties are deemed essential for compounds under evaluation as potential drugs for the cure of chronic pulmonary diseases such as emphysema and cystic fibrosis.[15] The activity of **4**,**5**,**7** and **12** in animal models of HLE-induced emphysema will be reported in due time.

Acknowledgments. We would like to express our deep thanks to Giovanni Franceschi and Giuseppe Cassinelli for continuous encouragement and discussion.

References and Notes

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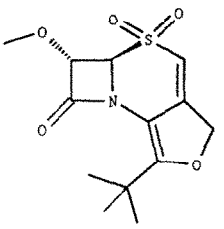
9. Preliminary attempts to isolate 4-cephem enol ether derivatives failed. However, we prepared the tricyclic structure shown aside, which can be considered a rigid enol ether derivative of the *tert*-butyl ketone 1. Peculiar features of this compound were the UV absorption (MeCN) at 312 nm (ϵ 16,500) and the very small coupling constant (J < 1 Hz) between H-6 and H-7 in the ^{1}H-NMR spectrum.



10. The UV spectra of the compounds were scrutinized in a variety of solvents, including ethanol and dimethylsulfoxide. The phenyl ketones display a shoulder at 280-290 nm beyond the main absorption of the indissociated species at 260 nm, and a shoulder or a relative maximum at 310-320 nm before the main enolate absorption at ca. 400 nm. However, the former is not compatible with the absorption expected for the free enol (see also ref. 9), and the latter seems part of the spectrum of the enolate molecule (as judged by the constant intensity ratio of the two bands).
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