Cephem Sulfones as Inactivators of Human Leukocyte Elastase. 5.[1] 7α-Methoxy- and 7α-Chloro-1,1-dioxocephem 4-Ketones

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Studies on cephem sulfones as inhibitors of human leukocyte elastase (HLE) have been extended to the new class of cephem 4-ketones. *tert*-Butyl and phenyl ketones were prepared from 4-carboxycephem derivatives, at either the sulfide or sulfone oxidation level, by chemoselective Grignard reaction. Obtained products were functionalized with heterocyclothio and acyloxy substituents at C-3′, C-2, or both positions. *tert*-Butyl ketones of the 7α-chlorocephem series were in general at least as potent as the corresponding esters at inhibiting the enzyme, but improvements in hydrolytic stability were only marginal. On the other hand, *tert*-butyl ketones of the 7α-methoxycephem series combined potent biochemical activity with acceptable hydrolytic stability, thus overstepping the esters, thiolesters, and amides reported previously. In particular, the *tert*-butyl ketones possessing a heterocyclothio group at C-3′ or C-2 were at least as active as the corresponding *tert*-butyl esters but 1 order of magnitude more stable in physiologic buffers (pH 7.4, 37 °C). Introduction of acyloxy groups at C-2 delivered the most potent HLE inhibitors of the cephem class ever reported, with inhibition parameters often outside the determination limits of our standard protocol (second-order rate constant k_{on} > 2 000 000 M^{-1} s^{-1}; K_i at steady state <2 nM). Keto–enol tautomerism was found to depress activity and boost hydrolytic stability. Thus, double substitution with heterocyclic thiols produced compounds with diverging properties, according to the extent of enolate formation at the investigated pH (7.4): the weakly acidic *tert*-butyl ketones ($pK_a \geq 5.8$) proved to be potent inhibitors (k_{on} over 10^4 M^{-1} s^{-1}) with reasonable hydrolytic stability ($t_{1/2}$ = 30–75 h), while the phenyl ketones ($pK_a < 4$) were fair inhibitors (k_{on} over 10^3 M^{-1} s^{-1}; K_i at steady state ≈ 50 nM) with hydrolytic half-lives exceeding 1000 h. Selected compounds efficiently inhibited the degradation of insoluble bovine neck elastin by HLE in a concentration-dependent manner. Intracellular HLE of polymorphonuclear leukocytes was in general unaffected; however, a lipophilic cephem sulfone apparently able to inactivate the enzyme in living cells was identified.

Over the last 3 decades, structural variation of natural cephalosporins has been a stimulating field of research for the discovery of new antibacterial agents.[2] Renewed interest in this chemistry ensued from the discovery[3] that cephalosporins can be modified to become time-dependent inhibitors of human leukocyte elastase (HLE), a serine endopeptidase believed to be involved in the pathogenesis of pulmonary emphysema and other connective tissue diseases.[4] Important sites for modification in the cephem ring were the C-7 position, with small α-oriented substituents (chloro and methoxy) being preferred, and the sulfur atom, the sulfones ranking among the most potent inhibitors.[5] The C-3′ position was extensively investigated, and a correlation was found between the electron-withdrawing ability of the substituent and the rate of HLE inactivation.[6] Finally, masking of the free carboxyl group at the C-4 position of cephalosporins was found mandatory; esters **I** and amides **II** were prepared and extensively evaluated.[7]

Modeling studies revealed the importance of the C-4 substituent, which in the binding process is positioned around the S1′–S2′ sites of HLE.[7a] In fact, inhibitory properties of esters and amides were strongly dependent on the shape and lipophilicity of the groups appended. We considered the possibility of extending structure–activity studies by new modification at the C-4 position. In the past, modification of the C-4 carboxyl of natural cephalosporins was discouraged by the early observation that a free 4-carboxyl group is strictly required for antibacterial activity.[8] The different substrate specificity for HLE (an endopeptidase, as opposed to the bacterial exopeptidases) opens new possibilities to the medicinal chemist. Changing the chemical nature of the substituent at C-4, in addition to changing its shape and lipophilicity, is expected to alter the reactivity of the β-lactam and possibly the structural reorganization ensuing from β-lactam cleavage, which is essential to the enzyme inactivation mechanism.[1,9,10] On this ground, a research program devoted to the synthesis and evaluation of cephem sulfones other than C-4 esters **I** and amides **II** was undertaken in our laboratories. We have already reported on the class of the cephem 4-thiolesters **III**[11] and wish to describe here the more innovative 4-ketones **IV**, systematically investigated after exploratory chemical studies.[12]

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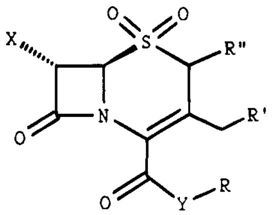
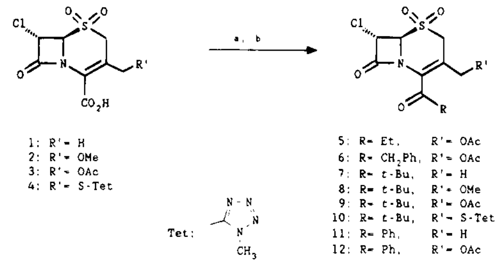


Chemistry. Our original synthetic plan[12a] entailed formation of the ketone moiety, as the last step, to be performed on cephem sulfones already carrying the desired C-7 and C-3′ substituents. The 4-carboxycephem sulfones **1–4** were prepared as previously reported[11] and coupled with Grignard reagents RMgCl (R = ethyl, benzyl, *tert*-butyl, phenyl) after activation of the carboxyl group under Vilsmeier conditions. Yields were poor (8–15%) and only marginally improved by the presence of copper(I) iodide; however, this straightforward strategy allowed the preparation of a representative set of 4-keto derivatives (**5–12**; Scheme 1) in the 7α-chlorocephem series.

The intrinsic reactivity of the 7-chloro-1,1-dioxocephem skeleton, further enhanced in **2–4** by the electron-withdrawing 3′-substituents, was held responsible for the modest yield of the Grignard reaction and for the hydrolytic lability of the products (see Discussion). Thence, we turned our attention to the 7α-methoxy analogs and examined a reversed sequence wherein the Grignard reaction is performed prior to oxidation at sulfur and functionalization at C-3′ (Scheme 2). The cheapest cephem template, 7β-amino-3-deacetoxycephalosporanic acid (7-ADCA), was converted in a single operation to the 7α-methoxy analog **13** using novel methodology.[13] Activation as the acid chloride **14** and reaction with *tert*-butylmagnesium chloride in the presence of CuI or, by an improved procedure, with lithium (phenylthio)(*tert*-butyl)cuprate afforded an inseparable mixture of Δ^2- and Δ^3-cephem ketones (**15**, **16**), which without purification could be oxidized by *m*-chloroperoxybenzoic acid (MCPBA) to provide the crystalline sulfone **17**. The overall yield of this pivotal intermediate from 7-ADCA did not exceed 25%, but the process is comparatively short and susceptible to scale up. The phenyl ketone **18** was prepared by the same sequence, using phenylmagnesium chloride in the presence of CuI.

The obtained 1,1-dioxocephem 4-ketones **17** and **18** were found amenable to regioselective functionalization at C-3′ (Scheme 2), C-2 (Scheme 3), or both positions (Scheme 4). Radical bromination with *N*-bromosuccinimide (NBS) under 1,1′-azobis(isobutyronitrile) (AIBN) catalysis occurred mainly at C-3′ to give the 3-(bromomethyl)cephem sulfones **19** and **20**, which reacted smoothly with heterocyclic thiols in the presence of triethylamine (TEA) to afford the corresponding 3-(heterocyclothio)methyl derivatives **22**, **23**, **25**, and **26** (Scheme 2). Compound **24**, bearing an acidic heterocycle (6-hydroxy-5-oxo-2,5-dihydro-1,2,4-triazine), was obtained by hydrolysis of the benzhydryloxy moiety of **23** with trifluoroacetic acid (TFA) in anisole; direct displacement of the bromine atom of **19** with the unprotected heterocyclic thiol was also possible, but in that case, purification was not trivial. Additionally, the 3-(acetyoxymethyl)cephem **21** was prepared by metathesis of the bromo precursor **19** with silver acetate in acetic acid.

Scheme 1. Synthesis of 7α-Chloro-1,1-dioxocephem 4-Ketones by Late Grignard Reaction[a]



[a] (a) Oxalyl chloride, THF, cat. DMF; (b) RMgCl, CuI, −70 °C.

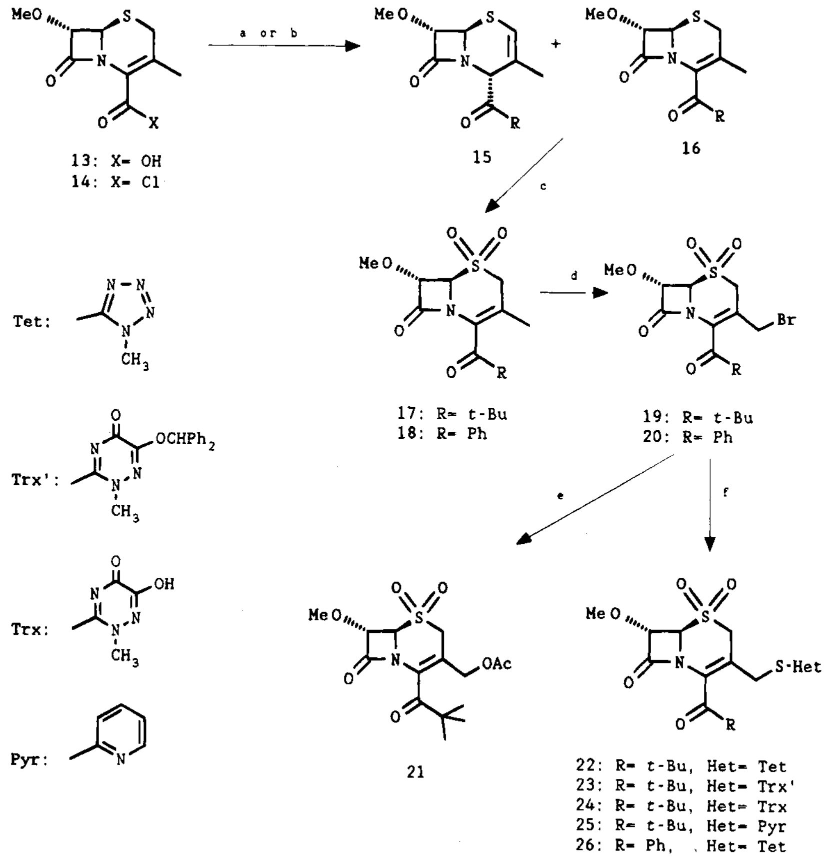
Bromination of the same pivotal 1,1-dioxocephem 4-ketones **17** and **18** under ionic conditions (stoichiometric amounts of NBS and TEA) afforded the corresponding 2-bromo derivatives of formula **28** and **29** (Scheme 3). Displacement of the halogen atom with several heterocyclic thiols was possible also in this case, providing the 2-(heterothio)cephems **31–35** in good to excellent yields. The analogy with 3′-bromocephems is only apparent because α-sulfonyl compounds were expected to be inert to S_N2 reactions[14] and 2-halocephem sulfoxides (in contrast to the corresponding sulfides[15]) were reported to be resistant to nucleophilic displacement.[15a] Indeed, we found that the bromide **30** resulting from the cephem ester **27** reacts sluggishly and undergoes reduction of the bromo atom rather than substitution. Reduction of the brominated ketones **28** and **29** occurred minimally during the preparation of **31–35** but became the main outcome of attempted displacement when the thiol reagent was 2-mercaptopyridine or thiophenol. In particular, upon reaction of **28** with 2 mol equiv of thiophenol and triethylamine, the reduced compound **17** (instead of **45**) and phenyl disulfide were isolated in quantitative yield.

Generation of the C-2 cephem carbanion by base treatment in the presence of a disulfide (or a synthetic equivalent) provided a novel and expedient route to the desired 2-substituted compounds in cases where displacement of 2-bromocephems failed. Thus, reaction of either ketone **17** or ester **27** with bis(2-pyridyl) disulfide in the presence of 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) provided **44** and **46**, respectively. The 2-phenylthio analog **45** was prepared by reaction of **17** with *S*-phenyl benzenethiosulfonate and DBN, but this compound was isolated in a mixture with the Δ^2-cephem isomer **47**. The single 2-phenylsulfonyl Δ^3-cephem **48** was obtained from both components by oxidation with excess MCPBA.

Displacement of 2-bromo-1,1-dioxocephem 4-ketones with silver salts of aliphatic and aromatic carboxylic acids in acetonitrile provided a novel type of cephem derivative, the 2-acyloxy compounds. From the *tert*-butyl ketone **28**, this reaction afforded **36–42** in fair yields. Minor byproducts were the 4-regioisomers **43**, which in few instances were isolated and characterized (e.g., **43**, R = Ph; 10%).[12c]

2,3′-Disubstituted cephem 4-ketones were obtained by a similar strategy (Scheme 4). Electrophilic bromination, occurring under extremely mild conditions, was compatible with the presence of an acetoxy and even a heterocyclothio group at C-3′. Thus, bromination of **21** afforded **50**, which underwent displacement with mercaptotetrazole to provide **55**. Similarly, **22** was brominated to **51** and then reacted with silver benzoate to afford **56**. The 2,3′-dibromo intermediates **49** and **57**

Scheme 2. Synthesis and 3′-Functionalization of 1,1-Dioxo-7α-methoxycephem 4-Ketones[a]



[a] (a) RMgX/CuI, THF, −70 °C; (b) PhSCu/t-BuLi, THF, −70 °C; (c) 55% MCPBA, CH_2Cl_2; (d) NBS, AIBN, reflux, CCl_4; (e) AgOAc, HOAc, MeCN; (f) HS-Het, TEA, MeCN.

were obtained from the unsubstituted precursors **17** and **18** either by electrophilic bromination followed by a second treatment with NBS under radical conditions or in a single step under forcing radical conditions. Displacement at both C-2 and C-3′ provided the bis(2,3′-benzoate) **54** and the bis(2,3′-heterocyclothio) derivatives **52**, **53**, **58**, and **59** in good yields. Extension of these procedures in the 7α-chlorocephem series provided a limited number of analogs (Scheme 5), represented here by the 2-substituted compounds **60** and **62** and the 2,3′-disubstituted derivative **61**.

Finally, Scheme 6 illustrates the preparation of the 4-(3-oxobutyl)cephem **66**, an isostere of cephem 4-ketones wherein the carbonyl group has been moved away from the dihydrothiazine ring. Michael addition to methyl vinyl ketone of the carbanion generated from the 1,1-dioxocephem p-methoxybenzyl ester **63** (TEA catalysis) gave the desired 4-adduct **64** (52%) along with the bis(2,4-adduct) **65** (24%).[12c] Acid hydrolysis (TFA) of the ester moiety of **64** was followed in alkaline solution (aqueous $NaHCO_3$) by spontaneous decarboxylation to afford **66**.

All of the Δ^3-cephem sulfones (either esters or ketones) described here adopt an "open" conformation for the dihydrothiazine ring. The ^1H NMR spectra of the 2-unsubstituted compounds exhibit long-range coupling for H-2α and H-6α ($J = 1.2-1.7$ Hz), which is associated with this conformation.[12c,16] This effect is absent in the spectra of the 2-bromo, 2-heterocyclothio, and 2-acyloxy derivatives, which were obtained as 2α-substituted isomers. However, in some cases, minor amounts of rapidly equilibrating 2β-epimers could be detected in $CDCl_3$ solution, typically, 5−7% for the 2-heterocyclothio 4-ketones (e.g., **31**, **44**) and up to 15% for the corresponding 4-esters (e.g., **46**). These isomers were characterized by the four-bond coupling between H-2 and H-6 ($J = 1.2-1.7$ Hz) and by a strong NOESY correlation for the same protons. A more extensive stereochemical analysis of 2-substituted cephem sulfones is presented elsewhere.[12c]

Results

Kinetics of HLE Inhibition. Inhibition of the amidolytic activity of HLE toward the chromogenic synthetic substrate MeO-Suc-Ala-Ala-Pro-Val-7-(4-methyl)coumarylamide was assayed on a routine basis. Product formation as a function of time was analyzed according to eq 1:

$$[P] = (v_0 - v_s)(1 - e^{-kt})/k + v_s t \qquad (1)$$

v_0 = initial reaction velocity

v_s = reaction velocity at steady state

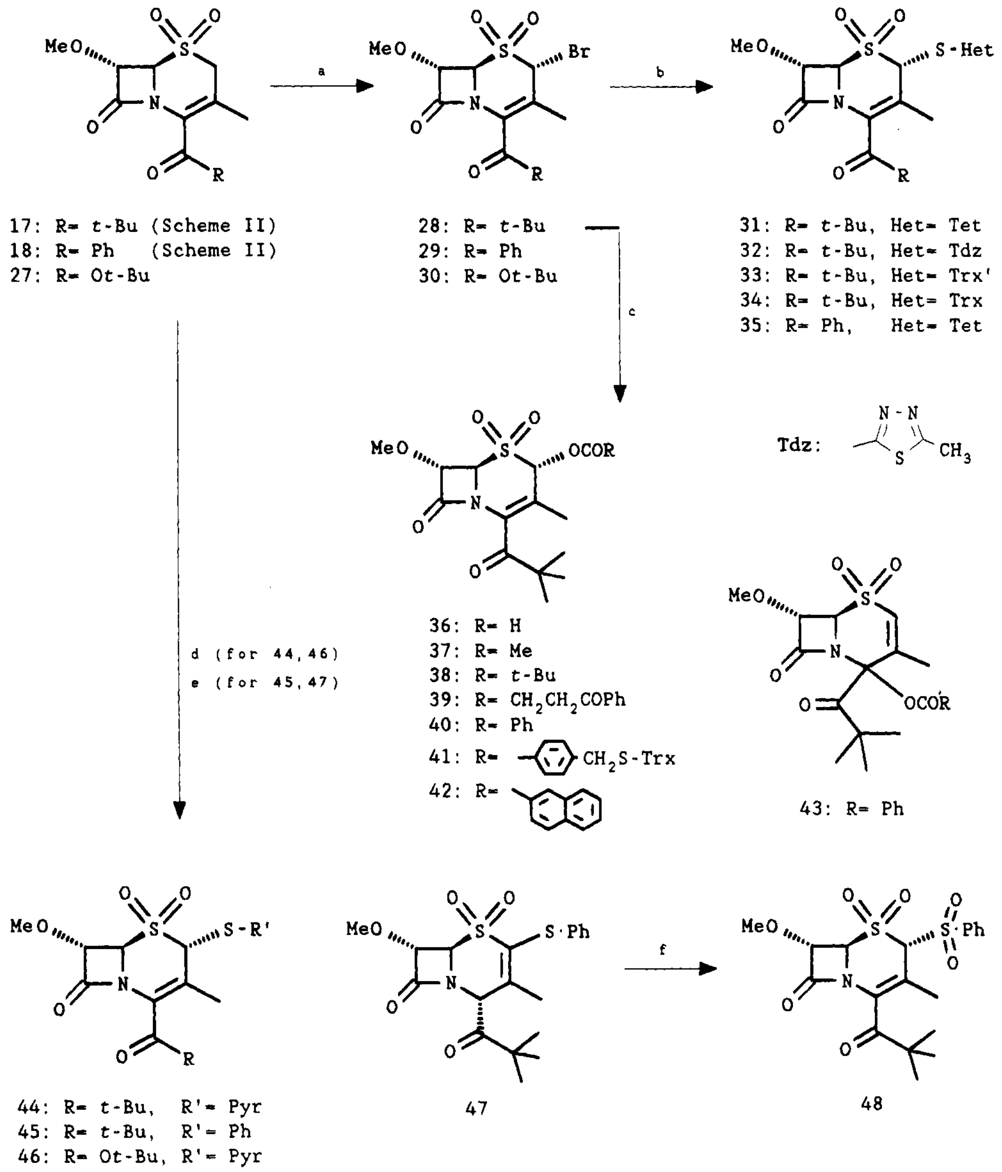
k = apparent first-order rate constant

In a few cases, dependence of the first-order rate constant k on inhibitor concentration followed that expected for a two-step (binding and reaction) mechanism:

$$k = k_{off} + k_{inact}/K_i \frac{[I]}{1 + [S]/K_m + [I]/K_i} \qquad (2)$$

More frequently, linear dependence was found ($K_i \gg [I]$),

Scheme 3. Synthesis of 2-Substituted 1,1-Dioxo-7α-methoxycephem 4-Ketones and the 4-Ester Reference Compound **46**[a]



[a] (a) NBS, TEA, CH_2Cl_2; (b) HS-Het, TEA, MeCN; (c) AgOCOR, MeCN; (d) Pyr-SS-Pyr, DBN, MeCN; (e) $PhSSO_2Ph$, DBN, MeCN; (f) MCPBA, CH_2Cl_2. Tet, Trx, Trx′, and Pyr: see Scheme 2.

and k_{inact}/K_i was the only experimentally accessible parameter. For consistence, only values of these second-order rate constants (k_{on}) are reported in Tables 1–3. For reasons which have been discussed in our previous report,[11] values of k_{off} are considered inaccurate and are not reported.

The dependence of the steady state velocity v_s on inhibitor concentration was analyzed with eq 3, the ordinary hyperbolic law for irreversible inhibition, and the steady state inhibition constant K_i(ss) was derived.

$$v_s = v_u \frac{1 + [S]/K_m}{1 + [S]/K_m + [I]/K_i(ss)} \quad (3)$$

v_u = reaction velocity in the absence of inhibitor

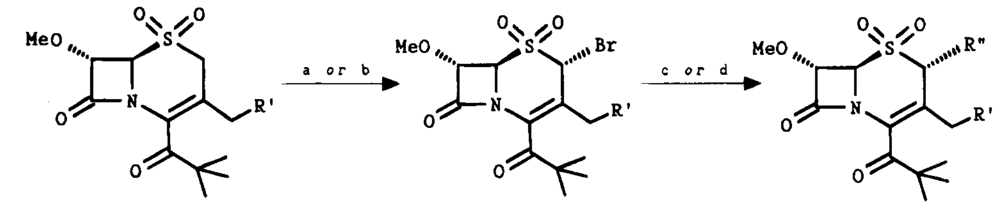
$$K_m = 1.2 \times 10^{-3}\ M$$

Systematic deviations from eq 3, as observed for the most potent inhibitors, indicate that K_i(ss) is not a true steady state parameter.

The two selected parameters of HLE inhibition, k_{on} and K_i(ss), are collected in Tables 1-3. The second-order rate constant k_{on} is directly comparable to previously reported parameters, k_2/K_i[11] and $k_{obs}/[I]$,[5,6] and combines information on both enzyme recognition and inhibitor reactivity. The steady state inhibition constant K_i(ss) contains further information on early reactivation of enzymatic activity and is reported as an empirical index of relative in vitro potency of the inhibitors.

Inhibition of the Elastinolytic Activity of HLE. Testing inhibitors of HLE in the presence of insoluble elastin rather than a synthetic peptide as the substrate is a closer model to the biological situation. Elastinolysis by HLE is a slow process, and the assay must be carried out with relatively high enzyme concentrations and long incubation periods to obtain reliable readings of released elastin peptides; as previously discussed,[11] an HLE concentration of 0.5 μM and an incubation time

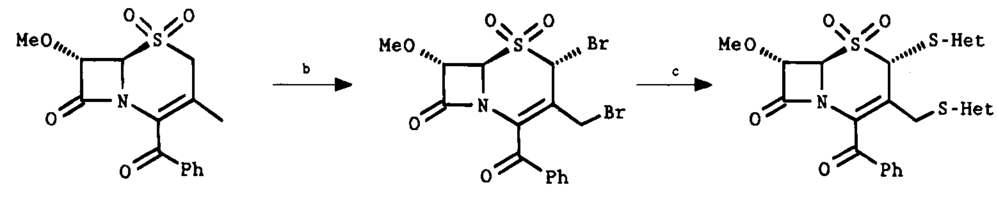
Scheme 4. Synthesis of 2,3′-Disubstituted 1,1-Dioxo-7α-methoxycephem 4-Ketones[a]



17: R′= H (Scheme II)
21: R′= OAc (Scheme II)
22: R′= S-Tet (Scheme II)

49: R′= Br
50: R′= OAc
51: R′= S-Tet

52: R′= S-Tet, R″= S-Tet
53: R′= S-Tdz, R″= S-Tdz
54: R′= OBz, R″= OBz
55: R′= OAc, R″= S-Tet
56: R′= S-Tet, R″= OBz



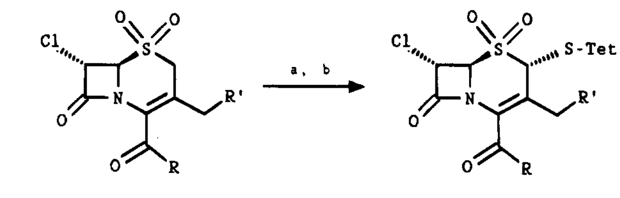
18 (Scheme II)

57

58: Het= Tet
59: Het= Tdz

[a] (a) NBS, TEA, CH_2Cl_2; (b) NBS, reflux, CCl_4; (c) HS-Het, TEA, MeCN; (d) AgOBz, MeCN. Tet and Tdz: see Schemes 1 and 3.

Scheme 5. Synthesis of Selected 7α-Chloro-1,1-dioxocephem 4-Ketones Incorporating a Mercaptotetrazole Group at C-2′ [a]



7: R= *t*-Bu, R′= H (Scheme I)
9: R= *t*-Bu, R′= OAc (Scheme I)
11: R= Ph, R′= H (Scheme I)

60: R= *t*-Bu, R′= H
61: R= *t*-Bu, R′= OAc
62: R= Ph, R′= H

[a] (a) NBS, TEA, CH_2Cl_2; (b) HS-Tet, TEA, MeCN. Tet: see Scheme 1.

of 2 h were selected as a good compromise. Results for the 3′-substituted ketones **10** and **22**, the 2-substituted ketones **31** and **35**, the 2,3′-disubstituted ketone **52**, and the reference amide **70** (Merck L-659,286;[7,17] structure in Table 3) are reported in Figure 1.

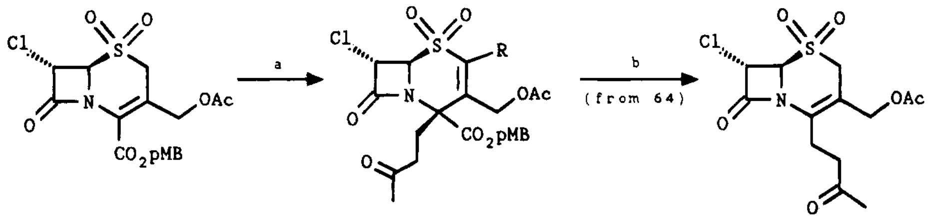
Inhibition of HLE in Living PMNs. For a few compounds (ketones **24**, **31**, **34** and the reference amide **70**), the ability to inhibit intracellular elastase in living polymorphonuclear leukocytes (PMNs) was tested according to a modification of the method of Bonney et al.[17] (see Experimental Section). Briefly, PMNs were prepared from freshly collected blood, incubated with the inhibitor, and washed with fresh incubation solvent before stimulation promoted by cytocalasin B and formylmethionyl-leucyl-phenylalanine (fMLP); elastase release in the supernatant was measured by a sensitive fluorimetric assay. Reduction of elastase activity expressed by treated cells (Table 4) is related to the ability of the compound at inhibiting the enzyme intracellularly or in the course of the degranulation process (see Discussion).

Hydrolytic Stability. Both the alkaline hydrolysis of β-lactam compounds and the process by which they may inactivate serine proteases involve attack of a nucleophilic oxygen upon the β-lactam carbonyl carbon. Not surprisingly, a broad correlation between hydrolytic reactivity and biological activity was observed for antibacterial cephalosporins.[18] A similar correlation is expected for cephalosporins modified to become HLE inhibitors (Figure 2 and Discussion); thus, recognition of structural features promoting HLE inhibition over chemical reactivity is crucial for the correct interpretation of biochemical data. On this ground, we undertook systematic determination of the hydrolytic stability of novel and reference cephem derivatives.

Because of analogy with the medium used in kinetic studies of HLE inhibition, hydrolytic stability was assayed at 37 °C in 50 mM pH 7.4 phosphate buffer in the presence of 2% (v/v) acetonitrile as solubilizing vehicle. The initial concentration of the inhibitors was 0.2 mM or less, depending on solubility. To prevent any catalytic effect of metal ions possibly present as impurities, 0.1 mM EDTA was included; EDTA itself, also checked at 1 mM levels, had no effect on the degradation rate of cephem sulfones. Stability of selected products was investigated over a wide range of pH values (3–12). In these cases, phosphate buffer concentration was reduced to 20 mM and the total ionic strength made constant ($\mu = 0.2$) by addition of KCl. The water-soluble product **52** was used as a model compound to check the influence of the medium and selected conditions. Degradation rate constants were found invariant in the range 0.05–0.5 mM initial concentration. Practically no kinetic primary salt effect was observed in the range $\mu = 0.05$–0.2 at 37 °C and pH 3, 7, and 11. A modest catalytic effect of phosphate was found, a 1–4% increase of degradation rate due to 20 mM phosphate at pH 3–4, 14–15% at pH 6–8, and 27% at pH 11–12. Finally, the presence of 2% acetonitrile was found to reduce the degradation rate of **52** in 20 mM phosphate buffers (pH 3, 7, and 11; $\mu = 0.2$; 37 °C) by a factor of 0.9.

Most of the examined cephem sulfones followed the usual pseudo-first-order exponential decay:

Scheme 6. Synthesis of the Homologated Cephem 4-Ketone **66**[a]



63 64: R= H 65: R= CH_2CH_2COMe 66

[a] (a) Methyl vinyl ketone, TEA; (b) TFA, anisole, CH_2Cl_2; then aq $NaHCO_3$. pMB: 4-methoxybenzyl.

$$A \xrightarrow{k_{st}} \text{products} \qquad (4)$$

$$[A] = [A_0]e^{(-k_{st}t)} \qquad (5)$$

where [A] and [A_0] are the concentrations of investigated compounds A at times t and zero. However, some products showed double-exponential decay, thus indicating that a second species, B, is involved in a general degradation pathway:

$$\text{products} \xleftarrow{k_1} A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} B \xrightarrow{k_2} \text{products} \qquad (6)$$

$$[A] = C_1e^{-\alpha t} + C_2e^{-\beta t} \qquad (7)$$

From experimental parameters, an empirical stability rate constant, k_{st}, can be defined as

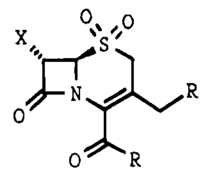
$$k_{st} = \alpha\beta(C_1 + C_2)/(\beta C_1 + \alpha C_2) \qquad (8)$$

This is related to the kinetic constants of mechanism 6 by

$$k_{st} = k_1 + k_2K_{eq}/(1 + k_2/k_4) \qquad (9)$$

where $K_{eq} = k_3/k_4$, [A] = [A_0] at t = 0, and [B] = 0 at t = 0. Complete assignment of values of k_1-k_4 cannot be made unless concentration of species B is followed over time together with concentration of A, which is generally not possible in the present case. Thus k_{st} values obtained from eq 8 currently represent the best possible estimates of hydrolytic stability. It should be noted that if decomposition of isomeric form B is fast with respect to equilibration with the original structure A ($k_4 \ll k_2$), then the steady state level of B is very low and time dependence of A approaches an apparent single-exponential decay with an observed rate constant of $k_{st} \approx k_1 + k_3$. Conversely, if a double-exponential decay is clearly observed and equilibrium is fast with respect to decomposition of B ($k_4 \gg k_2$), then B accumulates and the apparent stability rate constant calculated according to eq 8 becomes $k_{st} \approx k_1 + k_2K_{eq}$. This was indeed observed with products showing double-exponential decay, when isomeric peaks could be detected in comparatively relevant amounts and clearly recognized (e.g., product **25**, just to mention a typical case), though their absolute quantitation was impossible due to lack of the reference standard products. Nonetheless, the value of k_{st} obtained from eq 8 corresponds to an estimation of the total β-lactam-opening rate, which is, in our opinion, the most appropriate parameter to describe hydrolytic stability even though two different isomeric forms of cephem sulfones may have different

Table 1. Kinetic Parameters of HLE Inhibition and Hydrolytic Stability of 3′-H and 3′-Substituted 1,1-Dioxocephem 4-Ketones



no.	R	R′	HLE inhibition[a] k_{on} (M^{-1} s^{-1})	HLE inhibition[a] K'_i(ss) (nM)	$t_{1/2}$[b] (h)
		7α-Chlorocephem 4-Ketones (X = Cl)			
5	Et	OAc	7.3×10^4	11	1.1
6	CH_2Ph	OAc	4.9×10^4	100	0.2
7	t-Bu	H	2.1×10^3	380	5.6
8	t-Bu	OCH_3	1.5×10^4	170	6.9
9	t-Bu	OAc	2.3×10^5	4	3.9
10	t-Bu	STet	1.2×10^5	7	3.0
11	Ph	H	8.0×10^3	130	2.3
12	Ph	OAc	1.5×10^5	12	1.5
		7α-Methoxycephem 4-Ketones (X = OCH_3)			
17	t-Bu	H	9.0×10^1	1300	106
18	Ph	H	1.3×10^2	2700	26
21	t-Bu	OAc	2.4×10^4	11	14*
22	t-Bu	STet	1.9×10^4	34	24*
24	t-Bu	STrx	4.5×10^4	27	16*
25	t-Bu	SPyr	2.3×10^4	12	28*
26	Ph	STet	1.8×10^4	110	17*

[a] Second-order rate constant for enzyme inactivation, k_{on}, and steady state inhibition constant, K_i(ss), are defined in Results and were determined at 37 °C, pH 7.4, with the substrate MeO-Suc-Ala-Ala-Pro-Val-7-(4-methyl)coumarylamide. [b] Chemical stability at 37 °C, pH 7.4, was expressed as $t_{1/2} = 0.693/k_{st}$. For a definition of k_{st}, see Results. Data of compounds showing double-exponential decay are marked with an asterisk.

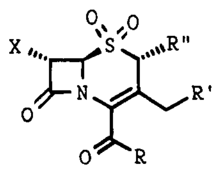
enzymatic activities. This closely resembles the pharmacokinetics of drugs in the blood, which are generally described by reporting and referring to the total drug concentration in the blood, though reversible binding to the serum proteins can affect their biological characteristics. Speculations about the chemical structure of isomeric forms B are attempted under Discussion.

Hydrolytic stability data were expressed as chemical half-lives, $t_{1/2}$ (h), obtained as $0.693/k_{st}$; these are included in Tables 1–3. A plot of log $t_{1/2}$ vs log k_{on} (the second-order rate constant for HLE inhibition, see above) for 7α-methoxycephem sulfones is presented in Figure 2 to illustrate the broad inverse correlation existing betwen hydrolytic stability and reactivity toward the enzyme at pH 7.4 and highlight interesting deviations (see Discussion). The stability profile of selected products (the 4-ketones **22**, **24**, **31**, **52**, **58**, the reference ester **69**, and the reference amide **70**) in a wide range of pH values is reported in Figures 3 and 4.

Discussion

Considerable experimental evidence indicates that

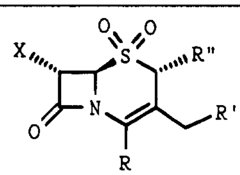
Table 2. Kinetic Parameters of HLE Inhibition and Hydrolytic Stability of 2-Substituted and 2,3′-Disubstituted 1,1-Dioxocephem 4-Ketones

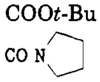
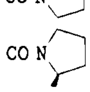


no.	R	R′	R″	HLE inhibition[a] k_{on} ($M^{-1} s^{-1}$)	K_i(ss) (nM)	$t_{1/2}$[b] (h)
			7α-Methoxycephem 4-Ketones (X = OCH_3)			
31	*t*-Bu	H	STet	1.0×10^5	8	18
32	*t*-Bu	H	STdz	4.7×10^4	12	27
34	*t*-Bu	H	STrx	5.3×10^4	26	21
35	Ph	H	STet	2.3×10^2	1400	200
36	*t*-Bu	H	OCHO	1.4×10^3	600	<0.5
37	*t*-Bu	H	OAc	6.5×10^4	4	6.3
38	*t*-Bu	H	$OCOCMe_3$	1.5×10^6	<2	54
39	*t*-Bu	H	$OCOCH_2CH_2COPh$	3.0×10^5	<2	13*
40	*t*-Bu	H	OBz	1.5×10^6	<2	24
41	*t*-Bu	H	$OCOC_6H_4CH_2STrx$	7.0×10^5	2	7.5
42	*t*-Bu	H	$OCOC_{10}H_7$	$>2 \times 10^6$	<2	33
44	*t*-Bu	H	SPyr	8.2×10^4	10	24
48	*t*-Bu	H	SO_2Ph	2.8×10^4	20	10*
52	*t*-Bu	STet	STet	2.6×10^4	93	31
53	*t*-Bu	STdz	STdz	2.1×10^4	21	75
54	*t*-Bu	OBz	OBz	1.6×10^6	<2	1.2*
55	*t*-Bu	OAc	STet	2.0×10^4	6	10
56	*t*-Bu	STet	OBz	$>2 \times 10^6$	<2	0.7
58	Ph	STet	STet	2.2×10^3	55	1400
59	Ph	STdz	STdz	3.9×10^3	50	1300
			7α-Chlorocephem 4-Ketones (X = Cl)			
60	*t*-Bu	H	STet	1.0×10^4	300	3.2
61	*t*-Bu	OAc	STet	5.7×10^3	140	1.8
62	Ph	H	STet	1.3×10^3	270	4.1

[a,b] See corresponding footnotes in Table 1.

Table 3. Kinetic Parameters of HLE Inhibition and Hydrolytic Stability of Model 1,1-Dioxocephem Derivatives



no.	R	R′	R″	HLE inhibition[a] k_{on} ($M^{-1} s^{-1}$)	K_i(ss) (nM)	$t_{1/2}$[b] (h)
		7α-Chlorocephems (X = Cl)				
66	CH_2CH_2COMe	OAc	H	2.1×10^2	ND	10
67	COOMe	OAc	H	1.3×10^5	12	0.9
		7α-Methoxycephems (X = OCH_3)				
46	COO*t*-Bu	H	SPyr	4.7×10^3	180	4.1*
68	COO*t*-Bu	SPyr	H	9.0×10^2	100	5.1*
69	COO*t*-Bu	STet	H	2.0×10^4	13	3.8
70[c]		STrx	H	9.2×10^3	75	25
71[d]		OAc	H	1.8×10^3	200	18

[a,b] See corresponding footnotes in Table 1. [c] Merck L-659,286 (refs 7, 17); sample prepared in our laboratories. [d] Merck L-658,758 (ref 7b); sample prepared in our laboratories. Lit. $k_{obs}/[I] = 3.8 \times 10^3\ M^{-1} s^{-1}$. ND: not determined.

cephalosporin sulfones are either substrates or mechanism-based suicide inhibitors of HLE. The proposed mechanism of inhibition, as inferred from crystallographic data,[6,9a] biochemical studies,[9b] and structural characterization of enzyme–inhibitor complexes and byproducts,[1,9b,10] involves β-lactam ring opening by the catalytic Ser-195 residue, expulsion of a leaving group at the 3′-position of the cephem moiety, and consequent covalent or noncovalent binding to the His-57 residue of the enzyme catalytic triad. In agreement with this proposal and with our previous observations on other cephem sulfone derivatives,[11] the cephem 4-ketones having no adequate leaving group attached on the dihydrothiazine ring (**7**, **11**, **17**, **18**) behaved as poor substrates rather than as inhibitors and were slowly but completely hydrolyzed by HLE. On the contrary, incubation of the 3′-activated cephem 4-ketones with mi-

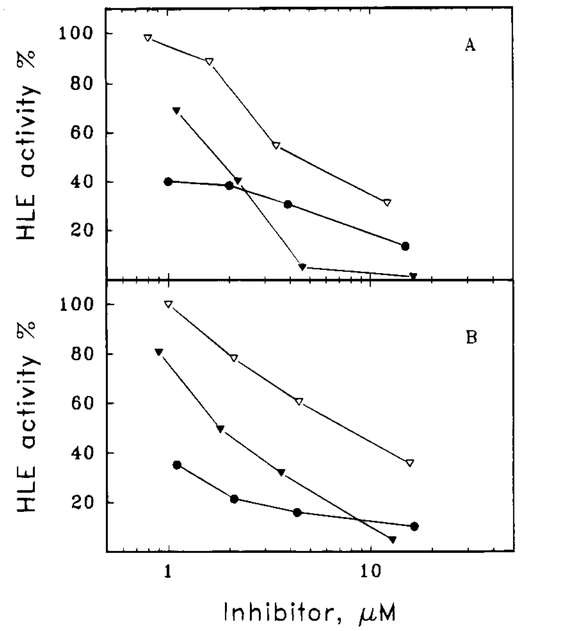
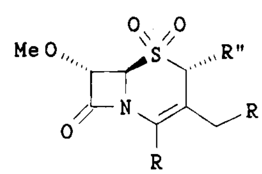
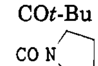
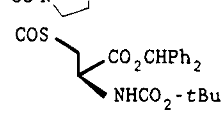


Figure 1. Inhibition of the lytic activity of HLE on elastin from bovine neck ligament by selected compounds. 3′-Substituted cephem derivatives (panel A) are represented by two *tert*-butyl ketones of the 7α-chloro and 7α-methoxy series, respectively **10** (▼) and **22** (●), and by the reference amide **70** (▽). 2-Substituted cephem derivatives (panel B) are represented by the *tert*-butyl ketone **31** (●) and the phenyl ketone **35** (▼), while 2,3′-disubstituted derivatives are represented by the *tert*-butyl ketone **52** (▽). Relative values refer to control experiments carried out in the absence of inhibitors.

Table 4. Inhibition of HLE in Living Polymorphonuclear Leukocytes



compound	R	R′	R″	HLE activity (%)[a]
24	CO*t*-Bu	STrx	H	70
31	CO*t*-Bu	H	STet	100
34	CO*t*-Bu	H	STrx	102
70[b]		STrx	H	98
FCE 26251[c]		STet	H	8

[a] Activity expressed by stimulated PMNs after incubation with inhibitors and washing relative to that expressed by cells exposed to the same treatment in the absence of inhibitors (see Experimental Section). [b] Merck L-659,286; sample prepared in our laboratories. Lit.:[17] 108%. [c] (*S*)-2-[(Benzhydryloxy)carbonyl]-2-[(*tert*-butoxycarbonyl)amino]ethyl 7α-methoxy-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-3-cephem-4-thiolcarboxylate 1,1-dioxide (compound **II–8c** in ref 11). This highly lipophilic compound was included as a positive control in the assay.

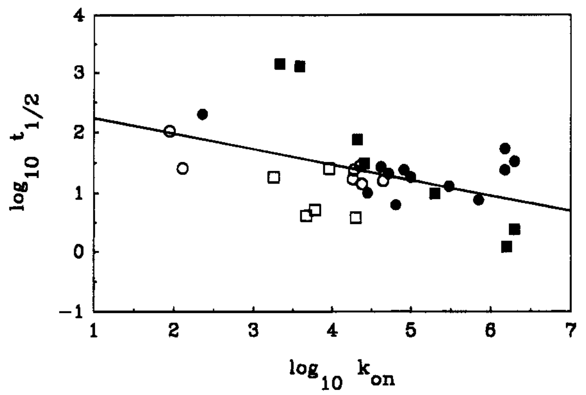


Figure 2. Correlation between biological activity ($\log_{10} k_{on}$ for HLE inhibition) and hydrolytic stability ($\log_{10} t_{1/2}$ at pH 7.4, 37 °C) for 7α-methoxycephem sulfones of Tables 1−3: 4-esters and 4-amides (□), 3′-substituted 4-ketones (○), 2-substituted 4-ketones (●), and 2,3′-disubstituted 4-ketones (■).

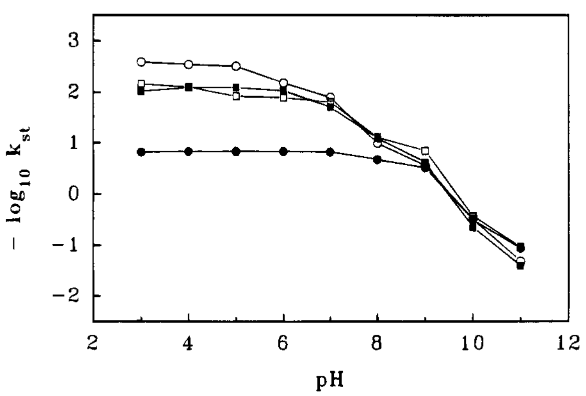


Figure 3. Hydrolytic stability profiles (plots of $-\log_{10} k_{st}$, see Experimental Section, vs pH) for 7α-methoxycephem sulfones representative of different substitution patterns at C-4: *tert*-butyl ketones **22** (○) and **24** (□), *tert*-butyl ester **69** (●), and amide **70** (■).

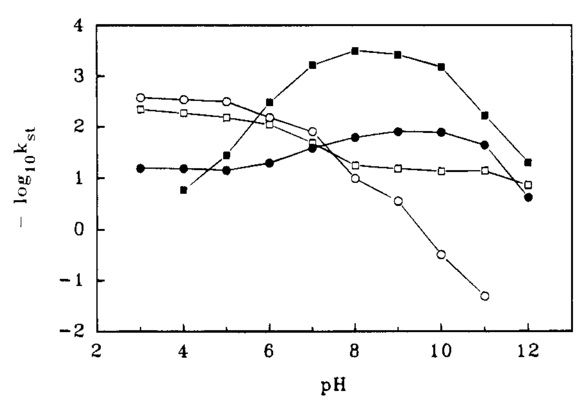


Figure 4. Hydrolytic stability profiles for 7α-methoxycephem sulfones representative of different enolization ability at the 4-keto moiety: 3′-substituted *t*-Bu ketone **22** (○; $pK_a = 11.6$), 2-substituted analog **31** (□; $pK_a = 7.9$), and 2,3′-disubstituted derivatives *t*-Bu ketone **52** (●; $pK_a = 6.0$) and Ph ketone **58** (■; $pK_a = 3.5$).

cromolar HLE for a moderately long time (30 min at 37 °C) confirmed the irreversible nature of inhibition and provided an estimate of the relative rate of side reactions leading to early recovery of enzyme activity (k_{hyd}) in comparison to that of the enzyme inactivation process (k_{inact}). This was obtained from the x-axis intercept, $[I_0]$, of linear plots of residual enzyme activity as a function of inhibitor concentration, [I]. The ratio r between $[I_0]$ and the total enzyme concentration $[E_t]$ is related to the two reaction rates by eq 10:

$$r = [I_0]/[E_t] = 1 + k_{hyd}/k_{inact} \qquad (10)$$

Values of r close to one, as obtained for representative compounds of the 7α-chloro and 7α-methoxy series (**10**, $r = 1.8$; **22**, $r = 1.6$), indicate extremely efficient enzyme inhibition by the 4-ketocephem derivatives. In comparison, values of r vary from 1.5 to 2.7 for 4-esters and thiolesters[11] and increase up to 3−5 for 4-amide derivatives.[9b]

Close analogy between the 4-ester class and the novel 4-ketones is immediately perceived in a comparison of kinetic data for the ester **67** (Table 3) and its isosteric ketone **5** (Table 1): the two compounds have similar values of both k_{on} and K_i(ss). Activation of the β-lactam carbonyl by the proximal C-4 ester or ketone carbonyl is a common feature of the two structural series. Its importance is confirmed by the considerable decrease of k_{on} in the model compound **66** (Table 3), which contains an aliphatic spacer between the carbonyl group and the dihydrothiazine ring. The analogy between 4-esters and 4-ketones in the 7α-chlorocephem series extends to previously recognized[6,11] structure−activity relationships: k_{on} increases with increasing electron-withdrawing ability of the 3′-substituent (from **7** to **10**; Table 1); bulky substituents (*t*-Bu, Ph) at the carbonyl group are preferred. When both hydrolytic stability and HLE inhibition efficiency are taken into account as selection criteria, 7α-chlorocephem 4-*tert*-butyl ketones perform marginally better than the corresponding 4-*tert*-butyl esters or 4-*tert*-butyl thiolesters. For instance, the ester analogs[11] of **9** and **10** both have $k_{on} = 1.5 \times 10^5$ M^{-1} s^{-1} and $t_{1/2} = 1.3$ and 1.2 h, respectively; the corresponding thiolesters[11] have better activity ($k_{on} = 6.4 \times 10^5$ and 10×10^5 M^{-1} s^{-1}) but lower stability ($t_{1/2} = 0.6$ and 0.7 h). Nevertheless, the hydrolytic stability of all of the 7α-chlorocephem sulfones remained unsatisfactory.

Hydrolytic stability is a prerequisite for stability in biological fluids, such as pulmonary epithelial lining fluid and plasma. Preliminary investigations[19] revealed that the degradation rates of representative cephem sulfones in human plasma are roughly 20−160 times greater than the corresponding rates in aqueous media at the same pH. Thus, hydrolytic half-lives (pH 7.4, 37 °C) were used as an initial screen before evaluation in vivo. Empirically, we set a lower limit of 12 h for compounds intended for topical administration (aerosol) and 100 h for prospective compounds for systemic (iv or oral) administration. Inhibitors fulfilling these hydrolytic stability criteria were found among the 7α-methoxycephem derivatives. In this series, the 2,3′-unsubstituted 4-ketones (**17** and **18**) were virtually inactive, but the 3′-substituted compounds (**21−26**, Table 1), in return for a moderate decrease of activity as compared to their 7α-chloro analogs, attained hydrolytic half-lives in the 20 h range. These compounds, with k_{on} always exceeding 10^4 M^{-1} s^{-1} and K_i(ss) in the 10−30 nM range, eclipsed the corresponding 4-esters[6,11] in terms of stability (**21** and **25**, Table 1, are 6 times more stable than **69** and **68**, Table 3) and the 4-amides[7] in terms of activity (**21**, **24** vs **70**, **71**)

Facile functionalization at C-2 with heterocyclic thiols in the cephem 4-ketone class made novel derivatives available for structure−activity investigation. The electronic equivalence of C-2 and C-3′ positions with respect to activation of the β-lactam bond can be easily recognized by a comparison between **31**, **34**, and **44** (Table 2) and **22**, **24**, and **25** (Table 1). Moreover, kinetic data[20] on **44** and **25** demonstrated similar release of the pyridylthio leaving group from both positions during reaction with HLE. All of the 7α-methoxycephem 4-*tert*-butyl ketones bearing a 2-heterocyclothio moiety and still unsubstituted at C-3′ (**31–34**, **44**) were at least as stable and active as the isosteric 3′-derivatives; compound **31**, in particular, combined both high activity and stability, inactivating HLE with extremely high efficiency ($r = 1.2$). The corresponding 4-*tert*-butyl esters (**46**, Table 3, vs **44**) and 4-phenyl ketones (**35** vs **31**) did not share this combination of favorable properties. The 7α-chlorocephem 4-ketones incorporating a heterocyclothio group at C-2 (**60** and **62**) were of no particular interest, mainly because of their limited hydrolytic stability, with little or no improvement in activity.

Substitution of 7α-methoxycephem 4-*tert*-butyl ketone by an acyloxy group at C-2 provided a superior class of compounds. Apart from compounds with small aliphatic chains (formyloxy **36** and acetoxy **37**), other derivatives, both aliphatic (**38**, **39**) and aromatic (**40–42**), were extremely potent, with K_i(ss) below the determination limit (2 nM) allowed by the experimental conditions of screening. The 2-naphthoyloxy compound **42** (together with the related 2-benzoyloxy 3′-heterocyclothio compound **56** discussed below), with second-order inhibition constant k_{on} also outside the determination limit (2 000 000 $M^{-1}\ s^{-1}$), is the most potent HLE inhibitor of the cephem sulfone class ever reported.

Multiple substitution (at C-2 and C-3′) was again of no particular value in the 7α-chloro series (compound **61**), but interesting trends were observed in the 7α-methoxy derivatives. The 2-heterocyclothio 3′-acyloxy compound **55** is a good inhibitor, and the reverse substitution pattern gave the superb inhibitor **56**. Two acyloxy groups, as in **54**, apparently increased the chemical reactivity to unacceptable levels. On the other hand, a good compromise between inhibitory activity and chemical reactivity was attained by the insertion of two heterocyclothio groups (**52**, **53** and **58**, **59**). The phenyl ketones of this class, **58** and **59**, underscore an apparently paradoxical effect. These compounds are endowed with extreme hydrolytic stability at pH 7.4, while previously investigated phenyl ketones were less stable than their *t*-Bu analogs (**11** vs **7**, **12** vs **9**, **18** vs **17**, **26** vs **22**). Multiple substitution by the electron-withdrawing heterocyclothio groups was expected to reduce hydrolytic stability, but **58** and **59** are actually more stable (and active) than **35**. Thus, these compounds (and possibly others) must benefit from some stabilization mechanism at the investigated pH.

At least two distinct processes by which hydrolytic degradation of 1,1-dioxocephem 4-ketones can be retarded are suggested by our study. Of the range of examined products, some showed double-exponential decay (starred data in Tables 1–3), thus revealing involvement of a second species in the degradation pathway. It has been found that the Δ^3-cephem structure of these compounds can undergo double-bond isomerism and equilibrate with the 3-*exo* and/or Δ^2-cephem structures.[12c] These isomeric products, which are generally eluted by HPLC in close proximity of the Δ^3-cephem peak, lack the characteristic chromophore of Δ^3-isomers but can be detected and recognized by the UV absorption spectrum. The occurrence of 3-*exo* and/or Δ^2-cephem protomerism may be regarded as a first mechanism of stabilization, since conjugative interaction of the unshared electron pair on nitrogen with the cephem double bond is competitive with the usual stabilization of the amide C–N bond.[21] The second, more important stabilization process is keto–enol tautomerism.[12b] By insertion of electron-withdrawing substituents at C-2 and C-3′, especially in the phenyl ketone series, the apparent pK_a of the C-2 proton(s) can be lowered and the equilibrium shifted toward the corresponding enolate forms even in aqueous media at physiological pH. There is an analogy with the 3-*exo* and Δ^2-protomerisms, in that all of the three mechanisms contribute to impairing mesomeric delocalization of the nitrogen lone pair out of the β-lactam ring. In keto–enol tautomerism, however, striking differences in stability are observed according to the pH of the buffer. This is illustrated in Figures 3 and 4, which report the pH dependence of $-\log k_{st}$, the stability rate constant. Figure 3 provides a comparison between the 3′-substituted *tert*-butyl ester **69** and the corresponding *tert*-butyl ketone **22**. This ketone ($pK_a = 11.6$), being unable to convert into appreciable amounts of the enolate in the investigated range of pH (3–11), behaves as the ester above pH 8, where the reaction is hydroxide ion catalyzed (slope = −1), but it is far more stable than **69** at pH 7 or below, where attack to the ester moiety might become the rate-limiting step in the hydrolytic degradation of the latter compound. The stability profile of **22** is, in fact, strikingly similar to that of the 3′-substituted amide **70** and the analogous *tert*-butyl ketone **24** (pK_a 12), two other cephem derivatives which do not give enolates and have a hydrolytically stable C-4 moiety. The peculiar features of cephem 4-ketones emerge when compounds able to give enolates are considered (Figure 4). The 2-substituted ketone **31** ($pK_a = 7.9$) behaves as the corresponding 3′-substituted analog **22** in the low range of pH but differs above pH 8, where enolate formation greatly protects against hydroxide-catalyzed β-lactam cleavage: at pH 11, for instance, **31** is 100 times more stable than **22**. The 2,3′-disubstituted ketone **52** ($pK_a = 6$) takes advantage of enolate stabilization in a more useful range of pH, with maximal stability at pH 9–10, though at the expenses of reduced stability below pH 7, where the increased activation of the β-lactam bond is not compensated by enolate stabilization. These features are intensified in the corresponding disubstituted phenyl ketone **58** ($pK_a = 3.5$), which shows an impressive increase of stability even in neutral buffers, with a maximum at pH 8.

Although chemical stability results often defied rational interpretation, some trends in activity–reactivity relationships could be examined by taking keto–enol tautomerism into account. The 2,3′-unsubstituted products **17** (*t*-Bu; $pK_a > 12$) and **18** (Ph; $pK_a = 9.6$) have similar activity, while stability of the latter at pH 7.4 is reduced by a factor of 4. The phenyl group has lowered the pK_a but not enough to promote formation of enolate at pH 7.4. Thus the net effect of the phenyl group is a reduction in stability, possibly due to increased mesomeric delocalization of the nitrogen lone pair. Substitution with mercaptotetrazole at C-3′ as in

26 largely increases the activity but gives a slightly less stable product, since the pK_a (8.4) is still not compatible with formation of enolate at pH 7.4. Substitution at C-2 is much more effective at reducing the pK_a, as appears in the comparison between the isomeric ketones with a tetrazolylthio group at C-3′ (**22**, $pK_a = 11.6$) or C-2 (**31**, $pK_a = 7.9$). However, in this series (*tert*-butyl ketones), the effect is still not sufficient for a significant stability gain at pH 7.4, and a second substituent is required to lower the pK_a below the neutral range (e.g., **52**, $pK_a = 6.0$) and thence substantially improve hydrolytic stability as determined at pH 7.4. The biological activity of **52**, which is similar to that of the monosubstituted compounds **22** and **31**, is likely to arise from a balance of two factors: greater activation of the β-lactam bond and slightly reduced acceptance by the enzyme of a negative charge close to the cleavable amide bond. The 2-substituted phenyl ketone **35** ($pK_a = 4.8$) exists largely as an enolate at neutral pH and is about 10-fold more stable than its 3′-isomer, **26**. Though elastase inhibition is unfavorably influenced, activity and stability of **35** actually improve with respect to the unsubstituted phenyl ketone **18**. What is more, a further improvement is obtained by converting the 2-substituted phenyl ketone **35** to the 2,3′-disubstituted analogs **58** and **59** ($pK_a = 3.5$): both stability and activity are 7−17 times better. The detrimental effect of the enolate charge on activity, already present in the monosubstituted compound **35**, is not worsened; thus, the second substitution activates the β-lactam bond for HLE inhibition while further reducing the pK_a, which confers improved stability to the molecule.

A general view of the activity−reactivity relationship for the 7α-methoxycephem inhibitors at pH 7.4 is provided in Figure 2. As pointed out before, a direct relationship was anticipated, since attack onto the β-lactam carbonyl by an oxygen nucleophile is a common event in the enzyme inactivation and the hydrolytic degradation process; that should translate into an inverse linear correlation between plotted parameters, log $t_{1/2}$ and log k_{on}. Data dispersion reveals that a reactive β-lactam is not the sole requirement for efficient HLE inhibition; the entire tridimensional structure of the compound plays important roles, first in a productive binding with the enzyme and subsequently in the stabilization of the initially formed acylenzyme against early return of enzymatic activity.[1,9b,10] Interesting inhibitors, combining good activity and stability, should be found toward the top right corner of the graph. Products emerging from the present study belong to the class of 2-substituted (filled circles) and 2,3-disubstituted (filled squares) cephem 4-ketones; 3′-substituted 4-ketones (open circles) are average, while most 4-ester and 4-amide reference compounds (open squares) are below.

Kinetic arguments have been raised on the limits of slow-acting proteinase inhibitors in the presence of the physiological protein substrates.[22] Analysis of the efficiency of a selected panel of cephem sulfones against HLE acting on insoluble elastin from bovine neck ligament revealed that elastinolysis by HLE is efficiently inhibited by the tested compounds in a concentration-dependent manner (Figure 1). Although much higher inhibitor concentrations were required than that used in the kinetic experiments, this increase corresponds to the increased enzyme concentration of the protocol (200−500 times). Performance correlated qualitatively with k_{on} and K_i(ss) data in Tables 1−3: the most potent inhibitors (**10**, **31**) performed best; the least potent ones (**35**, **70**) required about 10-fold higher concentrations. However, it is interesting to observe that the 2-substituted 4-phenyl ketones **35**, though inferior to the reference amide **70** (Merck L-659,286)[7,17] at protecting the synthetic substrate, was as active as the latter in the elastinolytic assay. Further, the 2,3′-disubstituted 4-*tert*-butyl ketone **52** attained virtually complete inhibition at a concentration lower than that of other assayed 7α-methoxycephem sulfones (**22**, **31**, **35**, **70**).

Testing 1,1-dioxocephem 4-ketones for their ability at inhibiting HLE in living human polymorphonuclear leukocytes revealed that the 2-heterocyclothio compounds **31** and **34** were unable to affect the intracellular enzyme; cells washed free of inhibitors before stimulation expressed the same proteolytic activity as cells not exposed to inhibitors (Table 4). In this respect, these compounds behaved as the reference amide **70**.[17] With two other compounds, elastase activity expressed by PMNs was decreased after incubation and washing: marginally in the case of the 3′-heterocyclothio 4-ketone **24** and substantially for a lipophilic 4-thiolester derivative, FCE 26251 (compound **II-8c** in ref 11; see Table 4 for structure), here included as a positive control. A positive result in this assay, however, does not necessarily imply intracellular inhibition of the stored enzyme; compound (especially lipophilic ones) might bind to the cellular membrane or other cellular components and inhibit the enzyme only in the degranulation process. There is considerable controversy on the pros and cons of intracellular activity by HLE inhibitors projected as therapeutic agents.[17,23] Our results suggest the possibility of modulating this property in the class of cephem sulfone derivatives by a proper choice of the substitution pattern and lipophilicity of the molecule.

Conclusion

Innovative chemistry at the dihydrothiazine ring of cephem sulfones, aimed at identifying mechanism-based inhibitors of HLE that could combine good biochemical activity and acceptable hydrolytic stability, resulted in the selection of 7α-methoxy 4-keto derivatives with distinctive substitution patterns. The *tert*-butyl ketones possessing a heterocyclothio group at C-3′ proved to be at least as active in vitro as the corresponding *tert*-butyl esters but are 1 order of magnitude more stable in physiologic buffers. The *tert*-butyl ketones possessing the same groups at C-2 gave similar or even better results (e.g., **31** vs **22**). Introduction of acyloxy groups at C-2 delivered the most potent HLE inhibitors of the cephem class ever reported, with inhibitory parameters outside the determination limits of our standard assay procedure (k_{on} higher than 2 000 000 M^{-1} s^{-1} and K_i(ss) lower than 2 nM). Hydrolytic half-lives (pH 7.4, 37 °C) equal to or exceeding 12 h, arbitrarily set as a selection criterion for compounds prospected for topical administration, were attained in conjunction with inhibition constant k_{on} exceeding 10^4 M^{-1} s^{-1} by several ketones of the 7α-methoxycephem sulfone structure: the 3′-substituted compounds **22** and **24**−**26**, the 2-substituted compounds **31**, **32**, **34**, **38**−**40**, **42**, and **44**, and the 2,3′-disubstituted compounds **52** and **53**. These properties were never obtained by any cephem 4-ester or 4-thiolester investigated by us[11] or, to our best knowledge, by others.[3,5-7] In particular, double substitution with heterocyclic thiols produced compounds with diverging properties, according to the extent of enolate formation at pH 7.4; either potent inhibitors with reasonable hydrolytic stability or exceptionally stable, moderate inhibitors could be designed. The *tert*-butyl ketone **53** (k_{on} = 2.1 × 10^4 M^{-1} s^{-1}; $t_{1/2}$ = 75 h) is a representative of the first group of products. The phenyl ketones **58** and **59**, representatives of the second group, combined stabilities exceeding 1000 h with HLE inhibition parameters k_{on} and K_i(ss) comparable to those of the cephem 4-amides ultimately selected at Merck, L-659,286[17] (reference compound **70**) and L-658,758[7b] (**71**). These ketones are actually the first HLE inhibitors of the cephem sulfone class to possess chemical stability levels adequate for prospected systemic administration. In particular, the half-life of **59** in human plasma in vitro was 28 h at 37 °C as compared to 1 h for **70**.[19] The favorable properties of selected compounds at inhibiting the amidolytic activity of HLE on synthetic fluorogenic peptides were reproduced by using bovine neck elastin as the substrate. On the whole, the 1,1-dioxo-7α-methoxycephem 4-ketones constitute a novel and interesting class of HLE inhibitors worthy of investigation in vivo.

Experimental Section

Chemistry. General Procedures. Purifications by flash chromatography were performed on columns packed with silica gel Merck 60 (230−400 mesh) and elution was carried out with a gradient of ethyl acetate in hexane unless otherwise stated. Melting points were determined on a Büchi apparatus and are uncorrected; all the tested compounds melted with decomposition. The ^{1}H NMR spectra (δ, ppm, tetramethylsilane as internal standard) were obtained on Varian EM-390 (90 MHz) or VXR-200 (200 MHz) spectrometers; the IR spectra were obtained on a Perkin-Elmer 1420 spectrophotometer. Analytical results for compounds followed by elemental symbols were ±0.4% of calculated values and were determined on a Carlo-Erba NA-1005 analyzer. FD mass spectra were recorded on a Varian Mat 311/A instrument equipped with a combined EI/FI/FD ion source using benzonitrile-activated emitters.

Products **68** and **69**, esters of cephem sulfones included in Table 3 for comparative purposes, were obtained as previously described.[11] Products **70** and **71**, further reference compounds of the 4-amide structure, were prepared by modifications of published procedures[7] and showed congruent physicochemical properties (^{1}H NMR, IR, elemental analyses). The following preparative protocols were routinely used.

Method A—Preparation of Cephem 4-Ketones from the Corresponding Cephem-4-carboxylic Acids. A solution of the carboxylic acid (1 mmol) in dry tetrahydrofuran (THF; 10 mL) was cooled to 0 °C under nitrogen and sequentially treated with oxalyl chloride (0.174 mL, 2 mmol) and a catalytic amount (0.02 mL) of *N,N*-dimethylformamide (DMF). After stirring for 2 h at a temperature ranging from 5 to 10 °C, the solvent and volatile products were removed in vacuo (bath temperature ≤ 25 °C). The crude residue was taken up with dry THF (10 mL) and treated with copper(I) iodide (380 mg, 2 mmol). The suspension was cooled to −70 °C, and a 2 M ethereal solution of the Grignard reagent (1 mL, 2 mmol) was added dropwise over 30 min under a nitrogen atmosphere. The reaction mixture was allowed to rise to −40 °C and then poured into a mixture of Et_2O, ice, and 30% aqueous NH_4Cl. After stirring for 10 min, the ethereal extracts (2 × 40 mL) were collected, washed with aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated. Obtained crude cephem 4-ketones were purified by flash chromatography or, in the case of sulfides, utilized as such in the oxidation step (method B below).

Method B—Oxidation of 4-Ketocephem Sulfides to the Corresponding Sulfones. To a solution of the crude cephem 4-ketone (1 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added 55% MCPBA (942 mg, 3 mmol). After the solution was stirred at 0 °C for 1 h and at room temperature overnight, CH_2Cl_2 (30 mL) was added and the solution was washed sequentially with 4% aqueous $NaHSO_3$ (1 × 15 mL), saturated $NaHCO_3$ (2 × 20 mL), and water. The solution was dried over Na_2SO_4 and concentrated by rotoevaporation. The residue was then purified by flash chromatography.

Method C—Bromination of 4-Ketocephem Sulfones at C-3′ (Radical Bromination). A solution of the 3-methylcephem (1 mmol) in CCl_4 (20 mL) containing NBS (196 mg, 1.1 mmol) and a catalytic amount of AIBN (5 mg) was heated under nitrogen at reflux for 4 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL), washed with 4% aqueous $NaHCO_3$, dried over Na_2SO_4, and rotoevaporated. Following purification by flash chromatography, pure 3-(bromomethyl)-cephem derivatives were obtained.

Method D—Bromination of 4-Ketocephem Sulfones at C-2 (Electrophilic Bromination). A solution of the cephem 4-ketone sulfone (1 mmol) in CH_2Cl_2 (30 mL) was cooled to 0 °C and sequentially treated with TEA (0.153 mL, 1 mmol) and NBS (214 mg, 1.2 mmol). In about 10 min, the reaction was over (TLC monitoring). The solution, diluted with additional CH_2Cl_2, was sequentially washed with 4% aqueous $NaHSO_3$, saturated $NaHCO_3$, and water. After drying over Na_2SO_4, removal of the solvent afforded the crude 2-bromocephem products, which were routinely used as such. Analytical samples could be obtained by flash chromatography.

Method E—Preparation of 3′-(Heterocyclothio)- and 2-(Heterocyclothio)cephem Sulfones from the Corresponding Bromo Derivatives. To a solution of the bromo derivative (1 mmol) in MeCN (40 mL) were sequentially added the heterocyclic mercaptan (1.2 mmol) and TEA (0.17 mL, 1.2 mmol). The mixture was stirred for 30 min and then partitioned between water and EtOAc. The organic layer was dried over Na_2SO_4 and rotoevaporated. The residue was purified by flash chromatography.

Method F—Preparation of 2-(Acyloxy)cephem Sulfones from the Corresponding Bromo Derivatives. A solution of the 2-bromocephem (0.5 mmol) in MeCN (10 mL) was treated with the proper silver carboxylate (0.75 mmol), prepared as described below. The mixture was stirred (30 min−2 h) in the dark, monitoring depletion of the starting bromocephem by TLC. The reaction mixture was partitioned between EtOAc and water; the insoluble material was filtered off, and the organic layer was separated, dried over Na_2SO_4, and rotoevaporated. Pure 2-(acyloxy)cephems were obtained by flash chromatography.

Typical Preparation of Silver Carboxylates. A mixture of the proper carboxylic acid (10 mmol) in water (50 mL) was treated with sodium methoxide (0.54 g, 10 mmol) and stirred until a clear solution appeared. Silver nitrate (1.7 g, 10 mmol) was then added in the dark, causing the immediate formation of a white precipitate. After stirring for few minutes, the mixture was filtered, and the solid was sequentially washed with water and ethyl ether. Following drying in an oven at 55 °C in vacuo, silver carboxylates were obtained as whitish or light gray powders in yields ranging from 60% to 95%.

3-(Acetoxymethyl)-7α-chloro-4-(ethylcarbonyl)-Δ^3-cephem 1,1-dioxide (5): obtained by sequential reaction of **3**[25] with oxalyl chloride and ethylmagnesium bromide (method A); white solid (11% yield); mp 168−169 °C; IR (KBr) ν_{max} 1810, 1730, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.17 (3H, t, J = 7.1 Hz), 2.08 (3H, s), 2.75 and 2.88 (2H, each q, J = 7.1 Hz), 3.74 (1H, d, J = 18.4 Hz), 4.02 (1H, br d, J = 18.4 Hz), 4.52 and 4.98 (2H, each d, J = 14.3 Hz), 4.86 (1H, m), 5.36 (1H, d, J = 1.7 Hz). Anal. ($C_{12}H_{14}ClNO_6S$) H, N; C: calcd, 42.93; found, 43.63.

Preparation of 6−12: obtained from acids **1−4**[11,25] according to method A, using as Grignard reagents benzylmagnesium chloride, *tert*-butylmagnesium chloride, or phenylmagnesium chloride.

3-(Acetoxymethyl)-4-(benzylcarbonyl)-7α-chloro-Δ^3-cephem 1,1-dioxide (6): yellowish powder (12% yield); mp 147−148 °C; IR (KBr) ν_{max} 1795, 1735, 1705 cm^{-1}; NMR

($CDCl_3$) δ 2.07 (3H, s), 3.67 (1H, d, J = 18.1 Hz), 3.88 (1H, br d, J = 18.1 Hz), 4.07 (2H, s), 4.17 (1H, br s), 4.53 and 5.00 (2H, each d, J = 14.4 Hz), 5.30 (1H, d, J = 1.7 Hz). Anal. ($C_{17}H_{16}ClNO_6S$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-chloro-3-methyl-Δ^3-cephem 1,1-dioxide (7): white solid (19% yield); mp 218–219 °C; IR (KBr) ν_{max} 1773, 1687 cm^{-1}; NMR ($CDCl_3$) δ 1.26 (9H, s), 1.72 (3H, s), 3.60 (1H, d, J = 18.1 Hz), 3.69 (1H, dd, J = 1.3 and 18.1 Hz), 4.76 (1H, dd, J = 1.3 and 1.7 Hz), 5.32 (1H, d, J = 1.7 Hz). Anal. ($C_{12}H_{16}ClNO_4S$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-chloro-3-(methoxymethyl)-Δ^3-cephem 1,1-dioxide (8): white solid (7% yield); mp 151–153 °C; IR (KBr) ν_{max} 1800, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.26 (9H, s), 3.28 (3H, s), 3.75 (2H, s), 3.80 (1H, d, J = 18.2 Hz), 4.08 (1H, br d, J = 18.2 Hz), 4.83 (1H, br s), 5.35 (1H, d, J = 1.7 Hz). Anal. ($C_{13}H_{18}ClNO_5S$) C, H, N.

3-(Acetoxymethyl)-4-*tert*-butylcarbonyl)-7α-chloro-Δ^3-cephem 1,1-dioxide (9): white powder (8% yield); mp 168–170 °C; IR (KBr) ν_{max} 1795, 1740, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 2.09 (3H, s), 3.74 (1H, d, J = 18.1 Hz), 3.99 (1H, br d, J = 18.1 Hz), 4.40 and 4.48 (2H, each d, J = 13.4 Hz), 4.84 (1H, m), 5.36 (1H, d, J = 1.7 Hz). Anal. ($C_{14}H_{18}ClNO_6S$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-chloro-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (10): white powder (6% yield); mp 162–164 °C; IR (KBr) ν_{max} 1790, 1690 cm^{-1} NMR ($CDCl_3$) δ 1.26 (9H, s), 3.76 and 4.08 (2H, each d, J = 14.3 Hz), 3.93 (3H, s), 4.01 (1H, d, J = 17.9 Hz), 4.26 (1H, dd, J = 0.9 and 17.9 Hz), 4.85 (1H, dd, J = 0.9 and 1.7 Hz), 5.33 (1H, d, J = 1.7 Hz). Anal. ($C_{14}H_{18}ClN_5O_4S_2$) C, H, N.

7α-Chloro-3-methyl-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (11): white solid (7% yield); mp 184–186 °C; IR (KBr) ν_{max} 1780, 1677 cm^{-1}; NMR ($CDCl_3$) δ 1.67 (3H, s), 3.66 (1H, d, J = 18.3 Hz), 4.03 (1H, dd, J = 1.4 and 18.7 Hz), 4.91 (1H, dd, J = 1.4 and 1.9 Hz), 5.33 (1H, d, J = 1.9 Hz), 7.5–8.0 (5H, m). Anal. ($C_{14}H_{12}ClNO_4S$) H, N; C: calcd, 51.62; found, 50.97.

3-(Acetoxymethyl)-7α-chloro-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (12): light yellow solid (13% yield); mp 58–61 °C; IR ($CHCl_3$) ν_{max} 1790, 1735, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.97 (3H, s), 3.80 (1H, d, J = 18.4 Hz), 4.11 (1H, br d, J = 18.4 Hz), 4.42 (2H, ABq, J = 13.5 Hz), 4.96 (1H, br s), 5.37 (1H, d, J = 2.0 Hz), 7.47–7.93 (5H, m). Anal. ($C_{16}H_{14}ClNO_6S$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-Dioxide (17). Standard Procedure (Methods A and B). A solution of **13**[13] (6.6 g, 28.8 mmol) in dry THF–C_6H_6, 1:1 (150 mL), was cooled to 0 °C and treated with oxalyl chloride (5.0 mL, 57.6 mmol) and a catalytic amount of DMF (0.15 mL). After stirring for 1.5 h at 10 °C, the reaction mixture was concentrated in vacuo. The residue was taken up with dry C_6H_6 and concentrated to dryness to give the crude acid chloride as a yellow foam. This product was dissolved in dry THF, and the solution was chilled to −70 °C under nitrogen. Copper(I) iodide (8 g, 42 mmol) was added followed by the dropwise addition (30 min) of 2 M *tert*-butylmagnesium chloride in THF (21 mL, 42 mmol). This mixture was stirred at −65 °C for 30 min and then poured into a mixture of ethyl ether and water under vigorous stirring. The organic phase was washed with aqueous $NaHCO_3$ and then dried over Na_2SO_4 and concentrated in vacuo. The oily residue (ca. 4 g) was dissolved in CH_2Cl_2 (100 mL) and treated at 0 °C with 55% MCPBA (10.5 g, 33.4 mmol of peracid). After stirring at room temperature for 6 h, the mixture was filtered, and the filtrate was sequentially washed with aqueous solutions of $NaHSO_3$, $NaHCO_3$, and NaCl. The organic layer was dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography gave the title product as a white solid (2.2 g, 25% yield): mp 152–154 °C; $[\alpha]_D$ −128.6° (EtOH); UV (EtOH) λ_{max} 255 nm (ϵ = 6370); IR (KBr) ν_{max} 1780, 1690, cm^{-1}; NMR ($CDCl_3$) δ 1.26 (9H, s), 1.70 (3H, s), 3.51 (2H, d, J = 18.1 Hz), 3.56 (3H, s), 3.93 (2H, br d, J = 18.1 Hz), 4.66 (1H, m), 5.16 (1H, d, J = 1.7 Hz). Anal. ($C_{13}H_{19}NO_5S$) C, H, N.

Improved Procedure. A suspension of copper(I) thiophenolate (5 g, 29 mmol) in dry THF (250 mL) was cooled to −20 °C, and under argon, a solution of 1.7 M *tert*-butyllithium in pentane (17.1 mL, 29 mmol) was added. The resulting solution was stirred for 5 min at −20 °C and then cooled to −70 °C and transferred into a flask containing a solution of 7α-methoxy-3-methyl-Δ^3-cephem-4-carbonyl chloride (prepared from 6.6 g of acid **13** as indicated above) in 100 mL of THF at −70 °C. The mixture was stirred at the same temperature for 30 min, diluted with ethyl ether, and shaken vigorously with saturated aqueous NH_4Cl. The precipitate was removed by filtration, and the organic phase was washed with brine and then dried (Na_2SO_4) and concentrated. A yellow oil was obtained (6.4 g) which was dissolved in CH_2Cl_2 (200 mL) and treated at 0 °C with 55% MCPBA (15.7 g, 50 mmol of peracid). The solution was stirred at room temperature for 6 h and then worked up as above under the standard procedure, affording the title product as a white powder (3.1 g, 36% yield).

7α-Methoxy-3-methyl-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (18): obtained by reaction of **13** with oxalyl chloride and phenylmagnesium chloride (method A) followed by oxidation (method B); white powder (32% yield); mp 136–138 °C; IR (KBr) ν_{max} 1770, 1690 cm^{-1}; NMR ($CDCl_3$) δ 1.94 (3H, s), 3.39 (1H, d, J = 18.1 Hz), 3.57 (3H, s), 4.57 (1H, br d, J = 18.1 Hz), 4.84 (1H, m), 5.21 (1H, d, J = 1.4 Hz), 7.10–7.36 (5H, m). Anal. ($C_{15}H_{15}NO_5S$) H, N; C: calcd, 56.06; found, 54.52.

(3-(Bromomethyl)-4-(*tert*-butylcarbonyl)-7α-methoxy-Δ^3-cephem 1,1-dioxide (19): obtained by allylic bromination of **17** (method C); white powder (55% yield); mp 157–158 °C; IR ($CHCl_3$) ν_{max} 1790, 1690 cm^{-1}; NMR ($CDCl_3$) δ 1.31 (9H, s), 3.56 (3H, s), 3.59 (1H, d, J = 17.8 Hz), 3.79 (1H, d, J = 11.4 Hz), 3.91 (1H, d, J = 11.4 Hz), 4.27 (1H, dd, J = 1.4 and 17.8 Hz), 4.77 (1H, m), 5.20 (1H, d, J = 1.4 Hz).

3-(Bromomethyl)-7α-methoxy-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (20): obtained by allylic bromination of **18** (method C); white powder (53% yield); IR (KBr) ν_{max} 1785, 1670 cm^{-1}; NMR ($CDCl_3$) δ 3.51 (3H, s), 3.76 (1H, d, J = 11.4 Hz), 3.81 (1H, d, J = 17.8 Hz), 3.91 (1H, d, J = 11.4 Hz), 4.23 (1H, dd, J = 1.4 and 17.8 Hz), 4.86 (1H, m), 5.20 (1H, d, J = 1.9 Hz), 7.4–8.0 (5H, m).

3-(Acetoxymethyl)-4-(*tert*-butylcarbonyl)-7α-methoxy-Δ^3-cephem 1,1-Dioxide (21). A solution of **19** (400 mg, 1.05 mmol) in MeCN (10 mL) and acetic acid (0.6 mL) was treated with silver nitrate (526 mg, 3.15 mmol). The reaction mixture was vigorously stirred for 1 h at room temperature and then poured into EtOAc–water. The organic phase was washed twice with water and then with aqueous $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed by rotoevaporation and the residue was purified by flash chromatography to afford the title product as a white powder (170 mg, 45% yield): mp 134 °C; IR (KBr) ν_{max} 1780, 1732, 1687 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 2.08 (3H, s), 3.57 (3H, s), 3.83 (1H, d, J = 18.5 Hz), 4.00 (1H, dd, J = 1.5 and 18.5 Hz), 4.43 (2H, ABq), 4.72 (1H, dd, J = 1.5 and 1.7 Hz), 5.18 (1H, dd, J = 1.7 Hz). Anal. ($C_{15}H_{21}NO_7S$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (22): obtained by reaction of **19** with 5-mercapto-1-methyl-1,2,3,4-tetrazole (method E); white powder (75% yield); mp 60–62 °C; IR (KBr) ν_{max} 1790, 1690 cm^{-1}; NMR ($CDCl_3$) δ 1.20 (9H, s), 3.78 (1H, d, J = 14.2 Hz), 4.05 (1H, br d, J = 14.2 Hz), 3.56 (3H, s), 3.93 (3H, s), 3.93 (1H, d, J = 17.8 Hz), 4.20 (1H, br d, J = 17.8 Hz), 4.75 (1H, m), 5.17 (1H, d, J = 1.7 Hz). Anal. ($C_{15}H_{21}N_5O_5S_2$) C, H, N.

3-[[[6-(Benzhydryloxy)-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl]thio]methyl]-4-(*tert*-butylcarbonyl)-7α-methoxy-Δ^3-cephem 1,1-dioxide (23): obtained by reaction of **19** with 6-(benzhydryloxy)-3-mercapto-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazine (method E); white powder (69% yield); mp 148–150 °C; IR (KBr) ν_{max} 1795, 1675 cm^{-1}; NMR ($CDCl_3$) δ 1.27 (9H, s), 3.55 and 4.22 (2H, each d, J = 14.2 Hz), 3.80 (1H, d, J = 18.0 Hz), 4.22 (1H, br d, J = 18.0 Hz), 4.75 (1H, br s), 5.18 (1H, d, J = 1.9 Hz), 6.75 (1H, s), 7.3–7.5 (10H, m). Anal. ($C_{30}H_{32}N_4O_7S_2$) C, H, N.

4-(*tert*-Butylcarbonyl)-3-[[(6-hydroxy-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)thio]methyl]-7α-methoxy-Δ^3-cephem 1,1-Dioxide (24). A solution of **23** (615 mg, 1 mmol) in CH_2Cl_2 (4 mL), anisole (2 mL), and TFA (4 mL) was allowed to stand at room temperature for 20 min and then rotoevaporated to dryness. The gummy residue was dissolved in CH_2Cl_2 (3 mL), and the resulting solution was dropped into isopropyl ether (50 mL), affording a white solid which was collected by filtration and dried in vacuo (426 mg, 93% yield): mp 148–150 °C; IR (KBr) ν_{max} 1795, 1705, 1645 (br) cm^{-1}; NMR ($CDCl_3$) δ 1.22 (9H, s), 3.56 (3H, s), 3.63 and 4.19 (1H, each d, J = 13.8 Hz), 3.75 (1H, s), 4.08 (1H, br d, J = 17.1 Hz), 4.76 (1H, br s), 5.18 (1H, d, J = 1.1 Hz). Anal. ($C_{17}H_{22}N_4O_7S_2$) C, H; N: calcd, 13.22; found, 12.51.

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-[[(2-pyridyl)thio]-methyl]-Δ^3-cephem 1,1-dioxide (25): obtained from **19** and 2-mercaptopyridine (method E); white powder (38% yield); mp 130–131 °C; IR (KBr) ν_{max} 1780, 1695 cm^{-1}; NMR ($CDCl_3$) δ 1.29 (9H, s), 3.54 (3H, s), 3.86 (1H, d, J = 14.6 Hz), 3.92 (1H, d, J = 18.0 Hz), 3.98 (1H, d, J = 14.6 Hz), 4.09 (1H, dd, J = 1.2 and 18.0 Hz), 4.67 (1H, m), 5.15 (1H, d, J = 1.7 Hz), 7.0–7.6 (3H, m), 8.5 (1H, m).

7α-Methoxy-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-methyl]-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (26): obtained from **20** and 5-mercapto-1-methyl-1,2,3,4-tetrazole (method E); yellow powder (78% yield); mp 169–171 °C; IR (KBr) ν_{max} 1800, 1670 cm^{-1}. NMR ($CDCl_3$) δ 3.49 (3H, s), 3.79 and 4.10 (2H, each d, J = 14.4 Hz), 3.85 (3H, s), 3.96 (1H, d, J = 18.1 Hz), 4.39 (1H, dd, J = 1.4 and 18.1 Hz), 4.88 (1H, dd, J = 1.7 and 1.4 Hz), 5.17 (1H, d, J = 1.7 Hz), 7.4–7.9 (5H, m). Anal. ($C_{17}H_{17}N_5O_5S_2$) C, H, N.

2α-Bromo-4-(*tert*-butylcarbonyl)-7α-methoxy-3-methylcephem 1,1-dioxide (28): obtained by electrophilic bromination of **17** (method D); white powder (92% yield); mp 125–127 °C; IR (KBr) ν_{max} 1800 (br), 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.26 (9H, s), 1.82 (3H, s), 3.57 (3H, s), 4.90 (1H, s), 5.17 and 5.32 (1H, each d, J = 2.0 Hz).

2α-Bromo-7α-methoxy-3-methyl-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (29): obtained by electrophilic bromination of **18** (method D); light yellow solid (80% yield); IR (KBr) ν_{max} 1800, 1670 cm^{-1}; NMR ($CDCl_3$) δ 1.80 (3H, s), 5.04 (1H, s), 5.34 (1H, d, J = 2.1 Hz), 5.50 (1H, d, J = 2.1 Hz), 7.53–7.92 (5H, m).

2α-Bromo-4-(*tert*-butoxycarbonyl)-7α-methoxy-3-methylcephem 1,1-dioxide (30): obtained from **27**[11] by electrophilic bromination (method D); yellowish waxy solid (65% yield); IR (KBr) ν_{max} 1810, 1720 cm^{-1}; NMR ($CDCl_3$) δ 1.55 (9H, s), 2.08 (3H, s), 3.58 (3H, s), 4.92 (1H, s), 5.14 (1H, d, J = 1.8 Hz), 5.25 (1H, d, J = 1.8 Hz).

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-Δ^3-cephem 1,1-dioxide (31): obtained from **28** and 5-mercapto-1-methyl-1,2,3,4-tetrazole (method E); white powder (91% yield); mp 74–76 °C; IR (KBr) ν_{max} 1800, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.24 (9H, s), 1.92 (3H, s), 3.54 (3H, s), 4.08 (3H, s), 4.98 (1H, s), 5.10 and 5.17 (1H, each d, J = 1.9 Hz). Anal. ($C_{15}H_{21}N_5O_5S_2$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-Δ^3-cephem 1,1-dioxide (32): obtained from **28** and 2-mercapto-5-methyl-1,3,4-thiadiazole (method E); white powder (90% yield); mp 116–117 °C; IR ($CHCl_3$) ν_{max} 1790, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.20 (9H, s), 1.89 (3H, s), 2.78 (3H, s), 3.53 (3H, s), 5.17 (1H, d, J = 1.8 Hz), 5.20 (1H, s), 5.24 (1H, d, J = 1.8 Hz). Anal. ($C_{16}H_{21}N_3O_5S_3$) C, H; N: calcd, 9.74; found, 9.22.

2α-[[6-(Benzhydryloxy)-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl]thio]-4-(*tert*-butylcarbonyl)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (33): obtained from **28** and 6-(benzhydryloxy)-3-mercapto-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazine (method E); white powder (57% yield); mp 143–148 °C; IR ($CHCl_3$) ν_{max} 1800, 1700 (sh), 1675 cm^{-1}; NMR ($CDCl_3$) δ 1.27 (9H, s), 1.79 (3H, s), 3.55 (3H, s), 3.69 (3H, s), 5.04 (1H, d, J = 1.8 Hz), 5.10 (1H, d, J = 1.8 Hz), 5.83 (1H, s), 6.71 (1H, s), 7.1–7.5 (10H, m). Anal. ($C_{30}H_{32}N_4O_7S_2$) C, H, N.

4-(*tert*-Butylcarbonyl)-2α-[(6-hydroxy-2-methyl-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)thio]-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (34): obtained from **33** by deprotection with TFA, as previously described for **24**; white powder (94% yield); mp 122–125 °C; IR (KBr) ν_{max} 1790, 1700, 1650 (br) cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 1.84 (3H, s), 3.56 (3H, s), 3.82 (3H, s), 4.98 (1H, d, J = 1.2 Hz), 5.19 (1H, d, J = 1.2 Hz), 5.91 (1H, s). Anal. ($C_{17}H_{22}N_4O_7S_2$) C, H, N.

7α-Methoxy-3-methyl-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-4-(phenylcarbonyl]-Δ^3-cephem 1,1-dioxide (35): obtained from **29** and 5-mercapto-1-methyl-1,2,3,4-tetrazole (method E); white powder (35% yield); mp 165–167 °C; IR (KBr) ν_{max} 1810, 1680 cm^{-1}; NMR ($CDCl_3$) δ 1.90 (3H, s), 3.51 (3H, s), 4.08 (3H, s), 5.18 (1H, s), 5.20 (2H, s). Anal. ($C_{17}H_{17}N_5O_5S_2$) C, H, N.

4-(*tert*-Butylcarbonyl)-2α-(formyloxy)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (36): obtained from **28** and silver formate (method F); white powder (22% yield, unstable on silica gel); mp 109–112 °C; IR (KBr) ν_{max} 1790, 1745, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 1.72 (3H, s), 3.56 (3H, s), 4.77 (1H, d, J = 1.8 Hz), 5.19 (1H, d, J = 1.8 Hz), 5.80 (1H, br s), 8.23 (1H, d, J = 0.9 Hz); FD-MS 345 (M^+).

2α-Acetoxy-4-(*tert*-butylcarbonyl)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (37): obtained from **28** and silver acetate (method F); waxy solid (61% yield); NMR ($CDCl_3$) δ 1.26 (9H, s), 1.68 (3H, s), 2.24 (3H, s), 3.54 (3H, s), 4.72 (1H, d, J = 1.8 Hz), 5.15 (1H, d, J = 1.8 Hz), 5.69 (1H, s); FD-MS 359 (M^+).

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-(pivaloyloxy)-Δ^3-cephem 1,1-dioxide (38): obtained from **28** and silver pivalate (method F); white powder (42% yield); mp 184–187 °C; IR (KBr) ν_{max} 1795, 1765, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.27 (9H, s), 1.29 (9H, s), 1.67 (3H, s), 3.56 (3H, s), 4.70 (1H, d, J = 1.8 Hz), 5.16 (1H, d, J = 1.8 Hz), 5.67 (1H, s); FAB-MS 402 (MH^+).

2α-[(3-benzoylpropionyl)oxy]-4-(*tert*-butylcarbonyl)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (39): obtained from **28** and silver 3-benzoylpropionate (method F); white powder (37% yield); mp 120–125 °C; IR (KBr) ν_{max} 1775, 1705, 1690 cm^{-1}; NMR ($CDCl_3$) δ 1.25 (9H, s), 1.78 (3H, s), 2.9–3.0 (2H, m), 3.3–3.5 (2H, m), 3.55 (3H, s), 4.76 (1H, d, J = 1.5 Hz), 5.17 (1H, d, J = 1.5 Hz), 5.70 (1H, s), 7.4–8.1 (5H, m).

2α-(Benzoyloxy)-4-(*tert*-butylcarbonyl)-7α-methoxy-3-methyl-Δ^3-cephem 1,1-dioxide (40): obtained from **28** and silver benzoate (method F); white powder (68% yield); mp 155–156 °C; IR (KBr) ν_{max} 1795, 1755, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (9H, s), 1.76 (3H, s), 3.57 (3H, s), 4.88 (1H, d, J = 1.8 Hz), 5.20 (1H, d, J = 1.8 Hz), 5.92 (1H, s), 7.4–7.8 (5H, m); FD-MS 421 (M^+).

4-(*tert*-Butylcarbonyl)-2α-[[4-[[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-1,2,4-triazin-3-yl)thio]methyl]benzoyl]-oxy]-7α-methoxy-3-methyl-Δ^3-cephem 1,1-Dioxide (41). By reaction of **28** and silver 4-[[[6-(benzhydryloxy)-2,5-dihydro-2-methyl-5-oxo-1,2,4-triazin-3-yl]thio]methyl]benzoate (method F), the benzhydryl derivative of the title product was obtained (white solid, 34% yield). This product (76 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (0.5 mL) and sequentially treated with anisole (0.03 mL) and trifluoroacetic acid (0.5 mL). After the mixture was stirred for 30 min at room temperature, the solvent was removed and isopropyl ether was added. The resulting white solid was collected and dried in vacuo (40 mg, 68% yield): IR (KBr) ν_{max} 1800, 1745, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (9H, s), 1.74 (3H, s), 3.57 (3H, s), 3.72 (3H, s), 4.55 (2H, s), 4.83 (1H, d, J = 1.8 Hz), 5.20 (1H, d, J = 1.8 Hz), 5.90 (1H, s), 7.59 (2H, d, J = 8.3 Hz), 8.03 (2H, d, J = 8.3 Hz).

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-(2-naphthoyloxy)-Δ^3-cephem 1,1-dioxide (42): obtained from **28** and silver 2-naphthoate (method F); white foam (19% yield); IR (KBr) ν_{max} 1790, 1740, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (9H, s), 1.79 (3H, s), 3.58 (3H, s), 4.93 (1H, d, J = 1.7 Hz), 5.22 (1H, d, J = 1.7 Hz), 5.97 (1H, s), 7.5–8.2 (7H, m); FD-MS 471 (M^+).

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-(2-pyridylthio)-Δ^3-cephem 1,1-Dioxide (44). Under a nitrogen blanket, a solution of **17** (452 mg, 1.5 mmol) in dry MeCN (12 mL) was sequentially treated with 2,2′-dithiodipyridine (340 mg) and DBN (0.185 mL). The resulting mixture was stirred for 90 min at room temperature and then poured into EtOAc and 2% aqueous HCl. The organic phase was dried over Na_2SO_4 and concentrated in vacuo. Flash chromatography of the residue gave the title product as a white solid (290 mg, 47%

yield): mp 149–150 °C; IR (KBr) ν_{max} 1798, 1696 cm^{-1}; NMR ($CDCl_3$) δ 1.27 (9H, s), 1.87 (3H, s), 3.54 (3H, s), 5.04 (1H, d, J = 1.8 Hz), 5.16 (1H, d, J = 1.8 Hz), 6.10 (1H, s), 7.1–7.3 (2H, m), 7.5–7.7 (1H, m), 8.5 (1H, m). Anal. ($C_{18}H_{22}N_2O_5S_2$) C, H, N.

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-(phenylthio)-Δ^3-cephem 1,1-Dioxide (45) and 4α-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2-(phenylthio)-Δ^2-cephem 1,1-Dioxide (47). Isomeric mixtures of the two compounds were obtained by reacting **17** with *S*-phenyl benzenethiosulfonate in the presence of DBN under the same experimental conditions described for compound **44** (95% yield). Isomerization occurring on silica gel prevented the isolation of pure individual samples. Equilibrium achieved after exposure to TEA in $CDCl_3$ for 2 h, as detected by NMR integration, was 2:1 in favor of **45**. NMR ($CDCl_3$) for compound **45**: δ 1.24 (9H, s), 1.97 (3H, s), 3.52 (3H, s), 4.21 (1H, s), 4.88 (1H, d, J = 1.9 Hz), 5.14 (1H, d, J = 1.9 Hz), 7.3–7.7 (5H, m). NMR ($CDCl_3$) for compound **47**: δ 1.44 (9H, s), 1.86 (3H, s), 3.56 (3H, s), 4.83 (1H, dd, J = 1.2 Hz), 4.96 (1H, br s), 5.24 (1H, d, J = 1.2 Hz).

4-(*tert*-Butoxycarbonyl)-7α-methoxy-3-methyl-2α-(2-pyridylthio)-Δ^3-cephem 1,1-dioxide (46): obtained from **27**[11] and 2,2′-dithiodipyridine by following a procedure analogous to that described for the preparation of **44**; white powder (46% yield); IR ($CHCl_3$) ν_{max} 1800, 1730 cm^{-1}; NMR ($CDCl_3$) δ 1.56 (9H, s), 2.14 (3H, s), 3.55 (3H, s), 4.97 (1H, d, J = 1.5 Hz), 5.13 (1H, d, J = 1.5 Hz), 6.20 (1H, s), 7.16, 7.27, 7.62, and 8.15 (each 1H, m).

4-(*tert*-Butylcarbonyl)-7α-methoxy-3-methyl-2α-(phenylsulfonyl)-Δ^3-cephem 1,1-Dioxide (48). An isomeric mixture of **45** and **47** (82 mg, 0.2 mmol) in CH_2Cl_2 (5 mL) was treated with 55% MCPBA (100 mg). The resulting solution was stirred at room temperature for 6 h and then diluted with the same solvent and washed sequentially with 1 M aqueous $NaHSO_3$, water, 4% aqueous $NaHCO_3$, and brine. After drying over Na_2SO_4, the solvent was removed and the residue purified by flash chromatography: white powder (63 mg, 61% yield); UV (CH_3CN) λ_{max} 274 nm (ϵ = 10 745); IR (KBr) ν_{max} 1805, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.32 (9H, s), 1.96 (3H, s), 3.54 (3H, s), 4.61 (1H, s), 5.18 (1H, d, J = 2.1 Hz), 5.67 (1H, d, J = 2.1 Hz), 7.4–8.1 (5H, m). Anal. ($C_{19}H_{23}NO_7S_2$) C, H, N.

2α-Bromo-3-(bromomethyl)-4-(*tert*-butylcarbonyl)-7α-methoxyΔ^3-cephem 1,1-dioxide (49): obtained by radical bromination of **28** (method C); white powder (80% yield); mp 130–135 °C; IR (KBr) 1805, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 3.57 (3H, s), 3.76 (1H, d, J = 11.7 Hz), 4.07 (1H, d, J = 11.7 Hz), 5.17 (1H, d, J = 2.1 Hz), 5.35 (1H, d, J = 2.1 Hz), 5.45 (1H, s).

Preparation by Direct Dibromination of 17. A solution of **17** (4.51 g, 15 mmol) in CH_2Cl_2 (120 mL) and CCl_4 (540 mL) was treated with NBS (5.34 g, 30 mmol) and a catalytic amount of AIBN (120 mg). The mixture was heated at reflux under argon for 6.5 h and then sequentially washed with 4% aqueous $NaHCO_3$ and water. Removal of the solvent and chromatographic purification of the residue afforded the title product as a white powder (5.09 g, 74% yield).

3-(Acetoxymethyl)-2α-bromo-4-(*tert*-butylcarbonyl)-7α-methoxy-Δ^3-cephem 1,1-dioxide (50): obtained from **21** by electrophilic bromination (method D); yellowish powder (57% yield); mp 107–109 °C; IR (KBr) ν_{max} 1795, 1745, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (9H, s), 2.13 (3H, s), 3.59 (3H, s), 4.39 (1H, d, J = 13.3 Hz), 4.63 (1H, d, J = 13.3 Hz), 5.20 (1H, d, J = 2.1 Hz), 5.26 (1H, s), 5.40 (1H, d, J = 2.1 Hz).

4-(*tert*-Butylcarbonyl)-2α-bromo-7α-methoxy-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (51): obtained from **22** by electrophilic bromination (method D); white solid (58% yield); mp 60–61 °C; IR (KBr) ν_{max} 1800, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (9H, s), 3.59 (3H, s), 3.70 (1H, d, J = 14 Hz), 3.93 (3H, s), 4.20 (1H, d, J = 14 Hz), 5.20 (1H, d, J = 1.9 Hz), 5.44 (1H, d, J = 1.9 Hz), 5.74 (1H, s).

4-(*tert*-Butylcarbonyl)-7α-methoxy-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (52): obtained from **49** according to method E by doubling the mole amount of 5-mercapto-1-methyl-1,2,3,4-tetrazole and TEA; yellowish powder (85% yield); mp 103–106 °C; IR (KBr) ν_{max} 1800, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.27 (9H, s), 3.53 (3H, s), 3.68 (1H, d, J = 14.2 Hz), 3.94 (3H, s), 4.11 (3H, s), 4.34 (1H, d, J = 14.2 Hz), 5.10 (1H, d, J = 1.9 Hz), 5.20 (1H, d, J = 1.9 Hz), 5.55 (1H, s). Anal. ($C_{17}H_{23}N_9O_5S_3$) C, H; N: calcd, 23.80; found, 23.18.

4-(*tert*-Butylcarbonyl)-7α-methoxy-2α-[(5-methyl-1,3,4-thiadiazol-4-yl)thio]-3-[[(5-methyl-1,3,4-thiadiazol-4-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (53): obtained from **49** according to method E by doubling the mole amount of 4-mercapto-5-methyl-1,3,4-thiadiazole and TEA; yellowish powder (85% yield); mp 112–115 °C; IR (KBr) ν_{max} 1795, 1690 cm^{-1}; NMR ($CDCl_3$) δ 1.21 (9H, s), 2.70 (3H, s), 2.78 (3H, s), 3.54 (3H, s), 3.62 and 4.46 (2H, each d, J = 14.2 Hz), 5.21 (1H, d, J = 2.0 Hz), 5.40 (1H, d, J = 2.0 Hz), 5.74 (1H, s). Anal. ($C_{19}H_{23}N_5O_5S_5$) H, N; C: calcd, 40.62; found, 40.19.

2α-(Benzoyloxy)-3-[(benzoyloxy)methyl]-4-(*tert*-butylcarbonyl)-7α-methoxy-Δ^3-cephem 1,1-Dioxide (54). A mixture of **49** (230 mg, 0.5 mmol) and silver benzoate (345 mg, 1.5 mmol) in MeCN (10 mL) was stirred at room temperature for 2 h. After partitioning between EtOAc and water, the upper layer was dried (Na_2SO_4) and rotoevaporated. The residue was purified by flash chromatography, affording the title product as a light yellow powder (76 mg, 28% yield): mp 58–60 °C; IR (KBr) ν_{max} 1800, 1755, 1740, 1700 (sh) cm^{-1}; NMR ($CDCl_3$) δ 1.36 (9H, s), 3.59 (3H, s), 4.73 (1H, d, J = 13.5 Hz), 4.80 (1H, d, J = 13.5 Hz), 4.99 (1H, d, J = 2.1 Hz), 5.13 (1H, d, J = 2.1 Hz), 6.26 (1H, s), 7.2–8.2 (10H, m); FD-MS 541 (M^+).

3-(Acetoxymethyl)-4-(*tert*-butylcarbonyl)-7α-methoxy-2-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-Δ^3-cephem 1,1-dioxide (55): obtained from **50** and 5-mercapto-1-methyl-1,2,3,4-tetrazole (method E); white powder (75% yield); mp 84–86 °C; IR (KBr) ν_{max} 1805, 1750, 1705 cm^{-1}; NMR ($CDCl_3$) δ 1.26 (9H, s), 2.14 (3H, s), 3.57 (3H, s), 4.08 (3H, s), 4.32 (1H, d, J = 13.2 Hz), 4.79 (1H, d, J = 13.2 Hz), 5.15 (1H, d, J = 2.0 Hz), 5.22 (1H, d, J = 2.0 Hz), 5.35 (1H, s).

2α-(Benzoyloxy)-4-(*tert*-butylcarbonyl)-7α-methoxy-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-Δ^3-cephem 1,1-dioxide (56): obtained from **51** (method F); white powder (47% yield); mp 101–104 °C; IR (KBr) ν_{max} 1805, 1750, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.35 (9H, s), 3.59 (3H, s), 3.84 (3H, s), 3.87 and 4.19 (2H, each d, J = 14.2 Hz), 4.99 (1H, d, J = 1.9 Hz), 5.24 (1H, d, J = 1.9 Hz), 6.25 (1H, s), 7.3–8.1 (5H, m).

2α-Bromo-3-(bromomethyl)-7α-methoxy-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (57): obtained from **18** (2.89 g, 9 mmol), NBS (3.21 g, 18 mmol), and AIBN (50 mg), as described for **49**; yellow powder (2.9 g, 67% yield); mp 63–64 °C; NMR ($CDCl_3$) δ 3.57 (3H, s), 3.79 (1H, d, J = 11.8 Hz), 4.07 (1H, d, J = 11.8 Hz), 5.24 (1H, d, J = 2.0 Hz), 5.45 (1H, d, J = 2.0 Hz), 5.59 (1H, s), 7.5–8.0 (5H, m).

7α-Methoxy-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-3-[[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]methyl]-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (58): obtained from **57** according to method E by doubling the mole amount of 5-mercapto-1-methyl-1,2,3,4-tetrazole and TEA; yellowish powder (79% yield); IR (KBr) ν_{max} 1800, 1675 cm^{-1}; NMR ($CDCl_3$) δ 3.46 (3H, s), 3.92 (3H, s), 3.87 (1H, d, J = 14.4 Hz), 4.10 (3H, s), 4.36 (1H, d, J = 14.4 Hz), 5.21 (1H, d, J = 2.0 Hz), 5.24 (1H, d, J = 2.0 Hz), 5.71 (1H, s), 7.4–7.8 (5H, m).

7α-Methoxy-2α-[(5-methyl-1,3,4-thiadiazol-4-yl)thio]-3-[[(5-methyl-1,3,4-thiadiazol-4-yl)thio]methyl]-4-(phenylcarbonyl)-Δ^3-cephem 1,1-dioxide (59): obtained from **57** according to method E by doubling the mole amount of 4-mercapto-5-methyl-1,3,4-thiadiazole and TEA; yellowish powder (63% yield); mp 125–130 °C; IR (KBr) ν_{max} 1800, 1675 cm^{-1}; NMR ($CDCl_3$) δ 2.69 (3H, s), 2.79 (3H, s), 3.50 (3H, s), 3.75 (1H, d, J = 14.5 Hz), 4.55 (1H, d, J = 14.5 Hz), 5.23 (1H, d, J = 1.9 Hz), 5.54 (1H, d, J = 1.9 Hz), 5.89 (1H, s), 7.4–8.0 (5H, m). Anal. ($C_{21}H_{19}N_5O_5S_5$) C, H; N: calcd, 12.03; found, 11.48.

4-(*tert*-Butylcarbonyl)-7α-chloro-3-methyl-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-Δ^3-cephem 1,1-dioxide (60): obtained from **7** by sequential electrophilic bromination and displacement with 5-mercapto-1-methyl-1,2,3,4-tetrazole (methods D and E); white powder (42% yield); mp 90–92 °C; IR (KBr) ν_{max} 1810, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.23 (9H, s), 1.90 (3H, s), 4.20 (3H, s), 5.05 (1H, s), 5.23 (1H, d, J = 1.9 Hz), 5.33 (1H, d, J = 1.9 Hz).

3-(Acetoxymethyl)-4-(*tert*-butylcarbonyl)-7α-chloro-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)thio]-Δ³-cephem 1,1-dioxide (61): obtained from **9** as described above; white powder (14% yield); IR ($CHCl_3$) ν_{max} 1815, 1735, 1700 cm^{-1}; NMR ($CDCl_3$) δ 1.25 (9H, s), 2.14 (3H, s), 4.08 (3H, s), 4.32 (1H, d, J = 13.3 Hz), 4.76 (1H, d, J = 13.3 Hz), 5.30 (1H, d, J = 2.0 Hz), 5.40 (1H, d, J = 2.0 Hz), 5.40 (1H, s).

7α-Chloro-3-methyl-2α-[(1-methyl-1,2,3,4-tetrazol-5-yl)-thio]-4-(phenylcarbonyl)-Δ³-cephem 1,1-dioxide (62): obtained from **11** as described above; light yellow powder (76% yield); mp 89–92 °C; IR ($CHCl_3$) ν_{max} 1810, 1675 cm^{-1}; NMR ($CDCl_3$) δ 1.92 (3H, s), 4.11 (3H, s), 5.28 (1H, s), 5.32 (1H, d, J = 2.0 Hz), 5.37 (1H, d, J = 2.0 Hz), 7.51–8.02 (5H, m). Anal. ($C_{16}H_{14}ClN_5O_4S_2$) C, H, N.

3-(Acetoxymethyl)-7α-chloro-4-[[(4-methoxybenzyl)-oxy]carbonyl]-Δ³-cephem 1,1-Dioxide (63). A mixture of 3-(acetoxymethyl)-7α-chloro-Δ³-cephem-4-carboxylic acid[25] (2.4 g, 8.2 mmol), 4-methoxybenzyl chloride (1.4 g, 9 mmol), TEA (1.25 mL, 9 mmol), and NaBr (926 mg, 9 mmol) in DMF (35 mL) was stirred overnight at room temperature and then poured into EtOAc–water. The organic phase was sequentially washed with 2% HCl, water, 4% aqueous $NaHCO_3$, and brine and then dried (Na_2SO_4) and rotoevaporated to give the crude cephem sulfide ester (mixture of Δ³- and Δ²-isomers) as a thick reddish oil. Following oxidation of this material with MCPBA according to method B, the title product was obtained as a whitish foam (1.3 g, 36% yield): IR (KBr) ν_{max} 1812, 1735 cm^{-1}; NMR ($CDCl_3$) δ 2.06 (3H, s), 3.77 (1H, d, J = 18.2 Hz), 3.81 (3H, s), 4.02 (1H, br d, J = 18.2 Hz), 4.70 (1H, d, J = 14.0 Hz), 4.78 (1H, m), 5.07 (1H, J = 14.0 Hz) 5.21 (1H, d, J = 11.9 Hz), 5.31 (1H, d, J = 1.9 Hz), 5.33 (1H, d, 11.9 Hz), 6.91 (2H, d, J = 8.9 Hz), 7.35 (2H, d, J = 8.9 Hz).

3-(Acetoxymethyl)-7α-chloro-4-[[(4-methoxybenzyl)-oxy]carbonyl]-4β-(3-oxo-1-butyl)-Δ²-cephem 1,1-Dioxide (64) and 3-(Acetoxymethyl)-7α-chloro-4α-[[(4-methoxy-benzyl)oxy]carbonyl]-2,4β-bis(3-oxo-1-butyl)-Δ²-cephem 1,1-Dioxide (65). Compound **63** (2.89 g, 6.99 mmol) was dissolved in a mixture of CH_2Cl_2 (6 mL) and methyl vinyl ketone (6 mL). After addition of TEA (0.29 mL), the solution was left at room temperature for 1 h and rotoevaporated. Fractionation by flash chromatography gave **64** (1.76 g, 52% yield) and **65** (0.93 g, 24%) as white waxy solids.

Compound 64: IR ($CHCl_3$) ν_{max} 1795, 1740, 1714 cm^{-1}; NMR ($CDCl_3$) δ 2.10 (3H, s), 2.14 (3H, s), 2.2–2.3 (4H, m), 3.81 (3H, s), 4.52 (1H, dd, J = 1.7 and 16.0 Hz), 4.69 (1H, d, J = 1.6 Hz), 4.70 (1H, dd, J = 1.7 and 16.0 Hz), 5.14 (1H, d, J = 11.6 Hz), 5.23 (1H, d, J = 1.6 Hz), 5.27 (1H, d, J = 11.6 Hz), 6.56 (1H, t, J = 1.7 Hz), 6.89 and 7.27 (each 2H, d, J = 8.7 Hz).

Compound 65: IR ($CHCl_3$) ν_{max} 1793, 1740–1710 cm^{-1}; NMR ($CDCl_3$) δ 1.97 (3H, s), 2.13 (3H, s), 2.15 (3H, s), 2.0–3.0 (8H, m), 3.81 (3H, s), 4.65 (1H, d, J = 1.4 Hz), 4.73 (2H, ABq, J = 13.6 Hz), 5.05 (1H, d, J = 11.7 Hz), 5.21 (1H, d, J = 1.4 Hz), 5.25 (1H, d, J = 11.7 Hz), 6.89 and 7.27 (each 2H, d, J = 8.7 Hz).

3-(Acetoxymethyl)-7α-chloro-3-methyl-4-(3-oxo-1-butyl)-Δ³-cephem 1,1-Dioxide (66). A solution of **64** (1.5 g) in CH_2Cl_2 (10 mL), anisole (0.5 mL), and TFA (4.5 mL) was allowed to stand for 15 min at room temperature. Removal of the solvent under reduced pressure left a residue which was dissolved in EtOAc and rapidly treated with 4% aqueous $NaHCO_3$. The aqueous phase was collected, fresh EtOAc was added, and the resulting mixture was vigorously stirred for 10 min (evolution of carbon dioxide). Following drying over Na_2SO_4, the organic phase was evaporated to a residue which crystallized from isopropyl ether (440 mg): mp 146–148 °C; IR (KBr) ν_{max} 1795, 1730, 1707 cm^{-1}; NMR ($CDCl_3$) δ 2.08 (3H, s), 2.18 (3H, s), 2.7–3.0 (4H, m), 3.68 (1H, d, J = 18.0 Hz), 3.94 (1H, br d, J = 18.0 Hz), 4.60 (1H, d, J = 13.0 Hz), 4.73 (1H, m), 4.74 (1H, d, J = 13.0 Hz), 5.29 (1H, d, J = 1.9 Hz). Anal. ($C_{13}H_{16}ClNO_6S$) C, H, N.

3-(Acetoxymethyl)-7α-chloro-4-(methoxycarbonyl)-Δ³-cephem 1,1-Dioxide (67). A solution of 3-(acetoxymethyl)-7α-chloro-Δ³-cephem-4-carboxylic acid 1,1-dioxide[25] (323 mg, 1 mmol) in dry THF (10 mL) was cooled to 0 °C under nitrogen and sequentially treated with oxalyl chloride (0.174 mL, 2 mmol) and a catalytic amount (0.02 mL) of DMF. After stirring for 2 h at 0 °C, the solvent was removed in vacuo (bath temperature ≤ 25 °C). The crude acid chloride was dissolved in 2:1 CH_2Cl_2–MeOH (15 mL) and treated with $CaCO_3$ (6 g). The mixture was vigorously stirred at room temperature for 2 h and then filtered and rotoevaporated. Purification of the raw material by flash chromatography gave the title product as a white solid (175 mg, 52% yield): mp 124–125 °C; IR (KBr) ν_{max} 1815, 1735 cm^{-1}; NMR ($CDCl_3$) δ 2.10 (3H, s), 3.79 (1H, d, J = 18.4 Hz), 3.93 (3H, s), 4.04 (1H, dd, J = 1.1 and 18.4 Hz), 4.73 (1H, d, J = 14.1 Hz), 4.81 (1H, m), 5.12 (1H, d, J = 14.1 Hz), 5.32 (1H, d, J = 1.8 Hz).

Biochemistry. Enzymes and Substrates. HLE (from human sputum) was purchased from Elastin Products; the peptide substrates were either from Sigma or Bachem (Bubendorf, Switzerland); bovine neck ligament elastin was a Sigma product. HLE was dissolved at a concentration of 1–2 mg/mL in 5 mM acetate buffer, pH 5, supplemented with 0.145 M NaCl and 0.01% Triton X-100, and then aliquoted in plastic tubes and stored frozen at −20 °C. The active site concentration of the enzyme was determined by spectrophotometric monitoring of residual activity after reaction with increasing amounts of the irreversible inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl (Sigma).

Inhibition of the Amidolytic Activity of HLE. Inhibition of HLE activity was investigated as previously described[11] with the fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-7-(4-methyl)coumarylamide at 37 °C and pH 7.4. Test solutions contained 1% DMSO and 1% MeCN, as solubilizer of substrate and inhibitor, respectively, and 0.01% Triton X-100. The active enzyme concentration ranged between 1 and 3 nM, while substrate and inhibitor concentrations varied as required for a proper determination of kinetic parameters. The Michaelis constant, K_m = 1.2 ± 0.1 mM, was independently determined for the same substrate under identical experimental conditions. Data fitting to eqs 1–3 (see Results) was carried out with the program MINSQ (Micro Math Scientific Software, Salt Lake City, UT).

Inhibition of the Elastinolytic Activity of HLE. Elastin from bovine neck ligament (Sigma) was incubated with HLE with or without added inhibitors at various concentrations. The finely powdered elastin substrate (2 mg) was suspended in 400 μL of 57 mM sodium/potassium phosphate buffer (pH 7.4, I = 0.15), and then 20 μL of inhibitor solution was added, diluted at the appropriate concentration in the same buffer supplemented with 40% (v/v) DMSO. The reaction was started by adding 10 μL of HLE prediluted in 0.1 M acetate buffer at pH 4.5 to give a 0.5 μM final concentration of elastase active sites. The mixture was incubated at 37 °C for exactly 120 min, and reaction was stopped by adding 100 μL of 25% (w/v) trichloroacetic acid. After centrifugation, a 0.1 mL portion of clear supernatant was mixed to 3.0 mL with 0.2 M sodium borate buffer (pH 8.5). A solution of fluorescamine in acetone (0.15 mg/mL, 1.0 mL) was then added under vigorous stirring, and the fluorescence of labeled peptides was monitored with λ_{ex} = 390 nm and λ_{em} = 480 nm. The fluorescence obtained in the absence of inhibitor was taken as reference (100% activity). Appropriate blanks were run to take into account the fluorescence developed by elastin, enzyme, and inhibitors alone. DMSO (final concentration = 1.9%) did not interfere with the enzyme activity, and the assay was linear with time up to 8 h in the absence of inhibitors.

Inhibition of HLE in Living PMNs. Human polymorphonuclear leukocytes (200–250 × 10^6 cells) under excellent conditions of vitality (trypan blue test) were obtained from fresh human venous blood (100 mL). Blood, treated with citrate to prevent coagulation, was centrifuged at 300g for 30 min in the presence of Mono-Poly resolving medium (M-PRM; Flow Laboratories; 3.5 mL of blood and 3.0 mL of M-PRM for each tube). The PMN-containing layer was collected by suction, and impurities from erythrocytes were removed by repeated hypotonic shock. Harvested cells were washed twice with Hank's balanced salt solution (HBSS), suspended in 1:1 HBSS and pH 7.2 phosphate buffer supplemented with 5 mM EDTA, and counted.

Incubations of PMNs with inhibitors were run according to the method of Bonney et al.[17] PMNs suspended at 5×10^6 cells/mL concentration in the above medium were divided into 1 mL aliquots, 0.01 mL of a solution of the inhibitor (10 μg) in DMSO was added, and the mixture was incubated for 15 min at 37 °C with shaking. Controls were obtained by adding DMSO alone. Cells were centrifuged at 300*g* for 10 min, washed three times by centrifugation with fresh suspension medium (2 mL), and resuspended in 0.5 mL of buffer without EDTA. A 2 μL aliquot of a cytochalasin B solution in DMSO (Serva; 1.25 mg/mL) was added to each sample. After a 5 min incubation at 37 °C, a 5 μL aliquot of 10^{-5} M fMLP (Bachem; 4.38 mg/mL in DMSO, diluted 1:100 with HBSS) was added and incubations were continued for 15 min. Samples were centrifuged at 400*g* for 5 min, and the supernatants were collected.

Elastase activity released by treated and untreated PMNs in the supernatants was assayed, using *N*-acetyl-Ala-Ala-Pro-Ala-7-(4-methyl)coumarylamide (Bachem) as a substrate, by a prolonged incubation procedure in order to maximize sensitivity. Aliquots (0.4 mL) of a stock solution of the substrate (0.5 mM) in 57 mM pH 7.4 sodium/potassium phosphate buffer supplemented with 1.4% (v/v) DMSO were mixed with the supernatants (50 μL) from stimulated PMNs and incubated at 25 °C for 6 h. The reaction was quenched with 86 mM phenylmethanesulfonyl fluoride (PMSF) in 2-propanol (10 μL), and fluorescence was determined with λ_{ex} = 383 nm and λ_{em} = 455 nm.

Stability Studies. Materials. Buffer salts and other chemicals were commercial products of analytical grade; solvents were HPLC grade. Water purified by a Milli-Q reagent grade water system (Millipore, Italy) was used throughout.

Preparation of Solutions. Cephem sulfones to be tested were dissolved in 2 volumes of MeCN and diluted with 8 volumes of water at room temperature to a final 2 mM concentration. Suspensions obtained from products not completely soluble under these conditions were filtered (Millex HV, 0.45 μm; Millipore). Kinetic runs were started by the addition of 0.1 mL aliquots of these solutions, preheated at 37 °C, to 0.9 mL aliquots of the aqueous phosphate buffers described above in clear glass HPLC vials, preheated and held at 37 °C, and immediately capped. The solutions were carefully inspected against any possible product precipitation, especially in the low-pH range, and, when in doubt, immediately filtered onto new preheated vials. Reaction vials were maintained in the dark in a controlled-temperature autosampler and product concentrations monitored over time by HPLC assay. Though total buffer concentration greatly exceeded the reacting product concentration, some phosphate buffers, especially those in the range of pH 9–11, have very low buffer capacity: pH invariance in the course of experiments was verified on separate but identical reaction mixtures.

Analytical Procedures. HPLC assay of cephem sulfones was carried out by reverse-phase high-speed chromatography using a Hewlett Packard HP1090A liquid chromatograph, equipped with an HP1040A diode array detector, a temperature-controlled autosampler, a programmable autoinjector, a heated column compartment, and a DR5 ternary solvent delivery system. Availability of three mobile phases on line allowed automatic switching between two different methods of elution. Phase A consisted of 50 mM aqueous phosphate buffer, adjusted to pH 3.0 with phosphoric acid and filtered (HATF, 0.45 μm; Millipore). Phase B was prepared by mixing 300 mL of phase A and 700 mL of MeCN. Phase C consisted of 50 mM aqueous phosphate buffer adjusted to pH 9.0 with sodium hydroxide and filtered. Aliquots (20 μL) of reaction mixtures were injected onto a Hypersil-ODS column (60 × 4.6 mm i.d., 3 μm spherical particles; Hewlett Packard) thermostated at 40 °C, and a gradient analysis was carried out at a 1 mL/min flow rate from 10% phase B (90% A) to 100% B over 4.5 min, holding 100% B up to 5.5 min, and reconditioning to 10% B for more than 2.5 min. The investigated cephem sulfones generally eluted in the range 4–5.5 min, completely resolved from degradation products and other byproducts, and were detected by UV absorption at 268 nm (bandwidth = 16 nm). Specificity of the assay was constantly checked by means of UV spectra acquired during peak elution. In just one case (compound **25**), achieving chromatographic resolution required isocratic elution at 60% B (40% A). Compounds **24**, **34**, and **70**, carrying the STrx group, could be eluted as narrow peaks only after increasing the pH of the mobile phase up to 9. To the aim, gradient elution was carried out from 10% phase B (90% C) to 100% B over 4.5 min.

Data collection at appropriate times was extended over more than 4 half-lives when possible, or at least 1 month for highly stable products (e.g., **58**, **59**). Reproducibility of the assay was checked over 1 month with standard solutions of benzoic acid: deviations of peak areas were within 1%. Experimental data of peak area values collected over time were fit to eq 5 (see Results) or, when required, eq 7. By a nonlinear weighted least squares procedure,[24] k_{st} was obtained either directly from fit to eq 5 or from the empirical definition (eq 8) in the case of double-exponential decay (eq 7). Half-life values listed in Tables 1–3 were calculated as $t_{1/2} = 0.693/k_{st}$. Stability data of a few compounds reported here have been previously communicated by us.[12b] These values, obtained under different conditions, are not directly comparable with those listed in Tables 1–3.

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