ligand affinity for the receptors (K_D) was held constant for each radioligand.

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Supplementary Material Available: Tables of fractional atomic coordinates, thermal parameters, and bond lengths and bond angles for (*S*)-**1b** (3 pages). Ordering information is given on any current masthead page.

Inhibition of Human Sputum Elastase by Substituted 2-Pyrones[1]

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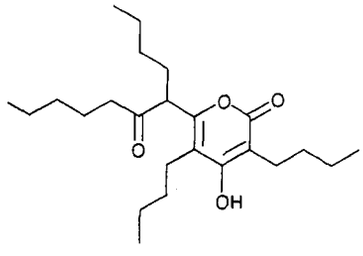
Nineteen 4-hydroxy- and 4-methoxy-2-pyrones related to elasnin (I) have been assayed for in vitro inhibition of human sputum elastase (HSE), porcine pancreatic elastase, α-chymotrypsin, and trypsin. Inhibition is reported as K_i and K_i'; percentage inhibition was dependent on [S] in a number of cases, making it unsuitable as a measure of relative inhibition. The 3-(1-oxoalkyl)-4-hydroxy-6-alkyl-2-pyrones were found to be most effective, the octyl homologue **11** being the most potent inhibitor (K_i = 4.6 μM, 30 times better than the lead compound). A further reduction in inhibition was observed when the hitherto hydrophobic 6-substituent was substituted by a branched functionality of hydrophilic nature. Conversely, methylation of the 4-hydroxy group of the 6-alkyl-2-pyrones increased inhibitory activity. The mechanism of inhibition varied from pure noncompetitive to mixed type to uncompetitive and was found to be dependent on the pattern of substitution. We believe that the 4-hydroxy-2-pyrone binds to the S_4 subsite, with the 6-substituent extending across the S_4–S_1 subsites and the 3-substituent occupying the S_5 subsite. The length of the inhibitor binding region was calculated to be approximately 24 Å. None of the hydrophobic compounds were found to have any appreciable inhibition (<10%) with porcine pancreatic elastase, bovine α-chymotrypsin, and bovine trypsin when tested at the limit of their solubility. The hydrophilic compounds were nonspecific, inhibiting all four enzymes. Dialysis was used to show that the interaction is fully reversible.

Human sputum elastase (HSE) has been implicated in many inflammatory disease states such as pulmonary emphysema, acute arthritis, and destruction of connective tissue.[2-5] The report by Omura et al.[6] that elasnin (I), an alkylated 2-pyrone of microbial origin, was a good inhibitor of HSE prompted us to use elasnin as a lead compound in the search for specific and potent inhibitors of HSE.



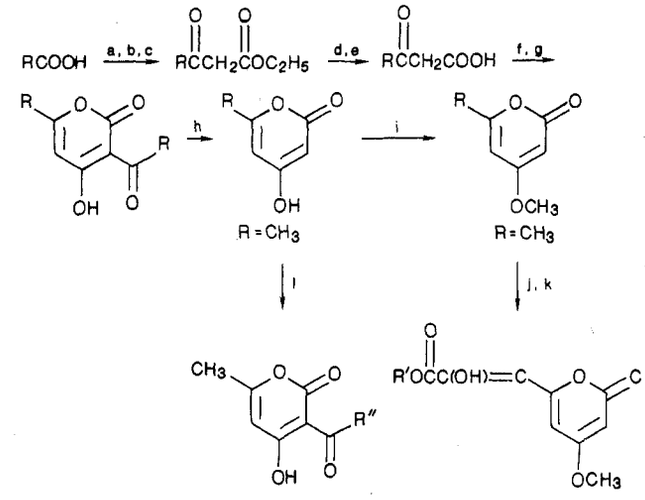
I

We report here the in vitro enzyme inhibitory properties of 19 elasnin analogues, their mechanism of action, the requirements of the inhibitor binding region, and its relationship to the substrate binding region.

Chemistry

The substituted 2-pyrones were prepared according to Scheme I. The 3-oxo carboxylate[7] was formed by reacting an acid imidazolide (formed in situ) with the neutral magnesium salt of ethyl hydrogen malonate.[8] Hydrolysis of the ester in 1 M NaOH afforded the 3-oxo carboxylic acid, which was cyclized by using 1.1 equiv of carbonyldiimidazole in THF[9] to afford the 3-(1-oxoalkyl)-4-hydroxy-6-alkyl-2-pyrones (pentyl to undecyl). Deacylation was easily carried out[10] by heating these 2-pyrones at 130 °C in 90% H_2SO_4. The 4-hydroxy group was methylated[11] by using dimethyl sulfate and anhydrous K_2CO_3

Scheme I[a]



[a] (a) Carbonyldiimidazole, THF, room temperature; (b) Mg(OOCCH$_2$COOC$_2$H$_5$)$_2$; (c) H$^+$; (d) 1 M NaOH; (e) H$^+$; (f) carbonyldiimidazole, THF, room temperature; (g) H$^+$; (h) 90% H_2SO_4, 130 °C; (i) $(CH_3)_2SO_4$, K_2CO_3, petroleum ether (60–80 °C); (j) $(R'OOC)_2$, Na; (k) H$^+$; (l) R″COCl, CF_3COOH.

(1) This is part 2 of a series. For part 1, see: Lentini, A.; Ternai, B.; Ghosh, P. *Biochem. Int.* **1985**, *10*, 221.
(2) Janoff, A. *Annu. Rev. Med.* **1972**, *23*, 177.
(3) Mittman, C., Ed. *Pulmonary Emphysema and Proteolysis*; Academic: New York, 1972.
(4) Lieberman, J. *Chest* **1976**, *70*, 62.
(5) Janoff, A.; Feinstein, G.; Malemud, C.; Elias, J. M. *J. Clin. Invest.* **1976**, *57*, 615.
(6) Omura, S.; Ohno, H.; Saheki, T.; Yoshida, M.; Nakagawa, A. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 704.

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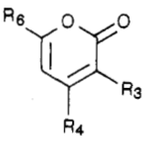
Table I. Inhibition of Human Sputum Elastase by Substituted 2-Pyrones



compd	R_3	R_4	R_6	mp, °C	K_i,[a] μM	α	mechanism
1	COC_5H_{11}	OH	CH_3	66.0–66.5	2000	1.0	noncompetitive
2	COC_7H_{15}	OH	CH_3	67.5–68.0	400	1.0	noncompetitive
3	COC_9H_{19}	OH	CH_3	74.0–74.5	250	1.0	noncompetitive[b]
4	$COC_{11}H_{23}$	OH	CH_3	82.0–82.5	54	1.0	noncompetitive
5	H	OH	C_5H_{11}	48.0–50.0	1700	0.2	mixed type[b]
6	H	OH	C_7H_{15}	71.0–71.5	1100	0.7	mixed type[b]
7	H	OH	C_9H_{19}	75.5–76.0	400	1.4	mixed type[b]
8	H	OH	$C_{11}H_{23}$	82.0–83.0	83	6.5	mixed type
9	COC_5H_{11}	OH	C_5H_{11}	59.5–60.0	67	1.0	noncompetitive
10	COC_7H_{15}	OH	C_7H_{15}	64.5–65.0	15	0.7[c]	mixed type[b]
11	COC_8H_{17}	OH	C_8H_{17}	68.5–69.0	4.6	0.8[d]	mixed type
12	COC_9H_{19}	OH	C_9H_{19}	73.5–74.0	7.9	0.7[e]	mixed type
13	$COC_{11}H_{23}$	OH	$C_{11}H_{23}$	81.5–82.0	ND[f]		
14	H	OCH_3	C_7H_{15}	44.0–45.0	140[g]		uncompetitive[b]
15	H	OCH_3	C_9H_{19}	56.5–57.0	130[g]		uncompetitive[b]
16	H	OCH_3	$CH{=}C(OH)COOC_2H_5$	168.0–170.0	120	0.9	mixed type
17	H	OCH_3	$CH{=}C(OH)COOC_4H_9$	129.5–130.5	350	0.4	mixed type
18	H	OCH_3	$CH{=}C(OH)COOC_5H_{11}$	118.5–119.5	410	0.5	mixed type
19	H	OCH_3	CH=C(OH)COOH	242.0–249.0	NIA[h]		

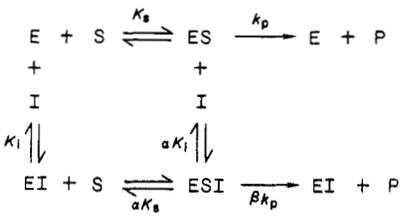
[a] ±10% error. [b] Result of two separate series of experiments. [c] $\beta = 0.1$. [d] $\beta = 0.2$. [e] $\beta = 0.3$. [f] ND = not determined precisely due to low solubility, $K_i > 8$ μM. [g] Uncompetitive inhibition; therefore, K_i' not K_i. [h] NIA = no inhibitory activity at 1000 μM.

in petroleum ether (60–80 °C). Acylation of 4-hydroxy-6-methyl-2-pyrone with the appropriate acid chloride in CF_3COOH yielded the desired 3-(1-oxoalkyl)-4-hydroxy-6-methyl-2-pyrone[12] (pentyl to undecyl). 4-Methoxy-6-methyl-2-pyrone was converted to the 6-(2-hydroxy-2-carboalkoxyvinyl) derivative by treatment with dialkyl oxalate in the presence of metallic sodium.[12] Acid hydrolysis of the ethyl ester[12] (**16**) yielded the carboxy compound (**19**).

Kinetic Analysis

Each compound was assayed at two or more concentrations within the following ranges: 15–250 μM for **1–8** and **14–18**, 5–25 μM for **9–12**, and 4–10 μM for **13**. The chromogenic substrate, *N*-(2-butoxycarbonyl)-L-alanine *p*-nitrophenyl ester was used at three to five different concentrations, in the range 50–750 μM (Boc-Ala-pNP, K_m = 340 μM (lit.[13] K_m = 300 μM)). Higher concentrations of substrate could not be used because then inhibition by the substrate was observed. To determine the kinetic mechanism of inhibition, the data were plotted as $[S]/v$ vs. [S],[14] $1/v$ vs. $1/[S]$,[15] $[S]/v$ vs. [I],[16] and $1/v$ vs. [I].[17] The data were preferentially plotted following the method of Hanes and Wilkinson, as this assured even distribution of uncertainty in the data. To determine whether the inhibition was pure (inactive ESI complex) or partial (active ESI complex), the data were plotted as $1/v_{max_i}$ against [I]. If the plot was linear then the inhibitor was considered to exhibit pure inhibition: that is, the ESI complex is inactive and hence cannot dissociate to EI and P ($\beta = 0$, Scheme II). For linear mixed-type inhibitors ($K_{s_{app}}$ and V_{max_i} change) and pure noncompetitive inhibitors (V_{max_i} changes), a plot of $1/K_{s_{app}}$ vs. [I] is linear. K_i was determined from these plots. A hyperbolic plot of $1/V_{max_i}$ vs. [I] indicated partial inhibition: ESI is active but less so than ES, that is, $\beta < 1$. In this case, a replot of ΔV_{max_i} vs. $1/[I]$ gave a linear plot ($\Delta V_{max_i} = (1/V_{max_i} - 1/V_{max})^{-1}$. Using this plot and $\Delta V_{max}/K_{s_{app}}$ vs. $1/[I]$, α, β, and K_i (and therefore K_i', since $K_i' = \alpha K_i$) could be calculated. All the equations and their derivations can be found in the excellent book by Segel.[18] If there was doubt as to the mechanism of inhibition, the experiment was repeated with fresh solutions of substrate and inhibitor and a new set of data were collected and plotted.

Scheme II[a]



[a] E = enzyme, S = substrate, I = inhibitor, P = *p*-nitrophenol, K_s = [E][S]/[ES], αK_s = [EI][S]/[ESI], K_i = [E][I]/[EI], αK_i = K_i' = [ES][I]/[ESI], k_p = catalytic constant. Pure noncompetitive inhibition: $K_i = K_i'$, $K_s = \alpha K_s$, $\beta = 0$. Mixed-type linear inhibition: $K_i \neq K_i'$, $K_s \neq \alpha K_s$, $\beta = 0$. Mixed-type hyperbolic inhibition: $K_i \neq K_i'$, $K_s \neq \alpha K_s$, $\beta \neq 0$. Pure uncompetitive inhibition: $K_i = \infty$, $K_s = \infty$, $\beta = 0$.

Results and Discussion

The major aspects to this work are mapping of the binding site of HSE and determination of its position in

(7) Brooks, D. W.; Lu, L. D.-L.; Masamune, S. *Angew. Chem. Int. Engl. Ed.* **1979**, *18*, 72.
(8) Hauser, C. R.; Breslow, D.; Baumgarten, E. *J. Am. Chem. Soc.* **1944**, *66*, 1286.
(9) Ohta, S.; Tsujimura, A.; Okamoto, M. *Chem. Pharm. Bull.* **1981**, *29*, 2762.
(10) Collie, J. N. *J. Chem. Soc.* **1891**, *59*, 607.
(11) Bu'Lock, J. D.; Smith, H. G. *J. Chem. Soc.* **1960**, 502.
(12) Douglas, J. L.; Money, T. *Can. J. Chem.* **1968**, *46*, 695.
(13) Visser, L.; Blout, E. R. *Biochim. Biophys. Acta* **1972**, *268*, 257.
(14) Hanes, C. S. *Biochem. J.* **1932**, *26*, 1406.
(15) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.
(16) Cornish-Bowden, A. *Biochem. J.* **1974**, *137*, 143.
(17) Dixon, M. *Biochem. J.* **1953**, *55*, 170.
(18) Segel, I. H. *Enzyme Kinetics Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; Wiley: New York, 1975; Chapters 3 and 4.

relation to the extended substrate binding region and the evaluation of these compounds as specific inhibitors of HSE.

Relationship between Structure and Inhibition. The inhibition constant of each compound, reported as K_i or K_i' (= αK_i), is included in Table I. K_i is of primary interest because it gives a measure of the affinity of I for E. However, for some inhibitors, an ESI and not an EI complex is formed, and therefore it is necessary to compare K_i' values within this group.

The effect of substitution in the 3-position was evaluated with 3-(1-oxoalkyl)-4-hydroxy-6-methyl-2-pyrones **1–4**, which have values of K_i ranging from 2000 to 54 μM. An increase in the number of methylenes was accompanied by a decrease in the magnitude of K_i; that is, an increase in hydrophobicity and molecular size resulted in greater affinity between E and I. Presumably the longer chain interacts better with the hydrophobic region. K_i did not reach a lower limit, so we presume that the binding region is at least large enough to accommodate a 2-pyrone nucleus and a dodecyl group. Insolubility of the higher homologues in the assay medium prevented us from carrying out further studies on longer chain analogues.

The effect of substitution in the 6-position was evaluated with 4-hydroxy-6-alkyl-2-pyrones **5–8**, which had values of K_i ranging from 1700 to 83 μM. An increase in the number of methylenes was accompanied by an increase in affinity between E and I, indicating that this binding region is also hydrophobic. Evidence from the kinetic mechanistic data (see below) indicates that the 3- and 6-substituents bind to hydrophobic regions that are independent of each other. Groutas et al.[19] and Spencer et al.[20] have found that the 3-substituent contributes most significantly to binding. Our results indicate that hydrophobic substituents at either the 3- or 6-positions can bind equally well to HSE.

On the basis of these results, compounds that featured a minimum of five methylene units at both the 3- and 6-positions were therefore of some interest. Compounds **9–12** were prepared and found to have K_i's ranging from 67 to 4.6 μM. The observed K_i values are much lower than those observed for the analogous 3-(1-oxoalkyl)- and 4-hydroxy-6-alkyl-2-pyrones. For example, **10** has a K_i of 15 μM, while **2** and **6** have K_i's of 440 and 1100 μM, respectively. The greater inhibitory activity of **10** may be due to the better fit between the enzyme surface and the 2-pyrone, resulting from the hydrophobic interactions formed by the alkyl chains. Groutas et al.[21] have shown that alkylated phenyl compounds do not bind, whereas the analogous 2-pyrone derivatives have considerable affinity for HSE. We have observed that saturated fatty acids, e.g., lauric acid, do not inhibit HSE. From these results we infer that both features are necessary for inhibition and work in unison. Another interesting feature of the dialkyl compounds (**9–13**) is that, unlike the monoalkyl compounds (**1–4** and **5–8**), K_i does reach a limit. Considering the results for the long-chain compounds, **4** and **8**, and the assumption that the 4-hydroxy-2-pyrone binds to a specific region of HSE, we would expect **13** to be the most potent compound. Due to the low solubility of compound **13**, we were unable to accurately determine the mode of inhibition and hence the K_i value. However, the K_i appears to be greater than that of compound **12**, making the shorter homologue, **11**, the most potent inhibitor. Assuming that the alkyl chains are extended while bound to HSE—and this is reasonable because K_i decreases monotonically with increased number of methylenes—the extended length of compound **11** gives an approximate upper limit to the length of the hydrophobic inhibitor binding region. With standard bond lengths and angles, this was calculated to be 24 Å. The long chain may be folded back, in which case the volume rather than the length of the chain would be relevant. However, the abrupt change in mechanism going from the 6-heptyl (**6**) to the 6-nonyl (**7**) homologue (see below) would seem to indicate that the inhibitor is in the extended conformation when bound to the enzyme.

An implicit assumption being made is that the 2-pyrone binds close to the center of the binding pocket with the two alkyl chains extending to either side. Validation of this assumption utilizing 2-pyrones combining substituents with both short and long alkyl chains, e.g., 3-(1-oxododecyl)-4-hydroxy-6-pentyl-2-pyrone, will be reported shortly. With our present knowledge, the results obtained for the long-chain derivatives, **4** and **8**, can be explained if we assume that the (potential) binding region for the 4-hydroxy-2-pyrone nucleus is somewhat extended. A slight shift of the 2-pyrone would allow all methylenes to bind to E, resulting in the maximum number of enzyme–inhibitor hydrophobic interactions and, hence, the good inhibitory activity.

Since hydrophobicity had been shown to be an important factor in the degree of binding of I to E, we wished to see whether inhibition could be improved by increasing the hydrophobicity by methylating the 4-hydroxy group. Methylation was not successful when the 3-(1-oxoalkyl) functionality was present, although it proceeded quite smoothly for the 6-alkyl derivatives. Therefore, for this initial study on the binding capability of the 4-methoxy group, two compounds were prepared, **14** and **15** with K_i' = 140 and 130 μM, respectively. Note that since these are uncompetitive inhibitors, it is necessary to compare values of K_i'. The analogous hydroxy compounds, **6** and **7**, have K_i' values of 800 and 570 μM, respectively, indicating that the methoxy compounds have greater affinity for ES. Compounds **14** and **15** are different from **6** and **7** in three ways: they are unable to bind to E, they have greater affinity for ES, and the affinity of I for ES appears to be independent of the number of methylenes at the 6-position. It is likely that the 4-hydroxy forms a hydrogen bond with the enzyme. Blocking of this group by methylation precludes such an interaction; only when the substrate molecule binds is there a change in conformation (of some crucial group) resulting in the compound being able to bind to the enzyme–substrate complex. The lack of dependence of K_i' with 6-alkyl chain length for these two 4-methoxy compounds indicates that the binding region might only be sufficiently large enough for a heptyl or nonyl residue. This result is interesting because it differs from that observed for the 4-hydroxy compounds; that is, that the binding space is large enough for an undecyl group. A possible explanation is that the 4-methoxy-2-pyrone binding site is somewhat displaced from the 4-hydroxy-2-pyrone binding site, possibly toward the substrate binding site.

Groutas et al.[19] reported that methylation of the 4-hydroxy group resulted in a compound with reduced inhibition. Our observations indicate the opposite and an explanation for this disparity may lie in their use of percentage inhibition as a measure of the relative order of inhibition. We have observed percentage inhibition to be

(19) Groutas, W. C.; Abrams, W. R.; Carrol, R. T.; Moi, M. K.; Miller, K. E.; Margolis, M. T. *Experientia* **1984**, *40*, 361.
(20) Spencer, R. W.; Copp, L. S.; Pfister, J. R. *J. Med. Chem.* **1985**, *28*, 1828.
(21) Groutas, W. C.; Stanga, M. A.; Brubaker, M. J.; Huang, T. L.; Moi, M. K.; Caroll, R. T. *J. Med. Chem.* **1985**, *28*, 1106.

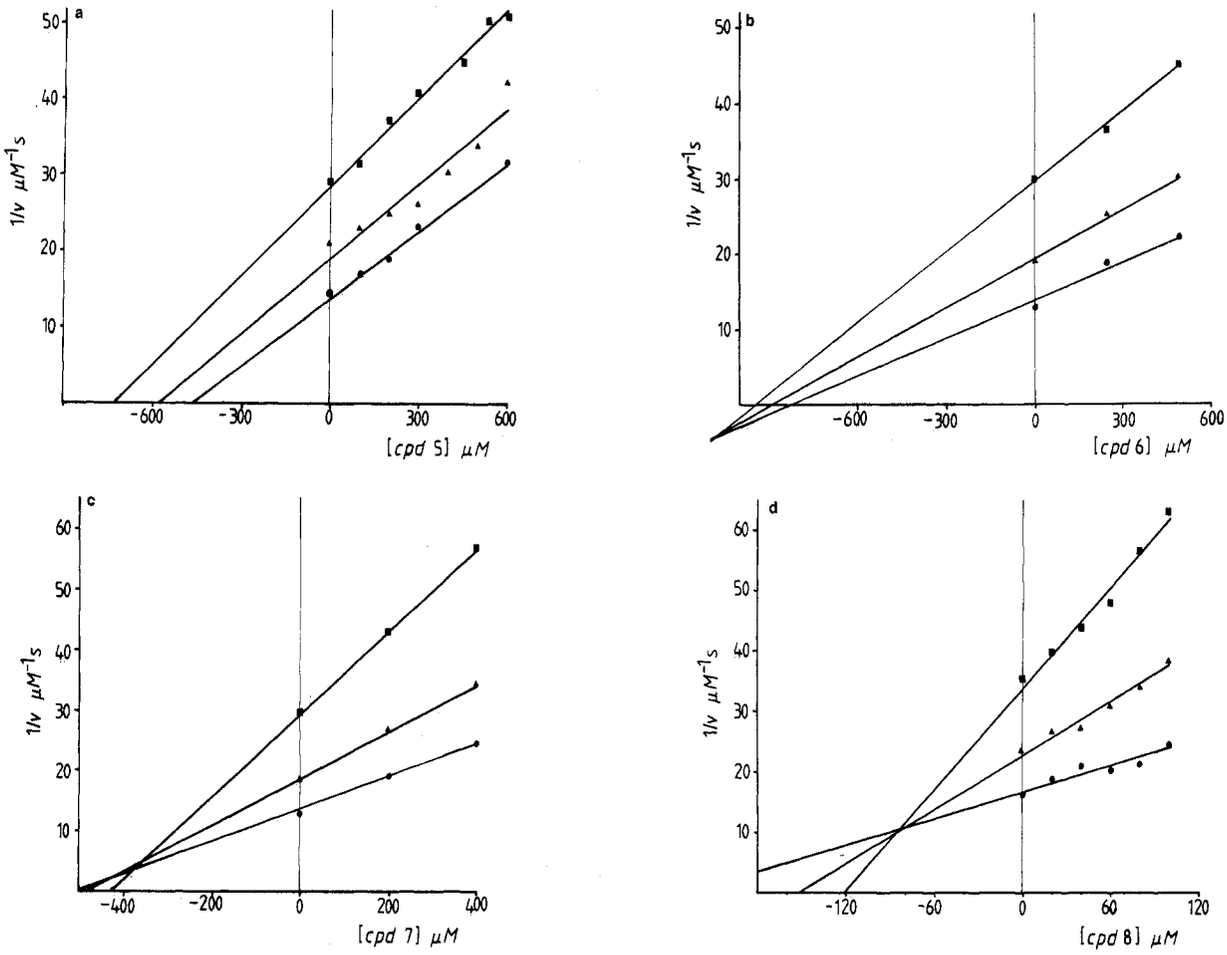


Figure 1. Dixon plot of the inhibition of HSE by 4-hydroxy-6-alkyl-2-pyrones. (a) Pentyl: (■) 200 μM, (▲) 400 μM, (●) 750 μM BAN. (b) Heptyl: (■) 100 μM, (▲) 250 μM, (●) 500 μM BAN. (c) Nonyl: (■) 100 μM, (▲) 250 μM, (●) 500 μM BAN. (d) Undecyl: (■) 200 μM, (▲) 400 μM, (●) 750 μM BAN. (For pentyl and heptyl, $K_i > K_i'$; for nonyl and undecyl, $K_1 < K_i'$.)

dependent on [S] for some enzyme–inhibitor interactions. Results for **7** and **14** demonstrate this quite well. At [S] = 250 μM (0.74 K_m), **7** ([I] = 250 μM) inhibits HSE by 24%, while **14** ([I] = 250 μM) inhibits HSE by only 13%. The conclusion arrived at is that the 4-hydroxy derivative (**7**) is the better inhibitor. However, if the experiment is performed at a higher [S] (750 μM for both cases, 2.5 K_m), then the reverse result is observed; **7** inhibits HSE by 34%, while **14** inhibits HSE by 42%. This result is, of course, expected because **14** is an uncompetitive inhibitor; that is, it can only bind to ES, and as [ES] is increased, the [ESI] increases and consequently the observed percentage inhibition is higher. Groutas et al. worked at 0.58 K_m and would therefore have had a low concentration of ES. These results indicate that using percentage inhibition as a measure of relative inhibitory activity can lead to incorrect conclusions. Determination of the dissociation constants, K_i and K_i', gives a reliable result, since they are independent of [S].

The substituent in the 6-position was then modified to a 2-hydroxy-2-carboalkoxyvinyl moiety while the 4-methoxy-2-pyrone nucleus was maintained. An increase in the number of ester methylenes resulted in a decrease in inhibitory activity (**16–18**; K_i' = 100, 140, and 206 μM, respectively). Previously, we suggested that the binding region for the 6-alkyl substituent of the 4-methoxy-2-pyrone might be large enough for seven to nine methylenes. The results obtained for this series of compounds indicate that the pocket is marginally smaller. Comparison between **15** and **18**, with K_i' = 130 and 200 μM, respectively, shows that the hydrophobic derivative has greater affinity for ES than does the oxygenated, and hence more hydrophilic, compound **18**. This indicates that the 6-substituent binds to a particularly hydrophobic region. Groutas et al.[19] have shown that introduction of a branched substituent increases inhibitory activity. Therefore, the lower inhibitory activity observed for **16–18** is presumably due to the increased hydrophilicity. The inactivity of the free carboxyl derivative, **19**, is then not surprising, since the results indicate that the binding region for the 6-substituent is hydrophobic.

Mechanism. A structure/inhibitory activity relationship has been established by comparison of the dissociation constants K_i and K_i', and on the basis of this, we predict that the hydrophobic inhibitor binding region extends for approximately 24 Å. From the mechanistic data, we can speculate where this region might lie. Our results show that these substituted 2-pyrones do not act directly at the active site. However, we do have reason to believe that they bind to the extended substrate binding region. A discussion of the relevant data is presented below.

Binding of the 3-(1-oxoalkyl)-4-hydroxy-2-pyrones **1–4** is unaffected by S binding, whereas binding of the 4-hydroxy-6-alkyl-2-pyrones **5–8** is influenced by the binding of S to E. The generally accepted nomenclature describes the former class of compounds as pure noncompetitive inhibitors, $K_i = K_i'$, while the latter are known as mixed-type inhibitors, $K_i \neq K_i'$. The mechanism of action differs significantly for the two types of compounds; therefore, we conclude that there is a specific binding region for the 4-hydroxy-2-pyrone nucleus and for each of the two alkyl fragments at positions 3 and 6. A further point of interest

is that, for **1–4**, the mechanism is not influenced by the number of methylenes, whereas, for **5–8**, the reverse is true (Figure 1). The long-chain homologues **7** and **8** have $K_i < K_i'$; that is, I has greater affinity for E than for ES. We interpret this to mean that the inhibitor and substrate binding regions overlap and that the long alkyl chain (alkyl ⩾ nonyl) cannot form as many hydrophobic interactions with E, when S is already bound, hence the decreased affinity for ES. For the shorter homologues (**5** and **6**), the reverse is true ($K_i > K_i'$); that is, they have greater affinity for ES. The change in conformation that arises when the enzyme–substrate complex is formed is apparently more favorable for these compounds. The 3-(1-oxoalkyl)-4-hydroxy-6-alkyl-2-pyrones **9–13** would by comparison with the 4-hydroxy-6-alkyl-2-pyrones **5–8** be expected to be mixed-type inhibitors. This was observed, although for the latter the ESI complex is active. The 4-methoxy-6-alkyl-2-pyrones **14** and **15** are uncompetitive inhibitors that can be considered to be an extreme form of mixed-type inhibition, where $K_i \gg K_i'$; that is, binding of I to ES is much more favorable, presumably because of the enhanced hydrophobic interactions and the inability of the methoxy species to bind to the enzyme. The differences that we have observed between the 4-methoxy and 4-hydroxy compounds could be attributed to tautomerism, since the 4-hydroxy-2-pyrone is able to tautomerize to the corresponding 2-hydroxy-4-pyrone, whereas the methylated species is fixed in the 2-pyrone form.[11] To be certain that this was not a contributing factor, we determined λ_{max} for some representative compounds (**3**, **7**, **12**, and **15**) and found no evidence for the presence of the 4-pyrone tautomer. Therefore, the trends that we observe are solely due to the changed characteristics brought about by blocking the potentially hydrogen bonding hydroxy group.

Interpretation of the relationship of mechanism to substituent size and position enables us to speculate on the location of this binding region. Marossy et al.[22] found that a tetrapeptide substrate, Z-D-Phe-Pro-Ala-pNA was a very good substrate for HLE and that removal or replacement of the P_4 residue,[23] benzyloxycarbonyl (Z), resulted in a decrease in affinity. Studies of tetrapeptide substrates show that Ala is preferred at the P_4 site.[24] However, the work of Marossy et al. indicates that even a phenyl can be accommodated at the S_4 subsite. We believe that the kinetic mechanistic data for the 6-alkyl derivatives **5–8** provides evidence that the 4-hydroxy-2-pyrone nucleus can bind at this site. The change in mechanism that is observed, as the number of methylenes is increased, suggests that, for the short-chain homologues, **5** and **6**, the alkyl chain is bound to S_4 and S_3, with the substrate residues Boc and Ala bound to S_2 and S_1, respectively. The alkyl chain of the longer homologues, **7** and **8**, would be bound at subsites S_2 and S_1 and so hindrance to S binding would be observed, hence the change in mechanism and the greater preference for binding to E and not to ES.

Spencer et al.[20] have suggested that the 2-pyrone carbonyl might be bound to the "oxyanion hole",[25] and if so, the substituent at position 3 could be placed on the P_1 site. Our data do not support such a suggestion. Instead, we think that the 3-substituent could be placed at the P_5 site, that is, bound at the S_5 subsite.

Specificity. The hydrophobic compounds were found to have no appreciable inhibition (<10%) when tested against three related serine proteases, porcine pancreatic elastase, bovine α-chymotrypsin, and bovine trypsin, at the approximate limit of their solubility (25 μM for **9–12**, 10 μM for **13**, 1000 μM for **19**, and 200 μM for the remaining compounds). The hydrophilic derivatives (**16–18**) were nonspecific, inhibiting all four enzymes, albeit to a different extent. These results indicate that HSE has a particularly hydrophobic binding cleft and that better inhibitors could be developed by increasing the hydrophobic bulk of the substituents.

Reversible Inhibition. The mechanism of action and a possible binding site have been established. These results indicate that it would be unlikely that these compounds are irreversible inhibitors. A further point in favor of reversible action is that the interaction is very rapid, as measured by UV spectroscopy, and is not dependent on incubation time of E and I, unlike the 6-chloropyrones.[26] However, we decided to prove that this was the case. Spencer et al.[20] used UV difference spectra to show that α-chymotrypsin was not acylated by an alkylated 2-pyrone, and hence they concluded that the interaction was reversible. We chose dialysis to study this problem. Two representative compounds were chosen: **10**, a 3,6-dialkyl-2-pyrone, and **17**, a 6-(2-hydroxy-2-carbobutoxyvinyl)-2-pyrone. In both cases full recovery of enzyme activity was observed after dialysis in buffer for 4 days, indicating that the interaction is fully reversible.

In summary, the present study has shown that these substituted 2-pyrones bind to a hydrophobic region that overlaps the extended substrate binding region. We have evidence that indicates that the 4-hydroxy-2-pyrone can bind to the S_4 subsite, with the 6-substituent binding to the S_4–S_1 subsites. Compound **11** was found to be the most potent inhibitor with a K_i of 4.6 μM, 30 times more potent than elasnin (K_i = 140 μM[20]). The hydrophobic compounds were found to be specific for HSE, and the interaction with the enzyme is fully reversible.

Experimental Section

Melting points were determined on a Reichert microscope melting point apparatus. The ^1H NMR spectra were recorded on a Perkin-Elmer R-32 spectrometer at 90 MHz with tetramethylsilane (Me_4Si) as internal standard. ^{13}C NMR spectra were recorded with a JEOL FX-200 NMR spectrometer at 50.1 MHz with $CDCl_3$ as internal lock. $CDCl_3$ (77.00 ppm) or Me_4Si (0.00 ppm) was used as internal standard. UV spectra were determined in 14.8% Me_2SO in HEPES buffer (see below, HSE assays). Elemental analyses were performed by AMDEL Australian Microanalytical Service (Fisherman's Bend, Victoria). *N*-(2-Butoxycarbonyl)-L-alanine *p*-nitrophenyl ester (BAN), *N*-(2-hydroxyethyl)piperazine-*N*'-ethanesulfonic acid (HEPES), porcine pancreatic elastase, α-chymotrypsin, and trypsin were purchased from Sigma and used without further purification. Spectrophotometric grade dimethyl sulfoxide (Me_2SO) was purchased from Aldrich. A. Lentini (this laboratory) isolated human sputum elastase from lung washings (kindly donated by the Department of Medicine, Austin Hospital, Victoria, Australia), using a modification[27] of the methods of Andrews et al.,[28] Viscarello et al.,[29] and Martodam et al.[30] No cathepsin G activity was observed

(22) Marossy, K.; Szabo, G. C.; Pozsgay, M.; Elödi, P. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 762.
(23) Nomenclature of Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
(24) Stein, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 5111.
(25) Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331.
(26) Westkaemper, R. B.; Abeles, R. H. *Biochemistry* **1983**, *22*, 3256.
(27) Lentini, A.; Farchione, F.; Ternai, B.; Kreua-Ongarjnukool, N.; Tovivich, P. *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 369.
(28) Andrews, J. L.; Ghosh, P.; Lentini, A.; Ternai, B. *Chem.-Biol. Interact.* **1983**, *47*, 157.
(29) Viscarello, B. R.; Stein, R. L.; Kusner, E. J.; Holsclaw, D.; Krell, R. D. *Prep. Biochem.* **1983**, *13*, 57.
(30) Martodam, R. R.; Baugh, R. J.; Twumasi, D. Y.; Leiner, I. E. *Prep. Biochem.* **1979**, *9*, 15.

when the enzyme was tested against the specific substrate *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Sigma). A Varian DMS 100 UV/visible spectrophotometer coupled with a DS-15 data station was used to store the initial velocity data (calculated with ϵ_{400nm} = 11 600 m^{-1} cm^{-1} for *p*-nitrophenol, determined under these assay conditions). Lines of best fit were calculated by linear regression. Varian software: Kinetics Storage P/N 85-100541-00 version 3 1984 and Kinetics Calculations (enhanced) P/N 85-100542-00 version 4 1984 were used.

Synthesis. Compounds **1–4** and **16–19** were prepared by using the method of Douglas and Money.[12] Compounds **17** and **18** were purified by triturating the crude brown oil with acetone, which yielded yellow crystals after filtration. Compounds **5–8** were prepared by Collie's method.[10] Compounds **14–15** were made by using the method of Bu'lock and Smith.[11] All compounds had satisfactory elemental analyses and ^1H and ^{13}C NMR spectra. Physical–chemical data for compounds **2–6**, **8–14**, and **16–19** are available as supplementary material. Representative syntheses of key compounds and their physical–chemical data are presented below.

Synthesis of $C_9H_{19}COCH_2COOC_2H_5$. Solid magnesium methoxide (10.8 g, 0.11 mol) was added to a solution of ethyl hydrogen malonate (26.4 g, 0.22 mol) in THF and the solution was stirred for 1 h. The solvent was removed under reduced pressure to give a white, slightly hygroscopic salt, Mg(OOCOCH$_2$COOEt), which was used directly. Carbonyldiimidazole (17.8 g, 0.11 mol) was added to decanoic acid (17.2 g, 0.10 mol) in THF. After the mixture was stirred at room temperature for 6 h, the prepared Mg(OOCCH$_2$COOEt)$_2$ was added. The mixture was stirred for 18 h at 25 °C, and the solvent was then removed at reduced pressure. The residue was partitioned between ether and aqueous 0.5 M HCl. The ether extract was washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated under reduced pressure to yield an oil in 95% yield.

Synthesis of $C_9H_{19}COCH_2COOH$. Crude $C_9H_{19}COCH_2COOC_2H_5$ (12 g, 0.05 mol) was stirred with 1 equiv of 1 M NaOH overnight (50 mL). Any remaining ester was removed by washing with ether. The aqueous layer was cooled and acidified with 32% HCl. The precipitated product was collected and thoroughly dried before being used in the next step (70% yield). Further purification was not necessary.

Synthesis of 3-(1-Oxodecyl)-4-hydroxy-6-nonyl-2-pyrone (12). Solid carbonyldiimidazole (5.9 g, 0.04 mol) was added to a THF solution of $C_9H_{19}COCH_2COOH$ (7 g, 0.03 mol). The reaction mixture was stirred under N_2 for 24 h and then acidified to pH 1 with 0.5 M HCl. The reaction mixture was then extracted with EtOAc, and the organic layer was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to yield the desired product as an orange solid in 85% yield. Recrystallization from MeOH yielded white needles: mp 73.5–74 °C (MeOH) (lit.[31] mp 65 °C (EtOH)); UV λ_{max} 310 nm (log ϵ = 4.07), 252 (3.82); ^1H NMR ($CDCl_3$) δ 0.88 (br t, 6 H, CH_3), 1.27 (br s, 24 H, CH_2), 1.71 (m, 4 H, CH_2), 2.49 (t, J = 7 Hz, 2 H, CH_2C=), 3.07 (t, J = 7 Hz, 2 H, CH_2CO), 5.90 (s, 1 H, CH=); ^{13}C NMR ($CDCl_3$) δ 14.13 (2 CH_3), 22.72, 24.03, 26.43, 28.99, 29.29, 29.43, 29.52, 31.89, 31.94, 34.34, 41.70 (16 CH_2), 99.63 (C_3), 100.74 (C_5), 161.03 (C_6), 172.57 (C_4), 181.30 (COO), 207.90 ($COCH_2$). Anal. ($C_{24}H_{40}O_4$) C, H.

Synthesis of 4-Hydroxy-6-nonyl-2-pyrone (7). Compound **12** (4.4 g, 8.5 mmol) was added to 5 equiv (by weight) of 90% H_2SO_4 (16.7 g) and the mixture heated at 130 °C for 15 min. Ice was added to the cooled solution with thorough stirring. The oily sludge was extracted with ether and concentrated under reduced pressure. Recrystallization of the residue from MeOH/H_2O (1:1) yielded white plates: mp 75.5–76 °C (MeOH/H_2O) (lit.[32] mp 78–79 °C (hexane)); UV λ_{max} 285 nm (log ϵ = 3.83); ^1H NMR ($CDCl_3$) δ 0.88 (br t, 3 H, CH_3), 1.27 (br s, 12 H, CH_2), 1.64 (m, 4 H, CH_2), 2.48 (t, J = 7 Hz, 2 H, CH_2C=), 5.62 (d, J = 2 Hz, 1 H, CH=, C_3), 6.00 (d, J = 2 Hz, 1 H, CH=, C_5); ^{13}C NMR ($CDCl_3$) δ 14.11 (CH_3), 22.70, 26.73, 29.00, 29.28, 29.46, 31.89, 33.67 (8 CH_2), 89.83 (C_3), 101.39 (C_5), 167.33 (C_6), 168.62 (C_2), 172.83 (C_4). Anal. ($C_{14}H_{22}O_3$) C, H.

Synthesis of 4-Methoxy-6-nonyl-2-pyrone (15). Dimethyl sulfate (320 mg, 2.52 mmol) was added to a methyl ethyl ketone solution of **7** (600 mg, 2.52 mmol) with anhydrous K_2CO_3 (1.1 g, 7.81 mmol). The reaction mixture was heated under reflux for 22 h. After cooling, the solids were removed by filtration, and the filtrate was concentrated under reduced pressure. The orange oil crystallized on cooling. The product was recrystallized from petroleum ether (60–80 °C) to yield pale yellow crystals: mp 56.5–57 °C (MeOH) (lit.[32] mp 58–59 °C (pentane); UV λ_{max} 285 nm (log ϵ = 3.83); ^1H NMR ($CDCl_3$) δ 0.88 (br t, 3 H, CH_3), 1.62 (br s, 8 H, CH_2), 1.62 (m, 2 H, CH_2), 2.44 (t, J = 8 Hz, 2 H, CH_2C=), 3.81 (s, 3 H, OCH_3), 5.42 (d, J = 2 Hz, 1 H, CH=, C_3), 5.78 (d, J = 2 Hz, 1 H, CH=, C_5); ^{13}C NMR ($CDCl_3$) δ 14.04 (CH_3), 22.60, 26.68, 28.94, 31.68, 33.64 (8 CH_2), 55.83 (OCH_3), 87.48 (C_3), 99.66 (C_5), 164.98 (C_2), 165.82 (C_6), 171.37 (C_4). Anal. ($C_{15}H_{24}O_3$) C, H.

Synthesis of 3-(1-Oxohexyl)-4-hydroxy-6-methyl-2-pyrone (1). 4-Hydroxy-6-methyl-2-pyrone (0.63 g, 0.005 mol) was added to CF_3COOH (3 mL) and hexanoyl chloride (1.3 g, 0.01 mol). The mixture was stirred and heated under reflux for 3 h, then cooled, and poured into ice–water (15 mL). The collected crude product was dissolved in dimethyl sulfoxide. Lead acetate in dioxane was added to complex with the free carboxylic acid. Addition of water precipitated the product, which was recrystallized from MeOH, yielding white needles: mp 66–66.5 °C (MeOH) (lit.[31] mp 65 °C (EtOH)); ^1H NMR (CCl_4) δ 0.86 (m, 3 H, alkyl-CH_3), 1.21–1.59 (m, 10 H, CH_2), 2.18 (s, 3 H, CH_3C=), 2.89 (t, 2 H, $COCH_2$), 5.59 (s, 1 H, CH=, C_5); ^{13}C NMR ($CDCl_3$) δ 13.98 (CH_3-alkyl), 20.56 (CH_3C=), 22.54, 29.06, 31.64 (CH_2), 41.58 ($COCH_2$), 99.44 (C_3), 101.47 (C_5), 160.86 (C_6), 168.71 (C_4), 181.21 (C_2), 207.96 ($COCH_2$), 160.86 (C_6), 168.71 (C_4), 181.21 (COO), 207.96 ($COCH_2$). Anal. ($C_{12}H_{16}O_4$) C, H.

Enzyme Inhibition Studies. Assay of HSE. HSE (1.8 μg/50 μL) was made up in 50 mM NaOAc and 0.45 M NaCl at pH 5.5. The assay conditions were 0.1 M HEPES, 0.5 M NaCl, and 14.8% Me_2SO at pH 7.5 and 37 °C. All inhibitor and substrate solutions were made up in 5% aqueous Me_2SO to retard hydrolysis. HSE (50 μL), Me_2SO/inhibitor (100 μL), and buffer (800 μL, preequilibrated to 37 °C) were added to the thermostated cuvette. The reference cell contained Me_2SO/inhibitor (100 μL) and buffer (850 μL). BAN (50 μL, final concentration 50–750 μM) was added to each cuvette with stirring. After a lag time of 10 s, the production of *p*-nitrophenol was followed at 400 nm.

Screening for Inhibitory Activity against Porcine Pancreatic Elastase, α-Chymotrypsin, and Trypsin. The assay conditions were identical with the HSE assays, except that each inhibitor was tested at one concentration with one concentration of substrate. Inhibitors were tested at their approximate limit of solubility: 25 μM for **9–12**, 10 μM for **13**, 1000 μM for **19**, and 200 μM for the remaining 2-pyrones. Each concentration was repeated at least twice. All compounds were tested at 0.29 K_m (porcine pancreatic elastase, K_m = 2600 μM; α-chymotrypsin, K_m = 210 μM; trypsin, K_m = 1100 μM).

Dialysis Experiment. To an aliquot of HSE (100 μL) was added 300 μL of Me_2SO (as control) or **10** (300 μM) or **17** (3000 μM). Sufficient inhibitor was added to inhibit the enzyme by at least 90%. The solutions were pipetted into presoaked dialysis tubing (retention 10 000 daltons). The enzyme–complex was dialyzed against 1 L of 50 μM NaOAc, 0.45 M NaCl, and 0.1% Brij 35 at pH 5.5 and magnetically stirred at 4 °C for 4 days. The activity of the enzyme was determined in the normal way with 100 μM BAN.

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Registry No. **1**, 27424-82-4; **2**, 107617-13-0; **3**, 107152-21-6; **4**, 96649-62-6; **5**, 81017-02-9; **6**, 90632-45-4; **7**, 75858-40-1; **8**, 81017-03-0; **9**, 107617-14-1; **10**, 107617-15-2; **11**, 107617-16-3; **12**, 107617-17-4; **13**, 107617-18-5; **14**, 107617-19-6; **15**, 75858-41-2; **16**, 18068-83-2; **17**, 107617-20-9; **18**, 107617-21-0; **19**, 18068-84-3; $C_9H_{19}COCH_2COOC_2H_5$, 67342-99-8; $C_9H_{19}COCH_2COOH$, 61058-75-1; $Me(CH_2)_4COCH_2COOH$, 13283-91-5; Me-

(31) Kogl, F.; Salemink, C. A. *Recl. Trav. Chim. Pays-Bas* **1952**, *71*, 779.
(32) Poulton, G. A.; Cyr, T. D. *Can. J. Chem.* **1980**, *58*, 2158.

$(CH_2)_6COCH_2COOH$, 13283-92-6; $Me(CH_2)_{10}COCH_2COOH$, 88222-72-4; $(C_2H_5OOC)_2$, 95-92-1; $(C_4H_9OOC)_2$, 2050-60-4; $(C_5H_4OOC)_2$, 20602-86-2; $(HOOC)_2$, 144-62-7; ethyl hydrogen malonate, 1071-46-1; decanoic acid, 334-48-5; octanoyl chloride, 111-64-8; decanoyl chloride, 112-13-0; dodecanoyl chloride, 112-16-3; 4-hydroxy-6-methyl-2-pyrone, 675-10-5; hexanoyl chloride, 142-61-0; sputum elastase, 9004-06-2; 4-methoxy-6-methyl-2-pyrone, 672-89-9.

Supplementary Material Available: Physical–chemical data for compounds **2–6, 8–14, 16–19** (4 pages). Ordering information is given on any current masthead page.

Cardiotonic Agents. 5. 1,2-Dihydro-5-[4-(1*H*-imidazol-1-yl)phenyl]-6-methyl-2-oxo-3-pyridinecarbonitriles and Related Compounds. Synthesis and Inotropic Activity[1]

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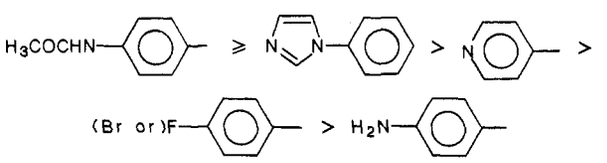
Several 1,2-dihydro-5-(substituted phenyl)-2(1*H*)-pyridinones were synthesized and evaluated for inotropic activity. 1,2-Dihydro-5-[4-(1*H*-imidazol-1-yl)phenyl]-6-methyl-2-oxo-3-pyridinecarbonitrile (**5a**) and the corresponding unsubstituted analogue **14a** were the most potent positive inotropic agents in this series. Although the 4,6-dimethyl analogue **6a** retained most of the activity of **5a**, the 4-methyl analogue **8a** was substantially less potent. The synthesis and structure–activity relationships are discussed.

Recently we have reported the positive inotropic activity of imazodan (CI-914, I), CI-930 (II), and related 4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-3(2*H*)-pyridazinones.[2,3] The inotropic activities of the rigidly fused tricyclic 2,4,4a,5-tetrahydro-7-(1*H*-imidazol-1-yl)-3*H*-indeno[1,2-*c*]pyridazin-3-ones (III)[4] derived from CI-930 (II) and 6-(substituted 1*H*-imidazol-4(5)-yl)-3-(2*H*)-pyridazinones[5] were also reported. In an attempt to define the structure–activity relationships of the 4,5-dihydro-3(2*H*)-pyridazinone moiety we have investigated other series of heterocyclic systems, such as, 2,4-dihydro-5-[4-(1*H*-imidazol-1-yl)phenyl]-3*H*-pyrazol-3-ones (IV)[6] and 2-[4-(1*H*-imidazol-1-yl)phenyl]-4*H*-1,3,4-oxadiazin-5-(6*H*)-ones (V)[7] (Chart I). These studies confirm the contribution of the (1*H*-imidazol-1-yl)phenyl moiety to superior inotropic activity in comparison with other more conventional aromatic substituents, such as halogen, alkyl, alkyloxy, nitro, amine, etc.[8] The rank order of potency[4] for the phenyl substituent across several series of compounds is

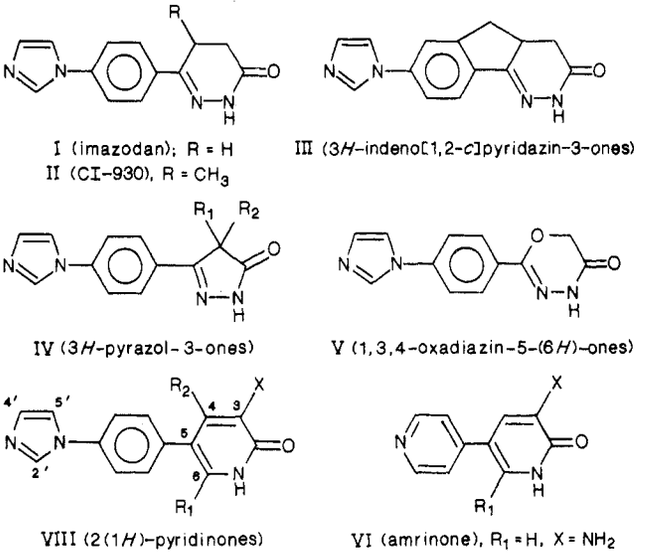


During the early stages of our investigation, amrinone (VI)[9] and milrinone (VII)[10] were reported to be promising compounds that possessed combined inotropic and vasodilator activities. The limited published data on the structure–activity relationship (SAR) of 5-substituted 2(1*H*)-pyridinones led us to investigate 5-[4-(1*H*-imidazol-1-yl)phenyl]-2(1*H*)-pyridinones (VIII, Chart I). This resulted in a series of potent inotropes,[11] the synthesis and biological activity of which is described in this paper.

Chemistry

The target compounds **5a–f** were synthesized from the requisite aldehydes **1a–g** according to Scheme I. Aldehydes **1a–g** (Table I) were prepared from 4-fluorobenzaldehyde and the requisite amines in refluxing pyridine in the presence of K_2CO_3 and Cu_2O by following the gen-

Chart I



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(1) Presented in part at the 187th National Meeting of the American Chemical Society, Miami Beach, April 1985. See *Abstracts of Paper*; American Chemical Society: Washington, DC, 1985; MEDI 40.
(2) Bristol, J. A.; Sircar, I.; Moos, W. H.; Evans, D. B.; Weishaar, R. E. *J. Med. Chem.* **1984**, *27*, 1099.
(3) Sircar, I.; Duell, B. L.; Bobowski, G.; Bristol, J. A.; Evans, D. B. *J. Med. Chem.* **1985**, *28*, 1405.
(4) Sircar, I.; Duell, B. L.; Cain, M. C.; Bristol, J. A.; Burke, S. E. *J. Med. Chem.* **1986**, *29*, 2142.
(5) Sircar, I.; Bobowski, G.; Bristol, J. A.; Weishaar, R. E.; Evans, D. B. *J. Med. Chem.* **1986**, *29*, 261.
(6) Morrison, G. C. U.S. Patent 4 526 982, July 2, 1985.
(7) Sircar, I.; Cain, M. C.; Topliss, J. G. U.S. Patent 4 508 718, April 2, 1985.
(8) Sircar, I. U.S. Patent 4 397 854, August 9, 1983.
(9) Farah, A. E.; Alousi, A. A. *Life Sci.* **1978**, *22*, 1139.
(10) Lesher, G. Y.; Philion, R. E. U.S. Patent 4 313 951, February 2, 1982.
(11) Bristol, J. A.; Sircar, I. U.S. Patent 4 503 061, March 5, 1985.

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