Efficient Inhibition of Human Leukocyte Elastase and Cathepsin G by Saccharin Derivatives

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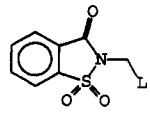
A series of saccharin derivatives I has been synthesized and evaluated for their inhibitory activity toward human leukocyte elastase and cathepsin G. Most of the compounds were found to be efficient and time-dependent inhibitors of elastase. Inactivated elastase was found to regain its activity almost fully after 24 h (80–90% activity) and the half-lives of reactivation ranged between 12–15 h. Addition of hydroxylamine to fully-inactivated enzyme led to rapid and complete recovery of enzymatic activity. A tentative mechanism of action is proposed on the basis of biochemical and model studies.

Introduction

Human leukocyte elastase (HLE) has been the focus of intense investigation in recent years[1] due to its likely involvememt in the pathophysiology of inflammatory diseases such as pulmonary emphysema,[2] cystic fibrosis,[3] and rheumatoid arthritis.[4] The influx of neutrophils at inflammatory loci is accompanied by the extracellular release of lysosomal enzymes, including elastase. Inefficient regulation of HLE by its physiological inhibitors can result in damage to connective tissue. The use of low molecular weight inhibitors of HLE may provide a way for alleviating these diseases.[5–7]

We have recently reported some preliminary findings related to the inhibition of HLE by saccharin derivatives I.[8] In this paper we wish to describe the results of



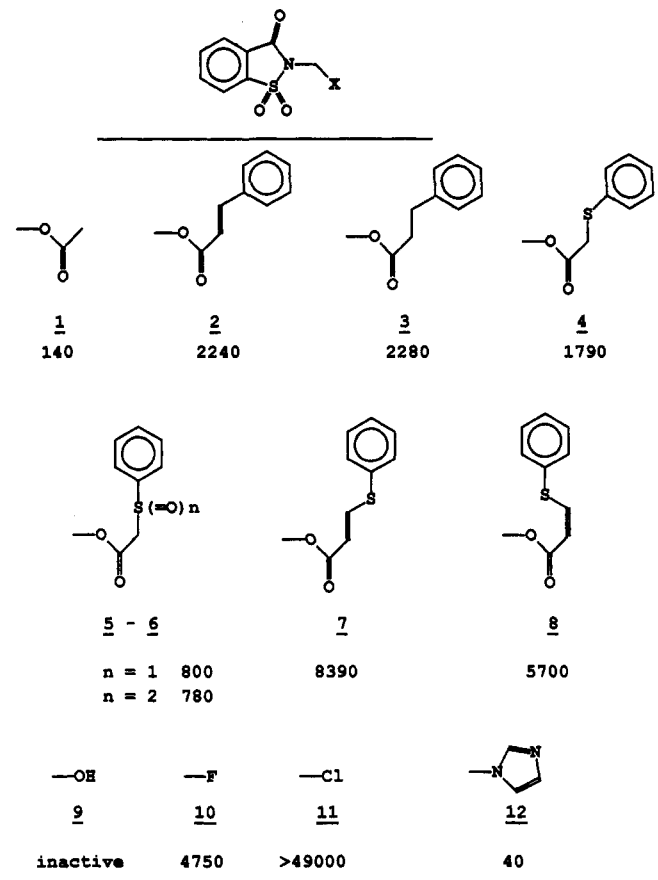
I

structure–activity relationship and inhibition studies, as well as relevant mechanistic studies with derivatives of I.

Chemistry. The synthesized compounds 1–12 are listed in Chart I. The physical constants and spectral data of the synthesized compounds are listed in Table I. Compounds 5 and 6 were prepared from 4 using *m*-chloroperbenzoic acid. Compounds 1 and 9 were prepared as described in the literature.[9]

Biochemical Studies. The enzyme assays and inhibition studies were carried out as described in detail elsewhere,[10,11] and the kinetics data were analyzed according to Kitz and Wilson. The apparent pseudo first-order inactivation rate constants were determined from the slopes of the semilogarithmic plots of ln $(E_t/E_o) = k_{obs}t$, where E_t/E_o is the amount of active enzyme remaining after time t and expressed in terms of the apparent second-order inactivation rate constants, $k_{obs}/[I]$ M^{-1} s^{-1} (listed in Chart I). The magnitude of the bimolecular rate constant $k_{obs}/[I]$ M^{-1} s^{-1} serves as an index of inhibitory prowess.

Chart I[a]



[a] $k_{obs}/[I]$ M^{-1} s^{-1} with HLE.

Results and Discussion

(a) Design Considerations and Structure–Activity Relationship Studies. On the basis of the known chemistry of saccharin and its derivatives toward nucleophiles,[12] it was hypothesized that compound I would likely inactivate HLE and related serine proteinases according to the mechanism shown in Scheme I, whereby binding to the active site of the target enzyme is followed by nucleophilic ring opening and tandem facile elimination of L to yield a Michael acceptor. Subsequent trapping by an active site nucleophile (His-57) was anticipated to lead to irreversible inactivation of the enzyme.

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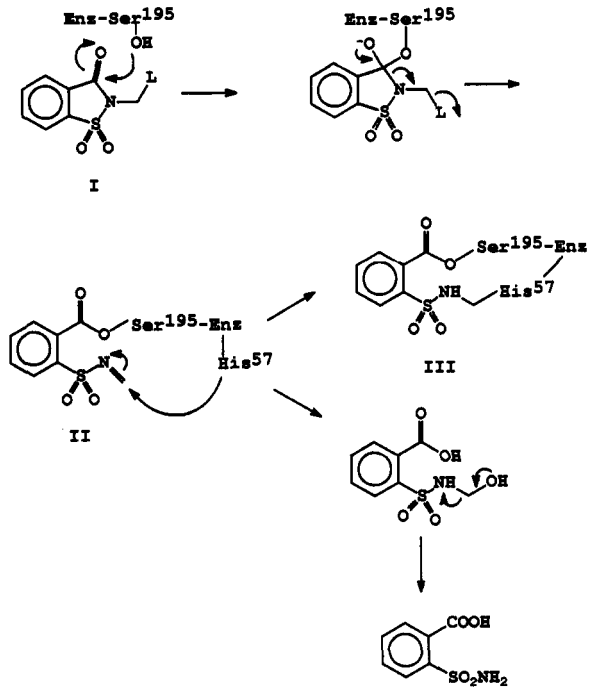
Table I. Physical Constants and Spectral Data of Inhibitors

compound	mp, °C	^{1}H NMR, ppm	mol formula (anal.)
2	126–7	6.0 (s, 2H), 6.7 (d, 1H), 7.5 (m, 3H) 7.8 (m, 3H), 8.0–8.3 (m, 3H), 8.2 (d, 1H)	$C_{17}H_{13}NO_5S$ (C,H,N)
3	68–9	2.7 (t, 2 H), 3 (t, 2H), 5.9 (s, 2H), 7.1–7.3 (m, 5H), 7.8–8.0 (m, 3H), 8.1 (d, 1H)	$C_{17}H_{15}NO_5S$ (C,H,N)
4	76–7	3.7 (s, 2H), 5.85 (s, 2H), 7.2–7.5 (m, 5H) 7.8–8.2 (m, 4H)	$C_{16}H_{13}NO_6S$ (C,H,N)
5	126–7	3.74 (d, 1H), 3.93 (d, 1H), 5.8 (s, 2H), 7.52 (m, 3H), 7.70 (m, 2H), 7.9–8.0 (m, 4H), 8.12 (d, 1H)	$C_{16}H_{13}NO_6S$ (C,H,N)
6	149–150	4.76 (s, 2H), 5.73 (s, 2H), 7.5–7.7 (m, 3H), 7.9 (m, 2H), 8.0–8.2 (m, 3H), 8.35 (d, 1H)	$C_{16}H_{13}NO_7S$ (C,H,N)
7	106–8 (trans)	5.62 (s, 1H), 5.87 (s, 2H), 7.4–7.5 (m, 5H), 7.83–7.98 (m, 4H), 8.12 (m, 1H)	$C_{17}H_{13}NO_5S_2$ (C,H,N)
8	143–6 (cis)	5.95 (d, 1H), 5.97 (s, 2H), 7.35–7.5 (m, 6H), 7.85–8.0 (m, 3H), 8.12 (m, 1H)	$C_{17}H_{13}NO_5S_2$ (C,H,N)
10	92–4	5.9 (d, 2H), 7.9–8.0 (m, 3H), 8.2 (d, 1H)	$C_8H_6FNO_3S$ (C,H,N)
12	173–4	6.1 (s, 2H), 6.9 (s, 1H), 7.3 (s, 1H), 7.8 (s, 1H), 8.0–8.2 (m, 3H), 8.3 (d, 1H)	$C_{11}H_9N_3O_3S$ (C,H,N)

Scheme I



Incubation of HLE with compound **10**, for example, led to rapid and progressive loss of enzymatic activity (Figure 1). This was followed by a slow but steady recovery of enzymatic activity. The regain in enzymatic activity after 24 h was 80–90% and the half-lives of reactivation ranged between 12–15 h. All inhibitors exhibited similar kinetics behavior. The observed differences in the $k_{obs}/[I]$ values appear to simply reflect differences in the acylation rate arising from the varying nature of L, while the deacylation rates are almost the same. This is indicative of the formation of an acyl enzyme that deacylates at a rate that is virtually independent of the nature of L. The observation that the addition of excess hydroxylamine to HLE that has been fully-inactivated by **10** or **2** leads to rapid and full recovery of enzymatic activity lends support to this hypothesis.

The lack of inhibitory activity of compound **9** and the low potency shown by **12** are not intuitively obvious. It was initially thought to be due to the instability of these compounds in buffer solution. Literature reports regarding the instability of compound **9** under mildly basic media[9,12] seemed to support this notion. Further investigation revealed, however, that compounds **9** and **12** remained unaffected upon stirring overnight in 0.1 M HEPES buffer, pH 7.20, in acetonitrile, as evidenced by thin-layer chromatography and NMR analysis. Leaving group ability may also account for these observations.

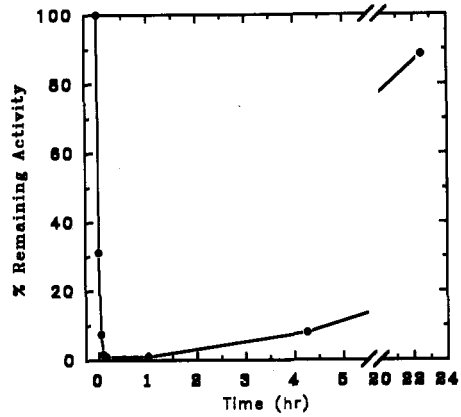


Figure 1. Time-dependent loss of enzymatic activity. Human leukocyte elastase (300 nM) was incubated with compound **10** (2.97 μM) in 0.1 M HEPES buffer, pH 7.25, and 1% DMSO. Aliquots were removed periodically and assayed for enzymatic activity using methoxysuccinyl Ala-Ala-Pro-Val *p*-nitroanilide.

Interestingly, *N*-methyl- and *N*-(*trans*-cinnamyl)saccharin were found to be devoid of any inhibitory activity when incubated with HLE under time-dependent or competitive inhibition conditions.

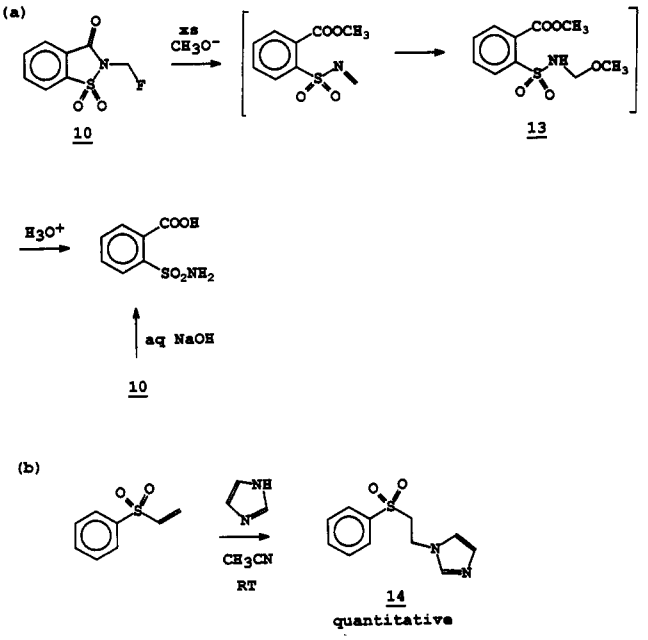
(b) Specificity. The interaction of compounds **2** and **12** with cathepsin G was also examined. Both compounds were found to be rather weak inhibitors of the enzyme ($k_{obs}/[I]$ values were 10 and 20 M^{-1} s^{-1}, respectively). Thus, these inhibitors are fairly specific for HLE.

(c) Mechanism of Action. The interaction of compound **10** with sodium methoxide resulted in the rapid disappearance of the characteristic doublet (5.8 ppm) for the methylene protons in **10**, as evidenced by NMR. NMR analysis of the crude solid obtained by evaporation of the solvent indicated the presence of a major and an unidentified minor component. NMR analysis of the crude mixture showed that the structure of the major component was consistent with that of compound **13** (Scheme IIa). Workup of the crude solid using 5% HCl yielded *o*-carboxybenzenesulfonamide. The same product was also obtained by stirring **10** with dilute base, followed by acidification.

Using phenyl vinyl sulfone as a model compound, we have verified that Michael addition with imidazole takes place very rapidly at room temperature in acetonitrile (Scheme IIb).

Taken together, these data suggest that the interaction of compound **I** with HLE very likely follows the pathway outlined in Scheme I, whereby the initial acyl enzyme **II** partitions between two likely pathways, one involving slow deacylation of the enzyme to yield active enzyme and *o*-carboxybenzenesulfonamide, while the second one involves Michael addition to the conjugated center to yield

Scheme II



III. It should be noted that either pathway by itself can account for the observed behavior of these compounds. For example, the reported inhibition of HLE by acyl saccharins via slow deacylation[13,14] lends support to the slow deacylation pathway. On the other hand, formation of adduct III can be followed by slow retro-Michael and deacylation, regenerating active enzyme. Definitive resolution of these questions will have to await the results of ongoing high-field NMR studies.

In summary, this study has shown that saccharin derivatives can function as inhibitors of the proteolytic enzymes elastase and cathepsin G. These compounds may be of value as pharmacological agents for the management of chronic obstructive pulmonary disease.

Experimental Section

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. The infrared and NMR spectra of the synthesized compounds were recorded on a Perkin-Elmer 1330 infrared spectrophotometer and a Varian XL-300 NMR spectrometer, respectively. A Gilford UV/vis spectrophotometer was used in the enzyme assays and inhibition studies. Human leukocyte elastase was purchased from Elastin Products Co., Owensville, St. Louis, MO. Human leukocyte cathepsin G was obtained from Athens Research and Technology Co., Athens, GA. Methoxysuccinyl Ala-Ala-Pro-Val *p*-nitroanilide and methoxysuccinyl Ala-Ala-Pro-Phe *p*-nitroanilide were purchased from Sigma Chemicals Co., St. Louis, MO.

Representative Syntheses. *N*-[(Phenylthioacetoxy)methyl]saccharin (4). A solution of (chloromethyl)saccharin (0.95 g; 4 mmol), thiophenoxyacetic acid (0.83 g; 4.93 mmol), and triethylamine (0.50 g; 4.94 mmol) in 20 mL of acetonitrile was refluxed for 2 h. The solvent was then removed and the residue was dissolved in 50 mL of methylene chloride. The solution was washed with water (2 × 50 mL), 5% sodium bicarbonate (2 × 35 mL), and 5% hydrochloric acid (34 mL). The solution was dried and evaporated to yield 1.23 g of a crude product which was purified on a chromatotron plate using a mixture of methylene chloride and hexane (3:1) as eluent. Pure compound 4 was obtained in 59% yield (0.85 g). Compounds 2 and 3 were synthesized using a similar procedure.

***N*-[(Phenylthioacetoxy)methyl]saccharin Sulfoxide (5).** A solution of sulfide 4 (5.45 g; 15 mmol) and *m*-chloroperbenzoic acid (2.59 g; 15 mmol) in 75 mL of methylene chloride was stirred overnight at room temperature. The precipitate was filtered off and the filtrate was washed with 5% sodium bicarbonate (2 × 30 mL), dried, and evaporated off *in vacuo*, leaving a pure product.

***N*-[(Phenylthioacetoxy)methyl]saccharin Sulfone (6).** A solution of sulfide 4 (1.82 g; 5 mmol) and *m*-chloroperbenzoic acid (2.16 g; 12.5 mmol) in 50 mL of methylene chloride was stirred overnight at room temperature. The precipitated solid was filtered off and the filtrate was washed with 2.5% sodium carbonate (30 mL). The solution was dried and evaporated, leaving 1.15 g (80% yield) pure product.

***trans*- and *cis*-*N*-[[3-(Phenylthio)acryloxy]methyl]saccharin (7 and 8).** A solution of *cis*-3-(phenylthio)acrylic acid (1.73 g; 9.6 mmol; Aldrich Co., 98% cis isomer), *N*-(chloromethyl)-saccharin (1.85 g; 8.0 mmol), and triethylamine (0.97 g; 9.6 mmol) in acetonitrile (15 mL) was refluxed for 4 h. The solution was concentrated under reduced pressure and the residue was taken up in 100 mL of methylene chloride. The solution was washed with water (75 mL) and 5% sodium bicarbonate (2 × 75 mL). After drying and evaporating off the solvent, there was obtained a mixture of cis and trans isomers. These were separated on a chromatotron plate using methylene chloride as the eluting solvent.

***N*-(Fluoromethyl)saccharin (10).** A solution of *N*-(hydroxymethyl)saccharin (9) (10.65 g; 50 mmol) in 100 mL of dry THF was added dropwise to a solution of DAST (8.06 g; 0.05 mol) in 50 mL of dry THF kept at −50 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was taken up in 150 mL of methylene chloride. Water (100 mL) was added cautiously with stirring. The layers were separated, and the organic layer was washed with water (2 × 100 mL), dried, and evaporated. The crude oil was purified by flash chromatography, yielding 5 g of pure product.

***N*-(Chloromethyl)saccharin (11).** *N*-(Hydroxymethyl)-saccharin (15.48 g; 0.073 mol) was suspended in 150 mL of dry ether and then treated with thionyl chloride (25.9 g; 0.22 mol). The reaction mixture was stirred for 2 days at room temperature. The solvent and excess thionyl chloride were removed *in vacuo*, and the residue was dissolved in 100 mL of methylene chloride. Undissolved impurities were removed by filtration. The product was further purified by flash chromatograhy using methylene chloride/hexane (3:1) as the eluent, yielding pure 11 (12.5 g; 74% yield).

***N*-(*N*-Imidazolylmethyl)saccharin (12).** A mixture of *N*-(chloromethyl)saccharin (1.16 g; 5 mmol), imidazole (0.34 g; 5 mmol), and anhydrous potassium carbonate (1.65 g; 10 mmol) in 10 mL of dry acetonitrile was refluxed for 3 h. Ethyl acetate (75 mL) was added to the cooled reaction mixture and the organic layer was washed with water (3 × 20 mL), dried, and evaporated *in vacuo*. The residue was washed with methylene chloride (30 mL), yielding pure 12 (0.52 g).

Alkaline Hydrolysis of Compound 10. Compound 10 (1.1 g; 5 mmol) in 50 mL of 5% aqueous sodium hydroxide and 2 mL of THF was stirred overnight, acidified with 20% HCl, and extracted with ethyl acetate (3 × 60 mL). Removal of the solvent yielded a solid which was recrystallized using acetone/methylene chloride (0.6 g, 59% yield): mp 150–2 °C; ^{1}H NMR (acetone-d_6) δ 6.6 (br s, 2H), 7.75 (m, 2H), 7.95 (m, 1H), 8.1 (m, 1H). Anal. Calcd for $C_7H_7NO_4S$: C, 41.79; H, 3.48; N, 6.97. Found: C, 42.00; H, 3.63; N, 6.74.

Reaction of Compound 10 with Excess Sodium Methoxide. Compound 10 (1.0 g; 4.5 mmol) was added to a solution of sodium methoxide (23.5 mmol) in 80 mL of methanol and the reaction mixture was stirred at room temperature for 50 min (the progress of the reaction was monitored by TLC). Methanol was removed *in vacuo*, leaving a solid (1.6 g). NMR analysis of the crude mixture indicated the presence of compound 13 (δ 8.2 (d, 1H), 7.8 (d, 2H), 7.1 (br s, 1H), 4.6 (d, 2H), 4.0 (s, 3H), 3.1 (s, 3H)). A sample (0.4 g) of this product was stirred for 1 h with 10 mL of saturated ammonium chloride and ethyl acetate (20 mL). The layers were separated, and the aqueous layer was acidified with 5% HCl and extracted with ethyl acetate (3 × 20 mL). Removal of the solvent left 100 mg of a white solid which was found to be identical to *o*-carboxybenzenesulfonamide.

Reaction of Phenyl Vinyl Sulfone with Imidazole. Imidazole (0.4 g, 6 mmol) was added to a solution of phenyl vinyl sulfone (0.5 g; 3 mmol) in 10 mL of acetonitrile. The reaction

mixture was stirred at room temperature for 2 h, water was added (20 mL), and the solution was extracted with ethyl acetate (2 × 20 mL). Removal of the solvent yielded a product (0.7 g; 100% yield). Further purification on a chromatotron plate yielded 0.6 g of adduct **13**: mp 80–81 °C; ^{1}H NMR ($CDCl_3$) 7.9 (m, 2H), 7.7–7.5 (m, 3H), 7.1 (s, 1H), 7.0 (s, 1H), 6.9 (s, 1H), 4.4 (t, 2H), 3.6 (t, 2H). Anal. Calcd for $C_{11}H_{12}N_2O_2S$: C, 55.93; H, 5.08; N, 11.86. Found: C, 55.74; H, 5.28; N, 11.64.

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