Dual Inhibition of Human Leukocyte Elastase and Lipid Peroxidation: *In Vitro* and *in Vivo* Activities of Azabicyclo[2.2.2]octane and Perhydroindole Derivatives

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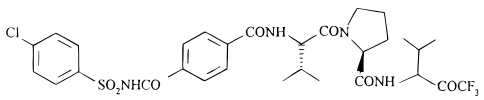
A series of potent and selective human leukocyte elastase (HLE) inhibitors of the Val-Pro-Val type has been developed. Initially, the central proline residue was replaced by nonnatural amino acids Phi ((2*S*,3a*S*,7a*S*)-perhydroindole-2-carboxylic acid) and Abo ((3*S*)-2-azabicyclo-[2.2.2]octane-3-carboxylic acid), and secondly several groups able to confer antioxidant properties to the molecule were introduced at the lipophilic N-terminal side chain. When compared to reference inhibitors, *in vitro* HLE inhibitory potency was maintained (10−100 nM) both with compounds containing the antioxidant moiety at the end of the N-terminal side chain and with compounds in which the N-terminal valine of the tripeptidic sequence had been replaced by a ϵ-substituted lysine. The lipidic peroxidation inhibitory potency of this series of inhibitors was found to be similar to that of the reference antioxidant compounds (around 1 μM). Moreover, HLE-induced hemorrhage in the hamster lung was effectively prevented (40−60% at 15 μg/kg) by most of the inhibitors tested when administered intratracheally 3 h before instillation of elastase. Among the most active analogs, compounds **11a**,**c**,**g** were still active when administered 18 h before elastase. Interestingly, compound **14a** was able to prevent HLE-mediated lung damage when administered 72 h prior to enzymatic challenge, indicating exceptional stability and retention in the lung. Finally, in a 14-day chronic model of emphysema in the hamster, **14a** significantly conserved alveolar spaces, a marker of lung tissue destruction, and was more potent than reference inhibitor ICI 200 880. This indicates that addition of peroxidation inhibitory properties to an HLE inhibitor can provide a powerful *in vivo* inhibitor of pulmonary tissue destruction.

Introduction

Human leukocyte elastase (HLE, EC 3.4.21.37) has been considered for over 2 decades as one of the most interesting biological targets in the research field of proteolytic destruction of lung tissue.[1] Subsequent to its secretion by polymorphonuclear neutrophils, one of the main actions of HLE is the degradation of structural proteins, including elastin, fibronectin, and collagen. Elastin is a highly cross-linked and flexible protein, mainly present in the macroscopic structure of pulmonary parenchyma. Under physiological conditions, the lung is protected from elastolytic activity by endogenous inhibitors such as α1-protease inhibitor (α1-PI); however, in the course of pathologies such as emphysema, cystic fibrosis, or adult respiratory distress syndrome (ARDS), the balance between the natural inhibitor and HLE is displaced in favor of the enzyme[2,3] (Scheme 1). Moreover, oxygen radical species (ORS) have been shown to be implicated in the development of emphysema and ARDS in two ways:[4] firstly, through a direct degradative action of cell structure and, secondly, through an indirect oxidation, by inactivation of antiproteases such α1-PI. In fact, ORS is contributing to lung destruction by increasing HLE degradative activity, suggesting that a synergestic action could exist between HLE and ORS. Furthermore, it is known that cigarette smoke is a source of active ORS:[5] it was reported to inactivate α1-PI and ceruloplasmine ferroxidase, an important oxygen radical scavenger.

When we started our investigations in the early 1990s, the tripeptidic trifluoromethyl ketone ICI 200 880 (or ZD 200 880) (**1**) was already described as a potent and selective HLE inhibitor.[6] Moreover, this compound presented a strong *in vivo* activity in several animal models of pulmonary lesions.[7]



ICI 200 880 1

Since then, several other pharmaceutical companies involved in research in the respiratory field have patented similar structures to the ICI compound,[8,9] all of them being derived from the tripeptidic sequence Val-Pro-Val. In our ongoing search for new and therapeutically useful low molecular weight inhibitors of serine proteinases, we have recently described the replacement of a proline residue by one of our original nonnatural amino acids in the generic structure of a known inhibitory peptidic sequence.[10,11] ICI 200 880 appeared at this time to be a suitable reference molecule in order to validate our structural hypothesis. Moreover, we were interested by introducing in the same molecule an anti-HLE activity and an antioxidant activity. With this aim, our chemical approach consisted of coupling modified Val-Pro-Val sequences with different groups able to confer an antioxidant property to the respective molecules.

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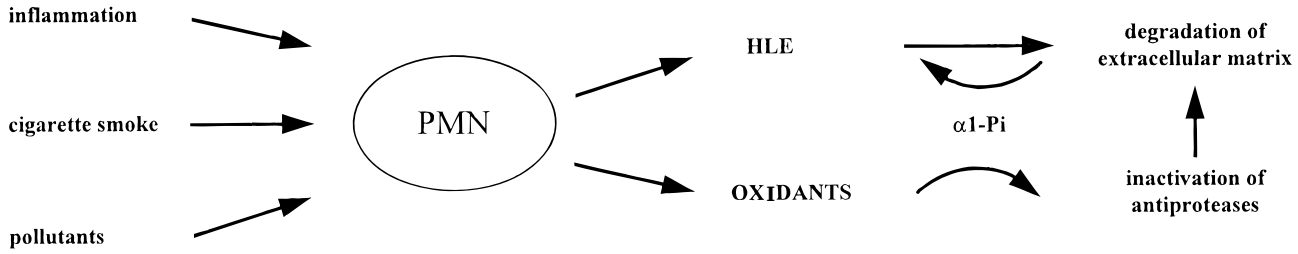
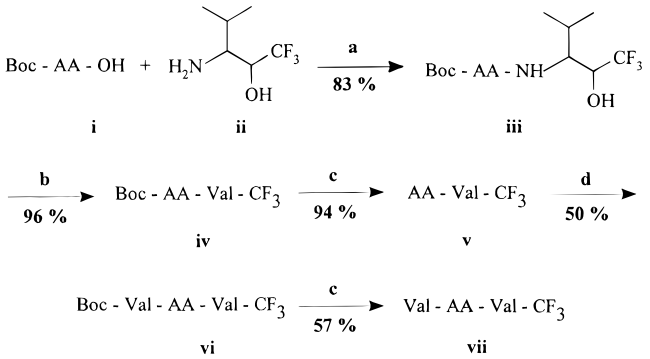
Scheme 1

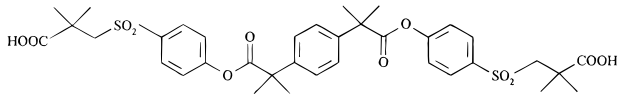


Scheme 2[a]



[a] **a**: AA = Phi. **b**: AA = Abo. Reagents: (a) isobutyl chloroformate, NMM; (b) Ac_2O, DMSO; (c) gaseous HCl, dioxane; (d) Boc-Val-OH, HOBT, DCC, DMF.

Few compounds appear in the literature with this dual inhibitory capacity. CT-1037 (**2**) is described as a potent elastase inhibitor (IC_{50} = 3 nM) embedded with an oxidative activation feature,[12] and P 1517 (**3**) is given as an elastase inhibitor with an IC_{50} on HLE around 0.1 mM but with *in vivo* radical scavenger properties superior to *N*-acetylcysteine.[13]



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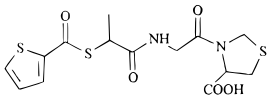
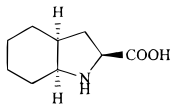


3

Chemistry

Nonnatural amino acids H-Phi-OH (Phi) (**4**) and H-Abo-OH (Abo) (**5**), containing a perhydroindole and an azabicyclo[2.2.2]octane ring, respectively, were prepared according to published procedures.[14−16]



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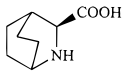

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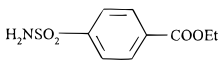
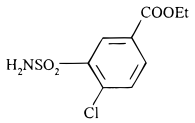
The compounds described in Tables 1 and 2 were prepared according to Schemes 2−5. The synthesis of the tripeptidic moiety is described in Scheme 2 and relies essentially on previously reported procedures: amino trifluoro alcohol **ii** was obtained in five steps from *N*-benzoylvaline as described by Peet.[17] Compound **ii** was conveniently reacted with either one of our Boc-protected amino acids **i** using mixed anhydride coupling methodology. In contrast to previous papers where oxidation of the alcohol was performed at the very last step of the synthesis of the inhibitors, we were able to prepare the dipeptidic trifluoromethyl ketone **iv** in satisfactory yields using Moffat's reagent (Ac_2O−DMSO). This reagent proved to be superior to other oxidants such as the Swern reagent or Dess−Martin periodinane. Deprotection of the N-terminal amino group of **iv** to give the dipeptide **v** was effected by treatment with dioxane, previously saturated with dry hydrogen chloride gas. A second coupling reaction with Boc-valine in the presence of DCC and HOBT in DMF followed, and the free tripeptidyl trifluoromethyl ketone **vii** was effectively isolated after treatment with dioxane previously saturated with hydrogen chloride gas. This ketone was obtained as a mixture of diastereomers. Isomer separation was not performed, because of a noted rapid epimerization observed *in vitro* as well as *in vivo*, most probably due to the propensity of the trifluoromethyl ketone to enolize and exist in a hydrated form.[18,19]

The suitable antioxidant functionalities were introduced through the use of different carboxylic acids **6**, which were either commercially available or prepared by standard methods according to Scheme 3. One of these acids (**6d**) necessitated the BOP, DIEA peptidic coupling methodology to react with tripeptide **vii-b** yielding inhibitor **7** (Scheme 4). However, the majority of the carboxylic acids **6** were reacted with sulfonamides **8a**,**b** via standard peptidic coupling conditions (DCC, DMAP) in DMF, to give esters of general formula **9** as illustrated in Scheme 4.



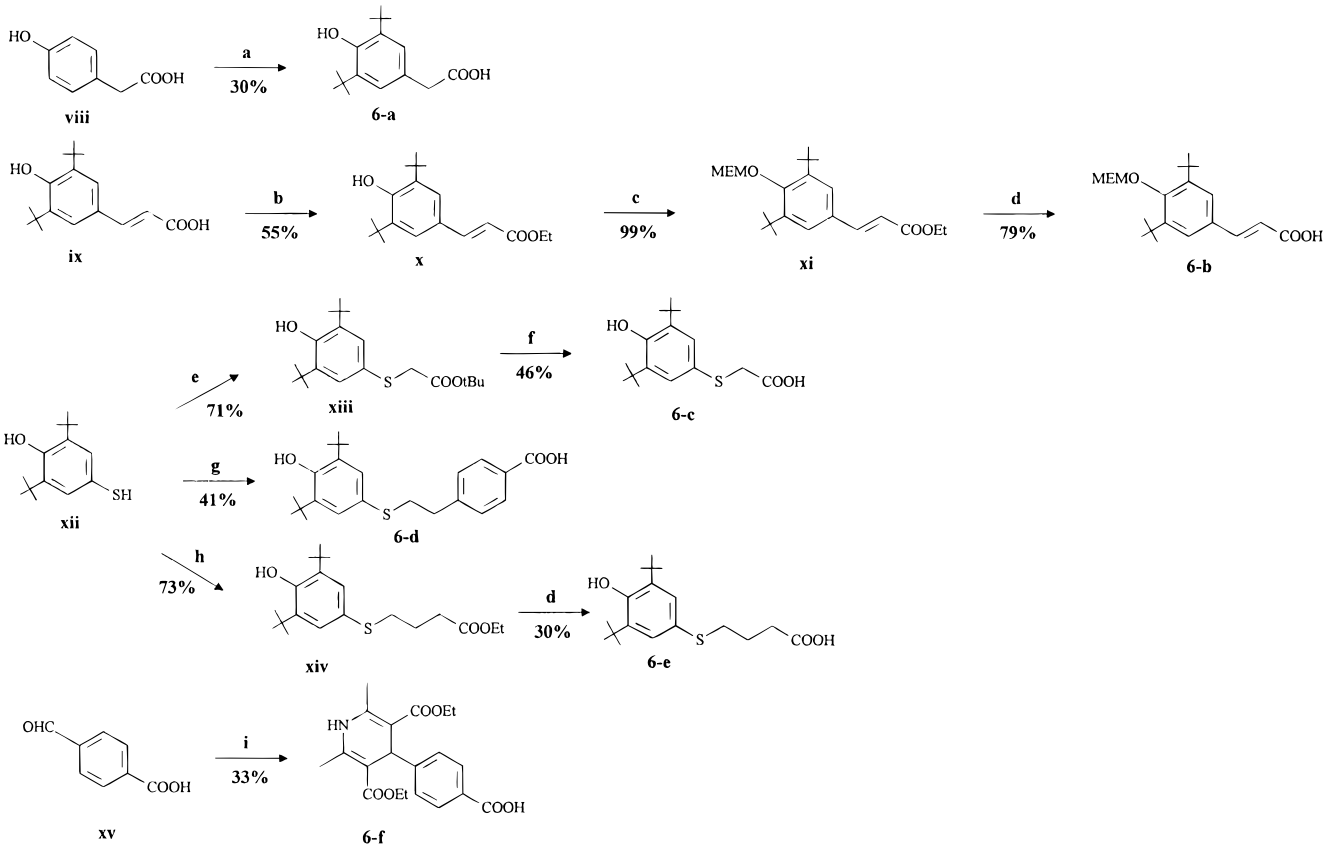
8-a



8-b

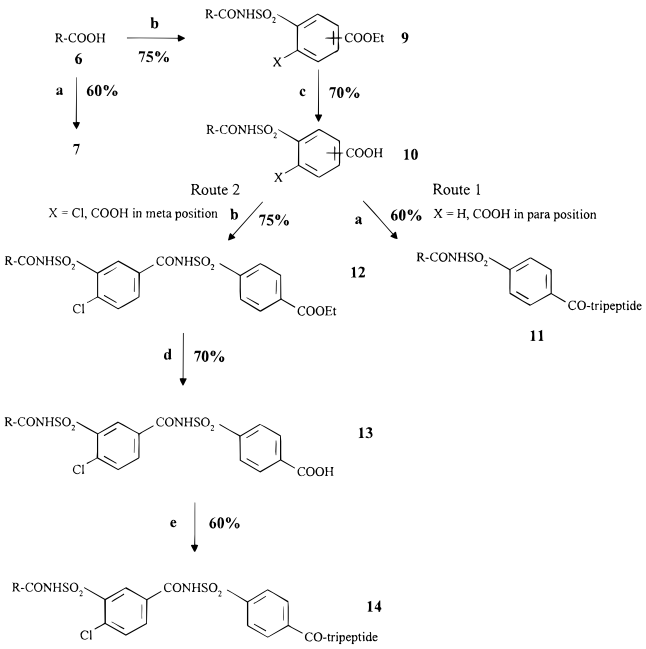
Ester **9c** was obtained as a secondary product during the preparation of ester **9b**. Both were used directly as a mixture and separated at the next step using reverse phase HPLC. Esters of generic formula **9** were converted to the corresponding carboxylic acids **10** in the presence of NaOH in EtOH followed by acidic workup (Scheme 4). In the case of X being hydrogen on generic formula **10** and the carboxylic acid function being *para* with respect to the sulfonamide (compound **8a**), compounds **10** were coupled directly with tripeptidic trifluoromethyl ketones **viia**,**b** using the BOP, DIEA methodology to give inhibitors **11** (route 1). The latter were generally purified by column chromatography on silica gel followed by trituration with ether or pentane. In the case of X being a chlorine atom and the carboxylic

Scheme 3[a]



[a] Reagents: (a) $CH_3COOtBu$, 70% $HClO_4$; (b) EtOH, H_2SO_4; (c) NaH, MEM-Cl, DMF; (d) NaOH, EtOH; (e) tBubromoacetate, K_2CO_3; (f) TFA, CH_2Cl_2; (g) EtONa, 4-(2-chloroethyl)benzoic acid; (h) ethyl 4-bromobutyrate, K_2CO_3; (i) CH_3COCH_2COOEt, NH_4OH, EtOH.

Scheme 4[a]



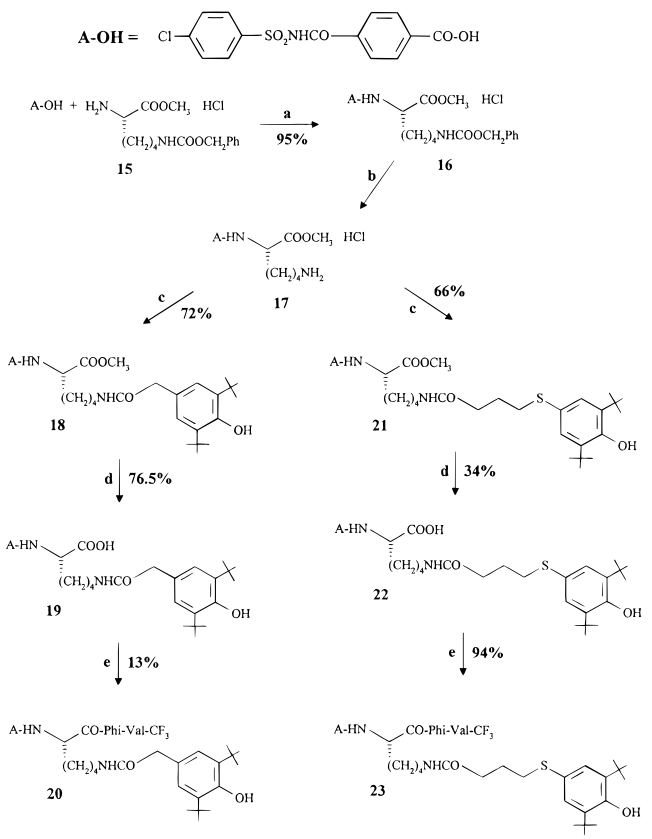
[a] Reagents: (a) **vii-b**, BOP, DIEA, DMF; (b) **8a** or **8b**, DCC, DMAP, DMF; (c) NaOH, EtOH; (d) **8a**, DCC, DMAP, DMF; (e) **vii-a**, BOP, DIEA, DMF.

acid moiety being *meta* to the sulfonamide (compound **8b**), a second coupling reaction was performed with **8a**, this time under DCC, DMAP coupling conditions, to give esters **12** (Route 2). Saponification followed by coupling with the tripeptidic entity **vii-a** as described above provided the two inhibitors **14a**,**b**.

Compounds in which the N-terminal valine was replaced by an ϵ-substituted lysine were prepared as outlined in Scheme 5. The hydrochloride of ϵ-*N*-(benzyloxycarbonyl)lysine methyl ester (**15**) was reacted with sulfonamide A-OH, already used by others,[6] to give intermediate **16**. Removal of the ϵ-amino protecting group was effected by treatment with HBr in AcOH. This was followed by condensation with carboxylic acids **6a**,**e** under classical peptidic coupling conditions (DCC, HOBT in DMF) to give intermediates **18** and **21**. Saponification was performed to provide acids **19** and **22** which were condensed with dipeptidic trifluoromethyl ketone Phi-Val-CF_3 (**v-a**) in the presence of DCC, HOBT, and triethylamine to give the desired inhibitors **20** and **23**, respectively.

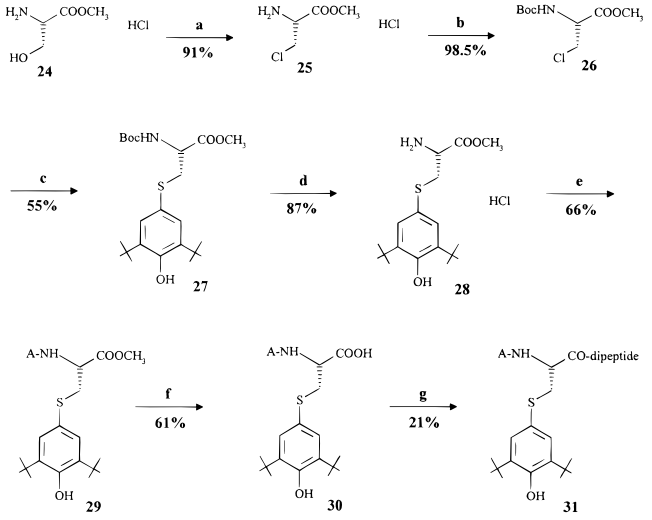
Finally, as illustrated in Scheme 6, the commercially available L-serine methyl ester (**24**) was converted into its chloro analog by treatment with PCl_5 in chloroform to give chloromethyl amino acid **25** as the hydrochloride salt.[20] After protection of the amino group (Boc_2O, Et_3N), condensation with 4-hydroxy-3,5-di-*tert*-butylthiophenol in the presence of potassium carbonate in acetonitrile was performed to provide cysteine derivative **27**. The required free amino group was obtained by deprotection with gaseous HCl in dioxane as described previously. Subsequent condensation with sulfonamidocarbonyl derivative A-OH, again using BOP, DIEA coupling methodology, gave intermediate **29**. Saponification of the ester function under usual conditions was followed by coupling with dipeptide Phi-Val-CF_3 (**v-a**) (DCC, HOBT, Et_3N) to give the final derivative **31**.

Scheme 5[a]



[a] Reagents: (a) BOP, DIEA, DMF; (b) HBr, CH_3COOH; (c) **6a** or **6c**, DCC, HOBT, DMF; (d) NaOH, CH_3OH; (e) **v-a**, HOBT, DCC, ET_3N.

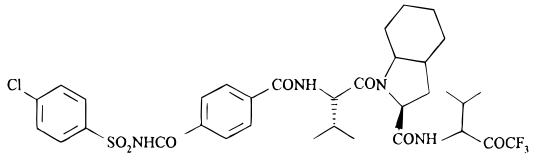
Scheme 6[a]



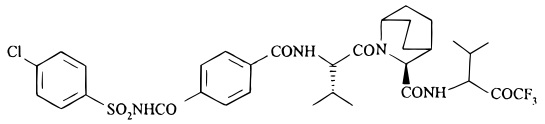
[a] Reagents: (a) PCl_5, $CHCl_3$; (b) Boc_2O, Et_3N; (c) 4-hydroxy-3,5-di-*tert*-butylthiophenol, K_2CO_3, CH_3CN; (d) gaseous HCl, dioxane; (e) A-OH, BOP, DIEA, DMF; (f) NaOH, CH_3OH; (g) **v-a**, DCC, HOBT, Et_3N.

Enzyme Inhibitory Activities

Previous studies in our laboratories had documented that it was possible to replace the central proline (Pro) of the tripeptidic backbone Val-Pro-Val found in ICI 200 880 (**1**) by our nonnatural amino acids Phi (**4**) and Abo (**5**) and obtain potent and specific HLE inhibitors.[10,11] This was exemplified herein by compounds **32** and **33** which displayed similar IC_{50} values against an HLE enzymatic preparation compared to their proline counterpart (in our hands, IC_{50} for ICI compound was 34 nM).



32 IC_{50} = 18 nM

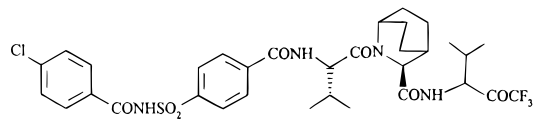


33 IC_{50} = 15 nM

The aim of our ongoing investigations was to add a complementary activity to the strict elastase inhibition. Among several possible synergistic activities (mucolytic, antiinflammatory, etc.), we turned to the hypothesis that it could be beneficial to embody our inhibitors with antioxidant properties.

As briefly mentioned by Stein et al.,[21] the minimum structural sequence necessary to obtain a strong *in vitro* anti-HLE inhibitory potency was derived from H-Val-AA-Val-CF_3: in our hands, the tripeptidic ketone **vii-b** (H-Val-Abo-Val-CF_3) was found to be moderately active (IC_{50} = 210 nM), which was in contrast to intermediate **vi-b** (Boc-Val-Abo-Val-CF_3) (IC_{50} = 32 nM), thus suggesting the possible importance of inhibitor interaction at the P3 region of the enzyme to obtain a good activity. The first inhibitor incorporating an antioxidant moiety (**7**) was quite atypical, mainly because the phenol ring was directly linked to the tripeptidic sequence through an alkylaryl chain (Table 1). Surprisingly this compound retained some *in vitro* inhibitory potency with an IC_{50} of 24 nM.

On the other hand, the (acylamino)sulfonyl derivative **34**, with an IC_{50} value of 24 nM, was equipotent to the reference inhibitor **1**. This observation opened up the field of introducing different carboxylic acids via coupling with *p*-sulfonamidobenzoic acid or a substituted analog.



34

When carboxylic acid **6a** was adjoined to the tripeptide through the *p*-sulfonamidophenyl moiety, the potencies of the corresponding Phi- and Abo-containing inhibitors **11a**,**b** were in the same range of potency (38 and 30 nM, respectively) as those of the reference inhibitors. If the methylene linker was removed to give **11c**, inhibition was still potent (IC_{50} = 36 nM), as was also observed with the bis di-*tert*-butylphenol derivative **11d** (IC_{50} = 46 nM). The vinyl analogs **11e**,**f** had similar potencies with an IC_{50} around 40 nM. The inhibitory potency was still robust when a 4-mercaptodi-*tert*-butylphenol group was linked to the acylsulfonamido

Table 1

B-Val-AA-Val-CF_3

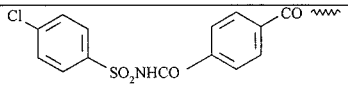
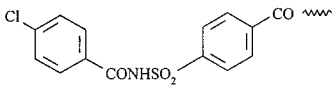
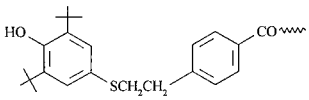
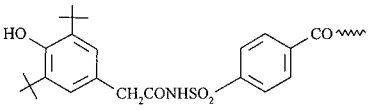
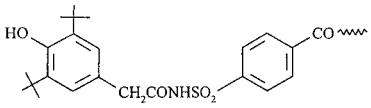
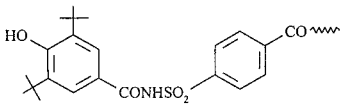
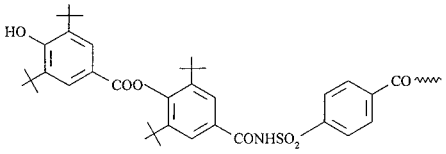
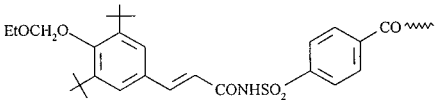
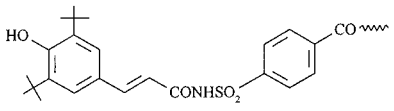
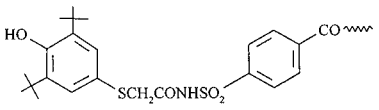
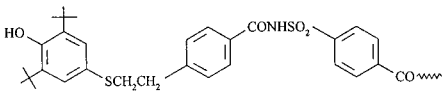
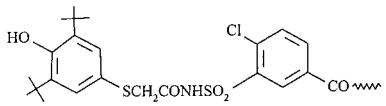
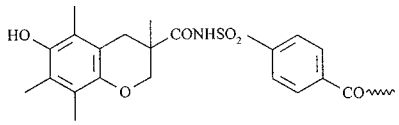
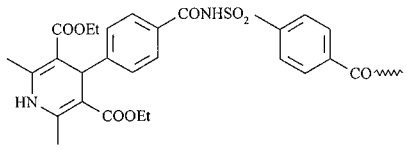
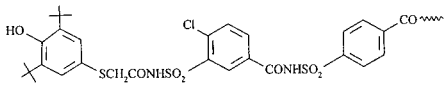
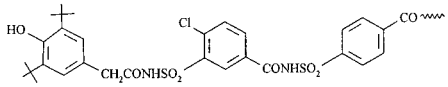
compd n°	B	AA	formula analysis [a]	mp (°C) recryst solv	IC_{50} (nM) [i] HLE	IC_{50} (μM) [j] Lipid Perox
1 ICI 200880		PRO	$C_{30}H_{34}ClF_3N_4O_7S$ ND[b]	amorphous	34	in[k]
32	id	PHI	$C_{34}H_{40}ClF_3N_4O_7S$ ND	amorphous	18	in
33	id	ABO	$C_{33}H_{38}ClF_3N_4O_7S$ ND	amorphous	15	in
34		ABO	$C_{33}H_{38}ClF_3N_4O_7S$ ND	amorphous	24	in
7		ABO	$C_{42}H_{58}F_3N_3O_5S$ C, H, N, S	amorphous	24	1.2-2.5
11-a		PHI	$C_{43}H_{59}F_3N_4O_8S$ C, H, N, S	amorphous	38	5-10
11-b		ABO	$C_{42}H_{57}F_3N_4O_8S$ ND	amorphous	30	2.5-10
11-c		PHI	$C_{42}H_{57}F_3N_4O_8S$ C[c], H, N, S	amorphous	36	10-20
11-d		PHI	$C_{57}H_{77}F_3N_4O_{10}S$ C, H, N, S[d]	amorphous	46	in
11-e		PHI	$C_{47}H_{65}F_3N_4O_9S$ C, H, N, S	150-152	36	in
11-f		PHI	$C_{44}H_{59}F_3N_4O_8S$ C, H, N, S	150-153 CH_2Cl_2-CH_3OH	38	2.5-5
11-g		PHI	$C_{43}H_{59}F_3N_4O_8S_2$ C, H, N, S[e]	amorphous	43	1.2-2.5
11-h		PHI	$C_{50}H_{65}F_3N_4O_8S$ C, H, N, S[f]	amorphous	40	1.2-2.5

Table 1 (Continued)

B-Val-AA-Val-CF_3

compd n°	B	AA	formula analysis [a]	mp (°C) recryst solv	IC_{50} (nM) [i] HLE	IC_{50} (μM) [j] Lipid Perox
11-i		PHI	$C_{43}H_{58}ClF_3N_4O_8S_2$ C, H, N, S, Cl	amorphous	46	0.6-1.2
11-j		ABO	$C_{40}H_{51}F_3N_4O_9S$ C, H, N, S	amorphous	20	1.2-2.5
11-k		ABO	$C_{46}H_{56}F_3N_5O_{11}S$ C[g], H, N, S	amorphous	41	in
14-a		PHI	$C_{50}H_{63}ClF_3N_5O_{11}S_3$ C, H, N, S, Cl	amorphous	26	0.6-1.2
14-b		PHI	$C_{50}H_{63}ClF_3N_5O_{11}S_2$ C, H[h], N, S, Cl	amorphous	34	5-10

[a] Compounds gave satisfactory analyses (±0.4%) unless otherwise indicated. [b] ND: not determined, but satisfactory results by high-resolution MS analysis were obtained. [c] C: found, 60.84; calcd 60.42. [d] S: found, 3.73; calcd, 3.00. [e] S: found, 7.93; calcd, 7.28. [f] S: found, 5.49; calcd. 6.60. [g] C: found, 57.86; calcd, 58.53. [h] H: found, 6.45; calcd, 5.95. [i] IC_{50} values are concentrations of compounds required to achieve 50% inhibition against HLE from human sputum. [j] IC_{50} values are concentrations of compounds required to achieve 50% inhibition against Fe^{3+} ascorbate-induced lipid peroxidation from liver microsomal preparation. [k] in: inactive.

moiety either by a methylene group as in **11g** (IC_{50} = 43 nM) or by an ethylphenyl group as in **11h** (IC_{50} = 40 nM). Compound **11i** which embodied a 4-chloro-3-sulfonamidobenzoic acid entity also retained a strong *in vitro* activity, having an IC_{50} value of 46 nM.

At this point, we selected the trolox group[22] as well as a 4-phenyldihydropyridyl entity as potential antioxidant functions, giving rise to inhibitors **11j** (IC_{50} = 20 nM) and **11k** (IC_{50} = 41 nM), respectively. It has been hypothesized that the sulfonamide functionality confers high *in vitro* and *in vivo* activity, probably through improved interactions with the S4−S5 enzymatic sites and also due to the acidic nature of this acylsulfonamide function which could prevent the rapid clearance of the compounds from the lungs.[23] We took this reasoning one step further by adding a second sulfonamide function to give the bis sulfonamide derivatives **14a**,**b**. Both compounds displayed potent *in vitro* inhibitory activity against HLE with IC_{50}s of 26 and 34 nM, respectively.

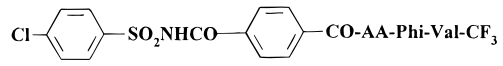
The preparation of an HLE inhibitor in which the P3 valine is replaced by a lysine has been documented in 1990 by Peet et al.[17] This compound was reported to have a K_i value of 0.58 nM on an HLE enzymatic preparation. As illustrated in Table 2, inhibitors **20** and **23** incorporated the tripeptidic trifluoromethyl ketone Lys-Phi-Val-CF_3. On the α-amino group of the lysine residue was appended the *p*-[*p*-Cl(C_6H_4)SO_2NHCO]-(C_6H_4)CO system (A), while the ϵ-amino group on the lysine side chain was derivatized with two different 4-mercaptodi-*tert*-butylphenol-containing carboxylic acids. These two compounds appeared to be slightly less potent against HLE than their related valine-containing counterparts, displaying IC_{50}s around 70−80 nM. Finally, the P3 valine was replaced by a S-substituted cysteine to give compound **31** which presented a potency similar to that of the reference inhibitors (IC_{50} = 59 nM).

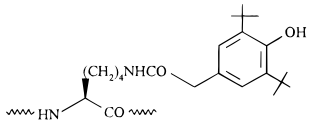
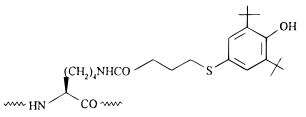
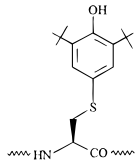
From these preliminary *in vitro* studies, it appears that (1) Phi and Abo are effective proline mimics in order to obtain potent HLE inhibitors *in vitro*. (2) The [(*p*-chlorophenyl)sulfonamido]carbonyl moiety used in the standard inhibitors can effectively be replaced by entities in which a chemical entity able to bring some additionnal antioxidant properties is incorporated: all the compounds tested presented *in vitro* anti-HLE potencies between 10 and 100 nM, indicating that the P4−P5 region of the enzyme has a high degree of tolerance with regard to the substituent group.

Selectivity Studies

Selectivity of inhibitor **14a** was studied on a panel of 22 receptors[24] where K_i values were higher than 10^{-5} M. Affinities for sodium (Veratridine) and calcium (Nifedipine) channels were superior to 10^{-4} and 2.4 × 10^{-6} M, respectively. Moreover, compound **14a** gave an IC_{50} of 1 μM against pig pancreatic elastase (PPE) and 2 μM against prolyl endopeptidase. No inhibitory activity was detected up to 33 μM on several factors of the coagulation and the fibrinolysis cascades, including thrombin, plasmin, factor Xa, kallikrein, activated protein C, urokinase, tPa, and trypsin, as well as on

Table 2



compd n°	AA	formula analysis [a, b]	IC_{50} (nM) [e] HLE	IC_{50} (μM) [f] Lipid Perox
1		$C_{30}H_{34}ClF_3N_4O_7S$		
ICI 200880	see text	ND[c]		
20		$C_{51}H_{65}ClF_3N_5O_9S$ C, H, N[d], S	70	2.5-5
23		$C_{53}H_{69}ClF_3N_5O_9S_2$ ND	84	2.5-5
31		$C_{46}H_{56}ClF_3N_4O_8S_2$ ND	59	2.5-5

[a] Compounds gave satisfactory analyses (±0.4%) unless otherwise indicated. [b] The four compounds were amorphous solids. [c] ND: not determined, but satisfactory results by high-resolution MS analysis were obtained. [d] C: found, 59.37; calcd, 60.26. N: found, 6.43; calcd, 6.89. [e] IC_{50} values are concentrations of compounds required to achieve 50% inhibition against HLE from human sputum. [f] IC_{50} values are concentrations of compounds required to achieve 50% inhibition against Fe^{3+} ascorbate-induced lipid peroxidation from liver microsomal preparation. [g] in: inactive.

acetylcholinesterase and cathepsin G. Finally, matrix metalloproteinases 1, 2, 3, and 9 were inhibited by compound **14a** with IC_{50} values of 58, 19, 38, and 24 μM, respectively.

Antioxidant Properties

In vitro antioxidant potential of our inhibitors was estimated by inhibition of the Fe^{3+} ascorbate-induced lipid peroxidation on a rat liver microsomal preparation. As expected, ICI 200 880 was completely devoid of activity in this assay. Inhibitor **11k** containing a dihydropyridine entity was virtually inactive on the peroxidation test. In contrast, the trolox group containing inhibitor **11j** gave potent inhibition with an IC_{50} of 1.2−2.5 μM. However, for the di-*tert*-butylphenol moiety-containing compounds, we found that the antioxidant potency depended on the link between the phenol ring and the acylsulfonamido group; as expected, the protected phenol **11e** was completely devoid of activity, and an ester function such as in **11d** was also deleterious for activity. Middle range potency was obtained with direct bonding (**11c**, 10−20 μM), a methylene spacer (**11a**,**b** or **14b**, 2.5−10 μM), or the vinylogous **11f** (2.5−5 μM). The strongest inhibition of peroxidation was obtained when the 4-mercaptodi-*tert*-butylphenol was used: it gave rise to IC_{50} values between 2.5 and 5 μM for inhibitors **20**, **23**, and **31**. Potency was further increased for inhibitors **7** and **11g**,**h** (IC_{50} = 1.2−2.5 μM) and culminated with compounds **11i** and **14a** with IC_{50}s ranging between 0.6 and 1.2 μM.

Furthermore, compound **14a** protected the purified human LDL from copper sulfate-induced oxidative modifications with an IC_{50} of 0.38 μM. In our hands, standard antioxidant compounds probucol and BHT gave IC_{50}s of 3 μM in this assay.

In Vivo Studies

The main goal of our investigations was to identify compounds affording a strong and long lasting *in vivo* activity after intratracheal administration. The animal model used along these studies was based on the fact that HLE induces an acute hemorrhage in the hamster lung when administered intratracheally (it).[25] This hemorrhage can be quantitated 3 h later by evaluation of the hemoglobin content in bronchial alveolar lavage fluid. All the compounds tested were administered it at various times prior to the HLE challenge at the dose of 15 nmol/animal. The results of this time−effect study are disclosed in Table 3.

In this assay, ICI 200 880 exhibited a potent activity, since 78% of the HLE-induced lung injury was inhibited at 3 h; this activity was maintained for at least 24 h. Several novel inhibitors with strong *in vitro* potencies were partially or even totally devoid of *in vivo* activity: compound **11j** in which the trolox group is appended on the (acylamino)sulfonyl linker showed no meaningful *in vivo* activity, as well as the bis di-*tert*-butylphenol derivative **11d**. As expected, compound **7** exhibited a modest activity when administered 3 h before the inflammatory challenge, probably due to the lack of acylsulfonamide function within its structure; more curiously, its extented analog **11h** was also poorly active. Inhibitor **11k** gave a good inhibition of the HLE-induced edema at 3 h but was devoid of activity at 6 h. More generally, incorporation of the (acylamino)sulfonyl functionality within the structure of the inhibitors

Table 3.

	% inhibition of HLE-induced hemorrhage in the hamster lung at					
compd no.	3 h	6 h	18 h	24 h	48 h	72 h
ICI 200 880	78	63	47	37	18	
7	24					
11a	66	65	26	3		
11b	28	0				
11c	53	71	40	11	0	
11d	0					
11e	19					
11f	63	58	49	0		
11g	60	60	11	0		
11h	20					
11i	41	52	23	0		
11j	0					
11k	56	0				
14a	68	66	65	76	73	50
14b	41	40	52	62	0	
20	40	41	45	14	0	
23	3					
31	42	49	12	6		

afforded, as anticipated, potent compounds *in vivo*. For example, compound **11a** was found to be very potent on the reduction of the HLE-induced hemorrhage when predosed 3 and 6 h before injection of elastase; however, its activity decreased when it was administered 18 h before HLE, and no activity subsisted when administered 24 h before HLE. Noteworthy, replacement of Phi by Abo (**11b**) in the tripeptidic sequence resulted in almost total loss of activity. This could perhaps be explained by a difference in lipophilicity between the two central amino acids (log *P*(Phi) = −1.19, log *P*(Abo) = −1.75), hence between the two corresponding inhibitors, indicating that lipophilicity could also be of importance for absorption or retention of the compounds in the lungs. Compounds **11c**,**f**,**g**,**i** presented an activity profile comparable to that of **11a** with more or less potency remaining at 18 h but without any activity left when dosed at 24 or 48 h before challenge. (Note that the protected analog of **11f**, **11e**, was very poorly active at 3 h.) All these compounds have in common the presence of two aryl rings linked together by an X-(acylamino)sulfonyl group, X being respectively a direct bond, CH=CH, CH_2, or SCH_2.

A similar profile of activity was observed with the bis acylsulfonamide containing inhibitor **14b**. More interestingly, analog **14a**, with only one more sulfur atom on the phenol ring, gave a strong and very long lasting *in vivo* potency: since at a fixed dose of 15 nmol it, the first administration time giving 50% protection against elastase-induced lung injury was 72 h for **14a** and only 18 h for ICI compound. As mentioned earlier, this long duration of action in the lung could at least in part be explained by the inability of the inhibitors to be cleared from the lung, although this was not investigated.[26] This property seems to be conferred to the molecule by the presence of the acylsulfonamido moiety and is probably exacerbated if two such groups are present, as in **14a**. The longer duration of action observed with **14a** as compared to **14b** can probably be rationalized in terms of differences in their *in vivo* metabolism.

Finally, the lysine and cysteine derivative-containing inhibitors **20** and **31** diplayed similar potencies as inhibitors **11c**,**f** suggesting that *in vitro* as well as *in vivo* activity can be obtained with compounds where the N-terminal valine of the tripeptidic sequence has been

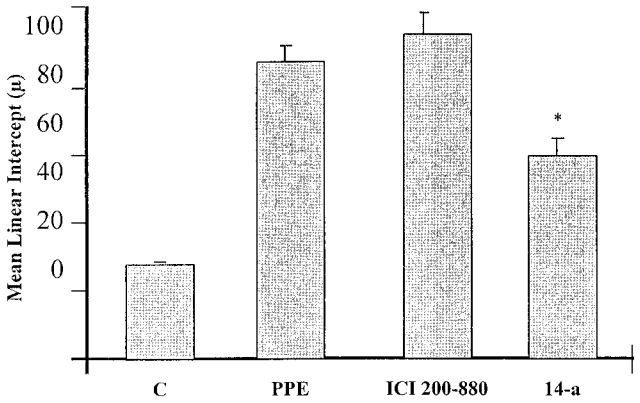


Figure 1. Effects of ICI 200 880 and **14a** on 14-day elastase-induced emphysema in the hamster. C = no treatment. PPE = 60 IU + 0.2 mL of saline of pig pancreatic elastase. ICI 200 880 and **14a** were administered it at 100 nmol in a 0.1 mL volume of saline, 3 h prior to PPE challenge.

replaced by another substituted polar amino acid. However, the length of the chain appended on the ϵ-nitrogen of the lysine residue seemed to be of prime importance since *in vivo* activity was completely lost when a CH_2 chain (as in **20**) was replaced by a $(CH_2)_3$ chain (as in **23**).

Furthermore, ICI 200 880 and compound **14a** were compared in a model of elastase-induced chronic lung injury in the hamster, where instillation of PPE caused microvascular damage and subsequent alveolar space enlargment, i.e. emphysema.[27] This was determined by quantifying the number of alveolar intercepts (mean linear intercept) on lung histological sections 14 days after the challenge (Figure 1). Intratracheal administration of ICI 200 880 just before HLE at the single dose of 100 nmol had no significant action on the increase of the alveolar spaces. In contrast, **14a** significantly inhibited pulmonary lesions associated with increased alveolar spaces.

Discussion and Summary

Among the many HLE inhibitors described in the recent literature, the tripeptidic trifluoromethyl ketone derivatives remain one of the most promising series for aerosol administration in man. We decided to extend the field of structural variations in this class of inhibitors, and several conclusions can be drawn from the foregoing results:

(1) Our proline analog amino acids have proven to be useful tools in order to obtain potent inhibitors as active as the reference compound ICI 200 880 in several assays of *in vitro* and *in vivo* reduction of HLE activity.

(2) We then hypothesized that it could be worthwhile to add to the strict antielastase property of our compounds a second activity, if possible acting synergistically. We decided to embody our compounds with antioxidant properties, since several studies had recently demonstrated that the destructive potential of HLE in the lung was enhanced by active ORS. This hypothesis was chemically expressed with the use of antioxidant groups such as the *tert*-butylphenol ring, which was appended in different ways on the modified tripeptidic sequence. This gave rise to inhibitors that were effective HLE inhibitors *in vitro* with IC_{50} values in the 10−100 nM range. Moreover, these compounds exhibited a real antioxidant potential, since they inhib-

ited lipid peroxidation on a rat liver microsomal preparation as well as on purified human LDL with an IC_{50} similar to or even better than IC_{50}s of reference antioxidant compounds. The elastase-induced acute lung injury was very discriminant for the selection of a leader compound in terms of duration of action in the lung: one of the most active compounds of the series, **14a**, showed significant activity in this assay, reducing the hemorrhage by 50% when predosed 72 h prior to HLE challenge. Furthermore, in a 14-day model of elastase-induced emphysema in the hamster, **14a** significantly inhibited alveolar space enlargement, when administered once just before instillation of HLE. In this model, ICI 200 880 was devoid of significant activity, indicating that the addition of an antioxidant moiety in the structure of an HLE inhibitor could be relevant in order to gain some *in vivo* potency in a chronic model of lung injury. Although promising, these results warrant some more *in vivo* confirmation: to adress the importance of the antioxidant effect of our compounds, it would be interesting to compare **14a** with a close analog devoid of antioxidant properties on an animal model of emphysema involving both elastase and reactive oxygen species.

Experimental Section

Melting points were determined on a Tottoli apparatus and were not corrected. Elemental analyses were carried out by the analytical department of the Institut de Recherches Servier; results obtained for specified elements are within ±0.4% of the theoretical values. IR spectra were recorded on a Bruker IFS 28 spectrophotometer. ^{1}H NMR spectra of deuteriochloroform or DMSO-d_6 solutions were recorded on a Bruker AC 200 or AM 300 spectrometer. Chemical shifts are given in ppm with TMS as the internal standard. Optical rotations were recorded with a 241 Perkin Elmer polarimeter. Abbreviations: DCC = *N, N′*-dicyclohexylcarbodiimide; DCU = *N, N′*-dicyclohexylurea; HOBT = 1-hydroxybenzotriazole; BOP = (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DMAP = 4-(dimethylamino)pyridine; DIEA = *N*,*N*-diisopropylethylamine; NMM = *N*-methylmorpholine.

(2*S*,3a*S*,7a*S*)-1-(*tert*-Butyloxycarbonyl)-2-[[(1-isopropyl-2-hydroxy-3,3,3-trifluoropropyl)amino]carbonyl]-perhydroindole (iii-a). To a mixture of 12.4 g (0.046 mol) of Boc-Phi-OH (**i**)[9−11] and 5 mL (0.046 mol) of *N*-methylmorpholine in 80 mL of THF was added dropwise at −10 °C a solution of 5.95 mL (0.046 mol) of isobutyl chloroformate in 10 mL of THF. After 10 min at −10 °C, a solution of the amino alcohol **ii** (9.5 g, 0.046 mol) and *N*-methylmorpholine (5 mL) in 60 mL of DMF was added dropwise at −10 °C. Stirring was continued overnight and temperature slowly raised to ambient. Solvents were evaporated, and the residue was taken up with isopropyl ether (150 mL); after filtration of an insoluble, the organic phase was washed with water and dried over calcium sulfate. Evaporation of the solvent was followed by trituration with pentane to give 16.8 g (83%) of the desired intermediate: mp 193 °C; IR (Nujol) 3600−3300, 1662 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (6H, m), 1.3−2.0 (9H, m), 1.4 (9H, s), 2.3 (3H, m), 3.75−4.4 (4H, m), 6.5 (1H, br s), 8.4 (1H, br s).

(3*S*)-2-Aza-2-(*tert*-butyloxycarbonyl)-3-[[(1-isopropyl-2-hydroxy-3,3,3-trifluoropropyl)amino]carbonyl]bicyclo-[2.2.2]octane (iii-b): IR (Nujol) 3600−3300, 1662, 1165, 1138, 1105 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.8 (6H, m), 1.35−2.0 (9H, m), 1.4 (9H, s), 2.15 (1H, m), 3.7−4.15 (4H, m), 6.35 (1H, br s), 7.2 and 7.6 (1H, br s).

Boc-Phi-Val-CF$_3$ (iv-a). Alcohol obtained at the previous step (15.8 g, 0.037 mol) was oxidized by the DMSO−Ac_2O mixture (160 and 130 mL, respectively). After stirring at room temperature during 48 h, 300 mL of water was added; stirring was continued for 3 more h and the resulting solid filtrated. This solid was taken up with dichloromethane; the organic layer was washed with a saturated sodium bicarbonate solution and then water. After drying on calcium sulfate and evaporation of the solvent, 15.1 g (96%) of an amorphous solid was obtained: mp 110 °C; IR (Nujol) 1764, 1680 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (6H, m), 1.3−2.0 (9H, m), 1.35 (9H, s), 2.3 (3H, m), 3.75 (1H, m), 4.2 (1H, m), 4.4 (1H, m), 8.4 (1H, br s).

Boc-Abo-Val-CF$_3$ (iv-b): IR (Nujol) 3311, 1761, 1670 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (6H, m), 1.35 (9H, s), 1.4−1.75 (7H, m), 1.9 (1H, m), 2.1 (1H, m), 2.2 (1H, m), 3.9 (1H, m), 4.15 (1H, dd), 4.6−4.7 (1H, 2m).

Phi-Val-CF$_3$ (v-a). General Method 1. The protected dipeptide obtained above (15.1 g, 0.036 mol) was dissolved in 350 mL of dioxane. Gaseous HCl was bubbled at 10 °C until saturation of the solution was obtained. Stirring was continued overnight at room temperature. After evaporation of the solvent, the crude solid was taken up with pentane to give 12 g of the desired hydrochloride (94%): mp 134 °C; IR (Nujol) 3600−2400, 1764, 1680, 1171 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.8−1.0 (6H, 2d), 1.2−1.9 (9H, m), 2.3 (3H, m), 3.6 (1H, m), 4.15 (1H, m), 4.4 (1H, m), 8.4 (1H, br s), 10.5 (1H, br s).

Abo-Val-CF$_3$ (v-b): mp 165−170 °C sublimation; IR (Nujol) 3600−2400, 1765, 1677, 1173 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.8−1.0 (6H, 2d), 1.3−2.0 (8H, m), 2.2 (1H, m), 2.3 (1H, m), 3.4 (1H, br s), 4.0−4.2 (2H, m), 8.5 (1H, br s), 9.8 (1H, br s).

Boc-Val-Phi-Val-CF$_3$ (vi-a). General Method 2. To 6 g (0.0168 mol) of Phi-Val-CF_3 in 5 mL of DMF were added 2.34 mL (0.0168 mol) of triethylamine, 3.65 g (0.0168 mol) of Boc-Val-OH in 3 mL of DMF, 2.57 g of HOBT (0.0168 mol) in 2 mL of DMF, and 3.46 g (0.0168 mol) of DCC in 3 mL of DMF. Stirring was continued for 20 h. DCU was filtered and DMF evaporated. The gummy residue was taken up with ethyl acetate; the organic solution was washed consecutively with brine, a saturated sodium bicarbonate solution, brine, a 10% citric acid solution, brine, and water. Solution was dried over calcium sulfate, filtered, and evaporated to give 9.1 g of a crude solid. This product was purified by chromatography (CH_2Cl_2−EtOH, 98:2) to give 4.4 g of the protected tripeptide: IR (Nujol) 3276, 1765, 1687, 1207−1150 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.8−1.0 (12H, m), 1.15−1.9 (8H, m), 1.4 (9H, s), 1.9 (1H, m), 2.2 (2H, m), 3.75 (1H, m), 4.0−4.4 (2H, m), 4.45−4.65 (1H, 2m).

Boc-Val-Abo-Val-CF$_3$ (vi-b): IR (Nujol) 3500−3300, 1793, 1697, 1624 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.8−1.1 (12H, m), 1.4 (9H, s), 1.5−1.7 (4H, m), 1.8−2.0 (4H, m), 2.4 (1H, m), 2.6 (2H, m), 3.95 (1H, m), 4.4 (1H, m), 5.02 (2H, m), 6.45 (1H, br s), 6.7 (1H, br s).

Val-Phi-Val-CF$_3$ (vii-a): see General Method 1; IR (Nujol) 1763, 1695 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.7−1.1 (12H, m), 1.1−2.5 (13H, m), 3.5−4.8 (4H, m).

Val-Abo-Val-CF$_3$ (vii-b): IR (Nujol) 1761, 1693, 1631 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.0 (12H, m), 1.3−1.8 (8H, m), 1.8−2.2 (3H, m), 4.0 (1H, m), 4.1 (1H, m), 4.4 (1H, dt), 4.6 (1H, 2m).

4-[2-(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]ethyl]benzoyl-(*S*)-Val-(*S*)-Abo-(*R*,*S*)-Val-CF$_3$ (7). General Method 3. Tripeptide **vii-b** (662 mg, 0.015 mol), carboxylic acid **6d** (580 mg, 0.015 mol), BOP (668 mg, 0.015 mol), and DIEA (0.79 mL, 0.045 mol) were stirred at room temperature for 18 h in 50 mL of DMF. After evaporation of the solvent, the residue was taken up with 100 mL of ethyl acetate. The solution was washed with water, dried over calcium sulfate, and evaporated. Purification by chromatography (CH_2Cl_2−AcOEt, 90:10) gave 0.7 g (60%) of the title compound: IR (Nujol) 1761, 1640−1700 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.95 (12H, m), 1.4 (18H, s), 1.5−2.0 (9H, m), 2.15 (2H, m), 2.9 (2H, t), 3.1 (2H, t), 4.1−4.7 (4H, m), 7.15 (2H, s), 7.25 (2H, d), 7.75 (2H, d).

Ethyl 4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)acetyl]-amino]sulfonyl]benzoate (9a). General Method 4. To a solution of 5 g (0.019 mol) of 4-hydroxy-3,5-di-*tert*-butylphenylacetic acid (**6a**) in 60 mL of DMF were successively added 4.35 g (0.019 mol) of ethyl 4-sulfonamidobenzoate (**8a**) in 10 mL of DMF, 2.32 g (0.019 mol) of DMAP in 10 mL of DMF, and DCC (3.92 g, 0.019 mol). The resulting solution was stirred at room temperature for 2 days. DCU was filtered and DMF was concentrated under reduced pressure. The residue was treated with ethyl acetate. The organic layer was washed consecutively with saturated aqueous NaCl, 10% aqueous citric acid, and again brine. The EtOAc was dried ($CaSO_4$) and

concentrated *in vacuo* to give the crude title compound as a dark solid. This compound was purified by chromatography over silica gel (CH_2Cl_2−CH_3OH−NH_4OH, 90:10:1) to give a colorless oil (6.8 g, 75.5%) that crystallized upon trituration with pentane: mp 150 °C; IR (Nujol) 3600, 3300−3000, 1726, 1711−1685, 1360, 1144 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (21H, t + s), 3.4 (2H, s), 4.35 (2H, q), 6.85 (2H, s), 6.9 (1H, br s), 8.0 (2H, d), 8.15 (2H, d), 12.45 (1H, br s).

Ethyl 4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carbonyl]amino]sulfonyl]benzoate (9b) and Ethyl 4-[[[[4-[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carboxy]-3,5-di-*tert*-butylphenyl]carbonyl]amino]sulfonyl]benzoate (9c). To a 100 mL toluene solution of the sulfonamide **3** (3.45 g, 0.015 mol) was added 2.1 mL (0.015 mol) of triethylamine; then at 80 °C the acid chloride of 4-hydroxy-3,5-di-*tert*-butylbenzoic acid (4.05 g, 0.015 mol) (itself prepared with 2 mol equiv of oxalyl chloride in toluene at 80 °C) was added. The reaction mixture was refluxed overnight and cooled down to room temperature, and water was added. The remaining starting sulfonamide was filtered, and the organic layer was washed several times with water. Drying ($CaSO_4$) and evaporation gave an amorphous solid (5.5 g). This compound was purified by chromatography (CH_2Cl_2−CH_3OH−NH_4OH, 90:10:0.5) to give 3.3 g of a mixture of both **9b**,**c**.

Ethyl 4-[[[4-(ethoxymethoxy)-3,5-di-*tert*-butylcinnamoyl]amino]sulfonyl]benzoate (9d): IR (Nujol) 3217, 1726, 1622, 1275, 1109 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.2 (3H, t), 1.35 (3h, t), 1.4 (18H, s), 3.8 (2H, q), 4.35 (2H, q), 4.9 (2H, s), 6.55 (1H, d), 7.5 (1H, d), 7.45 (2H, s), 8.15 (4H, dd), 12.3 (1H, br s).

Ethyl 4-[[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]amino]sulfonyl]benzoate (9e): mp 188 °C; IR (Nujol) 3600, 3245, 1722, 1699 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (18H, s), 1.35 (3H, t), 3.55 (2H, s), 4.35 (2H, q), 6.95 (2H, s), 7.10 (1H, br s), 7.55 (1H, br s), 8.00−8.2 (4H, m).

Ethyl 3-[[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]amino]sulfonyl]-4-chlorobenzoate (9g): IR (Nujol) 3500−3000, 1718, 1593 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (21H, m), 3.35 (2H, s), 4.35 (2H, q), 6.9 (1H, s), 7.05 (2H, s), 7.55 (1H, d), 7.95 (1H, dd), 8.5 (1H, d).

Ethyl 3-[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]sulfonyl]-4-chlorobenzoate (9h): IR (Nujol) 3610−3583, 3097, 1726, 1684 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.45 (3H, t), 1.45 (18H, s), 3.55 (2H, s), 4.4 (2H, q), 5.3 (1H, br s), 6.95 (2H, s), 7.55 (1H, d), 8.1 (1H, br s), 8.2 (1H, dd), 8.9 (1H, d).

Ethyl 4-[[[(2(*R*,*S*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)carbonyl]amino]sulfonyl]benzoate (9i): IR (Nujol) 3700−2500, 1707, 1649, 1375, 1141 cm^{-1}; ^1H NMR (CH_3OH-d_4) δ 1.4 (3H, t), 1.45 (3H, s), 1.65 (1H, m), 1.9−2.15 (9H, 3s), 2.35 (3H, m), 4.35 (2H, q), 7.65 (2H, d), 7.9 (2H, d).

Ethyl 4-[[[(2,6-dimethyl-3,5-dicarbethoxy-1,4-dihydropyridin-4-yl)carbonyl]amino]sulfonyl]benzoate (9j): mp 202 °C; IR (Nujol) 3500−3000, 1737−1676, 1136, 1130 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.1 (6H, t), 1.3 (3H, t), 2.25 (6H, s), 4.0 (4H, m), 4.3 (2H, q), 4.85 (1H, s), 7.05 (2H, d), 7.7 (2H, d), 7.9 (4H, m), 8.75 (1H, br s).

4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]-sulfonyl]benzoic Acid (10a). General Method 5. A 5 g (0.0105 mol) portion of ester **9a** was dissolved in 125 mL of ethanol. Nitrogen was bubbled through the solution for 1 h; then 22 mL (0.021 mol) of 1 N NaOH was added. The reaction mixture was stirred at room temperature for 2 h, followed by addition of 25 mL of water. The reaction mixture was then heated at 60 °C for 20 h under nitrogen. After cooling, the solvents were evaporated under reduced pressure, and the residue was taken up with water. The aqueous layer was extracted with ethyl acetate, acidified with 1 N HCl (30 mL), and extracted with ethyl acetate. The organic layer was washed with water to neutral pH, dried over calcium sulfate, and evaporated to give 4.2 g of a crude solid. This material was crystallized from pentane to give 4 g (70%) of the title compound: mp 228 °C; IR (Nujol) 3635, 3300−2400, 1707 cm^{-1}; ^1H NMR (DMSO-d_6) 1.3 (18H, s), 3.4 (2H, s), 6.85 (2H, s), 7.5 (1H, br s), 7.95 (2H, d), 8.1 (2H, d), 12.4 (1H, br s), 13.3 (1H, br s).

4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carbonyl]amino]sulfonyl]benzoic Acid (10b) and 4-[[[[4-[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carboxy]-3,5-di-*tert*-butylphenyl]carbonyl]amino]sulfonyl]benzoic Acid (10c). Separation of the two compounds (on a 2 g scale) was performed by reverse phase HPLC on a RP 18 15/25 column (5 mL/min) with CH_3-CN−H_2O−CH_3COOH (70:30:1) as eluent. After evaporation of the eluent, the crude powder was triturated in pentane to give 450 mg of **10b** and 700 mg of **10c**: mp 272 °C; IR (Nujol) 3626, 3298, 3000−2400, 1685 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (18H, s), 7.2 (1H, br s), 7.5 (1H, br s), 7.7 (2H, s), 7.95 (4H, m), 12.7 (1H, br s); IR (Nujol) 3570, 3257, 1716, 1685 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.25 (18H, s), 1.4 (18H, s), 7.8 (2H, s), 7.9 (2H, s), 8.15 (4H, m), 13.5 (1H, br s).

4-[[[4-(Ethoxymethoxy)-3,5-di-*tert*-butylcinnamoyl]amino]sulfonyl]benzoic acid (10d): IR (Nujol) 3101, 1687, 1612 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.2 (3H, t), 1.4 (18H, s), 3.8 (2H, q), 4.9 (2H, s), 6.55 (1H, d), 7.5 (2H, s), 7.55 (1H, d), 8.15 (4H, dd).

4-[[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]-amino]sulfonyl]benzoic acid (10e): mp 240°C; IR (Nujol) 3607, 3279, 3200−2450, 1703 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (18H, s), 3.55 (2H, s), 7.0 (2H, s), 7.1 (1H, br s), 7.95−8.2 (4H, 2d), 13−13.5 (1H, br s).

4-[[[4-[2-[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]ethyl]benzoyl]amino]sulfonyl]benzoic acid (10f): IR (Nujol) 3610, 3500−2400, 1703, 1360, 1171 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (18H, s), 2.85 (2H, t), 3.15 (2H, t), 7.0 (1H, br s), 7.05 (2H, s), 7.35 (2H, d), 7.8 (2H, d), 8.15 (4H, m).

3-[[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]-amino]sulfonyl]-4-chlorobenzoic acid (10g): IR (Nujol) 3600, 3500−3000, 1709 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (18H, s), 3.55 (2H, s), 7.0 (2H, s), 7.1 (1H, s), 7.8 (1H, d), 8.15 (1H, dd), 8.55 (1H, d), 12.5 (1H, br s).

3-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]-sulfonyl]-4-chlorobenzoic acid (10h): IR (Nujol) 3641, 3350−2500, 1709, 1682, 1377, 1109 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (18H, s), 3.45 (2H, s), 6.8 (1H, br s), 6.85 (2H, s), 7.75 (1H, d), 8.15 (1H, dd), 8.55 (1H, d), 12.8 (1H, br s), 13.6 (1H, br s).

4-[[[(2(*R*,*S*)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-yl)carbonyl]amino]sulfonyl]benzoic acid (10i): IR (Nujol) 3650−2300, 1724−1700, 1354, 1184 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (3H, s), 1.65 (1H, m), 1.85 (3H, s), 2.0−2.4 (3H, m), 2.1 (6H, 2s), 7.5 (1H, br s), 7.80 (2H, d), 8.05 (2H, d), 11.9 (1H, br s), 13.5 (1H, br s).

4-[[[(2,6-Dimethyl-3,5-dicarbethoxy-1,4-dihydropyridin-4-yl)carbonyl]amino]sulfonyl]benzoic acid (10j): IR (Nujol) 3600−2500, 1685, 1655, 1336, 1128 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.1 (6H, m), 2.25 (6H, s), 3.95 (4H, m), 4.85 (1H, s), 7.05 (2H, d), 7.7 (2H, d), 7.75−7.9 (4H, 2d), 8.8 (1H, br s).

Ethyl 4-[[[[4-chloro-3-[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]sulfonyl]phenyl]carbonyl]amino]-sulfonyl]benzoate (12b): IR (Nujol) 3627, 3232, 1705 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (3H, t), 1.3 (18H, s), 3.45 (2H, s), 4.35 (2H, q), 6.8 (1H, br s), 6.85 (2H, s), 7.7 (1H, d), 8.1 (5H, m), 8.5 (1H, d), 12.8 (1H, br s).

4-[[[[4-Chloro-3-[[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)-thio]acetyl]amino]sulfonyl]phenyl]carbonyl]amino]-sulfonyl]benzoic acid (13a): IR (Nujol) 3500−3000, 3500−2500, 1705 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.25 (18H, s), 3.55 (2H, s), 7.0 (2H, s), 7.1 (1H, br s), 7.75 (1H, s), 7.8 (1H, d), 8.15 (5H, m), 8.55 (1H, d), 12.8 (1H, br s).

4-[[[[4-Chloro-3-[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)-acetyl]amino]sulfonyl]phenyl]carbonyl]amino]sulfonyl]-benzoic acid (13b): IR (Nujol) 3627−3221, 2600, 1703 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3 (18H, s), 3.5 (2H, s), 6.9 (2H, 2s), 7.8 (1H, d), 8.15 (5H, m), 8.5 (1H, d), 12.9 (1H, br s).

4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]-sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11a): prepared according to General Method 3 from **10a** and tripeptide **vii-a**; IR (Nujol) 3639, 3400−3100, 1763−1650, 1360, 1673 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.8−0.95 (12H, m), 1.2−1.8 (8H, m), 1.3 (18H, s), 1.8−2.0 (2H, m), 2.15−2.3 (3H, m), 3.35 (2H, s), 4.2−4.5 (3H, m), 4.7 (1H, m), 6.8 (1H, br s), 6.85 (2H, s), 7.9−8.05 (4H, d), 8.5−8.7 (1H, 2d), 8.9 (1H, br s), 12.3 (1H, br s).

4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)acetyl]amino]-sulfonyl]benzoyl-(*S*)-Val-(*S*)-Abo-(*R*,*S*)-Val-CF_3 (11b): IR (Nujol) 3700−2500, 1761, 1700−1620, 1370, 1173 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.3 (9H, s), 1.4−1.8 (8H, m), 2.0−2.25 (3H, m), 3.4 (2H, s), 4.0-4.7 (4H, m), 6.8 (1H, br s), 6.85 (2H, s), 7.5 (1H, d), 7.9 (2H, d), 8.05 (2H, d), 8.4 (1H, d), 8.7 (1H, d), 12.4 (1H, br s).

4-[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carbonyl]amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11c): IR (Nujol) 3624, 3278, 1763−1622 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.1−2.0 (8H, m), 1.4 (18H, s), 1.95 (2H, m), 2.2 (3H, m), 4.25−4.5 (4H, m), 7.55 (2H, s), 8.0 (4H, q), 8.4 (1H, br s), 8.9 (1H, br s), 12.5 (1H, br s).

4-[[[[4-[(4-Hydroxy-3,5-di-*tert*-butylphenyl)carboxy]-3,5-di-*tert*-butylphenyl]carbonyl]amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11d): IR (Nujol) 3626, 3359, 1763−1620 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.95 (12H, m), 1.25 (4H, m), 1.3 (18H, s), 1.4 (18H, s), 1.7 (4H, m), 2.0 (3H, m), 2.2 (3H, m), 4.2−4.5 (3H, m), 7.8 (2H, s), 7.95 (2H, s), 8.1 (4H, dd), 8.5 (2H, br s), 8.9 (2H, br s).

4-[[[4-(Ethoxymethoxy)-3,5-di-*tert*-butylcinnamoyl]amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11e): IR (Nujol) 3269, 1763, 1668−1624 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.25 (3H, t), 1.4 (18H, s), 1.7−1.9 (11H, m), 2.2 (2H, m), 3.8 (2H, q), 4.35−4.8 (4H, m), 4.9 (2H, s), 6.6−7.5 (2H, d), 7.5 (2H, s), 7.7 (1H, d), 8.1 (4H, dd), 8.8 (1H, d), 12.2 (1H, br s).

4-[[(4-Hydroxy-3,5-di-*tert*-butylcinnamoyl)amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11f): prepared according to General Method 1 from **11e**; IR (Nujol) 3627, 3276, 1763−1622 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.2−2.3 (13H, m), 1.4 (18H, s), 4.3−4.7 (4H, m), 6.5 (1H, d), 7.3 (2H, s), 7.5 (1H, d), 7.6 (1H, br s), 8.05 (4H, dd), 8.6−8.9 (1H, br s).

4-[[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]-amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11g): IR (Nujol) 3633, 3300, 1763, 1720, 1700, 1660, 1622, 1527 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.0−2.3 (13H, m), 1.80 (18H, s), 3.1 (1H, m), 3.5 (2H, s), 3.9 (3H, m), 4.7 (1H, t), 6.7 (1H, d), 7.0 (2H, s), 7.05 (1H, s), 8.0 (4H, m), 8.60 (1H, d).

4-[[[4-[2-[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]ethyl]benzoyl]amino]sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11h): IR (Nujol) 3633, 3500−3200, 1761, 1720−1650, 1350, 1159 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.2−1.7 (8H, m), 1.4 (18H, s), 1.8−2.3 (5H, m), 2.8 (2H, t), 3.1 (2H, t), 4.0−4.5 (3H, m), 4.3−4.7 (1H, 2m), 7.05 (1H, br s), 7.1 (2H, s), 7.15 (2H, s), 7.8 (2H, d), 7.9 (4H, d), 8.5−8.7 (1H, 2d).

3-[[[[(4-Hydroxy-3,5-di-*tert*-butylphenyl)thio]acetyl]-amino]sulfonyl]-4-chlorobenzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (11i): IR (Nujol) 3633, 3307, 1763−1620 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.3 (18H, s), 1.5−2.3 (12H, m), 3.6 (2H, s), 4.5 (4H, m), 7.0 (2H, s), 7.1 (1H, s), 7.8 (1H, d), 8.25 (1H, d), 8.6 (1H, d).

4-[[[(2-(*R*,*S*)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-yl)carbonyl]amino]sulfonyl]benzoyl-(*S*)-Val-(*S*)-Abo-(*R*,*S*)-Val-CF_3 (11j): IR (Nujol) 3700−3000, 1761, 1730−1626, 1354, 1150 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.8−1.0 (12H, m), 1.3−1.75 (8H, m), 1.35 (3H, s), 1.8−2.3 (6H, m), 1.9 (3H, s), 2.1 (6H, 2s), 2.4 (1H, m), 4.0−4.7 (4H, 4m), 6.6−6.9 and 7.1−7.4 (1H, m), 7.4 (1H, br s), 7.8−7.95 (4H, 2d), 8.3−8.6 (1H, 2d), 11.8 (1H, br s).

4-[[[(2,6-Dimethyl-3,5-dicarbethoxy-1,4-dihydropyridin-4-yl)carbonyl]amino]sulfonyl]benzoyl-(*S*)-Val-(*S*)-Abo-(*R*,*S*)-Val-CF_3 (11k): IR (Nujol) 3330, 1730−1650, 1350, 1122 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.8−1.0 (12H, n), 1.1 (6H, t), 1.2−1.7 (8H, m), 1.8−2.2 (3H, m), 2.25 (6H, s), 3.95 (4H, m), 4.0−4.4 (3H, m), 4.65 (1H, m), 4.85 (1H, s), 7.1 (2H, d), 7.3−7.5 (2H, m), 7.65 (2H, d), 7.80 (4H, m), 8.5 (1H, 2m), 8.75 (1H, s).

4-[[[[4-Chloro-3-[[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)-thio]acetyl]amino]sulfonyl]phenyl]carbonyl]amino]-sulfonyl]benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (14a): IR (Nujol) 3340, 1761−1622 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.9 (12H, m), 1.2−2.3 (8H, m), 1.3 (18H, s), 3.6 (2H, s), 4.4 (4H, m), 7.0 (2H, s), 7.1 (1H, s), 7.8 (1H, d), 8.1−8.2 (5H, m), 8.6 (1H, d).

4-[[[[4-Chloro-3-[[[(4-hydroxy-3,5-di-*tert*-butylphenyl)-acetyl]amino]sulfonyl]phenyl]carbonyl]amino]sulfonyl]-benzoyl-(*S*)-Val-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (14b): IR (Nujol) 3500−3000, 1763−1622, 1377, 1173 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.85 (12H, m), 1.1−2.2 (8H, m), 1.3 (18H, s), 1.75 (2H, dd), 2.15 (2H, m), 2.25 (1H, m), 3.45 (2H, s), 4.15−4.7 (4H, m), 6.8 (2H, s), 7.7 (1H, d), 8.0 (5H, m), 8.1 (1H, br s), 8.45 (1H, d), 8.8 (1H, br s), 12.8 (1H, br s).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-(benzyloxycarbonyl)-(*S*)-Lys-OCH_3, hydrochloride (16): was prepared according to General Method 3 and directly treated without any further purification.

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-(*S*)-Lys-OCH_3, Hydrochloride (17). A 1.2 g (0.0019 mol) sample of the hydrochloride obtained in the previous step was dissolved in 50 mL of acetic acid. A 33% HBr in acetic acid solution (50 mL) was added, and the reaction was continued for 4 h. After evaporation of the solvent under reduced pressure, the residue was taken up with 80 mL of ethyl acetate. The organic layer was washed with a saturated sodium bicarbonate solution to neutral pH and evaporated. Treatment of the residue with dry methanol to eliminate traces of inorganic salts was followed by column chromatography (CH_2Cl_2−CH_3OH, 60:40) to give 1 g of the deprotected lysine derivative, probably containing a small amount of inorganic salts: IR (Nujol) 3600−3163, 1736, 1645, 1333, 1132 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.2−1.85 (6H, m), 2.75 (2H, t), 3.6 (3H, s), 4.45 (1H, t), 7.2 (1H, br s), 7.8 (4 + 1H, 2d + br s), 7.9 (2H, d), 8.7 (1H, br s).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[(4-hydroxy-3,5-di-*tert*-butylphenyl)acetyl]-(*S*)-Lys-OCH_3 (18): prepared from carboxylic acid **6a** and lysine derivative **17** described above according to General Method 2 and directly treated without any further purification.

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[[(4-hydroxy-3,5-di-*tert*-butylphenyl)thio]butyroyl]-(*S*)-Lys-OCH_3 (21): IR (Nujol) 3636, 3329, 1740, 1639, 1339, 1129 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.3−1.8 (8H, m), 1.35 (18H, s), 2.2 (2H, t), 2.8 (2H, t), 3.05 (2H, m), 3.65 (3H, s), 4.4 (1H, t), 5.55 (1H, br s), 7.0 (1H, br s), 7.05 (2H, s), 7.45 (2H, d), 7.85 (4H, m), 7.95 (2H, d), 8.7 (1H, d).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[(4-hydroxy-3,5-di-*tert*-butylphenyl)acetyl]-(*S*)-Lys-OH (19): prepared according to General Method 5 and directly treated without any further purification.

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[(4-hydroxy-3,5-di-*tert*-butylphenyl)thio]butyroyl]-(*S*)-Lys-OH (22): IR (Nujol) 1705, 1647, 1541, 1350, 1169 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (18H, s), 1.40 (4H, m), 1.7 (2H, q), 1.8 (2H, m), 2.15 (2H, t), 2.75 (2H, t), 3.0 (2H, q), 4.35 (1H, m), 6.95 (1H, s), 7.05 (2H, s), 7.75 (2H, d), 7.95 (4H, s), 8.05 (2H, d), 8.70 (1H, d), 12.6 (1H, br s).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[(4-hydroxy-3,5-di-*tert*-butylphenyl)acetyl]-(*S*)-Lys-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (20): prepared according to General Method 2, using the intermediate dipeptide Phi-Val-CF_3 (**v-a**) described above; IR (Nujol) 3639, 3315, 1741, 1645 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.0−2.0 (16H, m), 1.1 (6H, d), 1.4 (18H, s), 2.3 (2H, m), 3.0 (2H, m), 3.2 (2H, s), 6.6 (1H, s), 6.9 (2H, s), 7.4 (2H, d), 8.0 (6H, m).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*N*-ϵ-[[(4-hydroxy-3,5-di-*tert*-butylphenyl)thio]butyroyl]-(*S*)-Lys-(2*S*,3a*S*,7a*S*)-Phi-(*R*,*S*)-Val-CF_3 (23): IR (Nujol) 3600−3000, 1639, 1541 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.7−1.0 (6H, m), 1.1−1.8 (19H, m), 1.35 (18H, s), 2.15 (3H, m), 2.75 (3H, m), 3.0 (2H, m), 4.0 (1H, m), 4.40 (1H, m), 4.55 (1H, m), 7.05 (2H, s), 7.50 (2H, d), 7.85 (4H, m), 7.92 (2H, d).

***N*-(*tert*-Butyloxycarbonyl)-*S*-(4-hydroxy-3,5-di-*tert*-butylphenyl)-(*S*)-Cys-OCH_3 (27).** A 8.2 g (0.039 mol) portion of α-[(*tert*-butyloxycarbonyl)amino]-β-chloropropionic acid (**26**) was reacted under nitrogen with thiophenol **xii** (11.9 g, 0.05 mol) and potassium carbonate (5.53 g, 0.04 mol) in dry acetonitrile (120 mL) at 65 °C for 20 h. After filtration and concentration under reduced pressure, the residue was dis-

solved in ethyl acetate. The organic layer was washed with water to neutral pH and then with brine. Drying ($CaSO_4$) and evaporation gave a crude solid, which was purified by column chromatography (cyclohexane−EtOAc, 90:10). The resulting solid was triturated with pentane to give 9.4 g (55%) of the title intermediate: IR (Nujol) 3600, 3336, 1732, 1695 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.4 (18H, s), 2.95−3.2 (2H, dd), 3.6 (3H, s), 4.1 (1H, m), 7.15 (2H, s), 7.3 (1H, d).

***S*-(4-Hydroxy-3,5-di-*tert*-butylphenyl)-(*S*)-Cys-OCH$_3$, hydrochloride (28):** prepared according to General Method 1; IR (Nujol) 3633, 3400−1980, 1751 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.4 (18H, s), 3.4 (3H, s), 3.6 (2H, m), 4.4 (1H, m), 5.3 (1H, s), 7.3 (2H, s).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*S*-(4-hydroxy-3,5-di-*tert*-butylphenyl)-(*S*)-Cys-OCH$_3$ (29): prepared according to General Method 3 and directly treated without any further purification.

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*S*-(4-hydroxy-3,5-di-*tert*-butylphenyl)-(*S*)-Cys-OH (30): prepared according to General Method 5; IR (Nujol) 3629, 3400−2500, 1707, 1653 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (18H, s), 3.1−3.4 (2H, m), 4.5 (1H, m), 7.1 (3H, m), 7.6−8.0 (8H, m), 8.8 (1H, d).

4-[[[(4-Chlorophenyl)sulfonyl]amino]carbonyl]benzoyl-*S*-(4-hydroxy-3,5-di-*tert*-butylphenyl)-(*S*)-Cys-(2*S*,3a*S*,-7a*S*)-Phi-(*R*,*S*)-Val-CF$_3$ (31): prepared according to General Method 2; IR (Nujol) 3650−2500, 1761, 1643, 1539 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.6−0.95 (6H, m), 1.1−2.5 (13H, m), 1.35 (18H, 2s), 3.10 (2H, m), 4.0 (1H, m), 3.35 (1H, m), 4.5 (1H, m), 7.15 (2H, m), 7.45 (2H, d), 7.85 (4H, m), 7.90 (2H, t).

Enzymatic Inhibition of HLE.[28] Inhibition of HLE was assayed spectrophotometrically at 37°C by continuous monitoring of the release of *p*-nitroaniline at 410 nm from the substrate methoxysuccinyl-Ala-Ala-Pro-Ala-*p*-nitroanilide. Results are listed in Tables 1 and 2 and are expressed in terms of IC_{50}.

Lipid Peroxidation Assay: (a) Hepatic Microsomes. Rat livers were homogenized in phosphate buffer (0.01 M, pH 7.4). After differential centrifugation, microsomal proteins were used at 0.5 mg/mL for the lipid peroxidation assay and incubated with $FeCl_3$ (100 μM) and ascorbate (10 μM) for 30 min.

Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) by mixing 200 μL of the sample with 2 mL of a 0.3% thiobarbituric acid solution in 15% trichloroacetic acid and 0.02% *tert*-butylhydroxytoluene. The mixture was kept at 95°C for 25 min, and the assessment of MDA−thiobarbituric acid complex was determined by measuring the absorbance of the solution at 532 nm.[29]

(b) Human LDL Oxidation. LDL were prepared from fresh normal human plasma by sequential ultracentrifugation according to Havel et al.[30] Oxidation was performed by incubating 0.2 mg of LDLwith 5 μM $CuSO_4$ in 1 mL of serum-free HamF10 medium for 24 h at 37°C with or without test products. The extent of lipid peroxidation was assessed by measuring the TBARS according to the same method as described for hepatic microsomes.

Elastase-Induced Hemorrhage in the Hamster Lung.[25] Ten male golden Syrian hamsters 10 weeks of age, weighing 100−120 g, were used in each group. Hamsters were anesthetized by ip injection of nembutal, and the trachea were surgically exposed. Drugs solubilized in 20% DMSO were injected intratracheally (it) at a dose of 15 nmol in a 0.1 mL volume. At different times (3, 18, 24, 48, and 72 h) purified human sputum elastase (Elastin Products Co., Owensvill, MO), 50 IU in 0.2 mL of saline, was administered directly into the trachea. Three hours later, animals were killed by an overdose of nembutal, and lung lavage was performed using a single 3 mL aliquot of 0.9% saline in a 5 mL syringe by gently expanding the lungs and then withdrawing the saline a total of five times, yielding a final volume of approximately 2.5 mL of BAL (bronchoalveolar lavage) fluid from each animal. Hemorrhage was quantified by the amount of blood in each BAL sample, determined by using the Boehringer Manheim Diagnostica kit for hemoglobin. Results are expressed as percent inhibition of pulmonary hemorrhage in treated animals as compared to control.

Fourteen-Day Emphysema Model.[27] Six male golden Syrian hamsters weighing 100−120 g were used in each group. Anesthesia and drug administration were performed as described above. Drugs were solubilized in water and injected it at a dose of 100 nmol in a 0.1 mL volume. Three hours later, purified pig pancreatic elastase (Elastin Products Co.), 60 IU in 0.2 mL of saline, was administered it. After 14 days, the animals were killed by administration of a nembutal overdose. Lungs were inflated with 4% neutral buffered formalin under a 22 cm formalin pressure. Lungs were embedded in paraffin (5 μm thick sections, stained with hematoxylineosin). Cross sections of the intact lungs were performed on each animal. Signs of pulmonary emphysema were estimated by measure of mean linear intercepts according to Dunnill.[31]

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