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Review Article

New phytoweapons to curb leukocyte elastase

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CONTENTS

Abstract

This review does not presume to be exhaustive in the field of leukocyte elastase inhibitors. Instead, a general picture will be sketched to give the reader a brief update and encourage advances in this continuously growing sector of pharmacology, drug discovery and drug design. Three compounds with which the authors have direct experimental experience will be considered in detail as examples of newly emerging therapeutics from the ever-generous plant kingdom.

Introduction

A number of inflammatory diseases show progressive modification of tissue architecture, eventually impairing organ function. In such pathologies, including cystic fibrosis, rheumatoid arthritis and emphysema, both serine proteases and metalloproteinases have been demonstrated to be instrumental in altering the extracellular matrix (1); one of the most aggressive serine types is leukocyte elastase (LE), which is released mainly upon stimulation by polymorphonuclear (PMN) leukocytes at the site of inflammation.

By preferential cleavage of Val-X bonds, and to a lesser extent the Ala-X bonds preferred by pancreatic elastase (PE), LE is able to cause preferential disruption of the elastic network, although natural substrates also include collagens I-IV, cartilage proteoglycans, fibronectin and other extracellular matrix and circulating molecules (2), and bacterial cell walls. LE can also activate a number of matrix metalloproteinases (MMPs) involved in angiogenesis and tumor invasion, and deactivate their tissue inhibitors (3). In addition to its proteolytic properties, LE acts as a potent secretagogue and may be proinflammatory.

In our search for inhibitors of the undesirable actions of LE in the human body, we have tested the polyphenol (–)-epigallocatechin-3-gallate (EGCG) contained in green tea, the prenylated acylphloroglucinol hyperforin from St. John's wort, and, in a hybrid drug design approach, we also sought to exploit the crucial moiety from EGCG, combining it with a β-lactam with good LE-inhibitory activity.

Chemical characteristics of LE

LE (EC 3.4.21.11, EC 3.4.4.7), also known as bone marrow serine protease, elaszym, granulocyte elastase, human neutrophil elastase (HNE), lysosomal elastase, polymorphonuclear elastase (PMNE) and serine elastase, is one of several hydrolytic enzymes contained in the azurophil granules of human neutrophils (Fig. 1). The molecule consists of a single basic polypeptide chain of 218 amino acid residues (29.5 kDa) (4), joined together by four disulfide bonds. It contains two asparagine-linked carbohydrate side-chains, and is synthesized as a series of isozymes each containing different amounts of carbohydrate (4). The isoforms do not differ in catalytic activity and are immunologically identical (2, 5). The *N*-terminal amino acid sequence is strongly homologous with that of PE (6), but there is only moderate sequence homology between the two enzymes; also, while PE is generated from the inactive precursor proelastase by tryptic cleavage of an *N*-terminal activation peptide, no such zymogen is known for LE (2).

The pI of LE ranges from 8.7 to 9.1, and its optimum pH is 8.5. Leukocyte cathepsin G stimulates the rate of solubilization of elastin by LE (7), and its activity is also enhanced by high salt concentrations and hydrophobic solvents (8).

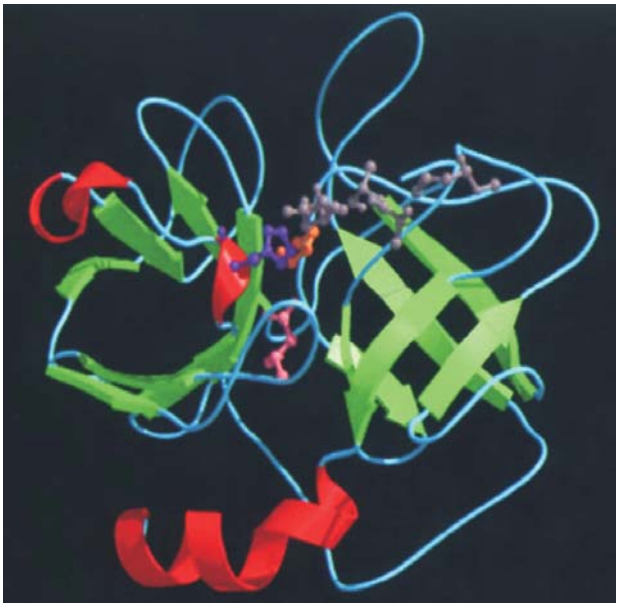

Fig. 1. Structure of leukocyte elastase.

Endogenous inhibitors

The enzymatic activity of LE is physiologically counterbalanced by endogenous serine protease inhibitors (Table I), *i.e.*, α_1-proteinase inhibitor (α_1-PI, also known as α_1-antitrypsin) (9), α_2-macroglobulin (10), elafin (11) (Fig. 2) and secretory leukoproteinase inhibitor (12). An enzyme/inhibitor imbalance may lead directly to increased lysis of extracellular matrix macromolecules and an increased risk of tissue injury in the immediate vicinity of activated neutrophils (13). In particular, α_1-PI deficiency is the most prevalent, potentially fatal hereditary disease in caucasians and an important risk factor for pulmonary emphysema (14). However, the elastase burden may also be elevated as a result of increased leukocyte recruitment to the lung induced by viral or bacterial pathogens. Also, there may be a functional deficiency of inhibitor(s) due to inactivation in the lung by oxidation (from cigarette smoke or oxygen radicals released from inflammatory leukocytes) (15).

Exogenous inhibitors

Exogenous elastase inhibitors would be first-choice drugs for treating many cases of inflammation, and airways inflammation in particular, and direct α_1-PI replacement is one potential therapeutic approach currently under investigation.

Very few LE inhibitors routinely used in biochemical research are suitable for use in humans, due to their limited solubility, stability, bioavailability or general toxicity. However, the search for new inhibitors has led to the design of new molecules. A number of heterocyclic inhibitors quite specific for LE have been developed, with K_i values in the range of 0.1-10 μM (16). Other recently synthesized inhibitors include sivelestat (Fig. 3), a compound reported to dramatically reduce mortality in patients with acute lung damage, although the extent of its effects has yet to be verified (17). Peptide chloromethylketones are also effective inhibitors in animal models of emphysema, and have served as comparison standards for newly developed inhibitors, although they present side effects that render them unsuitable for therapeutic use in humans (18).

However, the search for synthetic drugs has very often taken the lead from compounds in the natural world (*e.g.,* podophyllotoxin, paclitaxel, corticosteroids). Indeed, mother nature offers a variety of alternatives from both the animal and plant kingdoms, as discussed below.

Inhibitors from animal sources

One example is a new LE inhibitor, guamerin, a low-molecular-weight, cysteine-rich polypeptide isolated from the Korean leech *Hyrudo nipponia* and which is stable over a wide range of pH, from 1 to 11, and up to 90 °C. It has been demonstrated to inhibit LE quite efficiently (19-21). Nevertheless, the use of the recombinant compound requires further investigation, since it shows a paradoxical effect *in vivo*; in wounds exposed to infiltrating microorganisms it hampers healing by interfering with other innate defensive factors and increasing the influx of PMNs to the inflamed wound site, while in fibrin-enclosed wounds it accelerates healing by inhibiting inflammation-generated proteases and acute inflammatory reactions.

Other peptidic LE inhibitors of animal origin were identified very recently in a mollusk (the gasteropod *Cenchritis muricatus*), with K_i values below 2 nM for the most potent. The specificity of these compounds (denoted CmPI-I, -II and -III) is not strict, since they also inhibit trypsin and pancreatic elastase (22). Their biological fate and *in vivo* effects have yet to be investigated.

Inhibitors from plant sources

Plant secondary metabolites, which play an important role in the interaction and adaptation of plants to their environment, also represent a rich source of novel compounds for the pharmaceutical industry. Protease inhibitors, for example, are widely distributed in reproductive, storage and vegetative tissues of most plant species (23, 24), where they have regulatory and defensive roles and act as storage proteins (25). Of the various groups of inhibitors, the serine protease inhibitors are the most extensively studied. Originally isolated from leguminous seeds (26-29), they have been shown to control the abnormal secretion of endogenous proteases characteristic of a number of diseases (30), including those mentioned above.

One of the most recently identified inhibitors is a 12-15-kDa proteinaceous compound known as PG-50, extracted from the seeds of the Tamarind tree (*Tamarindus indica* L. Leguminosae), with selective inhibitory activities against human LE and bovine trypsin. This compound preferentially affects elastase release, indicating selective inhibition of the receptors involved in

Table I: LE inhibitors mentioned in the text.

		Source	Ref.
Endogenous inhibitors			
	α_1-Proteinase inhibitor (α_1-PI)	Various tissues	9
	α_2-Macroglobulin (α_1-MG)	Hepatocytes	10
	Aprotinin	Lung	51
	Elafin	Epithelia	11
	Secretory leukoproteinase inhibitor (SLPI)	Parotid	12
Exogenous inhibitors			
Synthetic	Cephalosporins	(First from microorganism)	48
	Heterocyclic inhibitors		16
	N-Galloyl-4-alkylidene β-lactam	(First from microorganism)	82
	Peptide chloromethylketones		18
	Phenylmethylsulfonylfluoride (PMSF)		52
	Sivelestat		17
	Trifluoromethylketones		49
Animals	CmPI-I, CmPI-II, CmPI –III	*Cenchritis muricatus*	22
	Elastinal	Actinomycetes	47
	Guamerin	*Hyrudo nipponia*	19
	Ovomucoid	Egg white	50
Plants	(–)-Epigallocatechin-3-gallate (EGCG)	*Camelia sinensis*	35
	Fucoidan	*Ascophyllum nodosum*	32
	Hyperforin	*Hypericum perforatum*	63
	Hyperoside	*Davidia involucrata*	33
	Proteinaceous compound (PG50)	*Tamarindus indica*	31

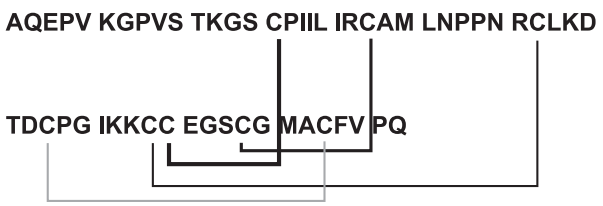

Fig. 2. Structure of elafin.

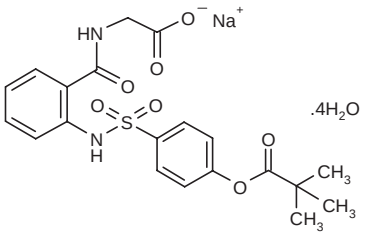

Fig. 3. Structure of sivelestat.

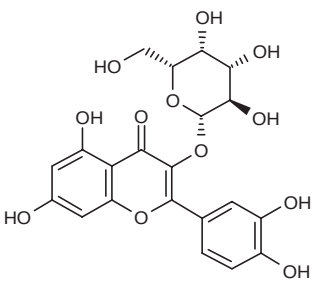

Fig. 4. Structure of hyperoside.

PMN activation (31); it shows an IC_{50} of 4 μM, but its efficacy *in vivo* has yet to be demonstrated.

The 16-kDa sulfated fucosylated polymer fucoidan, from the brown algae *Ascophyllum nodosum,* is a potent modulator of connective tissue proteolysis. It inhibits gelatinase (MMP-2), as well as LE, resulting in protection of the human skin elastic fiber network against enzymatic proteolysis *ex vivo* (32). Topical application is likely to be effective, but its bioavailability after systemic administration has yet to be demonstrated.

Of the polyphenol class (over 5,000 members) and derivatives, those contained in common foods, beverages or edible extracts may be responsible for some of the beneficial effects that fruit/vegetable-rich diets are claimed to exert in a number of pathological conditions where LE is involved, including inflammation and cardiovascular diseases. After examining 40 phenolic compounds, a German team found that those with a catecholic moiety showed particularly strong inhibition of LE activity, the strongest being hyperoside (Fig. 4), with an IC_{50} of about 0.3 μM (33); however, we are not aware of any subsequent investigations testing the efficacy of this compound *in vivo*.

Extensive studies by our team on the two compounds described below —EGCG (with a catecholic structure) and hyperforin (with a fluoroglucinol structure)— have confirmed their beneficial effects in *in vivo* model systems.

1. (–)-Epigallocatechin-3-gallate (EGCG)

In 1999, good inhibition of metalloproteinases was reported for noncytotoxic concentrations of some natural polyphenol compounds (34). One abundant source is green tea (*Camelia sinensis* Kuntze [Theaceae]) (35), whose polyphenols are mainly flavanols, or "catechins". (–)-Epigallocatechin-3-gallate (EGCG) (Fig. 5) is the most prevalent polyphenol in this plant, and after moderate green tea consumption plasma concentrations reach 0.1-0.3 μM (36). These levels, as confirmed by recent *in vivo* experiments, are sufficient to lower body and liver fat accumulation (37) and inhibit angiogenesis (38) and tumor cell invasion *in vitro* (39-42). In particular, EGCG is a direct inhibitor of MMP-2 and MMP-9, the two gelatinases most frequently overexpressed in cancer and angiogenesis, and instrumental in cutting through basement membrane barriers (34, 43, 44) and degrading extracellular matrix proteins in human pulmonary emphysema (45).

In 2002, our group showed that the readily achievable plasma concentrations of EGCG mentioned above (35) inhibit the activity of human LE in a concentration-dependent, noncompetitive manner, with K_i values below 0.4 μM (46). As evident from the IC_{50} values, this catechin is only 30 times less potent than α_1-PI, the endogenous inhibitor most involved in balancing the elastolytic burst in inflamed lungs (13, 14). On the other hand, EGCG exerts superior inhibition compared to a variety of natural and synthetic inhibitors; its IC_{50} is 1/40th that reported for the microbial elastase-like protease inhibitor elastatinal (47) (Fig. 6) and 1/50th-1/200th that of some substituted cephalosporins, β-lactams and trifluoromethylketones, the latter recently proposed for the treatment of diseases characterized by the involvement of PMNs and LE (48, 49). The inhibition is much greater than that exerted by certain standard class-specific serine protease inhibitors, *i.e.*, ovomucoid (50), aprotinin (51) and PMSF (52), which are also moderately active against LE (53, 54), and is constistently maintained over a 2-h period, suggesting a durable effect at body temperature.

Comparative studies on a cohort of flavonoids showed the presence of the galloyl residue to be crucial for the anti-LE potency (55); this was exploited to create a new galloyl/β-lactam hybrid (see below).

EGCG is also effective in PMN cultures. Noncytotoxic concentrations inhibit the elastolytic activity of freshly isolated PMNs in a concentration-dependent manner, although less efficiently than in biochemical assays (46). Most of the elastolytic activity expressed by PMNs is attributable to serine proteases, and since of the two serine proteases released predominantly by neutrophils, cathepsin G is comparatively insensitive to EGCG (1/2500th), the prevalent serine protease inhibited by micromolar concentrations of flavanol should be LE. It is worth mentioning that the differential effect of EGCG on the two PMN serine proteases may prove useful in clinical application, considering the need to preserve a number of cathepsin G-mediated reactions (coagulation, immune response, wound debridement, etc.) (3).

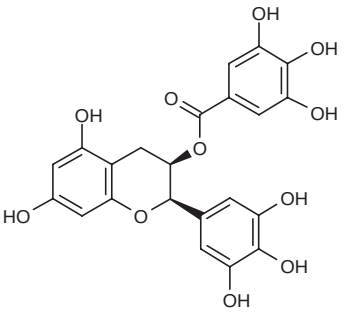

Fig. 5. Structure of EGCG.

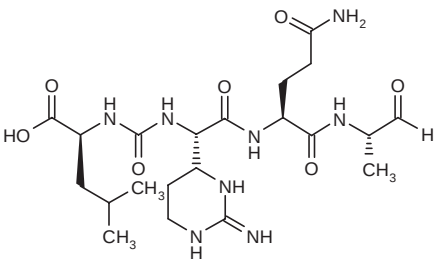

Fig. 6. Structure of elastatinal.

In addition to LE, the abundant MMP-9 secreted by neutrophils may also be partly responsible for the elastolytic metalloproteinase activity. Regardless of the respective contribution to elastin degradation, both MMP-9 and LE are extracellularly blocked by the catechin, which thus has the potential to contain the degradative neutrophil activity in an *in vivo* context in the case of endogenous inhibitor failure. In fact, EGCG has been reported to inhibit MMP-2 and MMP-9 (43, 44), the activity of which is necessary for endothelial and tumor cells to cut through extracellular matrix barriers during angiogenic and invasive/metastatic processes and pulmonary emphysema (45). We reported that the IC_{50} of EGCG for these MMPs lies in the range 10-30 μM (44), while the inhibition of LE is 50 times stronger (0.4 μM) (46). This inhibition occurs at concentrations approximately 25-fold and 2 orders of magnitude lower than the cytotoxic threshold already reported for transformed and normal endothelial cells, respectively (44), and similar to those in the plasma of moderate green tea drinkers (0.1-0.3 μM) (35).

When purified LE is incubated with pro-MMP-9 in a cell-free system, the LE-triggered conversion of this zymogen to its activated form is substantially inhibited in the presence of EGCG (46). In addition, the activation of pro-MMP-9 secreted by freshly isolated PMNs is inhibited to some extent when they are briefly cultured in the presence of the catechin. This reduced activation may play an important role in both airways inflammation and tumors; it could contribute to downregulation of local proinflammatory IL-1β activity (56), inhibition of TGF-β-induced tumor cell invasion and angiogenesis (57), preservation of the most potent inhibitor of LE (58) and inhibition of neutrophil

recruitment by chemoattractant fragments of α_1-PI (59), preservation of the underlying elastin structure of the lung (45, 60), and containment of degradation of the bone marrow molecular scaffold, a prerequisite for angiogenesis (38) and tumor cell invasion. As regards the latter, a direct lytic effect of LE on collagen IV (3) must also be considered as a potential contributor to the documented inhibition of tumor invasive processes (34, 38).

Although the precise mechanism of EGCG's action on LE has not yet been elucidated, mainly due to technical problems in obtaining crystals for X-ray diffraction analysis, recent *ex vivo* and *in vivo* experiments have provided clues. These include the demonstration that micromolar concentrations of EGCG repress reactive oxygen species and inhibit apoptosis of activated neutrophils, and dramatically inhibit chemokine-induced neutrophil chemotaxis *in vitro*; moreover, both oral EGCG and green tea extract (GTE) block neutrophil-mediated angiogenesis *in vivo* in an inflammatory angiogenesis model, and oral administration of GTE enhances resolution in a pulmonary inflammation model, significantly reducing the consequent fibrosis (61). These results provide molecular and cellular insights into the purported beneficial properties of green tea, and indicate that EGCG is a potent anti-inflammatory compound with therapeutic potential.

2. Hyperforin

While a full assessment of the clinical potential of EGCG is not yet possible and must await clarification of issues such as absorption, bioavailability and metabolic fate (62), the pharmacokinetics of hyperforin have been thoroughly investigated. This prenylated acylphloroglucinol is contained in the extract of St. John's wort (*Hypericum perforatum* L. Guttiferae), and its use has its origins in traditional Western medicine well before the 1600s (Fig. 7). The extracts are a rich source of unique natural products, the prenylated acylphloroglucinol hyperforin having emerged as key for the antidepressant activity (63); the compound is orally available, with therapeutic antidepressant doses of St. John's wort extract (3 x 300 mg/day) providing serum levels of hyperforin of up to 0.45 μM (64).

Hyperforin was first discovered in the sixties to have remarkable antibacterial activity against several Gram–positive bacteria, including methicillin-resistant *Staphylococcus aureus* (65), and it was later shown to be able to exert a concentration-dependent antiproliferative effect *in vitro* in PHA-stimulated peripheral blood lymphocytes (66) and in several tumor cell lines. Inhibition of proliferation has also been reported *in vivo*, and the cytotoxic effect of hyperforin on human malignant cells correlated with the induction of caspase-driven apoptosis (67).

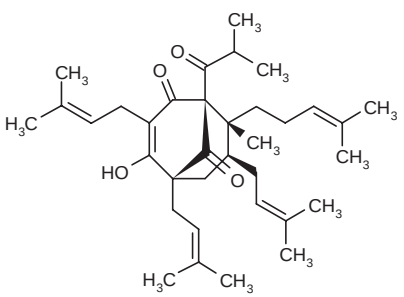

Fig. 7. Structure of hyperforin.

Encouraged by these findings, we were spurred to investigate whether hyperforin could also be useful in an oncological context, and found that it exerts antiinvasive and antimetastatic properties predictive of clinical activity. Hyperforin inhibited in a concentration-dependent, non-competitive manner various proteinases involved in extracellular matrix degradation; the greatest activity was seen against LE (IC_{50} = 3 μM), followed by cathepsin G and urokinase-type plasminogen activator (u-PA), while little activity was seen against the metalloproteinases MMP-2 and MMP-9 (IC_{50} > 100 μM) (68).

Although the precise mechanism of action of hyperforin on LE has not yet been investigated, marked inhibition of tumor cell chemoinvasion through reconstituted basement membrane was observed at these concentrations, and in mice injected i.v. with transformed cells, daily i.p. administration of hyperforin at doses below the antitumor blood levels markedly reduced inflammatory infiltration, neovascularization, lung weight and the number and size of experimental metastases, while being devoid of apparent toxicity (68).

We then tested whether hyperforin might also effectively inhibit both PMN recruitment and subsequent detrimental tissue responses. The results showed that, while it had no effect *in vitro* on human PMN viability and chemokine receptor expression, hyperforin concentration-dependently inhibited PMN chemotaxis (induced through gelatin) and chemoinvasion (induced through reconstituted basement membrane), with an IC_{50} 1 μM for both effects (69); this was associated with a reduced expression of the adhesion molecule CD11b by fMLP-stimulated neutrophils, blockade of LE-triggered activation of MMP-9 (instrumental to their extravasation) (70) and upregulation of ERK1/2 activation. PMN-triggered angiogenesis in an IL-8-induced murine model was also blocked by both local injection and daily i.p. administration of hyperforin; when administered i.p., hyperforin also induced improvement in a bleomycin-induced pulmonary inflammation model, significantly reducing consequent fibrosis (69).

These results indicate that hyperforin is a potent anti-inflammatory compound with therapeutic potential for preventing and combating inflammation-triggered cancer onset, spread and metastatic growth.

3. Phytomoieties for drug design

A comparative study of several flavonoids for their effectiveness in inhibiting LE activity led to the identification of the chemical moieties underpinning the anti-MMP and anti-LE activity (55). This resulted in the design of

new molecules combining the galloyl moiety of the best LE inhibitor (EGCG) with β-lactams, compounds with better bioavailability, widely used as drugs in humans and extensively studied for use as LE inhibitors.

β-Lactams, initially developed and employed as antimicrobial agents, have recently been used as inhibitors of certain serine enzymes produced by viruses, fungi and mammals. Biological activity of β-lactams has been demonstrated against human LE, cytomegalovirus protein, prostate-specific antigen (PSA), thrombin, herpesvirus, co-enzyme A-independent transacylase, γ-aminobutyric acid (GABA) aminotransferase and cytosolic phospholipase A_2 ($cPLA_2$) (71). As regards monocyclic β-lactam derivatives, some structural features have been recognized as crucial for inhibitory activity (72); however, the possibility of activating the azetidinone ring through unsaturated systems directly linked to the β-lactam ring has been investigated very little (73-75).

In their search for biologically active β-lactams (76-80), an organic chemistry team collaborating with our group succeeded in synthesizing a series of 4-alkylidene β-lactams exhibiting activity against LE, as well as the gelatinases MMP-2 and MMP-9, at micromolar concentrations (81). We demonstrated that C-4 insaturation on the β-lactam ring determines the degree of biological activity, with selectivity for LE shown by 3-[1-(*tert*-butyldimethylsilyloxy)ethyl] derivatives (lowest IC_{50} = 4 μM). The compounds tested showed no cytotoxicity against NIH/3T3 murine fibroblasts.

Following this initial work, a series of compounds combining the β-lactam with the galloyl moiety were prepared and evaluated for inhibition of human LE. The most potent compound against LE was an *N*-galloyl-4-alkylidene β-lactam, [3-[1-(*tert*-butyldimethylsilanyloxy)ethyl]-4-oxo-1-(3,4,5-trisbenzyloxybenzoyl)azetidin-2-ylidene]acetic acid ethyl ester (Fig. 8), with an IC_{50} of 0.5 μM, 1 order of magnitude lower than the original β-lactam (82).

This compound, which exerts marked inhibition of LE activity but weak inhibition of cathepsin G, protease-3, MMP-2 and MMP-9, efficiently inhibits the extravasation of PMNs (IC_{50} 1-2 μM) by blocking the LE-triggered activation of pro-MMP-9, without affecting chemotactic response and viability (83). Daily i.p. injection of the compound enhances resolution in a pulmonary inflammation model, significantly reducing consequent fibrosis. These initial results indicate that the new β-lactam could be a potent antiinflammatory compound with therapeutic potential.

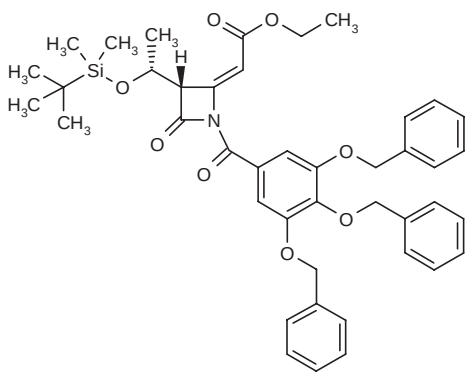

Fig. 8. Structure of an *N*-galloyl-4-alkylidene β-lactam.

Conclusions

This brief overview concentrates on just a few antielastolytic compounds in Nature's treasure trove, although research has only just begun to exploit the riches. This snapshot of these intriguing resources should encourage efforts to unravel the mechanisms of action at the biochemical and biological level, test their efficacy in other pathological model systems and design new hybrid compounds combining molecules showing good stability and bioavailability with phytomoieties identified as crucial by comparative analyses.

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