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Towards the first class of suicide inhibitors of kallikreins involved in skin diseases

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ABSTRACT: The inhibition of kallikreins 5 and 7, and possibly kallikrein 14 and matriptase, (that initiates the kallikrein proteolytic cascade) constitutes an innovative way to treat some skin diseases such as Netherton syndrome. We present here the inhibitory properties of coumarin-3-carboxylate derivatives against these enzymes. Our small collection of these versatile organic compounds was enriched by newly synthesized derivatives in order to obtain molecules selective against one, two, three enzymes or acting on the four ones. We evidenced a series of compounds with IC_{50} values in the nanomolar range. A suicide mechanism was observed against kallikrein 7 whereas the inactivation was either definitive (suicide type) or transient for kallikreins 5 and 14, and matriptase. Most of these potent inhibitors were devoid of cytotoxicity towards healthy human keratinocytes. *In situ* zymography investigations on skin sections from human kallikrein 5 transgenic mouse revealed significant reduction of the global proteolytic activity by several compounds.

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INTRODUCTION

Kallikrein-related peptidases (KLKs) constitute a family of fifteen mammalian serine proteases.[1] A variety of endogenous inhibitors that control their activities have been identified. Failure in their inhibitory capacities is implicated in several pathologies.[2, 3] One of the most relevant examples is Netherton syndrome (NS), a rare autosomal recessive skin disease due to the absence of the lympho-epithelial Kazal type related inhibitor (LEKTI-1) expressed in stratified epithelia.[4, 5] Loss-of-function mutations of *SPINK5* encoding LEKTI-1 result in unopposed KLK5 activity. KLK5 is a key player of the epidermal proteolytic cascade that also implicates KLK7 and KLK14. This unopposed proteolytic activity has profound effects on skin barrier integrity and permeability and also induces skin inflammation and allergy compromising the vital forecast of the newborn.[3] In fact, epidermal proteases show a high degree of cooperation leading to the activation of the kallikrein activation cascade, KLK5 activating pro-KLK7 and pro-KLK14, pro-KLK5 being activated by KLK14 and itself, and matriptase being required for the activation of KLK5 and KLK7.[2, 6] Recently, mesotrypsin has also been shown to activate pro-KLK5 and pro-KLK7 and to degrade LEKTI inhibitory fragments.[7] Protease-inhibitor imbalances can also be involved in some forms of psoriasis and in common eczema.[8, 9] Specific inhibitors of the implicated proteases having appropriate pharmacokinetic and pharmacodynamic properties may potentially be of great interest to develop new treatments of these conditions.

Currently, there are only symptomatic treatments for NS which comprise the use of emollients, topical steroids and calcineurin inhibitors to prevent severe dehydration and to reduce skin inflammation and allergy.[3, 10] In severe cases, intravenous immunoglobulins and anti-TNF alpha treatment were shown to bring clinical benefit.[11, 12] The inhibition of the KLKs by synthetic inhibitors may constitute an innovative way to treat NS by potentially restoring the functions of the epidermis barrier and preventing deleterious immunological

responses since these enzymes play a critical role in corneodesmosome degradation and skin inflammation. Relatively few inhibitors of KLKs and matriptase have been described, especially those acting through the formation of covalent complexes with the targeted enzymes. Peptidic inhibitors have been derived from LEKTI[13] and sunflower trypsin inhibitor (SFTI-1)[14] as KLKs inhibitors; others were obtained by drug design approach.[15] In addition to isocoumarins from Brazilian plants[16] and isomannide derivatives[17, 18], acyl-1,2,4-triazoles were described as transient inactivators of human KLK5, KLK7, KLK14 and also matriptase.[19] *In silico* screening of a collection of organic compounds led to the identification of reversible and structurally diverse inhibitors of these enzymes.[20] Several inhibitors of matriptase have been described based for example on macromolecular inhibitors [21-24] or on a structure-based approach.[25]

Our former studies on coumarin-3-carboxylic acid derivatives as potent inhibitors of some serine proteases[26-28] prompted us to obtain new families of non-peptidic inhibitors susceptible to selectively target KLKs. This study was also extended to matriptase, an activator of pro-KLK5 that is considered as an initiator of the KLK cascades in the skin.[6]

Coumarin derivatives display diverse pharmacological activities supporting their potential therapeutic[29] use as antitumor[30], antibacterial[31], anti-HIV[32-34], anti-inflammatory[35, 36] and antioxidant agents.[37] 6-Substituted coumarin-3-carboxylate derivatives constitute remarkable nonpeptidic protease inhibitors characterized by an easy access to organic synthesis, a high inhibitory potency and an extreme sensitivity to structural variations at the 3- and 6-positions, that not only influence the specificity but also the inhibition mechanism of the targeted enzyme.[26, 28] The general structure of our collection of previously synthesized as well as newly synthesized coumarin derivatives is presented in Figure 1. Most compounds are aromatic esters, thioesters or amides of 6-substituted coumarin-3-carboxylic acids, thus having an electron-withdrawing group at the 3-position, which increases the electrophilicity

of the conjugated lactonic carbonyl group, facilitating its nucleophilic attack by the hydroxy group of the protease active serine (Ser195) (Figure 2).[38] This attack leads to the formation of an acyl-enzyme. If the substituent at the 6-position is a methylene radical bearing a good leaving group (i.e. -CH_2W), a methylene quinone intermediate may be formed displaying a very high sensitivity to nucleophilic addition. The alkylation of a nucleophilic active-site residue (histidine 57 in the case of α-chymotrypsin)[28] leads to definitive inactivation of the enzyme (suicide mechanism; pathway a, Figure 2). A selective and very efficient inactivation of chymotrypsin by 6-chloromethylated coumarin-3-carboxylate aryl esters *via* this mechanism was observed[27] with no or poor inhibition of human leukocyte elastase and thrombin but a major difference between chymotrypsin and HLE: the inactivation of HLE was transient (stable acyl-enzyme; pathway b, Figure 2). Moreover, this transient inhibition of HLE was demonstrated to be specific when the latent alkylating function CH_2-W was absent.[26]

Thus, coumarin-3-carboxylic acid aryl esters appear as time-dependent versatile general inhibitors of serine proteases able to discriminate between different classes of serine proteases, depending on the nature of the substituents, through two major distinct mechanisms: suicide substrates (chymotrypsin for example) or alternate substrates inhibitors (HLE for example). Owing to the renewal of interest for covalent drugs[39-41], these coumarin derivatives appeared as good candidates to obtain for the first time suicide substrates of KLKs and matriptase. We present here the screening of our collection of coumarin-3-carboxylic acid derivatives against human KLK5, KLK7, KLK14 and matriptase and their chemical optimization. The mechanism of enzyme inhibition and the toxicity of a selection of compounds with respect to healthy keratinocytes were analyzed. In addition *in situ* zymography experiments were performed on skin-specific human KLK5 transgenic mouse model as this model replicates the major cutaneous and systemic hallmarks of NS.[42]

RESULTS AND DISCUSSION

Synthesis. The synthesis of several tested compounds was previously described.[26-28] New 2-oxo-2*H*-1-benzopyran-3-carboxylic acid derivatives bearing a fluoromethyl or a bromomethyl group at the 6-position were obtained according to the chemical pathway described in Scheme 1. The reaction of the appropriate di(halo)phenyl ester of malonic acid **2** with 5-hydroxymethylsalicylaldehyde[27, 43] in the conditions of the Knoevenagel reaction provided the (halo)phenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylates **3**. The latter 6-(hydroxymethyl)-substituted intermediates were converted into the final 6-(fluoromethyl)- (**4a**) or 6-(bromomethyl)-substituted (halo)phenyl 2-oxo-2*H*-1-benzopyran-3-carboxylates (**4b-e**) after reaction with the Ishikawa's reagent (**56**) (fluorination) or with thionyl bromide (bromination). The naphthyl esters of 2-oxo-2*H*-1-benzopyran-3-carboxylic acid **6** and **8** were obtained after reaction of the appropriate dinaphthyl malonate (**5** and **7**, respectively) with salicylaldehyde in the conditions of the Knoevenagel reaction (Scheme 2). The isoquinol-1-yl ester of 2-oxo-2*H*-1-benzopyran-3-carboxylic acid (**56**) resulted from the reaction of 1-hydroxyisoquinoline with the acid chloride of the commercially available 2-oxo-2*H*-1-benzopyran-3-carboxylic acid. Other unpublished monosubstituted phenyl esters of 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (compounds **18**, **19**, **28**, **29**, **30**, **33**) were synthesized from the reaction of 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carbonyl chloride with the appropriate phenol according to a previously described process.[27]

Enzyme inhibition, structure-activity relationships and selectivity. Before testing the coumarin-3-carboxylic acid derivatives against the targeted enzymes, their stability in aqueous medium was evaluated in the absence of enzyme at pH 7.5 or 8.0 by following their UV spectrum evolution. Some compounds were stable showing no spectral evolution within 4 h such as compounds **15**, **17**, **23**, **31, 56** and **4d**. According to the chemical structure, different half-life times were observed: for example, > 100 min (**11**, **24**, **25**), ranging from 40 to 50 min

(**21**, **45**, **46**) or from 25 to 30 min (**53-55**); some nitro-substituted compounds being less stable ($t^{1/2}$ = 10 min for **51**). The *in vitro* inhibitory potency of the collection of coumarin-3-carboxylic acid derivatives towards human KLK5, KLK7, KLK14 and matriptase was examined by measuring the hydrolysis of the appropriate fluorogenic substrate at 37 °C and pH 8. When an inhibition was observed, the IC_{50} values (inhibitor concentrations giving 50 % inhibition) were determined after 15 min incubation of the tested enzyme with various inhibitor concentrations (Figure 2S). The results are summarized in Tables 1-5. Several compounds belonging to different chemical series (46 out of 260) were found to be active against at least one of the tested enzymes.

Active and potent compounds against KLKs and matriptase were found amongst aryl esters (Z = O) and aryl thioesters (Z = S) of 6-(halomethyl)-substituted 2-oxo-2*H*-1-benzopyran-3-carboxylic acids (i.e. **11**, **4d** and **14**), whereas the corresponding amides such as **13** were less efficient inhibitors (Table 1). Looking at the nature of the substituent at the 6-position of the coumarin ring, it was evidenced that the presence of a latent alkylating function with a good leaving group (X = CH_2Cl or CH_2Br) was required to observe noticeable inhibitions of the four tested enzymes (**4d**), or only (**11**) and more specifically (**14**) KLK7. Comparatively, the fluoromethyl-substituted compound (**4a**) almost devoid of an alkylating character was found to be inefficient, while the presence of the poor acetate leaving group induced modest inhibitory activity on KLKs (**12** compared to **11** and **4d**). In the absence of leaving group (X = H or CH_3), the inhibitory potency of esters was lost. As a result, the rank order of potency on KLK7 was found to be directly correlated to the potential alkylating character of the substituent at the 6-position (-CH_2Br > -CH_2Cl > -CH_2OCOCH_3 > -CH_2F).

On the other hand, cycloalkyl (cyclododecyl; X = CH_2Cl) ester[28] and amide[28] derived from 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid were found inactive on the selected KLKs and matriptase. It was also the case for the corresponding methyl[27] and allyl[27]

amides. Methyl[27] and ethyl[27] esters (X = CH_2Cl) were poorly active: IC_{50} = 54 ± 2 μM against KLK5 and 27 ± 2 μM against KLK7 for the methyl compound; IC_{50} = 21 ± 1 μM against KLK7 for the ethyl compound. No inhibition was observed against KLK14 and matriptase for both compounds, and against KLK5 for the allyl one. We previously found that the most potent inhibitors of α-chymotrypsin and human leucocyte elastase possessed a phenyl or a pyridyl ester substituent at the 3-position, whereas alkyl esters were inactive.[26, 27] The same structural feature seems to hold against the tested kallikreins and matriptase. Consequently, we focused our attention on series of phenyl (Table 2), pyrid-2-yl and pyrid-3-yl (Table 3) esters bearing a chloromethyl or a bromomethyl group at the 6-position, and bicyclic naphtyl or isoquinolyl esters devoid of substituent at this position (Table 4). Noticeable differences in the inhibitory potency against the various targeted enzymes were obtained according to the nature and position of substituents on the aromatic ring.

We observed that the chymoptryptic serine protease KLK7 was more efficiently inhibited by the 6-chloromethyl phenyl esters than the tryptic ones (KLK5, KLK14 and matriptase) (Table 2) with IC_{50} values ranging from 63 nM to 8.4 μM. A single halogen atom located in the *ortho* position (**16** for Cl, **17** for I) was less favorable to inhibit KLK7 than in the *meta* (**11** for Cl, **23** for I) or *para* (**31** for Cl) positions. The strongest KLK7 inhibition was observed with Br (IC_{50} = 65 ± 3 nM for **22**) and I (IC_{50} = 77 ± 3 nM for **23**) in the *meta* position, the corresponding chloro and fluoro compounds being less efficient by a factor of ≈1.4 and 3.6, respectively (rank order of potency: Br ≥ I > Cl > F). For the bulkier methoxycarbonyl substituent (ester function), the *ortho* position (**19**) was more favorable than the *para* one (**33**). The monosubstitution by other kinds of substituents (CH_3, CF_3, $CONH_2$, COOH, NO_2 and $N(CH_3)_2$ groups) was usually less favorable.

The presence of a second substituent on the phenyl ring of the chloromethyl derivatives did not significantly improve the inhibitory potency towards KLK7 (**34**, **43**).

However, this activity was increased with the 2,3-dichloro-substituted derivative **34** (R_1 = 2'-Cl; R_2 = 3'-Cl) with IC_{50} = 63 ± 4 nM compared to the activity of the respective 2-chloro- and 3-chloro-substituted analogues (IC_{50} values were 209 ± 7 nM and 103 ± 5 nM for the monosubstituted **16** (R_1 = 2'-Cl) and **11** (R_1 = 3'-Cl), respectively). As a result, chlorine atoms at the 2,3-positions (*ortho* and *meta* positions) appeared to be the best substituent combination in order to induce a marked inhibitory activity on KLK7. Interestingly, the presence of chlorine atoms at the 2,5-positions (also *ortho* and *meta* positions) (**42**) was responsible for a noticeable inhibitory activity on KLK5 (1.3 ± 0.1 μM) and KLK7 (0.19 ± 0.02 μM) with a moderate activity on KLK14 (54 ± 6 μM). A second substituent different from a halogen atom was tolerated, especially when the first one was a halogen atom.

KLK5 was inhibited by a few 6-halomethyl-substituted compounds with IC_{50} values ranging from 0.92 μM (**4d**, X = CH_2Br; R_1 = 3'-Cl) to about 80 μM (**42**, X = CH_2Cl; R_1 = 3'-Cl, R_2 = 5'-Cl). Some of these compounds were also found to be active on KLK14, but usually with a lower efficiency, the most potent one on this enzyme being compound **4d** with an IC_{50} value of 2.9 ± 0.1 μM.

Only three compounds were able to inhibit the four tested enzymes in the micromolar range: the 3'-$CONH_2$-substituted 6-chloromethylcoumarin-3-carboxylate phenyl ester **30** (IC_{50} values: 21.6 μM on KLK5; 7.8 μM on KLK7; 30 μM on KLK14; 10.5 μM on matriptase) and, with a higher efficiency, the 3'-chloro- and 3'-bromo-substituted 6-bromomethylcoumarin-3-carboxylates phenyl esters **4d** and **4e** (IC_{50} values for **4d**: 0.92 μM on KLK5; 64 nM on KLK7; 2.9 μM on KLK14; 1.42 μM on matriptase). Matriptase was the less sensitive enzyme to the inhibitory effect by coumarin derivatives.

The pyrid-3-yl (**44**-**50**) and pyrid-2-yl (**51**-**55**) esters of 6-chloromethylcoumarin-3-carboxylic acid were generally inefficient or less efficient than the phenyl esters against KLK5, KLK14 and matriptase (Table 3). The unsubstituted or halomethyl-monosubstituted

pyrid-3-yl cycle was the best choice to inhibit KLK7 (IC_{50} = 0.46-12.6 μM). Only the 6-chloro-substituted pyrid-2-yl ester **54** was found to express an inhibitory activity on KLK5, KLK7, KLK14 and matriptase.

The influence of a bulky aromatic group was analyzed by introducing isoquinolyl and naphthyl ester groups at the 3-position of coumarin-3-carboxylic acid (Table 4). When a bicyclic isoquinol-1-yl group was introduced (**56**), KLK5 and KLK14 were inhibited with a preference for KLK5 (IC_{50} = 7.0 ± 0.4 μM and 13 ± 1 μM, respectively). On the opposite, the isoquinol-5-yl (**57**) or the isoquinol-3-yl (**58**) esters were totally inactive. The naphth-1-yl (**6**) and naphth-2-yl (**8)** esters orientated against KLK7 but with a very poor efficiency.

Mechanism of inhibition. Detailed mechanistic studies were performed for the best inhibitors acting on the various targeted proteases. All of the 6-halomethyl-substituted coumarin-3-carboxylic acid phenyl esters acting on KLK7 in the nanomolar range behaved as time-dependent inhibitors indicating that the inhibitory effects are slowly elicited on the time scale of the enzyme turnover. No spontaneous reactivations of the inhibited enzymes (> 98%) occurred within 12 h as observed for **11**, **23**, **24**, **34**, **35**, **42**, **4d**, **4e** (against KLK7), **36**, **4d**, **4e** (against KLK5), and **4d** and **4e** (against KLK14 and matriptase). Hydroxylamine did not accelerate reactivations of the treated enzymes confirming the irreversible character of the inhibitions by these inhibitors and their mechanism-based behavior as previously observed for several of related derivatives against α–chymotrypsin[26, 28] and thrombin.[44] If the inactivation was induced by the formation of a stable acyl-enzyme, such stable acyl-enzyme would have been deacylated by hydroxylamine leading to enzyme activity recovery. Due to the leaving group properties of the 6-chlorine and 6-bromine atoms, an electrophilic quinone methide may be formed after the lactone ring opening induced by Ser195 nucleophilic attack as shown in Figure 2. The progress curve method in which the inhibitor competes with the fluorogenic

substrate was used to evaluate the k_i/K_I ratios[45], which are indexes of the inhibitory potency for this category of inhibitors (Figure 3; Table 5).

The values obtained for k_i/K_I varied from 97 M^{-1} s^{-1} for the weaker inhibitor **35** to 5443 $M^{-1}.s^{-1}$ for the most potent inactivator **42**. The presence of the CH_2Cl group at the 6-position poorly influenced inactivation efficacy as shown by the results obtained with CH_2Br group located at this position (**17**, **11** and **22** *vs* **4c**, **4d** and **4e**, respectively). For the monosubstituted phenyl compounds (except for R_1 =3'-$COOCH_3$), there was little effect on the inactivation efficiency of the substituent nature and position. The situation was more contrasted for disubstituted phenyl molecules with the following decreasing efficiency order:

3'-Cl, 5'-Cl > 2'-Cl, 5'-Cl > 2'-Cl, 3'-Cl > 2'-Cl, 6'-Cl > 3'-Cl, 5'-OCH_3 > 2'-Br, 5'-F

For example, the nature of a second substituent may be critical: a 8-fold decrease of activity was observed when the 5'-chlorine atom of **42** (3'-Cl, 5'-Cl) was replaced by a methoxy group in **43** (3'-Cl, 5'-OCH_3).

Several identified covalent inhibitors of KLK7 were also able to inhibit irreversibly KLK5, KLK14 and matriptase. However, some transient inactivations were observed for the tryptic KLK5, KLK14 and matriptase (**18**/KLK14; **19**/matriptase; **28**/ KLK14, matriptase; **30**/KLK5, matriptase; **40**/KLK5). Monosubstitutions at position 2' or 3' by a carboxyl or ester or amide group seemed to favor the transient inactivation of these tryptic enzymes.

Besides covalent inhibitors, we also have identified a reversible inhibitor (**56**) of KLK5 and KLK14 belonging to a new subfamily. This inhibitor was characterized by the absence of a substituent at the 6-position and the presence of a bicyclic substituent (isoquinoline) on the Y-position (see Table 4). The mechanism of inhibition of compound **56** on KLK5 was shown to be competitive with an inhibition constant $K_i = 5.4 \pm 0.6$ µM (Figure 2S).

Models inhibitor/protease complexes have been built using the CLC Drug Discovery Workbench software to corroborate the mechanistic data. We compared the possible binding modes of two inhibitors in the active site of the targeted proteases (Figure 4): the suicide inhibitor **4d** that was active on all proteases and the noncovalent inhibitor **56** that acted competitively against KLK5. It was found that these molecules can adopt two preferential conformations in agreement with optimal electronic delocalization within the plane of the intracyclic lactonic and exocyclic ester groups.[28] All docking calculations were performed using both conformations as starting points and revealed no differences on the resulting binding modes. The analysis of compound **4d** positioning within the active sites of KLK5, KLK7 and matriptase showed that in all cases the aryl ester moiety at the 3-position was accommodated within the S_1 subsite (Figure 4A-C). This may explain why compound **4d** displayed higher efficiency for chymotryptic KLK7 than for the tryptic enzymes for which the negatively charged Asp189 is located at the bottom of their S1 subsite instead of Asn189 for KLK7. The distance between the oxygen atom of the OH group of Ser195 and the carbon atom of the carbonyl group of the lactone function was compatible with a nucleophilic attack by the active serine. The substituent at the 6-position (CH_2Br) pointed towards His57 indicating a possible formation of a covalent bond between this residue and the methylene group formed after the bromine departure in agreement with the suicide mechanism of inhibition identified *in vitro.* In the case of compound **56** bound within the active site of KLK5, a reverse orientation of the coumarin ring compared to that of compound **4d** was observed in about ten poses (Figure 4D). The nitrogen atom of the isoquinoline ring was found sufficiently close to the Ser195 OH group to establish a hydrogen bond. The **56**-KLK5 complex might potentially be stabilized by three additional putative hydrogen bonds between the coumarin ring and Ser195 and Gln192 residues. This latter observation was in agreement with the competitive mechanism evidenced by kinetic data. The original binding mode of the

bicyclic isoquinol-1-yl group may constitute a valuable starting point for the elaboration of noncovalent inhibitors with improved affinity for KLK5.

Biological evaluation

Cytotoxicity on keratinocytes. In order to validate the effect of our coumarin inhibitors in a biological context, we firstly tested their toxicity on normal human keratinocytes isolated from healthy donors. In this context, compounds were selected according to their inhibitory capacity and structural diversity and tested at two different concentrations, 1 and 10 µM (Figure 5). In all cases, coumarinic derivatives were found to be non-cytotoxic at the tested concentrations that were 10-100 fold higher than the corresponding *in vitro* IC_{50} values. The usual lack of toxicity of our compounds on human keratinocytes is a favorable characteristic for a potential therapeutical use.

In situ zymography analyses. The transgenic mouse model in which human KLK5 is overexpressed in the granular layer of the epidermis (Tg-h*KLK5*) is a relevant model to study cutaneous and systemic phenotypic aspects of NS.[42, 46] Indeed human KLK5 overexpression in the epidermis leads to enhanced protease activity of KLK7 and KLK14, and downstream targets of the proteolytic cascade, thus increasing the total protease activity in the skin. In our *in situ* zymography experiment, the skin sections from Tg-hKLK5 mice were treated with selected coumarinic inhibitors prior to evaluation of total protease activity using a fluorogenic protein substrate (casein coupled to FITC). The results obtained with compounds **4d**, **11**, **26**, **34**, **36** and **56** are shown in Figure 6. The presence of **4d** (the most potent inhibitor of KLK5, 7, 14 and matriptase), **36** (preferential inhibition of KLK5 and KLK7) and **56** (selective inhibitor of KLK5 and KLK14) induced a drastic reduction of the total protease activity of the skin compared to the control. This reduction of the protease activity was particularly marked in the epidermis. In this transgenic model, molecules inhibiting only KLK7 (**11** and **34**) or none of the targeted enzymes (**26**), thus devoid of any inhibitory potency against KLK5, had

no effect. However **4d** also appeared to exhibit its inhibitory effect in the dermis, probably because of its broad spectrum of action on targeted proteases. These results indicate that the inhibitory activities of coumarin-3-carboxylates aryl esters on several KLKs are still effective in a tissue context replicating phenotypic characteristics of NS.

Conclusion

In conclusion, we have identified several subfamilies of coumarin derivatives that differentially inhibit key proteases whose activity is deregulated in NS. They act mainly as suicide substrates. Such inhibitors are likely to be extremely selective *in vitro* and *in vivo* since their inhibitory activity requires discrimination in the binding steps, the demasking of the latent electrophilic function during one step of the catalytic cycle of the target enzyme, and the irreversible modification of the active site facilitated by the formation of the acyl-enzyme maintaining part of the inhibitor within the active site.[38] Their inhibitory effects on KLKs and matriptase have been validated by both *in vitro* enzymatic studies and *in situ* zymography using Tg-h*KLK5* mouse as a model of NS. Molecular modeling was used to better understand their binding modes to the catalytic site of different proteases. We found that appropriate structural variations may lead to preferential inhibition of KLK7 or orientation towards the trypsin-like KLK5 and KLK14. These findings are expected to facilitate a further rational optimization of these compounds.

EXPERIMENTAL SECTION

Compounds **11**, **15**-**17**, **23**-**25**, **31**, **32** and **42**[27], **45**-**56**[26], **9**, **10**, **12**, **13**, **14**, **21**-**23**, **27**, **28**, **34**, **36**, **40**, **41** and **43**[28], **35**, **37**, **38** and **39** [47] were previously reported by our laboratory. The synthesis of new compounds **4a-e**, **6**, **8**, **18-20**, **29**, **30, 33**, and **56** is described hereafter. The purity of the compounds established by elemental analysis was equal to or greater than

95%. Stock solutions (10 mM) were prepared by dissolving the corresponding pure solid compounds in DMSO and stored at -20 °C. The kallikreins (KLK5, KLK7, KLK14 and matriptase) were purchased from R&D Systems Inc (Minneapolis, USA). The fluorogenic substrates used in enzymatic assays were purchased from Bachem (France) and hydroxylamine from Serva (Germany). Enzymatic studies were performed using a BMG Fluostar microplate reader (black 96-well microplates, Costar 3915, Corning International, USA).

Chemical synthesis

Reagents and solvents were purchased from usual commercial suppliers (Sigma-Aldrich, Beigium; Acros Organics, Belgium) and were used without further purification. All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck) and visualization was performed by UV light (254 nm). Melting points were determined on a Stuart SMP3 apparatus in open capillary tubes and were uncorrected. IR spectra were recorded as KBr pellets on a Perkin-Elmer Spectrum GX FTIR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance (^{1}H: 500 MHz; ^{13}C: 125 MHz) instrument using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard; chemical shifts were reported in δ values (ppm) relative to internal TMS. The abbreviation s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet and b = broad signal were used throughout. Exact mass were obtained with a Bruker APEX-Qe 9.4 Tesla FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA) in positive ion mode. Elemental analyses (C, H, N, S) were determined on a Thermo Flash EA 1112 series elemental analyzer and were within ± 0.4% of the theoretical values. This analytical process ensures for each target compound a purity equal or greater than 95 %).

General synthetic pathway to di-3-halophenyl esters of malonic acid (2). The mixture of malonic acid (2 g, 19.2 mmol), the appropriate 3-halophenol (2 eq., 38.4 mmol)

and phosphoryl chloride (2 eq., 38.4 mmol) was heated at 100°C for 90 min. The reaction mixture was then carefully poured on a cooled 1% w/v aqueous solution of sodium hydroxide (50 mL). The resulting precipitate of the title compound was collected by filtration, washed with water, dried and recrystallized in methanol (yields: 65-80%).

The following malonate esters were obtained according to this procedure and were found to be identical those reported in the literature (diphenyl malonate[48]), di-3-fluorophenyl malonate[49], di-3-chlorophenyl malonate[50]).

Di-3-bromophenyl malonate (**2d**); yields: 74%; m.p.: 64-66 °C; IR (KBr) 3070, 1770, 1583, 1468, 1364, 1190, 1141, 1120 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 4.12 (s, 2H, CH_2), 7.24 (dd, J = 8.1 Hz/1.6 Hz, 2H, 6-H, 6'-H), 7.44 (t, J = 8.1 Hz, 2H, 5-H, 5'-H), 7.50 (t, J = 1.9 Hz, 2H, 2-H, 2'-H), 7.53 (d, J = 8 Hz, 2H, 4-H, 4'-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 40.8; 121.0; 121.5; 124.8; 129.3; 131.5; 150.8; 164.9. Anal. ($C_{15}H_{10}Br_2O_4$) theoretical (%): C: 43.51; H: 2.43. Found (%): C: 43.70; H: 2.00.

General synthetic pathway to (3- halo)phenyl 6-(hydroxymethyl)-2-oxo-2H-1-benzopyran-3-carboxylates (3). The solution of 5-hydroxymethylsalicylaldehyde[43] (0.4 g, 2.62 mmol) and the appropriate di-(3-halo)phenyl malonate (**2**) (1.5 eq., 3.94 mmol) in dioxane (12 mL) was supplemented with 8 drops of piperidine and 4 drops of glacial acetic acid and stirred for 30 min at room temperature. At the end of the reaction, the solvent was removed by distillation under reduced pressure and the residue was triturated with cold methanol (10-20 mL). The resulting precipitate was collected by filtration, washed with cold methanol, dried and recrystallized in chloroform (yields: 25-75%).

According to this method, the following compounds were obtained:

Phenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**3a**): yields: 25%; m.p. : 187-188 °C; IR (KBr) 3264, 3067, 1760, 1621, 1574, 1493, 1379, 1247, 1215, 1200 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 4.59 (d, J = 5.1 Hz, 2H, CH_2); 5.42 (t, J = 5.4 Hz,

1H, OH); 7.28 (d, J = 7.8 Hz, 2H, 2'-H, 6'-H); 7.33 (t, J = 7.4 Hz, 1H, 4'-H); 7.45-7.51 (m, 3H, 8-H, 3'-H, 5'-H); 7.73 (d, J = 8.5 Hz, 1H, 7-H); 7.90 (s, 1H, 5-H); 9.07 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 61.9; 116.0; 116.5; 117.4; 121.8; 126.2; 127.7; 129.7; 133.4; 139.5; 150.3; 150.5; 153.7; 156.0; 161.1. HRMS for $C_{17}H_{12}O_5$ + H^+ calcd, 297.0757; found, 297.0754. Anal. ($C_{17}H_{12}O_5$) theoretical (%): C: 68.92 ; H: 4.08. Found (%): C: 68.57 ; H: 4.09.

3-Fluorophenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**3b**): yields: 30%; m.p. : 173-178°C; IR (KBr) 3253, 3057, 1763, 1621, 1606, 1574, 1486, 1377, 1242, 1214, 1132 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.59 (d, J = 5.4 Hz, 2H, CH_2); 5.43 (t, J = 5.6 Hz, 1H, OH); 7.17 (dd, J = 8.1 Hz/1.7 Hz, 1H, 6'-H), 7.21 (td, J = 8.6 Hz/2.3 Hz, 1H, 4'-H); 7,27 (dt, J = 9.8 Hz/2.2 Hz, 1H, 2'-H); 7.46 (d, J = 8.6 Hz, 1H, 8-H); 7.56 (dd, J = 15.1 Hz/8.2 Hz, 1H, 5'-H);7.74 (dd, J = 8.6 Hz/1.8 Hz, 1H, 7-H); 7.91 (s, 1H, 5-H); 9.09 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 61.8; 109.9; 113.2; 116.0; 116.1; 117.4; 118.2; 127.8; 130.9; 133.6; 139.5; 150.9; 151.2; 153.8; 155.9; 160.7; 161.2-163.2. HRMS for $C_{17}H_{11}FO_5$ + H^+ calcd, 315.0663; found, 315.0660. Anal. ($C_{17}H_{11}FO_5$) theoretical (%): C: 64.97 ; H: 3.53. Found (%): C: 64.53 ; H: 3.51.

3-Chlorophenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**3c**): yields: 50%; m.p. : 162-165 °C ; IR (KBr) 3526, 3058, 1732, 1621, 1574, 1471, 1371, 1239, 1195 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm) : 4.58 (d, J = 5.1 Hz, 2H, CH_2); 5.43 (t, J = 5.5 Hz, 1H, OH); 7.30 (dd, J = 8.1 Hz/1.6 Hz, 1H, 6'-H); 7.42 (dd, J = 8.1 Hz/1.1 Hz, 1H, 4'-H); 7.45-7.48 (m, 2H, 8-H, 2'-H); 7.52 (t, J = 8.1 Hz, 1H, 5'-H); 7.73 (dd, J = 8.6 Hz/1.8 Hz, 1H, 7-H); 7.90 (s, 1H, 5-H); 9.10 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm) : 61.8; 116.0; 116.1; 117.4; 120.9; 122.3; 126.3; 127.8; 131.1; 133.4; 133.6; 150.9; 151.0; 153.8; 155.9; 160.7. HRMS for $C_{17}H_{11}ClO_5$ + H^+ calcd, 331.0368; found, 331.0363. Anal. ($C_{17}H_{11}ClO_5$) theoretical (%): C: 61.74; H: 3.35. Found (%): C: 62.00 ; H: 3.33.

3-Bromophenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**3d**): yields: 75%; m.p.: 157-159°C ; IR (KBr) 3504, 3059, 1748, 1621, 1571, 1473, 1397, 1376, 1245, 1196, 1175, 1115 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 4.59 (d, J = 5.2 Hz, 2H, CH_2); 5.42 (t, J = 5.5 Hz, 1H, OH); 7.34 (dd, J = 8.1 Hz/1.4 Hz, 1H, 6'-H); 7.45-7.48 (m, 2H, 8-H, 5'-H); 7.55 (d, J = 8.1 Hz, 1H, 4'-H); 7.61 (t, J = 1.9 Hz, 1H, 2'-H); 7.74 (dd, J = 8.6 Hz/1.8 Hz, 1H, 7-H); 7.90 (s, 1H, 5-H); 9.10 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 61.8; 116.0; 116.1; 117.4; 121.2; 121.5; 125.1; 127.8; 129.2; 131.4; 133.6; 139.5; 150.9; 151.0; 153.8; 155.9; 160.7. HRMS for $C_{17}H_{11}BrO_5 + H^+$ calcd, 374.9863; found, 374.9865. Anal. ($C_{17}H_{11}BrO_5$) theoretical (%): C: 54.42 ; H: 2.96. Found (%): C: 53.98 ; H: 2.96.

3-Chlorophenyl 6-(fluoromethyl)-2-oxo-2H-1-benzopyran-3-carboxylate (4a). 3-Chlorophenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**3c**) (0.4 g, 0.68 mmol) was dissolved in methylene chloride (10 mL) and the resulting solution cooled on an ice bath was supplemented with Ishikawa's reagent (2.5 eq.). The reaction mixture was stirred for 30 min at 0°C and then heated under reflux for 90 min. After cooling, the reaction medium was poured onto water (20 mL). The organic layer was collected, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue of the title compound was purified by column chromatography on silicagel (eluent: methylene chloride) and then recrystalized in a mixture of methylene chloride and hexane: yields: 54%; m.p. : 178-179 °C; IR (KBr) 3081, 1777, 1720, 1625, 1578, 1378, 1247, 1221, 1205, 1174, 1112 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm) : 5.52 (d, J = 47.6 Hz, 2H, CH_2); 7.30 (dd, J = 8.1 Hz/1.9 Hz, 1H, 6'-H); 7.43 (dd, J = 7.9 Hz/1.4 Hz, 1H, 4'-H); 7.48 (t, J = 2 Hz, 1H, 2'-H); 7.54 (t, J = 8 Hz, 1H, 5'-H); 7.56 (d, J = 8.5 Hz, 1H, 8-H); 7.85 (d, J = 8.6 Hz, 1H, 7-H); 8.05 (s, 1H, 5-H); 9.12 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 82.5-83.8; 116.5; 116.7; 117.7; 120.9; 122.3; 126.4; 129.8; 131.1; 132.0-133.1; 133.4; 134.6; 150.5; 150.9; 154.9; 155.6; 160.6. HRMS for $C_{17}H_{10}ClFO_4 + H^+$ calcd, 333.0324; found, 333.0329. Anal. ($C_{17}H_{10}ClFO_4$) theoretical (%): C:

61.37 ; H: 3.03. Found (%): C: 60.68 ; H: 3.06.

General synthetic pathway to (3-halo)phenyl 6-(bromomethyl)-2-oxo-2H-1-benzopyran-3-carboxylates (4b-e). The appropriate (3-halo)phenyl 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (0.4 g, ~0.7 mmol) was dissolved in methylene chloride (10 mL) and then supplemented with pyridine (1.0 eq.) and thionyl bromide (1.15 eq.). The reaction mixture was heated under reflux for 90 min. After cooling, the reaction medium was poured onto water (20 mL). The organic layer was collected, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue of the title compound was recrystalized in a mixture of methylene chloride and hexane (yields: 15-50%).

According to this method, the following compounds were obtained:

Phenyl 6-(bromomethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**4b**): yields: 15%; m.p. : 186-187 °C; IR (KBr) 3065, 1760, 1621, 1574, 1377, 1249, 1225, 1195 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.82 (s, 2H, CH_2); 7.28 (d, J = 7.7 Hz, 2H, 12'-H, 6'-H); 7.34 (t, J = 7.4 Hz, 1H, 4'-H); 7.50 (m, 3H, 8-H, 3'-H, 5'-H); 7.86 (dd, J = 8.6 Hz/2.1 Hz, 1H, 7-H); 8.06 (d, J = 2 Hz, 1H, 5-H); 9.06 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 33.0; 116.9; 117.0; 117.9; 121.8; 126.2; 129.7; 130.8; 135.0; 135.9; 149.9; 150.3; 154.4; 155.7; 161.0. HRMS for $C_{17}H_{11}BrO_4 + H^+$ calcd, 358.9913; found, 358.9917. Anal. ($C_{17}H_{11}BrO_4$) theoretical (%): C: 56.85 ; H: 3.09. Found (%): C: 56.82 ; H: 3.15.

3-Fluorophenyl 6-(bromomethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**4c**): yields: 15%; m.p.: 189-190 °C ; IR (KBr) 3071, 1754, 1709, 1620, 1572, 1487, 1379, 1227 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.82 (s, 2H, CH_2); 7.22 (m, 3H, 2'-H, 4'-H, 6'-H); 7.51 (d, J = 8.6 Hz, 1H, 8-H); 7.55 (m, 1H, 5'-H); 7.87 (dd, J = 8.6 Hz/2.1 Hz, 1H, 7-H); 8.06 (d, 2 Hz, 1H, 5-H); 9.08 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 33.0; 109.8-110.0; 113.1-113.3; 116.5; 116.9; 117.8; 118.2; 130.9; 131.0; 135.0; 136.1; 150.3; 151.1; 154.4; 155.6; 160.5; 161.2; 163.2. HRMS for $C_{17}H_{10}BrFO_4 + H^+$ calcd, 376.9819; found, 376.9823. Anal.

($C_{17}H_{10}BrFO_4$) theoretical (%): C: 54.14 ; H: 2.67. Found (%): C: 53.88 ; H: 2.71.

3-Chlorophenyl 6-(bromomethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**4d**): yields: 50%; m.p. : 186-187 °C ; IR (KBr) 3088, 1776, 1725, 1619, 1574, 1376, 1247, 1224, 1206, 1112 cm^{-1}; ^1H NMR (DMSO-d_6) δ (ppm): 4.83 (s, 2H, CH_2); 7.30 (dd, J = 8.1 Hz/1.4 Hz, 1H, 6'-H); 7.43 (dd, J = 8 Hz/1.2 Hz, 1H, 4'-H); 7.48 (t, J = 2.1 Hz, 1H, 2'-H); 7.52 (m, 2H, 8-H, 5'-H); 7.87 (dd, J = 8.6 Hz/2.1 Hz, 1H, 7-H); 8.06 (d, J = 2.1 Hz, 1H, 5-H); 9.08 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 33.0; 116.5; 116.9; 117.8; 120.9; 122.3; 126.4; 130.9; 131.1; 133.4; 135.1; 136.1; 150.3; 150.9; 154.4; 155.6; 160.6. HRMS for $C_{17}H_{10}BrClO_4 + H^+$ calcd, 392.9524; found, 392.9526. Anal. ($C_{17}H_{10}BrClO_4$) theoretical (%): C: 51.87 ; H: 2.56. Found (%): C: 51.54 ; H: 2.55.

3-Bromophenyl 6-(bromomethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**4e**): yields: 16%; m.p.: 185-186 °C; IR (KBr) 3088, 1773, 1726, 1619, 1573, 1470, 1375, 1246, 1222, 1202, 1112 cm^{-1}; 1H NMR (DMSO-d_6) δ (ppm): 4.82 (s, 2H, CH_2); 7.34 (dd, J = 8.1 Hz/1.3 Hz, 1H, 6'-H); 7.47 (t, J = 8.1 Hz, 1H, 5'-H); 7.51 (d, J = 8.6 Hz, 1H, 8-H); 7.56 (d, J = 7.9 Hz, 1H, 4'-H); 7.61 (t, J = 1.9 Hz, 1H, 2'-H); 7.88 (dd, J = 8.6 Hz/2.1 Hz, 1H, 7-H); 8.06 (d, J = 2 Hz, 1H, 5-H); 9.07 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 33.0; 116.5; 116.9; 117.8; 121.2; 121.5; 125.1; 129.2; 130.9; 131.4; 135.1; 136.1; 150.3; 150.9; 154.4; 155.6; 160.6. HRMS for $C_{17}H_{10}Br_2O_4 + H^+$ calcd, 436.9019; found, 436.9024. Anal. ($C_{17}H_{10}Br_2O_4$) theoretical (%): C: 46.61 ; H: 2.30. Found (%): C: 46.32 ; H: 2.32.

General synhetic pathway to dinaphthyl esters of malonic acid. The mixture of malonic acid (2 g, 19.2 mmol), the appropriate naphthol (1-naphthol or 2-naphthol) (2 eq., 38.4 mmol) and phosphoryl chloride (2 eq., 38.4 mmol) was heated at 100°C for 90 min. The reaction mixture was then carefully poured on a cooled 1% w/v aqueous solution of sodium hydroxide (50 mL). The resulting precipitate of the title compound was collected by filtration, washed with water, dried and recrystallized in methanol (yields: 25-30%). The white solid of

the title compound (di-1-naphthyl malonate or di-2-naphthyl malonate) was used in the next step without further purification.

General synthetic pathway to naphthyl 2-oxo-2H-1-benzopyran-3-carboxylates 6 and 8. The solution of salicylaldehyde (0.4 g, 3.28 mmol) and the appropriate dinaphthyl malonate (1.5 eq., 4.92 mmol) in dioxane (12 mL) was supplemented with 8 drops of piperidine and 4 drops of glacial acetic acid and stirred for 30 min at room temperature. At the end of the reaction, the solvent was removed by distillation under reduced pressure and the residue was triturated with cold methanol (20 mL). The resulting precipitate was collected by filtration, washed with cold methanol and dried. The white solid of the title compound was recrystallized in a mixture of methylene chloride and hexane (yields: 75-80%).

According to this method, the following compounds were obtained:

1-Naphthyl 2-oxo-2*H*-1-benzopyran-3-carboxylate (**6**): yields: 80-85%; m.p.: 159-160 °C; IR (KBr) 3066, 1743, 1611, 1565, 1305, 1235, 1222, 1210 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm) : 7.48 (m, 2H, $H_{arom.}$); 7.54 (d, J = 8.4 Hz, 1H, $H_{arom.}$); 7.61 (m, 3H, $H_{arom.}$); 7.83 (t, J = 7.2 Hz, 1H, $H_{arom.}$); 7.93 (d, J = 8.2 Hz, 1H, $H_{arom.}$); 8.06 (m, 3H, $H_{arom.}$); 9.22 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 116.3; 116.6; 117.9; 118.6; 121.3; 125.0; 125.8; 126.3; 126.8; 126.9; 128.0; 130.7; 134.2; 135.1; 146.1; 150.6; 154.9; 156.1; 161.5. HRMS for $C_{20}H_{12}O_4$ + H^+ calcd, 317.0808; found, 317.0806. Anal. ($C_{20}H_{12}O_4$) theoretical (%): C: 75.94 ; H: 3.82. Found (%): C: 75.54 ; H: 3.81.

2-Naphthyl 2-oxo-2*H*-1-benzopyran-3-carboxylate (**8**): yields: 75-80%; m.p.: 166-167 °C; IR (KBr) 3047, 1761, 1715, 1610, 1568, 1454, 1377, 1304, 1236, 1210, 1128, 1152 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.47 (m, 2H, $H_{arom.}$); 7.52 (d, J = 8.4 Hz, 1H, $H_{arom.}$); 7.57 (m, 2H, $H_{arom.}$); 7.82 (m, 1H, $H_{arom.}$); 7.84 (d, J = 2.1 Hz, 1H, 1'-H); 7.99 (m, 2H, $H_{arom.}$); 8.03 (dd, J = 7.8 Hz/1.2 Hz, 1H, $H_{arom.}$); 8.05 (d, J = 8.9 Hz, 1H, $H_{arom.}$); 9.16 (s, 1H, 4-H). ^{13}C NMR (DMSO-d_6) δ (ppm): 116.3; 116.4; 117.9; 118.7; 121.5; 125.0; 126.0; 126.8; 127.6; 127.8;

129.5; 130.6; 131.1; 133.3; 135.1; 147.9; 150.6; 154.8; 155.9; 161.2. HRMS for $C_{20}H_{12}O_4$ + H^+ calcd, 317.0808; found, 317.0804. Anal. ($C_{20}H_{12}O_4$) theoretical (%): C: 75.94 ; H: 3.82. Found (%): C: 75.53 ; H: 3.84.

Isoquinol-1-yl 2-oxo-2*H*-1-benzopyran-3-carboxylate (**56**): 1.0 g of the commercially available 2-oxo-2*H*-1-benzopyran-3-carboxylic acid and 10 mL of thionyl chloride were refluxed for 3 h. The resulting solution was evaporated under reduced pressure. The residue was suspended in 10 mL anhydrous toluene. The solvent was eliminated by distillation under reduced pressure. The two last steps were repeated twice. The residue was dispersed in 10 mL dioxane. To this suspension were added 1-hydroxyisoquinoline (1.1 eq.) and anhydrous pyridine (1.1 eq.). After 90 min stirring at room temperature, the solvent was removed by distillation under reduced pressure. The residue was solubilized in chloroform and the organic phase was washed three times with HCl 0.1N, then dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue obtained was recrystallized in ethyl acetate. Yields: 15%. White solid: m.p. 197-200°C; IR (KBr) 3076, 1726, 1706, 1676, 1641, 1599, 1293, 1271, 1247, 1228, 1208, 1170, 1138 cm^{-1}; ^1H NMR (DMSO-d_6) d (ppm): 6.87 (d, J = 7.7 Hz, 1H, 4-$H_{isoquin.}$), 7.47 (t, J = 7.2 Hz, 1H, 6-$H_{coumar.}$), 7.52 (d, J = 8.2 Hz, 1H, 8-$H_{coumar.}$), 7.58 (t, J = 7.3 Hz, 1H, 7-$H_{isoquin.}$), 7.75 (m, 2H, 7-$H_{coumar.}$ + 5-$H_{isoquin.}$), 7.84 (m, 2H, 6-H + 3-$H_{isoquin.}$), 7.93 (d, J = 7.2 Hz, 1H, 5-$H_{coumar.}$), 8.16 (d, J = 7.7 Hz, 1H, 8-$H_{isoquin.}$), 8.56 (s, 1H, 4-$H_{coumar.}$). ^{13}C NMR (DMSO-d_6) d (ppm): 108.7; 116.4; 118.2; 124.8; 125.2; 125.6; 125.7; 126.8; 128.0; 128.1; 129.9; 133.9; 134.6; 136.6; 143.7; 153.7; 157.3; 162.1; 166.4. HRMS for $C_{19}H_{11}NO_4$ + H^+ calcd, 318.0761; found, 318.0547. Anal. ($C_{19}H_{11}NO_4$) theoretical (%): C: 71.92; H:3.49; N: 4.41. Found (%): C: 71.46; H: 3.45; N: 4.29.

General synthetic pathway to the monosubstituted phenyl esters of 2-oxo-2H-1-benzopyran-3-carboxylic acid 18, 19, 28, 29, 30 and 33. 6-(Hydroxymethyl)-2*H*-1-benzopyran-3-carboxylic acid (1.0 g, 4.54 mmol) obtained as previously described [27] was suspended in

thionyl chloride (10 mL) and refluxed for 3 h. The resulting solution was evaporated to dryness under reduced pressure, and the residue of crude 6-(chloromethyl)-2*H*-1-benzopyran-3-carbonyl chloride was dispersed in dry toluene (10 mL). The solvent was eliminated under reduced pressure. Dispersion in dry toluene and solvent elimination was repeated twice according to the previously described protocol. [27] The crude acid chloride was dispersed in anhydrous dioxane (10 mL) and supplemented under stirring with the appropriate phenol (5.0 mmol) and anhydrous pyridine (10.0 mmol). After 12-48 h stirring at room temperature, the solvent was eliminated by distillation under reduced pressure. The residue was partitioned between chloroform (60 mL) and 0.1 N HCl (30 mL), and the organic layer was decanted, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The crude final compound was then recrystallized in the appropriate solvent.

According to this method, the following compounds were obtained:

2-Carboxyphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**18**): the compound was recrystallized in methanol/water and then in dioxane/water; yields: 45%; m.p.: 152-155 °C; IR (KBr) 3081, 1775, 1673, 1624, 1575, 1487, 1374, 1246 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.88 (s, 2H, CH_2Cl), 7.36 (d, J = 2.1 Hz, 1H, 6'-*H*), 7.47 (td, J = 7.6 Hz/1.0 Hz, 1H, 4'-*H*), 7.53 (d, J = 8.6 Hz, 1H, 8-*H*), 7.73 (td, J = 7.9 Hz/1.7 Hz, 1H, 5'-*H*), 7.87 (dd, J = 8.6 Hz/2.2 Hz, 1H, 7-*H*), 8.01 (dd, J = 7.8 Hz/1.6 Hz, 1H, 3'-*H*), 8.09 (d, J = 2.1 Hz, 1H, 5-*H*), 9.09 (s, 1H, 4-*H*), 13.19 (bs, 1H, COO*H*). ^{13}C NMR (DMSO-d_6) δ (ppm): 45.0; 116.7; 116.8; 117.7; 123.9; 124.0; 126.6; 130.6; 131.6; 134.1; 134.6; 135.6; 149.8; 150.2; 154.5; 155.6; 160.6; 165.4. Anal. ($C_{18}H_{11}ClO_6$) theoretical (%): C: 60.27; H: 3.09. Found (%): C: 59.94; H: 3.07.

2-Methoxycarbonylphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**19**): the compound was recrystallized in acetone; yields: 46%; m.p.: 152-155 °C ; IR (KBr) 3069, 1769, 1716, 1622, 1575, 1485, 1445, 1376, 1217, 1202 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.76 (s,

3H, OC*H*$_3$), 4.89 (s, 2H, C*H*$_2$Cl), 7.41 (dd, J = 8.1 Hz/0.9 Hz, 1H, 6'-*H*), 7.50 (td, J = 7.7 Hz/1.1 Hz, 1H, 4'-*H*), 7.53 (d, J = 8.6 Hz, 1H, 8-*H*), 7.77 (td, J = 7.9 Hz/1.7 Hz, 1H, 5'-*H*), 7.87 (dd, J = 8.6 Hz/2.2 Hz, 1H, 7-*H*), 8.02 (dd, J = 7.8 Hz/1.7 Hz, 1H, 3'-*H*), 8.10 (d, J = 2.1 Hz, 1H, 5-*H*), 9.11 (s, 1H, 4-*H*). ^{13}C NMR (DMSO-d_6) δ 45.0; 52.4; 116.6; 116.8; 117.8; 122.9; 124.1; 126.8; 130.6; 131.4; 134.5; 134.6; 135.6; 149.6; 150.3; 154.6; 155.6; 160.6; 164.4. HRMS for $C_{19}H_{13}ClO_6$ + H^+ calcd, 373.0473; found, 373.0509. Anal. ($C_{19}H_{13}ClO_6$) theoretical (%): C: 61.22; H: 3.51. Found (%): C: 61.17; H: 3.57.

3-Carboxyphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**28**): the compound was recrystallized in methanol/water; yields: 56%; m.p.: 231-235 °C ; IR (KBr) 3078, 1770, 1685, 1622, 1577, 1490, 1374 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.89 (s, 2H, C*H*$_2$Cl), 7.53 (d, J = 8.6 Hz, 1H, 8-*H*), 7.57 (dd, J = 8.1 Hz/1.2 Hz, 1H, 6'-*H*), 7.64 (t, J = 7.9 Hz, 1H, 5'-*H*), 7.83 (s, 1H, 2'-*H*), 7.86 (dd, J = 8.6 Hz/2.0 Hz, 1H, 7-*H*), 7.91 (d, J = 7.6 Hz, 1H, 4'-*H*), 8.06 (d, J = 1.8 Hz, 1H, 5-*H*), 9.12 (s, 1H, 4-*H*), 13.25 (bs, 1H, COO*H*). ^{13}C NMR (DMSO-d_6) δ 45.0; 116.8; 116.8; 117.8; 122.6; 126.4; 127.0; 130.1; 130.5; 132.4; 134.6; 135.5; 150.2; 150.2; 154.5; 155.7; 160.8; 166.4. HRMS for $C_{18}H_{11}ClO_6$ + H^+ calcd, 359.0317; found, 359.0350. Anal. ($C_{18}H_{11}ClO_6$) theoretical (%): C: 60.27; H: 3.09. Found (%): C: 60.62; H: 3.12.

3-Methoxycarbonylphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**29**): the compound was recrystallized in acetone; yields: 32%; m.p.: 128-133 °C ; IR (KBr) 3076, 1761, 1724, 1622, 1574, 1486, 1432, 1377, 1220, 1204 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.89 (s, 3H, OC*H*$_3$), 4.89 (s, 2H, C*H*$_2$Cl), 7.53 (d, J=8.6 Hz, 1H, 8-*H*), 7.61 (ddd, J = 8.1 Hz/2.3 Hz/1.1 Hz, 1H, 6'-*H*), 7.67 (t, J = 7.9 Hz, 1H, 5'-*H*), 7.86 (m, 2H, 7-*H*/2'-*H*), 7.93 (dt, J = 7.7 Hz/1.3 Hz, 1H, 4'-*H*), 8.06 (d, J = 2.1 Hz, 1H, 5-*H*), 9.12 (s, 1H, 4-*H*). ^{13}C NMR (DMSO-d_6) δ 45.0; 52.4; 116.7; 116.8; 117.8; 122.5; 126.8; 126.9; 130.3; 130.5; 131.1; 134.6; 135.6; 150.2; 150.3; 154.5; 155.7; 160.7; 165.4. HRMS for $C_{19}H_{13}ClO_6$ + H^+ calcd, 373.0473; found,

373.0513. Anal. ($C_{19}H_{13}ClO_6$) theoretical (%): C: 61.22; H: 3.51. Found (%): C: 60.89; H: 3.47.

3-Carbamoylphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**30**): the compound was recrystallized in chloroform/petroleum ether (40-60 °C) and then in dioxane/water; yields: 82%; m.p.: 190-192 °C ; IR (KBr) 3367, 3187, 3074, 1772, 1754, 1652, 1622, 1575, 1491, 1380, 1241, 1210 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.89 (s, 2H, CH_2Cl), 7.46 (ddd, J = 8.1 Hz/2.3 Hz/0.8 Hz, 1H, 6'-*H*), 7.51 (bs, 1H, N*H*), 7.53 (d, J = 8.6 Hz, 1H, 8-*H*), 7.58 (t, J = 7.9 Hz, 1H, 5'-*H*), 7.78 (m, 1H, 2'-*H*), 7.86 (m, 2H, 7-*H*/4'-*H*), 8.06 (d, J = 2.1 Hz, 1H, 5-*H*), 8.09 (bs, 1H, N*H*), 9.11 (s, 1H, 4-*H*). ^{13}C NMR (DMSO-d_6) δ 45.0; 116.8; 116.8; 117.8; 121.0; 124.7; 125.2; 129.6; 130.6; 134.6; 135.6; 135.9; 150.1; 150.2; 154.5; 155.7; 160.9; 166.7. HRMS for $C_{18}H_{12}ClNO_5 + H^+$ calcd, 358.0477; found, 358.0511. Anal. ($C_{18}H_{12}ClNO_5$) theoretical (%): C: 60.43 ; H: 3.38 ; N: 3.91. Found (%): C: 59.92; H: 3.72; N: 3.73.

4-Methoxycarbonylphenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (**33**): the compound was recrystallized in chloroform/petroleum ether (40-60 °C) and then in acetone/petroleum ether (40-60°C); yields: 35%; m.p.: 212-215 °C ; IR (KBr) 3069, 1777, 1718, 1623, 1576, 1491, 1433, 1377, 1224, 1208 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.88 (s, 3H, OCH_3), 4.89 (s, 2H, CH_2Cl), 7.46 (d, J = 8.6 Hz, 2H, 2'-*H*/6'-*H*), 7.53 (d, J = 8.6 Hz, 1H, 8-*H*), 7.86 (dd, J = 8.6 Hz/1.8 Hz, 1H, 7-*H*), 8.06 (d, J = 1.4 Hz, 1H, 5-*H*), 8.09 (d, J = 8.6 Hz, 2H, 3'-*H*/5'-*H*), 9.12 (s, 1H, 4-*H*). ^{13}C NMR (DMSO-d_6) δ 44.6; 52.3; 117.5; 117.6; 117.7; 121.6; 128.2; 129.5; 131.3; 134.7; 135.3; 149.8; 153.9; 155.2; 156.0; 160.9; 166.2. HRMS for $C_{19}H_{13}ClO_6 + H^+$ calcd, 373.0473; found, 373.0510. Anal. ($C_{19}H_{13}ClO_6$) theoretical (%): C: 61.22; H: 3.51. Found (%): C: 60.97; H: 3.50.

Enzyme and inhibition assays. Kallikreins activities were determined by monitoring the hydrolysis of the appropriate fluorogenic substrate (λ_{exc} = 355 nm, λ_{em} = 460 nm for AMC

substrates) at 37 °C in the presence of untreated kallikreins (control) or kallikreins that had been incubated with a test compound. Substrates and compounds were previously dissolved in DMSO, with the final solvent concentration kept constant at 2 % (v/v). The composition of the activity buffers was (pH 8.0): 50 mM Tris-HCl, Tween 20 0.01 % (v/v), and 150 mM NaCl for KLK5, 1M NaCl for KLK7 and KLK14. The final concentrations were 0.6 nM (KLK5), 100 μM (Boc-Val-Pro-Arg-AMC); 7.6 nM (KLK7), 40 μM (Suc-Leu-Leu-Val-Tyr-AMC); 0.2 nM (KLK14), 10 μM (Boc-Val-Pro-Arg-AMC); 0.1 nM (matriptase), 10 μM (Boc-Gln-Ala-Arg-AMC). Compounds (0.1-100 μM) were tested in triplicate for each inhibitor concentration to detect their inhibitory potential. The enzyme and the inhibitors were incubated for 15 min before the determination of the enzyme activity. Initial rates determined in control experiments (V_0) were considered to be 100 % of the proteinase activity; initial rates (V_i) that were below 100 % in the presence of a tested compound were considered to be inhibitions. The inhibitory activity of compounds was expressed as IC_{50} (inhibitor concentrations giving 50% inhibition). The values of IC_{50} were calculated by fitting the experimental data either to equation 1: % Inhibition = 100 x $(1-V_i/V_0) = 100\ [I]_0/(IC_{50} + [I]_0)$, or equation 2, % Inhibition = $100\ [I]_0^{nH}/(IC_{50}^{nH} + [I]_0^{nH})$ with n_H = Hill number.

Mechanistic studies. Reversibility was evaluated either by dilution or by treatment with hydroxylamine. The reaction mixtures were usually diluted 40-fold after 15 and 60 min preincubation of the enzyme with inhibitor. Aliquots of reaction mixtures (2.5 μL) were added to 97.5 μL of buffer containing the fluorogenic substrate (experimental conditions identical to the routine protocol used for a given enzyme) or eventually immediately treated by 0.5 M of hydroxylamine at pH 8.0 during 60 min before determination of remaining activity. For non-covalent inhibitors, the mechanisms of inhibition were determined by varying substrates and inhibitors concentrations and using classical representations (Lineweaver-Burk). For suicide inhibitors, inactivation can be represented by the minimum

kinetic scheme (eq. 3)[26], where E and I are the free forms of enzyme and inhibitor, E*I a kinetic chimera of the Michaelis complex and the acyl-enzyme, E_i the inactivated enzyme, and P the products.

(eq. 3)

The kinetic constants k_i and K_I were determined for KLK7 using the progress curve method. [26] The ratio k_i/K_I was obtained by fitting the experimental data to the equations (F.U., fluorescence unit):

$$F.U. = \int_0^t v_i \cdot dt + F.U._0 = \frac{-v_0 * e^{-\pi * t} + v_0}{\pi} + F.U._0 \quad \text{(eq. 4)}$$

with $\pi = \frac{k_i * [I]'}{K_I + [I]}$ and $[I]' = \frac{[I]}{1 + [S]/K_m}$

Linear and non-linear regression fits of the experimental data to the equations were performed with Kaleidagraph Software. The experimental conditions were: $[KLK7]_0 = 7{,}6$ nM , $[S]_0 = 40$ µM, $[I]_0 = 0$-200 nM.

Cell culture assay. Human skin was obtained from healthy donors undergoing abdominal plastic surgery. Samples were obtained after informed consent and used according to the guidelines of the Declaration of Helsinki Principles. Healthy keratinocytes are spread at a rate of 4,000 cells per well of 96-well plates in 125 µL of medium/well consisting of: 50% of complete culture medium Green containing 60% of DMEM (Dulbecco's Modified Eagle Media), 30% of Ham's F12, 10% of FCS, 180 mM of adenine, 5 mg/mL of insulin, 0.4 mg/mL of hydrocortisone, 10 nM of cholera toxin, 2 nM of triiodothyronine, 10 ng/mL of human recombinant EGF, 100 U /mL of penicillin G / streptomycin, and 50% of basal medium (Epilife - Cascade Biologics) for the culture of human keratinocytes without feeder layer. 48 h after seeding the cells, 125 µL of fresh medium were mixed with the inhibitor in

DMSO to give a concentration of 2 or 20 μM, and then this solution was added to the wells to obtain a final concentration of 1 or 10 μM. For each condition, at least three wells are realized. After 48 h incubation, cells were washed with PBS. Then 250 μL of NR medium (culture medium containing neutral red 33 μg/mL) were added, and cells were incubated at 37°C and 5% of CO_2 for 3 h. Then, the medium containing neutral red was removed; the cells were washed with PBS. Then, 100 μL of NR desorbed solution (1% acetic acid, 50% ethanol, 49% H_2O) were added, the plate was shaked for 20-40 min and the absorbance at 540 nm read. Relative cell viability was expressed as a percentage of the viability of cells treated with DMSO alone.

In situ *zymography.* *In situ* zymography was carried out on skin sections of transgenic mice overexpressing human KLK5.[46] Cryosections of non lesional skin (thickness of 5 μm) were washed with a PBS solution containing 2% of Tween 20 during 2 min, then 5 min with PBS. The skin sections were then incubated (or not) with the inhibitors at 37 ° C overnight with 100 uL of BODIPYFL casein (10 μg /mL) using the Ultra EnzChek Protease Assay kit (Invitrogen) in a buffer containing Tris-HCl 100 mM, pH 8. Sections were rinsed with PBS. The inhibition of the release of fluorochrome was then analyzed, visualized and quantified using a Leica TCS SP5 AOBS confocal microscope.

ASSOCIATED CONTENT

Supporting information. Three figures showing the postulated mechanisms for the inhibition of serine proteases by coumarin-3-carboxylic acid derivatives, IC_{50} curves and Lineweaver-Burk plot; molecular docking specification; references of supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AMC, 7-amino-4-methylcoumarin; KLK5, kallikrein-related peptidase 5; KLK7, kallikrein-related peptidase 7; KLK14, kallikrein-related peptidase 14; LEKTI, lymphoepithelial Kazal-type-related inhibitor; SFTI-1, sunflower tryspin inhibitor-1; HLE, human leukocyte elastase; NS, Netherton syndrome; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; TLC, thin-layer chromatography; DMSO, dimethylsulfoxide; TMS, tetramethylsilane; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; Boc, tert-butyloxycarbonyl; PDB, Protein Data Bank.

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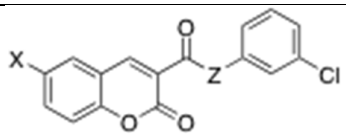
TABLES

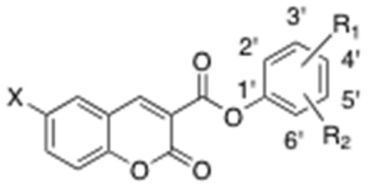
Table 1. Inhibition of human KLK5, KLK7, KLK14 and matriptase by coumarinic derivatives at pH 8 and 37 °C.



			IC_{50} (μM) or *% inhibition at 50 μM*			
Z	**X**	Compound	KLK5	KLK7	KLK14	Matriptase
O	-H	**9**	ni[a]	ni	ni	ni
	$-CH_3$	**10**	ni	ni	ni	ni
	$-CH_2F$	**4a**	ni	ni	ni	ni
	$-CH_2Cl$	**11**	ni	0.103 ± 0.005	ni	ni
	$-CH_2Br$	**4d**	0.92 ± 0.07	0.064 ± 0.002	2.9 ± 0.1	1.42 ± 0.05
	$-CH_2OCOCH_3$	**12**	*31%*	*56%*	31 ± 6	ni
NH	$-CH_2Cl$	**13**	ni	*60%*	ni	ni
S	$-CH_2Cl$	**14**	ni	0.35 ± 0.02	*34%*	ni

[a]ni: < 30% inhibition at 50 μM.

Table 2. Inhibition of human KLK5, KLK7, KLK14 and matriptase by diversely substituted phenyl esters of 6-halomethylcoumarin-3-carboxylic acid at pH 8 and 37 °C.



IC_{50} (μM) or

% inhibition at 50 μM

X = $-CH_2Cl$

R_1	R_2	Compound	KLK5	KLK7	KLK14	Matriptase
H	H	**15**	ni[a]	0.235 ± 0.009	ni	ni
2'-Cl	H	**16**	ni	0.209 ± 0.007	41 ± 4	ni
2'-I	H	**17**	ni	1.95 ± 0.03	ni	ni
2'-COOH	H	**18**	ni	ni	43 ± 4	ni
2'-$COOCH_3$	H	**19**	26.3 ± 1.4	2.7 ± 0.1	38 ± 5	*33%*
2'-NO_2	H	**20**	ni	1.44 ± 0.01	ni	ni
3'-F	H	**21**	ni	0.25 ± 0.01	ni	ni
3'-Cl	H	**11**	ni	0.103 ± 0.005	ni	ni
3'-Br	H	**22**	ni	0.065 ± 0.003	ni	ni
3'-I	H	**23**	ni	0.077 ± 0.003	ni	ni
3'-NO_2	H	**24**	ni	≈ 50 μM	ni	ni
3'-CH_3	H	**25**	ni	0.33 ± 0.03	ni	ni
3'-CF_3	H	**26**	ni	ni	ni	ni
3'-OCH_3	H	**27**	ni	8.4 ± 0.2	ni	ni
3'-COOH	H	**28**	ni	ni	ni	61 ± 5
3'-$COOCH_3$	H	**29**	ni	0.68 ± 0.01	ni	ni
3'-$CONH_2$	H	**30**	21.6 ± 5	7.8 ± 0.6	30 ± 8	10.5 ± 0.3
4'-Cl	H	**31**	ni	0.13 ± 0.03	ni	ni
4'-CH_3	H	**32**	ni	0.48 ± 0.02	ni	ni
4'-$COOCH_3$	H	**33**	ni	ni	ni	ni
2'-Cl	3'-Cl	**34**	ni	0.063 ± 0.004	ni	ni
2'-F	5'-F	**35**	ni	0.49 ± 0.04	39 ± 4	ni
2'-Cl	5'-Cl	**36**	1.3 ± 0.1	0.19 ± 0.02	54 ± 6 μM	ni

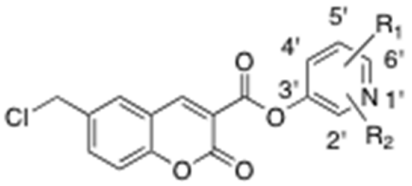
2'-Br	5'-F	**37**	ni	0.38 ± 0.06	ni	ni
2'-NO_2	5'-F	**38**	ni	0.93 ± 0.4	ni	ni
2'-NO_2	5'-NO_2	**39**	ni	ni	ni	ni
2'-Cl	6'-Cl	**40**	59 ± 9	0.49 ± 0.04	ni	ni
3'-CH_3	4'-Cl	**41**	ni	ni	ni	ni
3'-Cl	5'-Cl	**42**	80 ± 8	0.14 ± 0.01	ni	ni
3'-Cl	5'-OCH_3	**43**	ni	0.129 ± 0.005	ni	ni

X = -CH_2Br

R_1	R_2	Compound	KLK5	KLK7	KLK14	Matriptase
H	H	**4b**	*34%*	0.249 ± 0.004	*53%*	*62%*
3'-F	H	**4c**	*42%*	0.198 ± 0.008	*48%*	*66%*
3'-Cl	H	**4d**	0.92 ± 0.07	0.064 ± 0.002	2.9 ± 0.1	1.42 ± 0.05
3'-Br	H	**4e**	1.47 ± 0.06	0.057 ± 0.002	3.0 ± 0.1	1.97 ± 0.05

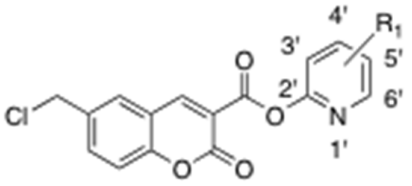
[a]ni: < 30% inhibition at 50 μM.

Table 3. Inhibition of human KLK5, KLK7, KLK14 and matriptase by diversely substituted 3-pyridyl esters of 6-chloromethylcoumarin-3-carboxylic acid at pH 8 and 37 ° C.



IC_{50} (μM)

R_1	R_2	Compound	KLK5	KLK7	KLK14	Matriptase
H	H	**44**	ni[a]	5.7 ± 0.3	ni	*30%*[a]
2'-Cl	H	**45**	ni	12.6 ± 0.5	ni	ni
2'-Br	H	**46**	ni	6.5 ± 0.4	ni	ni
5'-Cl	H	**47**	ni	0.463 ± 0.007	ni	ni
5'-CH_3	H	**48**	ni	1.45 ± 0.07	ni	ni
6'-CH_3	H	**49**	ni	ni	ni	ni
2'-NO_2	6'-CH_3	**50**	ni	ni	ni	ni



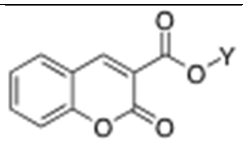
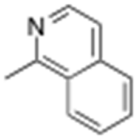
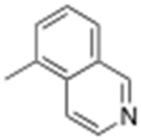
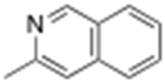
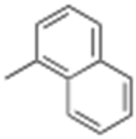
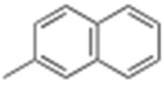
IC_{50} (μM) or

% inhibition at 50 μM

R_1	Compound	KLK5	KLK7	KLK14	Matriptase
H	**51**	ni	ni	ni	ni
4 -CH_3	**52**	ni	ni	ni	ni
5 -Cl	**53**	ni	*62%*[a]	ni	ni
6 -Cl	**54**	78 ± 12	4.9 ± 0.3	30 ± 2	*38%*
6 -CH_3	**55**	ni	ni	ni	ni

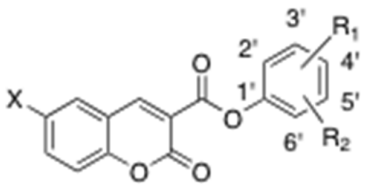
[a]ni: < 30% inhibition at 50 μM.

Table 4. Inhibition of KLK5, KLK7, KLK14 and matriptase by naphthyl, quinolyl or isoquinolyl esters of coumarin-3-carboxylic acid at 37 ° C and pH 8.

		IC_{50} (μM) or *% inhibition at 50 μM*			
Y	Compound	KLK5	KLK7	KLK14	Matriptase
	**56**	7.0 ± 0.4	ni[a]	13 ± 1	ni
	**57**	ni	ni	ni	ni
	**58**	ni	ni	ni	ni
	**6**	ni	*50%*	ni	ni
	**8**	ni	*30%*	ni	ni

[a]ni: < 30% inhibition at 50 μM.

Table 5. Kinetic parameters (k_i/K_I) for the inactivation of human KLK7 by diversely substituted phenyl esters of 6-halomethylcoumarin-3-carboxylic acid at pH 8 and 37 °C.

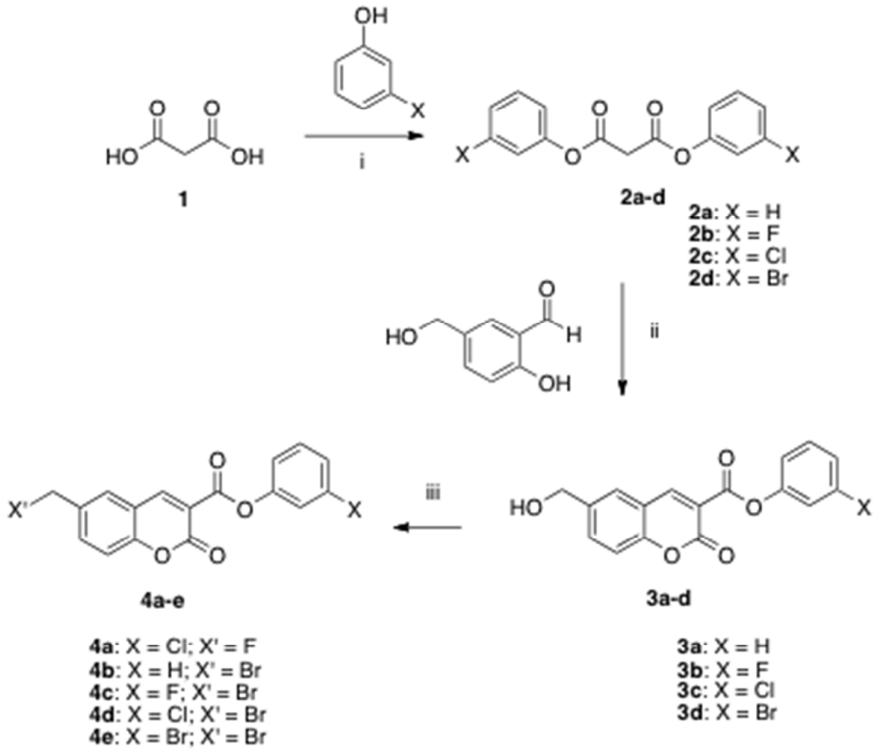


X	R_1	R_2	Compound	k_i/K_I^* ($M^{-1}.s^{-1}$)
-CH_2Cl	H	H	**15**	613
	2'-Cl	H	**16**	891
	3'-F	H	**17**	1198
	3'-Cl	H	**11**	1980
	3'-Br	H	**22**	1657
	3'-I	H	**23**	1471
	3'-CH_3	H	**25**	1134
	4'-Cl	H	**31**	1557
	4'-CH_3	H	**32**	569
	2'-Cl	3'-Cl	**34**	2330
	2'-F	5'-F	**35**	97
	2'-Cl	5'-Cl	**36**	3218
	2'-Br	5'-F	**37**	563
	2'-Cl	6'-Cl	**40**	1130
	3'-Cl	5'-Cl	**42**	5443
	3'-Cl	5'-OCH_3	**43**	697
-CH_2Br	H	H	**4b**	649
	3'-F	H	**4c**	572
	3'-Cl	H	**4d**	1424
	3'-Br	H	**4e**	1205

*k_i/K_I ratios were evaluated through at least three independent experiments; standard errors ≤ 10%.

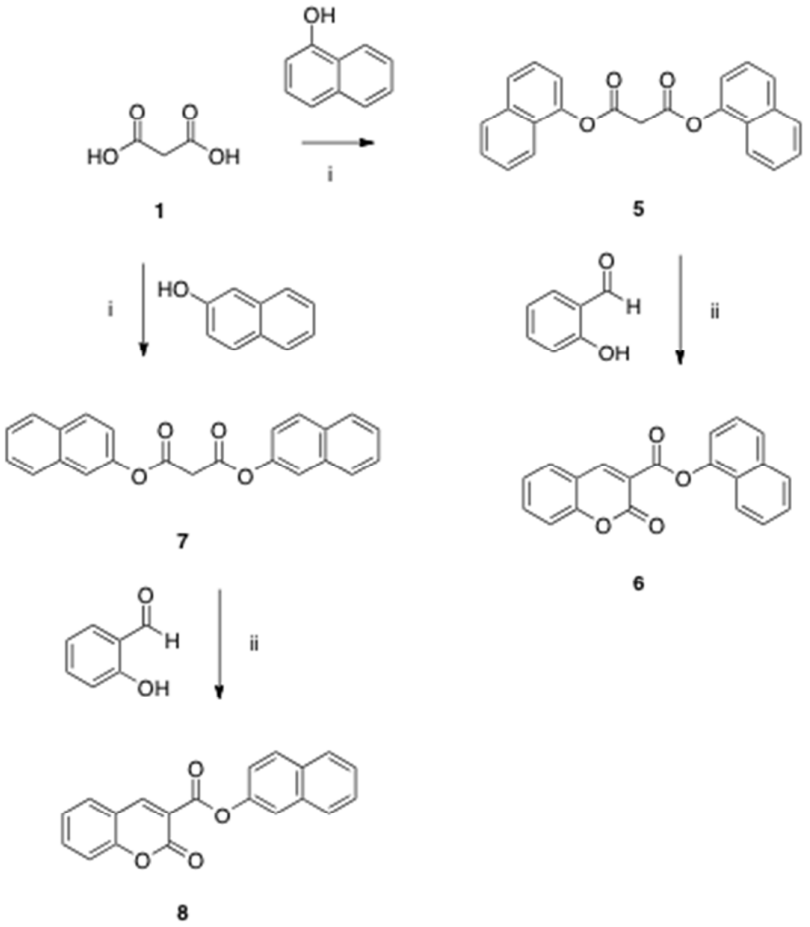
SCHEMES

Scheme 1. Synthesis of 6-(halomethyl)-coumarin-3-carboxylic acid derivatives **4a**-**e**.



Reagents: i: 3-halophenol (2 eq.), $OPCl_3$ (2 eq.), 100 °C, 90 min; ii: 5-hydroxymethyl-salicylaldehyde (0.7 eq.), piperidine, HOAc, dioxane, r.t., 30 min.; iii: X' = F: Ishikawa's reagent (2.5 eq.), CH_2Cl_2, 0 °C, 30 min., then reflux, 90 min.; X' = Br: $SOBr_2$ (1.15 eq.), pyridine (1 eq.), CH_2Cl_2, reflux, 90 min.

Scheme 2. General synthetic pathway to naphthyl 2-oxo-2*H*-1-benzopyran-3-carboxylates **6** and **8**.



Reagents: i: naphtol (2 eq.), $OPCl_3$ (2 eq.), 100 °C, 90 min. ii: salicylaldehyde (0.7 eq.), piperidine, HOAc, dioxane, r.t., 30 min.

FIGURES

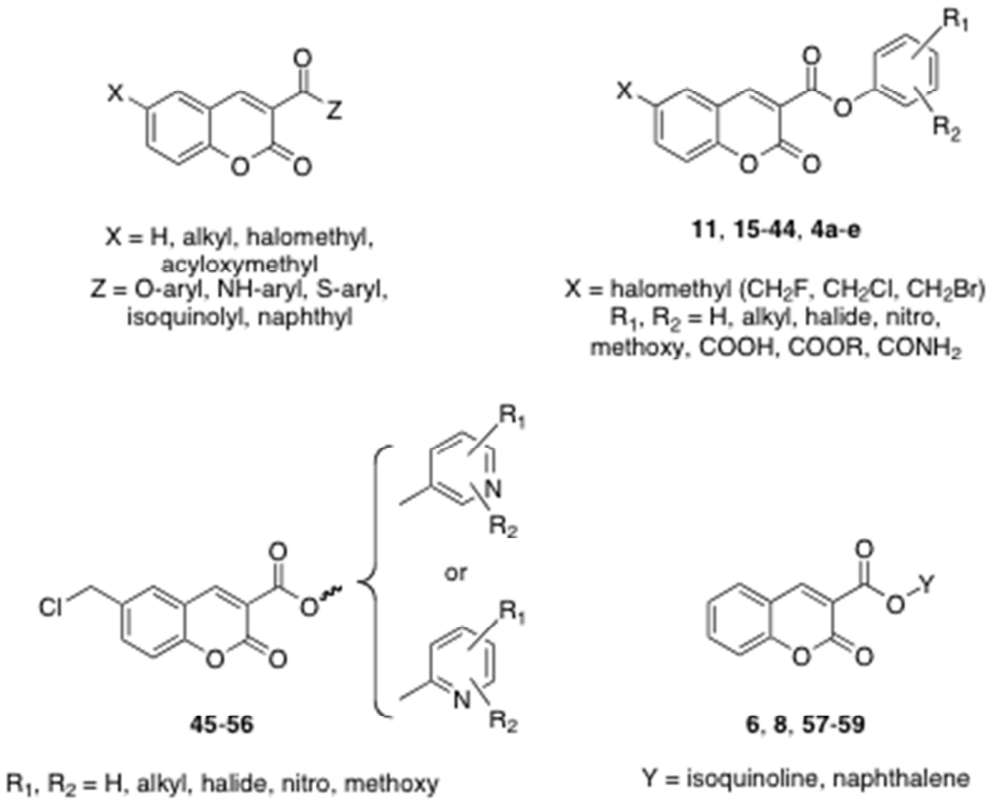


Figure 1. General chemical structures of previously and newly synthesized coumarin derivatives.

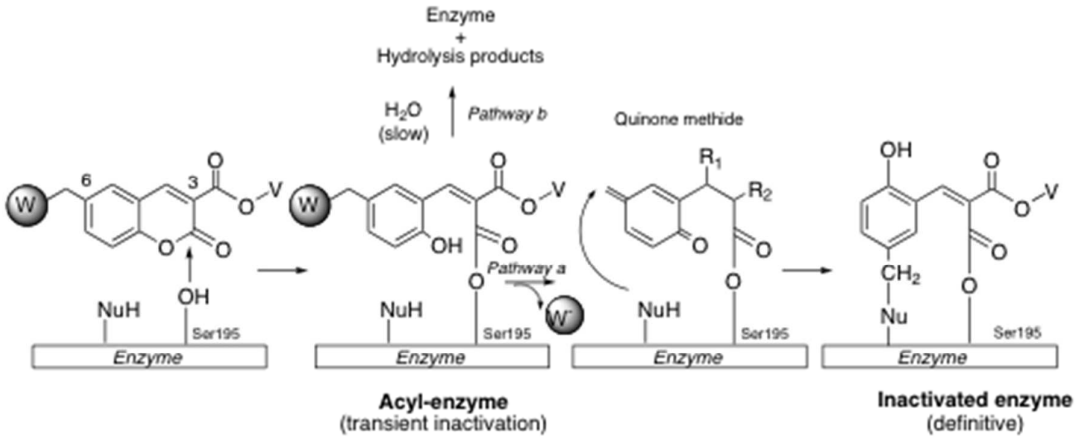


Figure 2. Postulated mechanisms for the inhibition of serine proteases by coumarin-3-carboxylic acid derivatives. NuH: nucleophile. Pathway a: suicide-type inactivation by the inhibitor acting as a suicide substrate. Pathway b: transient inactivation by formation of a stable acyl-enzyme (alternate subtrate-inhibitor). W: good leaving group; V: aryl or pyridyl group.

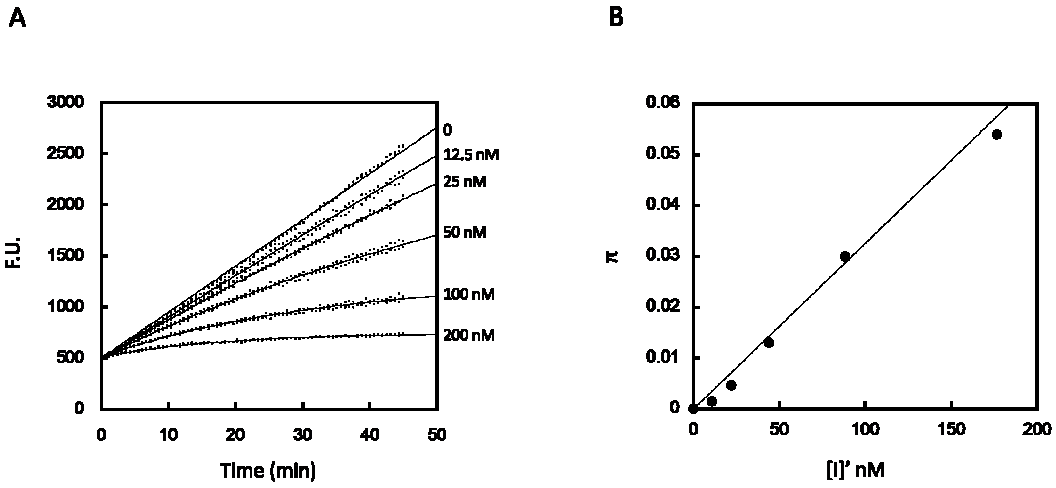


Figure 3. Inactivation of KLK7 by compound **43** characterized by the continuous kinetic method at pH 8 and 37 °C. A. Fluorescent product formed over time in the presence of different concentrations of compound **43** ($[E]_0$ = 7,6 nM; $[S]_0$ = 40 μM). B. Secondary graph for the determination of k_i/K_I. The expressions of π and [I]' are given in equation 4.

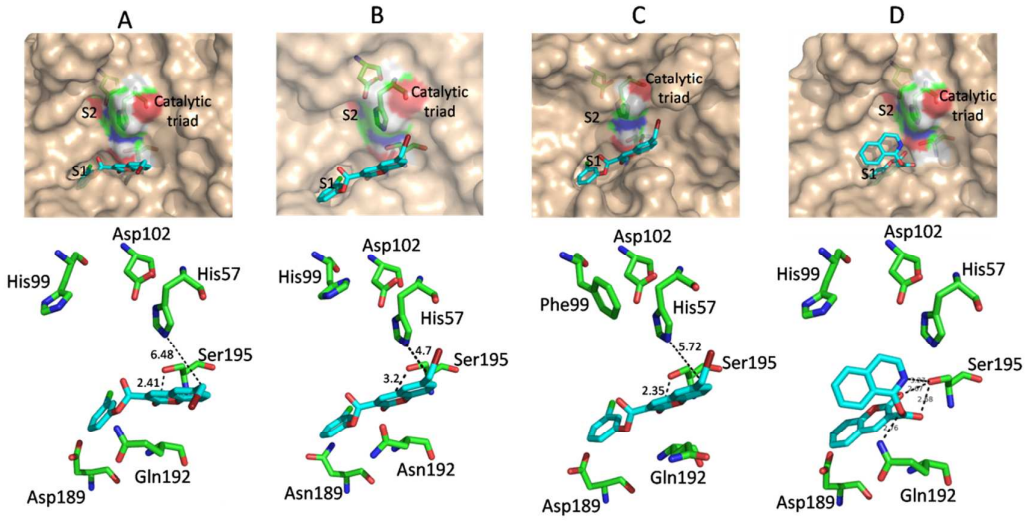


Figure 4. Comparison of possible binding modes of the suicide inhibitor **4d** in the active site of KLK5 (A), KLK7 (B) and matriptase (C), and of the competitive inhibitor **57** in the active site of KLK5. The enzyme structures were retrieved from PDB (file 2PSX for KLK5[51], file 2QXH for KLK7[52] and file 1EAX for matriptase[53]). The protease surfaces are colored in light brown and the ligands are shown in stick representation. Subsites S1 and S2 are indicated. The chymotrypsinogen numbering is used; the residues Ser195, His57 and Asp102 form the catalytic triad. The distances (Å) between the oxygen atom of the Ser195 OH group and the carbon atom of the lactonic carbonyl group of the coumarin ring, and the nitrogen atom of the His57 imidazole ring and the carbon atom of the CH_2Br substituent are indicated (A, B and C). The hydrogen bond network formed between inhibitor **57** and the KLK5 active-site residues is also indicated (D).

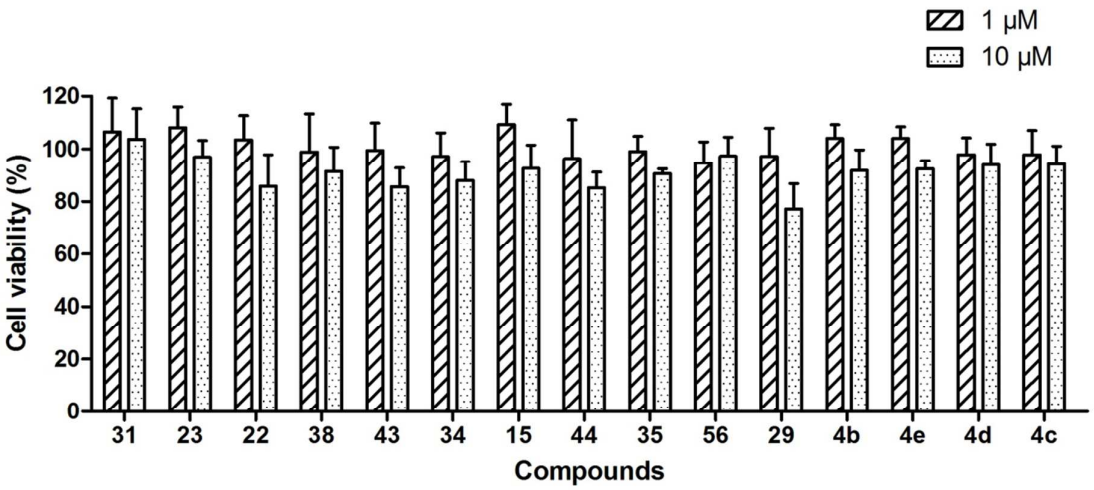


Figure 5. Evaluation of cytotoxicity of the coumarinic derivatives at 1 and 10 µM on healthy human keratinocytes after 48 h treatment. Results are mean +/- SD of five experiments performed in triplicate.

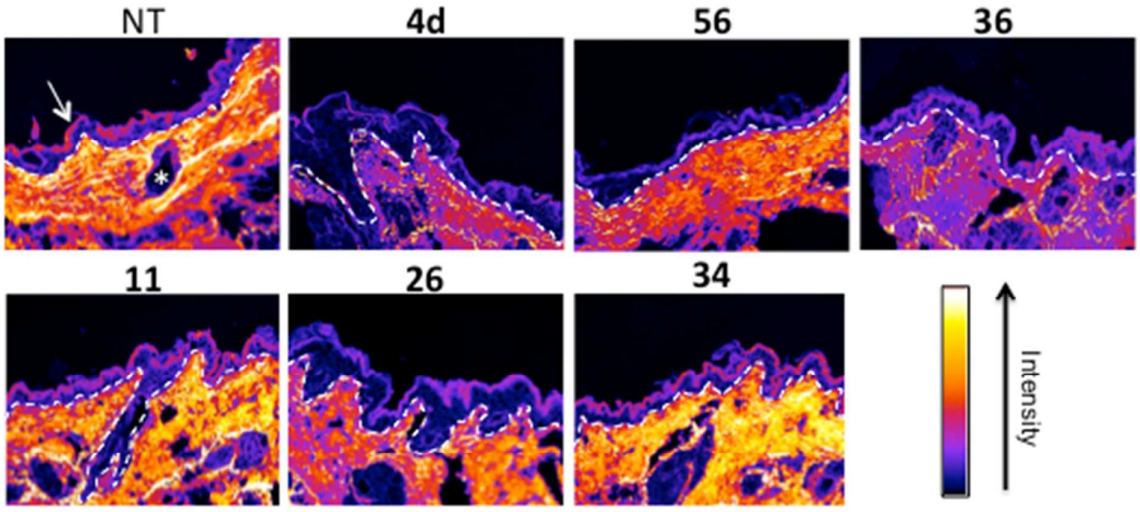


Figure 6. *In situ* zymography studies of inhibitory effects of molecules **4d**, **56**, **36**, **11**, **26** and **34** on protease activity of skin sections of Tg-h*KLK5* mice (NT, non treated). [Compounds] = 5 µM. The fluorescence intensity is correlated with the cleavage of the substrate (casein-FITC). Fluorescence intensity data was transformed to color gradient (as shown) using ImageJ software. Dashed white lines represent the dermal-epidermal junction, arrow the *stratum corneum* and asterisk a hair follicle.

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