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Discovery of Novel Guanidinophenylpyrazole Human Acrosin Inhibitors by Molecular Hybridization

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Abstract

Human acrosin is a promising target for male contraceptives. A series of novel guanidinophenylpyrazole derivatives were rationally designed by molecular hybridization of the phenylpyrazole and guanidinobenzoate inhibitors. Most of the target compounds showed potent human acrosin inhibitory activities. In particular, compound **F3** was a highly active human acrosin inhibitor (IC_{50} = 1.26 μM) with good selectivity over trypsin. Molecular docking studies revealed that compound **F3** formed hydrophobic and hydrogen bonding interactions with the active site of human acrosin. Compound **F3** represents a promising lead compound for the development of novel male contraceptives.

Introduction

Birth control, also known as contraception and fertility control, is an important global issue because of unintended pregnancy and overpopulation. It is highly desirable to develop effective contraceptives with good safety profile.[1] The development of hormonal-based oral contraceptives for women represents an important breakthrough for preventing pregnancies. Although women contraceptives have protected millions of couples worldwide from unwanted pregnancy and allowed them to take control over their reproduction, most of them have serious side effects, such as breakthrough menstrual bleeding, weight gain, breast tenderness and headaches.[2] In contrast, the development of male contraceptive has lagged far behind and there is no effective drug available in clinic. The discovery of effective, orally active, safe and reversible male contraceptive remains a significant challenge.[3]

Human acrosin is a serine protease that is located in epididymis and plays an important role in the reproduction process.[4] Recent studies revealed that human acrosin affected the motility of sperm, dispersed acrosomal matrix and facilitated the penetration of spermatozoa into the egg.[5] The decrease of acrosin level can lead to infertility and contraception can be achieved through inhibition of acrosin activity.[6] Due to its special location and multi-function during fertilization, human acrosin is becoming an attractive target for developing novel male contraceptive agents. Up to now, several classes of small molecule human acrosin inhibitors, such as TLCK [7], DV-1006, *p*-aminobenzamidine (pAB) [8], isoxazolecarbaldehydes [9] (**Fig. 1**), have been reported. However, most of them showed relatively low inhibitorty activity and also had several drawbacks such as lack of selectivity, poor metabolic stability and high toxicity.

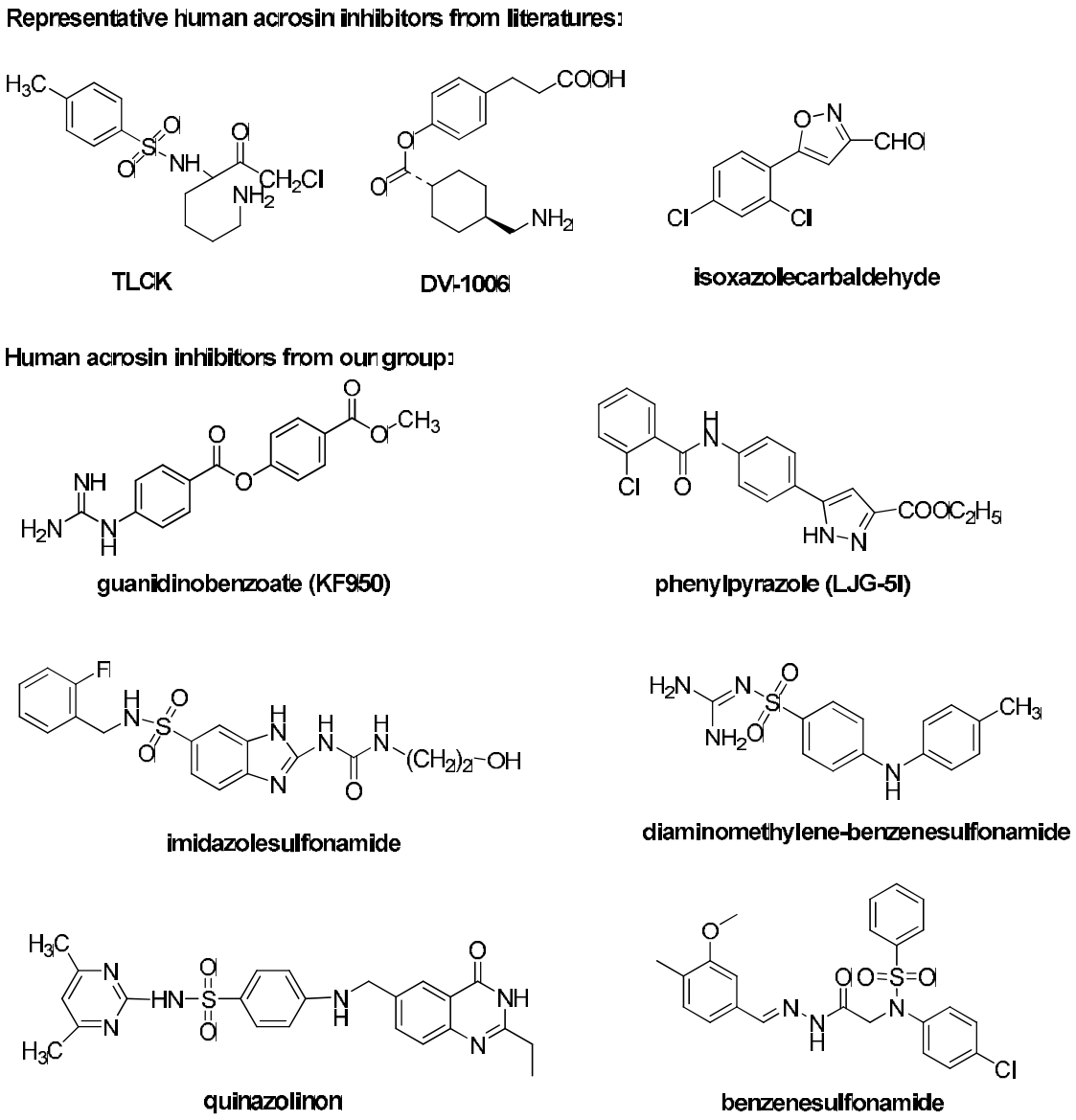


Figure 1. Chemical structures of representative acrosin inhibitors.

In 2000, Tranter *et al*. solved the crystal structures of ram and boar β-acrosins in complex with *p*-aminobenzamidine.[6] Based on these crystal structures, our group constructed structural model of human acrosin using homology modeling[10], which facilitated rational inhibitor design. A number of novel acrosin inhibitors, such as 4-guanidinobenzoates[10], phenylpyrazoles[11], imidazolesulfonamides[12],

benzenesulfonamides[13], quinazolinons[14] and diaminomethylene-benzenesulfonamides[15] were successfully designed by our group (**Fig. 1**). More recently, we discovered a series of potent phenylisoxazole acrosin inhibitors by structure-based inhibitor design.[16] Continuing our efforts in structure-based rational design of novel acrosin inhibitors, a molecular hybridization strategy was used to design a series of novel guanidinophenylpyrazole inhibitors (**Fig. 2**). *In vitro* assay indicated that several compounds showed potent human acrosin inhibitory activity.

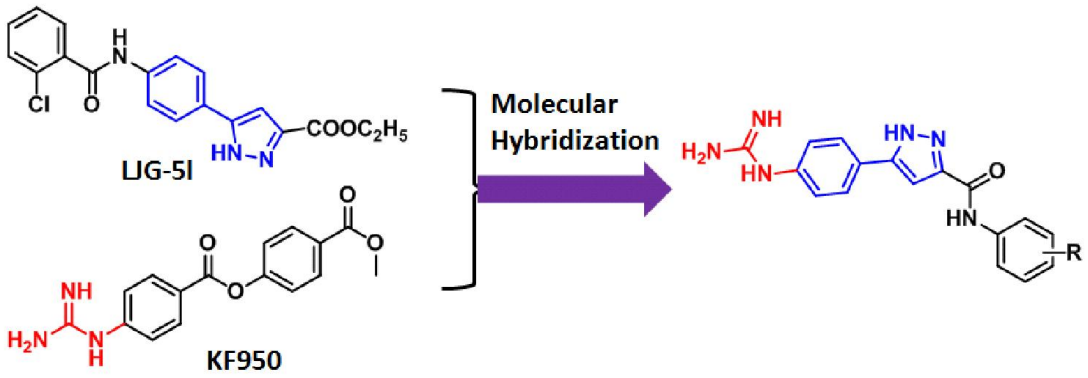


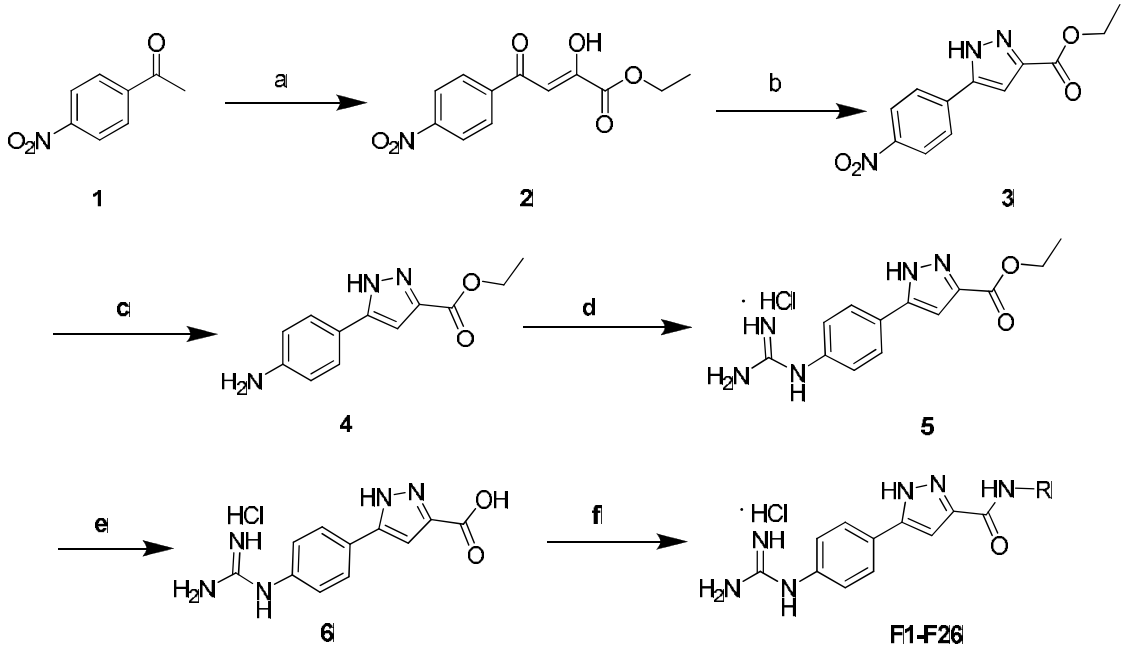
Figure 2. Rational design of guanidinophenylpyrazole human acrosin inhibitors by molecular hybridization of phenylpyrazole and guanidinobenzoate inhibitors.

Results and Discussion

Chemistry

The synthetic route of the target compounds was outlined in **Scheme 1**. Intermediate **4** was prepared according to our reported procedure.[11] Briefly, in the presence of EtONa, 1-(4-nitrophenyl)ethanone (**1**) was reacted with diethyl oxalate to afford intermediate (**2**) with good yield, which was condensed with hydrazine hydrate to give pyrazole **3**. The nitro group of compound **3** was reduced to amine in the presence of $SnCl_2$. The guanidino

intermediate **5** was prepared by reacting the amino group of compound **4** with NH_2CN using EtOH as the solvent and HCl as the acid. Catalyzed by NaOH, the ester group of compound **5** was hydrolyzed and finally condensed with various amine in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC·HCl) and *N*-hydroxybenzotriazole (HOBt) to give the target compounds **F1-F26**.



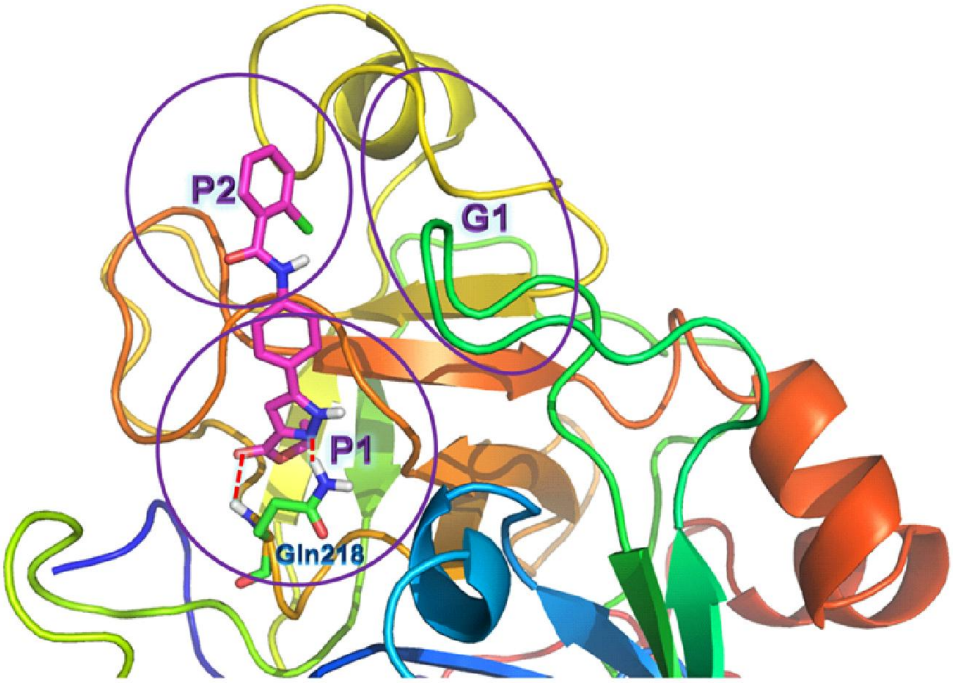
Scheme 1. Reagents and conditions: (a) $(COOC_2H_5)_2$, C_2H_5ONa, rt, 24 h, yield 90.6%; (b) NH_2NH_2, EtOH, reflux, 4 h, yield 88.1%; (c) $SnCl_2{\cdot}2H_2O$, EtOAc, reflux, 6 h, yield 82.3%; (d) H_2NCN, HCl, EtOH, reflux, 8h, yield 84.0%; (e) NaOH, CH_3OH/H_2O (1:1), reflux, 2 h, yield 92.4%; (f) RNH_2, EDC·HCl, HOBt, rt, 48h,yield 44%-84%.

Design Rationale of the Guanidinophenylpyrazole Human Acrosin Inhibitors

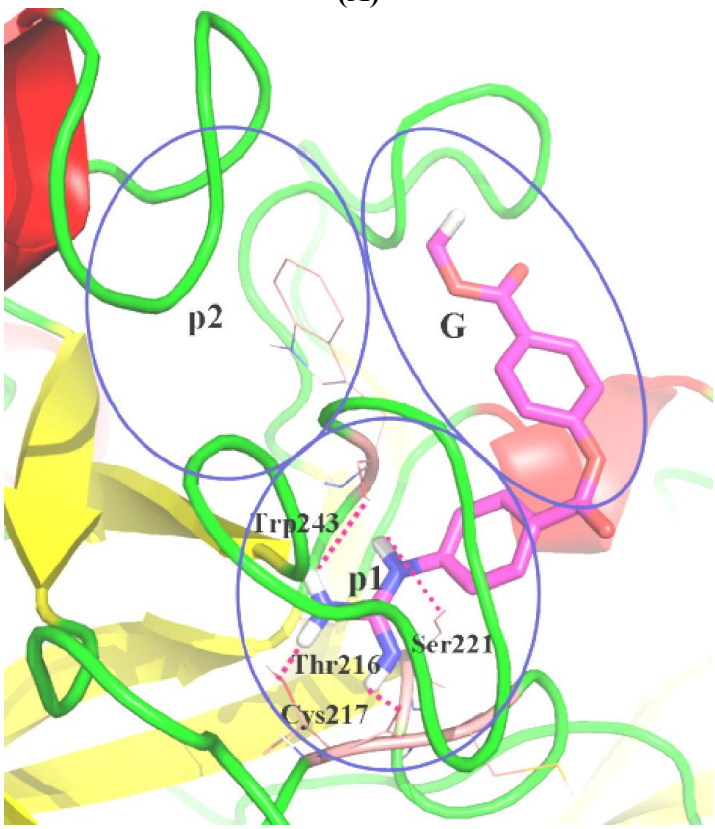
Computational analysis of the active site of human acrosin indicated that there three important sub-sites (P1, P2 and G) for inhibitor binding (**Fig. 3**).[17] Docking model of the phenylpyrazole inhibitor **LJG-5l** with human acrosin revealed that its phenylpyrazole scaffold was located in the P1 pocket and its ester carbonyl oxygen atom and pyrazole

nitrogen atom formed two hydrogen bonds with Thr216 and Gln218, respectively (**Fig. 3A**). Notably, phenylpyrazole inhibitor **LJG-5l** was only moderately active and its human acrosin inhibitory activity remained to be further improved. On the other hand, guanidinobenzoate inhibitor **KF950** was one of the most active human acrosin inhibitor reported to date. However, **KF950** has two metabolically unstable ester groups and tends to be hydrolyzed into inactive metabolites *in vivo*. Molecular docking studies revealed that its guanidyl group was an important pharmacophore, which formed four hydrogen bonds with Ser211, Thr216, Cys217 and Trp243, respectively (**Fig. 3B**).

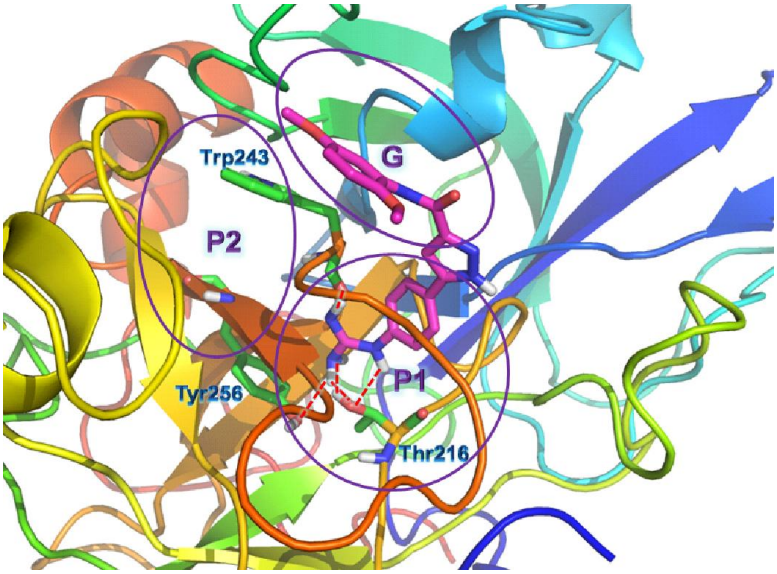
Molecular hybridization is a relatively new concept in drug design, which generates new class of hybrid compounds by combining pharmacophoric moieties of two or more known bioactive derivatives.[18] Herein, we envisioned that the phenylpyrazole pharmacophore of inhibitor **LJG-5l** and the guanidine pharmacophore of **KF950** could be combined to design a novel class of human acrosin inhibitors. As a result, a series of guanidinophenylpyrazole derivatives were designed and synthesized. In order to validate the design rationale, a representative guanidinophenylpyrazole derivative (**F3**) was docked into the active site of human acrosin using LibDock within Discovery Studio 2.5 software package (DS 2.5)[19]. As shown in **Fig. 3C**, the general conformation of compound **F3** was similar to that of KF950. Compound **F3** had suitable length to fit well with the P1 and G pocket. As expected, the guanidyl group was located deep into the P1 pocker and formed five hydrogen bonds with Trp243, Thr216, and Val225, respectively. Moreover, the dimethoxylphenyl group also formed π-π interaction with Trp243.



(A)



(B)



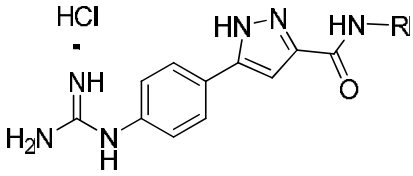
(C)

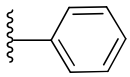
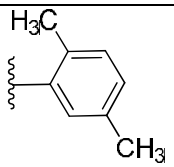
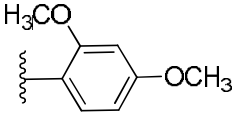
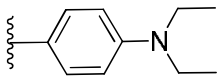
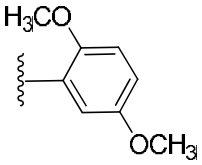
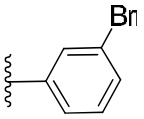
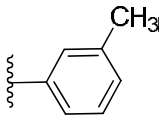
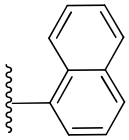
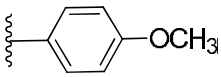
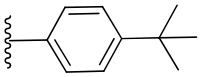
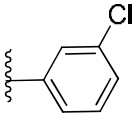
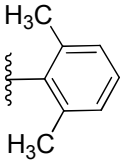
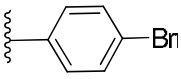
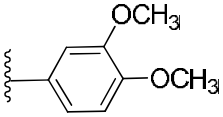
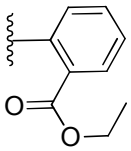
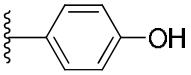
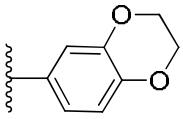
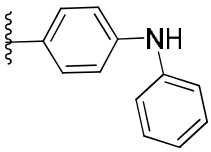
Figure 3. The binding modes of compounds LJG-**5l** (**A**), **KF950** (**B**), and **F3** (**C**) with the active site of human acrosin. The figures were generated using PyMol (http://pymol.sourceforge.net/).

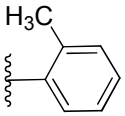
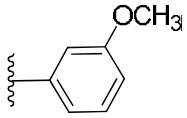
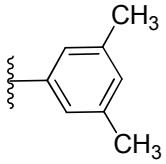
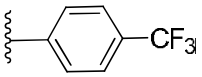
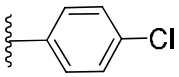
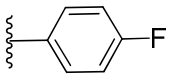
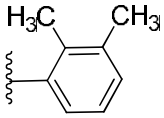
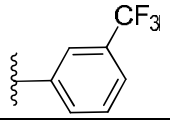
Human Acrosin Inhibitory Activities and Selectivity

As shown in **Table 1**, guanidinophenylpyrazole derivatives **F1-F26** generally showed moderate to good human acrosin inhibitory activities with IC_{50} values ranging from 1.26 μM to 28.33 μM. Notably, all the compounds were more potent than the phenylpyrazole lead compound **LJG-5l**. The IC_{50} value of 11 compounds, namely **F2**, **F3**, **F4**, **F8**, **F11**, **F14**, **F16**, **F20**, **F20**, **F22** and **F25**, was lower than 10 μM. In particular, compound **F3** showed the best activity (IC_{50} = 1.26 μM), which was more active than the lead compound **LJG-5l** (IC_{50} = 110 μM) and **KF950** (IC_{50} = 2.41 μM). In order to investigate the selectivity of compound **F3**, we determined its inhibitory activity against pig trypsin (a member of the serine protease family). The IC_{50} value was 2100 μM, indicating that compound **F3** exhibited good specificity toward human acrosin.

Table 1. Chemical structures and human acrosin inhibitory activities of the guanidinophenylpyrazole derivatives



Compds	R	IC_{50} (μM)	Compds	R	IC_{50} (μM)
F1		15.14	**F16**		7.47
F2		2.38	**F17**		25.91
F3		1.26	**F18**		13.11
F4		9.43	**F19**		24.38
F5		11.32	**F20**		5.87
F6		17.74	**F21**		4.32
F7		16.13	**F22**		3.44
F8		5.73	**F23**		28.33
F9		25.43	**F24**		26.77

F10		10.86	**F25**		2.42
F11		9.46	**F26**		16.55
F12		23.87	**5**		>100
F13		24.53	**6**		>100
F14		8.45	**KF950**		2.41
F15		23.74	**LJG-5l**		110

Structure-activity Relationships

First, the guanidinophenylpyrazole derivatives showed significantly better activity than the phenylpyrazole lead compound **LJG-5l**, highlighting the importance of the guanidine group. Second, ester intermediate **5** and carboxylic acid intermediate **6** were inactive, indicating that the substituted aromatic amide group was also important for the human acrosin inhibitory activity. Third, the type and position of the substitutions played an important role for the inhibitory activities. As compared with the unsubstituted phenyl derivative **F1** (IC_{50} = 15.14 μM), the introduction of a halogen atom (**F7**, **F12**, **F13**), hydroxyl (**F23**), trifluoromethyl (**F26**), or N,N-diethylamine group (**F17**) on the *ortho*-position of the phenyl group led to the decrease of the inhibitory activities. Moreover, the trifluoromethyl group (**F15**, IC_{50} = 23.74 μM) and chloro substitution (**F15**, IC_{50} = 17.74 μM) on the *meta*-position was also unfavorable for the activity. In contrast, improvement of the activity was observed for the 4-*tert*-butyl (**F20**, IC_{50} = 5.87 μM) and

4-methoxyl (**F5**, IC_{50} = 11.32 μM) derivatives. Movement of the 4-methoxyl group of compound **F5** to the *meta*-position (**F25**, IC_{50} = 2.42 μM) led to significant increase of activity. Interestingly, compounds with di-methoxyl substitutions, namely **F2**, **F3**, and **F22**, showed good inhibitory activities at low micromolar concentrations. Among them, the 2,5-dimethoxyl derivative **F3** (IC_{50} = 1.26 μM) was the most active compound in this series. When the 2,5-dimethoxyl group of compound **F3** was replaced by the 2,5-dimethyl group, compound **F16** (IC_{50} = 7.47 μM) showed decreased activity. The same trend was observed for the 3-methoxyl derivative **F25** (IC_{50} = 2.42 μM) and 3-methyl derivative **F4** (IC_{50} = 9.43 μM). Last, when the phenyl group was enlarged to dihydrobenzodioxine (**F9**) and naphthalene (**F19**) or extended to phenylaniline (**F24**), the human acrosin inhibitory activity was also decreased.

Conclusion

In summary, a new molecular hybridization strategy was successfully used to design novel guanidinophenylpyrazole human acrosin inhibitors. Most of the target compounds showed good human acrosin inhibitory activities. In particular, compound **F3** was discovered as a highly potent human acrosin inhibitor with good selectivity toward trypsin. Molecular docking studies revealed that it formed hydrophobic and hydrogen bonding interactions with the active site of human acrosin. Compound **F3** represented a good lead structure to develop novel male contraceptives. Further structural optimization studies are in progress.

Experimental Section

Chemistry

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300, 500 or 600

spectrometer with TMS as an internal standard and *d*6-DMSO as the solvent. Chemical shifts (δ values) and coupling constants (*J* values) are given in ppm and Hz, respectively. ESI mass spectra were performed on an API-3000 LC-MS spectrometer. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Silica gel column chromatography was performed with Silica gel 60 G (200-300 mesh, Qindao Haiyang Chemical, China). Commercial solvents were used without any pretreatment.

Chemical synthesis of ethyl 5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxylate hydrochloride (5)

Compound **4** [11] (23.1 g, 0.1 mol) was dissolved in EtOH (150 mL) under reflux and was added NH_2CN (12.6 g, 0.15 mol). Then, concentrated HCl (3.6 mL) was added and the resulting mixture was refluxed for 8 h. The mixture cooled down to room temperature and the solids were collected and filtrated to afford compound **5** as yellow solids (26.0 g, yield 84.0%). ^{1}H NMR (300 MHz, DMSO) δ 10.19 (s, 1H), 7.92 (d, *J* = 8.5 Hz, 2H), 7.63 (br s, 4H), 7.29 (d, *J* = 8.7 Hz, 3H), 4.30 (q, *J* = 7.0 Hz, 2H), 1.31 (t, *J* = 7.1 Hz, 3H). MS (ESI) m/z: 274 (M+1).

Chemical synthesis of 5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxylic acid hydrochloride (6)

Compound **5** (15.5 g, 0.05 mol) was dissolved in MeOH/H_2O (100 mL, MeOH: H_2O = 1:1) under reflux and NaOH (2.5g, 0.108 mol) was added. The resulting mixture was refluxed for 3 h and cooled down to room temperature. The pH value of the mixture was adjusted to 3 by HCl and the solids were collected and filtrated to afford compound **6** as white solids (13.0 g, yield 92.4 %). ^{1}H NMR (300 MHz, DMSO) δ 10.24 (s, 1H), 7.90 (d, *J* = 8.5 Hz, 2H), 7.65 (br s, 4H), 7.28 (d, *J* = 8.5 Hz, 2H), 7.22 (s, 1H). MS (ESI) m/z: 246 (M+1).

General procedure for the synthesis of target compound F1-F26.

Compound **6** (0.7g, 2.5 mmol) was dissolved in 5 mL of DMSO and was added various substituted aromatic amine (3.75 mmol), EDC·HCl (0.58g, 3 mmol), HOBt (0.4g, 3 mmol). The resulting mixture was stirred at room temperature for 48 h. Then, 20 mL of H_2O was added into the reaction mixture, the solid was filtrated and washed with H_2O (20 mL × 3). The resulting solid was dissolved in CH_3OH (30 mL), filtrated and evaporated to dryness. The residue was purified by reverse silica gel column chromatography using 30% methanol/water as the eluent.

5-(4-Guanidinophenyl)-*N*-phenyl-1*H*-pyrazole-3-carboxamide hydrochloride (F1) White solid; yield: 80.5%.^{1}H NMR (600 MHz, DMSO) δ 10.20 (s, 1H), 8.44 (s, 1H), 8.02 (br s, 4H), 7.88 (d, *J* = 8.5 Hz, 2H), 7.81 (d, *J* = 7.7 Hz, 2H), 7.37 - 7.26 (m, 5H), 7.09 (t, *J* = 7.4 Hz, 1H). MS (ESI) m/z: 321 (M +1), 319 (M-1).

***N*-(2,4-Dimethoxyphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F2)** Pinky solid; yield: 73%.^{1}H NMR (600 MHz, DMSO) δ 9.24 (s, 1H), 8.06 (s, 1H), 7.92 (d, *J* = 8.4 Hz, 2H), 7.64 (br s, 4H), 7.33 (d, *J* = 8.5 Hz, 2H), 7.25 (s, 1H), 6.68 (d, *J* = 2.6 Hz, 1H), 6.55 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.88 (s, 3H), 3.76 (s, 3H). MS (ESI) m/z: 381 (M +1), 379 (M-1).

***N*-(2,5-Dimethoxyphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F3).** Pinky solid; yield: 76%. ^{1}H NMR (600 MHz, DMSO) δ 9.41 (s, 1H), 8.43 (s, 1H), 8.30 – 7.50 (br s,4H), 7.99 (d, *J* = 2.5 Hz, 1H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.29 (d, *J* = 8.6 Hz, 2H), 7.24 (s, 1H), 7.02 (d, *J* = 9.0 Hz, 1H), 6.65 (dd, *J* = 8.9, 3.1 Hz, 1H), 3.86 (s, 3H), 3.72 (s, 3H). MS (ESI) m/z: 381 (M +1), 379 (M-1).

5-(4-Guanidinophenyl)-*N*-(3-methylphenyl)-1*H*-pyrazole-3-carboxamide

hydrochloride (F4). White solid; yield: 84%. ^{1}H NMR (600 MHz, DMSO) δ 7.91 (d, *J* = 7.6 Hz, 2H), 7.72 – 7.35 (br s, 4H), 7.62 (m, 3H), 7.32 (d, *J* = 8.4 Hz, 2H), 7.22 (t, *J* = 7.8 Hz, 1H), 6.91 (d, *J* = 7.3 Hz, 1H), 2.30 (s, 3H). MS (ESI) m/z: 335 (M +1), 333(M-1).

5-(4-Guanidinophenyl)-*N*-(4-methoxyphenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F5). White solid; yield: 81%. ^{1}H NMR (600 MHz, DMSO) δ 10.09 (s, 1H), 8.44 (s, 1H), 8.30 – 7.40 (br s, 4H), 7.88 (d, *J* = 8.5 Hz, 2H), 7.70 (d, *J* = 9.0 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 3H), 6.91 (d, *J* = 9.1 Hz, 2H), 3.73 (s, 3H). MS (ESI) m/z: 351 (M +1), 349 (M-1).

***N*-(3-Chlorophenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F6).** White solid; yield: 74%. ^{1}H NMR (600 MHz, DMSO) δ 10.42 (s, 1H), 8.02 (t, *J* = 2.0 Hz, 1H), 7.90 (d, *J* = 8.6 Hz, 2H), 7.78 (m, 1H), 7.52 (br s, 4H), 7.37 (t, *J* = 8.1 Hz, 2H), 7.31 (d, *J* = 8.6 Hz, 2H), 7.17 – 7.12 (m, 1H). MS (ESI) m/z: 355 (M +1), 353 (M-1).

***N*-(4-Bromophenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F7).** White solid; yield: 77%. ^{1}H NMR (600 MHz, DMSO) δ 10.34 (s, 1H), 8.46 (s, 1H), 8.13 (br s, 4H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.81 (d, *J* = 8.9 Hz, 2H), 7.52 (d, *J* = 8.9 Hz, 2H), 7.29 (d, *J* = 8.4 Hz, 3H). MS (ESI) m/z: 399 (M +1).

Ethyl 2-(5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamido)benzoate hydrochloride (F8). White solid; yield: 64%. ^{1}H NMR (600 MHz, DMSO) δ 12.15 (s, 1H), 8.76 (d, *J* = 8.3 Hz, 1H), 8.44 (s, 1H), 8.40 – 7.90 (br s, 4H), 8.03 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.86 (d, *J* = 8.6 Hz, 2H), 7.69 – 7.64 (m, 1H), 7.28 (d, *J* = 8.6 Hz, 2H), 7.24 (s, 1H), 7.21 – 7.17 (m, 1H), 4.38 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 7.1 Hz, 3H). MS (ESI) m/z: 393 (M +1), 391 (M-1).

***N*-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carb oxamide hydrochloride (F9).** Pinky solid; yield: 79%. ^{1}H NMR (600 MHz, DMSO) δ 10.10 (s, 1H), 7.91 (d, *J* = 8.3 Hz, 2H), 7.63 (s, 4H), 7.44 (d, *J* = 2.4 Hz, 1H), 7.32 (d, *J* = 8.5 Hz, 3H), 7.25 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.81 (d, *J* = 8.7 Hz, 1H), 4.21 (dd, *J* = 12.8, 5.1 Hz, 4H). MS (ESI) m/z: 379 (M +1), 377 (M-1).

5-(4-Guanidinophenyl)-*N*-(2-methylphenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F10). White solid; yield: 75%. ^{1}H NMR (600 MHz, DMSO) δ 7.93 (d, *J* = 6.5 Hz, 2H), 7.66 (br s, 5H), 7.34 (d, *J* = 8.5 Hz, 3H), 7.27 (d, *J* = 7.5 Hz, 1H), 7.22 (t, *J* = 7.6 Hz, 1H), 7.14 (t, *J* = 7.0 Hz, 1H), 2.28 (s, 3H). MS (ESI) m/z: 335 (M +1), 333 (M-1).

***N*-(3,5-Dimethylphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F11).** White solid; yield: 82%. ^{1}H NMR (600 MHz, DMSO) δ 9.96 (s, 1H), 8.44 (s, 1H), 8.25 – 7.50 (br s, 4H) 7.87 (d, *J* = 8.5 Hz, 2H), 7.43 (s, 2H), 7.30 (d, *J* = 8.6 Hz, 3H), 6.74 (s, 1H), 2.25 (s, 6H). MS (ESI) m/z: 349 (M +1), 347 (M-1).

***N*-(4-Chlorophenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F12).** White solid; yield: 67%. ^{1}H NMR (600 MHz, DMSO) δ 10.35 (s, 1H), 8.45 (s, 1H), 8.08 (br s, 4H), 7.86 (dd, *J* = 8.7, 5.3 Hz, 4H), 7.39 (d, *J* = 8.9 Hz, 2H), 7.32 – 7.25 (m, 3H). MS (ESI) m/z: 355 (M +1), 353 (M-1).

***N*-(4-Fluorophenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F13).** White solid; yield: 74%. ^{1}H NMR (600 MHz, DMSO) δ 10.30 (s, 1H), 8.43 (s, 1H), 7.89 (d, *J* = 8.4 Hz, 2H), 8.06 – 7.60 (br s, 4H), 7.84 (dd, *J* = 8.3, 5.2 Hz, 2H), 7.31 (t, *J* = 9.4 Hz, 3H), 7.18 (t, *J* = 8.9 Hz, 2H). MS (ESI) m/z: 339 (M +1), 337 (M-1).

***N*-(2,3-Dimethylphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F14).** White solid; yield: 84%. ^{1}H NMR (600 MHz, DMSO) δ 9.79 (s, 1H), 7.91 (d, *J* = 8.4 Hz, 2H), 7.66 (s, 4H), 7.32 (d, *J* = 8.6 Hz, 3H), 7.26 (s, 1H), 7.14 – 7.03 (m, 2H), 2.27 (s, 3H), 2.13 (s, 3H). MS (ESI) m/z: 349 (M +1), 347 (M-1).

5-(4-Guanidinophenyl)-*N*-(3-(trifluoromethyl)phenyl)-1*H*-pyrazole-3- carboxamide hydrochloride (F15). White solid; yield: 64%. ^{1}H NMR (600 MHz, DMSO) δ 10.55 (s, 1H), 8.43 (s, 1H), 8.30 (s, 1H), 8.28 – 7.90 (br s, 4H), 8.11 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 8.5 Hz, 2H), 7.58 (t, *J* = 8.0 Hz, 1H), 7.43 (d, *J* = 7.7 Hz, 1H), 7.30 (t, *J* = 7.0 Hz, 3H). MS (ESI) m/z: 389 (M +1), 387 (M-1).

***N*-(2,5-Dimethylphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F16).** White solid; yield: 82%. ^{1}H NMR (600 MHz, DMSO) δ 9.59 (s, 1H), 8.44 (s, 1H), 8.08 (br s, 4H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.37 (s, 1H), 7.27 (d, *J* = 8.6 Hz, 2H), 7.25 (s, 1H), 7.13 (d, *J* = 7.7 Hz, 1H), 6.94 (d, *J* = 7.7 Hz, 1H), 2.28 (s, 3H), 2.21 (s, 3H). MS (ESI) m/z: 349 (M +1), 347 (M-1).

***N*-(4-(Diethylamino)phenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F17).** White solid; yield: 52%. ^{1}H NMR (600 MHz, DMSO) δ 10.10 (s, 1H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.91 (s, 2H), 7.70 (d, *J* = 8.4 Hz, 2H), 7.58 (br s, 4H), 7.55 – 7.50 (m, 2H), 7.42 – 7.37 (m, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 3.41 – 3.30 (br m, 4H), 1.06 (t, *J* = 7.1 Hz, 6H). MS (ESI) m/z: 392 (M +1), 390 (M-1).

***N*-(3-Bromophenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F18).** White solid; yield: 77%. ^{1}H NMR (600 MHz, DMSO) δ 10.44 (s, 1H), 8.16 (t, *J* = 1.9 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 2H), 7.84 (d, *J* = 8.4 Hz, 1H), 7.66 (br s, 4H), 7.38 (s, 1H), 7.35 – 7.23 (m, 4H). MS (ESI) m/z: 399 (M +1), 397 (M-1).

5-(4-Guanidinophenyl)-*N*-(naphthalen-1-yl)-1*H*-pyrazole-3-carboxamide hydrochloride (F19). Pinky solid; yield: 54%. ^{1}H NMR (600 MHz, DMSO) δ 10.33 (s, 1H), 8.44 (s, 1H), 8.27 – 7.98 (m, 4H), 7.98 (m, 2H), 7.89 (d, *J* = 8.5 Hz, 2H), 7.85 (d, *J* = 8.3 Hz, 1H), 7.70 (d, *J* = 7.3 Hz, 1H), 7.55 (m, 3H), 7.38 (s, 1H), 7.30 (d, *J* = 8.5 Hz, 2H). MS (ESI) m/z: 371 (M +1), 369 (M-1).

***N*-(4-(*Tert*-butyl)phenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F20).** White solid; yield: 69%. ^{1}H NMR (300 MHz, DMSO) δ 10.11 (s, 1H), 8.44 (s, 1H), 8.40 – 7.90 (br s,4H), 7.86 (d, *J* = 8.3 Hz, 2H), 7.70 (d, *J* = 8.7 Hz, 2H), 7.35 (d, *J* = 8.8 Hz, 2H), 7.28 (d, *J* = 8.6 Hz, 3H), 1.27 (s, 9H). ^{13}C NMR (300MHz, DMSO) δ 170.15, 164.95, 161.41, 158.47, 148.26, 139.05, 138.40, 129.70, 128.69 (2C), 127.55 (2C), 126.27 (2C), 122.37 (2C),105.19, 36.36 , 33.51 (3C). MS (ESI) m/z: 377 (M +1), 375 (M-1).

***N*-(2,6-Dimethylphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F21).** White solid; yield: 86%. ^{1}H NMR (500 MHz, DMSO) δ 9.76 (s, 1H), 8.43 (s, 1H), 8.25 – 7.55 (br s,4H), 7.88 (d, *J* = 7.8 Hz, 2H), 7.31 (d, *J* = 7.8 Hz, 3H), 7.12 (s, 3H), 2.20 (s, 6H). MS (ESI) m/z: 349 (M +1), 347 (M-1).

***N*-(3,4-Dimethoxyphenyl)-5-(4-guanidinophenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F22).** Pinky solid; yield: 73%. ^{1}H NMR (500 MHz, DMSO) δ 10.16 (s, 1H), 8.39 (s, 1H), 8.27 – 7.75 (br s,4H), 7.90 (d, *J* = 8.2 Hz, 2H), 7.53 (d, *J* = 1.7 Hz, 1H), 7.42 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.31 (d, *J* = 8.3 Hz, 3H), 6.93 (d, *J* = 8.7 Hz, 1H), 3.75 (d, *J* = 7.3 Hz, 6H). MS (ESI) m/z: 381 (M +1), 379 (M-1).

5-(4-Guanidinophenyl)-*N*-(4-hydroxyphenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F23). White solid; yield: 44%. ^{1}H NMR (500 MHz, DMSO) δ 9.99 (s,

1H), 8.42 (s, 1H), 8.30 – 7.65 (br s,4H), 7.88 (d, *J* = 8.0 Hz, 2H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.30 (d, *J* = 7.9 Hz, 3H), 6.75 (d, *J* = 8.6 Hz, 2H). MS (ESI) m/z: 337 (M +1), 335 (M-1).

5-(4-Guanidinophenyl)-*N*-(4-(phenylamino)phenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F24). White solid; yield: 69%. ^{1}H NMR (500 MHz, DMSO) δ 10.06 (s, 1H), 8.45 (s, 1H), 8.10 (s, 1H), 8.07 – 7.75 (br s,4H), 7.89 (d, *J* = 8.1 Hz, 2H), 7.68 (d, *J* = 8.6 Hz, 2H), 7.29 (d, *J* = 8.1 Hz, 3H), 7.21 (t, *J* = 7.6 Hz, 2H), 7.06 (dd, *J* = 18.2, 8.3 Hz, 4H), 6.78 (t, *J* = 7.1 Hz, 1H). MS (ESI) m/z: 412 (M +1), 410 (M-1).

5-(4-Guanidinophenyl)-*N*-(3-methoxyphenyl)-1*H*-pyrazole-3-carboxamide hydrochloride (F25). White solid; yield: 81%. ^{1}H NMR (300 MHz, DMSO) δ 10.15 (s, 2H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.63 (br s, 4H), 7.53 (s, 1H), 7.45 (d, *J* = 8.1 Hz, 1H), 7.34 (d, *J* = 8.5 Hz, 2H), 7.25 (t, *J* = 8.2 Hz, 1H), 6.69 (dd, *J* = 8.2, 1.9 Hz, 1H), 3.76 (s, 3H). MS (ESI) m/z: 351(M +1), 349 (M-1).

5-(4-Guanidinophenyl)-*N*-(4-(trifluoromethyl)phenyl)-1*H*-pyrazole-3- carboxamide hydrochloride (F26). White solid; yield: 64%. ^{1}H NMR (300 MHz, DMSO) δ 10.64 (s, 1H), 10.23 (s, 1H), 8.11 (d, *J* = 8.5 Hz, 2H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.79 – 7.58 (m, 6H), 7.35 (d, *J* = 8.5 Hz, 2H). MS (ESI) m/z: 389 (M +1), 387 (M-1).

Molecular docking

Docking studies were performed using the Libdock method within DS 2.5 software package. Structural model of human acrosin was obtained from our previous report [10]. In the process of Libdock docking, the docking preferences parameter was set to "high quality" and the conformation method was set to "best". Other parameters were used as default.

Human acrosin inhibitory activity assay

The acrosin inhibitory activities of the purchased compound were determined by the method of Kennedy *et al* [20]. Briefly, 0.1 mL of liquefied human semen was carefully layered over 0.5 mL of 11% Ficoll solution containing NaCl (0.12 mol/L) and HEPES buffer (pH = 7.4, 0.025 mol/L) in a conical centrifuge tube and centrifuged at 1000 g for 30 min. After carefully removing the seminal plasma and Ficoll solution without disturbing the sperm pellet, 0.1 mL of test compound solution (0.1 mL of buffer in control tubes) was added and mixed. TLCK was used as positive control. Finally, 0.8 mL of buffer–substrate solution (pH = 8.0) containing *N*-α-benzoyl-*L*-arginine p-nitroanilide (BAPNA, 0.1%), Triton X-100 (0.01%), HEPES buffer (0.055 mol/L) and NaCl (0.055 mol/L) was added, mixed thoroughly and incubated at 37.8℃ for 3 h. At the end of the incubation period, 100 mL of TLCK solution (0.5 mol/L) was added to stop the reaction and the tubes were centrifuged at 1000 g for 30 min. The optical density (OD) of the supernatant was read at 410 nm in a spectrophotometer. The inhibition activities were calculated according to the OD values. The inhibitory activity of compound **F3** against trypsin were determined according to our reported procedures [13].

Supplementary Material: Original spectra of the key compound F3.

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