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DESIGN, SYNTHESIS, AND EVALUATION OF ORALLY ACTIVE FIBRINOGEN INHIBITORS

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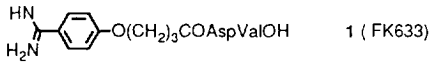
Abstract: Low molecular weight and orally active fibrinogen inhibitors are described. The compounds studied in this work were rationally designed based on a metabolic study of a peptidic fibrinogen inhibitor, 4-(4-amidinophenoxy)butanoylaspartylvaline (**1**, FK633), which led to the synthesis of a potent and orally active anti-platelet agent, 4-(4-amidinophenoxy)butanoylaspartylvalylthiomorpholine 1,1-dioxide (**3f**, FR158999).

Uncontrolled platelet aggregation and platelet adhesion to the subendothelium of damaged blood vessels causes life-threatening diseases such as myocardial infarction,[1] transient ischemic attack,[1] and unstable angina.[2] Binding of a ArgGlyAsp (RGD) sequence in fibrinogen to activated glycoprotein IIb/IIIa (GPIIb/IIIa) on the surface of platelet is the final step in the platelet aggregation cascade, and is thought to be the most important step for platelet aggregation.[3] Thus, many fibrinogen inhibitors, RGD mimics, have been studied and reported to be clinically useful anti-platelet agents.[4] We have also reported a design using computer simulations and synthesis of low molecular weight and peptidic fibrinogen inhibitors, ω-(4-amidinophenoxy)alkanoylaspartylvaline derivatives, in a previous paper, which led to the synthesis of a potent anti-platelet agent, 4-(4-amidinophenoxy)-butanoylaspartylvaline (**1**, FK633).[5a] Compound **1** displayed potent *in vivo* anti-platelet activity, inhibiting platelet-dependent coronary artery thrombosis in dog following intravenous infusion,[6] which indicated that **1** is potentially clinically useful for acute treatment.

Scheme 1.



1 (FK633)

On the other hand, orally active anti-platelet drugs are often necessary for treatment of chronic diseases. Thus, we assessed the bio-availability of **1** in a follow-up study, in which anti-platelet activities of **1** were measured after oral administration in guinea pigs, rats, and dogs (Table 1).

Table 1. Inhibitory Activities *Ex Vivo* of 1 in Guinea Pigs, Rats, and Dogs After Oral Administration[7]

dogs				rats				guinea pigs			
dose, po	Inhibition (%)			dose, po	Inhibition (%)			dose, po	Inhibition (%)		
(mg/kg)	1	2	3 h	(mg/kg)	0.5	1	2 h	(mg/kg)	1	3	6 h
3.2	19±17*	30±13	10±6	10	24±6	18±6	4±7	0.32	70±19	–	–
32	58±7	29±5	18±13	20	45±8	33±8	20±17	1.0	99±15	34±20	–
								3.2	100±8	64±18	19±18

*mean ± standard derivation.

Interestingly **1** showed potent anti-platelet activity even at a dose of 0.32 mg/kg in guinea pigs although it is generally thought that peptidic compounds such as **1** are not effective after oral administration. The bio-availability of **1** in rats and dogs, however, is not sufficient; that is, **1** showed only weak anti-platelet activity

even at dosages of 20 mg/kg in rats and 32 mg/kg in dogs, respectively. Thus, our purpose in this study was focused on obtaining compounds which are effective after oral administration not only in guinea pigs but also in rats and dogs.

We here describe the rational design of novel peptidic and orally active fibrinogen inhibitors effective in guinea pigs, rats, and dogs, based on a hypothesis that the low-bioavailability of **1** in rats and dogs could due to hydrolysis by peptidase in intestinal juice. We also show their synthesis and anti-platelet activities *in vitro* and *ex vivo* after oral administration

Results and Discussion: The anti-platelet activity of **1** in rats after oral administration was increased 3 times[8] by co-administration of the known peptidase inhibitor, bacitracin.[9] Based on this result, we set a hypothesis at the beginning of this study that the low bio-availability of **1** in rats and in dogs arose from hydrolysis by peptidase in intestinal juice and/or cells; that is, **1** is decomposed in rats and in dogs after oral administration in intestinal juice and/or intestine cells, causing the low bio-availability in rats and dogs, while **1** is stable in that of guinea pigs, resulting in the high bio-availability in guinea pigs. In order to assess this supposition, we tested the stability of **1** in intestinal juice and homogenate of intestinal cells of rats and those of guinea pigs (Figure 1). This study showed that **1** was decomposed only in intestinal juice of rats while **1** was completely stable in that of guinea pigs and in homogenates of rats and guinea pigs, which supports our supposition[11] and indicated that design of novel **1** derivatives which are stable in intestinal juice of rats could afford the desired compounds.

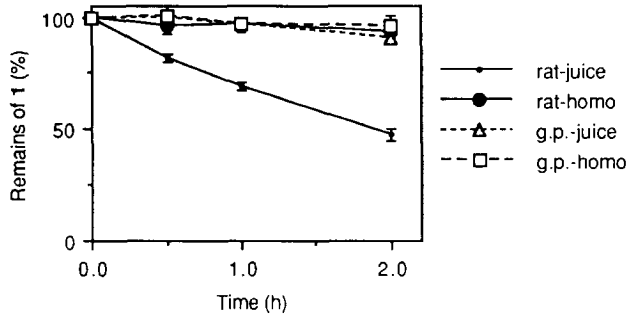


Figure 1. Stability of **1** at 37 °C in intestinal juice and homogenate of intestine of rats and guinea pigs.[10]

As regards to the structure of **1**, there are three moieties (**A**, **B**, and **C**) which could be responsible for hydrolysis in intestinal juice (Figure 2). In order to know the structure-stability relationships of these moieties for rational design of the desired compounds, we first synthesized some compounds whose carboxyl group (**C**) at the carboxy terminal (C-terminal) of **1**, which could be recognized by peptidases such as carboxypeptidases, was removed (**2a-b**) or replaced with carboxamide (**3a**), or whose amide bond (**A**), which could be easily hydrolyzed by peptidases, was replaced with ureido (**4**), and then tested their stabilities in intestinal juice of rats (Table 2).

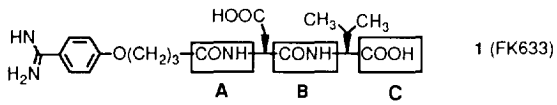


Figure 2. Putative candidates for being responsible for hydrolysis (**A**, **B**, and **C**) of **1** in intestinal juice of rats.

These studies showed that removal (**2a-b**) and replacement with carboxamide (**3a**) of a carboxyl moiety at the C-terminal are effective, while replacement of the amide moiety with ureido bond (**4a**) showed little effect. These

results suggested that the decomposition of **1** derivatives in intestinal juice may be due to peptidases which recognize the carboxyl moiety at the C-terminal and hydrolyzed the amide bond at position **B**.[11]

Table 2. Stability of **1** Derivatives in Intestinal Juice of Rats

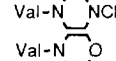
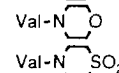
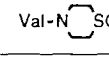
cmpd.	R*	Remains (%) (n=1) 0 h	2 h	5 h	7 h
1 (FK633)	Val-OH	100	61.3	25.6	19.2
2a	$NHCH_2CH(CH_3)_2$	100	80.4	83.6	86.2
2b	$NHCH_2CH_2Ph(4\text{-}OMe)$	100	100.5	105.7	NT
3a	$ValNH_2$	100	105.6	107.3	NT
4*	ValOH	100	63.8	45.1	NT

* See the Scheme 2.

Based on these results, we next synthesized several **2** and **3** derivatives, and tested their *in vitro* activities using human platelets because it was known[5a] that the anti-platelet activity of these derivatives depends upon the structure of the side chain etc. (Table 3).[12]

They all showed potent inhibitory activity with greater than micromolar IC_{50} values. Most of the inhibitory values are, however, weaker than that of **1**. However, two of the compounds possessing either morpholine (**3e**) and thiomorpholine 1,1-dioxide (**3f**) at the C-terminal exhibited potent anti-platelet activity with 10^{-8} (M) range IC_{50} values, which are two or three times more potent than that of **1**. Because *in vitro* potency is sometimes species dependent, compound **1**, **3e** and **3f** were also assessed in the dogs (Table 3); within experimental error the potency was the same.

Table 3. *In vitro* Activities[12] and Yields of Compounds Studied in This Study

	R[a]	Anti-platelet Activities(*in vitro*) human, ADP IC_{50} (μM)	dog, ADP IC_{50} (μM)	ratio (dog/human)	Yield[b] (%)
1	ValOH	0.10±0.01[5a]	0.38±0.12	3.8	43.5
2a	$NHCH_2CH(CH_3)_2$	1.2±0.3	-		85.5
2b	$NHCH_2CH_2Ph(4\text{-}OCH_3)$	0.44±0.11	-		71.2
2c	$NHCH(CH_3)CH(CH_3)_2$	1.4±0.5	-		75.6
2d	$NHCH_2CH_2Ph(4\text{-}O\text{-}iPr)$	0.37±0.09	-		88.2
2e	$NHCH_2CH_2Ph(4\text{-}OEt)$	0.34±0.12	-		55.1
2f	$NHCH_2CH_2Ph(3,4\text{-}diOCH_3)$	0.54±0.18	-		31.8
3a	$Val\text{-}NH_2$	0.67±0.20	-		35.3
3b	$Val\text{-}NH\text{-}cC_6H_{11}$	0.21±0.10	-		11.5
3c	$Val\text{-}NEt_2$	0.25±0.08	-		22.2
3d		0.18±0.06	-		42.0
3e		0.079±0.020	0.14±0.06	1.8	38.1
3f		0.030±0.010	0.11±0.03	3.7	73.8

[a] See the Scheme 2. [b] Yield of the last reaction in the Scheme 2.

We assessed *ex vivo* activities of these two compounds (**3e**, **3f**) in dogs, rats, and guinea pigs to evaluate their bio-availabilities (Table 4). Gratifyingly they showed potent *ex vivo* activity after oral administration not only in guinea pigs but also in dogs and rats; that is, **3e** and **3f** are effective at 3.2 mg/kg (po) both in dogs and rats while **1** showed only a weak anti-platelet activity even at the dosage of 20 mg/kg in rats and at the dosage of 32 mg/kg in dogs. The effect of **3e** and **3f** in guinea pigs are similar to that of **1**.

These potent *ex vivo* activities in three different species indicated that the novel peptidic fibrinogen inhibitors,

3e and **3f**, are orally active and could be clinically useful for treatment of chronic diseases. Finally we selected compound **3f** (FR158999) to be further evaluated in a clinical study because the *ex vivo* activity in dogs of **3f** was slightly more potent than that of **3e**.

Table 4. *Ex Vivo* Activities of **3e**, and **3f** After Oral Administration in Dogs, Rats, and Guinea pigs

	dogs					rats				guinea pigs			
	dose, po	Inhibition (%)				dose, po	Inhibition (%)			dose, po	Inhibition (%)		
	(mg/kg)	1	3	6	8 h	(mg/kg)	0.5	1	2 h	(mg/kg)	1	2	3 h
3e	3.2	53±28	45±13	20±11		3.2	95±5	97±2	95±5		NT		
	10	96±32	75±25	59±17									
3f	1.0	40±8	11±15	NT	NT	3.2	76±13	97±3	44±14	0.1	49±23	–	–
	3.2	85±4	65±8	46±7	0±11					0.32	69±19	50±13	–
	10	NT	80±4	65±2	45±16					1.0	100±5	59±28	21±19

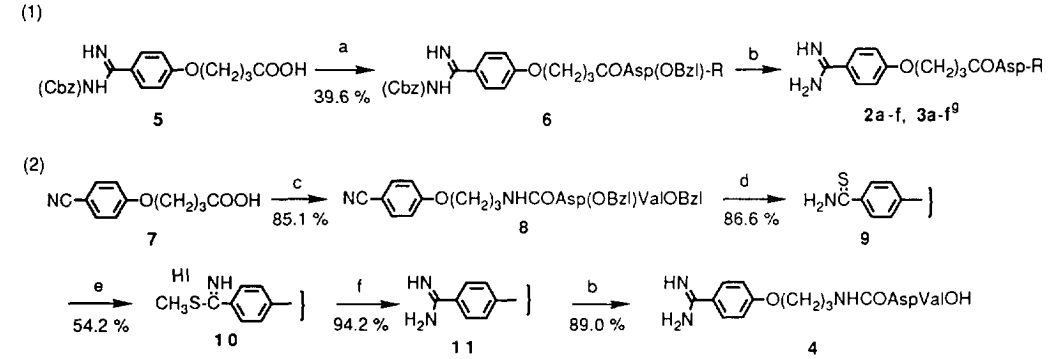
These results also supported our hypothesis that the weak activity of **1** in rats and dogs after oral administration was due to hydrolysis by peptidase in the intestinal luminal.

Synthesis:[13,14] The general preparation of the compounds **2a-f**, **3a-f**, and **4** is outlined in Scheme 2. We utilized benzyloxycarbonyl (Cbz) and benzyl (Bzl) ester for protecting groups.

Coupling of **5** with protected Asp derivatives by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) gave **6**, which were deprotected via hydrogenation using 10 % palladium on charcoal to give crude desired compounds (**2a-f**, **3a-f**), which were purified *via* reverse phase HPLC for bioassay.

Reaction of 4-(4-cyanophenoxy)butyric acid (**7**) with diphenylphosphoryl azide in the presence of triethylamine (NEt_3) gave a isocyanate intermediate, which was immediately coupled with a protected Asp derivative to give the ureido derivative **8**. Conversion of the cyano moiety of **8** via thioamide (**9**) to amidino moiety (**11**), followed by deprotection by hydrogenation gave **4**.

Scheme 2.



Reagents: (a) EDC, HOBt/DMF, rt; (b) H_2, 10 % Pd-C/THF, 1 N HCl, rt and purified *via* RP-HPLC; (c) diphenylphosphoryl azide, NEt_3/DMF,70 °C and then H-Asp(OBzl)ValOBzl, NEt_3, rt; (d) H_2S, NEt_3/pyridine, rt; (e) CH_3I/acetone, reflux; (f) $AcONH_4$/ MeOH, 60 °C; (g) Yields of the last reaction are shown in Table 3.

In summary, we reported a peptidic fibrinogen inhibitor, 4-(4-amidinophenoxy)butanoylaspartylvaline (**1**) which displayed a potent *in vivo* anti-platelet activity after intravenous infusion in a previous report. In order to obtain orally active fibrinogen inhibitors, the *ex vivo* activity of **1** was evaluated in our continuing study.

Compound **1**, however, showed only weak anti-platelet activities in rats and in dogs after oral administration. In this study, a metabolic study *in vitro* using intestinal juice of rats etc. on **1** was first carried out, which led us to a hypothesis where the low bio-availability of **1** could be due to the hydrolysis of **1** in intestinal juice. Thus, our purpose in this study was focused on compounds which are stable against intestinal juice.

Some derivatives were synthesized and their stabilities in the intestinal juice were tested to know the structure-stability relationships on **1** in intestinal juice. This study suggested that **1** may be hydrolyzed by peptidases which recognize a carboxyl moiety at the C-terminal, causing the low bio-availability of **1**. Based on these results, we synthesized several derivatives having no carboxyl group (**2a-f**) or having carboxamide (**3a-f**) at the C-terminal for protection from the peptidases, and assessed their *in vitro* anti-platelet activities using human blood. Among them, compounds possessing morpholine (**3e**) and thiomorpholine 1,1-dioxide (**3f**) at the C-terminal exhibited potent *in vitro* anti-platelet activity with 10^{-8} (M) range IC_{50} values, which are two or three times more potent than that of **1**. **3e** and **3f** showed potent *ex vivo* activity after oral administration as expected; that is, they are effective at 3.2 mg/kg (po) in dogs and rats while **1** showed only weak anti-platelet activity even at dosages of 20 mg/kg in rats and 32 mg/kg in dogs, respectively. Finally compound **3f** (FR158999) has been selected for further evaluating for a clinical study. These results also supported our hypothesis that the weak activity of **1** after oral administration was due to hydrolysis by peptidase in the intestinal luminal.

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7. Bioassay methods: see the previous papers (*in vitro*[5a] and *ex vivo*[5b]).
8. The activity of **1** in rats at the dosage of 50 mg/kg after oral administration was increased 3 times by co-administration of the known peptidase inhibitor, bacitracin[9] (32 mg/kg, data not shown).
9. Storm, D. R., *Ann. N. Y. Acad. Sci.* **1974**, *235*, 387.
10. A typical procedure for measurement of stability in intestinal juice: under ethyl acetate (AcOEt) anesthesia, the pylorus was ligated and 2 ml of distilled water was administered into the stomach. Two hrs after administration of water, the small intestine was excised from the duodenum to the ileum and rinsed on the inside with 10 ml of pH 6.4 isotonic phosphate buffer. The rinse was centrifuged and the supernatant was used as intestinal juice. To 3.9 ml of intestinal juice placed in a 10 ml glass stoppered test tube, 0.1 ml of solubilized test compound in water (1 mg/ml) was added. After agitation, the sample solution were incubated in a water bath at 37 ℃. At the appropriate time, 0.1 ml of sample solution was collected, then was assessed by a HPLC. HPLC conditions: Column, YMC-PACK R-ODS-15 120A ODS (YMC Co., Ltd.), 4.6ϕ x 250 mm; Elution, 20 % CH_3CN in 0.1 % TFA aq.; Flow, 118 ml/min; Detection, 254 nm; Retention time, 9.8 min.
11. Decomposition of **1** by intestinal juice of rats gave a compound whose Val moiety was removed, which was confirmed by HPLC analysis (data not shown).

12. The inhibitory activities of these compounds against human and dogs platelet aggregation induced by ADP were measured according to our previous paper.[5a]
13. A typical experimental procedure for compounds for biological assays (**2a**, R=$NHCH_2CH(CH_3)_2$) except for compound **4**: to a mixture of **5** (0.90 g, 2.54 mmol), H-Asp(OBzl)$NHCH(CH_3)_2$ hydrochloride (0.80 g, 2.54 mmol), and 1-hydroxy-1 *H*-benzotriazole (HOBt, 0.39 g, 2.54 mmol) in DMF (7 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 0.46 ml, 2.54 mmol), and was stirred at room temperature for 2 h. The reaction mixture was poured into a mixture of water and ethyl acetate (AcOEt), and was adjusted to pH 9.5 with a saturated aqueous solution of $NaHCO_3$. The resulting precipitate was collected by filtration, was washed with water and AcOEt, and dried *in vacuo* to give **6** (0.57 g, 39.6 %); mass spectrum *m/e* 565 ($M+H^+$). A mixture of the above crude intermediate (0.50 g, 0.885 mmol) and 10 % Pd-C (0.15 g) in a mixture of 1 N HCl (0.5 ml) and THF (5 ml) was stirred under H_2 atmosphere at room temperature for 2 h. After filtration, the filtrate was evaporated *in vacuo*. The resulting oil was subjected to preparative HPLC under the below conditions to give **2a** (0.38 g, 85.5 %): IR(Nujol) 3300, 3100, 1730, 1660, 1620, 1605 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ 0.80 (d *J*=6.6, 6 H), 1.63 (m, 1 H), 1.96 (m, 2 H), 2.30 (t *J*=6.7, 2 H), 2.4-2.8 (m, 2 H), 2.86 (t *J*=6.4, 2 H), 4.09 (t *J*=6.3, 2 H), 4.56 (m, 1 H), 7.14 (d *J*=8.8, 2 H), 7.82 (m, 3 H), 8.18 (d *J*=8.0, 1 H), 9.16 (brs, 4 H), 12.3 (brs, 1 H); mass spectrum *m/e* 393 ($M+H^+$). HPLC conditions: Column, YMC-PACK R-ODS-15 S-15 120A ODS (YMC Co., Ltd.), 50φ x 250 mm; Elution, 20 % CH_3CN in 0.1 % TFAaq.; Flow, 118 ml/min; Detection, 254 nm; Retention time, 9.8 min.

Compounds **2b–f**, and **3a–f** were prepared in a manner similar to that of **2a**. The yields of the last reaction of these compounds were shown in Table 3.

14. Compound **4** was synthesized according to the following manner. A mixture of 4-(4-cyanophenoxy)butyric acid (**7**, 3.00 g, 14.6 mmol), diphenylphosphoryl azide (3.47 ml, 16.1 mmol), triethylamine (2.24 ml, 16.1 mmol), and DMF (30 ml) was stirred at 70 ℃ for 4 h. After cooling to room temperature, H-Asp(OBzl)Val-OBzl hydrochloride (7.22 g, 16.1 mmol) and triethylamine (2.24 ml, 16.1 mmol) were added thereto, and the reaction mixture was stirred at rt overnight. The reaction mixture was poured into a mixture of ice-water and ethyl acetate (AcOEt), and was adjusted to pH 10 with a saturated aqueous solution of $NaHCO_3$. The AcOEt layer was washed with 0.25 N HCl, a saturated aqueous solution of $NaHCO_3$, water, and brine, dried over $MgSO_4$. After filtration, the filtrate was evaporated *in vacuo*. The resulting precipitate was collected by filtration, and washed with Et_2O to give **8** (7.65 g, 85.1 %): mp 106-107 ℃ (dec.); mass spectrum *m/e* 615 ($M+H^+$). To a mixture of **8** (8.70 g, 14.2 mmol), triethylamine (70 ml), and pyridine (500 ml) was bubbled H_2S gas at 0~10 ℃ for 2 h, and it was allowed to stand at rt overnight. The reaction mixture was poured into a mixture of ice-water and AcOEt. The AcOEt layer was washed with water and brine, dried over $MgSO_4$. After filtration, the filtrate was evaporated *in vacuo*. The resulting precipitate was collected by filtration, and washed with Et_2O to give **9** (7.95 g, 86.6 %); mass spectrum *m/e* 649 ($M+H^+$). A mixture of **7** (7.80 g, 12.0 mmol), CH_3I (22.5 ml, 0.361 mol), and acetone (390 ml) was stirred at reflux for 2 h. The resulting precipitate was collected by filtration, and was washed with acetone and dried *in vacuo* to give **10** hydroiodide (5.15 g, 54.2 %); mass spectrum *m/e* 791 ($M+H^+$). A mixture of **10** (5.00 g, 6.32 mmol), $AcONH_4$ (1.17 g, 15.2 mmol), and MeOH (500 ml) was stirred at 60 ℃ for 17 h. After cooling to rt, Et_2O (1 l) was added to the reaction mixture. The resulting precipitate was collected by filtration, and washed with Et_2O to give **11** hydroiodide (4.52 g, 94.2 %); mass spectrum *m/e* 760 ($M+H^+$). A mixture of **9** hydroiodide (0.40 g, 0.536 mmol) and 10 % Pd-C (0.16 g) in a mixture of 1 N HCl (1.5 ml) and THF (15 ml) was stirred under H_2 atmosphere at room temperature for 2 h. After filtration, the filtrate was evaporated *in vacuo*. The resulting oil was subjected to preparative HPLC under the above conditions to give **4** (0.27 g, 89.0 %); ^{1}H-NMR (DMSO-d_6) δ 0.84 (d *J*=6.6 Hz, 6 H), 1.8-2.2 (m, 3 H), 2.4-2.8 (m, 2 H), 3.17 (brs, 2 H), 4.0-4.6 (m, 4 H), 6.42 (m, 2 H), 7.14 (d *J*=8.8 Hz, 2 H), 7.75 (d *J*=8.8 Hz, 2 H), 7.85 (d *J*=8.0 Hz, 1 H), 9.00 and 9.23 (each s, 4 H); mass spectrum *m/e* 452 ($M+H^+$).

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