

**NOVEL SPECIFIC PUROMYCIN-SENSITIVE
AMINOPEPTIDASE INHIBITORS: 3-(2,6-DIETHYLPHENYL)-
2,4(1*H*, 3*H*)-QUINAZOLINEDIONE AND *N*-(2,6-DIETHYLPHENYL)-
2-AMINO-4*H*-3,1-BENZOXAZIN-4-ONE**

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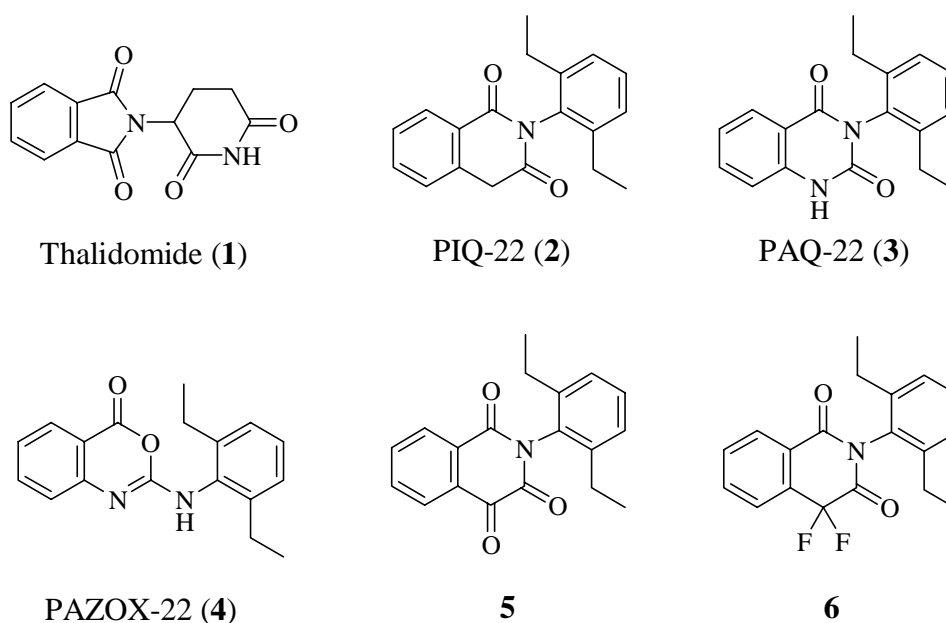
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Abstract - Novel specific PSA (puromycin-sensitive aminopeptidase) inhibitors, 3-(2,6-diethylphenyl)-2,4(1*H*, 3*H*)-quinazolinedione (**3**: PAQ-22) and *N*-(2,6-diethylphenyl)-2-amino-4*H*-3,1-benzoxazin-4-one (**4**: PAZOX-22), were designed and synthesized. These compounds are chemically much more stable than the known specific PSA inhibitor PIQ-22 (**2**), and the enzyme specificity and inhibitory activity to PSA are similar to those of **2**. The inhibitory manner of these compounds was found to be a non-competitive mode by Lineweaver- Burk plot analysis.

Puromycin-sensitive aminopeptidase (PSA) is a neutral aminopeptidase with a similar substrate specificity to that of aminopeptidase N (APN), which was first found as a candidate aminopeptidase that primarily is relevant for degrading enkephalins, and is present in the brain in much higher amounts than APN.¹⁻³ We have reported the possibility that PSA is a novel molecular target of inhibitors of tumor cell invasion.⁴ Though increased anxiety and impaired pain response in PSA

gene-deficient mice obtained by a mouse gene-trap method were recently reported,⁵ the physiological functions of PSA remain unclear, and specific inhibitors to PSA are expected for candidates as bioprobes for PSA. Through our structural development studies of thalidomide (**1**) (Figure 1),⁶⁻⁸ novel nonpeptide aminopeptidase inhibitors with a cyclic imide skeleton were found.^{4,9-12} Among them, *N*-(2,6-diethylphenyl)homophthalimide (PIQ-22 (**2**); Figure 1) particularly showed a specific and potent inhibiting activity to PSA.⁴ However, **2** is chemically labile with oxidation at the benzylic position.¹³ In this communication, novel specific PSA inhibitors PAQ-22 (**3**) and PAZOX-22 (**4**) are reported as a new entry for chemically stable analogs (Figure 1).

Figure 1

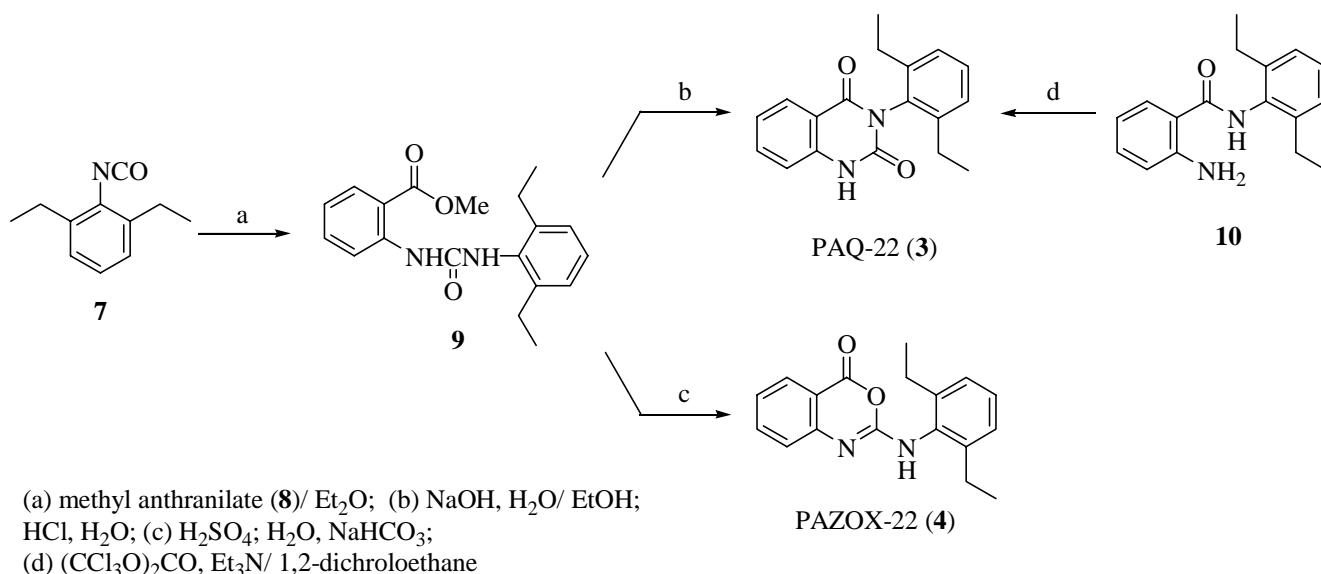


In spite of potent and specific PSA inhibitory activity, PIQ-22 (**2**) could not be a bioprobe to PSA because of its chemical stability. Its auto-oxidized product; tricarboxylic analog (**5**) showed no activity.¹³ Then, we prepared the difluoro-substituted analog (**6**) in order to prevent oxidation at the benzylic position of **2**, but this difluorinated analog (**6**) had no inhibitory activity either (Figure 1).^{4,13} Disappearance of inhibitory activity by changing the benzylic protons to an oxygen or fluorine let us know the possible importance of the benzylic protons. Thus, we designed aza analogs, 3-(2,6-diethylphenyl)-2,4(1*H*,3*H*)-quinazolin-1-one: PAQ-22 (**3**) and *N*-(2,6-diethylphenyl)-

2-amino-4*H*-3,1-benzoxazin-4-one: PAZOX-22 (**4**) having a proton at the position corresponding to the benzylic moiety of **2** (Figure 1). (In the case of **4**, it is possible to get a benzylic mimic proton by protonation or isomerization from imine to amine.) These newly designed analogs (**3** and **4**) were also expected to be stable to the oxidation at the benzylic position.

PAQ-22 (**3**) and PAZOX-22 (**4**) were prepared as shown in Scheme 1.¹⁴ 2,6-diethylphenyl isocyanate **7**, prepared from 2,6-diethylaniline, triphosgene and triethylamine in 1,2-dichloroethane, reacted with methyl anthranilate (**8**) in ether at room temperature to give urea (**9**). Treatment of this urea (**9**) with 2*N* sodium hydroxide solution gave **3**¹⁵ in 65% yield (2 steps) after acidic work-up with hydrochloric acid. Alternative preparation of **3** was performed with reaction of the amide (**10**), triphosgene and triethylamine in 1,2-dichloroethane in 79% yield (Scheme 1). On the other hand, **4** was prepared from the common intermediate (**9**) under acidic conditions in 40% yield (2 steps).¹⁵ The stability of **3** and **4** was examined with ¹H NMR spectroscopy. In CD₃OD solution, **3** and **4** were stable and were not oxidized at all. In contrast, PIQ-22 (**2**) was oxidized completely at room temperature after 2 days.¹³

Scheme 1



With PAQ-22 (**3**) and PAZOX-22 (**4**) in hand, aminopeptidase inhibitory activities of these new analogs were evaluated in a system using L-Ala-AMC with MOLT-4 cells for PSA-inhibitory, L-Ala-AMC with HL60 cells for APN-inhibitory, Gly-Pro-AMC with MOLT-4 cells for dipeptidyl peptidase type IV (DPP-IV)-inhibitory, respectively.⁴ All experiments were performed at least in duplicate, and these results are summarized using IC₅₀ in Table 1. As shown in Table 1, inhibitory activity to PSA of **3** was more potent than that of PIQ-22 (**2**), and that of **4** showed a similar level of activity to **2**. With other inhibitory assay systems, both new analogs did not show any inhibitory activities, and these were recognized to be specific to PSA. Inhibition manners of these compounds were examined by Lineweaver-Burk plot analysis and found to be non-competitive (Figure 2-a, 2-b). Since this inhibition manner is the same as that of **2**,^{10,12} **3** and **4** were recognized not to act as substrate-mimics, but to bind at a specific site of PSA. Determination of the binding site is under investigation.

In conclusion, we have synthesized novel/stable/potent PSA inhibitors with a 3-(2,6-diethylphenyl)-2,4(1*H*,3*H*)-quinazolinedione (PAQ-22; **3**) and a *N*-(2,6-diethylphenyl)-2-amino-4*H*-3,1-benzoxazin-4-one (PAZOX-22; **4**). These compounds would be useful for analysis of the physiological function of PSA, and superior lead compounds for inhibitors of cell invasion.

Table 1

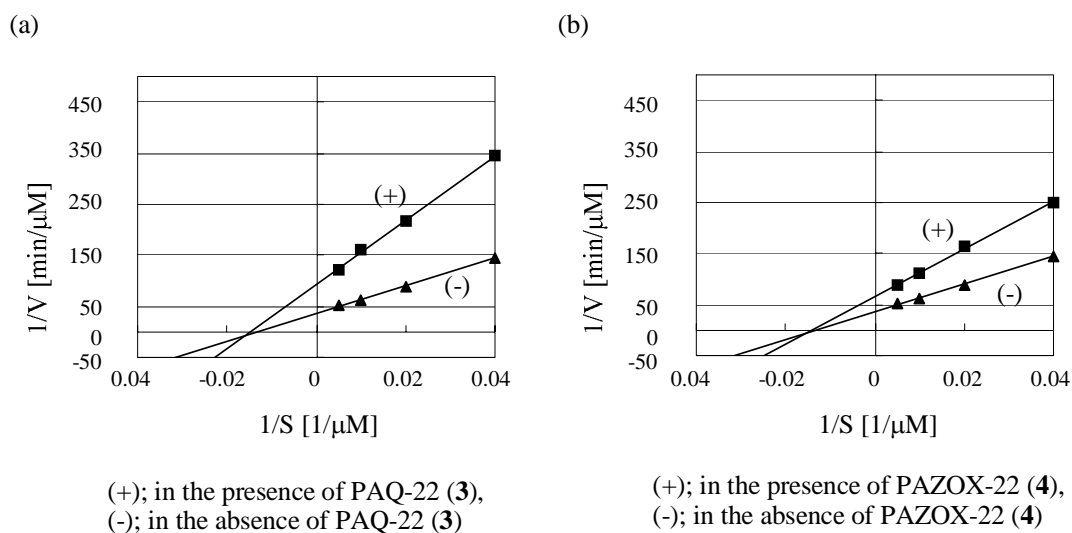
Compound	PSA ^a IC ₅₀ [μM]	APN ^b IC ₅₀ [μM]	DPP-IV ^c IC ₅₀ [μM]
2 : PIQ-22	7.8	>100	>100
3 : PAQ-22	3.8	>100	>100
4 : PAZOX-22	9.8	>100	>100
5	>100	>100	>100
6	>100	>100	>100

^aPSA-inhibitory activity was assayed by the L-Ala-AMC with MOLT-4 method.

^bAPN-inhibitory activity was assayed by the L-Ala-AMC with HL60 method.

^cDPP-VI-inhibitory activity was assayed by the Gly-L-Pro-AMC with MOLT-4 method.

Figure 2: Lineweaver-Burk plot of PAQ-22 (**3**) and PAZOX-22 (**4**)



ACKNOWLEDGEMENT

The work described in this paper was partially supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and funds for the Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety Research.

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15. **3-(2,6-diethylphenyl)-2,4(1H, 3H)-quinazolinedione (PAQ-22: 3).**

colorless cube from AcOEt; mp: 226 °C; ¹H NMR (500 MHz, CDCl₃) δ : 9.19 (br s, 1H), 8.17 (d, 1H, *J* = 8.0 Hz), 7.59 (td, 1H, *J* = 8.5, 2.0 Hz), 7.42 (t, 1H, *J* = 8.0 Hz), 7.27 (d, 2H, *J* = 8.0 Hz), 7.25 (t, 1H, *J* = 8.0 Hz), 6.94 (d, 1H, *J* = 8.0 Hz), 2.47 (q, 4H, *J* = 8.0 Hz), 1.17 (t, 6H, *J* = 7.5 Hz); HRMS (FAB) calcd for C₁₈H₁₈N₂O₂+H 295.1447, found 295.1463; IR (KBr, cm⁻¹) 3200, 1720, 1660; Anal. Calcd for C₁₈H₁₈N₂O₂: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.55; H, 6.41; N, 9.26.

***N*-(2,6-diethylphenyl)-2-amino-4H-3,1-benzoxazin-4-one (PAZOX-22: 4).**

white powder from CH₂Cl₂/Hexane; mp: 185 ~ 187 °C; ¹H NMR (500 MHz, CDCl₃) δ : 8.06 (dd, 1H, *J* = 8.5, 1.5 Hz), 7.61 (t, 1H, *J* = 8.0 Hz), 7.31 (t, 1H, *J* = 8.0 Hz), 7.22 (td, 1H, *J* = 8.0, 1.5 Hz), 7.20 (d, 1H, *J* = 7.5 Hz), 7.19 (d, 2H, *J* = 8.0 Hz), 2.67 (q, 4H, *J* = 7.5 Hz), 1.22 (t, 6H, *J* = 8.0 Hz); HRMS (FAB) calcd for C₁₈H₁₈N₂O₂+H 295.1447; found 295.1461; IR (KBr, cm⁻¹) 3000, 1780, 1670. Anal. Calcd for C₁₈H₁₈N₂O₂: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.15; H, 6.15; N, 9.37.