Cyclic GMP Phosphodiesterase Inhibitors. 1. The Discovery of a Novel Potent Inhibitor, 4-((3,4-(Methylenedioxy)benzyl)amino)-6,7,8-trimethoxyquinazoline

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A newly synthesized compound, 4-((3,4-(methylenedioxy)benzyl)amino)-6,7,8-trimethoxyquinazoline (6), had a potent (IC_{50} = 0.36 μM) inhibitory action on cyclic GMP phosphodiesterase (cGMP-PDE) isolated from porcine aorta; its inhibitory activities toward other PDE isozymes were at least 10-fold weaker. In addition, 6 relaxed porcine coronary arteries precontracted with $PGF_{2\alpha}$ (EC_{50} = 1.96 ± 0.58 μM). At the concentration of 30 μM, 6 caused elevation of the intracellular cGMP level in porcine coronary arteries without any change in cAMP level. Various other 4-substituted 6,7,8-trimethoxyquinazolines were also synthesized and evaluated for cGMP-PDE inhibitory activity. From their structure–activity relationships, we concluded that the 4-((3,4-(methylenedioxy)benzyl)-amino) group is essential for potent inhibition of cGMP-PDE.

Introduction

Phosphodiesterases (PDEs) catalyze the hydrolysis of cyclic nucleotides such as cAMP and cGMP. Five PDE isozymes are known.[1-4] One of them, cGMP-PDE (type V), hydrolyzes cGMP specifically and is insensitive to Ca^{2+}–calmodulin. Zaprinast (M&B 22948) and MY-5445 are available as selective inhibitors of cGMP-PDE[3-5] (Chart I).

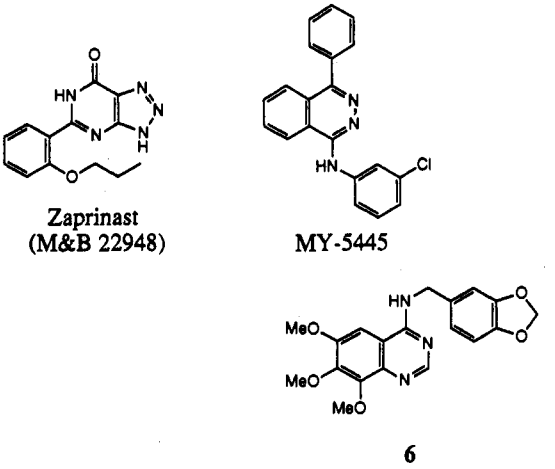
The structure–activity relationships (SARs) of inhibitors of cAMP-PDE isozymes have been extensively studied, with some success in the cases of the inhibitors of PDEs III and IV.[6] On the other hand, there is little information on the SARs of the cGMP-PDE inhibitors because even the most potent inhibitors have very diverse molecular structures, making identification of the most important sites of interaction difficult.[2] Furthermore, little is known about the *in vivo* actions and clinical effects of selective cGMP-PDE inhibitors.[7]

Structurally novel inhibitors of cGMP-PDE would be useful in studies to elucidate the pharmacological and physiological roles of cGMP-PDE and to develop novel therapeutic agents. In this paper we report the discovery of a potent inhibitor, 4-((3,4-(methylenedioxy)benzyl)-amino)-6,7,8-trimethoxyquinazoline (6, Chart I), through structural transformation based on the results of our screening program for cGMP-PDE inhibitory activity. The SARs of 4-substituted 6,7,8-trimethoxyquinazolines and the results of a pharmacological investigation of 6 are also reported.

Enzyme Source and Screening Assay

The methods for the preparation of each PDE isozyme and the inhibition assay were described in the previous paper.[4] We separated cGMP-PDE from the other isozymes, Ca^{2+}–calmodulin-dependent PDE (CaM-PDE, type I), cGMP-stimulated PDE (type II), and two cAMP-PDEs (types III and IV), in porcine aorta. [^{3}H]cGMP or [^{3}H]cAMP at a concentration of 1 μM was used as a substrate. The tested compounds were dissolved in DMSO and then diluted with assay buffer, at concentrations ranging from 10^{-8} to 10^{-4} M. The final concentration of DMSO was less than 0.4% (v/v), which had no interferences in the screening assay system. Zaprinast and MY-5445 were used for comparison as selective cGMP-PDE inhibitors.

Chart I



Screening and Structural Transformation

During the screening program, we found that FPL-55712 (antiallergic agent) possesses a slight inhibitory activity toward cGMP-PDE as one of its pharmacological actions.[8] Accordingly, we investigated the essential pharmacophore of this compound for cGMP-PDE inhibition and obtained the following SAR information: (1) the chromone ring (part A, Figure 1) is essential for the activity, and the phenyl ring on the other side (part B) is also necessary (data not shown); and (2) the central hydroxyl group is not essential, and it is possible to shorten the central $(CH_2)_3$ linking unit (part C, Table I). Synthetic transformation based on these results led to 3, which possesses a 3,4-(methylenedioxy)phenyl group and exhibits a slightly improved activity.

On the other hand, we found in our screening program that 5-[(6,7,8-trimethoxy-4-quinazolinyl)amino]-1-pentanyl nitrate maleate (KT-1) is also a potent inhibitor of cGMP-PDE (Table II). Although KT-1 has a coronary-dilating action based on the activation of guanylate cyclase induced by its nitrate ester,[9,10] its cGMP-PDE inhibitory

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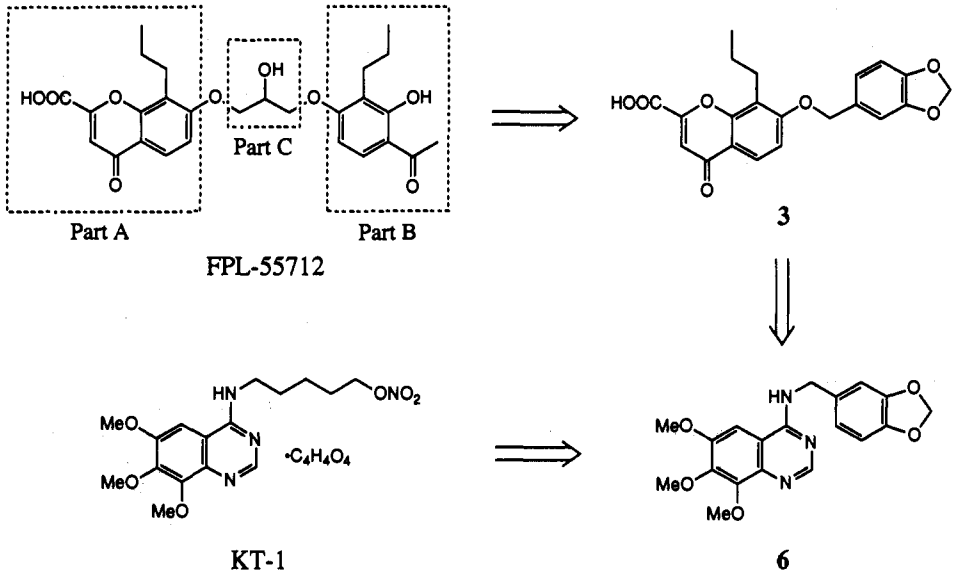
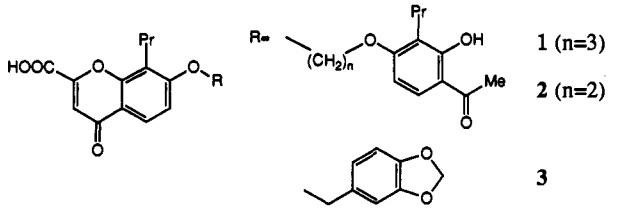


Figure 1. Structural transformations.

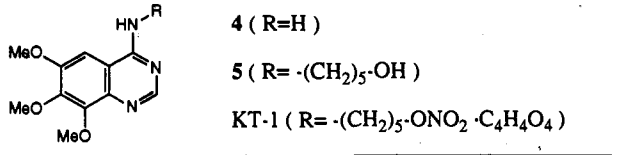
Table I. FPL-55712 and Related Compounds



compd	formula[a]	mp, °C	IC_{50}, μM[b]
FPL-55712			1.58 ± 0.39 (n = 4)
1			1.5
2	$C_{26}H_{28}O_8 \cdot 0.4H_2O$	244–245 dec	1.5
3	$C_{21}H_{18}O_7$	180–181	0.65

[a] Analyses for C and H were within ±0.4% of the expected values for the formula. [b] IC_{50} values were determined from the logarithmic concentration–inhibition curve (at least three points). An averaged value is expressed as the mean ± SEM (number of experiments).

Table II. KT-1 and Related Compounds



compd	formula[a]	mp, °C	IC_{50}, μM[b]
4	$C_{11}H_{13}N_3O_3 \cdot 0.1C_2H_5OH$	194–195	9.6
5			5.5
KT-1			1.47 ± 0.42 (n = 4)

[a] Analyses for C, H, and N were within ±0.4% of the expected values for the formula. [b] See footnote *b* in Table I.

activity had not been discovered. This may have been because it is difficult to distinguish cGMP-PDE inhibitory activity from the activation of guanylate cyclase in *in vivo* experiments. We synthesized a new molecule, **6**, combining the structural features of KT-1 and **3**. Compound **6** was found to be a potent inhibitor of cGMP-PDE, with a structure different from those of known selective inhibitors (Figure 1).

Synthesis

FPL-55712 and related compounds were synthesized according to the literature[11] and usual synthetic methods. 6,7,8-Trimethoxyquinazolin-4(3*H*)-one (**30**) and its chlorinated derivative (**31**) were synthesized by reference to the literature.[9] Reaction of **31** with ethanolic ammonia afforded **4**. Compound **6** and related compounds (**7-25**) were obtained by the reaction of **31** with the appropriate amines in the presence of triethylamine or Na_2CO_3. Reaction of **31** with 3,4-(methylenedioxy)benzyl alcoxide gave **26**. Compound **30** was alkylated with the appropriate chloride or tosylate to give **27-29** (Scheme I).

Table III. Inhibitory Activities of **6** and Other cGMP-PDE Inhibitors on Five PDE Isozymes[a]

	IC_{50} (μM)				
compd	V	I	II	III	IV
6	0.36 ± 0.09	5.5 ± 2.5	8.7 ± 1.2	>100	>100
zaprinast	0.45 ± 0.12	32.2 ± 5.3	46.8 ± 1.7	>100	79.6 ± 20.2
MY-5445	1.8 ± 0.4	>100	>100	>100	37.2 ± 13.5
dipyridamole	0.52 ± 0.17	>100	3.6 ± 0.2	>100	6.4 ± 1.1
papaverine	8.8 ± 4.2	21.6 ± 3.8	2.3 ± 0.2	0.62 ± 0.07	1.7 ± 0.7

[a] (I) Ca^{2+}–calmodulin-dependent PDE; (II) cGMP-stimulated PDE; (III) cGMP-inhibited PDE (selectively inhibited by a large number of positive inotropic agents such as milrinone); (IV) cAMP-specific PDE (selectively inhibited by rolipram); (V) cGMP-PDE. Substrate in the inhibition assay for I, cGMP; II, cAMP; III, cAMP; IV, cAMP; V, cGMP. All IC_{50} values of zaprinast, dipyridamole, and papaverine were cited from the previous paper.[4] IC_{50} values were determined from the logarithmic concentration–inhibition curve (at least three points) and are indicated as means ± SEM from at least three experiments; >100 means that the IC_{50} was greater than 100 μM.

Pharmacological Results and Discussion

The IC_{50} of **6** for cGMP-PDE was 0.36 μM, whereas the IC_{50} values for types III and IV were over 100 μM (Table III). Compound **6** also showed an inhibitory activity toward CaM-PDE (type I), but its potency was approximately 10 times lower than that toward cGMP-PDE. Furthermore, we found that the mode of inhibitory action of **6** is competitive, and **6** does not activate guanylate cyclase (data not shown). Zaprinast exhibited approximately the same inhibitory activity as that of **6** toward cGMP-PDE and more remarkable selectivity for inhibition of cGMP-PDE. On the other hand, dipyridamole, which also has been regarded as a selective inhibitor of cGMP-PDE, was a rather nonselective one.

Scheme I

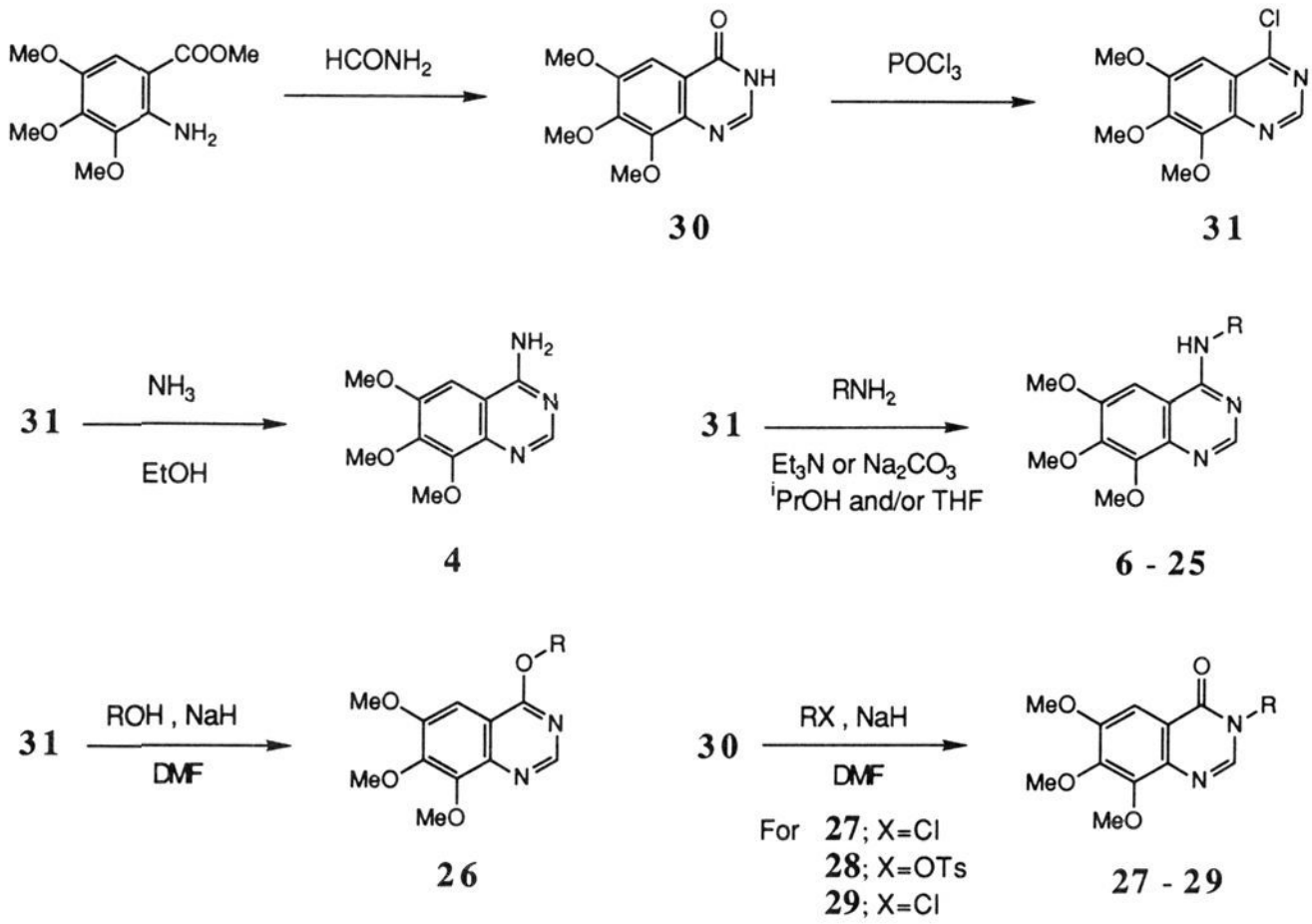


Table IV. Relaxing Effects of **6** and Zaprinast on Isolated Pig Coronary Arteries Precontracted with $PGF_{2\alpha}$

compd	EC_{50},[a] μM
6	1.96 ± 0.58 (n = 13)
zaprinast	4.75 ± 1.12 (n = 7)

[a] EC_{50} values were determined from the logarithmic cumulative concentration–relaxation curve and are indicated as means ± SEM. The n values in parentheses are the number of experiments. The methods are described in the Experimental Section.

We confirmed the relaxing effect of **6** on isolated porcine coronary arteries precontracted with $PGF_{2\alpha}$ (10^{-5} M). Its potency (EC_{50} = 1.96 μM) was of the same order as that of zaprinast (Table IV).

In order to confirm the cGMP-PDE inhibitory effect of **6** more directly, we investigated the effects of **6** and zaprinast on the cyclic nucleotide levels in isolated porcine coronary arteries. The time courses of cGMP and cAMP levels after exposure to the inhibitors are shown in Figure 2. Compound **6** and zaprinast (30 μM) significantly elevated the intracellular cGMP level without causing any change in cAMP level. In the similar experimental conditions, however, dipyridamole elevated both cGMP and cAMP levels[12] (data not shown), which seems to be due to its nonselective inhibitory activities toward types V, II, and IV.

We think that these effects of **6** described above are caused through its inhibition of cGMP-PDE, since we also separated cGMP-PDE from the other PDE isozymes in porcine coronary arteries and **6** exhibited the similar potent inhibitory activity toward the cGMP-PDE isozyme (IC_{50} = 0.13 μM).

As for the *in vitro* SARs at the 4-position of the 6,7,8-trimethoxyquinazoline (Table V), the potency was attenuated when we changed the nitrogen atom (**6**) at the linking region to oxygen (**26**) or the methylene group ($-(CH_2)_n-$, **6**; n = 1) to n = 0 (**7**) or n = 2 (**8**). Therefore, we mainly investigated the effect of varying the (arylmethyl)amino moiety. The results were as follows: (1) an appropriate distance is required between the quinazoline and the phenyl ring at the 4-position of the quinazoline (**6-8**), (2)

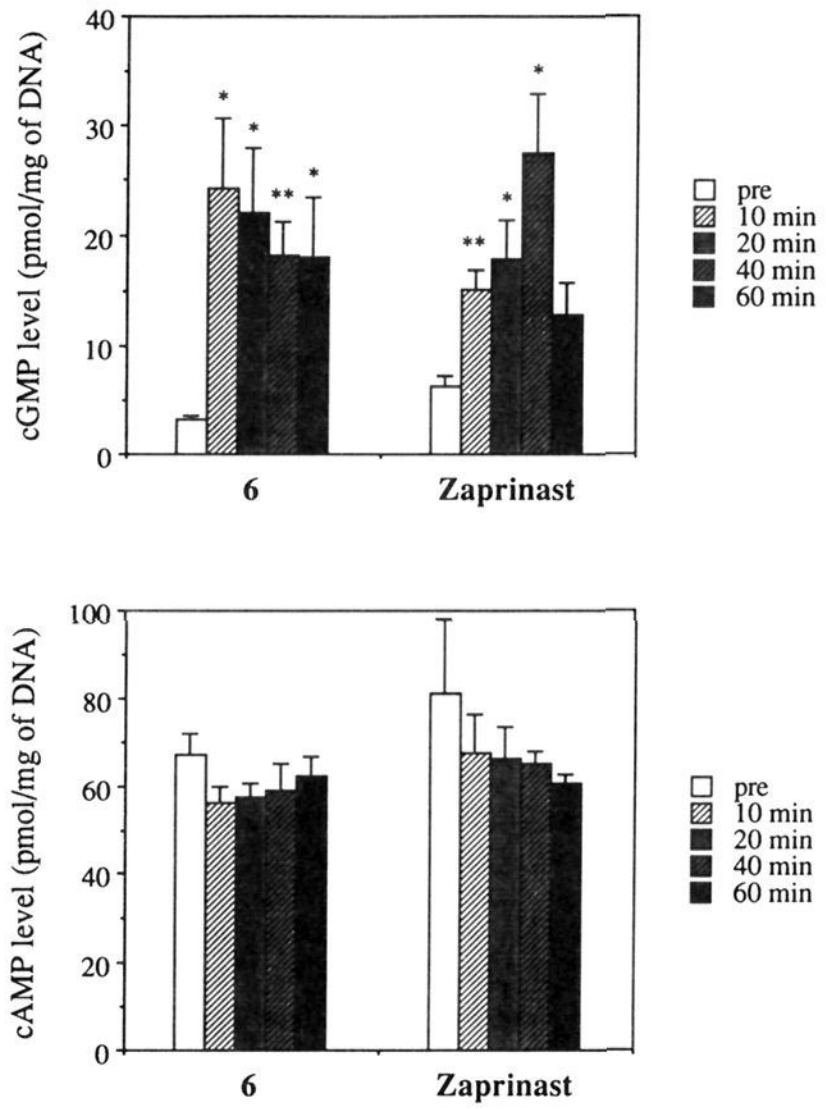
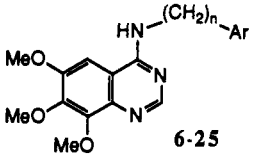


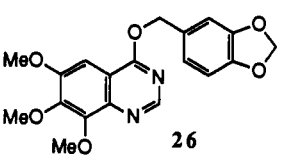
Figure 2. Effects of **6** and zaprinast on the intracellular cGMP and cAMP levels in isolated porcine coronary arteries. The methods are described in the Experimental Section. the concentration of the drug was 30 μM in each case. All values are indicated as means ± SEM (n = 5 or 6), expressed in pmol/mg of DNA. *, **: $p < 0.05$, $p < 0.01$ vs pre, respectively (ANOVA followed by t-test or Cochran-Cox test).

on the phenyl ring, the 3,4-(methylenedioxy)phenyl group is essential for potent activity, and (3) indan-5-yl (**21**), 3,4-dimethoxyphenyl (**19**), and even 3,4-(ethylenedioxy)-phenyl (**20**) groups cannot adequately replace it. These results suggest that it is necessary to substitute both the

Table V. Structures, Properties, and cGMP-PDE Inhibitory Activities of 6,7,8-Trimethoxyquinazolines



6-25



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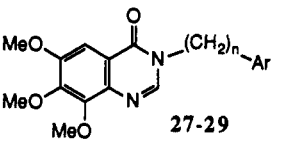
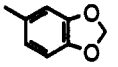
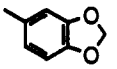
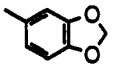
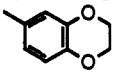
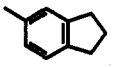
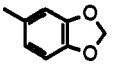
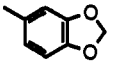


27-29

compd	*n*	Ar	formula[a]	mp, °C (recrystn solv)[b]	yield, %[c]	IC_{50}, μM[d]
6	1		$C_{19}H_{19}N_3O_5$	197–198 (C)	69	0.36 ± 0.09 (n = 3)
7	0[e]		$C_{18}H_{17}N_3O_5$	254–255 (D)	58	11
8	2		$C_{20}H_{21}N_3O_5 \cdot 0.3H_2O$	193–194 (A)	68	2.2
9	1	C_6H_5	$C_{18}H_{19}N_3O_3$	180–181 (A)	91	2.0
10	1	3-$MeOC_6H_4$	$C_{19}H_{21}N_3O_4 \cdot 0.2H_2O$	142–143 (A)	89	1.7
11	1	4-$MeOC_6H_4$	$C_{19}H_{21}N_3O_4$	174–175 (A)	97	1.0
12	1	3-ClC_6H_4	$C_{18}H_{18}ClN_3O_3 \cdot 0.1H_2O$	161–162 (A)	85	1.3
13	1	4-ClC_6H_4	$C_{18}H_{18}ClN_3O_3$	181–182 (A)	61	1.3
14	1	3-$O_2NC_6H_4$	$C_{18}H_{18}N_4O_5 \cdot 0.2H_2O$	159–160 (A)	30	3.5
15	1	4-$O_2NC_6H_4$	$C_{18}H_{18}N_4O_5 \cdot 0.2H_2O$	210–212 (A)	28	>100
16	2	4-$O_2NC_6H_4$	$C_{18}H_{20}N_4O_5 \cdot 0.5H_2O$	152–153 (A)	58	22
17	1	4-EtC_6H_4	$C_{20}H_{23}N_3O_3$	195–196 (A)	88	26
18	1	2-$PrOC_6H_4$	$C_{21}H_{25}N_3O_4$	139–140 (B)	80	2.0
19	1	3,4-$(MeO)_2C_6H_3$	$C_{20}H_{23}N_3O_5 \cdot 0.2H_2O$	171–172 (A)	32	4.6
20	1		$C_{20}H_{21}N_3O_5 \cdot 0.3H_2O$	217–219 (A)	92	0.78
21	1		$C_{21}H_{23}N_3O_3 \cdot 0.2H_2O$	198–199 (A)	61	1.9
22	1	2,4,6-$(MeO)_3C_6H_2$	$C_{21}H_{25}N_3O_6 \cdot 0.5H_2O$	213–215 dec (A)	64	>100
23	1	3,4,5-$(MeO)_3C_6H_2$	$C_{21}H_{25}N_3O_6$	153–154 (B)	60	8.9
24	1		$C_{17}H_{18}N_4O_3 \cdot 0.4H_2O$	166–168 (A)	76	32
25	1		$C_{16}H_{17}N_3O_4 \cdot 0.2H_2O$	198–199 (A)	81	4.0
26			$C_{19}H_{13}N_2O_6$	141–142 (A)	39	1.0
27	1		$C_{19}H_{18}N_2O_6$	115–116 (A)	74	0.53
28	2		$C_{20}H_{20}N_2O_6 \cdot 0.1H_2O$	115–116 (A)	44	32
29	1	2-$PrOC_6H_4$	$C_{21}H_{24}N_2O_5 \cdot 0.2H_2O$	99–100 (B)	40	11

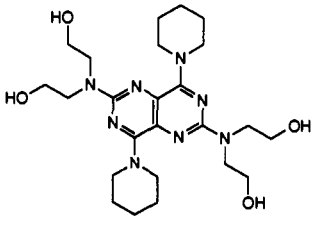
[a] Analyses for C, H, and N were within ±0.4% of the expected values for the formula. [b] Key: A = $CHCl_3$/hexane; B = AcOEt/hexane; C = AcOEt; D = MeOH. [c] Yields were not optimized. [d] See footnote *b* in Table I. [e] 0 means the anilino derivative.

meta and para positions on the phenyl ring by heteroatoms, and the substituents must be sterically compact for potent inhibitory activity. These requirements may imply that the methylenedioxy group interacts with the catalytic site of cGMP-PDE, if we assume that the quinazoline moiety has a structural similarity to the guanine moiety of cGMP.

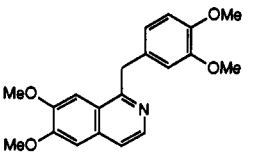
3-(3,4-(Methylenedioxy)benzyl)-6,7,8-trimethoxyquinazolin-4(3*H*)-one (**27**) also had a comparatively potent inhibitory activity toward cGMP-PDE. Although **27** is less potent than **6**, the SARs of **27** and its derivatives are seemingly similar to those of **6** and its derivatives, though the data are incomplete (**27-29**). In any case, the application of these SARs to the structures of other cGMP-PDE inhibitors, such as zaprinast, MY-5445, and dipyridamole, is difficult at present. Only papaverine, which is a rather nonselective PDE inhibitor, bears a structural resemblance to **6** (Chart II).

In conclusion, 4-((3,4-(methylenedioxy)benzyl)amino)-6,7,8-trimethoxyquinazoline (**6**) was synthesized and proven to be a potent inhibitor of cGMP-PDE. We confirmed

Chart II



Dipyridamole



Papaverine

that **6** has a relaxing effect on porcine coronary arteries and elevates intracellular cGMP. This compound should be a useful experimental tool for studies on the pharmacological and physiological roles of cGMP-PDE, and it could be a novel therapeutic agent. The SARs of **6** and related compounds imply a rigid steric limitation on the substituent at the 4-position of the quinazoline, but they are not yet sufficiently explored, especially because we

fixed the 6,7,8-substituents on the quinazoline ring as trimethoxy groups at first and chose a quinazoline ring as the heteroaromatic moiety. Therefore, further studies may lead to even more potent cGMP-PDE inhibitors, once the SARs of related compounds are established in more detail.

Experimental Section

Melting points (mp) were determined on an electrothermal capillary melting point apparatus and are uncorrected. All ^1H NMR spectra were measured on a Varian (400 MHz) spectrometer with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) and elemental analyses were performed by the Analytical Chemistry Section of Eisai Tsukuba Research Laboratories.

Ethyl 7-((3,4-(Methylenedioxy)benzyl)oxy)-4-oxo-8-propyl-4*H*-1-benzopyran-2-carboxylate (Ethyl Ester of 3). To a mixture of 3.5 g (13 mmol) of ethyl 7-hydroxy-8-propyl-4-oxo-4*H*-1-benzopyran-2-carboxylate[11] and 2.6 g (15 mmol) of piperonyl chloride in 30 mL of dimethylformamide was added 2.5 g (18 mmol) of K_2CO_3. The mixture was stirred at 60 °C for 2 h, poured into water, and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered through a small amount of silica gel, and concentrated under reduced pressure. The residual solid was recrystallized from ethyl acetate–hexane to give 4.0 g (78%) of the title compound as pale brown crystals: mp 151–152 °C; ^1H NMR ($CDCl_3$) δ 0.97 (3H, t, J = 7.4 Hz), 1.43 (3H, t, J = 7.2 Hz), 1.67 (2H, sextet, J = 7.4 Hz), 2.94 (2H, t, J = 7.4 Hz), 4.45 (2H, q, J = 7.2 Hz), 5.12 (2H, s), 5.99 (2H, s), 6.83 (1H, d, J = 8.0 Hz), 6.89 (1H, dd, J = 8.0, 1.6 Hz), 6.92 (1H, d, J = 1.6 Hz), 7.05 (1H, s), 7.06 (1H, d, J = 9.0 Hz), 8.03 (1H, d, J = 9.0 Hz); MS *m/e* (FAB) 411 (MH^+). Anal. ($C_{23}H_{22}O_7$) C, H.

7-((3,4-(Methylenedioxy)benzyl)oxy)-4-oxo-8-propyl-4*H*-1-benzopyran-2-carboxylic Acid (3). The above ethyl ester (1.0 g, 2.4 mmol) was dissolved in tetrahydrofuran (20 mL) and ethanol (20 mL), and 1 N aqueous NaOH (20 mL) was added. The solution was stirred at room temperature for 2 h, neutralized with 1 N aqueous HCl (20 mL), concentrated, and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. Recrystallization from ethyl acetate–hexane gave 0.48 g (51%) of **3** as pale brown crystals: mp 180–181°C; ^1H NMR (DMSO-d_6) δ 0.89 (3H, t, J = 7.4 Hz), 1.61 (2H, sextet, J = 7.4 Hz), 2.85 (2H, t, J = 7.4 Hz), 5.20 (2H, s), 6.03 (2H, s), 6.82 (1H, s), 6.93 (1H, d, J = 8.0 Hz), 6.97 (1H, dd, J = 8.0, 1.6 Hz), 7.02 (1H, d, J = 1.6 Hz), 7.33 (1H, d, J = 8.8 Hz), 7.90 (1H, d, J = 8.8 Hz); MS *m/e* (FAB) 383 (MH^+). Anal. ($C_{21}H_{18}O_7$) C, H.

4-((3,4-(Methylenedioxy)benzyl)amino)-6,7,8-trimethoxyquinazoline (6). General Procedure. A mixture of 21.2 g (0.083 mol) of **31**, 17.0 g (0.112 mol) of piperonylamine, and 13.5 g (0.127 mol) of Na_2CO_3 in 2-propanol (400 mL) was refluxed overnight. After cooling of the mixture, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography (eluted with ethyl acetate) and recrystallization from ethyl acetate to give **6** as pale yellow needles (21.3 g, 69%): mp 197–198 °C; ^1H NMR ($CDCl_3$) δ 3.94 (3H, s), 4.03 (3H, s), 4.12 (3H, s), 4.76 (2H, d, J = 4.8 Hz), 5.55 (1H, br s), 5.97 (2H, s), 6.64 (1H, s), 6.80 (1H, d, J = 8.0 Hz), 6.87 (1H, d, J = 8.0 Hz), 6.91 (1H, s), 8.66 (1H, s); MS *m/e* (FAB) 370 (MH^+). Anal. ($C_{19}H_{19}N_3O_5$) C, H, N.

4-((3,4-(Methylenedioxy)benzyl)oxy)-6,7,8-trimethoxyquinazoline (26). To a suspension of 0.18 g (4.5 mmol) of 60 wt % sodium hydride in 20 mL of dimethylformamide was added 0.70 g (4.6 mmol) of piperonyl alcohol, and the mixture was stirred at 50 °C for 10 min. Then 1.0 g (3.9 mmol) of **31** was added, and the reaction mixture was stirred at 70 °C for 3 h. After cooling, water was added and the aqueous mixture was extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (eluted with ethyl acetate–hexane) and recrystallization from chloroform–hexane to give **26** as white crystals (0.57 g, 39%): mp 141–142 °C; ^1H NMR ($CDCl_3$) δ 3.97 (3H, s), 4.05 (3H, s), 4.13 (3H, s), 5.53 (2H, s), 5.99 (2H, s), 6.84 (1H, d, J = 8.0 Hz), 7.00 (1H, dd, J = 8.0, 2.0 Hz), 7.02 (1H, d, J = 2.0 Hz), 7.20 (1H, s), 8.74 (1H, s); MS *m/e* (FAB) 371 (MH^+). Anal. ($C_{19}H_{18}N_2O_6$) C, H, N.

3-((3,4-(Methylenedioxy)benzyl)-6,7,8-trimethoxyquinazolin-4(3*H*)-one (27). General Procedure. To a mixture of 2.9 g (12 mmol) of **30** and 2.5 g (15 mmol) of piperonyl chloride in 30 mL of dimethylformamide was added 0.56 g (14 mmol) of 60 wt % sodium hydride, and the mixture was stirred at 60 °C for 3 h. After cooling, water was added and the whole was extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography (eluted with ethyl acetate–hexane) and recrystallization (chloroform–hexane) to give 3.4 g (74%) of **27** as pale yellow crystals: mp 115–116 °C; ^1H NMR ($CDCl_3$) δ 3.97 (3H, s), 4.01 (3H, s), 4.06 (3H, s), 5.09 (2H, s), 5.94 (2H, s), 6.77 (1H, d, J = 8.0 Hz), 6.84 (1H, d, J = 8.0 Hz), 6.86 (1H, s), 7.50 (1H, s), 8.03 (1H, s); MS *m/e* (FAB) 371 (MH^+). Anal. ($C_{19}H_{18}N_2O_6$) C, H, N.

Relaxing Effect on Isolated Coronary Arteries Precontracted with $PGF_{2\alpha}$. Porcine coronary arteries were removed, cleaned of adjacent tissues, and cut into rings with special care not to damage the endothelium. The rings were longitudinally opened and mounted in organ baths containing 10 mL of Krebs-Henseleit solution (37 °C, pH 7.4, bubbled with 95% O_2–5% CO_2). The coronary arterial strips were allowed to equilibrate under a resting tension of 1 g. The presence of intact endothelial cells was confirmed by bradykinin (final concentration, 7×10^{-9} M)—induced relaxation of strips precontracted with KCl (final concentration, 50 mM). The strips were contracted with $PGF_{2\alpha}$ (final concentration, 10^{-5} M), and after the attainment of a plateau contraction, cumulative concentration–relaxation curves for **6** and zaprinast were constructed. Relaxation was calculated as a percentage of the contractile response to $PGF_{2\alpha}$.

Measurement of the Intracellular cGMP and cAMP Levels in Smooth Muscle Cells of Isolated Coronary Arteries. Porcine coronary arteries were removed, cleaned of adjacent tissues, cut transversally into rings, denuded of endothelial cells, and incubated in Krebs-Henseleit solution (37 °C, pH 7.4, bubbled with 95% O_2–5% CO_2). The coronary arterial strips were preincubated for 40 min, during which period the incubation medium was changed three times. After this, $PGF_{2\alpha}$ (final concentration, 10^{-5} M) was added and the strips were incubated for 60 min, and then a PDE inhibitor (final concentration, 30 μM) was added for a period of 0, 10, 20, 40, or 60 min. The pieces of artery were quickly frozen in liquid N_2 and stored at −80 °C, before being homogenized in 1 mL of 10% TCA. Each homogenate was centrifuged, and the supernatant was extracted with water-saturated ethyl ether. The organic solution was subjected to radioimmunoassay for cyclic nucleotides.[13] Cyclic nucleotide levels were expressed with respect to DNA, which was extracted from the pellets and assayed fluorometrically by previously described methods.[14]

After Smirnoff's elimination analysis, data were subjected to analysis of variance (ANOVA). The estimated variance of error was used for the calculation of standard errors of mean values and statistical analysis of the difference between two values by the t-test (variances: uniform) or Cochran-Cox test (variances: not uniform).

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