1,4:3,6-Dianhydrohexitol Nitrate Derivatives. I. Synthesis and Antianginal Activity of Alkylpiperazine Derivatives

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A series of 5-deoxy-5-(4-substituted piperazin-1-yl)-1,4:3,6-dianhydro-L-iditol 2-nitrates was prepared and evaluated for oral anti-ischemic activities. Inhibition of lysine-vasopressin-induced T-wave elevation in the electrocardiogram (ECG) of rats (angina pectoris model) served as a primary assay. Optimum activity was observed for the compounds with the aryl-heteroatom (O, S, or N)-propyl group. Among them, the phenylthiopropyl-substituted compound 13 exhibited the most potent activity. Furthermore, intraduodenal administration (i.d.) of 13 tended to decrease left ventricular end-diastolic pressure (LVEDP) in a propranolol-induced heart failure model (dogs) and showed a potent protective effect against reperfusion arrhythmia in rats. Thus, 13 (KF 14124) is under further study as an orally active nitrate.

Keywords nitrate; dianhydrohexitol; vasodilator; heart failure; anginal pectoris; antiarrhythmic activity

Among modern cardiovascular therapeutic agents, organic nitrates are still of major importance.[1] They are highly effective in a variety of forms of ischemic heart disease, such as angina pectoris and congestive heart failure. Angina pectoris is a clinical syndrome caused by transient myocardial ischemia resulting from an imbalance between myocardial oxygen supply and demand,[1,2] and organic nitrates mainly exert their effects by reducing the preload *via* venodilation, followed by a decrease in myocardial oxygen consumption. Although the mechanism has not been fully elucidated at the molecular level, it seems very likely that nitric oxide or a closely related compound is released. This then activates guanylate cyclase, giving rise to an increase in cyclic guanosine monophosphate (c-GMP), resulting in vascular smooth muscle relaxation.[3] The so-called endothelium-derived relaxing factors (EDRF) have been demonstrated to act in a similar way.[4,5] Accumulating evidence suggests that at least one of the EDRFs is nitric oxide.[6] In this respect, organic nitrates can be considered as prodrugs of EDRF (endogenous vasodilators), thus making nitrate research of much interest.[4]

Although several nitrate derivatives have been investigated,[7] the classical compounds such as glyceryl trinitrate (GTN) (**1**) and isosorbide dinitrate (ISDN) (**2**) are still in general clinical use. However, these drugs have a number of disadvantages such as low oral bioavailability, first-pass effect, and development of tolerance during chronic administration.[8] Isosorbide-5-mononitrate (IS-5-MN) (**3**), which is an active metabolite of ISDN, is now marketed as a nitrate having only a weak first-pass effect.[9] Moreover, new nitrates, such as nicorandil (**4**, potassium channel opener)[10] and nipradilol (**5**, β-blocker),[11] having other action mechanisms have recently been developed. In this report, we describe the synthesis and oral anti-ischemic activity of some new nitrates, a series of 5-deoxy-5-(4-substituted piperazin-1-yl)-1,4:3,6-dianhydro-L-iditol 2-nitrate derivatives.[12]

Design In the group of dianhydrohexitol dinitrates such as ISDN (**2**) (*exo, endo*), there are two other stereoisomers, which are isomannide dinitrate (IMDN) (**6**) (*endo, endo*) and isoidide dinitrate (IIDN) (**7**) (*exo, exo*). IIDN is the most active among them.[13] Further, mononitrate derivatives might be favorable in order to avoid the first-pass effect of ISDN. Thus, isoidide mononitrate (IIMN) was selected as a parent pharmacophore, having basic skeleton of IIDN, and a piperazine ring was introduced as shown in **8** in order to increase the solubility of the compound. The lipophilicity of nitrates is important for their pharmacological action since the soluble isoenzyme of guanylate cyclase is located in the cytoplasm.[14] Consequently, a lipophilic group was introduced into the piperazine ring in order to obtain good membrane permeability

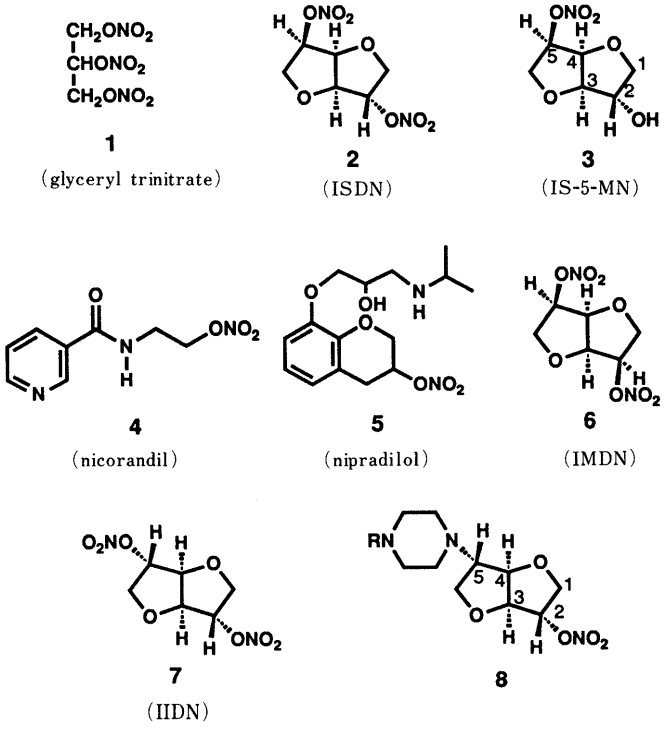


Chart 1

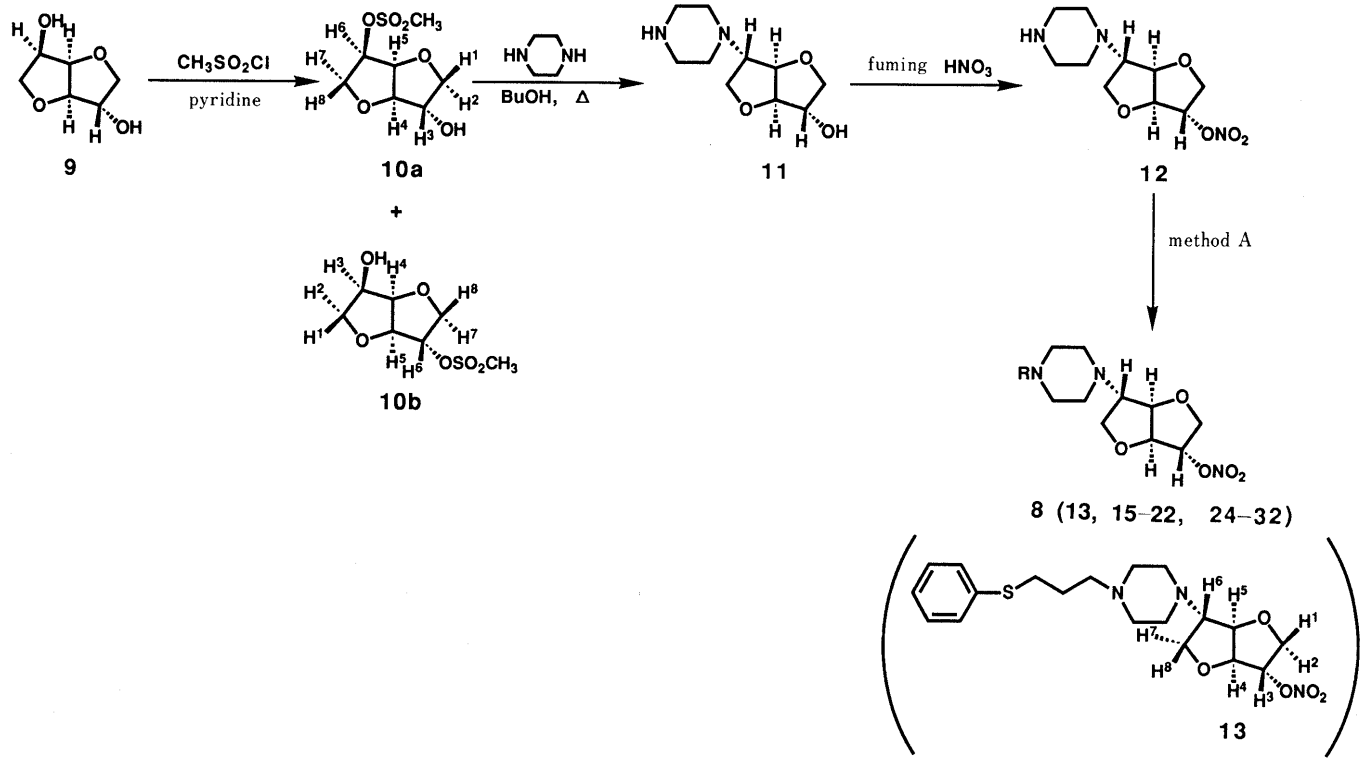


Chart 2

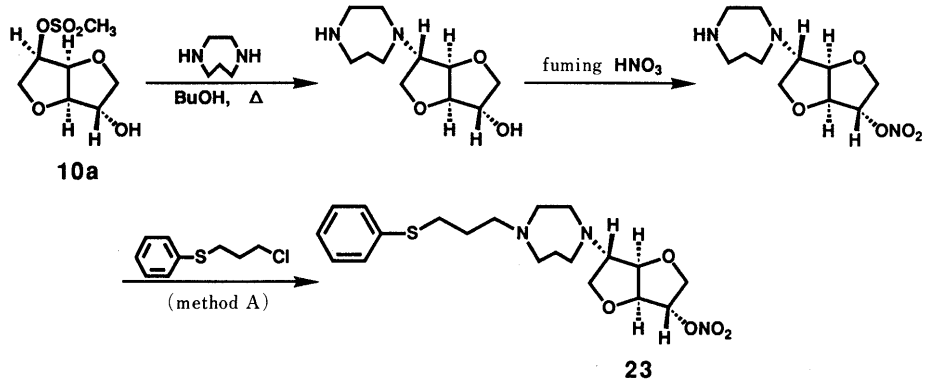


Chart 3

and oral absorption.

Chemistry The general procedure for preparation of the target compounds is shown in Chart 2. Reaction of 1,4 : 3,6-dianhydro-D-glucitol (isosorbide (**9**)) with methanesulfonyl chloride gave 1,4 : 3,6-dianhydro-D-glucitol 5-methanesulfonate (**10a**, 40%), along with 1,4 : 3,6-dianhydro-D-glucitol 2-methanesulfonate (**10b**, 6%) and 1,4 : 3,6-dianhydro-D-glucitol 2,5-dimethanesulfonate (10—20%).[15,16] Compounds **10a** and **10b** were purified by fractional crystallization. Then, **10a** was reacted with piperazine to afford crude **11** which was purified with porous polymer resin. Compound **11** was then nitrated with fuming HNO_3 to give the key intermediate **12**, which was *N*-alkylated to afford the target compounds **8** (**13**, **15**—**22**).

The stereochemistry of compounds **10a** and **10b** was determined by the aid of ^{1}H-NMR analysis. Here, we defined a CH proton adjacent to the methanesulfonyloxy group as H^6 and that adjacent to the hydroxyl group as H^3, while the other protons are numbered as shown in Chart 2. In compound **10a**, the ^{1}H–^{1}H coupling constant between H^5 and H^6 (J_{H^5,H^6}) was 4.5 Hz and the ^{1}H–^{1}H coupling constant between H^3 and H^4 (J_{H^3,H^4}) was 0 Hz. On the other hand, in compound **10b**, J_{H^5,H^6} was 0 Hz and J_{H^3,H^4} was 4.6 Hz. In nuclear Overhauser effect (NOE) experiments with **10a**, irradiation at δ 5.07 (H^6) enhanced the intensity of the signal at δ 4.70 (H^5) by 12.9%, but irradiation at δ 4.09 (H^3) enhanced the intensity of the signal at δ 4.28 (H^4) by only 3.7%. From these results, we identified the structures of **10a** and **10b** as shown in Chart 2. The stereochemistry of the target compounds **8** (**13**—**22**, **24**—**32**) was determined similarly. For example, in compound **13**, NOE was observed between H^3 and H^4 (6.2%) but not between H^5 and H^6 (0%) (Chart 2).

The carboxylic acid **19** was prepared by hydrolysis of the corresponding ethyl ester **18**. A homopiperazine ring instead of a piperazine ring was similarly introduced to afford **23** (Chart 3).

The target compounds can be alternatively obtained by

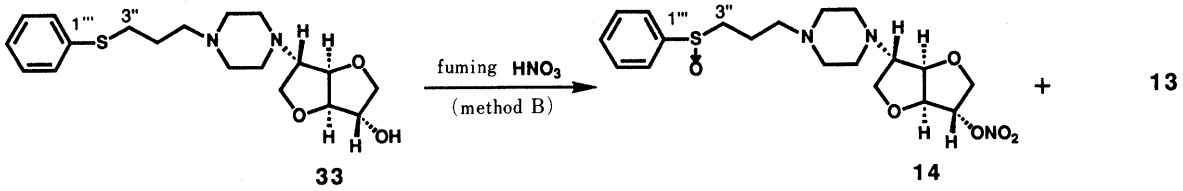
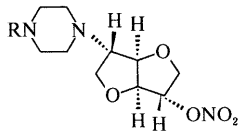
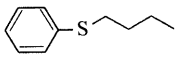
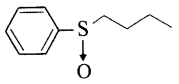
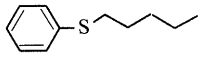
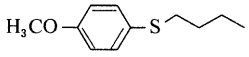
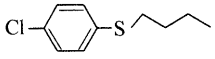
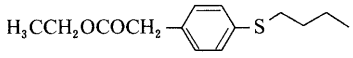
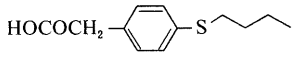
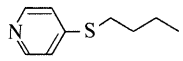
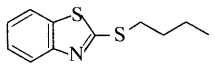
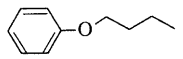
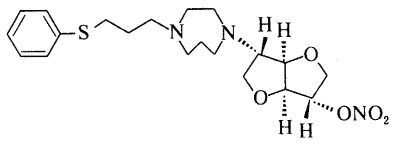


Chart 4

TABLE I. Physical Properties of Arylthioalkyl-Type Compounds



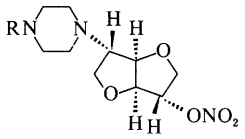
Compd. No.	R	Yield (%)	Method	mp (°C)[a]	Formula	Analysis (%) Calcd (Found) C	H	N
13		34	A	171.5—173.0	$C_{19}H_{27}N_3O_5S \cdot 2HCl \cdot 0.5H_2O$[b]	46.43 (46.61)	6.15 6.09	8.55 8.45)
14		35	B	179—181	$C_{19}H_{27}N_3O_6S \cdot 2HCl \cdot H_2O$[c]	44.18 (44.48)	6.05 6.01)	
15		64	A	195.5—197.5	$C_{20}H_{29}N_3O_5S \cdot 2HCl$	48.39 (48.29)	6.29 6.36	8.46 8.23)
16		62	A	203.5—204.5	$C_{20}H_{29}N_3O_6S \cdot 2HCl \cdot 0.5H_2O$[d]	46.06 (46.31)	6.18 6.18	8.05 8.04)
17		67	A	201.5—202.0	$C_{19}H_{26}ClN_3O_5S \cdot 2HCl \cdot H_2O$[e]	42.66 (42.43)	5.65 5.32	7.85 7.48)
18		70	A	194—195	$C_{23}H_{33}N_3O_7S \cdot 2HCl$	48.59 (48.76)	6.20 6.25	7.39 7.39)
19		78	A[f]	129—130	$C_{21}H_{29}N_3O_7S$	53.95 (53.96)	6.25 6.27	8.99 8.82)
20		50	A	207.7—208.5	$C_{18}H_{26}N_4O_5S \cdot 2HCl \cdot H_2O$[g]	40.19 (40.21)	5.81 5.64	10.42 10.17)
21		31	A	213.5—214.0	$C_{20}H_{26}N_4O_5S_2 \cdot 2HCl \cdot 2H_2O$[h]	41.73 (42.01)	5.60 5.30	9.73 9.75)
22		73	A	207.3—208.5	$C_{19}H_{27}N_3O_6 \cdot 1.5HCl \cdot H_2O$[i]	48.95 (49.33)	6.59 6.41	9.01 8.98)
23		53	A	Hygroscopic	$C_{20}H_{29}N_3O_5S \cdot 2HCl \cdot 4H_2O$[j]	42.25 (41.92		7.39 7.15)

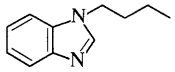
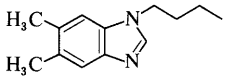
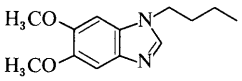
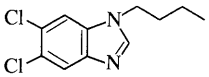
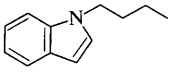
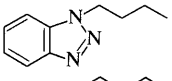
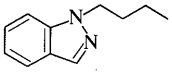
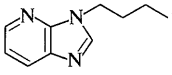
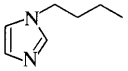
a) After purification by silica gel chromatography, the evaporation residue was dissolved in $CHCl_3$. To this solution was added 10 ml of EtOAc saturated with HCl. The resulting precipitate was filtered, washed with ethyl acetate, and dried to afford an analytical sample. b) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{28}N_3O_5S$: 410.1749. Found: 410.1746. c) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{28}N_3O_6S$: 426.1700. Found: 426.1686. d) HR FAB-MS ($M^+ + 1$) Calcd for $C_{20}H_{30}N_3O_6S$: 440.1855. Found: 440.1848. e) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{27}ClN_3O_5S$: 444.1360 (^{35}Cl). Found: 444.1368 (^{35}Cl). f) Synthesized by hydrolysis of compound **18**. g) HR FAB-MS ($M^+ + 1$) Calcd for $C_{18}H_{27}N_4O_5S$: 411.1702. Found: 411.1701. h) HR FAB-MS ($M^+ + 1$) Calcd for $C_{20}H_{27}N_4O_5S_2$: 467.1423. Found: 467.1425. i) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{28}N_3O_6$: 394.1978. Found: 394.1980. j) HR FAB-MS ($M^+ + 1$) Calcd for $C_{20}H_{30}N_3O_5S$: 424.1908, Found: 424.1918.

nitration of an alkylpiperazine derivative **33**. However, nitration of **33** with fuming HNO_3 was accompanied by oxidation of sulfur to afford the sulfoxide **14** (Chart 4). The structure of compound **14** was confirmed by spectroscopic analyses. The fast atom bombardment mass spectrum (FAB-MS) of **14** showed ($M^+ + 1$) at m/z 426 and that of **13** showed ($M^+ + 1$) at m/z 410. Thus, the presence of the oxygen in **14** was suggested. In the ^{1}H-NMR spectrum, the signals of aromatic protons of **14** were observed at lower fields than δ 7.5, and those of **13** at higher field than δ 7.5. Further, the signal of the aromatic carbon (^{13}C) adjacent to the S atom of **14** (C-1‴) was seen at δ 143.7 and that of the methylene carbon adjacent to the S atom (C-3″) was at δ 46.6. On the other hand, these signals of **13** were at δ 135.4 and δ 29.4, respectively, both at higher field than those observed in **14**. These results indicate that the sulfur atom was oxidized to the sulfoxide as shown in Chart 4.

The physical, analytical, and spectral data of the compounds are listed in Tables I, II and III.

TABLE II. Physical Properties of Benzimidazolylalkyl-Type Compounds



Compd. No.	R	Yield (%)	Method	mp (°C)[a]	Formula	Analysis (%) Calcd (Found)		
						C	H	N
24		35	A	216.5—217.5	$C_{20}H_{27}N_5O_5 \cdot 3HCl \cdot 0.5H_2O$[b]	44.83 (44.88)	5.83 5.65	13.07 13.03)
25		44	A	225.8—226.8	$C_{22}H_{31}N_5O_5 \cdot 3HCl \cdot H_2O$[c]	46.12 (46.16	6.33 6.22	12.22 11.96)
26		36	A	224.5—226.0	$C_{22}H_{31}N_5O_7 \cdot 3HCl$	45.02 (44.94	5.84 5.86	11.93 11.83)
27		52	A	228.0—229.5	$C_{20}H_{25}Cl_5N_5O_5 \cdot 3HCl$	40.32 (40.36	4.74 4.80	11.76 11.79)
28		44	A	>300	$C_{21}H_{28}N_4O_5 \cdot 2HCl \cdot 1.5H_2O$[d]	48.84 (49.10	6.44 6.14	10.84 10.51)
29		51	A	206.5—208.0	$C_{19}H_{26}N_6O_5 \cdot 2HCl \cdot 0.3H_2O$[e]	45.94 (46.01	5.80 5.83	16.92 16.70)
30		29	A	197.0—198.5	$C_{20}H_{27}N_5O_5 \cdot 2HCl \cdot H_2O$[f]	47.25 (47.28	6.15 6.07	13.78 13.74)
31		24	A	208.3—208.8	$C_{19}H_{26}N_6O_5 \cdot 3HCl \cdot 0.5H_2O$[g]	42.50 (42.51	5.63 5.59	15.65 15.34)
32		26	A	Hygroscopic	$C_{16}H_{25}N_5O_5 \cdot 3HCl \cdot 1.5H_2O$[h]	38.14 (38.52	6.20 6.01	13.90 13.63)

a) See footnote *a*) in Table I. *b*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{20}H_{28}N_5O_5$: 418.2090. Found: 418.2108. *c*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{22}H_{32}N_5O_5$: 446.2403. Found: 446.2412. *d*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{21}H_{29}N_4O_5$: 417.2138. Found: 417.2152. *e*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{27}N_6O_5$: 419.2043. Found: 419.2039. *f*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{20}H_{28}N_5O_5$: 418.2091. Found: 418.2101. *g*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{19}H_{27}N_6O_5$: 419.2043. Found: 419.2056. *h*) HR FAB-MS ($M^+ + 1$) Calcd for $C_{16}H_{26}N_5O_5$: 368.1934. Found: 368.1946.

Results and Discussion

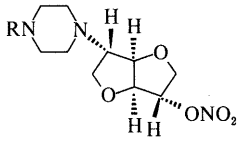
To avoid possible inconsistency between *in vitro* and *in vivo* results, we used an *in vivo* model for the initial screening. Lysine-vasopressin elevates the T-wave in the electrocardiogram (ECG) of the anesthetized rat.[17] Although this model is very useful for the evaluation of antianginal activity, its disadvantage is the inter-rat variability of response. This had to be compensated for by increasing the number of experiments. The activity data for this series of compounds are shown in Table IV. ISDN (**2**) and IS-5-MN (**3**) (30 mg/kg, *p.o.*) were not active in this assay, presumably due to the first-pass effect and weak vasodilatory activity,[13,17] respectively. However, nicorandil (**4**) potently inhibited the elevation.

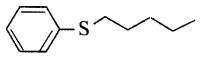
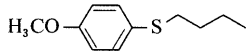
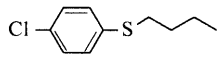
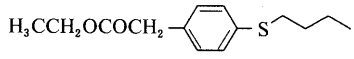
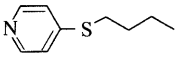
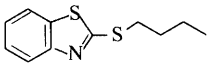
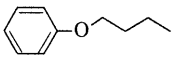
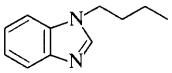
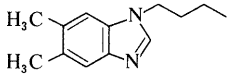
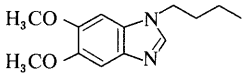
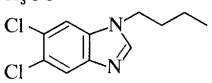
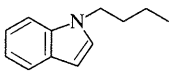
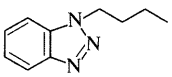
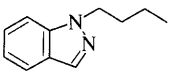
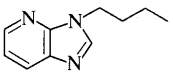
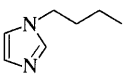
Initially, well-known lipophilic substituents in vasodilators were introduced into the piperazine moiety (R) in **8** and the activities of the derivatives were examined, but highly active compounds were not discovered (data not shown). After exhaustive screening, the phenylthiopropyl-substituted compound **13**, was found to exhibit potent activity comparable to that of nicorandil (**4**) (Table IV). Thus, a series of compounds **8**, having an arylthio- or aryloxy group (R) was examined. Extension of an alkyl group attached to piperazine caused loss of activity (**15**). The sulfoxide **14** was inactive. Substitution on the benzene ring in **13** usually led to a decrease in or loss of activity (**16**—**19**). 3-(Pyridin-4-yl)thiopropyl or 3-(benzothiazol-2-yl)thiopropyl substitution (**20** or **21**) decreased or eliminated the activity. Replacement of the S atom by an O atom (**22**) tended to reduce the activity. Further, replacement of piperazine by homopiperazine (**23**) also eliminated the activity. Thus, none of these modifications led to the increase in the activity associated with **13**.

Then, the phenylthio group was replaced by benzimidazole, indole, benzotriazole, indazole, azabenzimidazole, and imidazole. Compound **24** showed activity. Potent inhibition was observed in the 3-(5,6-dimethylbenzimidazol-1-yl)-propyl-substituted compound (**25**). Introduction of electron-donating groups or electron-withdrawing groups on the benzene ring of **25** resulted in loss of activity (**26**, **27**). Substitution by indole (**28**) or imidazole (**32**) led to the retention of activity, but other heterocyclic derivatives (**29**—**31**) lost the activity.

With respect to the selected compounds (**13**, **25**, **28**, and **32**) and nicorandil (**4**), the activity at lower doses was examined using the same model. Most of these compounds showed no activity at a dose of 10 mg/kg (*p.o.*). Only compound **13** exhibited activity at this dose (40.2%

TABLE III. Spectral Data for Compounds **15**—**18**, **20**—**22**, and **24**—**32**

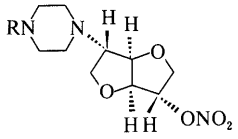


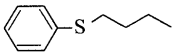
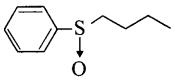
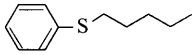
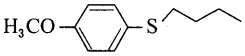
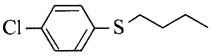
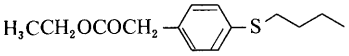
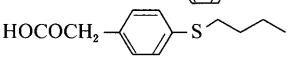
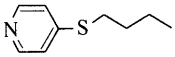
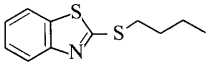
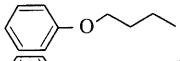
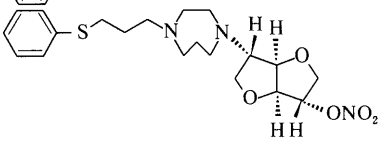
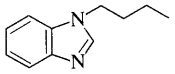
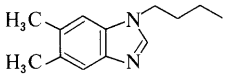
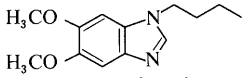
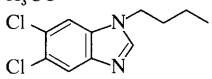
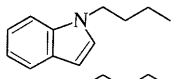
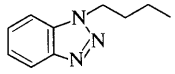
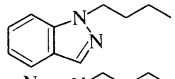
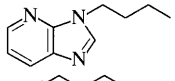
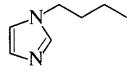
Compd. No.	R	IR (KBr) ν (cm^{-1})	MS m/z (M^+)	^{1}H-NMR (DMSO-d_6) δ (J=Hz)
15		2960, 2400, 1648, 1278	423	7.25—7.41 (4H, m), 7.19 (1H, m), 5.42 (1H, m), 5.05 (1H, m), 4.80 (1H, m), 4.09—4.21 (1H, m), 4.01—4.09 (2H, m), 3.86—3.99 (1H, m), 3.14 (2H, t, J=7.9), 3.05—3.80 (9H, m), 3.00 (2H, t, J=7.2), 1.84 (2H, m), 1.61 (2H, m)
16		2956, 2346, 1643, 1272	439	7.38 (2H, d, J=8.8), 6.93 (2H, d, J=8.8), 5.42 (1H, m), 5.03 (1H, m), 4.80 (1H, m), 4.08—4.19 (1H, m), 4.03—4.09 (2H, m), 3.86—3.97 (1H, m), 3.75 (3H, s), 3.4—4.5 (5H, m), 3.1—3.4 (6H, m), 2.92 (2H, t, J=7.0), 1.93 (2H, m)
17		2882, 2400, 1644, 1275	443 (^{35}Cl)	7.39 (4H, s), 5.42 (1H, m), 5.01 (1H, m), 4.79 (1H, m), 4.08—4.19 (1H, m), 4.03—4.09 (2H, m), 3.84—3.96 (1H, m), 3.4—4.5 (5H, m), 3.1—3.4 (6H, m), 3.07 (2H, t, J=7.2), 2.00 (2H, m)
18		2980, 2410, 1731, 1638, 1276	495	7.33 (2H, d, J=8.3), 7.23 (2H, d, J=8.3), 5.41 (1H, m), 5.01 (1H, m), 4.79 (1H, m), 3.64 (2H, s), 3.4—4.5 (11H, m), 3.1—3.4 (6H, m), 3.04 (2H, t, J=7.1), 2.01 (2H, m), 1.18 (3H, t, J=7.1)
20		2920, 2550, 1627, 1276	410	8.66 (2H, d, J=6.8), 7.96 (2H, d, J=6.8), 5.42 (1H, m), 5.06 (1H, m), 4.81 (1H, m), 4.1—4.2 (1H, m), 4.0—4.1 (2H, m), 3.85—4.00 (1H, m), 3.0—4.5 (13H, m), 2.17 (2H, m)
21		2960, 2368, 1635, 1275	466	8.02 (1H, d, J=7.7), 7.87 (1H, d, J=7.5), 7.48 (1H, m), 7.38 (1H, m), 5.42 (1H, m), 5.09 (1H, m), 4.82 (1H, m), 4.10—4.22 (1H, m), 4.03—4.10 (2H, m), 3.90—4.03 (1H, m), 3.49 (2H, t, J=7.1), 3.0—5.1 (11H, m), 2.27 (2H, m)
22		2954, 2362, 1643, 1278	393	7.30 (2H, m), 6.85—7.05 (3H, m), 5.42 (1H, m), 5.04 (1H, m), 4.80 (1H, m), 4.10—4.20 (1H, m), 4.00—4.11 (2H, m), 3.85—3.99 (1H, m), 3.0—5.1 (13H, m), 2.19 (2H, m)
24		2950, 2500, 1641, 1277	417	9.66 (1H, s), 8.05—8.14 (1H, m), 7.87—7.98 (1H, m), 7.60—7.75 (2H, m), 5.40 (1H, m), 4.80—4.87 (1H, m), 4.69—4.75 (1H, m), 4.64 (2H, t, J=7.2), 4.00—4.13 (1H, m), 2.7—3.9 (14H, m), 2.40 (2H, m)
25		2970, 2356, 1635, 1275	445	9.66 (1H, s), 7.92 (1H, s), 7.66 (1H, s), 5.42 (1H, m), 5.03 (1H, m), 4.79 (1H, m), 4.61 (2H, t, J=6.6), 3.0—4.5 (15H, m), 2.42 (3H, s), 2.41 (3H, s), 2.3—2.5 (2H, m)
26		2970, 2420, 1642, 1274	477	9.56 (1H, s), 7.72 (1H, s), 7.31 (1H, s), 5.42 (1H, m), 5.05 (1H, m), 4.80 (1H, m), 4.65 (2H, t, J=6.8), 4.09—4.20 (1H, m), 4.02—4.10 (2H, m), 3.94 (3H, s), 3.88 (3H, s), 3.0—4.1 (12H, m), 2.41 (2H, m)
27		2965, 2350, 1642, 1276	485 (^{35}Cl)	9.15 (1H, s), 8.38 (1H, s), 8.08 (1H, s), 5.43 (1H, m), 5.09 (1H, m), 4.81 (1H, m), 4.54 (2H, t, J=7.0), 4.11—4.21 (1H, m), 4.03—4.11 (2H, m), 3.92—4.04 (1H, m), 3.22 (2H, t, J=7.4), 3.00—3.85 (9H, m), 2.34 (2H, m)
28		2980, 2410, 1640, 1278	416	7.49—7.61 (2H, m), 7.39—7.48 (1H, m), 7.14 (1H, m), 7.03 (1H, m), 6.45 (1H, m), 5.41 (1H, m), 5.05 (1H, m), 4.80 (1H, m), 4.30 (2H, t, J=7.1), 3.15 (2H, t, J=7.9), 3.0—5.1 (13H, m), 2.23 (2H, m)
29		2982, 2360, 1639, 1278	418	8.05 (1H, d, J=8.4), 7.95 (1H, d, J=8.4), 7.58 (1H, m), 7.42 (1H, m), 5.41 (1H, m), 4.94 (1H, m), 4.84 (2H, t, J=7.0), 4.75 (1H, m), 2.8—4.2 (15H, m), 2.42 (2H, m)
30		2950, 2420, 1642, 1275	417	8.09 (1H, s), 7.77 (1H, d, J=8.1), 7.72 (1H, d, J=8.6), 7.41 (1H, m), 7.15 (1H, m), 5.41 (1H, m), 4.94 (1H, m), 4.75 (1H, m), 4.52 (2H, t, J=6.9), 2.8—4.3 (15H, m), 2.31 (2H, m)
31		2970, 2400, 1640, 1274	418	9.62 (1H, s), 8.65 (1H, d, J=4.8), 8.33 (1H, d, J=8.2), 7.61 (1H, dd, J=8.2, 4.8), 5.43 (1H, m), 5.13 (1H, m), 4.82 (1H, m), 4.61 (2H, t, J=6.6), 4.11—4.22 (1H, m), 4.08 (2H, m), 3.95—4.05 (1H, m), 3.81 (1H, m), 3.34—3.74 (8H, m), 3.28 (2H, t, J=7.7), 2.47 (2H, m)
32		2950, 2550, 1642, 1278	367	9.26 (1H, s), 7.85 (1H, m), 7.72 (1H, m), 5.43 (1H, m), 5.09 (1H, m), 4.82 (1H, m), 4.38 (2H, t, J=6.8), 4.10—4.22 (1H, m), 4.04—4.11 (2H, m), 3.90—4.03 (1H, m), 3.17 (2H, t, J=7.1), 3.0—5.0 (9H, m), 2.33 (2H, m)

inhibition) comparable to that of nicorandil (50.4% inhibition). Three stereoisomers ((*endo*, *exo*), (*exo*, *endo*), (*endo*, *endo*)) of **13** lacked the activity.[16] Thus, compound **13** (KF 14124) was selected for further studies.

Classical nitrates are known to reduce venous return by increasing venous capacitance.[1] Preload reduction resulting from this action is thought to play an important role in their therapeutic effects on angina pectoris and congestive

TABLE IV. Activity in a Lysine-Vasopressin-Induced Angina Model



Compd.	R	Dose, mg/kg	T wave Elevation (%)[a]		Δ (Inhibition, %)
			Control (%)	Treated (%)	
13		30 (*p.o.*)	111.2±21.2	37.3±11.2[b]	+66.5
		10 (*p.o.*)	128.5±18.5[d]	76.9± 8.6[b,d]	+40.2
14		30 (*p.o.*)	92.3±17.1	106.2±31.7	−15.1
15		30 (*p.o.*)	90.5±14.4	103.7± 5.8	−14.6
16		30 (*p.o.*)	68.6±11.6	72.4±15.6	−5.5
17		30 (*p.o.*)	68.6±11.6	51.0±10.8	+25.7
18		30 (*p.o.*)	111.2±21.2	112.4±14.2	−1.1
19		25 (i.p.)	111.2±21.2	66.4±13.2	+40.3
20		30 (*p.o.*)	77.8±10.9	52.1±11.8	+33.0
21		25 (*p.o.*)	83.4±13.2	74.7±13.8	+10.4
22		30 (i.p.)	101.7±15.5	74.8±18.6	+26.4
23		30 (*p.o.*)	77.8±10.9[e]	69.8±12.5	+10.3
24		30 (*p.o.*)	77.8±10.9[e]	33.9±14.6	+56.4
25		30 (*p.o.*)	111.2±21.2	36.3±10.5[c]	+67.4
26		30 (*p.o.*)	101.7±15.5	76.3±22.6	+24.9
27		30 (*p.o.*)	84.4±14.0	89.3±14.9	−5.7
28		30 (*p.o.*)	111.2±21.2	41.2± 6.6[b]	+63.0
29		30 (*p.o.*)	101.7±15.5	93.8±10.1	+7.7
30		25 (*p.o.*)	90.1±11.3[f]	75.7±21.9	+16.0
31		25 (i.p.)	90.1±11.3[f]	105.5±29.8	−17.0
32		30 (*p.o.*)	111.2±21.2	50.6± 9.2[b]	+54.5
2 (ISDN)		30 (*p.o.*)	84.3±17.8	106.5±17.3	−26.3
3 (IS-5-MN)		30 (*p.o.*)	68.6±11.6	79.0±15.1	−15.2
4 (Nicorandil)		30 (*p.o.*)	141.1±28.9[f]	33.5±14.3[c,f]	+76.3
		10 (*p.o.*)	141.1±28.9[f]	70.0±13.9[b,f]	+50.4

a) Results are expressed as mean values±S.E.M. (unless otherwise mentioned, the number of experiments is 4 for i.p., and 6 for *p.o.*). *b, c*) Significant difference from control group (*b*) $p<0.05$, *c*) $p<0.01$; unpaired Student's *t* test). *d*) Number of experiments is 8. *e*) Number of experiments is 4. *f*) Number of experiments is 10.

TABLE V. Activity in a Propranolol-Induced Heart Failure Model

Compd.	Dose, mg/kg (i.d.)	% decrease in LVEDP[a]		
		15 min[b]	30 min[b]	60 min[b]
13	0.3[c]	29.5±11.5	31.9±8.1	12.4± 7.6
	0.1[c]	17.3± 9.7	26.0±8.0	7.1±21.7
2 (ISDN)	1[d]	21.0± 2.4	17.7±3.2	11.7± 5.1
	0.3[d]	14.9± 6.5	13.1±5.6	1.6± 5.0
4 (Nicorandil)	0.3[d]	9.7± 7.1	9.4±6.9	2.1±10.3

a) Results are expressed as mean values±S.E.M. *b*) Time after administration of the compound. *c*) Number of experiments is 5. *d*) Number of experiments is 6.

TABLE VI. Antiarrhythmic Activity in a Reperfusion Arrhythmia Model

Compd.	Dose, mg/kg (i.v.)	*n*	Score					
			0	1	2	3	4	Mean[a]
Control (Saline)		15				3	12	3.80±0.01
2 (ISDN)	0.5	7					7	4.00
4 (Nicorandil)	3	9			3	2	4	3.11±0.31[b]
Control (Saline)		31		1	7	6	17	3.26±0.17
13	1	12	1	1	3	2	5	2.75±0.39
	3	8			7	1		2.13±0.33[c]

a) Results are expressed as mean values±S.E.M. Significant difference from the corresponding control groups (*b*) $p<0.05$, *c*) $p<0.01$; Mann–Whitney's test).

heart failure. Propranolol produces several of the hemodynamic characteristics of heart failure; it produces significant and sustained decreases in cardiac contractile force and cardiac output, resulting in the increase in left atrial pressure and left ventricular end-diastolic pressure (LVEDP), but it does not produce a significant alteration of stroke volume.[18,19] Inhibition of the increase in LVEDP can be used as an index of preload reduction. Thus, compound **13** was evaluated using this heart failure model in dogs (Table V). ISDN (**2**) at a dose of 0.3 mg/kg (i.d.) did not inhibit the increased LVEDP, but compound **13** tended to decrease LVEDP at a dose of 0.3 or 0.1 mg/kg. On the other hand, nicorandil (**4**) which was characterized as being predominantly an arteriolar vasodilator,[20] showed weak activity in this assay. Thus, compound **13** (KF14124) was proved to be an orally-active, nitrate-type vasodilator.

Then, compound **13** was evaluated for antiarrhythmic activity in a reperfusion arrhythmia model in the coronary-ligated rat. This model is useful for the evaluation of activity against ischemia-reperfusion arrhythmia, which is often observed in patients with angina pectoris.[21] To obtain a quantitative measure, the arrhythmias were graded according to their severity. The occurrence of premature beat was assigned the score 0, and the scores 1, 2, 3, and 4 were applied when nothing, tachycardia, fibrillation, and death occurred, respectively. Activity was estimated in terms of the mean score (Table VI). This model depends on the degree of cornonary ligature and the individual rat. Thus, the control value was obtained in each experiment. Compound **13** showed potent activity at 3 mg/kg (i.v.) and the activity was more potent than that of nicorandil (**4**).

Regarding acute lethal toxicity (mice), the LD_{50} value (the 50% lethal dose) of **13** was 550 mg/kg (*p.o.*) [nicorandil; >1000 mg/kg (*p.o.*): ISDN; >1000 mg/kg (*p.o.*)]. In addition, compound **13** showed several undesired side effects, such as central nervous system depression (reflex depression, behavioral depression, muscle relaxation, *etc.*) at oral doses of 50—100 mg/kg. The pharmacological and toxicological results will be reported in detail elsewhere.

In summary, we have described a novel series of vasodilators, 5-deoxy-5-(4-substituted alkyl)piperazinyl-1,4:3,6-dianhydro-L-iditol 2-nitrates. Although compound **13** showed potent activity in a lysine-vasopressin-induced angina model and a reperfusion arrhythmia model, it caused several side effects.

Experimental

Proton nuclear magnetic resonance spectra (^{1}H-NMR) and carbon-13 nuclear magnetic resonance spectra (^{13}C-NMR) were recorded on a JEOL JNM-GX270 FT NMR or a Hitachi R-90H FT NMR spectrometer with Me_4Si as an internal standard. Mass spectra were measured on a JMS-SX102 instrument. Melting points were determined with a Büchi-510 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a JASCO IR-810 spectrometer. Elemental analyses were performed by the analytical department of our laboratories. Most of the the compounds are very hygroscopic.

Chemistry The following procedures are representative of the general methods that are described in the text.

1,4:3,6-Dianhydro-D-glucitol 5-Methanesulfonate (10)[15,16] Methanesulfonyl chloride (31 ml, 0.401 mol) was added dropwise to a mixture of 1,4:3,6-dianhydro-D-glucitol (isosorbide (**9**), 48.2 g, 0.330 mol) and pyridine (240 ml) under ice-salt cooling over several hours. The mixture was stirred at room temperature for 10 h and concentrated under reduced pressure. The residue was taken up in water (150 ml) and the mixture was heated under reflux. After cooling, the precipitated crystals (1,4:3,6-dianhydro-D-glucitol 2,5-dimethanesulfonate) were filtered off, followed by addition of NaOH (15 g) under cooling. The mixture was concentrated under reduced pressure, and the residue was subjected to Soxhlet extraction (solvent, $CHCl_3$). After cooling, the precipitated crystals were collected by filtration and dried to give compound **10a** (35.5 g, 40%), mp 124.1—125.3 °C. ^{1}H-NMR (DMSO-d_6) δ: 5.19 (1H, br s, OH), 5.07 (1H, m, H^6), 4.70 (1H, dd, J=4.5, 4.5 Hz, H^5), 4.28 (1H, d, J=4.5 Hz, H^4), 4.09 (1H, m, H^3), 3.87 (1H, dd, J=9.5, 6.1 Hz, H^8), 3.73—3.78 (2H, m, H^1 and H^2), 3.64 (1H, dd, J=9.5, 6.0 Hz, H^7), 3.22 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6) δ: 87.9 (d, C-5), 79.8 (d), 79.2 (d), 75.4 (t, C-6), 74.9 (d), 69.1 (t, C-1), 37.6 (q, CH_3). The filtrate was concentrated under reduced pressure, and the residue was recrystallized from EtOH. Recrystallization from $CHCl_3$ gave compound **10b** (4.6 g, 6%), mp 137.2—138.7 °C. ^{1}H-NMR (DMSO-d_6) δ: 5.06 (1H, d, J=3.4 Hz, H^6), 4.92 (1H, br s, OH), 4.57 (1H, d, J=4.6 Hz, H^5), 4.47 (1H, dd, J=4.6, 4.6 Hz, H^4), 4.15 (1H, m, H^3), 4.02 (1H, d, J=11.2 Hz, H^7), 3.92 (1H, dd, J=11.2, 3.4 Hz, H^8), 3.76 (1H, dd, J=8.6, 6.5 Hz, H^1 or H^2), 3.36 (1H, m, H^1 or H^2), 3.26 (3H, s, CH_3).

5-Deoxy-5-(piperazin-1-yl)-1,4:3,6-dianhydro-L-iditol (11) A mixture of 1,4:3,6-dianhydro-D-glucitol 5-methanesulfonate (**10a**, 20.2 g, 90.1 mmol), piperazine (84.8 g, 984.4 mmol) and BuOH (240 ml) was refluxed for 36 h, then concentrated under reduced pressure. The residue was azeotropically evaporated with toluene several times to remove piperazine as thoroughly as possible. The residue was purified by column chromatography on Diaion SP 207® (Mitsubishi Kasei Co.,) with H_2O to MeOH–H_2O (3:7) as the eluent. After azeotropic distillation with iso-PrOH, the residue was recrystallized from EtOAc to give compound **11** (13.6 g, 70%). ^{1}H-NMR (DMSO-d_6) δ:5.35 (1H, m), 4.50 (1H, m), 4.19 (1H, m), 2.2—4.1 (14H, m).

5-Deoxy-5-(piperazin-1-yl)-1,4:3,6-dianhydro-L-iditol 2-Nitrate (12) A solution of **11** (19.4 g, 90.5 mmol) in H_2O (10.6 ml) was treated dropwise with concentrated H_2SO_4 (5.6 ml) under ice-cooling (solution A). A solution of urea (2.47 g, 41.1 mmol) in concentrated H_2SO_4 (55.6 ml) was added dropwise to fuming HNO_3 (86%, 37 ml) at −15 °C with stirring. The solution A was added dropwise to this mixture at −15 °C over 30 min to 1 h. The reaction mixture was stirred at the same temperature for a further 2 h, then slowly poured into H_2O (300 ml) with stirring. A solution of NaOH (120 g, 3.0 mmol) in H_2O (370 ml) was gradually added to neutralize this mixture under ice-cooling followed by extraction with $CHCl_3$ 5 to 10 times. The organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (10:1 to 0:1) as the eluent to give compound **12** (8.9 g, 38%). IR (KBr): 3420 (br), 1634,

1278 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 5.44 (1H, m), 4.86 (1H, m), 4.70 (1H, m), 1.9—4.6 (14H, m).

Method A. 5-Deoxy-5-[4(3-phenylthiopropyl)piperazin-1-yl]-1,4:3,6-dianhydro-L-iditol 2-Nitrate Dihydrochloride (13) A mixture of compound **12** (1.25 g, 4.82 mmol), 1-chloro-3-phenylthiopropane (0.91 g, 4.87 mmol), Et_3N (0.70 ml, 5.02 mmol), and EtOH (30 ml) was refluxed for 24 h. The mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (40:1) as the eluent. The product was dissolved in EtOH, and EtOAc saturated with HCl was added to this solution. The mixture was poured into cold Et_2O with stirring and the precipitated crystals were collected by filtration and dried to give compound **13** as the hydrochloride (0.80 g, 34%). IR (KBr): 2985, 2410 (br), 1643, 1275 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 11.65 (1H, br s), 7.28—7.45 (4H, m), 7.21 (1H, m), 5.42 (1H, m, H^3), 5.04 (1H, m, H^5), 4.80 (1H, m, H^4), 4.14 (1H, dd, J=9.9, 3.4 Hz, H^7), 4.01—4.10 (2H, m, H^1 and H^2), 3.92 (1H, dd, J=9.9, 3.7 Hz, H^8), 3.64 (1H, m, H^6), 3.1—4.6 (10H, m), 3.06 (2H, t, J=7.2 Hz), 2.02 (2H, m). ^{13}C-NMR (DMSO-d_6) δ: 135.4 (s, C-1‴), 129.0 (2C, d), 128.4 (2C, d), 125.9 (d, C-4‴), 86.2 (d), 84.1 (d), 83.6 (d), 68.9 (t), 68.8 (d, C-5), 68.2 (t), 54.1 (2C, t, C-2′ and C-6′), 48.8 (2C, t, C-3′ and C-5′), 46.7 (t), C-1″, 29.4 (t, C-3″), 23.0 (t, C-2″). FAB-MS m/z: 410 (M^++1). $[\alpha]^{20}$: +23.0° (c=0.10, H_2O). Compounds **15**—**18**, **20**—**22**, and **24**—**32** were prepared in a similar manner using the respective alkyl bromide or iodide instead of phenylthiopropyl chloride, and their spectral data are listed in Table III.

5-Deoxy-5-[4-(3-phenylthiopropyl)homopiperazin-1-yl]-1,4:3,6-dianhydro-L-iditol 2-Nitrate Dihydrochloride (23) Compound **23** was prepared in a manner similar to that described for **13** using homopiperazine instead of piperazine (total yield, 6% from **10**). IR (KBr): 2940, 2580 (br), 1636, 1276 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 7.28—7.46 (4H, m), 7.17—7.28 (1H, m), 5.44 (1H, m), 5.20 (1H, m), 4.85 (1H, m), 3.1—4.3 (15H, m), 3.05 (2H, t, J=7.2 Hz), 2.22 (2H, m), 1.99 (2H, m).

Method B. 5-Deoxy-5-[4-(3-phenylsulfinylpropyl)piperazin-1-yl]-1,4:3,6-dianhydro-L-iditol 2-Nitrate Dihydrochloride (14) 5-Deoxy-5-[4-(3-phenylthiopropyl)piperazin-1-yl]-1,4:3,6-dianhydro-L-iditol (**33**) was prepared in a similar manner to that described for **13** using **11** instead of **12** in the alkylation reaction (method A). Total yield from **11** was 27%. IR (KBr): 3410 (br), 2975 (br), 1442, 1073 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 11.71 (1H, br), 7.29—7.45 (4H, m), 7.17—7.27 (1H, m), 4.99 (1H, m), 4.36 (1H, m), 3.99—4.13 (2H, m), 3.87 (1H, m), 3.15—3.82 (13H, m), 3.06 (2H, t, J=7.2 Hz), 2.01 (2H, m). Fuming HNO_3 (0.88 ml) was cooled to 0 °C and CH_3CN (1.76 ml) and Ac_2O (4.40 ml) were added dropwise thereto (solution A). Solution A was then added dropwise to a solution of compound **33** (1.76 g, 4.83 mmol) in CH_3CN (4.4 ml) with stirring under ice cooling, and the mixture was stirred at 0 °C for an additional 1.5 h. Then, the pH of the reaction mixture was adjusted with 2.2 N NaOH to about 10, followed by extraction with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (25:1) as an eluent. The product was dissolved in EtOH, and EtOAc saturated with HCl was added. The mixture was poured into cold Et_2O with stirring and the precipitated crystals were collected by filtration and dried to give compound **14** as the hydrochloride (0.81 g, 35%). IR (KBr): 2960, 2415, 1639, 1444, 1277, 1085, 956, 852, 750, 690 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 11.53 (1H, br s), 7.70 (2H, m), 7.50—7.68 (3H, m), 5.42 (1H, m, H^3), 5.01 (1H, m, H^5), 4.79 (1H, m, H^4), 4.13 (1H, dd, J=9.9, 6.4 Hz, H^7), 4.01—4.10 (2H, m, H^1 and H^2), 3.90 (1H, dd, J=9.9, 6.3 Hz, H^8), 3.0—4.7 (12H, m), 2.85 (1H, m), 2.11 (1H, m), 1.95 (1H, m). ^{13}C-NMR (DMSO-d_6) δ: 143.7 (s, C-1‴), 130.8 (d, C-4‴), 129.2 (2C, d), 123.9 (2C, d), 86.1 (d), 84.1 (d), 83.5 (d), 68.9 (t), 68.7 (d, C-5), 68.1 (t), 53.9 (2C, t, C-2′ and C-6′), 52.2 (t, C-1″), 48.6 (2C, t, C-3′ and C-5′), 46.6 (t, C-3″), 16.7 (t, C-2″). FAB-MS m/z: 426 (M^++1).

5-[4-[3-[4-(Carboxymethyl)phenylthio]propyl]piperazin-1-yl]-5-deoxy-1,4:3,6-dianhydro-L-iditol 2-Nitrate (19) A mixture of compound **18** (1.87 g, 3.77 mmol), NaOH (0.40 g, 10.0 mmol), H_2O (25 ml), EtOH (25 ml), and THF (50 ml) was stirred at room temperature for 20 min, and then the pH of the reaction mixture was adjusted with 1 N HCl to 6—7. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (5:1) as an eluent and recrystallized from iso-PrOH–H_2O. The crystals were collected by filtration and dried to give compound **19** (1.38 g, 78%). IR (KBr): 3420 (br), 2922 (br), 1695, 1636, 1275 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 7.26 (2H, d, J=8.3 Hz), 7.19 (2H, d, J=8.3 Hz), 5.35 (1H, m), 4.62 (2H, m), 3.85—4.10 (3H, m), 3.53 (2H, s), 3.5—3.6 (1H, m), 2.94 (2H, t, J=7.3 Hz), 2.78 (1H, m), 2.15—2.65 (10H, m), 1.68 (2H, m).

Biology Lysine-Vasopressin-Induced Angina Pectoris Model in the Rat[17] Male Wistar rats weighing 210—250 g were used as experimental animals. The electrocardiogram (ECG lead II) was measured with an electrocardiograph and recorded on a polygraph (RPM-6200, Nihon Kohden, Tokyo, Japan). Oral (*p.o.*) and intraperitoneal (i.p.) administrations of the test compound to rats were performed at 20 and 10 min before anesthetization, respectively. Ten minutes after anesthetization with urethane (1.2 g/kg, i.p.), lysine-vasopressin (Sigma Co., Ltd.; V-2875, 0.3 I.U./kg) was intravenously injected into the rats. Then, T-wave elevation was observed in the ECG. The T-wave heights were measured before and at 15 to 30 s after the lysine-vasopressin injection in rats with and without test compound treatment, and the T-wave elevation % was calculated in each rat. Inhibition % (Δ) was calculated as follows: [mean control (%) − mean treated (%)] × 100/mean control (%). The control value was obtained in each experiment.

Propranolol-Induced Heart Failure Model in the Dog[19] Adult mongrel dogs of either sex, weighing 8—22 kg, were used for the experiments. The animals were anesthetized with sodium pentobarbital (35 mg/kg, i.v.) and artificially ventilated with a respirator (made by Takashima Co., for large animals) following tracheal intubation. The right common carotid artery was cannulated and the manometer for measurement of left ventricular pressure (Millar Tip) was advanced to the left ventricular cavity. Left ventricular pressure (LVP), the maximum rate of change of left ventricular pressure (LV dP/dt_{max}), and left ventricular end-diastolic pressure (LVEDP) were measured by the Millar Tip transducer. The systemic blood pressure (BP) was measured with a pressure transducer (MPU-0.5, Nihon Kohden) attached to a catheter placed in the femoral artery, and heart rate (HR) was measured with a cardiotachometer (AT610-G, Nihon Kohden) from BP. All measurements were recorded on a polygraph (RPM-6200, Nihon Kohden) or on a pen-recorder (RAT-1200, Nihon Kohden). After values of all parameters had stabilized, a bolus intravenous injection of propranolol at a dose of 2 mg/kg was performed. Thereafter, propranolol (0.05 mg/kg/min, i.v.) was continuously infused to evoke heart failure.[19a] An increase of 5 to 15 mmHg in LVEDP was considered as a sign of heart failure. After occurrence of heart failure, the test compound was intraduodenally administered. After administration of the test compound, LVEDP, LVP, LV dP/dt_{max}, BP, and HR were recorded every 15 min. Percent decrease in LVEDP was calculated as follows: [mean value before injection of drug − mean value after injection of drug] × 100/mean value before injection of drug.

Reperfusion Arrhythmia Model in the Coronary-Ligated Rats[21] Male Wistar rats weighing 175—245 g were used for the experiments. Each animal was anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and artificially respirated with room air *via* a tracheal cannula. Ligation of the coronary artery was performed according to the modified procedure described by Selye *et al.*[22] The coronary ligature was untied after 5 min and reperfusion was done for 10 min. The test compounds were injected intravenously immediately before reperfusion. The arrhythmias were observed during 10 min of the reperfusion time. The control value was obtained in each experiment.

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