Selective Inhibition of Cyclic Nucleotide Phosphodiesterases by Analogues of 1-Methyl-3-isobutylxanthine†

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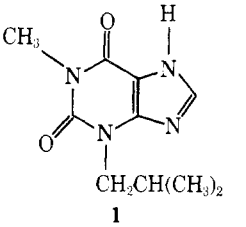
ABSTRACT: A series of 8-substituted 1-methyl-3-isobutylxanthines and 1,3-disubstituted xanthines was designed and prepared in an attempt to increase the specificity of the 1-methyl-3-isobutylxanthine (MIX) structure for one of two forms of cyclic nucleotide phosphodiesterase isolated from pig coronary arteries. The 8-substituted derivatives were in general much less potent than MIX as inhibitors of peak II phosphodiesterase activity; if the 8-substituent did not withdraw electrons, the compounds were as potent or more potent than MIX as inhibitors of peak I phosphodiesterase activity. 1-Isoamyl-3-isobutylxanthine and 1-*n*-amyl-3-isobutylxanthine, on the other hand, were about equipotent with MIX as inhibitors of peak II phosphodiesterase activity but were $\frac{1}{10}$ to $\frac{1}{15}$ as potent as MIX as inhibitors of peak I phosphodiesterase activity. Since different cell types may contain different forms of phosphodiesterase or different ratios of the forms, the xanthines were studied as inhibitors of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate hydrolysis by crude extracts from pig sperm, sea urchin sperm, and pig coronary arteries. 1-Isoamyl-3-isobutylxanthine is about twofold more potent as an inhibitor of cAMP than of cGMP hydrolysis by the coronary artery supernatant fraction, but is three- to fourfold more potent as an inhibitor of cGMP than cAMP hydrolysis by the supernatant fraction from a homogenate of pig sperm. Thus the selectivity of these inhibitors may vary from one cell type to another.

Adenosine 3′,5′-monophosphate (cAMP) has been implicated as an intracellular mediator of the actions of many hormones in a variety of tissues (Robison et al., 1971) and an increasing body of evidence suggests a regulatory role for this nucleotide in vascular and other smooth muscle (Anderson et al., 1972; Triner et al., 1971). The levels of guanosine 3′,5′-monophosphate (cGMP) are increased by cholinergic and other agents that produce contraction of smooth muscle (Lee et al., 1972; Schultz et al., 1973a–c; Goldberg et al., 1973) but the significance of these changing levels of cGMP is not clear. 1-Methyl-3-isobutylxanthine (MIX, **1**), a potent inhibitor of cyclic nucleotide phosphodiesterases in many tissues including coronary arteries (Wells et al., 1975a), produces relaxation of smooth muscle but increases the levels of both cAMP and cGMP in these tissues (Schultz et al., 1973b; Sutherland et al., 1973).

We have separated and partially characterized cyclic nucleotide phosphodiesterase activities from the intima + media layer of pig coronary arteries (Wells et al., 1975b) and from pig and sea urchin sperm (Wells and Garbers, 1976). Two fractions of phosphodiesterase activity and a heat-stable, nondialyzable activator were separated by DEAE[1]-cellulose chromatography of the 40 000*g* supernatant fraction of pig coronary arteries. Peak I phosphodiesterase activity catalyzes the hydrolysis of both cGMP and cAMP and exhibits classical kinetic behavior with both substrates, although it has a much lower apparent K_m for cGMP (2–4 μM) than for cAMP (40–100 μM). Peak I activity is stimulated three- to eightfold by a heat-stable, nondialyzable activator, while peak II activity is unaffected by this material. Peak II exhibits apparent negatively cooperative kinetic behavior and is relatively selective for cAMP (the lower limit of the apparent K_m value, which can be only roughly estimated from curvilinear plots, is approximately 1 μM for cAMP). The activity of peak II with cGMP as a substrate is too low to permit practical kinetic studies.



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We have recently shown that MIX (**1**) shows some selectivity for inhibition of cGMP over cAMP hydrolysis by crude phosphodiesterase preparations from pig coronary arteries and for peak I phosphodiesterase activity over peak II (Wells et al., 1975a). In addition, we have shown that substitution on the 7-position of MIX greatly reduces the potency of the xanthine to inhibit peak II activity. One 7-substituted derivative, 7-benzyl-MIX (**2**), is about 25-fold more potent as an inhibitor of peak I with 1 μM cGMP as substrate than of peak II activity with 1 μM cAMP as substrate (Garst et al., 1976).

It has been reported that some 8-substituted theophylline analogues are more potent than theophylline as inhibitors of cyclic nucleotide phosphodiesterase activity from beef heart at high (mM) cAMP concentrations (Goodsell et al., 1971). We, therefore, studied the effect of substitution on the 8-position of MIX, which is about 30-fold more potent than theophylline as an inhibitor of cyclic nucleotide phosphodiesterase activity from pig coronary arteries. We also prepared a series of xanthines in which the substituents on the 1- and 3-positions were varied. The primary aim of the present study was to study these analogues and derivatives of MIX to ascertain if increased selectivity for inhibition of the hydrolysis of cGMP or cAMP by the separated phosphodiesterases of coronary arte-

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; DMF, dimethylformamide.

ries is possible. In addition we compared the potency of some of these compounds as inhibitors of the blood vessel phosphodiesterases with their potencies as inhibitors of phosphodiesterases from sea urchin sperm and pig sperm. The latter experiments were carried out because the phosphodiesterases from sperm differ from the blood vessel phosphodiesterases in several ways such as substrate specificity, distribution, and kinetic parameters (Wells and Garbers, 1976). It seemed possible that the specificity and/or potency of inhibition by the MIX derivatives would also differ in these systems.

Experimental Procedures

Melting points were determined in open glass capillary tubes using a Laboratory Devices Mel-Temp and are reported uncorrected. Infrared spectra were obtained using a Perkin-Elmer Model 257 spectrophotometer. Nuclear magnetic resonance spectra were recorded on a Jeol Model JNM-MH-100 spectrometer using tetramethylsilane as an internal standard. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. In all cases spectral data were consistent with the proposed structures and elemental analyses were within ±0.4% of the calculated values.

Enzyme Preparation. The crude supernatant and DEAE-cellulose-separated phosphodiesterase activities from pig coronary arteries were obtained as described previously (Wells et al., 1975b). Briefly, the right coronary, anterior descending, and circumflex arteries from fresh cold pig hearts were everted, and an inner layer which consisted predominantly of microscopically identifiable intima + media tissue was removed and homogenized in 4 mL of buffer (20 mM Tris-HCl, pH 7.5, 2 mM MgOAc, and 1 mM dithiothreitol) per g of tissue (wet weight). After centrifugation at 40 000*g* for 30 min at 0 °C, the supernatant fraction was used directly or was subjected to DEAE-cellulose chromatography as described previously (Wells et al., 1975b).

Sea urchin (*Strongylocentrotus purpuratus*) and pig sperm were collected and washed as previously described (Wells and Garbers, 1976; Garbers et al., 1971) and suspended to 20% (wet w/v) in a solution containing 20 mM Tris-HCl buffer at pH 7.5, 1 mM dithiothreitol, and 2 mM $MgCl_2$. This suspension was then homogenized with an Ultra-Turrax homogenizer (Jahnke and Kunkel, Staufen, Germany). The homogenate was either used directly or was centrifuged at 40 000*g* for 30 min at 0–4 °C.

Phosphodiesterase Assay. The compounds were assayed for inhibitor activity against the crude homogenate, the 40 000*g* supernatant fraction, or the separated forms of phosphodiesterase. The assay procedures have been reported (Wells et al., 1975a,b). Assays were performed with 1 μM substrate at 30 °C for 30 min at enzyme dilutions which gave 10–20% hydrolysis of substrate in the absence of inhibitor. MIX (10^{-5} M) was included in each experiment to assure that the enzyme preparation was responding in a constant manner. The compounds were dissolved in 30% Me_2SO, and 25 μL of this solution was added to the assay tube (final volume was 250 μL). All activities (including control and no-enzyme blank) were measured in the presence of 3% Me_2SO, and product accumulation was linear for at least 30 min under the conditions of the assay. None of the compounds altered the efficacy of the nucleotidase step or subsequent steps in the assay. Concentrations of the compounds that inhibited by 50% the hydrolysis of 1 μM substrate (I_{50}) were determined from concentration–percent inhibition curves, utilizing concentrations of the compounds from 10^{-7} to 10^{-4} M (or 10^{-7} to 10^{-3} M if the compound was sufficiently soluble). The presence or absence of the calcium-binding protein activator and/or calcium ion did not affect the activity of the compounds studied. Each compound was assayed in the presence and absence of the protein activator. Since no differences were observed, the values given in Table II are composites of data obtained in the presence and absence of the activator.

Synthesis. 1,3-Disubstituted Xanthines. General Procedures. The appropriate 1-monosubstituted 6-aminouracil was prepared and alkylated following the procedure of Papesch and Schroeder (1953) using the indicated alkylating agent (Table I) to give the 1,3-disubstituted 6-aminouracil. This was converted to the xanthine using the following modification of the method of Speer and Raymond (1953). The crude 1,3-disubstituted 6-aminouracil (5–10 mmol) was suspended or dissolved with warming in 20% HOAc (25 mL). Sodium nitrite (15 mmol) in H_2O (5 mL) was added dropwise to give the 5-nitrosouracil as a red or purple precipitate. The crude 5-nitrosouracil was dissolved by warming in concentrated NH_4OH (10 mL), and $Na_2S_2O_4$ (30 mmol) in H_2O (20 mL) was added slowly with stirring and warming. The mixture was warmed and stirred for ca. 30 min and then cooled in an ice bath. The resulting precipitate was separated by filtration and air-dried for a short time. If no precipitate was obtained, the cold mixture was extracted with CH_2Cl_2. The CH_2Cl_2 solution was dried ($MgSO_4$) and then evaporated to give the 1,3-disubstituted 5,6-diaminouracil. This crude product was dissolved in 98% formic acid (10 mL) and heated under reflux for 1 h. The formic acid was removed in vacuo. To the resulting oil was added 10% NaOH (10 mL), and the mixture was heated under reflux for ca. 15 min. The reaction mixture was cooled in an ice bath and acidified with concentrated HCl. The resulting precipitate was separated by filtration and purified by recrystallization from the solvent indicated in Table I or sublimed to give compounds **3–9.**

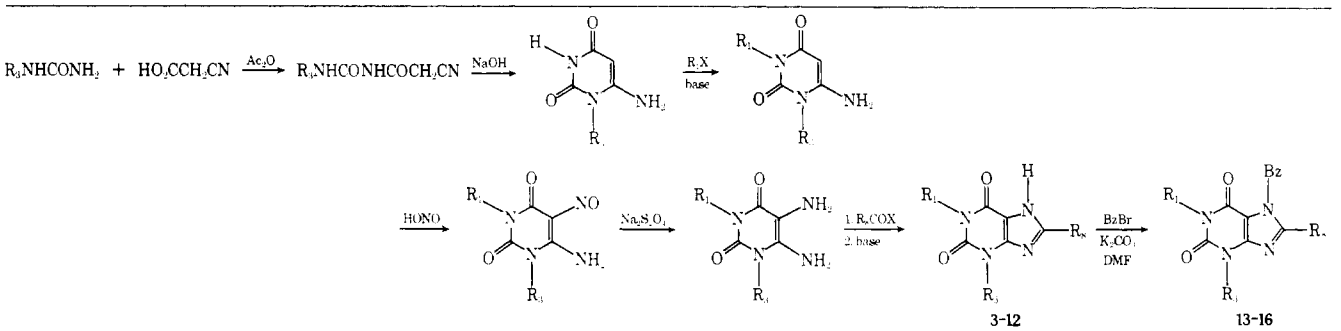
8-Substituted 1-Methyl-3-isobutylxanthines. General Procedure. I-Isobutyl-3-methyl-5,6-diaminouracil (Papesch and Schroeder, 1952) (10 mmol) was heated under reflux in acetic anhydride (25 mL, 2 h), trifluoroacetic anhydride (25 mL, 2 h), or with pivaloyl chloride (12 mmol) in pyridine (25 mL, 6 h). The resulting solid (after washing with H_2O (25 mL) to remove pyridine salts where necessary) was dissolved with heating and stirring in 10% NaOH (50 mL). The resulting solution was filtered and allowed to cool. The solution was acidified to pH 3–4 by addition of 6 N HCl with rapid stirring. The solid was collected by filtration and washed with H_2O (25 mL) to give **10, 12,** or **11,** respectively. The products were then recrystallized from the solvent indicated in Table I.

8-Substituted 1-Methyl-3-isobutyl-7-benzylxanthines. General Procedure. Benzylation of **10, 11, 12,** and **17** was carried out according to the previously reported method (Garst et al., 1976) by treatment with benzyl bromide and K_2CO_3 in DMF for 2 h. The reaction mixture was then poured into H_2O and cooled overnight to give **13, 14, 15,** and **16,** respectively. The products were recrystallized from the solvent indicated in Table I.

1-Methyl-3-isobutyl-8-bromoxanthine (**17**). Bromine (5 mmol) was added to a solution of 1-methyl-3-isobutylxanthine (5 mmol) and NaOAc (5 mmol) in glacial HOAc (57.5 g) heated at 40–50 °C. Heating was continued for 20 min and the solution was allowed to cool and the crude product collected by filtration. Purification was carried out by dissolution of the crude product in base followed by reprecipitation by addition of acid. The resulting solid was recrystallized from EtOH–H_2O: 95%; mp 228–229 °C. Anal. ($C_{10}H_{13}BrN_4O_2$) C, H.

1-Benzyl-3-isobutylxanthine (**18**). To a solution of 1-iso-

TABLE I: Preparative Data and Physical Properties of Substituted Xanthines.



No.	R_3	R_1X	R_8	Mp	Recrystn solvent or method	Yield (%)	Formula	Anal.
3	Benzyl	(None) R_1 = H	H	314d	Acidify basic solution	41[a]	$C_{12}H_{10}N_4O_2$	C, H
4[b]	Benzyl	Me_2SO_4	H	261.5–263.5	EtOH–H_2O	32[a]	$C_{13}H_{12}N_4O_2$	C, H
5[b]	Isoamyl	Me_2SO_4	H	186–187.5	Subl [145 °C (1 mm)]	30[a]	$C_{11}H_{16}N_4O_2$	C, H
6	*i*-Bu	*i*-BuOTs[c]	H	187–189	Benzene	5[a]	$C_{13}H_{20}N_4O_2$	C, H
7	*i*-Bu	*n*-AmOTs[c]	H	181.5–183.5	Benzene	18[a]	$C_{14}H_{22}N_4O_2$	C, H
8	*i*-Bu	*i*-AmOTs[c]	H	189–191	Benzene	12[a]	$C_{14}H_{22}N_4O_2$	C, H
9	*i*-Bu	*n*-OctOTs[c]	H	150–151	Column chromatog[d] recryst hexane	2[a]	$C_{17}H_{22}N_4O_2$	C, H
10	*i*-Bu	Me_2SO_4	Me	221.5–223	EtOH–H_2O	65[e]	$C_{17}H_{16}N_4O_2$	C, H
11	*i*-Bu	Me_2SO_4	*t*-Bu	208.5–210	EtOH–H_2O	68[e]	$C_{14}H_{22}N_4O_2$	C, H
12	*i*-Bu	Me_2SO_4	CF_3	216–217	EtOH–H_2O	52[e]	$C_{11}H_{13}F_3N_4O_2$	C, H
13	*i*-Bu	Me_2SO_4	Me	149–150.5	95% EtOH	80[f]	$C_{18}H_{22}N_4O_2$	C, H
14	*i*-Bu	Me_2SO_4	*t*-Bu	135.5–137	95% EtOH	78[f]	$C_{21}H_{28}N_4O_2$	C, H
15	*i*-Bu	Me_2SO_4	CF_3	87–89	95% EtOH	95[f]	$C_{18}H_{19}F_3N_4O_2$	C, H
16	*i*-Bu	Me_2SO_4	Br	132.5–134	95% EtOH	82[f]	$C_{17}H_{19}BrN_4O_2$	C, H

[a] Yield from 1-alkyl-6-aminouracil. [b] Previously prepared but physical data not given (Wooldridge and Slack, 1962). [c] OTs = *p*-toluenesulfonate. [d] Neutral alumina eluted with benzene then Et_2O–MeOH. [e] Yield from 1-methyl-3-isobutyl-5,6-diaminouracil. [f] Yield for benzylation step from appropriate xanthine.

butyl-6-aminouracil (Papesch and Schroeder, 1953) (87 mmol) in concentrated H_2SO_4 (15 mL) was added fuming HNO_3 (3.2 mL) dropwise with stirring (Brown et al., 1955). The solution was cooled on an ice bath and then poured onto ice. The precipitate, 1-isobutyl-5-nitro-6-aminouracil, was separated by filtration, air-dried, and used without further purification: 80%. A solution of this compound (10 mmol), KOH (11 mmol), and benzyl chloride (12 mmol) in DMF (15 mL) was heated at 100 °C for 1 h. The mixture was allowed to cool and the NaCl precipitate was removed by filtration. The filtrate was evaporated in vacuo to give the crude alkylated product. This crude product was reduced with $Na_2S_2O_4$, formylated, and cyclized according to the general procedure for 1,3-disubstituted xanthines to give 1-benzyl-3-isobutylxanthine: 20%. An analytical sample was obtained by sublimation (145 °C, 1 mm): mp 175–176 °C. Anal. ($C_{16}H_{18}N_4O_2$) C, H.

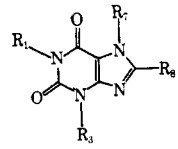
1,9-Dimethyl-3-isobutylxanthine (**20**). 1-Isobutyl-3-methyl-5,6-diaminouracil was prepared following the general procedure for 1,3-disubstituted xanthines through the reduction step with $Na_2S_2O_4$. This product was converted to 1,9-dimethyl-3-isobutyl-8-mercaptoxanthine by the method of Blicke and Schaaf (1956). This compound (2 mmol) was dissolved in glacial HOAc (10 mL) with warming and $NaNO_2$ (29 mmol) in H_2O (20 mL) was added dropwise with stirring. The reaction mixture was made basic (pH 9) with NH_4OH and diluted with water to 100 mL total volume. The solution was cooled overnight to give a crystalline precipitate. The solid was separated by filtration and washed with water: 80%; mp 164–166.5 °C. Anal. ($C_{11}H_{16}N_4O_2$) C, H.

Results and Discussion

The most demanding assessment of potency and selectivity of inhibition of the coronary artery phosphodiesterases is to compare the potency of a compound as an inhibitor of cGMP hydrolysis by peak I with the potency of the compound as an inhibitor of cAMP hydrolysis by peak II, since these are the high-affinity substrates for the respective enzyme preparations (Wells et al., 1975b). The 1 μM substrate concentration used in this study is near the apparent K_m's of the enzymes being studied. All of the compounds in this study were competitive inhibitors of peak I phosphodiesterase activity. Although K_i values could be obtained, the potencies are given as I_{50} values (the concentrations required to inhibit by 50% the hydrolysis of 1 μM substrates), because the purpose of the study was to compare the potencies of the compounds as inhibitors of peak I activity with the potencies to inhibit peak II. Since peak II displays anomalous kinetics, it is difficult or impossible to estimate meaningful K_i values from graphical analysis of the data (Wells et al., 1975a).

A number of generalizations are suggested by the data in Table II concerning structural requirements for potency of xanthines as inhibitors of pig coronary artery phosphodiesterases. It would appear that alkyl substitution larger than methyl in position 3 of the xanthine nucleus enhances potency against either peak of activity. Some substitution other than H at position 1 is apparently also required for potent inhibition of either form of phosphodiesterase. Substitution of electron-withdrawing groups at position 8 reduces the potency against either peak. Alkyl substitution at position 7 (Garst et al., 1976) or 8 has little effect on potency against peak I but greatly re-

TABLE II: Inhibition of Separated Phosphodiesterases from Pig Coronary Arteries.



Compound No.	R_1	R_3	R_7	R_8	I_{50} (μM)[a] and % inhibition at 100 μM: Peak I cGMP	Peak I cAMP	Peak II cAMP
1[b]	CH_3	*i*-Bu	H	H	6.3 ± 0.7 (6)	3.9 ± 0.3 (6)	15 ± 2 (3)
10	CH_3	*i*-Bu	H	CH_3	1.9 ± 0.1 (5)	2.3 ± 0.6 (7)	68 ± 4 (6)
12	CH_3	*i*-Bu	H	CF_3	>100 (38% ± 5) (6)	84 ± 5 (6)	>100 (21% ± 5) (3)
11	CH_3	*i*-Bu	H	$C(CH_3)_3$	1.8 ± 0.2 (7)	1.2 ± 0.2 (9)	58 ± 6 (6)
17	CH_3	*i*-Bu	H	Br	55 ± 9 (8)	35 ± 4 (6)	>100 (24% ± 5) (4)
2[b]	CH_3	*i*-Bu	Benzyl	H	4.4 ± 0.7 (7)	3.6 ± 0.8 (7)	108 ± 14 (4)
13	CH_3	*i*-Bu	Benzyl	CH_3	2.2 ± 0.3 (10)	3.2 ± 0.6 (10)	24 ± 4 (4)
14	CH_3	*i*-Bu	Benzyl	$C(CH_3)_3$	6.2 ± 0.6 (7)	6.0 ± 0.4 (7)	>10
15	CH_3	*i*-Bu	Benzyl	CF_3	11 ± 1 (6)	17 ± 4 (6)	78 ± 6 (5)
16	CH_3	*i*-Bu	Benzyl	Br	2.2 ± 0.4 (6)	2.9 ± 0.5 (6)	16 ± 1 (3)
19[c]	H	*i*-Bu	H	H	>100 (43% ± 6) (5)	>100 (34% ± 2) (6)	>100 (58% ± 3) (4)
6	*i*-Bu	*i*-Bu	H	H	>100 (41% ± 7) (5)	>100 (38% ± 4) (5)	44 ± 7 (3)
7	*n*-Amyl	*i*-Bu	H	H	>100 (46% ± 5) (10)	>100 (42% ± 4) (12)	40 ± 10 (4)
8	Isoamyl	*i*-Bu	H	H	86 ± 5 (7)	>100 (44% ± 2) (7)	17 ± 3 (6)
9	*n*-Octyl	*i*-Bu	H	H	>100 (33% ± 6) (6)	>100 (29% ± 7) (6)	>100 (38% ± 2) (3)
18	Benzyl	*i*-Bu	H	H	22 ± 4 (3)	25 ± 8 (3)	36 ± 8 (6)
21	Isoamyl	CH_3	H	H	>100 (43% ± 3) (4)	>100 (54% ± 4) (6)	50 ± 9 (4)
Theophylline[d]	CH_3	CH_3	H	H	100 ± 9	58 ± 4	190 ± 6
4	CH_3	Benzyl	H	H	26 ± 5 (7)	20 ± 3 (7)	32 ± 4 (4)
5	CH_3	Isoamyl	H	H	6.0 ± 1 (4)	6.3 ± 2 (5)	17 ± 2 (4)
3	H	Benzyl	H	H	>100 (26% ± 3) (8)	>100 (30% ± 2) (8)	90 ± 8 (4)
20	CH_3	*i*-Bu	9-CH_3	H	>100 (38% ± 4) (6)	>100 (55% ± 5) (5)	>100 (9.0% ± 2) (3)

[a] I_{50} value is the concentration (μM) of agent required to inhibit by 50% the hydrolysis of 1 μM substrate. Values are ± standard errors of the number of determinations given in parentheses. Some of the agents were insoluble above 100 μM; therefore, % inhibition at 100 μM is given if I_{50} is above 100 μM. [b] Garst et al. (1976). [c] Wooldridge and Slack (1962). [d] Wells et al. (1975a).

duces potency against peak II. Substitution of certain saturated alkyl groups larger than methyl at position 1 has little effect on potency against peak II but greatly reduces potency against peak I. These generalizations would indicate that there are relatively strict electronic requirements at the site of inhibition of both forms of phosphodiesterase and that both forms have rather specific requirements for substitution at position 3 of the xanthine nucleus. Further, there appears to be a tolerance or preference for alkyl bulk in positions 7 or 8 at the peak I site that is absent at the peak II site. Alkyl bulk in position 1, however, is tolerated at the peak II site but not by peak I.

Substitution of a benzyl (**4**), isoamyl (**5**), or isobutyl (**1**) moiety for the methyl group in the 3-position of theophylline brings about a 4- to 16-fold increase in potency against either peak of activity. Comparison of MIX (**1**) or **4** with **19** and **3**, respectively, shows that replacement of the 1-methyl group with H reduces potency against either peak from 3- to 16-fold. Such a replacement would be expected to bring about relatively large changes in the electronic and tautomeric properties of the xanthine ring. Another indication of the importance of the electronic properties of the xanthine is the effect of substitution of electron-withdrawing groups in the 8-position. The methyl and trifluoromethyl groups are quite similar in size (van der Waals radii of H and F are 1.2 and 1.35 Å, respectively; Hine, 1956) but they have distinctly different electronic properties (the pK_a of acetic and trifluoroacetic acid are 4.76 and 0.23, respectively; Albert and Serjeant, 1962). Thus, the 8-trifluoromethyl group of **12** has a much greater tendency to withdraw electrons from the xanthine nucleus than does the 8-methyl group of **10**. A comparison of the I_{50} values in Table II for 8-methyl-MIX (**10**) and 8-trifluoromethyl-MIX (**12**) shows the greater potency of **10** against either peak of activity. Similar results are found with an 8-bromo group (**17**) which is also electron withdrawing and is slightly smaller in size than methyl (van der Waals radii for bromo and methyl are 1.95 and 2.0 Å, respectively; Hine, 1956). This effect of electron-withdrawing groups is, for reasons not immediately obvious, attenuated or eliminated if a benzyl moiety is substituted in the 7-position (Table II, compounds **15** and **16**).

8-Methyl-MIX (**10**) and 8-*tert*-butyl-MIX (**11**) are about one-fourth as potent as MIX as inhibitors of peak II activity but are slightly more potent than MIX against peak I. The

TABLE III: Inhibition of the Cyclic Nucleotide Phosphodiesterase Activities in Crude Supernatant Fractions or Homogenate of Pig Coronary Arteries, Pig Sperm, and Sea Urchin Sperm.

No.	Compound	I_{50}[a] Coronary artery supernatant fraction cAMP	Coronary artery supernatant fraction cGMP	Pig sperm supernatant fraction cAMP	Pig sperm supernatant fraction cGMP	Urchin sperm homogenate cAMP	Urchin sperm homogenate cGMP
1	MIX	11 ± 2	5.6 ± 0.8	65 ± 7	4.7 ± 0.5	5.4 ± 0.2	0.9 ± 0.2
2	7-Benzyl MIX	30 ± 6	3.9 ± 0.6	84 ± 10	4.5 ± 0.6	21 ± 2	>100
11	8-*t*-Bu MIX	18 ± 4	1.9 ± 0.4	>100	2.0 ± 0.7	7.7 ± 1.7	0.2 ± 0.01
10	8-Methyl MIX	21 ± 5	2.0 ± 1	9.8 ± 0.2	1.6 ± 0.4	1.4 ± 0.3	0.08 ± 0.01
8	1-Isoamyl-3-isobutylxanthine	40 ± 4	97 ± 3	>100	28 ± 6	19 ± 1	42 ± 2

[a] Values are means ± standard errors of four determinations with four different preparations.

reduction in potency as inhibitors of peak II would seem to be because the binding site for the xanthines on peak II cannot tolerate a group larger than H on the 8-position of MIX. The catalytic site of peak I, however, tolerates or prefers relatively large substituents in this position. Similarly, substitution of relatively large groups (e.g., *n*-hexyl) on the 8-position of theophylline increased the potency of the compounds as inhibitors of a beef heart phosphodiesterase as measured at high (mM) substrate concentration (Goodsell et al., 1971).

Substitution of an isobutyl, *n*-amyl, or isoamyl group for the 1-methyl group of MIX (compounds **6, 7,** and **8**) has a relatively small effect on potency against peak II but significantly reduces potency against peak I. Thus, while 8-methyl-MIX (**10**) and 8-*tert*-butyl-MIX (**11**) were 30- to 35-fold more potent as inhibitors of 1 μM cGMP hydrolysis by peak I than of hydrolysis of 1 μM cAMP by peak II, compounds **6, 7,** and **8** are 2- to 5-fold more potent as inhibitors of peak II than of peak I under the same conditions. It appears that large alkyl groups in the 1-position of the xanthine molecule cannot be tolerated by the catalytic site of peak I, while the site to which the xanthines bind on peak II can accommodate a group at least as large as isoamyl. That steric size of the groups may not be the only factor that determines the effect of substitution at the 1-position is indicated by the data for 1-octyl-3-isobutylxanthine (**9**) and 1-benzyl-3-isobutylxanthine (**18**). Compound **9** is quite inactive against both peaks, indicating the limits of the tolerance for bulk in this position by peak II. The relatively large benzyl group in **9,** however, has less effect on peak I inhibition than might be expected and leaves this compound equipotent with the isobutyl (**6**) and *n*-amyl (**7**) compounds against peak II. Data for 1-isoamyl-3-methylxanthine (**21**) suggest some affinity for the 1-isoamyl group since this compound is almost fourfold more potent against peak II than is theophylline.

We have previously shown that substitution of a benzyl group for the 7-H of MIX yields a compound (**2**) with increased selectivity for peak I phosphodiesterase (Garst et al., 1976). We, therefore, prepared a series of 7-benzyl 8-substituted MIX derivatives (**13–16**). 7-Benzyl-8-methyl-MIX (**13**) and 7-benzyl-8-*tert*-butyl-MIX (**14**) were less potent as inhibitors of peak I activity and less selective between peak I and peak II activity than were the corresponding 8-substituted compounds (**10** and **11**). On the other hand, substitution of a benzyl moiety for the 7-H of 8-trifluoromethyl-MIX and 8-bromo-MIX gave compounds (**15** and **16**) with greater potency as inhibitors of peak I activity than the corresponding 8-substituted compound (**12** and **17**).

9-Methyl-MIX (**20**), although not as potent as 7-methyl-MIX (Garst et al., 1976) as an inhibitor of peak I, has very low potency as an inhibitor of peak II; therefore, proper substitution in the 9-position might yield compounds with high specificity for inhibition of peak I.

The use of separated forms of phosphodiesterase allows a more valid and straightforward interpretation of the selectivity and nature of inhibition than does the use of whole homogenates. However, in examining only the separated enzymes, it might be possible to overlook inhibition of significant activities that are not present among the separated forms. Moreover, the patterns of inhibition seen in crude systems may be more representative of the composite patterns that occur in intact cells. Table III gives the inhibitory potency of some of these xanthines as inhibitors of total cGMP and cAMP phosphodiesterase activities of crude 40 000*g* supernatant fractions from coronary arteries and pig sperm and in the whole homogenate of sea urchin sperm. Whole homogenate of sea urchin sperm was used because the phosphodiesterase activity is predominately located in the particulate fraction, whereas most of the activity of the coronary arteries and pig sperm is in the supernatant fraction (Wells et al., 1975b; Wells and Garbers, 1976). At 1 μM cAMP, total phosphodiesterase activity of peak I from coronary artery is approximately equal to the total activity of peak II; therefore, a compound which selectively inhibits peak I will have a significant effect on the hydrolysis of cAMP by the crude supernatant fraction. This point is demonstrated by a comparison of the data in Table II with the data of Table III.

The relative potencies of these compounds to inhibit cAMP and cGMP hydrolysis changes or even reverses from the coronary artery system to pig or sea urchin sperm systems. For example, 7-benzyl-MIX (**2**), which is about eightfold more potent as an inhibitor of cGMP than cAMP hydrolysis by the crude artery supernatant fraction, is at least fourfold more potent as an inhibitor of cAMP than cGMP hydrolysis by the crude sea urchin sperm homogenate (Table III).

The data in Table III indicate that these compounds are, in general, much more potent as inhibitors of sea urchin sperm phosphodiesterase activities than activities from the two other sources studied and that 8-*tert*-butyl-MIX (**11**) and 8-methyl-MIX (**10**) are extremely potent (I_{50} = 0.2 and 0.08 μM, respectively) inhibitors of 1 μM cGMP hydrolysis by the crude preparation from sea urchin sperm.

The data presented demonstrate that modification of the xanthine molecule can give rise to highly selective inhibitors of cyclic nucleotide phosphodiesterases. The data also emphasize that the selectivity and/or potency of a given compound for inhibition of the hydrolysis of cAMP or cGMP may vary from one cell type to another. Therefore, any studies that rely upon the pharmacological manipulation of cyclic nucle-

otide levels by phosphodiesterase inhibition should be based upon a knowledge of the potency of the agent to inhibit the phosphodiesterase activities of the cell type being studied.

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