Biochemical Pharmacology, Vol. 28, pp. 1227–1231.

0006-2952/79/0401-1227 $02.00/0

INHIBITION OF ALKALINE PHOSPHATASE BY SUBSTITUTED XANTHINES *

MARTIN A. CROCE†, GEORGE L. KRAMER and DAVID L. GARBERS‡
Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A.

(*Received* 6 *July* 1978; *accepted* 1 *September* 1978)

Abstract—Various xanthine derivatives were synthesized and tested as inhibitors of calf intestinal and beef hepatic alkaline phosphatases. Three derivaties, 1-carboxymethyl-3-isobutylxanthine, theophylline and 1-carboxymethyl-3-methylxanthine, were potent uncompetitive inhibitors of calf intestinal alkaline phosphatase. 1-Carboxymethyl-3-methylxanthine, theophylline and 1-carboxymethyl-3-isobutylxanthine, at 80 μM concentrations, inhibited intestinal alkaline phosphatase by 50.0, 21.3 and 38.7 per cent respectively. Theophylline was also a potent inhibitor of beef hepatic alkaline phosphatase (53.2 per cent inhibition at 80 μM). The most potent inhibitors of the beef liver enzyme were 1-isoamyl-3-methylxanthine, 1-*n*-propyl-3-methylxanthine and 1-phenethyl-3-methylxanthine, which inhibited the beef liver enzyme by 56.6, 46.4 and 45.5 per cent respectively, at 80 μM. These inhibitors of the beef liver enzyme, however, except for theophylline, were ineffective inhibitors of the calf intestine enzyme.

Various inhibitors of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC3.1.3.1) have been introduced and studied [1–8]. Very few inhibitors of intestinal alkaline phosphatase, however, are known, except for theophylline [8]. The only other potent inhibitors of intestinal alkaline phosphatase that have been reported are inorganic phosphate and arsenate [8].

Substituted xanthines have been utilized in inhibition studies of cyclic 3′,5′-nucleotide phosphodiesterase [9–11]. It is possible, therefore, that various methylxanthines could have a pharmacological effect by the inhibition of either the phoshodiesterase or alkaline phosphatase. In this study, various substituted xanthines, including potent 3′,5′-nucleotide phosphodiesterase inhibitors, were synthesized and tested for their effects on beef liver and calf intestine alkaline phosphatases.

MATERIALS AND METHODS

The alkaline phosphatases extracted from beef liver [suspension in 2.6 M $(NH_4)_2SO_4$–0.02 M Mg $(Ac)_2$–0.001 M Zn $(Ac)_2$ solution; type IX] and calf intestinal mucosa (suspension in ammonium sulfate solution, stabilized with 0.001 M $MgCl_2$ and 0.0001 M $ZnCl_2$; type VII), *p*-nitrophenylphosphate, xanthine, 8-chlorotheophylline, 7-(β-hydroxypropyl)-theophylline, and L- and D-*p*-bromotetramisole oxalate were purchased from the Aldrich Chemical Co., Milwaukee, WI; 7- and 9-methylxanthine, 1,7- 1,9- and 3,9-dimethylxanthine, and 1,3,9-trimethylxanthine were purchased from the Adams Chemical Co., Round Lake, IL. Theophylline was purchased from Mallinkrodt, St. Louis, MO. All other materials were analytical reagent grade.

Glycine (100 mM) was adjusted to pH 9.5 with 5 N HCl and was used as the buffer solution throughout this study. All the agents tested were prepared in 1 mM stock solutions in 30% dimethylsulfoxide (DMSO). For enzymatic activity studies, *p*-nitrophenylphosphate (PNP) was added to the 100 mM glycine buffer to give a 5 mM concentration. Varying amounts of substrate (5, 2, 1, 0.5, 0.2 and 0.1 mM substrate in the glycine buffer) were used for kinetic studies.

Enzyme assay

Each assay tube contained 1 ml of the substrate solution and either 200 μl of 30% DMSO, or 100 μl of the 1 mM test agent and 100 μl of 30% DMSO. Fifty μl of diluted enzyme was added to initiate the reaction. At the concentration used, DMSO was found to inhibit the enzyme by less than 5 per cent. The percentage inhibition due to theophylline was about the same in the presence or absence of DMSO. The calf intestine enzyme was diluted to give 25 units of enzyme in the final assay volume, and the beef liver enzyme was diluted to give 7.5 units of enzyme in the final assay volume, where 1 unit (by definition) will hydrolyze 1.0 mole PNP/min at pH 10.4 at 37°. The solution was incubated for 30 min at 30°; readings were taken on a Beckman spectrophotometer at 410 nm (extinction coeffecient = 0.0182 M^{-1}).

Kinetic studies

The buffer and enzyme solutions were the same for both activity and kinetic studies. Varying amounts of substrate mentioned above were used in the glycine buffer. The enzymatic activity was determined for each substrate concentration in the presence of 0, 20, 40, 80 and 150 μM concentrations of each inhibitor at a constant reaction volume of 1.25 ml. The enzyme was added to start the reaction, and formation of the *p*-nitrophenol was calculated by determination of the absorbance change every 5 sec. The initial velocities were used to calculate the kinetic data.

Synthesis

General procedures for xanthine preparation. All appropriately substituted 6-aminouracils were pre-

* Supported by NIH Grants AMO7462, HD10254, GM16811 and HL 19325.
† Trainee, Howard Hughes Medical Institute.
‡ Investigator for the Howard Hughes Medical Institute.

pared according to the procedure of Papesch and Schroeder [12]. To 5 m-moles of 1,3-disubstituted-6-aminouracil in 20 ml of 20% HOAc was added 10 m-moles $NaNO_2$ in 5 ml H_2O dropwise with stirring. The volume was reduced *in vacuo*. The solution was cooled to give a reddish precipitate which was removed by filtration. The solid was added to 10 ml of concentrated NH_4OH, heated, and stirred. This mixture, after adding 10 m-moles $Na_2S_2O_4$ in 20 ml H_2O, was heated for 1 hr. The water was evaporated and the salts were triturated with either EtOH or CH_2Cl_2. The solvent was evaporated and 10 ml formic acid was added to the residue. The solution was heated at reflux for 1 hr. The formic acid was evaporated and 20 ml of 10% NaOH was added; the solution was heated for 1 hr and then cooled and acidified with concentrated HCl. The resulting precipitate was collected by filtration and recrystallized from the appropriate solvent.

1-Methyl-3-(2-acetoxyethyl)-xanthine (compound 4). 1-(β-Hydroxyethyl)-3-methyl-6-aminouracil [12] was treated as described under General Procedures. The xanthine derivative was recrystallized from EtOH–H_2O. To 1.0 g (5 m-moles) of the xanthine derivative was added 5 ml (5.5 m-moles) acetyl chloride; the mixture was stirred and heated for 1 hr. To this mixture was added 10 ml H_2O. The resulting precipitate was collected by filtration and recrystallized from EtOH. Yield: 18%; m.p. 196.5–198°; anal. $C_{10}H_{12}O_4N_4$(C,H).

1-Methyl-3-allylxanthine (compound 5). 1-Allyl-3-methyl-6-aminouracil was prepared [12] and treated as described under General Procedures. The xanthine derivative was recrystallized from 50% EtOH. Yield: 30 per cent; m.p. 209.5–211°; anal. $C_9H_{10}O_2N_4$(C,H).

1-Carboxymethyl-3-isobutylxanthine (compound 7). To a solution of 2.3 g (10 m-moles) 1-isobutyl-6-aminouracil [12] in 15 ml concentrated H_2SO_4 was added 3.2 ml of fuming HNO_3 dropwise with stirring. The solution was cooled in an ice bath and poured onto ice. The precipitate was separated by filtration. To this solid was added 20 ml CH_3OH and 0.5 KOH. The mixture was heated for 15 min and CH_3OH was removed *in vacuo*. To the residue was added 15 ml dimethyl formamide (DMF) and 3 g (15 m-moles) ethyl bromoacetate. The reaction mixture was heated and stirred at 100° for 1 hr. The DMF was removed *in vacuo*. The reduction and ring closure were carried out according to the General Procedures. The xanthine derivative was treated with concentrated HCl. The mixture was added to 25 ml of saturated $NaHCO_3$ solution and filtered. The solution was acidified with concentrated HCl and cooled. The precipitate was recrystallized from EtOH–H_2O. Yield: 15 per cent; m.p. 308° (d); anal. $C_{11}H_{14}O_4N_4$(C,H).

1-n-Propyl-3-methylxanthine (compound 9). 1-Methyl-3-*n*-propyl-6-aminouracil was prepared [12] and treated as described under General Procedures. The xanthine derivative was recrystallized from EtOH. Yield: 15 per cent; m.p. 206.5–208°; anal. $C_9H_{12}O_2N_4$(C,H).

1-Phenethyl-3-methylxanthine (compound 10). A mixture of 1-methyl-6-aminouracil [12] (1.4 g, 10 m-moles), NaOH (1.4 g, 35 m-moles) and phenethyl bromide (5.6 g, 30 m-moles) in 15 ml EtOH and 10 ml H_2O was heated at reflux for 3 hr. The volume was reduced *in vacuo* and 30 ml H_2O was added. The solution was cooled and the precipitate was collected by filtration. The product was treated as described under General Procedures. The resulting compound was recrystallized from EtOH. Yield: 10 per cent; m.p. 245–247°; anal. $C_{14}H_{14}O_2N_4$(C,H).

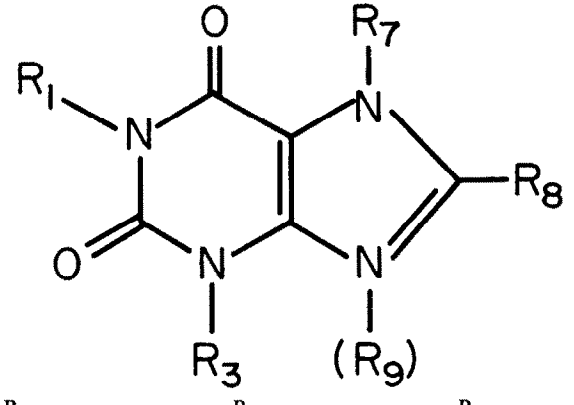
1-Carboxymethyl-3-methylxanthine (compound 11). 1-Methyl-6-aminouracil [12] was nitrated in the same manner as 1-isobutyl-6-aminouracil in the preparation of compound 7. The potassium salt was formed as in compound 7. To this solid (1.8 g, 10 m-moles) was added 4 g (20 m-moles) ethyl bromoacetate in 15 ml DMF. The mixture was heated and stirred overnight. The DMF was removed *in vacuo*. To the residue was added 50 ml CH_3OH and 0.2 g of 5% Pd-C. The mixture was shaken on a Parr hydrogenator under 20 psig H_2 until no pressure drop was noted (about 1 hr). The ring closure was carried out according to the General Procedures. The solution was neutralized with concentrated HCl. The mixture was added to 25 ml of saturated $NaHCO_3$ solution and filtered. The solution was acidified with concentrated HCl and cooled overnight. The precipitate was recrystallized from EtOH. Yield: 15 per cent; m.p. 316° (d); anal. $C_8H_8O_4N_4$(C,H).

Other compounds. 1-Methyl-3-isobutylxanthine (compound 3), 1-methyl-3-benzylxanthine (compound 6) and 1-isoamyl-3-methylxanthine (compound 8) were prepared according to the procedure of Kramer *et al.* [11].

RESULTS

A number of xanthine derivatives with varied potencies as phosphodiesterase inhibitors [10, 11], such as 1-methyl-3-isobutyl-8-methylxanthine, 1-methyl-3-isobutyl-8-bromoxanthine, 1-methyl-3-isobutyl-7-benzylxanthine, 3-isobutylxanthine and 1-*n*-amyl-3-isobutylxanthine, failed to inhibit significantly either the intestinal or hepatic alkaline phosphatases (data not shown). In addition, 1-methyl-3-isobutyl-7-benzyl-8-methylxanthine and 1-methyl-3-isobutyl-7-benzyl-8-bromoxanthine, which cause 50 per cent inhibition of phosphodiesterase at 2–4 μM [11], failed to inhibit the alkaline phosphatases at 80 μM (data not shown). Various other substituted xanthines that were effective inhibitors of beef liver and calf intestine alkaline phosphatses are shown in Table 1. Inhibitions at a 80 μM concentration of agents are reported because many of the agents were not soluble at higher concentrations. Most of the drugs which were effective inhibitors of beef liver alkaline phosphatase were not effective against the calf intestinal enzyme. A clear example is the potent inhibition of beef liver alkaline phosphatase by the levorotatory isomer of *p*-bromotetramisole (compound 20) and the failure of this compound to inhibit the intestinal enzyme. Three isomers of theophylline also were tested, and these three compounds (1,7-, 1,9- and 3,9-dimethylxanthine—compounds 16, 17 and 18, respectively) either acted as slight activators or were ineffective as inhibitors against both enzymes. The same can be said for both 7- and 9-methylxanthine (compounds 14 and 15, respectively) and 1,3,9-trimethylxanthine (compound 19). Xanthine itself was ineffective against both enzymes. 1-Methyl-3-isobutylxanthine (compound 3), which is an excellent phosphodiesterase inhibitor [9], also had no effect on

Table 1. Xanthine analogues and bromotetramisole inhibition of beef liver and calf intestine alkaline phosphatase*



No.	Compound	R_1	R_3	R_7	R_8	R_9	Beef liver enzyme† (% activity)	Calf intestine enzyme† (% activity)
1	Xanthine	—H	—H	—H	—H		104.0	101.0
2	Theophylline	—CH_3	—CH_3	—H	—H		3.2 ± 2.6	78.7 ± 4.1
3	1-Methyl-3-isobutylxanthine	—CH_3	$CH_2CH(CH_3)_2$	—H	—H		97.0	99
4	1-Methyl-3-(2-acetoxyethyl)-xanthine	—CH_3	—$CH_2CH_2O_2CCH_3$	—H	—H		86.3 ± 2.7	83.5 ± 1.2
5	1-Methyl-3-allylxanthine	—CH_3	—CH_2CH—CH_2	—H	—H		84.3 ± 2.1	78.3 ± 2.8
6	1-Methyl-3-benzylxanthine	—CH_3	—$CH_2C_6H_5$	—H	—H		91.3 ± 3.4	79.0 ± 5.0
7	1-Carboxymethyl-3-isobutylxanthine	—CH_2CO_2H	—$CH_2CH(CH_3)_2$	—H	—H		94.3 ± 1.7	60.3 ± 4.4
8	1-Isoamyl-3-methylxanthine	—$CH_2CH_2CH(CH_3)_2$	—CH_3	—H	—H		43.4 ± 3.1	87.8 ± 1.9
9	1-*n*-Propyl-3-methylxanthine	—$CH_2CH_2CH_3$	—CH_3	—H	—H		53.6 ± 4.5	94.0 ± 7.0
10	1-Phenethyl-3-methylxanthine	—$CH_2CH_2C_6H_5$	—CH_3	—H	—H		55.5 ± 3.3	94.0 ± 0.9
11	1-Carboxymethyl-3-methylxanthine	—CH_2CO_2H	—CH_3	—H	—H		100.0 ± 1.2	50.0 ± 2.1
12	8-Chlorotheophylline	—CH_3	—CH_3	—H	—Cl		109.5 ± 8.5	101.0
13	7-(β-Hydroxypropyl)-theophylline	—CH_3	—CH_3	—$CH_2CH(OH)CH_2$	—H		96.0	101.0
14	7-Methylxanthine	—H	—H	—CH_3	—H		105.0	102.0
15	9-Methylxanthine	—H	—H		—H	—CH_3	107.0	99.0
16	1,7-Diemthylxanthine	—CH_3	—H	—CH_3	—H		125.0	107.0
17	1,9-Dimethylxanthine	—CH_3	—H		—H	—CH_3	103.0	101.0
18	3,9-Dimethylxanthine	—H	—CH_3		—H	—CH_3	120.0	98.0
19	1,3,9-Trimethylxanthine	—CH_3	—CH_3		—H	—CH_3	112.5 ± 27.5	105.0
20	L-*p*-Bromotetramisole oxalate						13.4 ± 0.6	103.7 ± 0.88
21	D-*p*-Bromotetramisole oxalate						79.5 ± 5.5	105.3 ± 1.33

* All drug concentrations are 80 μM. The control was run in the presence of DMSO.

† Values represent the means ± S. E. M. for six to eight observations except for those numbers without a S. E. M.; these are the means of two observations.

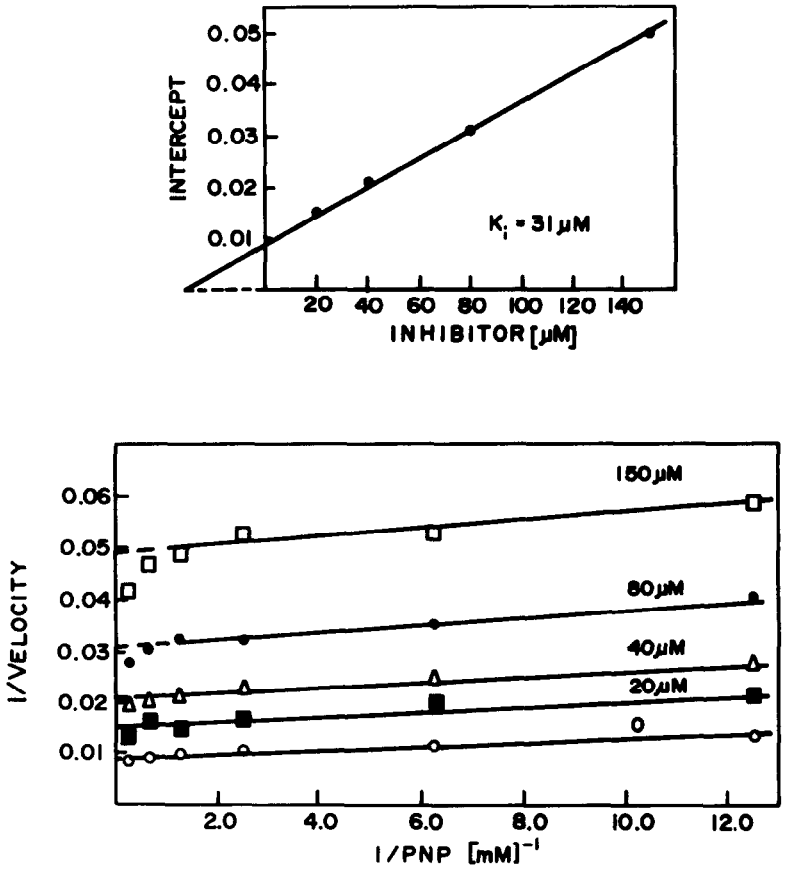


Fig. 1. Uncompetitive inhibition of calf intestinal alkaline phosphatase by 1-carboxymethyl-3-methylxanthine. Alkaline phosphatase was incubated with various concentrations of *p*-nitrophenylphosphate and 0, 20, 40, 80 or 150 μM of the inhibitor. The reciprocal velocity is expressed as (nmoles of *p*-nitrophenol formed/min)$^{-1}$ at 30°. The K_i was calculated by a replot of the intercepts.

either enzyme. A potent inhibitor of the calf intestinal enzyme, 1-carboxymethyl-3-isobutylxanthine (compound 7), did not inhibit the beef liver enzyme. The reverse was true for the compound 1-isoamyl-3-methylxanthine (compound 8). This drug was an excellent inhibitor of the beef liver type, but exhibited only a slight inhibitory effect on the intestinal enzyme. The same was observed for two other disubstituted xanthines: 1-*n*-propyl-3-methylxanthine (compound 9) and 1-phenethyl-3-methylxanthine (compound 10). The most effective compound against the calf intestinal enzyme, 1-carboxymethyl-3-methylxanthine (compound 11), was not an effective inhibitor of the liver enzyme. Two theophylline analogues, 8-chlorotheophylline (compound 12) and 7-(β-hydroxypropyl)-theophylline (compound 13), were ineffective as inhibitors toward both enzymes. Other drugs exhibited a slight inhibitory effect (Table 1).

The most potent inhibitor of calf intestinal alkaline phosphatase was compound 11. This compound caused 50 per cent inhibition at an 80 μM concentration. Theophylline reached 50 per cent inhibition at a concentration of approximately 150 μM. The K_i values for compound 11, theophylline and compound 7 were 31, 45 and 82 μM respectively.

An example of the type of kinetics obtained with these inhibitors is shown for compound 11 in Fig. 1. Some deviation from linearity was found at high substrate concentrations. The K_i values were determined by a replot of the intercepts. The inhibition is uncompetitive, evident by the parallel lines on the graph.

DISCUSSION

It is clear from the data that the potencies of xanthine derivatives to inhibit alkaline phosphatase activities do not parallel their potencies to inhibit cyclic nucleotide phosphodiesterases. Thus, pharmacological effects of low concentrations of potent phosphodiesterase inhibitors, such as 1-methyl-3-isobutylxanthine (compound 3), are probably not attributable to effects on alkaline phosphatases. On the other hand, the observation that theophylline inhibits phosphodiesterases and also alkaline phosphatases in the same concentration range indicates the necessity for caution in interpreting the results of pharmacological studies using this agent. Few agents other than theophylline were shown to be effective against intestinal alkaline phosphatase. Compounds 7 and 11 were more potent than theophylline as inhibitors of the calf intestinal enzyme while being considerably less potent as inhibitors of phosphodiesterases and of other types of alkaline phosphatases, of which the beef

liver enzyme is representative. Thus, the availability of these compounds may prove quite useful in the investigation of intestinal alkaline phosphatases.

None of the xanthine derivatives display the marked selectivity of compound 20 toward inhibition of beef liver or calf intestine enzymes. It is of interest, however, that by varying substituents on the xanthine nucleus the maximum selectivities obtained can be effectively reversed (i.e. compound 11 as compared with compound 9). It appears, therefore, that there is a potential in the xanthine system not only for potent and selective alkaline phosphatase inhibitors but also for obtaining information concerning both the differences and the similarities between the inhibitory sites of the beef liver and intestinal enzyme types. Little such information, however, is available from the limited series of compounds presented here. Two generalizations do seem possible concerning the structural requirements of xanthine derivatives for potent inhibition of the beef hepatic alkaline phosphatase. Alkyl groups larger than methyl in the R_3 position decrease the inhibitory potency against this enzyme whereas increased nonpolar alkyl bulk in the R_1 position has little effect. The presence of the carboxymethyl group in the R_1 position greatly decreases the potency against the beef liver enzyme but enhances the potency against the calf intestinal alkaline phosphatase.

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