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α-Hydroxytropolones: A New Class of Potent Inhibitors of Inositol Monophosphatase and Other Bimetallic Enzymes

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Abstract: Mono- and polyhydroxytropolones are potent competitive inhibitors of inositol monophosphatase. Modeling studies indicate that this inhibition occurs most probably through a novel mode of action involving the chelation of the two magnesium ions in the active site. This is consistent with experimental data. Inhibition occurs when at least three oxygen atoms are present on the seven-membered ring, and only if they are contiguous to one another. In addition, those oxygens should not be protected. The corresponding six-membered rings showed no activity. Other bimetallic enzymes such as alkaline phosphatase (APase) or dopamine β-monooxygenase (DBM) are also inhibited (in a competitive or uncompetitive manner) by hydroxytropolones.

Inositol monophosphatase (IMPase, EC 3.1.3.25) hydrolyzes all *myo*-inositol monophosphates arising from the second messenger *myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) in the phosphoinositide cycle, as well as from the *de novo* synthesis of L-*myo*-inositol 1-phosphate (L-Ins(1)*P*) from glucose 6-phosphate.[1] The human enzyme has been cloned and expressed[2a] and was recently characterized by X-ray crystallography.[2b−d] It requires at least two magnesium ions per subunit for activation.[3] The uncompetitive inhibition of IMPase by lithium ion has led to the hypothesis that the enzyme might be the target of lithium therapy.[4] However the narrow therapeutic window and the side effects often associated with lithium treatment have somewhat detracted from the value of the drug.[5] In addition, other antipsychotic drugs are often required for patients suffering from acute mania, during the 7−10 days that it takes for lithium to exert its antimanic effect. Thus several laboratories have initiated the search for other inhibitors of the enzyme.[6] For example, bisphosphonic acids were described as potent competitive inhibitors; however their bioavailability was low.[6a,7] We herein report the discovery of a new class of potent inhibitors of IMPase and other bimetallic enzymes, via a novel mode of action.

Puberulonic acid **1a** is a natural product that can be isolated from several *Penicillium* strains.[8] Structurally, the compound is characterized by a cycloheptatrienone flanked by three

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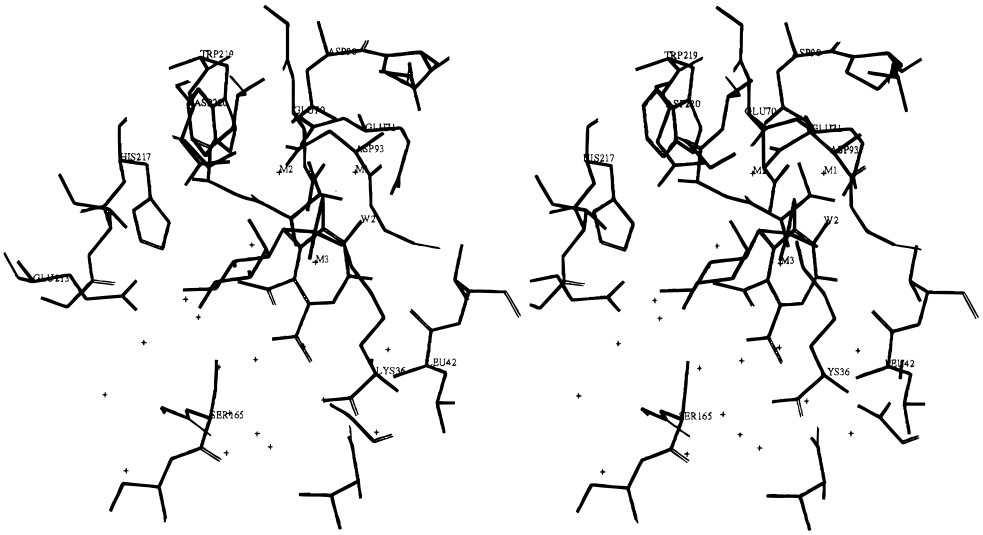

Figure 1. Stereoview of the active site of the inositol monophosphatase complex with Ca^{2+} and D-inositol 1-phosphate.[11] M1, M2, and M3 are the positions of the three Ca^{2+} ions. W2 is the potential nucleophilic water molecule.[2c] Glu-70, Asp-90, Asp-93, and Asp-220 are metal ion ligands. In our model for the binding of puberulonic acid oxygen 2 (superimposed on a phosphate oxygen) chelates both M1 and M2, oxygen 3 (superimposed on the phosphoester oxygen) chelates M2, and oxygen 1, by displacing water W2, chelates M1, and makes a hydrogen bond with Glu-70. The plane of the molecule was estimated to be around 70° from the mean plane of the inositol ring of D-Ins(1)*P*, thereby precluding the third metal ion M3 from positioning itself in the active site.

hydroxyl groups and a carboxylic anhydride function. It was recently reported as an inhibitor of IMPase with an IC_{50} value in the low micromolar range, but of unknown mode of action.[9] The original assumption was that, as the anhydride function is most probably hydrolyzed at physiological pH, the biscarboxylic moiety would chelate the magnesium ions in the active site, mimicking the binding of a phosphate moiety, and the hydroxyl groups would play the same role as those in D-*myo*-inositol 1-phosphate (D-Ins(1)*P*). Consequently, various biscarboxylic acids were tested as inhibitors of IMPase, but were found to be inactive at concentrations of 1 mM.[10] To shed more light on the precise mode of action of puberulonic acid, modeling studies based on the X-ray structure of the human enzyme complexed with Ca^{2+} and D-Ins(1)*P* were carried out.

Figure 1 shows that D-*myo*-inositol 1-phosphate binds to the active site in such a way that the ester oxygen interacts with metal ion M2 and one of the three phosphate oxygens with both metal ions M1 and M2. In addition, a third metal ion (M3) was found.[11] Additional hydrogen bonds are present between the hydroxyl groups in position 2 and 4 on the cyclitol ring of D-Ins(1)*P* and, respectively, Ala196/Asp93 and Glu213 of the enzyme. Hydrolysis of the phosphate ester most probably occurs through the direct attack of a water molecule (W2) which is coordinated to the active site metal ion M1 and to Glu70, and is ideally positioned to be in line with the leaving group during the transition state.[2c] An alternative mechanism of hydrolysis involving in-line attack of another water molecule has also been discussed.[3d,12] For the docking studies with puberulonic acid (**1a**), both the anhydride and the opened form (diacid) were considered. Although the seven-membered ring in tropolone and derivatives is nonaromatic as can be seen from the different bond lengths in known X-ray structures, its conformation is completely planar.[13] This largely facilitated the docking experiments, as only one rigid conformation had to be considered for puberulonic acid, while the opened form contained only two restricted rotational bonds linking the ring with the carboxylic acid groups. Docking was done manually within the active site of the X-ray structure of IMPase/D-Ins(1)*P* after removal of the ligand. No satisfactory results could be obtained when attempting to obtain direct interactions between the anhydride function (puberulonic acid) or the diacid (the opened form), and the metallic centers in the enzyme. Positioning of the hydroxyl groups on the seven-membered ring in a similar way as the cyclitol hydroxyl groups in the enzyme substrate also led to steric clashes. However, a nearly perfect superimposition of three of the four seven-membered ring oxygens of puberulonic acid (**1a**) with the ester oxygen, the phosphate oxygen which simultaneously binds to metal ion sites M1 and M2, and water W2 was obtained (Figures 1 and 3). This model suggests that neither the anhydride function in puberulonic acid nor its opened form play an important role in its recognition by IMPase. Additionally, a mode of action is suggested which could be general for enzymes containing a bimetallic center with a similar arrangement as in IMPase.

To verify this model, mono- and polyhydroxytropones were prepared and tested as inhibitors of IMPase (Figure 2, Table 1). Hydroxytropolones **1c**, **1d**, and **1e** were prepared according to literature procedures, using a bromination−acetolysis−hydrolysis sequence of reactions.[14] An improved bromination procedure was worked out for compound **1i** (R^1 = OH, R^2 = R^5 = Br, R^3 = R^4 = H) and **1j** (R^1 = OH, R^2 = R^4 = R^5 = Br,

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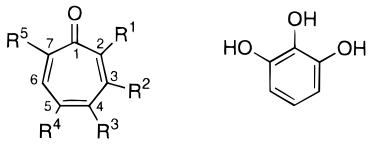

1 **2**

Figure 2.

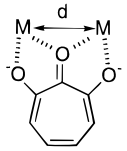

Figure 3. Chelation of two metal ions by the dianion of 7-hydroxytropolone as it might happen in the active site of IMPase. The distance (*d*) between the two metal ions is 3.73 Å as measured from the IMPase/D-Ins*P* X-ray structure.

Table 1. Inhibition of IMPase, Alkaline Phosphatase (APase), and DBM by Tropolones

						$IC_{50}(\mu M)$		
	R^1	R^2	R^3	R^4	R^5	IMPase (Mg-Mg)	APase (Zn-Zn)	DBM (Cu-Cu)
1a	OH	OH	CO−O−CO		OH	10 ± 2	nd[a]	nd
1b	H	H	H	H	OH	>1000	>1000	2
1c	OH	H	H	H	OH	75 ± 10	15	2
1d	OH	OH	H	H	OH	8 ± 1	60	3
1e	OH	OH	H	OH	OH	17 ± 2	nd	nd
1f	H	OH	CO−O−CO		OH	>1000	nd	nd
1g	OMe	OMe	H	H	OMe	>1000	nd	nd
1h	OAc	OAc	H	H	OAc	>1000	nd	nd

[a] nd: not determined.

R^3 = H) (see Experimental Section). 2,3,7-Trimethoxytropone (**1g**) was prepared by reacting 3,7-dihydroxytropolone with freshly prepared diazomethane.

Tropolone (**1b**) was found to be inactive at concentrations of 1 mM while 7-hydroxytropolone (**1c**) inhibited the enzyme with an IC_{50} value of 75 μM. The addition of a third, contiguous hydroxyl group produced compound **1d** characterized by an IC_{50} value essentially identical to that of puberulonic acid, as is 3,5,7-trihydroxytropolone **1e**. The results thus obtained clearly demonstrate that the carboxylate functions of puberulonic acid (**1a**) play little role, if any, in the inhibitory properties of the compound. This is confirmed by the fact that stipitatonic acid (**1f**) does not inhibit IMPase. It can be concluded that inhibition occurs when at least *three oxygen atoms* are present on the seven-membered ring, and only if they are *contiguous* to one another. The difference in potency between **1c** and **1d** probably results from an interaction of the additional hydroxyl group (OH in position 7) with the carbonyl group of amino acid residue Leu42 (distance 2.4 Å, Figure 1).

The rate of substrate hydrolysis by IMPase was measured in a coupled spectrophotometric assay[15a] by varying the concentrations of inositol-1-phosphate at different fixed concentrations of **1d**. A plot of 1/rate *versus* 1/[inositol 1-phosphate] gave a series of straight lines that intersected in a common point on the *y* axis, indicating competitive inhibition of **1d** with respect to the substrate (data not shown). A K_i value of 5 ± 0.5 μM was determined with the use of the computer program COMP.[15b] This result is consistent with the model depicted in Figure 1, where the hydroxytropolone unit takes the place of the phosphate moiety.

Pyrogallol (**2**) at 1 mM did not inhibit the enzyme despite the fact that superimposition with **1c** indicated an almost perfect match of the oxygens of the two molecules. Comparison of the pK_{a1}s of **1c** (6.7) and **2** (9.3) shows that the tropolone derivative is markedly more acidic.[16] The net result at neutral pH is the deprotonation of the first hydroxyl group in **1c** (but not in **2**), leading to an increased chelating potency.[17] In this context it is noteworthy that neither **1g**, nor **1h** showed inhibition of the enzyme at 1 mM concentration.

Other bimetallic enzymes with similar arrangements of active site metal ions may also be inhibited by tropolones, in particular zinc and copper enzymes, since these metal ions are known to form stable complexes with tropolones in solution.[18] Alkaline phosphatase contains two catalytic zinc ions that are about 4 Å apart,[19] and the table shows that this enzyme is also inhibited by hydroxytropolones. Again, inhibition by **1d** was competitive with respect to the substrate (data not shown). It is noteworthy that with IMPase inhibition becomes more efficient with an increasing number of oxygens, **1d** being the most active compound. In contrast, **1c** has the highest affinity for alkaline phosphatase and inhibitory activity decreases when more hydroxyl groups are added. This may reflect the difference in size of the active sites of the two enzymes. IMPase has a large water-filled cavity with many possibilities for hydrophilic interactions,[2b−d] whereas the active site of alkaline phosphatase consists of a small surface pocket, just large enough to accommodate a phosphate moiety.[19]

Dopamine β-monoxygenase (DBM) has a requirement for two copper ions,[20a] and its inhibition by unsubstituted tropolone (**1b**) has been described.[20b] This effect on DBM was confirmed and extended to hydroxytropolones **1c** and **1d**. IC_{50} values of 2−3 μM were obtained. Inhibition was uncompetitive versus dopamine, indicating that, in this case, the tropolone may take the place of the cofactor ascorbate. The inhibitory potency is independent of the number of hydroxyl groups which may suggest that the metal chelating properties alone are responsible for inhibition and tropolones bind to the active-site copper ions only with no additional interactions to enzymic residues.[21]

A more detailed interpretation of these results will have to wait until X-ray crystallography data of enzyme−tropolone complexes become available. However, they show that, in principle, selectivity toward a given target enzyme may be achieved through rational inhibitor design.

Conclusion. Tropolones substituted by at least one hydroxyl function constitute a new class of efficient competitive inhibitors of IMPase on the necessary condition that the array of oxygen

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(17) Experiments with pyrogallol were also carried out at pH = 9, i.e. under deprotonation conditions (pyrogallol pK_a = 9.3). No inhibition could be measured at 1 mM concentration; technical problems with the enzymatic assay and the instability of deprotonated pyrogallol, however, complicate those experiments; a clear answer could not be obtained.

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(21) Given their properties as complexing agents, tropolones may just reduce the free metal ion concentration in the assay mixture, thereby causing inhibition in a nonspecific fashion. However, inhibition of IMPase is observed in the presence of a large excess of Mg^{2+} (2 mM) and APase and DBM contain tightly bound metal ions that are difficult to remove by complexing agents.[22,20b] In addition, the competitive or uncompetitive nature of inhibition with respect to the substrate argues against depletion of free metal ions, which is expected to give rise to noncompetitive inhibition. Finally, the different selectivity profiles, at least with IMPase and APase, clearly show that inhibition occurs through binding at the enzyme active site.

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atoms branched on the cycle be contiguous. In addition, the hydroxyl groups of the seven-membered ring must not be protected as deprotonation occurs under physiological conditions leading to more efficient chelators. Potentially, hydroxytropolones may also find applications as inhibitors of other bimetallic enzymes, in particular those that recognize phosphoesters such as retroviral or bacterial RNase H, DNA polymerases or serine/threonine protein phosphatases.[23]

Experimental Section

Unless otherwise stated, starting materials and solvents were obtained from commercial sources and used without further purification. Diazomethane was freshly prepared according to the procedure of de Boer and Backer and was used immediately.[24] Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium/benzophenone immediately *prior* to use. Drying of the organic extract was carried out using Na_2SO_4. Chromatography was performed using Merck 60 (230−400 ASTM) silica gel according to the procedure published by Still.[25] Melting points were determined on a Büchi 535 apparatus and are uncorrected. Unless otherwise stated, ^{1}H-NMR spectra were recorded in deuterated chloroform at 200 MHz and proton-decoupled ^{13}C-NMR spectra were recorded at 50 MHz; chemical shifts are expressed in ppm downfield from internal or external tetramethylsilane and deuterated chloroform, respectively; coupling constants (*J*) are expressed in Hertz. Low-resolution mass spectra were recorded using the positive or negative ions thermospray method.

3-Hydroxytropolone (1c). Prepared in three steps from tropolone by the method of Takeshita.[14] A colorless crystalline material was obtained whose analytical data were in accordance with those published: mp 89−90 °C (lit.[14] mp 90−91 °C). Anal. Calcd for $C_7H_6O_3$: C, 60.87; H, 4.38. Found: C, 60.75; H, 4.25.

3,7-Dibromotropolone (1i). Tropolone (1.22 g, 10 mmol) was dissolved in a 1:1 mixture of MeOH/CH_2Cl_2 (200 mL). Calcium carbonate (2.052 g, 20.5 mmol, 2.05 equiv) was added while stirring, followed by benzyltrimethylammonium tribromide (7.8 g, 20 mmol, 2.0 equiv). The solution was stirred at room temperature for 24 h and filtered, and the filtrate was evaporated. The residue was taken up in water (180 mL) and heated at 65 °C for 3 h while stirring to dissolve the ammonium salts. Filtration and washing with hot (65 °C) water yielded a crystalline, yellow material. Absence of ammonium salts should be checked at this point, and the last step can be repeated if needed. The solid (2.8 g), when free from ammonium salt, is then dissolved in ethylene glycol dimethyl ether (DME) (14 mL) and stirred overnight at room temperature. Filtration and drying under reduced pressure gave the compound as a yellow, crystalline solid (2.46 g (64%); a 1:1 mixture with DME by ^{1}H-NMR spectroscopy): mp 157−158 °C (lit.[26] mp 157−158 °C); ^{1}H-NMR δ (CD_3OD) 6.18 (t, 1H, ^3J = 10.6), 7.97 ppm (d, 2H, ^3J = 10.6). This compound was used as such in the acetolysis reaction.[14]

3,5,7-Tribromotropolone (1j). The hereabove procedure was followed with 3 equiv of benzyltrimethylammonium tribromide (11.7 g, 30 mmol) to give the title compound in 98% yield as a yellow, crystalline powder, pure by ^{1}H-NMR spectroscopy: mp >320 °C (lit.[26] mp 122−123 °C);[27] ^{1}H-NMR δ (CD_3OD) 8.26 (s, 2H); (DMSO-d_6) 8.11 (s, 2H); MS (TSP^-) 355, 357, 359, 361 (M − H). Recrystallization from DME gave large yellow crystals as a 1:0.33 mixture of product/DME: ^{1}H-NMR δ (500 MHz, DMSO-d_6) 3.23 (s, 6H), 3.42 (s, 4H), 8.14 (s, 2H); ^{13}C-NMR δ (500 MHz, DMSO-d_6) 58.0, 71.0, 103.9, 122.7, 140.0, 172.9. Anal. Calcd for $C_7H_3Br_3O_2\cdot$0.33DME: C, 25.73; H, 1.64. Found: C, 25.76; H, 1.58.

2,3,7-Triacetoxytropone (1g) and **2,3,5,7-tetraacetoxytropone (1k)** were obtained by acetolysis of **1i** and **1j**, respectively, as a pale yellow oil (**1g**) and a creamy solid [**1k**, mp 131−132 °C (lit.[14] mp 133−134 °C)], identical in every respect with literature data.[14]

3,7-Dihydroxytropolone (1d) was obtained as creamy crystals by hydrolysis of **1g**:[14] mp 236−237 °C (lit.[14] mp 237−238 °C). Anal. Calcd for $C_7H_6O_4$: C, 54.55; H, 3.92. Found: C, 54.54; H, 3.89.

3,5,7-Trihydroxytropolone (1e) was obtained as colorless crystals by hydrolysis of **1g**:[14] mp 266−267 °C (lit.[14] mp 263−266 °C). Anal. Calcd for $C_7H_6O_5\cdot H_2O$: C, 44.68; H, 4.29. Found: C, 44.56; H, 4.20.

2,3,7-Trimethoxytropone (1h). To a slurry of the 3,7-dihydroxytropolone (462 mg, 3 mmol) in THF (15 mL) at 0 °C was added slowly a freshly prepared solution of diazomethane[24] (CAUTION) (56 mL of a 0.4 M solution in diethyl ether, 18.09 mmol), and stirring was continued overnight at room temperature. Acetic acid (2 mL) was added slowly to the stirring mixture cooled at 0 °C. Evaporation of the volatiles, chromatography of the residue, and elution (ethyl acetate) delivered the desired compound as a pale yellow oil (288 mg, 49% yield):[28] ^{1}H-NMR δ (500 MHz) 3.84 (s, 3H), 3.89 (s, 3H), 3.94 (s, 3H), 6.61 (d, 1H, J = 9.3), 6.84 (d, 1H, J = 10.9), 6.93 (dd, 1H, J = 9.3, 10.9); ^{13}C-NMR δ (125 MHz) 56.4, 57.9, 59.4, 109.6, 118.3, 127.9, 153.1, 160.0, 164.8, 173.7; MS (TSP^+) 197 (MH^+). Anal. Calcd for $C_{10}H_{12}O_4\cdot{}^1/_2H_2O$: C, 58.53; H, 6.38. Found: C, 58.61; H, 6.05.

Enzyme Assays. Recombinant human IMPase was produced in *Escherichia coli* and purified to homogeneity as previously described.[3d] IC_{50} values were determined at 37 °C and pH = 7.5 with 0.2 mM (≈2 × K_m) DL-Ins(1)*P* using a colorimetric assay, which detects the formation of a phosphomolybdate complex in the presence of malachite green,[3d] or a radiochemical assay measuring the release of [^{3}H]inositol.[30] Similar results were obtained with both assay systems. Alkaline phosphatase from calf intestine was assayed at 37 °C and pH 8.0 in 0.5 M Tris-Cl, with 50 μM (≈5 × K_m) 4-nitrophenyl phosphate. Release of 4-nitrophenolate was measured at 405 nm. Dopamine β-monooxygenase purified from beef adrenals was assayed as previously described.[31]

Modeling Experiments. Modeling was done using SYBYL 5.32.[32] No energy optimization was performed since neither the conformation of tropolones nor interactions with metal ions are well handled by standard force field. This is not a problem in this case as the docked compounds are conformationally rigid and metal binding distances could be taken from the known IMPase complexes X-ray structures.[11]

Acknowledgment. This is dedicated to Clayton H. Heathcock on the occasion of his 60th birthday.

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(27) All the analytical data obtained from our compound were in accordance with the structure of 3,5,7-tribromotropolone (**1j**); furthermore, it was successfully transformed into 3,5,7-trihydroxytropolone (**1e**). Hence we do not explain the discrepancy in the melting points.

(28) Isomeric 2,3,4-trimethoxytropone was also isolated in 40% yield. Assignment of the correct structure was achieved by using ^{1}H and ^{13}C NMR in 1-D and 2-D modes at 500 MHz and 125 MHz. Heteronuclear multiple quantum correlation (HMQC) and Heteronuclear multiple bond correlation (HMBC) were run to establish direct and long-range ^{13}C−^{1}H connectivities.[29]

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