Alkaloids from the Tunicate *Polycarpa aurata* from Chuuk Atoll

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Two new alkaloids, polycarpine (**1**) and *N*,*N*-didesmethylgrossularine-1 (**4**), have been isolated from extracts of the ascidian *Polycarpa aurata* collected in Chuuk, Federated States of Micronesia. Three degradation products of **1** were also isolated. The structures of **1**, **2**, and **4** were determined by X-ray crystallography. The dimeric disulfide **1** inhibited the enzyme inosine monophosphate dehydrogenase, but the inhibition could be reversed by addition of excess dithiothreitol suggesting that **1** reacts with sulfhydryl groups on the enzyme.

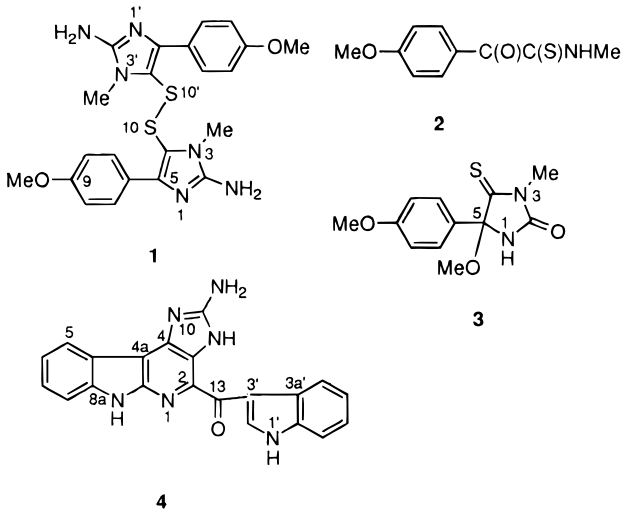
Introduction

Studies to date have amply demonstrated that ascidians are an excellent source of novel and biologically active compounds.[1,2] In our continuing search[3] for anticancer drug candidates, we found that extracts of the solitary ascidian *Polycarpa aurata* (Quoy and Gaimard, 1834) collected in Chuuk, Federated States of Micronesia, strongly inhibited the enzyme inosine monophosphate dehydrogenase (IMPDH)[4] which was being used as a screen to detect potential antiproliferative drugs. We report here the isolation of two new alkaloids, polycarpine (**1**)[5] and *N*,*N*-didesmethylgrossularine-1 (**4**), from bioactivity-guided fractionation of these extracts. IMPDH catalyzes the rate-limiting reaction in the *de novo* pathway of guanine nucleotides, IMPDH activity has been shown to be increased in hepatomas,[6] and enzyme protein expression is elevated in certain leukemic cell lines.[7]

Of the very few known inhibitors of IMPDH, the fungal metabolite mycophenolic acid (MPA)[8,9] is the most potent, $K_i = 10$ nM, and hence it serves as a rigorous standard for discovery of new IMPDH inhibitors. Tiazofurin, an IMPDH inhibitor, is being evaluated in human clinical trials as an antileukemic agent.[10]

Results and Discussion

Methanol and methanol−methylene chloride extracts of freshly thawed *P. aurata* (Family Styelidae) were concentrated and partitioned between 30% aqueous methanol and methylene chloride. Chromatography of the organic solubles twice over Sephadex LH-20 using methanol−methylene chloride concentrated the activity in early fractions (see Experimental Section), and chromatography of these active fractions ($IC_{50} = 0.06$ μg/mL) by vacuum flash silica gel chromatography[11] using gradient elution yielded four fractions. The first fraction contained quite pure orthorhombic sulfur. From the second fraction oleic acid, methyl (*p*-methoxyphenyl)-glyoxalate, and amides **2** and **3** were obtained by further purification using silica gel HPLC. The third and most active fraction was difficult to purify by silica chromatography, but did yield some pure red crystalline polycarpine, **1**, $IC_{50} = 0.015$ μg/mL, after numerous crystallization attempts from MeOH. The last fraction from the LH-20 chromatography yielded alkaloid **4** after repeated chromatography over LH-20. *p*-Methoxybenzoic acid was present in many of the LH-20 chromatography fractions.



The low-resolution FAB mass spectrum of polycarpine, **1**, showed an $(M + 1)^+$ peak at *m*/*z* 469 corresponding to its molecular formula, while the highest mass ion observed in the EI mass spectrum was at *m*/*z* 235. The structure of **1** was derived by X-ray analysis (Figure 1). The molecule consists of a pair of non-coplanar phe-

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(4) Weber, G. *Bioscience Soc. Trans.* **1990**, *18*, 74. Weber, G. *Cancer Res.* **1983**, *43*, 3466.
(5) Alkaloid **1** and decomposition product **3** were discovered concurrently and independently by two other groups, one at Scripps Institution of Oceanography, La Jolla, CA (W. Fenical), and the other at the Australian Institute of Marine Science (P. Murphy). To avoid confusion in the literature, we adopt the name polycarpine assigned by H. Kang and W. Fenical, *Tetrahedron Lett.*, submitted, personal communication from W. Fenical.
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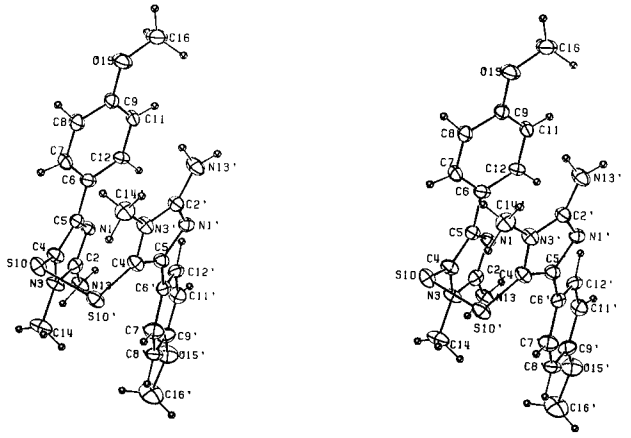

Figure 1. Stereoview of an ORTEP plot of a single molecule of compound **1**.

nylimidazole systems linked together through a disulfide bridge. The phenyl group of one half of the molecule stacks on the top of the imidazole group of the other half, and vice versa, thus giving the molecule the appearance of a cage. This stacking interaction may be cause for the long S−S distance (2.215 Å), the short C−S distances (1.703 and 1.718 Å) and the unusual C−S−S−C conformation (51°). NMR analysis of **1** was rendered somewhat difficult because of noticeable decomposition of the sample in solution, especially during the longer times required for ^{13}C data accumulation. The ^{1}H NMR spectrum at ambient temperature was unusual in that one of the aromatic proton signals, 6.83 ppm, was a sharp doublet, $J = 8.4$ Hz, as expected while the other aromatic proton resonance appeared as a very broad singlet at 7.51 ppm. Irradiation of the doublet at 6.83 ppm clearly sharpened the 7.51 ppm signal. The general appearance of the spectrum was the same in $CDCl_3$ or in CD_3OD and at 24 and 50 °C. However, when the temperature was lowered to −20 °C, the 6.83 ppm signal was resolved into the expected 8.4 Hz doublet. We ascribe the signal broadening to an equilibrium between the tautomeric forms of the quanidine moieties, for which slightly different chemical shifts could be expected for the protons adjacent to the heterocyclic ring. However, the crystal structural results from low temperature data do not indicate any tautomerism.

The structure of thioamide **2** was confirmed by spectral and X-ray diffraction analysis. A stereo ORTEP plot of the two independent molecules is shown in Figure 2. The thioamide group and the carbonyl group are approximately perpendicular to one another showing the lack of conjugation between the two groups. A similar conformation has been observed in a related compound,1-(4-chlorophenyl)-2-morpholino-2-thioxoethanone.[12] These conformations parallel the orthogonal relationship found to exist between the carbonyl groups of the α-keto amide moiety in the immunosuppressive agents FK506 and rapamycin.[13,14]

The formula of lactone **3** was derived from high-resolution EIMS as $C_{12}H_{14}N_2O_3S$. The *p*-methoxyphenyl group was evident from the ^{1}H NMR spectrum (two proton doublets at 7.47 and 6.90 ppm, three proton singlet at 3.78 ppm). A quaternary ^{13}C NMR resonance at 94.0 ppm suggested the presence of an acetal or aminal function. The presence of a one-proton broad exchangeable proton signal at 5.94 ppm when considered together with IR absorption at 3342 and 1664 cm^{-1} indicated the presence of an amide moiety. The presence of a second methoxy group and an *N*-methyl group was indicated by three-proton singlets at 3.32 and 3.30 ppm and ^{13}C NMR signals at 50.5 and 29.2 ppm. The remaining carbon atom in the formula was accounted for by a ^{13}C NMR signal at 201.7 ppm and was assigned to a thioamide functionality.[15] The phenyl group, thioamide carbonyl, and amide carbonyl account for six of the seven degrees of unsaturation dictated by the formula and hence an additonal ring must be present. This could be accounted for by structure **3**. The ureide group fits well with the ^{13}C NMR signal at 156.0 ppm. This structure is also logically related to thioamide **2**. Irradiation of the signal at 5.94 ppm (NH) caused a NOE on the aromatic proton doublet at 7.47 ppm, thus verifying that the exchangeable proton is at position 1 rather than 3.

Amides **2** and **3** and methyl (*p*-methoxyphenyl)glyoxalate are presumed to be decomposition products of **1**.

The structure of the yellow alkaloid **4** was confirmed by X-ray diffraction and spectral analysis. The single successful crystallization of **4** (see Experimental Section) yielded crystals of a 1:1 complex of the alkaloid with acetone (Figure 3). An internal hydrogen bond is formed between the NH group of the imidazole ring and the carbonyl group, as was observed in the related alkaloid grossularine II.[16] The tetracyclic and bicyclic ring systems are approximately coplanar (7°). ^{1}H and ^{13}C NMR signals were assigned through evaluation of COSY, HMQC, and HMBC data, see Table 1. Alkaloid **4**, which was not active in the IMPDH assay, is the *N*,*N*-didesmethyl derivative of grossularine-1 which was isolated from the tunicate *Dendrodoa grossularia*.[17]

Although disulfide **1** was very active in the high throughput IMPDH screen, $IC_{50} = 0.03$ μM, the enzyme inhibition was time-dependent and could be reversed by addition of excess dithiothreitol (DTT). Thus **1** appears to be a reversible sulfhydryl reagent and is not a good lead as an enzyme inhibitor. Recent evidence suggests that Cys-331 of IMPDH resides in the active site of the enzyme. Alkylation of this residue with iodoacetamide or 6-Cl-IMP completely inactivated enzyme activity.[18] On the basis of these observations, we suggest that sulfhydryl reagents can modify Cys-331 leading to enzyme inhibition. This activity appears to be nonspecific: sulfur itself ($IC_{50} = 0.19$ μM) and compounds **2** and **3** ($IC_{50} =$ 5.3 and 6.7 μM, respectively) also inhibit IMPDH activity. On the basis of this information, the IMPDH high throughput screen was modified to include 1 mM DTT in order to eliminate sulfhydryl reagents early in the screening process. The reference inhibitory compound, MPA, is unaffected by the presence of DTT.

Experimental Section

NMR spectra were obtained at 300 and 500 MHz for ^{1}H and 75 or 125 for ^{13}C. IR spectra were obtained from KBr discs or

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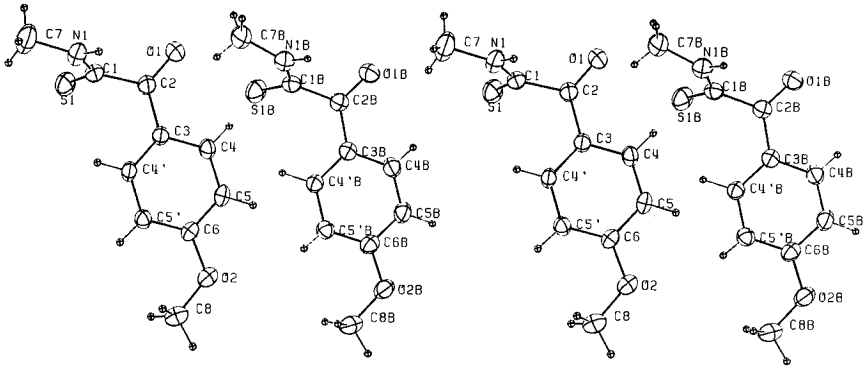

Figure 2. Stereoview of an ORTEP plot of the two independent molecules of compound **2**.

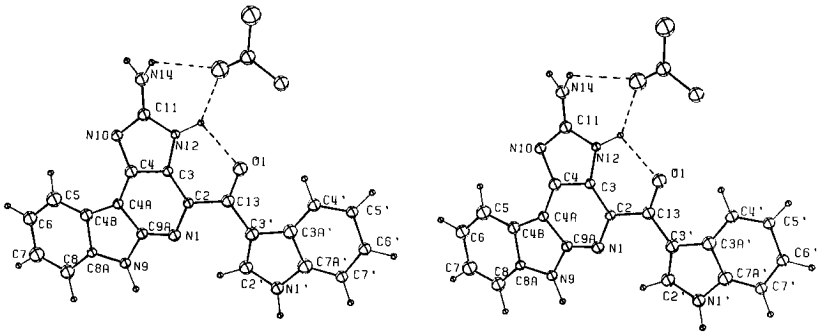

Figure 3. Stereoview of an ORTEP plot of compound **4**. Dashed lines indicate hydrogen bonds.

Table 1. NMR Data for 4a

	^{1}H, δ		^{13}C		
C			δ	mult[b]	HMBC corr
1					
2			146.6	C	
3			132.4	C	
4			126.5	C	
4a			105.4	C	
4b			119.8	C	
5	8.19	(d, 7.8)	122.7	CH	C-7
6	7.21	(td, 7.8, 1.2)	118.9	CH	C-4b/C-7/C-8
7	7.41	(td, 7.8, 1.2)	127.4	CH	C-6/C-8a
8	7.51	(d, 7.8)	110.6	CH	C-4b
8a			137.5	C	
9	11.6	(s)			C-4a/C-4b
11				159.4	C
12	11.3	(s)			
13			187.0	C	
1′	12.2	(s)			
2′	9.6	(s)	139.7		C-3′/C-7a′
3′			114.5		C
3a′			125.7		C
4′	8.54	(m)	122.3	CH	C-4′/C-9′ C-3a′/C-7a′
4b					
5′	7.26	(m)	121.8[c]	CH	
6′	7.27	(m)	121.9[c]	CH	C-7′/C-7a′
7′	7.57	(m)	112.2	CH	C-6′/C-7a′
7a′			135.9	C	

[a] DMSO, 300 MHz. [b] Multiplicities and CH assignments by HMQC. [c] Assignments may be interchanged.

thin films prepared by evaporation of $CHCl_3$ solutions on NaCl plates. Vacuum flash chromatography was carried out on silica gel 60 H (230−400 mesh).

Freshly thawed ascidian, *P. aurata* (12.5 kg wet wt; 0.3 kg dry wt after extraction), collected at Chuuk, Federated States of Micronesia, at −3 to −12 m in July 1992, was extracted three times with MeOH (10 L) overnight and then three times with CH_2Cl_2−MeOH (1:1) (10 L). The extracts were concentrated separately. The aqueous concentrate from the MeOH extract was diluted with MeOH to give a 7:3 mixture of MeOH−water, and this solution (6 L) was extracted three times with CH_2Cl_2 (6 L). The combined CH_2Cl_2 extracts, 6.9 g (IC_{50} for IMPDH = 0.28 μg/mL), were filtered through a small LH-20 column using the solvent sequence: CH_2Cl_2−MeOH (1:1) → MeOH and finally pyridine. All the material obtained prior to pyridine elution was pooled (5.8 g) (IC_{50} for IMPDH = 0.24 μg/mL) and chromatographed again over a large LH-20 column ($2^1/_4$ in. × 24 in.) using gradient elution (CH_2Cl_2 → MeOH). Seventeen fractions (~150 mL each) were collected. The fifth fraction, IC_{50} = 0.06 μg/mL, was resolved by vacuum flash chromatography (silica gel) using gradient elution (hexane → EtOAc → MeOH). Four fractions (A−D) were collected. The material obtained from fraction A, hexane elution, IC_{50} = 0.05 μg/mL, was identified as orthorhombic sulfur, S8, mp 114 °C, *m*/*z* 256, 192, 160, 128, 96, 64. Fraction B was rechromatographed using normal phase HPLC with EtOAc−hexane to give four compounds, oleic acid, methyl (*p*-methoxyphenyl)-glyoxalate, **2**, and **3**. Crystallization of the third fraction (C) from CH_2Cl_2/MeOH (1:2) afforded the most active compound, **1**, IC_{50} = 0.015 μg/mL. The 17th fraction of the LH-20 chromatography (MeOH elution) afforded compound **4** upon repetitive LH-20 chromatography using CH_2Cl_2−MeOH mixtures.

Compounds **1** and **4** were difficult to crystallize. Alkaloid **1** tended to decompose slowly in solution, but in one crystallization attempt from CH_2Cl_2−MeOH (1:1), ~20 mg of red, rod-like crystals was obtained by slow evaporation of the solvent while under refrigeration. These crystals were used for mp and X-ray analysis. Crystals of **4** were obtained only once (MeOH/acetone) although multiple attempts were made.

Methyl (4-methoxyphenyl)glyoxylate: white, amorphous solid (6 mg); mp 55 °C (lit.[19] mp 49−50 °C); IR (film) ν_{max} 1731, 1656 cm^{-1}; UV (EtOH) λ_{max} 226 nm (ϵ 10 857), 294 (19 520); EIMS *m*/*z* (rel intensity) 194 (M^+, 6) 135 (100), 107 (10), 92 (10); FAB$^+$ MS *m*/*z* 195 $(M + H)^+$; ^{1}H NMR ($CDCl_3$) δ 7.99 (2H, d, J = 9 Hz), 6.95 (2H, d, J = 9 Hz) 3.94, 3.87 (each 3H, s), ^{13}C NMR ($CDCl_3$) δ 184.4, 165.0, 164.3, 132.6, 125.5, 114.2, 55.6, 52.6.

Polycarpine (1): red rods from MEOH−CH_2Cl_2 (1:1) (20 mg); mp 201−204 °C; IR (KBr) ν_{max} 3342 (br) 1664, 1633, 1607,

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1543, 1495 cm^{-1}; UV (EtOH) λ_{max} 226 nm (ϵ 21 820), 264 (23 708), 364 (9323); EIMS *m/z* (rel intensity) 235 ($M/2^+$, 100), 202 (25), 192 (60), 151 (60), 133 (79); FAB$^+$ MS *m/z* 471 $(M + H)^+$; ^{1}H NMR ($CDCl_3$, rt) δ 7.51 (4H, br s), 6.83 (4H, d, J = 8.4 Hz), 3.76 (6H, s), 2.98 (6H, s); (CD_3OD) δ 7.45 (4H, br s), 6.81 (4H, d, J = 8.4 Hz), 3.82 (6H, s), 3.13 (6H, br s); at −20 °C in CD_3OD, 7.45 (4H, d, J = 8.4 Hz); ^{13}C NMR (CD_3OD) δ 160.8, 153.3, 130.6, 129.3, 128.65, 126.7, 114.0, 55.7, 28.7.

***N*-Methyl-(4-methoxyphenyl)-2-oxothioacetamide (2)**: yellow crystals from $CHCl_3$/MeOH (9:1) (2 mg); IR (film) ν_{max} 3266, 1653, 1596 cm^{-1}; EIMS *m/z* (rel intensity) 209 (M^+, 17), 135 (100), 107 (10), 92 (14); FAB$^+$ MS *m/z* 210 $(M + H)^+$; ^{1}H NMR ($CDCl_3$) δ 8.12 (2H, d, J = 8.5 Hz), 6.92 (2H, d, J = 8.5 Hz) 3.88 (3H, s), 3.33 (3H, d, J = 5.1 Hz) 8.3 (1H, brs); ^{13}C NMR δ 194.9, 186.6, 164.2, 133.5, 130.54, 113.5, 55.5, 32.0.

Compound 3: yellow, amorphous solid (6.5 mg); mp 124−126 °C; IR (film) ν_{max} 3283, 1761, 1740 cm^{-1}; UV (EtOH) λ_{max} 224 (ϵ 8822), 282 (7640); EIMS *m/z* (rel intensity) 266 (M^+, 11), 192 (100), 164 (17), 134 (28); HREIMS *m/z* 266.0691 (obs), calcd for $C_{12}H_{14}N_2O_3S$, 266.0726; ^{1}H NMR ($CDCl_3$) δ 7.47 (2H, d, J = 9 Hz), 6.90 (2H, d, J = 9 Hz), 5.94 (1H, br s) 3.78, 3.32, 3.30 (each 3H, s); ^{13}C NMR ($CDCl_3$) δ 201.7, 160.3, 156.0, 131.0, 127.1, 113.8, 94.0, 55.3, 50.5, 29.2.

***N,N*-Didesmethylgrossularine-1 (4)**: crystallized from MeOH containing some acetone by slow evaporation; yellow needles (18 mg); mp 333−335 °C; IR (KBr) ν_{max} 3421, 1714 (w), 1651 (s), 1613 cm^{-1}; UV (EtOH) λ_{max} 214 nm (ϵ 27 857), 232 (22 177), 256 (16 012) 326 (9102), 370 (13 555); EIMS *m/z* 366 (M^+ 100) 249 (M^+-C_8H_6N), 221 (M^+-C_9H_6NO), 144 ($C_9H_6NO^+$), 117 ($C_8H_6N^+$); HREIMS *m/z* 366.1270 (obs), calcd for $C_{21}H_{14}ON_6$, 366.1231; FABMS *m/z* 367 $(M + H)^+$. **and 4.**[23] All the X-ray measurements were carried out on an Enraf-Nonius CAD-4 automatic diffractometer equipped with a liquid N_2 low-temperature device using Cu Kα radiation. All three structures were solved by the direct methods using the program SHELXS-86[20] and refined by a full-matrix least-squares routine[21] where the quantity $\sum\omega(F_o - F_c)^2$ was minimized.

Polycarpine (**1**) was crystallized from MeOH−CH_2Cl_2 as thin red rods. The unit cell parameters were obtained from a least-squares fit to $\pm 2\theta$ values of 48 reflections measured at 163 K. Crystal data: $C_{22}H_{24}O_2N_6S_2 + 2CH_3OH + H_2O$, FW = 550.6, monoclinic, $P2_1/a$, a = 11.563(2) Å, b = 22.871(2) Å, c = 10.564-(1) Å, β = 98.90(1)°, V =2760.1(5) Å^3, Z = 4, λ = 1.54178 Å, μ(Cu Kα) = 20.1 cm^{-1}, D(calc) = 1.329 g/cm^3. Intensity data were measured at 163 K employing the $\theta-2\theta$ scan technique. A total of 5198 reflections ($2\theta_{max}$ = 130°) were measured of which 2188 were considered observed ($I > 2\sigma(I)$). The hydrogen atoms were located from a difference Fourier map, and they were refined with isotropic temperature factors. Hydrogen atoms belonging to the methanol solvent were kept fixed during the refinement. R = 5.5%, Rω = 5.3%, S = 1.30, $\Delta\rho$-(max) = 0.40 e/Å^3, Δ/σ(max) = 0.07.

Compound **2** was crystallized from $CHCl_3$/MeOH as yellow blocks. The unit cell parameters were obtained from a least-squares fit to $\pm 2\theta$ values of 48 reflections measured at 293 K. Crystal data: $C_{10}H_{11}O_2NS$, FW = 209.2, triclinic, $P\bar{1}$, a = 7.2521(4) Å, b = 13.7502(6) Å, c = 11.2933(7) Å, α = 106.63-(1)°, β = 91.77(1)°, γ = 97.72(1)°, V = 1066.4(4) Å^3, Z = 4, λ = 1.54178 Å, μ(Cu Kα) = 23.7 cm^{-1}, D(calc) = 1.303 g/cm^3. Intensity data were measured at 293 K employing the $\theta-2\theta$ scan technique. A total of 4058 reflections ($2\theta_{max}$ = 140°) were measured of which 3078 were considered observed ($I > 2\sigma(I)$). The asymmetric unit contains two independent molecules. The non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were located from a difference Fourier map, and these were refined isotropically. R = 4.9%, Rω = 5.9%, S = 1.90, $\Delta\rho$(max) = 0.25 e/Å3, Δ/σ(max) = 0.03.

N,N-Didesmethylgrossularine-1 (**4**) was crystallized from MeOH as yellow needles which diffracted poorly. The unit cell parameters were obtained from a least-squares fit to $\pm 2\theta$ values of 24 reflections measured at 163 K. Crystal data: $C_{21}H_{14}N_6O + C_3H_6O$, FW = 424.5, monoclinic, $P2_1/c$, a = 12.087(3) Å, b = 5.406(2) Å, c = 30.161(11) Å, β = 97.30(3)°, V = 1954.8(6) Å^3, Z = 4, λ = 1.54178 Å, μ(Cu Kα) = 6.8 cm^{-1}, D(calc) = 1.442 g/cm^3. Intensity data were measured at 163 K and employing the $\theta-2\theta$ scan technique. A total of 3323 reflections ($2\theta_{max}$ = 130°) were measured of which only 547 were considered observed ($I > 3\sigma(I)$). All non-hydrogen atoms were refined isotropically. The hydrogen atoms were placed in their calculated positions and were not refined. R = 6.6%, Rω = 7.0%, S = 2.10, $\Delta\rho$(max) = 0.30 e/Å^3, Δ/σ(max) = 0.02.

IMPDH Activity Assay. This assay is a continuous spectrophotometric assay which monitors the appearance of absorbance (340 nm) due to formation of the reduced form of β-nicotinamide adenine dinucleotide (NADH) from NAD+ accompanying conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) using IMP dehydrogenase (IMPDH) as the catalyst. The enzyme was isolated from *Escherichia coli* engineered with the cDNA for human type II IMPDH and purified as described previously.[22]

Reactions (250 μL) to determine IMPDH inhibition are performed at 40 °C in a disposable plastic 96-well microtiter plate. Compounds are initially assayed from a concentration range of 16−0.128 μg/mL. The reactions contain 0.1 M Tris•-HCl, pH 8.0, 0.1 M KCl, 3.0 mM EDTA, 10 μg/mL BSA, 0.05 mM IMP, 0.1 mM NAD, 1.0 mM DTT. The reaction is initiated by the addition of IMPDH. Mycophenolic acid is used as the positive inhibition control.

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Supporting Information Available: ^{1}H NMR spectra of **1**−**4** (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(23) The author has deposited atomic coordinates for these structures with the Cambridge Crystllographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.