Biochemical Properties and Crystal Structure of Ethylmethylglyoxal Bis(guanylhydrazone) Sulfate – an Extremely Powerful Novel Inhibitor of Adenosylmethionine Decarboxylase

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Ethylmethylglyoxal bis(guanylhydrazone) (EMGBG) sulfate, an analog of the well-known antileukemic drug methylglyoxal bis(guanylhydrazone), was synthesized. It was shown to be an extremely powerful competitive inhibitor of eukaryotic S-adenosylmethionine decarboxylase, with an apparent K_i value 12 nM. Thus, it appears to be the most powerful known inhibitor of the enzyme, being almost an order of magnitude more powerful than the corresponding ethylglyoxal derivative. It neither inhibited the proliferation of mouse L1210 leukemia cells *in vitro,* nor did it potentiate the growth inhibition produced by α-difluoromethyl ornithine. In this respect, its properties are closely related to those of dimethylglyoxal, ethylglyoxal and propylglyoxal bis(guanylhydrazones), while in striking contrast to those of the antiproliferative glyoxal and methylglyoxal analogs. EMGBG also inhibited intestinal diamine oxidase activity (K_i 0.7 μM). EMGBG sulfate was crystallized from water, giving orthorhombic crystals (space group Pbcn). Their crystal and molecular structure was determined by X-ray diffraction methods. The carbon-nitrogen double bonds between the ethylmethylglyoxal part and the aminoguanidine moieties were found to have the same configuration as they are known to have in the salts of glyoxal, methylglyoxal and propylglyoxal bis(guanylhydrazones). The glyoxal bis(guanylhydrazone) chain of the EMGBG cation deviated strongly from planarity, thus differing dramatically from the corresponding chains of the glyoxal, methylglyoxal and propylglyoxal analogs.

Introduction

In 1958, Freedlander and French [1] reported the antileukemic activity of the bis(guanylhydrazones) GBG [2] and MGBG [2] (Fig. 1). Since that time, a great number of derivatives were screened for antitumor activity (for references, see [3] and [4]), but it was found that minor modifications, such as dialkylation of the glyoxal moiety, resulted in the loss of antitumor activity [3, 4]. In 1972, Williams-Ashman and Schenone [5] found that MGBG powerfully inhibits S-adenosylmethionine decarboxylase and hence the synthesis of spermidine and spermine. This discovery drastically stimulated the interest in this class of compounds, but it soon became evident that no relationship existed between the inhibition of this enzyme and the antileukemic activity. Thus, although EGBG [2] and DMGBG [2] were known to be devoid of antitumor activity [3], they were found to be much more effective inhibitors of S-adenosylmethionine decarboxylase than were GBG and MGBG [6, 7]. The lack of antitumor activity is possibly due to the fact that EGBG and DMGBG are only poorly taken up by mammalian cells [7, 8] *via* the

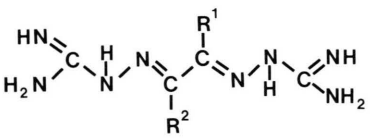

Fig. 1. The structural formulas of GBG ($R^1 = R^2 = H$), MGBG ($R^1 = H$, $R^2 = CH_3$), EGBG ($R^1 = H$, $R^2 = CH_2CH_3$), PGBG ($R^1 = H$, $R^2 = CH_2CH_2CH_3$), DMGBG ($R^1 = R^2 = CH_3$) and EMGBG ($R^1 = CH_3$, $R^2 = CH_2CH_3$).

Abbreviations: DMGBG, dimethylglyoxal bis(guanylhydrazone); EGBG, ethylglyoxal bis(guanylhydrazone); EMGBG, ethylmethylglyoxal bis(guanylhydrazone); GBG, glyoxal bis(guanylhydrazone); MGBG, methylglyoxal bis(guanylhydrazone); PGBG, propylglyoxal bis(guanylhydrazone).

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putative polyamine carrier [9] inducible by polyamine depletion [10].

On the basis of the data so far accumulated, it appears that compounds such as EGBG and DMGBG are much more specific inhibitors of polyamine biosynthesis than is the parent compound GBG and do not display the profound antimitochondrial activity typical of MGBG [8, 11–13]. Thus, the compounds are valuable tools in polyamine research. When S-adenosylmethionine decarboxylase inhibition is considered, the K_i values of monoalkylglyoxal bis(guanylhydrazones) decrease from GBG (6 μM) through MGBG (0.2 μM) to EGBG (0.06 μM) [14]. After the monoethyl stage, however, the K_i value begins to increase again, the K_i value of PGBG [2] being 0.2 μM [15], and it is obvious that among all members of the series of monoalkyl derivatives, EGBG is the most powerful inhibitor of S-adenosylmethionine decarboxylase. On the other hand, the addition of a second methyl substituent to the glyoxal moiety of MGBG also leads to a decrease of the K_i value. Thus, DMGBG is a clearly stronger inhibitor of the enzyme than is MGBG [7]. On this basis, we concluded that the addition of a methyl group to the glyoxal part of EGBG might considerably increase the inhibitory power of the compound. Therefore, we have now synthesized the sulfate salt of that compound, EMGBG [2], and have studied its biochemical properties and determined the crystal and molecular structure of the compound. The results of these studies are reported here.

Materials and Methods

Synthesis of EMGBG sulfate

Aminoguanidine bicarbonate (28.69 g, 0.211 mol; Aldrich, Steinheim, West-Germany) was dissolved in 0.42 M aqueous sulphuric acid (400 ml) at room temperature. This solution was then added with stirring to a solution containing 2,3-pentanedione (10.44 g, 0.104 mol; Aldrich) in absolute ethanol (200 ml). With continuous stirring, the mixture was warmed to about 90 °C and was then allowed to cool. Thus, the yellow color of the mixture disappeared and a small white precipitate appeared. Stirring of the mixture gave a further precipitate. As it was not known, whether the precipitate contained unreacted aminoguanidine sulfate, the mixture was gently refluxed for 30 minutes, allowed to cool to room temperature and then cooled in an ice bath. The white precipitate was collected by filtration, washed with a small amount of water and air-dried (yield 27.5 g, 81%). (More product could be obtained from the combined filtrate and washings after standing at room temperature for several days and cooling in an ice bath.) For recrystallization, the crude product was dissolved in water (1500 ml) at 90 °C. 1300 ml of the solution obtained was cooled in an ice/water bath, the precipitate formed was filtered off, washed with a small amount of water and air-dried (yield 10 g, 42% recovery). *Analysis:* found C, 25.51%, and H, 5.93%; calculated for $C_7H_{16}N_8 \cdot H_2SO_4 \cdot H_2O$: C, 25.60%, and H, 6.14%. This lot was used for the biochemical studies. (More product (7 g) was obtained from the filtrate as it was allowed to concentrate at room temperature.) The remaining 200 ml lot of the recrystallization solution was used for crystal growing for the X-ray study. Thus, it was allowed to concentrate at 65 °C during 4 days to the volume of about 120 ml, the crystals obtained were filtered off, washed with a small amount of cold water, dried in a desiccator for 2 days and transferred to an airtight bottle. The product had a white to very faintly greenish appearance.

Biochemical measurements

S-adenosylmethionine decarboxylase inhibition was studied according to previously published procedures [7, 16]. Intestinal diamine oxidase inhibition was studied according to the method of Tryding and Willert [17].

Mouse L1210 leukemia cells were grown in Gibco's medium RPMI 1640 supplemented with 5% (v/v) of pooled human serum (Finnish Red Cross Transfusion Service), 2 mM glutamine, and 50 mg of the sodium salt of penicillin G and 50 mg of streptomycin per litre.

Crystal data

$C_7H_{18}N_8SO_4$, M_r = 310.3, orthorhombic, space group Pbcn, a = 22.347(4) Å, b = 9.789(2) Å, c = 13.010(4) Å, V = 2846.0 Å^3, Z = 8, D_m = 1.45 Mg m^{-3}, D_c = 1.45 Mg m^{-3}, λ(MoKα) = 0.71069 Å, μ(MoKα) = 2.6 cm^{-1}, F(000) = 1312.

Crystallographic measurements

The colourless crystal selected for intensity data collection had the dimensions of 0.50 × 0.30 × 0.30 mm. The cell parameters were determined by least-

squares treatment of the adjusted angular settings of 20 reflections measured on a Nicolet P3F diffractometer. The intensity measurements were carried out at room temperature (21 ± 2 °C) with graphite monochromatized MoKα radiation and the ω-scan technique. The scan rate varied from 2.5 to 29.3° min^{-1}, depending on the number of counts measured in a fast preliminary scan. Up to a maximum value of 2 θ = 50°, 2868 reflections were measured, resulting to a set of 2504 unique reflections. Of these 1472 were considered as observed ($I > 2\sigma(I)$). Standard reflections ($\bar{1}\bar{1}\bar{3}$, $1\bar{7}\bar{2}$, $43\bar{6}$) monitored periodically exhibited no significant variation. The intensities were corrected for Lorenz and polarization effects but corrections for absorption and extinction were considered unnecessary.

Systematic absences (0kl, k = 2*n* + 1; h0l, l = 2*n* + 1; hk0, h + k = 2*n* + 1; hkl, no restrictions) established the space group as orthorhombic Pbcn (No. 60). The density was measured by flotation in a toluene-CCl_4 mixture.

The structure was solved by direct methods [18] and successive Fourier syntheses [19]. The hydrogen atoms were not determined. Full-matrix least-squares refinement with all atoms as anisotropic led to $R = \Sigma||F_o|-|F_c||/|F_o| = 0.070$ and $R_w = (\Sigma w(|F_o|-|F_c|)^2/\Sigma w|F_o|^2)^{1/2} = 0.072$, where $w = 1/\sigma(F_o)^2$. After the last cycle, the $\Delta/\sigma_{av} = 0.0002$ and $\Delta/\sigma_{max} = 0.001$. In the final difference map the Δϱ peaks were between 0.58 and −0.50 $eÅ^{-3}$. Scattering factors were from Cromer and Mann [20] and the anomalous dispersion corrections were applied [21]. All calculations were carried out on a UNIVAC 1100/61 E1 computer.

Results and Discussion

As shown in Fig. 2, EMGBG acted as a competitive inhibitor of S-adenosylmethionine decarboxylase with an apparent K_i value as low as 12 nM. Thus, its inhibitory power is almost an order of magnitude greater than that of EGBG, and it seems to constitute the most powerful known inhibitor of the enzyme. The compound also inhibited intestinal diamine oxidase activity, yet the K_i value (0.7 μM) was much higher than that for adenosylmethionine decarboxylase.

At the concentrations tested (5 to 200 μM), EMGBG did not inhibit the growth of cultivated L1210 mouse leukemia cells, nor did it potentiate the

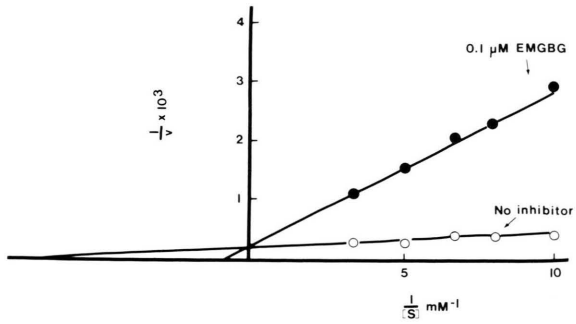

Fig. 2. Inhibition of S-adenosylmethionine decarboxylase by EMGBG.

growth inhibition produced by α-difluoromethyl ornithine (results not shown). These findings are in line with those given in the only previous report on EMGBG [22], which states that EMGBG dihydrochloride dihydrate is inactive against KB cells in culture and against leukemia L1210 *in vivo*. In this respect, the compound clearly falls in the category constituted by DMGBG, EGBG and PGBG, and is distinctly different from the anti-proliferative analogs GBG and MGBG.

The X-ray study on EMGBG sulfate revealed that the carbon-nitrogen double bonds between the ethylmethylglyoxal part and the aminoguanidine moieties had the same configuration as they are known to have in the salts of GBG, MGBG and PGBG [23–25]. Thus, the biochemical differences between these compounds cannot be due to differences in the configurations of double bonds. An ORTEP drawing [26] and the numbering scheme of the EMGBG cation are shown in Fig. 3 and a perspective view in

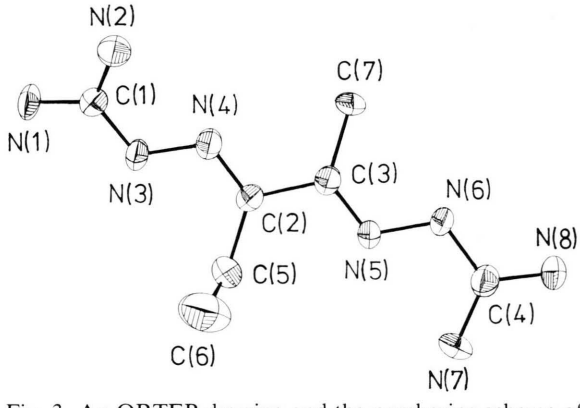

Fig. 3. An ORTEP drawing and the numbering scheme of the EMGBG cation. The thermal ellipsoids are drawn at the 50% probability level.

Fig. 4. Bond lengths and angles are shown in Fig. 5 and the packing in Fig. 6. The atomic coordinates and the equivalent values of the anisotropic temperature coefficients [27] are listed in Table I.

The bond lengths and angles of the EMGBG cation agree well with those of other glyoxal bis(guanylhydrazone) cations [23–25]. The main difference between EMGBG sulfate and other bis(guanylhydrazone) salts so far studied is the strong deviation from planarity of the glyoxal bis(guanylhydrazone) moiety of EMGBG. (The corresponding moieties of GBG, MGBG and PGBG are known to be practically planar [23–25].) Especially the N(1)–N(2)–C(1)–N(3)–N(4) part of the EMGBG cation is strongly distorted. As a result of this distortion the angle between the N(1)–C(1)–N(2) plane and the N(7)–C(4)–N(8) plane is 34(1)°. The terminal CN_3 groups are distinctly different in the sense that while

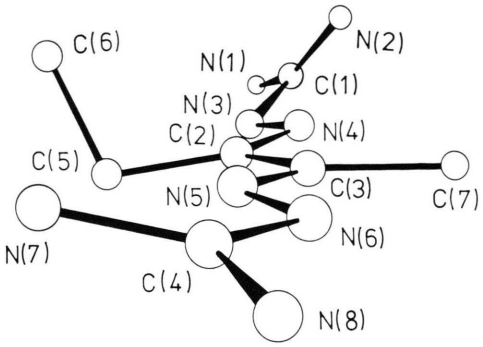

Fig. 4. A perspective view of the EMGBG cation, showing the non-planarity of the cation.

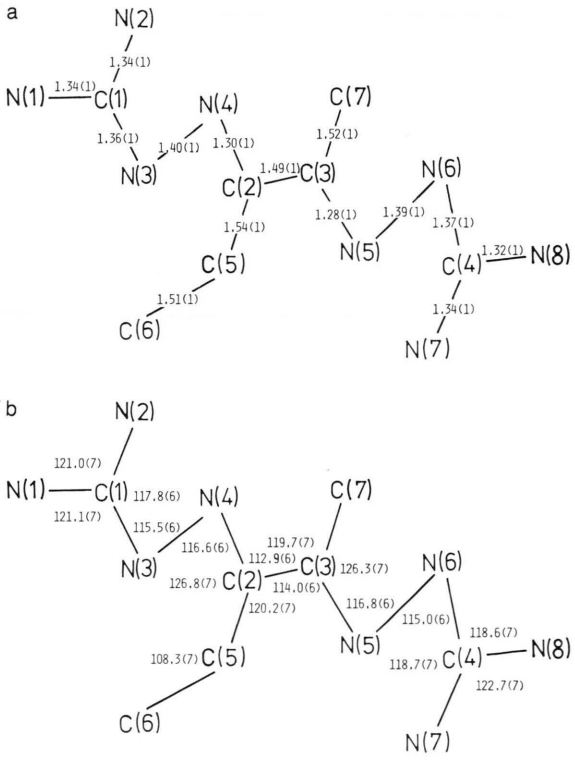

Fig. 5. (a) The bond lengths (Å), and (b) the angles (°) of the EMGBG cation, with estimated standard deviations in parentheses.

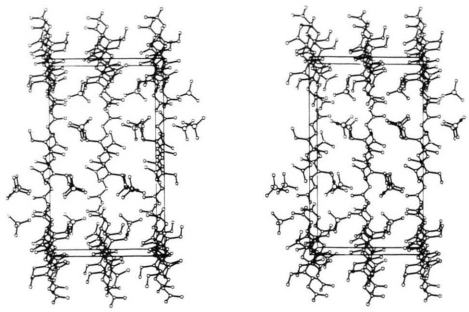

Fig. 6. The packing of ions in the EMGBG sulfate crystal viewed down *b*.

Table I. Fractional atomic coordinates ($\times 10^4$) and equivalent values of the anisotropic temperature coefficients U_{eq} (Å^2) ($\times 10^4$)[a] of EMGBG sulfate, with standard deviations in parentheses.

	X	*Y*	*Z*	U_{eq}
S1	0.1582(1)	0.3073(2)	0.2103(1)	2.9(1)
01	0.1541(2)	0.1581(5)	0.2002(3)	4.4(3)
02	0.0980(2)	0.3679(5)	0.1922(3)	3.3(3)
03	0.1774(2)	0.3422(6)	0.3149(4)	5.4(4)
04	0.1998(3)	0.3615(6)	0.1337(5)	5.9(4)
N1	0.2913(3)	0.4825(7)	0.3527(5)	4.2(4)
N2	0.2597(3)	0.3301(7)	0.4798(4)	3.9(4)
C1	0.2961(3)	0.4302(8)	0.4478(6)	3.4(5)
N3	0.3368(3)	0.4800(7)	0.5157(4)	4.1(4)
N4	0.3834(3)	0.5561(7)	0.4729(5)	3.9(4)
C2	0.4163(3)	0.6256(8)	0.5365(6)	4.0(4)
C3	0.4677(3)	0.6953(9)	0.4851(5)	3.6(5)
N5	0.4969(3)	0.7766(7)	0.5440(5)	4.1(4)
N6	0.5473(3)	0.8393(7)	0.5022(4)	4.0(4)
C4	0.5757(4)	0.9302(8)	0.5662(5)	3.6(5)
N7	0.5499(3)	0.9620(7)	0.6562(5)	4.8(5)
N8	0.6279(3)	0.9806(7)	0.5374(5)	4.5(4)
C5	0.4075(4)	0.6384(10)	0.6533(6)	5.5(6)
C6	0.3601(5)	0.7456(12)	0.6731(7)	8.2(8)
C7	0.4821(3)	0.6634(9)	0.3731(5)	4.4(5)

[a] $U_{eq} = (1/3)\sum_i\sum_j U_{ij} a_i^* a_j^* \mathbf{a}.\mathbf{a}$

the N(6)–C(4)–N(7)–N(8) group is planar within standard deviations, the N(3)–C(1)–N(2)–N(1) group is slightly pyramidal, C(1) being 0.119(8) Å out of the N(1)–N(2)–N(3) plane. At this stage, it is difficult to predict, whether this deviation from planarity is an intrinsic property of the EMGBG cation or whether it is a result of packing effects or of the hydrogen bond system in EMGBG sulfate crystals.

The crystal structure consists of stacked pairs of EMGBG cations (Fig. 6). The mean distance between the EMGBG cations is about 4.1 Å. The crystal system is held together by an extensive hydrogen bond network through the SO_4^{2-} ions.

It would be very important to find out the reason for the striking deviation from planarity of the EMGBG cation in EMGBG sulfate, because such a deviation probably has profound effects on the degree of delocalization of π-electrons in the ion. Such changes may in turn affect the biochemical properties of the compound. Therefore, studies on the crystal structures of other salts of EMGBG are warranted, as they probably would reveal, whether the deviation from planarity is an intrinsic property of the EMGBG cation.

Supplementary material available

Temperature factor and structure factor tables of EMGBG sulfate are available from the authors (H. E. and I. M.) on request.

Acknowledgements

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