The Structure of Guanine Propionate*

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The isolation of guanine propionate was described in the previous paper. This paper reports the physical and chemical properties of this compound and the evidence that it has the structure, 2(α-propionamino)-6-hydroxypurine.

EXPERIMENTAL PROCEDURES

Guanine propionate was isolated as described previously (1). 7-Methylguanine and N*-methylguanine were gifts from Dr. Gertrude Elton, Wellcome Research Laboratories (Tuckahoe, New York). Phosphatase was determined according to the method of Fiske and SubbaRow (2). The ornithin determination of pentose was carried out by the procedure of Kerr et al. (3). The Pauly reaction was run according to the procedure of MacPherson (4). The assay by the phenol reagent was that of Folin and Ciocalteau (5). L-Lactic acid was assayed with crystalline rabbit muscle lactic dehydrogenase (Boehringer). The conditions of the assay were according to Horn et al. (6). Total lactic acid was determined by the method of Barker and Summerson (7). The preparation of the chromatographic solvents designated with numbers is described in the previous paper (1). A quantitative measure of amino acids after paper chromatography was obtained by spraying with 1% ninhydrin and 1% 2,6-dichlorophenolindophenol (Boehringer). The conditions of the assay were according to Korn et al. (8). The colored area was cut out and eluted with 70% ethanol and the optical density of the resulting solution determined at 540 mp. Carboxylic acids were separated by paper chromatography with n-propanol-water-diethylamine in the proportions 85:15:1 (volume for volume). The standard error of deviation was less than ±5%. n-Alanine was determined by coupling partially purified n-amino acid oxidase (9) with lactic dehydrogenase and DPNH and following the decrease in optical density at 340 mp. Carboxylic acids were determined according to the method of Barker and Summerson (7). The pH of the solution after each incremental addition of acid or base. These volume changes were always less than 1%.

Nitrous Acid Treatment—The nitrous acid treatment was a modification of the method of Davoll (11). To a solution of the compound in 0.2 n NaNO₂ which had been preheated to 50° was added 0.1 volume of glacial acetic acid. After 10 minutes at 50°, 0.5 volume of 0.2 n urea was added to destroy the excess nitrous acid. For more vigorous conditions, deaminations were occasionally carried out using a final concentration of 3 n HCl instead of 1.5 n acetic acid.

RESULTS

Spectra—The ultraviolet spectra of guanine propionate were presented in the previous paper (1). By spectrophotometric titration the compound showed pKₐ values of 1.4, 3.2, and 10.2 (Fig. 1). Table I compares these values with those of known guanine derivatives. The infrared spectrum of guanine propionate is very similar to that of guanine (Fig. 2). The fluorescence spectrum in 0.1 n HCl also is similar to those of guanine derivatives. However, the intensity of the fluorescence is lower. With excitation at 285 mp and at a pH of 1, the maximum for guanine propionate was 350 mp; for guanine, 355 mp. The intensity of guanine propionate was 0.64 relative to guanine.

Various Qualitative and Quantitative Assays—Purified guanine propionate reacted negatively to the following tests: organic solvent, orcinol, reaction, Bratton-Marshall reaction (12), Bratton-Marshall reaction after treatment with 6 n HCl for 1 hour at 100°, reduction with the FeCl₃-NH₃ reagent (13), reduction of 2,6-dichlorophenol-indophenol (13), and the Folin uric acid reagent reaction (14). In all these cases a standard compound was added at the end of the reaction and was shown to give the reaction in the presence of guanine propionate.

The compound was not acted upon by guanase and xanthine oxidase, singly or in combination. This finding was utilized to determine guanine in the presence of guanine propionate (15). Guanine propionate was not deaminated by treatment with nitrous acid under various conditions, nor did it form a nitrosoguanine derivative. Instead it was isolated unchanged after treatment.

Guanine propionate reacted with the Folin phenol reagent to give the same molar extinction coefficient as guanine. The compound also gave a reaction with the Pauly reagent (4). Table
Fig. 1. The spectrophotometric determination of the ionizing groups in guanine propionate. Details of the determination are given under "Experimental Procedures."

Table I
Dissociation constants of amino and hydroxy groups in certain guanine derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>NH*</th>
<th>NH-</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine</td>
<td>3.3</td>
<td>9.2</td>
<td>26</td>
</tr>
<tr>
<td>N\textsuperscript{7} Methylguanine</td>
<td>2.3</td>
<td>9.8</td>
<td>†</td>
</tr>
<tr>
<td>7-Methylguanine</td>
<td>3.7</td>
<td>9.3</td>
<td>†</td>
</tr>
<tr>
<td>Guanosine</td>
<td>1.6</td>
<td>9.2</td>
<td>27</td>
</tr>
<tr>
<td>Guanine acid (2' and 3')</td>
<td>2.4</td>
<td>9.3</td>
<td>27</td>
</tr>
<tr>
<td>Guanosine-5'-phosphate</td>
<td>2.3</td>
<td>9.4</td>
<td>28</td>
</tr>
<tr>
<td>Guanine propionate</td>
<td>1.4</td>
<td>10.2</td>
<td>†</td>
</tr>
</tbody>
</table>

* Probable assignment.
† Dissociation from the 1 6 position.
‡ Determined spectrophotometrically.

II describes the chromophores formed by various purines with this reagent.

Identity of Products of Acid Hydrolysis of Guanine Propionate —When guanine propionate was refluxed for 60 hours in 6 N HCl, xanthine, alanine, glycine, and lactic acid were identified as products of the hydrolysis.

Xanthine—The ultraviolet absorption spectra of the hydrolysate at different pH values were identical with those of xanthine. Table III compares the R\textsubscript{f} values of authentic xanthine with those of xanthine derived from the acid hydrolysis. When the solution was neutralized, diluted, and treated with xanthine oxidase, the resulting absorption spectrum was indistinguishable from that of uric acid. When this reaction was followed in the Cary spectrophotometer recording the entire spectrum at intermediate times, all the curves crossed at the two isosbestic points which are characteristic of the conversion of xanthine to uric acid. These curves suggest that xanthine oxidase was acting on a single compound and converting it to a single product. In two experiments the enzymatic assay for xanthine showed that at least 90% of the ultraviolet-absorbing material present in the acid hydrolysate was xanthine.

Alanine and Glycine—The acid-hydrolyzed solution of guanine propionate was examined by paper chromatography in four solvent systems. After being sprayed with ninhydrin, two spots developed corresponding in R\textsubscript{f} values and colors to glycine hydrochloride and alanine hydrochloride run simultaneously (Table IV).

Stoichiometry of Acid Hydrolysis—Table V presents two experiments in which guanine propionate was refluxed for 60 hours with 6 N HCl. After hydrolysis the sum of the molar quantities of the remaining intact purine ring (xanthine) and of alanine was greater in each case than the original molar quantity of guanine.

Table II
Reaction of guanine propionate and various xanthine and guanine derivatives with Pauly reagent
The spectra of the reaction mixtures were run in the Cary recording spectrophotometer between 350 and 650 m\textsubscript{u}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Max.</th>
<th>O.D.\textsubscript{max} Pauly reaction/O.D.\textsubscript{max} original compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine</td>
<td>440</td>
<td>0.72</td>
</tr>
<tr>
<td>Guanosine</td>
<td>417</td>
<td>0.27</td>
</tr>
<tr>
<td>N\textsuperscript{7} Methylguanine</td>
<td>450</td>
<td>0.033</td>
</tr>
<tr>
<td>Guanine propionate</td>
<td>460</td>
<td>0.80</td>
</tr>
<tr>
<td>1,7-Dimethylxanthine</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylxanthine</td>
<td>420</td>
<td>0.18</td>
</tr>
<tr>
<td>Xanthine</td>
<td>495</td>
<td>0.65</td>
</tr>
</tbody>
</table>
TABLE III
Chromatographic identification of zanthine as product of acid hydrolysis of guanine propionate

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Xanthine</th>
<th>Hydrolyzed guanine propionate</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isobutyric-NH₃</td>
<td>0.17</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Butanol-ethanol-water</td>
<td>0.37</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Butanol-acetic acid</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>77% ethanol</td>
<td>0.34</td>
<td>0.35</td>
<td>0.34</td>
</tr>
<tr>
<td>Propanol-NH₃OH</td>
<td>0.29</td>
<td>0.29</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* See preceding paper (1).

TABLE IV
Chromatographic identification of glycine and alanine as products of acid hydrolysis of guanine propionate

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Alanine hydrochloride</th>
<th>Glycine hydrochloride</th>
<th>Glycine + xanthine</th>
<th>Xanthine (xanthine oxidase) as a product of acid hydrolysis of guanine propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>77% ethanol</td>
<td>0.61</td>
<td>0.31</td>
<td>0.32, 0.02</td>
<td></td>
</tr>
<tr>
<td>Propanol-diethylamine*</td>
<td>0.43</td>
<td>0.36</td>
<td>0.33, 0.42</td>
<td></td>
</tr>
<tr>
<td>Methanol-pyridine†</td>
<td>0.50</td>
<td>0.34</td>
<td>0.34, 0.50</td>
<td></td>
</tr>
<tr>
<td>Butanolic-acetic acid†</td>
<td>0.43</td>
<td>0.34</td>
<td>0.33, 0.43</td>
<td></td>
</tr>
</tbody>
</table>

* Seventy percent n-propanol-diethylamine (100:1).
† Freshly prepared n-butanol-glacial acetic acid-water (2:1:1).

TABLE V
Hydrolysis of guanine propionate by 0 N hydrochloric acid

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Starting guanine propionate</td>
<td>22.0</td>
</tr>
<tr>
<td>Xanthine (O.D. 265 mµ)</td>
<td>9.3</td>
</tr>
<tr>
<td>Xanthine (xanthine oxidase)</td>
<td>8.5</td>
</tr>
<tr>
<td>Glycine*</td>
<td>13.3</td>
</tr>
<tr>
<td>Alanine (total)*</td>
<td>18.2</td>
</tr>
<tr>
<td>d-Alanine†</td>
<td></td>
</tr>
<tr>
<td>Lactic acid†</td>
<td>1.7</td>
</tr>
<tr>
<td>Glycine + xanthine</td>
<td>21.8 (99%)$</td>
</tr>
<tr>
<td>Alanine + lactic acid</td>
<td>19.9 (91%)$</td>
</tr>
</tbody>
</table>

* By quantitative paper chromatography.
† By D-amino acid oxidase and lactic dehydrogenase.
‡ Colorimetric determination (7).
§ The recovery of the purine moiety (the figure in parentheses is the percentage of recovery in terms of starting guanine propionate).
¶ The recovery of the 3-carbon acid moiety.

propionate. Therefore, the nitrogen of alanine could not have been derived from a ring nitrogen and the propionate residue must have been on the amino group.

D-Alanine accounted for 49% of the total alanine in two analyses of one of the hydrolysates of guanine propionate. This suggested that the alanine produced by acid hydrolysis was a racemic mixture.

Guanine and L-Lactic Acid, Products of Neutral Hydrolysis of Guanine Propionate—At all pH values from 3 to 12, solutions of guanine propionate break down to guanine and L-lactic acid. With different preparations of the compound, the rate of the reaction was different, suggesting that it might be catalyzed by an impurity. However, in contrast to the breakdown of 8-succinaminopurine and some of its derivatives (16), the addition of calcium or ferric ions did not increase the rate.

Guanine—Guanine was identified by its behavior on Dowex 50 columns, by paper chromatography with solvent 2, by its ultraviolet absorption spectra at various pH values, and by its conversion to uric acid by treatment with guanase followed by xanthine oxidase. Finally, carrier guanine was mixed with C¹⁴-guanine propionate of high specific activity. The mixture was subjected to hydrolysis and separated on Dowex 50 columns, adsorbed to and eluted from charcoal, and separated by paper chromatography in two solvents. The spots on the paper corresponding to guanine were cut out and the radioactivity determined. The specific activity expected by the dilution with carrier guanine was 1100 counts per minute per µmole. The specific activity of the guanine isolated by chromatography with solvent 2 was 1000 counts per minute per µmole, and that of guanine with solvent 3 was 1040.

L-Lactic Acid—Lactic acid was identified by its Rₚ value on paper in 85% propanol-diethylamine, 100:1 (volume for volume) (Rₚ, 0.54; authentic lactic acid, 0.53), and by its reaction with crystalline lactic dehydrogenase and the specific colorimetric test (7).

In addition, in one experiment lactic acid was extracted from a paper chromatogram and measured by lactic dehydrogenase and DPN. The 2,4 dinitrophenylhydrazones of the product was prepared (17) and the reaction mixture subjected to paper chromatography in two solvents. With 0.5 m NaOH as the solvent, phenylhydrazones spots were observed at Rₚ values of 0.46 and 0.60 (authentic pyruvic acid treated in the same way, 0.46 and 0.60). In n-butanol saturated with 3% NH₃OH the phenylhydrazones spots were at Rₚ values of 0.32 and 0.46 (authentic pyruvic derivative, 0.31 and 0.45).

Stoichiometry—Upon refluxing 2.5 µmoles of guanine propionate for 88 hours in 0.05 m KHCO₃, 0.91 µmoles of guanine (assayed by guanase and xanthine oxidase), 1.12 µmoles of the lactic acid (colorimetric), and 0.91 µmoles of L-lactic acid (enzyme assay) were produced.

DISCUSSION

The compound is considered to be a propionic acid derivative of guanine (identified as 2(α-propionamino)-6-hydroxypurine) on the basis of (a) the formation of guanine and lactic acid on neutral hydrolysis and the formation of xanthine and alanine by hydrolysis in 0 N HCl, (b) the correspondence to the empirical formula, and (c) the measurement of equivalent weight by determination of the carboxyl group. Evidence for the assignment of the 3-carbon unit to position 2 on guanine is summarized as follows: (a) the ultraviolet spectra of the compound at all pH values except in 6 N HCl agree more closely with those of N³-
methylguanine than with any known guanine derivative; (b) a balance of the products of the hydrolysis by 6 N HCl excludes all other possibilities (see acid hydrolysis under "Results"); and (c) the inability to deaminate guanine propionate is strong evidence for substitution on the amino group of guanine.

In support of this evidence are the following additional findings. (a) Guanine propionate and N²-methylguanine are the only two guanine derivatives studied or found in the literature which show a significant elevation of the pK' of the —CO—NH group (Table II). It appears that the amino group and its electron-donating substituents in some manner interact with the 1—6 grouping to repress the ionization. (b) Guanine propionate and N²-methylguanine both show suppression of the 1690 cm⁻¹ band in the infrared absorption spectrum. This band is assigned to the C—O bond stretching in position 6 of guanine (18). In turn the N—H stretching band, which is very pronounced in guanine, is almost completely suppressed in N²-methylguanine and guanine propionate (e). The reaction of the compound with the phenol reagent is taken as evidence against attachment to one of the imidazole ring nitrogens. While the nature of the reacting group is not known, 7 or 9 substituted guanine derivatives do not give this reaction. In contrast to N²-methylguanine, guanine propionate did not show a shift in its ultraviolet absorption spectrum between pH 1 and 6 N HCl, was strongly positive to the Pauly reaction, and did not form a nitroso derivative upon treatment with nitric acid (21). The fluorescence spectrum of guanine propionate was also unlike that of N²-methylguanine. The reasons for these differences are not known, but possibly they are related to the low pKₐ of the amino group in guanine propionate.

The group with a pK' 10.2 corresponds to the group with a dissociation constant of 9.2 in guanine, since the dissociations of the two groups show very similar effects on the spectra. The pK' of 9.2 generally is assigned to the —CO—NH— group in position 1—6 (22). The groups with pK' values of 3.2 and 1.4 are tentatively considered to be the carboxyl and amino groups, respectively. The lack of absorption at 1845 cm⁻¹ when guanine propionate was prepared by precipitation from concentrated formic acid followed by drying under vacuum suggests the absence of a charged amino group. The method of preparation should lead to the isolation of the compound at its isoelectric point. Thus if the pK' of the amino group were higher than that of the carboxyl group, both groups would be expected to be charged. The presence of a broad peak at 1300 cm⁻¹ in guanine propionate also suggests an uncharged carboxyl group.

With the present data it is not possible to ascertain the configuration of the α-carbon of the 3-carbon acid residue. The formation of lactic acid predominantly with the L-configuration by the hydrolysis of the guanine propionate does not answer the question, since it is not known whether the hydrolysis involves an inversion. However, the fact that predominantly one optical isomer of lactic acid is produced shows that the guanine propionate could not have been a racemic mixture originally. Because

The results of the chlorine and permanganate oxidations (19, 20) were not conclusive. These degradations should, in theory, yield derivatives of propionic acid which are characteristic of its point of attachment to the ring.

Guanine, N²-methylguanine, 1,3-dimethylxanthine, and 2,4,5-triamino-6-hydroxyxymidine were found to give the reaction, whereas guanosine, 7-methylguanine, and 1,7-dimethylxanthine did not.

![GUANINE PROPIONATE](image)

**Fig. 3.** The hydrolysis of guanine propionate in 6 N HCl and in "neutral" solution.

of the production of DL-alanine upon hydrolysis of guanine propionate with 6 N acid and the apparently close association of the carboxyl group with the 1—6 grouping, it is conceivable that this hydrolysis might have occurred through the intermediate formation of a hydantoin, perhaps produced by the condensation of the carboxyl group with the nitrogen in position 1 in the purine ring. Hydantoins are known to racemize easily (23). Such a cyclization could be analogous to that occurring during the acid breakdown of argininosuccinate (24) and postulated for 6-ureidopurine (25).⁴

**SUMMARY**

This paper reports on the structure of guanine propionate, a compound isolated from cell-free incubation mixtures of *Bremtheclum ashbyii*.

1. Its ultraviolet, infrared, and fluorescence spectra and its reaction with various reagents were compared with other guanine derivatives.

2. In water at 100° guanine propionate is hydrolyzed to guanine and L-lactic acid stoichiometrically, and in 6 N HCl at 100° forms xanthine, DL-alanine, and glycine.

3. On the basis of the stoichiometry of the hydrolytic degradation, the lack of deamination by nitrous acid, and some of its physical properties, the compound was assigned the structure 2(α-propionamino)-6-hydroxyxypurine.

**REFERENCES**


⁴More recently 2(α-propionamino)-6-hydroxyxypurine has been synthesized by an unambiguous route and has been found to be identical with the natural compound. The configuration at the α-position of the propionic acid residue is not yet resolved. These studies will be the subject of a separate report (D. H. Hayes, unpublished results).
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