Structural Studies on Anthglutin, an Inhibitor of γ-Glutamyl Transpeptidase, from *Penicillium Oxalicum*

Takeshi KINOSHITA* and Sadamasa MINATO†

Central Research Laboratories, Sankyo Co., Ltd., Hiromachi, Shinagawa-ku, Tokyo 140

† *Fermentation Research Laboratories, Sankyo Co., Ltd., Hiromachi, Shinagawa-ku, Tokyo 140*

(Received February 23, 1978)

Anthglutin, a new γ-glutamylphenylhydrazine and an inhibitor of γ-glutamyl transpeptidase, has been isolated from the cultured medium of *Penicillium oxalicum*. Spectral studies indicate that it has the structure of 1-γ-L-glutamyl-2-(2-carboxyphenyl)hydrazine.

γ-Glutamyl transpeptidase [EC 2.3.2.2] is an enzyme widely distributed in the tissues and body fluids of animals,[1] microorganisms,[2] plants,[3] and coelenterates.[4] Serum γ-glutamyl transpeptidase activity is assayed in clinical diagnosis.[5] A strong inhibitor of γ-glutamyl transpeptidase has been isolated from the cultured medium of *Penicillium oxalicum* SANK 10477 and named anthglutin.[6] The new compound anthglutin is a competitive and specific inhibitor for γ-glutamyl transpeptidase. This paper deals with the structure determination of anthglutin.

Experimental

Spectra. IR spectra were obtained with a JASCO IRA-2 spectrometer, and NMR spectra with a Varian A-60D spectrometer using tetramethylsilane as an internal standard. GLC analyses were carried out on a JEOL JGC-20KFP gas chromatograph and GC-MS analyses on a JEOL JMS D-100 gas chromatograph mass spectrometer. Field desorption mass spectra (FD-MS) were obtained with a double focusing JEOL JMS-OlSG instrument equipped with a combined f.d.f.i.e.i. ion source. Conventional low and high resolution mass spectra were taken on a JEOL JMS-OlSG spectrometer, using a direct insertion probe and an ionization energy of 75 eV.

Material. Anthglutin was obtained by the method described in a previous paper.[6] The result of elemental analysis agreed with the formula $C_{12}H_{15}N_3O_5$.

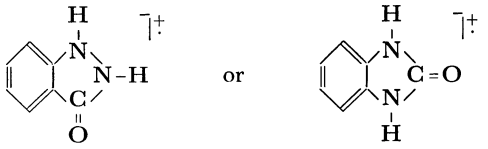
Found: C, 51.07; H, 5.51; N, 14.65; O, 28.87%. Calcd for: C, 51.24; H, 5.38; N, 14.94; O, 28.44%.

Preparation of Derivatives. Trimethylsilyl (TMS), perdeuteriotrimethylsilyl (TMS-d_9), and trifluoroacetyl (TFA) derivatives of anthglutin (I) were prepared according to methods in literature.[7-10] *N*-[(Dimethylamino)methylene] methyl ester derivatives of amino acids were also prepared according to the reported method.[11]

Results and Discussion

The formula $C_{12}H_{15}N_3O_5$ of anthglutin (I) was substantiated by high resolution mass measurement on the quasi-molecular ion $(M+H)^+$ at *m/e* 282 in the field desorption mass spectrum (Fig. 1). The NMR spectrum of I in D_2O at basic pD is shown in Fig. 2. The signals at δ 6.8—8.2 correspond to four aromatic hydrogens, indicating the presence of an *ortho*-disubstituted benzene. The remaining signals consist of a triplet at δ 3.90 (1H, J=6 Hz) and a complex pattern centered at δ 2.5 (4H), corresponding to those of L-glutamic acid whose occurence in I was expected by bioassay and amino acid analysis.[6] The IR spectrum (Nujol) of I exhibits absorption bands which can be attributed to >N-H (3290 cm^{-1}), >C=O (1675, 1655 cm^{-1}), and *ortho*-disubstituted benzene (760 cm^{-1}).

Major structure analysis was carried out by means of fragmentation analysis of the mass spectra. A strong peak is seen at *m/e* 134 ($C_7H_6N_2O$, *M.W.* obsd=134.0475, *M.W.* calcd=134.0480) corresponding to $[M-C_5H_9NO_4]^+$ ion (Fig. 3). Since the atomic composition of $C_5H_9NO_4$ is identical to that of glutamic acid and the NMR spectrum of I suggests the presence of an *ortho*-disubstituted benzene ring, the structure of this fragment ion at *m/e* 134 should be one of the following:



II or III

As shown in Fig. 2, the signals in the down-field of the NMR spectrum of I resemble those of *o*-aminobenzoic acid very closely in chemical shifts and spectral

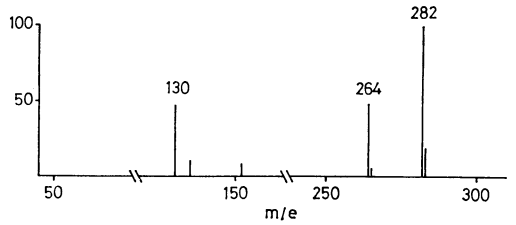


Fig. 1. The field desorption mass spectrum of **I**: heating current 17 mA, *m/e* 282=$C_{12}H_{16}N_3O_5$ (*M.W.* calcd=282.1088, *M.W.* obsd=282.1079).

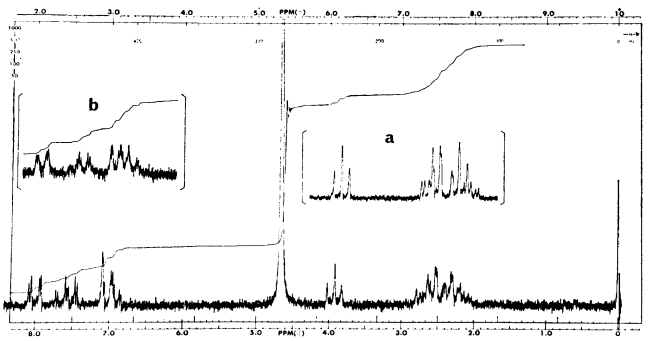


Fig. 2. The NMR spectrum of **I** in D_2O containing a small amount of NaOH; the spectra in brackets: (a) L-glutamic acid in D_2O and (b) *o*-aminobenzoic acid in $(CD_3)_2CO$: D_2O (3:1).

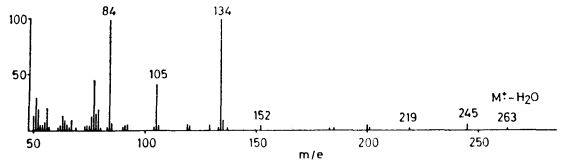
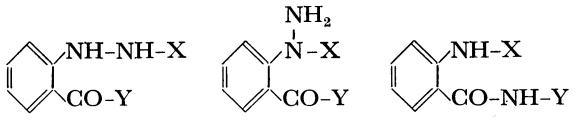


Fig. 3. Mass spectrum of **I**.

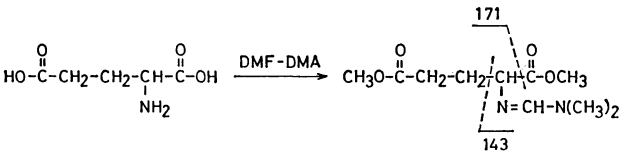
patterns. Thus, structure II seems more likely. The fragment ion at *m/e* 134 can be derived from one of the structural units of the following types in which X plus Y should be $C_5H_9NO_4$.



IV V VI

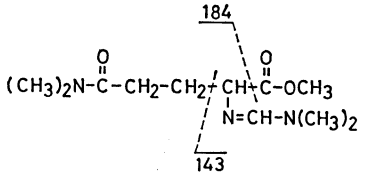
In order to determine the whole structure, the reactions of I with some reagents were carried out. The product derived from the reaction of I with *N,N*-dimethylformamide dimethyl acetal (DMF–DMA)[11] is labile even at 230 °C (Fig. 4). Gas chromatography mass spectrometry (GC-MS) was applied for further analysis to the products derived from the partial pyrolysis at the injection port of a gas chromatograph.

The mass spectrum of GLC peak A in Fig. 4 is identical to that of the product (VII) from the reaction of L-glutamic acid with DMF–DMA (Fig. 5-(a)).



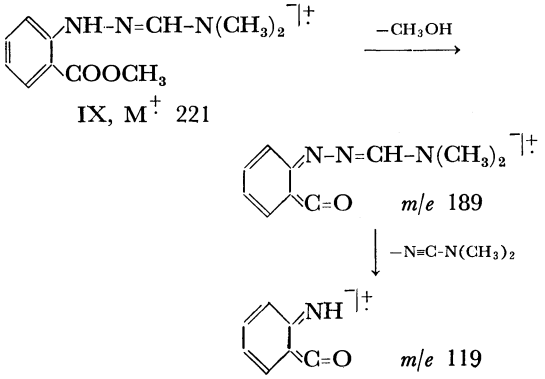
VII, M=230

The structural assignment for peak B is based on its mass spectrum which is analogous in fragmentation patterns to that of VII as shown below.



VIII, M=243

Peak C seems to be derived from compound IX as indicated by its mass spectrum (Fig. 5-(b)).



IX, M^+ 221

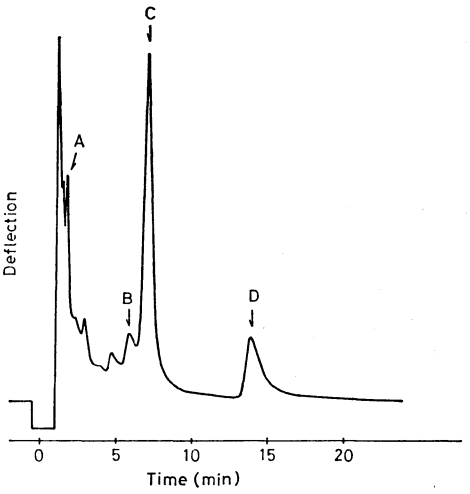


Fig. 4. Gas chromatogram of the reaction products of **I** with DMF–DMA taken by total ion current of GC-MS. Column of 2% Silicone OV-1 on Chromosorb G, 1 m×2 mm i.d. glass column, col. temp: 145 °C, inj. temp: 230 °C, flow rate: 23 ml/min He.

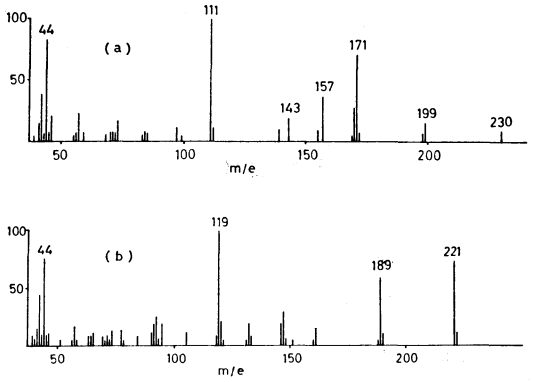
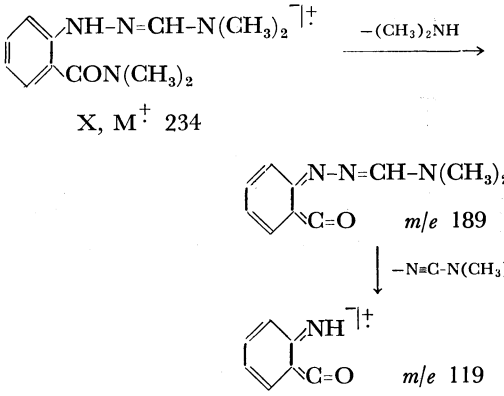


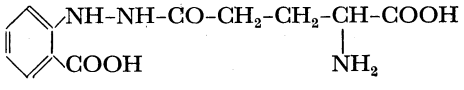
Fig. 5. Mass spectra of peak A (a) and peak C (b).

For peak D, the assignment was done by the analysis of the corresponding GC-MS spectrum which is analogous in fragmentation modes to that of IX.

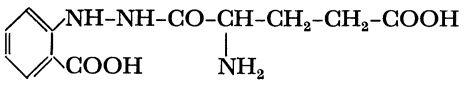


X, M^+ 234

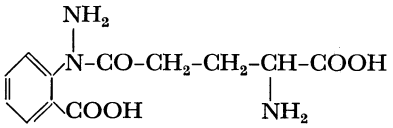
The above data suggest that the structure of I should be one of the following five structures XI—XV.



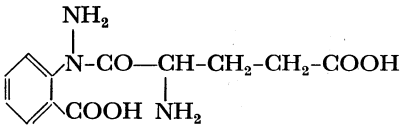
XI



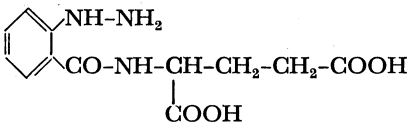
XII



XIII

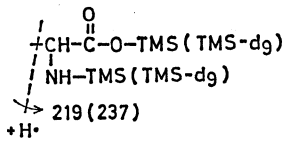


XIV



XV

Further information was obtained by a study of the mass spectra of the trimethylsilyl (TMS) and the perdeuteriotrimethylsilyl (TMS-d_9) derivatives of I (Figs. 6 and 7). The occurrence of the fragment ions at *m/e* 219 and 237 (Figs. 6 and 7) suggests that I has an α-amino acid moiety as part of its structure.



The result of the color reaction of I with ninhydrin, a strong purple color being obtained at elevated temperature, also suggests that a free α-amino acid moiety might occur in I. Thus, of the five possible structures, only structures XI and XIII seem likely. The information for differentiating XI and XIII was obtained from the mass spectrum of the *N*-trifluoroacetyl-*O*-trimethylsilyl (*N*-TFA-*O*-TMS) derivative of I. *N*-TFA-*O*-TMS derivatives are easily prepared by trimethylsilylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) followed by selective *N*-acylation with *N*-methylperfluorodiacetamide (MPFDA).[9]

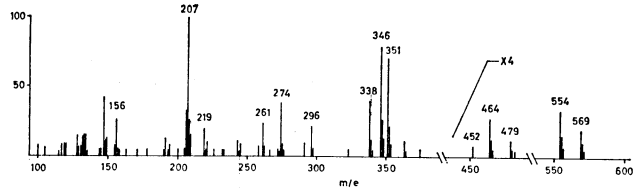


Fig. 6. Mass spectrum of the TMS derivative of **I**.

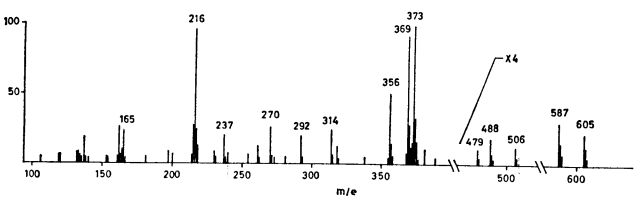
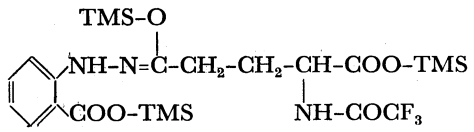


Fig. 7. Mass spectrum of the TMS-d_9 derivative of **I**.

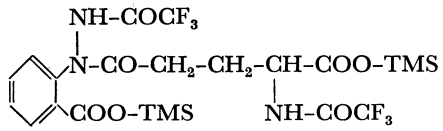
TABLE 1. HIGH RESOLUTION MASS MEASUREMENTS OF PRINCIPAL IONS IN MASS SPECTRUM OF TMS DERIVATIVE OF I

Observed	Calculated	Formulae
569.2619	569.2591	$C_{24}H_{47}N_3O_5Si_4$
554.2370	554.2356	$C_{23}H_{44}N_3O_5Si_4$
479.2067	479.2090	$C_{21}H_{37}N_3O_4Si_3$
464.1832	464.1855	$C_{20}H_{34}N_3O_4Si_3$
452.2201	452.2219	$C_{20}H_{38}N_3O_3Si_3$
351.1573	351.1559	$C_{16}H_{27}N_2O_3Si_2$
346.1709	346.1729	$C_{14}H_{32}NO_3Si_3$
338.1574	338.1587	$C_{15}H_{26}N_2O_3Si_2$
296.1394	296.1375	$C_{13}H_{24}N_2O_2Si_2$
274.1315	274.1294	$C_{11}H_{24}NO_3Si_2$
261.1066	261.1059	$C_{13}H_{17}N_2O_2Si$
219.1093	219.1109	$C_8H_{21}NO_2Si_2$
207.0962	207.0954	$C_{10}H_{15}N_2OSi$
206.0885	206.0876	$C_{10}H_{14}N_2OSi$
156.0832	156.0844	$C_7H_{14}NOSi$

Subsequent acylation of the TMS derivative of I with MPFDA gave a mass shift of the molecular ion of 24 mass units as compared with the original derivative (Fig. 8). This indicates that the TMS derivative of I has only one *N*-trimethylsilyl group. It can be concluded that the *N*-TFA-*O*-TMS derivative of I is XVI and not XVII.



XVI, M=593



XVII, M=617

Consequently, the proposed structure of anthglutin (I) is 1-γ-L-glutamyl-2-(2-carboxyphenyl)hydrazine (XI). The structural assignments of fragment ions of the TMS derivative of I (Fig. 6) were confirmed by high resolution mass measurements (Table 1) and by comparison with the mass spectrum of the TMS-d_9 derivative of I. The peaks at *m/e* 569, 452, 351, 346, 338, and 219 in the mass spectrum of the TMS derivative shifted to *m/e* 605, 479, 369, 373, 356, and 237, respectively, in that of the TMS-d_9 derivative (Fig. 7). These peaks decide the selection of structure XI as the most likely structure for I. The formation of these ions can be illustrated as shown in Fig. 9.††

The major fragmentation routes of I in its mass spectrum (Fig. 3) are shown in Scheme 1. High resolution measurements of these fragment ions have been carried out (Table 2). A similar strong ion

†† Substituting a TMS group for the hydrogen atom of –NH–N=C< in the TMS derivative of I seems to be difficult because of the steric hindrance of the bulky group, –CO–O–TMS, at the *ortho* position in the phenyl group.

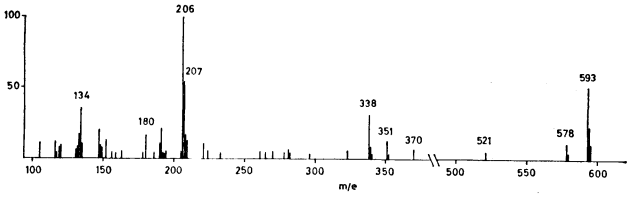


Fig. 8. Mass spectrum of the TMS derivative of **I** followed by *N*-acylation with MPFDA.

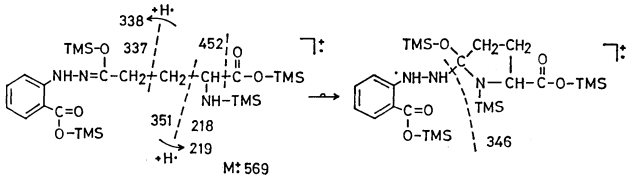
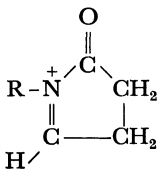


Fig. 9. Mass spectral fragmentation modes of the TMS derivative of **I**.

peak at *m/e* 84 (C_4H_6NO) was also observed in the mass spectra of glutamic acid and its derivatives such as glutamine, 4-(γ-L-glutaminyl)phenol,[12] and glutamic acid diethyl ester.[13]

The proposed structure of the fragment ion at *m/e* 84 is in line with the results showing that in the case of the TMS and the TMS-d_9 derivatives of I, the corresponding ions appear at *m/e* 156 and 165, respectively.



R=TMS, *m/e* 156
R=TMS-d_9, *m/e* 165

It can be said that the structural assignment for the strongest fragment ion of *m/e* 134 derived from the ion of *m/e* 152 as shown in Scheme 1 is supported by the fact that *o*-hydrazinobenzoic acid is easily converted into indazolone.[14]

A synthetic investigation of 1-γ-L-glutamyl-2-(2-carboxyphenyl)hydrazine (XI) is in progress.

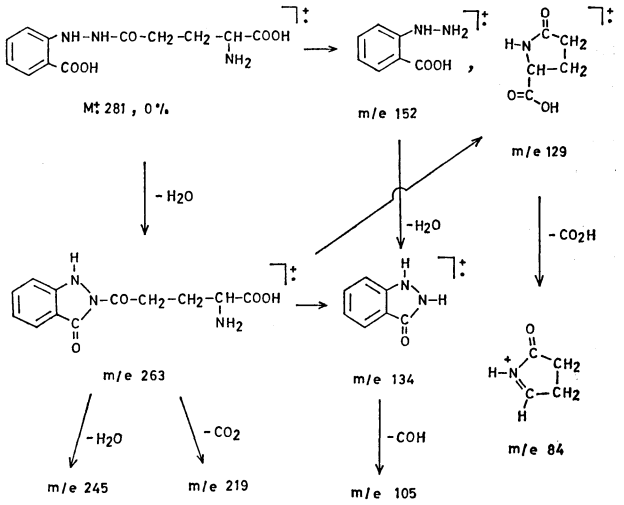
We are grateful to Dr. Chihiro Tamura for his valuable discussions. Thanks are also due to Mr. Hidemi Nagaki for his assistance in obtaining field desorption spectra.

References

1) S. B. Rosalki, "Advances in Clinical Chemistry," ed by O. Bodansky and A. L. Latner, Academic Press, New York (1975), Vol. 17, p. 53.
2) W. J. Williams and C. B. Thorne, *J. Biol. Chem.*, **210**, 203 (1954).
3) M. Y. Goore and J. F. Thompson, *Biochim. Biophys. Acta*, **132**, 15 (1967).
4) J. Danner, H. M. Lenhoff, M. Houston-Cobb, W. Heagy, and G. R. Marshall, *Biochem. Biophys. Res. Commun.*, **73**, 180 (1976).
5) S. Minato, H. Tamaoki, S. Takei, and K. Fujisawa, *Clin. Chim. Acta*, **65**, 21 (1975).
6) S. Minato, A. Terahara, and T. Kinoshita, Japan Patent, Application No. (77)-41166., 1977.
7) F. Shahrokhi and C. W. Gehrke, *J. Chromatogr.*, **36**, 31 (1968).
8) K. Bergström, J. Gürtler, and R. Blomstrand, *Anal. Biochem.*, **34**, 74 (1970).
9) M. Donike, *J. Chromatogr.*, **115**, 591 (1975).
10) H. Miyazaki, M. Ishibashi, M. Itoh, and T. Nambara, *Biomed. Mass Spectrom.*, **4**, 23 (1977).
11) J. P. Thenot and E. C. Horning, *Anal. Lett.*, **5**, 519 (1972).
12) R. F. Weaver, K. V. Rajagopalan, P. Handler, D. Rosenthal, and P. W. Jeffs, *J. Biol. Chem.*, **246**, 2010 (1971).
13) W. Vetter, "Biochemical Applications of Mass Spectrometry," ed by G. R. Waller, Wiley-Interscience, New York (1972), p. 387.
14) E. C. Horning, *Org. Synth.*, Coll. Vol. III, 475 (1955).

Table 2. High resolution mass measurements of principal ions in mass spectrum of I

Observed	Calculated	Formulae
263.0889	263.0905	$C_{12}H_{13}N_3O_4$
245.0795	245.0800	$C_{12}H_{11}N_3O_3$
219.1021	219.1008	$C_{11}H_{13}N_3O_2$
152.0597	152.0585	$C_7H_8N_2O_2$
134.0475	134.0480	$C_7H_6N_2O$
129.0454	129.0427	$C_5H_7NO_3$
105.0438	105.0452	$C_6H_5N_2$
84.0433	84.0449	C_4H_6NO
56.0479	56.0500	C_3H_6N



Scheme 1.