G1549, A NEW CYCLIC HYDROXAMIC ACID ANTIBIOTIC, ISOLATED FROM CULTURE BROTH OF *PSEUDOMONAS ALCALIGENES*

W. R. BARKER, C. CALLAGHAN, L. HILL and D. NOBLE

Biochemistry Department, Glaxo Group Research Ltd.,
Sefton Park, Stoke Poges, Buckinghamshire, England

P. ACRED, P. B. HARPER and M. A. SOWA

Chemotherapy Department, Glaxo Group Research Ltd.,
Greenford, Middlesex, England

R. A. FLETTON

Physical Chemistry Department, Glaxo Group Research Ltd.,
Greenford, Middlesex, England

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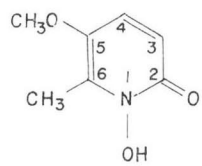
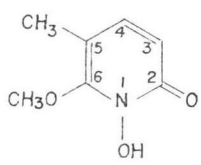
Antibiotic G1549, isolated from culture broth of *Pseudomonas alcaligenes*, is a new cyclic hydroxamic acid with a 1-hydroxy-2(1H)-pyridinone structure that complexes with metals. The structure of G1549 is suggested to be 1-hydroxy-5-methoxy-6-methyl-2(1H)-pyridinone. *In vitro*, G1549 and its copper and ferric complexes show moderate activity against Gram-positive bacteria, fungi and *Trichomonas vaginalis*. Topical application of G1549 and its copper and ferric complexes protect guinea pigs against cutaneous infection with *Microsporum canis*. The compounds, however, have some systemic toxicity in mice.

Antibiotic G1549 was isolated from culture broth of *Pseudomonas alcaligenes*. Physical and chemical measurements of G1549 suggest it to be either 1-hydroxy-5-methoxy-6-methyl-2(1H)-pyridinone (**I**) or 1-hydroxy-6-methoxy-5-methyl-2(1H)-pyridinone (**II**). Physical and chemical measurements of a reduction product of G1549, however, favour structure **I**. G1549 may thus be grouped with cyclic hydroxamic acid antibiotics[1].

This paper describes the production, isolation and physical, chemical and biological properties of G1549 and its copper and ferric complexes.



I



II

Experimental

Organism

The antibiotic is produced by *Pseudomonas alcaligenes*[2], isolated as a laboratory contaminant. It has been deposited in the National Collection of Industrial Bacteria, Aberdeen, Scotland and designated as NCIB 11492.

Fermentation

Growth of the organism from a slope of nutrient agar (Oxoid Ltd., London, England) was suspended in 5 ml sterile distilled water and 0.5 ml of the suspension was transferred to 100 ml nutrient broth (Oxoid Ltd.) in a 250-ml conical flask. The flask was shaken (220 rev/minute on an orbital shaker with a 2-cm travel) for 18 hours at 28°C.

A portion (40 ml) of growth liquor from the shake flask was transferred to 4 litres of medium (yeast extract, Oxoid L21, 0.9%; neutralised peptone, Oxoid L34, 0.18%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; glycerol, 2.0%; made up in distilled water and adjusted to pH 7.0 with sodium hydroxide) in a 5-litre fermentor. The mixture was stirred (550 rev/minute) and aerated (6 litres air/minute) for 24 hours at 22°C.

A portion (1.5 litres) of growth liquor from the 5-litre fermentor was transferred to 150 litres of the same medium in a 230-litre fermentor. The mixture was stirred (50 rev/minute) and aerated (283 litres air/minute) for 68 hours at 22°C. Antifoam (polypropylene glycol) was added as required throughout the fermentation.

Antibiotic isolation

Broth (135 litres) at harvest was adjusted to pH 7.0 with hydrochloric acid and centrifuged at 10~20°C for a throughput time of 45 minutes (Westfalia KO 2006 chamber bowl centrifuge, Oelde, Westfalia, Germany) to separate aqueous solution from cells. Chilled aqueous solution (125 litres) was adjusted to pH 3.0 with hydrochloric acid and extracted with 3 one-third volumes of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness.

The residue was extracted with methanol, filtered to remove inactive solid and the filtrate was fractionated with methanol on a column of Sephadex LH20 (100×9 cm; Pharmacia Fine Chemicals AB., Uppsala, Sweden). Fractions active against both *Staphylococcus aureus* and *Escherichia coli* were collected in two pools well separated from each other. The first pool to elute from the column was purified as given below, the second was a mixture of metal complexes of the antibiotic present in the first pool and was not purified further.

The first pool was evaporated; the residue was extracted with acetonitrile - water (7: 3 by volume), filtered and the filtrate was fractionated with the same solvent on a column of Sephadex LH20 (150× 4 cm). Fractions active against both test organisms were combined and evaporated to a solid.

Preparation of G1549 copper complex

The solid obtained from the second Sephadex column was dissolved in the minimal volume of methanol and a solution of saturated copper sulphate in water added. Green crystalline solid (8 g), the copper complex of G1549, was collected and dried under reduced pressure.

Preparation of G1549 ligand and its ethanolamine salt

Hydrogen sulphide was passed into a chloroform solution of G1549 copper complex for 30 minutes. Copper sulphide precipitate was removed by filtration (filter aid) and the filtrate was evaporated to dryness to give G1549 ligand as a brown solid.

An aqueous solution of ligand was neutralised with aqueous ethanolamine and the neutral solution freeze-dried to give the ethanolamine salt of G1549 ligand as a brown solid.

Preparation of G1549 ferric complex

G1549 ligand was dissolved in the minimal volume of methanol; ferric chloride, dissolved in a small volume of distilled water, was added to the solution of ligand to give a molar ratio of ligand to $FeCl_3$ of 10: 1. The solution was left for 24 hours at −20°C and red crystalline solid that formed was filtered and dried under reduced pressure.

Preparation of reduction product of G1549 ligand

G1549 ligand (100 mg), 2 M hydrochloric acid (35 ml) and excess zinc granules were heated under reflux for 5 hours. The mixture was filtered and the filtrate (pH 1.2) neutralised with sodium hydroxide solution to pH 7. A precipitate of zinc hydroxide was removed by filtration and the clear filtrate (pH 7) was extracted three times with an equal volume of butan-1-ol. The combined butan-1-ol extracts were evaporated to dryness to yield 20 mg of white solid, the reduction product of G1549 ligand.

Thin-layer chromatography

The methods used were as described by NOBLE *et al.*[3]

Spectroscopy

Proton NMR and ^{13}C NMR spectra were recorded on Jeol MH100 and Jeol FX100 spectrometers.

Mass spectra (electron ionisation and field desorption) were recorded on a Varian MAT 311A spectrometer.

In vitro assays

Fermentation broths and extracts: Production of antibiotic activity during growth of the organism was followed by an agar diffusion cup-plate assay. Antibiotic extracts were assayed by an agar diffusion method in which samples were applied to Whatman 3MM paper. Test organisms were *Staphylococcus aureus* Oxford H strain Vl and *Escherichia coli* 573E (Glaxo strain). Details of the methods have been given by NOBLE *et al.*[3]

Minimum inhibitory concentration: The minimum inhibitory concentration (MIC) of test compounds was determined against a range of Gram-positive and Gram-negative bacteria using a broth dilution method in microtitre plates[3] and against a range of pathogenic fungi using a tube dilution method[3].

Anti-trichomonas: Activity against *Trichomonas vaginalis* was determined by a tube dilution method using *Trichomonas* medium No. 2 (Oxoid Ltd.) as modified by BUSHBY and COPP[4] and SQUIRES and MCFADZEAN[5].

In vivo assays

Antifungal: The activity of test compounds was assessed after their topical application to guinea pig (female, 300~350 g Porcellus strain) skin infected with *Microsporum canis* (DO 3185, kindly supplied by Prof. J. C. GENTLES, Glasgow University, Scotland).

An area (2.5 cm^2) on the flank of each animal was closely clipped and the site lightly abraded. *Microsporum canis* was grown on rice medium (8 g rice to 25 ml water) and 0.2 ml of a suspension in distilled water containing 2.5×10^5 macroconidia/ml was rubbed into the abraded area of skin. The inoculated site was covered with an occlusion dressing (cling-film) secured with adhesive tape and left 2~3 days before removal. After 5 days, each site was treated twice daily with 0.2 ml aqueous cream (emulsifying ointment BP, 30% in distilled water) containing either 1% or 5% (w/v) of test compound. Treatment was continued for 10 days. Infected control animals were treated similarly with cream alone. The infection of each cutaneous site was assessed on the basis of the extent and intensity of erythema and the degree of fluorescence under ultraviolet light (340 nm).

Anti-candida: Activity of test compounds against *Candida albicans* C316 was determined in mice infected with either a lethal system infection or a localised abscess infection.

Anti-trichomonas: Activity against *Trichomonas vaginalis* was determined by the method of LYNCH *et al.*[6] in subcutaneously infected mice.

Acute mouse toxicity: The ligand was dissolved in methanol - water (1: 1 by volume), the copper complex was dissolved in dimethylsulphoxide - water (1: 1 by volume) and the ferric complex was suspended in saline (pH 7.0) containing 3 drops surfactant (Nonex). LD_{50} values were obtained by the method given by NOBLE *et al.*[3]

Results

Physical and Chemical Properties

G1549 ligand

The ligand is a brown solid melting at 94~96°C. It is soluble in methanol, ethanol and acetone; slightly soluble in ethyl acetate, chloroform and water. Rf values obtained after thin-layer chromatography are given in Table 1.

The mass spectrum (electron ionisation, Fig. 1) of the ligand shows a base peak-molecular ion at *m/e* 155; an accurate mass measurement for this peak gave a value 155.0583, indicating a molecular formula of $C_7H_9NO_3$. Fragmentation ions at *m/e* 140 $(M-15)^+$, 139 $(M-16)^+$ and 124 $(M-31)^+$ suggest the loss of CH_3, O[13] and OCH_3 fragments from the molecule.

Elemental analysis gave: C, 53.7; H, 5.8; N, 8.7; $C_7H_9NO_3$ requires C, 54.1; H, 5.8; N, 9.0%.

Table 1. Rf values of G1549 and its metal complexes on t.l.c. plates.

Solvent	Support	Rf		
		Ligand	Copper complex	Ferric complex
Benzene	Silica	0.0	0.0	0.0
Chloroform		0.0	0.4	0.1
Ethyl acetate		0.0	0.5 (streak)	0.2
Acetone		0.0	0.8 (streak)	0.9
Acetonitrile		0.0	0.8 (streak)	0.8
Methanol		0.7	0.7 (streak)	0.7
Propan-1-ol - water (7: 1)	Cellulose	0.7	0.8 (streak)	1.0
Butan-1-ol - acetic acid - water (3: 1: 1)		0.9	0.9 (streak)	1.0
Butan-1-ol - methanol - water (4: 1: 2)		0.8	0.8 (streak)	1.0

Fig. 1. Mass spectrum of G1549 ligand.

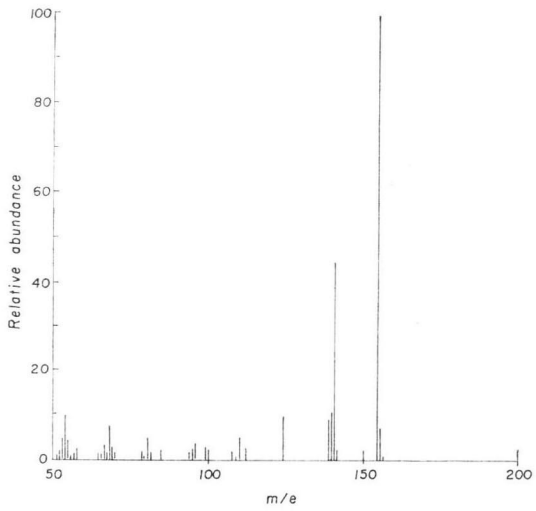
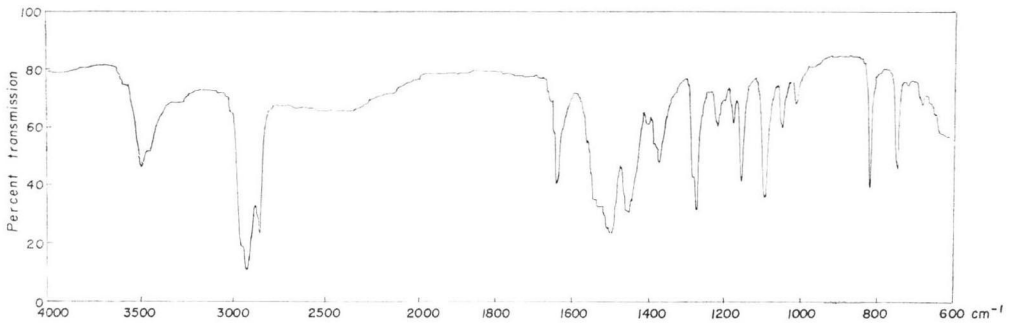


Fig. 2. Infrared spectrum of G1549 ligand (Nujol).



The ultraviolet absorption spectrum of the ligand in methanol gave absorption maxima (nm; $E^{1\%}_{1cm}$ within brackets) at 229 (560) and 328 (364). The infrared spectrum of a Nujol mull of the ligand (Fig. 2) shows peaks at 3480 (–OH), 1640 (–C=O), 1275 (–NO) and 825 (aromatic CH).

The results of a proton N.M.R. spectrum of ligand is shown in Table 2. Addition of $Eu(Fod)_3$ to the ligand induced upfield shifts of the signals at τ 7.55 and τ 6.22, indicating abnormal complexing. The carbon-13 N.M.R. spectrum of the ligand ($CDCl_3$, TMS) gave carbon resonances at δ 10.7 ($C-CH_3$), 58.7 ($O-CH_3$) and 111.6, 126.1, 135.2, 140.7, 155.2 (sp_2 ring carbons).

Reduction Product of G1549 Ligand

The ligand reduction product is a white solid, melting at 146.5~147°C.

Mass spectrometry (electron ionisation) of the ligand reduction product gave a molecular ion at *m/e* 139.0623 indicating a molecular formula of $C_7H_9NO_2$.

The proton N.M.R. spectrum of the ligand reduction product ($CDCl_3$) gave signals at τ 7.67, 3H, s ($C-CH_3$); τ 6.28, 3H, s, ($O-CH_3$); τ 3.58, 1H, d, 9Hz and τ 2.63, 1H, d, 9Hz, (*ortho* aromatic

protons). Addition of $Eu(Fod)_3$ to the reduction product induced downfield shifts of the signals at τ 7.67 and τ 6.28 with the largest downfield shift observed for the τ 7.67 signal.

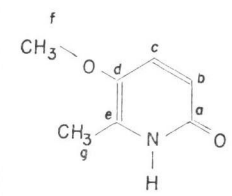
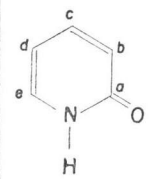
The carbon-13 N.M.R. spectrum of the ligand reduction product is compared with that of 2(1H)-pyridinone in Table 3.

Table 2. 100 MHz proton N.M.R. spectrum of G1549 ligand ($CDCl_3$).

Chemical shift (τ)	Multiplicity	Proton integration	Attribution
7.55	s	3H	CH_3 substitution at either C5 or C6
6.22	s	3H	OCH_3 substitution at either C5 or C6
3.42	d (9Hz)	1H	Proton at C3*
2.80	d (9Hz)	1H	Proton at C4*
1.24 (signal removed by addition D_2O)	s	1H	OH

* Attribution of the *ortho* ring protons to C3 and C4 is by comparison of the observed shift values with those quoted for ring protons of 2(1H)-pyridinone[7].

Table 3. Carbon-13 N.M.R. spectra of the reduction product of G1549 ligand and of 2(1H)-pyridinone ($CDCl_3$, TMS).

Reduction product of G1549 ligand		2(1H)-pyridinone*	
Chemical shift (δ)	Attribution	Chemical shift (δ)	Attribution
162.9 (a)		164.9 (a)	
116.2 (b)		119.9 (b)	
133.2 (c)		141.3 (c)	
140.7 (d)		106.6 (d)	
134.8 (e)		134.6 (e)	
58.9 (f)			
13.5 (g)			

* 2(1H)-pyridinone was obtained from Koch-Light Ltd., Colnbrook, Buckinghamshire, England.

The mass spectrum (electron ionisation) showed molecular ions at *m/e* 371 and 373, consistent with the presence of one copper atom with an atomic weight of either 63 or 65/molecule ligand, indicating a molecular formula of $(C_7H_8NO_3)_2Cu$.

Elemental analysis gave: C, 45.3; H, 4.5; N, 7.8; Cu, 17.0; ($C_7H_8NO_3)_2Cu$ requires C, 45.2; H, 4.3; N, 7.5; Cu, 17.1%.

The ultraviolet absorption spectrum of the copper complex in methanol showed absorption maxima (nm; $E^{1\%}_{1cm}$ within brackets) at 223.5 (494) and 327.5 (112). The infrared spectrum of the copper complex is similar to that of the ligand, but showed extra absorptions at 710, 790, 870 cm^{-1} and no –OH absorption.

Rf values of the copper complex obtained after thin-layer chromatography are given in Table 1.

G1549 copper complex

The copper complex is a green, crystalline solid, melting point 308~310°C with decomposition. It is soluble in chloroform, slightly soluble in methanol and acetone and insoluble in water.

G1549 ferric complex

The ferric complex is a red solid, melting point 135~137°C; soluble in chloroform, acetone and methanol and insoluble in water.

The mass spectrum (field desorption) showed a molecular ion of *m/e* 518, with no other significant ions, indicating a molecular formula of $(C_7H_8NO_3)_3Fe$.

Elemental analysis gave: C, 48.5; H, 5.0; N, 7.4; Fe, 10.6; ($C_7H_8NO_3)_3Fe$ requires C, 48.7; H, 4.6; N, 8.1; Fe, 10.8%.

The ultraviolet absorption spectrum of the ferric complex in methanol showed absorption maxima (nm; $E^{1\%}_{1cm}$ within brackets) at 224 (1216) and 318 (291). The infrared spectrum of the ferric complex

is similar to that of the ligand, but showed extra absorptions at 708, 805 (shoulder), 880 cm^{-1} and no –OH absorption. Rf values of the ferric complex obtained after thin-layer chromatography are given in Table 1.

Biological Properties

Antibacterial spectra of G1549 ligand, the ethanolamine salt of the ligand and the copper and ferric complexes of G1549 are given in Table 4. Antifungal and anti-*Trichomonas* spectra are given in Table 5. All four compounds show moderate activity against Gram-positive bacteria, fungi and *Trichomonas vaginalis*.

In vivo tests showed the compounds to be inactive against systemic and cutaneous *Candida albicans* infections in mice and inactive against subcutaneous *Trichomonas vaginalis* infections in mice.

In guinea pigs infected cutaneously with *Microsporum canis* topical treatment with the ligand, its ethanolamine salt and the copper complex proved as effective as 1% tolnaftate standard (Table 6). It was not possible to assess the effect of the ferric complex due to the brown-red colour of the cream masking the sites of infection.

Administered i.p. to mice the LD_{50} of the ligand, its ethanolamine salt, copper complex and ferric complex was 250, 500, 50 and 350 mg/kg body weight respectively.

Table 4. Antibacterial spectra of G1549 ligand, its ethanolamine salt and copper and ferric complexes of G1549.

Organism	MIC* (μg/ml)			
	Ligand	Ligand ethanolamine salt	Copper complex	Ferric complex
Staphylococcus aureus 853E	31	31	250	8
〃 〃 663E	2	8	62	1
Micrococcus sp. 1810E	4	4	62	2
Streptococcus faecalis 850E	250	125	16	8
Streptococcus pneumoniae 5372	1	<0.5	<0.5	<0.5
Streptococcus haemolyticus 618	4	4	16	1
Bacillus subtilis NCIB 9883	0.5	1	8	1
Bacillus cereus 11778	4	4	125	1
Haemophilus influenzae 1184E	125	31	>250	125
Escherichia coli 1193E	125	125	>250	250
〃 〃 1507E	62	125	>250	250
〃 〃 1852E	125	125	>250	250
〃 〃 1343	125	125	250	250
Klebsiella aerogenes 1082E	125	125	>250	250
〃 〃 1522E	125	125	250	125
Enterobacter cloacae 1051E	125	125	250	125
〃 〃 1321E	62	125	250	250
Proteus mirabilis 431E	250	250	>250	250
Proteus morganii 235	250	125	>250	250
Serratia marcescens 1324E	125	125	>250	250
Bacteroides fragilis 9326	>250	>250	250	62
Pseudomonas aeruginosa 1371E	250	>250	250	250
Flavobacterium sp. C1980	16	31	>250	16

* MIC values were determined by a dilution method in microlitre plates (see Experimental).

Table 5. Antifungal and anti-Trichomonas spectra of G1549 ligand, its ethanolamine salt and copper and ferric complexes of G1549.

Organism	MIC* (μg/ml)			
	Ligand	Ligand ethanolamine salt	Copper complex	Ferric complex
Candida albicans C316	125	31	31	>250
Candida albicans 1195E	125	62	31	>250
Saccharomyces cerevisiae NCYC 81	31	31	31	>250
Saccharomyces carlsbergensis NCYC 4228	62	31	16	>250
Neurospora crassa Glaxo 34486	16	31	62	31
Neurospora sitophila IMI 21944/2	16	>250	>250	>250
Fusarium oxysporum Glaxo G1967	250	250	>250	250
Trichophyton mentagrophytes Glaxo 687E	16	31	62	8
Microsporum canis Glaxo 764E	8	31	31	250
Epidermophyton floccosum SC 108	125	62	250	250
Trichomonas vaginalis	16	16	16	40

* MIC values were determined by a tube dilution method (see Experimental).

Table 6. Topical activity of G1549, its ethanolamine salt and copper complex against a cutaneous *Microsporum canis* infection in guinea pigs.

	G1549							
	Days	Aqueous cream	1% Ligand	5% Ligand	5% Ethanolamine salt of ligand	1% Copper complex	5% Copper complex	1% Tolnaftate
Expt. 1*	5~9 days	106	72.4	61.2	79.4	95	93.6	76.8
	10~14 〃	78	21.4	20	15.4	23.3	72	19
	15~20 〃	52.5	10	10.5	9	19	70.5	10
	21~26 〃	65.3	8	12	7	16	33	2
Expt. 2**	10~14 days	79	15	10	18	32	51	26
	15~20 〃	55	16	22	26	45	61	9
	21~26 〃	32	10	21	35	39	19	8

* Average daily lesion score for treated and control cream base groups expressed as a % of the infected control lesion score. Treatment was begun on day 5 and terminated on day 14.

** Average daily fluorescence score for treated and control cream base groups expressed as a % of the infected control fluorescence score. Treatment was begun on day 5 and terminated on day 14.

Discussion

The physical and chemical data (Figs. 1 and 2, Table 2 and text) obtained for G1549 ligand is consistent with either structure **I** or **II**.

$Eu(Fod)_3$ shift reagent studies with the reduction product of G1549 showed a greater downfield shift of the methyl substituent compared with that of the methoxy substituent. This suggests that the methyl group is adjacent to the ring N and thus structure **I** rather than **II** is favoured for G1549. Also, comparison of the carbon-13 N.M.R. spectrum of 2(1H)-pyridinone with that of G1549 reduction product shows the greatest deshielding to occur at carbon d (Table 3), suggesting that the methoxy group is attached to this carbon atom. This again favours structure **I** for G1549. Finally, the melting point of G1549 reduction product is in agreement with that quoted for 5-methoxy-6-methyl-2(1H)-pyri-

dinone[14].

G1549 belongs, therefore, to a group of cyclic hydroxamic acids produced by microorganisms that includes antibiotics and growth factors[1,8]. Two pigment compounds, tenellin and bassianin, isolated from *Beauveria* species[9,10], contain a 1-hydroxy-2(1H)-pyridinone ring in their structure.

A series of synthetic 6-substituted, 1-hydroxy-4-methyl-2(1H)-pyridinone compounds with topical antifungal activity *in vivo* has been reported[11,12]. The activity of these was increased by increasing the lipophilicity of the substituting group and by application of the compounds as ethanolamine salts. In contrast, the ethanolamine salt of G1549 showed no increase in topical antifungal activity compared with G1549.

G1549 forms stable metal complexes with copper (Cu^{2+}) and iron (Fe^{3+}). These complexes, however, did not appear markedly to increase the biological effectiveness of G1549 in the tests used.

Acknowledgement

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