ISOLATION AND STRUCTURE OF ANTIBIOTIC U-68,204, A NEW THIOLACTONE

LESTER A. DOLAK*, THOMAS M. CASTLE, S. E. TRUESDELL and OLDRICH K. SEBEK†

Infectious Diseases Research, The Upjohn Company
Kalamazoo, Michigan 49001, U.S.A.

(Received for publication July 23, 1985)

A new thiolactone-containing antibiotic U-68,204 was found to be produced by a soil actinomycete identified as *Streptomyces thiolactonus* UC 8478 (NRRL 15,439). The production, isolation, structure determination as well as the physical, spectroscopic and antibacterial properties of this $C_{13}H_{17}NO_3S$ compound are here reported. On the basis of these data, the antibiotic was identified as the 10-carboxamide of thiotetromycin.

The recent literature reports on the isolation of thiolactone antibiotics which are of interest because of their broad antibacterial spectra and low toxicity; thiolactomycin[1,2], thiotetromycin[3,4] and citreothiolactone[5]. In addition, the syntheses of racemic thiolactomycin[6] and of a number of thiotetromycin[7] analogues have been published. In the course of our search for new antibiotic substances, we have isolated a new compound of this group and identified it as thiotetromycin 10-carboxamide or 5-(2-methyl-1,3-butadienyl)-5-ethyl-2,5-dihydro-4-hydroxy-2-oxo-3-thiophene acetamide (**1b**).

Fermentation

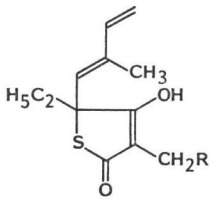
The antibiotic-producing actinomycete was isolated in a routine manner from a soil sample collected in the western United States and identified as a new species of *Streptomyces thiolactonus* UC 8478 (NRRL 15,439)††. The active substance was detected by means of a mutant of *Pseudomonas aeruginosa* UC 6954, highly sensitive to β-lactams†††.

S. thiolactonus was maintained on Hickey-Tresner agar and stored as agar plugs above liquid nitrogen[8]. To prepare the inoculum, the homogenized suspension of one agar plug in 2~4 ml of sterile water was transferred into 100 ml of a seed medium (glucose 0.5%, Bacto-Tryptone 0.5% and Bacto-Yeast Extract 0.3%) in a 250-ml Erlenmeyer flask; the seed medium was incubated on a New Brunswick rotary shaker (250 rpm) at 28°C for 3 days. Five ml of the heavily grown seed was then used to inoculate 500-ml Erlenmeyer flasks, each containing 100 ml of the production medium (Cerelose 2%, corn steep liquor 2%, Pharmamedia 1%, $(NH_4)_2SO_4$ 0.3%, $CaCO_3$ 0.4%, $ZnSO_4 \cdot 7H_2O$ 0.003% in tap water, pH 7.2). The fermentation was carried out in these shaken flasks (250 rpm, 28°C) and the antibiotic production monitored by pipeting 80 μl volumes of centrifuged culture on 13 mm-paper discs, which were applied to agar plates seeded with *Bacteroides fragilis* UC 6513 and *P. aeruginosa* UC 6955. The maximum antibiotic production was reached within 2~3 days during which time the reaction medium increased from pH 7.2 to pH 8.6. The contents of the flasks were pooled and processed as below.

† Department of Biology and Biomedical Sciences, Western Michigan University, Kalamazoo, MI 49008, U.S.A.

†† The taxonomy was done by A. DIETZ of The Upjohn Company.

††† The screening methods are due to A. L. LABORDE of The Upjohn Company.



Thiotetromycin (**1a**) R=CH_3
Antibiotic U-68,204 (**1b**) R=$CONH_2$

Isolation

Broth grown as described above is filtered over a bed of filter aid at harvest pH (pH 8.2). The filtrate is percolated over a bed of XAD-4 resin (Rohm & Haas, 1 liter resin/7 liters broth). The column is washed with deionized water and eluted with acetone - water, 1: 1. The first bed volume of eluate contains all the antibiotic and is concentrated to an aqueous on a rotary evaporator. The aqueous is adjusted to pH 3 with 1 N HCl and extracted twice with equal volumes of CH_2Cl_2. The combined organic phases are dried with magnesium sulfate, filtered and concentrated on a rotary evaporator. The oily residue is suspended in methylene chloride and the suspension is loaded atop a bed of Silica gel 60 (10 g per gram of oil used) in a sintered glass funnel. The antibiotic is eluted with CH_2Cl_2 - MeOH, 1: 1. The active fractions are combined and concentrated on a rotary evaporator. The brown, oily product (0.9 g/liter of broth) is about 30% pure.

A comparable product can be obtained by passing the aqueous concentrate from the XAD-4 column over a bed of DEAE cellulose (Whatman DE-52) elution with 0.3 M NaCl and desalting over XAD-4. One gram of this oil is dissolved in 100 ml of each phase of a solvent system consisting of $CHCl_3$ - MeOH - 0.1 M acetate buffer (pH 4.2), 6: 5: 4. This solution is equally divided into the first ten tubes of a 500-tube CCCD apparatus using 10 ml per phase per tube. After 500 transfers using the aqueous phase as the mobile phase, 0.5 ml aliquots of each phase from every tenth tube are added to 9 ml of a solution containing 25 ml of concentrated NH_4OH solution per 500 ml of MeOH. The UV maxima at 303 nm are read and used to calculate a theoretical curve. The peak tube is No. 206 so that K=0.70. The experimental curve differs from the theoretical curve in that it skews toward higher mobility but material isolated from the "skewed" tubes (No. 221~260) is identical to that obtained from the tubes within the theoretical curve (tubes 185~220).

The pool from tubes 185~220 is acidified with HCl and the phases are separated. The upper phase is extracted twice with equal volumes of $CHCl_3$. The combined organic phases are dried with magnesium sulfate, filtered, and concentrated on a rotary evaporator. The oily residue is dissolved in 0.1 N NH_4OH solution and lyophilized. The yield is 73.5 mg (*ca.* 3.6 mg/liter of broth) of slightly yellow solid. The same work-up applied to tubes 221~260 gives an additional 67.9 mg (3.4 mg/liter) of comparable product.

The ammonium salt would not crystallize. The free acid crystallized after dissolving the salt (500 mg/50 ml) in 0.01 N NH_4OH, filtering the solution through a 5 μm syringe filter, and adjusting the filtrate to pH 2 with 1 N HCl in a separatory funnel. The milky white precipitate is extracted 3 times with equal volumes of CH_2Cl_2. The aqueous phase remains yellow but shows no maximum at 303 nm. The combined organic layers are dried with magnesium sulfate, filtered and concentrated to 50 ml. To the solution is added 200 ml of cyclohexane in a 250-ml beaker. The solution is warmed to 60°C in a water bath until the volume is about 175 ml. At this point the cloudiness is persistent. The solution is removed from the bath and allowed to stand (3 hours at room temperature, 16 hours at 5°C).

The resulting white plates are collected, washed with cold cyclohexane, and dried in a Abderhalen

apparatus at 1 μm over refluxing cyclohexane for 48 hours. The plates turned opaque during the drying and showed a trace of cyclohexane in the ^{13}C NMR.

Physico-chemical Properties

In Table 1 are summarized the physico-chemical properties of antibiotic U-68,204.

Table 1. Physico-chemical properties of antibiotic U-68,204.

Appearance		Opaque, slightly yellow plates
MP		99~103°C
$[\alpha]_D^{25}$		+177° (*c* 0.8925, EtOH)
Anal Found:		C 58.32, H 6.68, N 5.01, S 10.91.
Calcd:		C 58.43, H 6.37, N 5.24, S 11.99.
FAB-MS, *m/z*		(M^++K) 306.0565 (calcd for $C_{13}H_{17}NO_3SK$: 306.0566)
EI-MS, *m/z*		Found: 267.0928; theory: 267.0929
GC-MS, *m/z*		Di-TMS 411; tri-TMS 483
Formula, free acid		$C_{13}H_{17}NO_3S$; MW=267
Paper chromatography		Free acid, Rf 0.6 with toluene - MeOH - H_2O (1:1:2), upper phase; salts show Rf 0.0.
TLC		Rf 0.25, $CHCl_3$ - MeOH, 9:1, Analtech silica gel plates
UV (H_2O) nm (ε)	pH 10:	303 (13,200), 238 (31,025)
	pH 2:	239 (31,800)
Solubility		Soluble in most organic solvents except slightly soluble in cyclohexane. Slightly soluble in H_2O.
IR (FMIR) (cm^{-1})		3320, 3200, 2965, 2920, 1640, 1580 (sh), 1460, 1415, 1370, 1340, 1300, 990, 910, 845
^{13}C NMR (DMSO-d_6) (δ)	Free acid:	195 (s), 182 (s), 175 (s), 143 (d), 140 (s), 132 (d), 116 (s), 115 (t), 56 (s), 47 (t), 17 (q), 14 (t), 13 (q)
	Salt form:	196 (s), 191 (s), 174 (s), 144 (d), 137 (d), 136 (s), 112 (t), 105 (s), 59 (s), 49 (t), 18 (q), 15 (t), 13 (q)
^1H NMR (DMSO-d_6) (δ)		7.7, 6.7 (1H each, br, exchangeable); 6.3 (dd, 1H, J=17.2, 10 Hz); 5.7 (s, 1H, br); 5.1 (d, 1H, J=17.2 Hz); 4.9 (d, 1H, J=10 Hz); 2.6 (AB, 2H, J=15.3 Hz); 1.9 (q, 2H, J=7.2 Hz); 1.6 (s, 3H); 0.8 (t, 3H, J=7.2 Hz)

Fig. 1. The FMIR spectrum of antibiotic U-68,204.

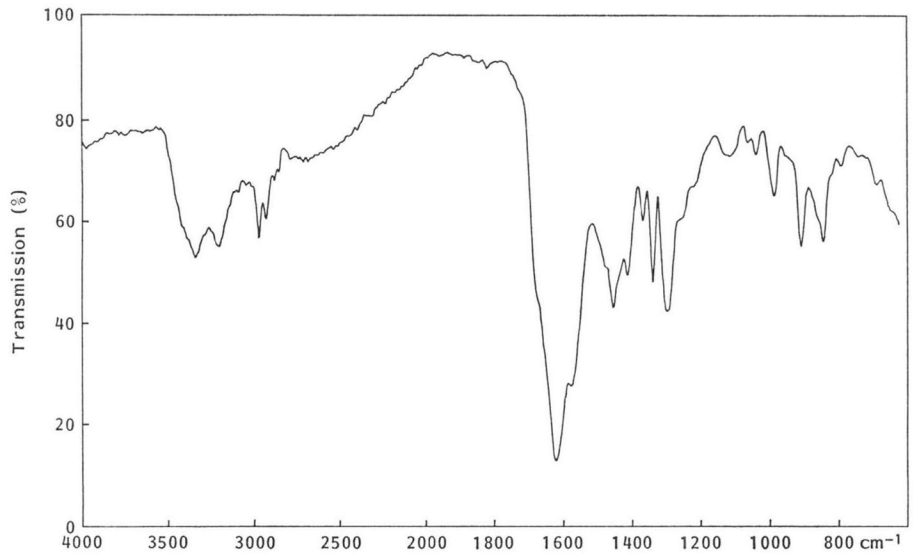
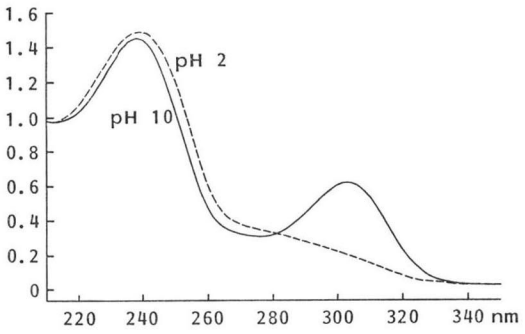


Fig. 2. The UV spectrum of antibiotic U-68,204 in 0.01 N NH_4OH (c 0.0125 mg/ml).

One drop of 1 N HCl was added to the cuvette to get the acidic spectrum (dotted line).



Mass spectroscopic studies established the molecular formula and the fact that three exchangeable protons were present. The shift with pH in the UV spectrum (Fig. 2) showed that one of the exchangeables had to be attached to the chromophore. The ^1H NMR spectrum showed two broad, exchangeable protons (Table 1) characteristic of NH moieties. The IR spectrum (Fig. 1) shows a broad carbonyl and other bands consistent with the presence of an amide group. The ^{13}C NMR spectrum (Fig. 3) showed 13 lines.

A detailed comparison of the spectra with those published for thiolactomycin[2] and thiotetromycin[3,4] led us to conclude that antibiotic U-68,204 must be related to the thiolactone antibiotics. In fact, it must have structure (**1**), in which the 10-methyl group of thiotetromycin has been oxidized to a carboxamido moiety (**1b**).

The IR spectrum shown in Fig. 1 was taken using the FMIR technique. The UV spectrum in acid and base is shown in Fig. 2. The ^{13}C NMR spectrum of the acid form is shown in Fig. 3.

Biological Activity

In the dipped disc assay, antibiotic U-68,204 showed activity against *Rhodopseudomonas spheroides* UC 3238, *Bacteroides fragilis* UC 6513 and *P. aeruginosa* UC 6955. Any of the three organisms was useful as an assay for the antibiotic. In Table 2 are summarized the MIC determinations for antibiotic U-68,204.

Fig. 3. The ^{13}C NMR spectrum of antibiotic U-68,204 in DMSO-d_6.

* The cyclohexane line is solvent of crystallization. See Table 1.

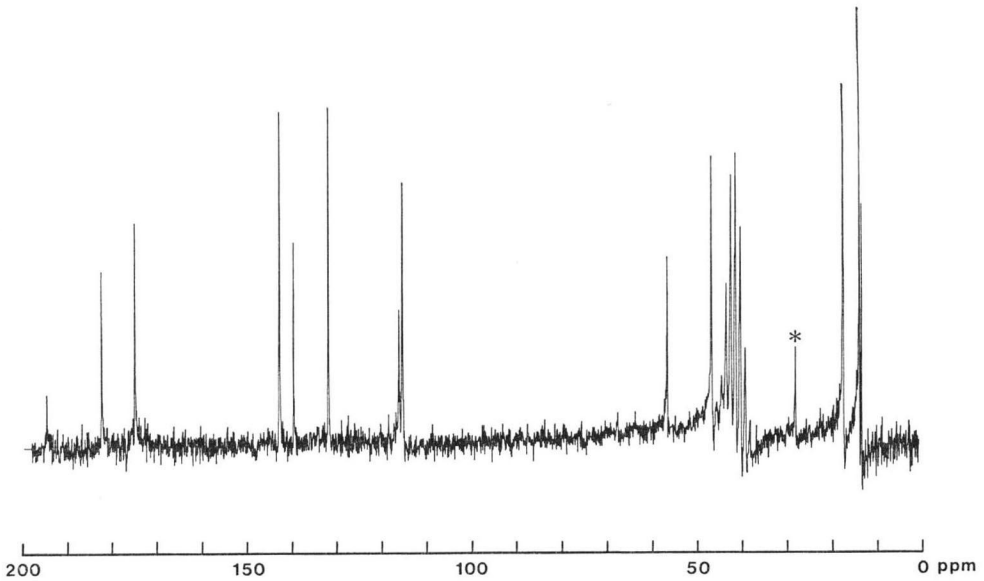


Table 2. *In vitro* activity of antibiotic U-68,204.

Organism	UC	MIC (μg/ml)	Organism	UC	MIC (μg/ml)
Staphylococcus aureus	76	75	*Serratia marcescens*	6888	>600
S. aureus	6675	75	*Citrobacter freundii*	3507	>600
S. aureus	3665	75	*Haemophilus influenzae*	6482	9.4
S. aureus	6685	600	*H. influenzae*	6483	19
Streptococcus pyogenes	152	>600	*Bacteroides fragilis*	6513	5
S. pneumoniae	41	>600	*B. distasonis*	6359	40
Enterococcus faecalis	694	>600	*B. thetaiotaomicron*	6512	80
Escherichia coli	311	600	*Fusobacterium nucleatum*	6324	>160
Klebsiella pneumoniae	58	300	*F. necrophorum*	6568	>160
Enterobacter cloacae	9382	>600	*Propionibacterium acnes*	6564	>160
Pseudomonas aeruginosa	6676	>600	*Clostridium difficile*	6858	>160
P. aeruginosa	6432	>600	*C. bifermentans*	6505	>160
P. aeruginosa	6954	>600	*C. sporogenes*	6308	>160
P. aeruginosa	6955	19	*C. ramosum*	6328	>160
P. aeruginosa	9027	37.5			

The *in vitro* spectrum of antibiotic U-68,204 is not significantly different from that published for thiotetromycin[3] but is apparently weaker than that of thiolactomycin[6].

Antibiotic U-68,204 protected mice infected with *Haemophilus influenzae* with a CD_{50} 50 mg/kg. It did not protect mice infected with multiply-resistant *Staphylococcus aureus* UC 6685 at 100 mg/kg. There was no acute toxicity noted at the higher dose.

Discussion

Application of super-sensitive *Pseudomonas* to soil screening has again led to the isolation of a new thiolactone antibiotic. Antibiotic U-68,204 is structurally related most closely to thiotetromycin and has bioactivity similar to that compound. Since it has an extra functional group in the carboxamido moiety, it is potentially a useful starting point for new analogs in this series of compounds.

Acknowledgments

The authors are indebted to Dr. C. W. FORD for the *in vivo* data, to Mr. G. E. ZURENKO for the *in vitro* data, to Ms. A. DIETZ for the taxonomy, to Dr. L. BACZYNSKYJ for the mass spectra, to Mr. S. MIZSAK for some of the NMR data and to Dr. A. L. LABORDE for some of the fermentations.

References

1) OISHI, H.; T. NOTO, H. SASAKI, K. SUZUKI, T. HAYASHI, H. OKAZAKI, K. ANDO & M. SAWADA: Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. J. Antibiotics 35: 391~395, 1982
2) SASAKI, H.; H. OISHI, T. HAYASHI, I. MATSUURA, K. ANDO & M. SAWADA: Thiolactomycin, a new antibiotic. II. Structure elucidation. J. Antibiotics 35: 396~400, 1982
3) ŌMURA, S.; Y. IWAI, A. NAKAGAWA, R. IWATA, Y. TAKAHASHI, H. SHIMIZU & H. TANAKA: Thiotetromycin, a new antibiotic. Taxonomy, production, isolation, and physicochemical and biological properties. J. Antibiotics 36: 109~114, 1983
4) ŌMURA, S.; A. NAKAGAWA, R. IWATA & A. HATANO: Structure of a new antibacterial antibiotic, thiotetromycin. J. Antibiotics 36: 1781~1782, 1983
5) SHIZURI, Y.; M. NIWA, H. FURUKAWA & S. YAMAMURA: Isolation and structure of citreothiolactone, a novel metabolite of *Penicillium citreo-viride* B. Tetrahedron Lett. 24: 1053~1054, 1983

6) WANG, C. L. J. & J. M. SALVINO: Total synthesis of (±) thiolactomycin. Tetrahedron Lett. 25: 5243~5246, 1984
7) TSUZUKI, K. & S. ŌMURA: Syntheses and biological activities of thiotetromycin analogs. J. Antibiotics 36: 1589~1591, 1983
8) DIETZ, A.: Nitrogen preservation of stock cultures of unicellular and filamentous microorganisms. Round Table Conference Cryog. Preserv. Cell Cultures, Nat'l Acad. Sci. 1974: 22~36, 1975
9) NOTO, T.; S. MIYAKAWA, H. OISHI, H. ENDO & H. OKAZAKI: Thiolactomycin, a new antibiotic. III. *In vitro* antibacterial activity. J. Antibiotics 35: 401~410, 1982