ISOLATION AND STRUCTURE DETERMINATION OF A NEW ANTIFUNGAL α-HYDROXYMETHYL-α-AMINO ACID

F. Aragozzini, P. L. Manachini and R. Craveri

Cattedra di Microbiologia Industriale. Universitá degli Studi. Via Celoria 2. 20133 Milano

B. Rindone and C. Scolastico

Instituto di Chimica Organica, Centro Nazionale della Chimica delle Sostanze Organiche Naturali del CNR, Via Saldini 50, 20133 Milano

Abstract— A new α-hydroxymethyl-α-amino acid, thermozymocidin showing antifungal activity, has been isolated from a thermophilic mold. Physical and chemical evidence suggest structure 1.

IN THE COURSE of screening work on biological activity of thermophilic fungi,[1] we have isolated from broth cultures of an eumycete strain, now included among *Mycelia sterilia*, a new substance showing strong inhibitory activity against a large number of yeasts and molds. The strain had proteolytic, amylolytic, lipolytic, milk clotting and ribonucleasic activity.[2] Thermozymocidin 1, the antibiotic isolated, is a white crystalline substance with molecular formula $C_{21}H_{39}NO_6$, m.p. 170–172°, m/e 383.[3] It is 'almost insoluble in water, slightly soluble in lower alcohols, $CHCl_3$, pyridine, and DMSO and soluble in N NaOH, N HCl or conc. HCOOH and AcOH.

HCl in MeOH transformed 1 into the aminolactone hydrochloride (2) which gave a rose colour with ninhydrin and had IR bands corresponding to the absorption pattern described by Weygand and Mayer[4] for some α-amino-γ-lactones. Since the characteristic fragmentation of such compounds[5] was also noted in the MS of 2 (Fig. 1), thermozymocidin (1) possessed an α-amino-γ-hydroxy system. The fragmentation pattern of 2 also indicated the presence of a carbonyl group and of a side-chain double bond.

m/e	%		
383	2	Parent peak	
256	41	$[.(CH_2)_5CH=CHCH_2-R]^{.*}$	(β cleavage)
200	46	$[.CH_2-CH=CH-CH-R]^{.*}$	(allylic fragmentation)
113	24	$[.OC(CH_2)_5CH_3]^{.}$	(α cleavage)
102	100	(2) → $CO_2 + CH_3(CH_2)_5CO(CH_2)_6CH=CHCH_2 +$	

$$\begin{bmatrix} H_2N & & CH_2OH \\ & \diagdown C \diagup & \\ & \diagup \quad \diagdown & \\ .CH & & CHOH \end{bmatrix}$$

$$* R = \quad -CH \underset{\diagdown O \diagup}{\overset{}{\quad}} CO \quad - C \overset{NH_2}{\underset{CH_2OH}{\diagup}} \quad HOCH$$

FIG 1. MS of thermozymocidine lactone hydrochloride 2

Hydrogenation of 1 with Pd/C in MeOH at room temperature and ambient pressure yielded a dihydroderivative (3); the disappearance of the 965 cm^{-1} band of $\pm$ in the IR of 3 indicated a *trans* configuration for the double bond. The NMR spectra of 1 and 3 were not recorded owing to low solubility.

Acetylation of thermozymocidin with excess Ac$_2$O in the presence of MeOH gave the N-acetyl-γ-lactone (4) whose NMR spectrum showed in particular the presence of four protons on carbons bearing oxygen atoms.

Acetylation of 4 or 1 with Ac$_2$O and pyridine produced the oily triacetyl-γ-lactone (5). The absence of OH bands in its IR spectrum and the number of acetyl groups in the NMR indicated that the remaining two oxygen atoms of 1 were alcoholic. Comparison of the NMR spectra of N-acetylderivative 4 with 5 showed that these alcoholic groups had primary and secondary natures respectively. The presence of an hydroxymethyl group at the carbon adjacent to the carboxyl was demonstrated by the isolation of formaldehyde dimedone derivative after sodium periodate oxidation.

In order to localize the position of the double bond in 1, O$_s$O$_4$ oxidation of 5 in pyridine yielded the corresponding diol 6 which gave, on sodium periodate treatment followed by silica-gel chromatography, two aldehydes (7) and (8). Unstable 8 was characterized as the corresponding acid 9 obtained through oxidation with alkaline Ag$_2$O. The MS of 9 and its methyl ester (10) allowed us to define the position of the keto group. Synthesis of 9 and 10 was accomplished by reaction of the cadmium Grignard reagent[6] from 1-bromohexane and ω-carbethoxyheptanoylchloride and subsequent hydrolysis of the condensation product.

Double resonance experiments showed that the aldehydic proton of 7 was adjacent to a methylenic group which was also coupled with one of the protons resonating at 4·88–5·16 δ. Complete elucidation of the structure of the polyfunctionalized part of the molecule of thermozymocidin was obtained by observation, in the NMR spectrum of 5 of an ABXY system of type.

$$
\begin{array}{ccc}
H_A & H_X & H_Y \\
| & | & | \\
C & C & C \\
| & | & | \\
H_B & O{-} & OAc
\end{array}
$$
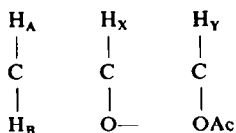
Here H$_Y$ appeared as a doublet at 5·75 δ (*J* 4·5 Hz) and H$_X$ as a sextet at 4·72 δ derived by the superposition of two triplets centered at 281 and 285·5 Hz respectively, belonging to the X part of an ABX system where $J_{AX} = J_{BX}$ 6·5 Hz. Double resonance confirmed this interpretation since irradiation at 2·48 δ transformed H$_X$ and H$_Y$ into two doublets with $J_{XY} = J_{YX}$ 4·5 Hz; irradiation at 4·72 δ reduced H$_Y$ to a singlet at 5·75 δ and irradiation at 5·75 δ transformed H$_X$ into a triplet at 4·72 δ with $J_{AX} + J_{BX}$ 13 Hz.

Chromic oxidation of dihydrothermozymocidin (3) in AcOH followed by methylation of the mixture with CH$_2$N$_2$ in ether, gave ketoester 12, ruling out the alternative formulation 11 for compound 5. Attempts failed to isolate aldehyde 13 through sodium periodate oxidation of 3 or the corresponding acid (14) derived from a further oxidation.

R—Y
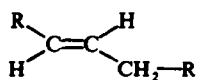
1
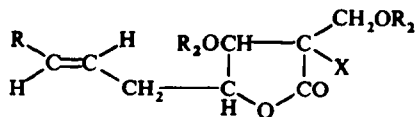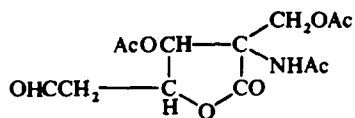
2 : R$_2$ = H, X = NH$_2$; HCl
4 : R$_2$ = H, X = NHAc
5 : R$_2$ = Ac, X = NHAc

8 : Y = CHO
9 : Y = COOH
10 : Y = COOCH$_3$

R—(CH$_2$)$_3$—R$_1$

3

7

R—(CH$_2$)$_3$COOCH$_3$

12

11

R—(CH$_2$)$_3$CHO   R—(CH$_2$)$_3$COOH

13   **14**

16

17

Where: R = CH$_3$(CH$_2$)$_5$CO(CH$_2$)$_6$— and R$_1$ = —CHOH—CHOH—C—COO$^-$ with CH$_2$OH above and NH$_3^+$ below
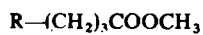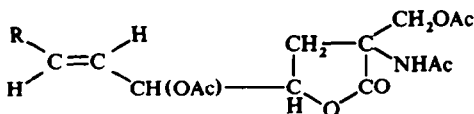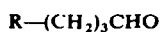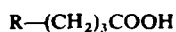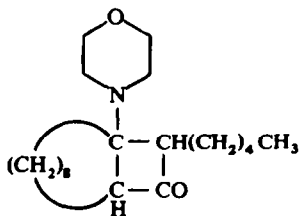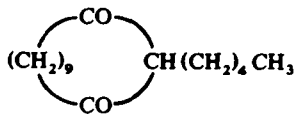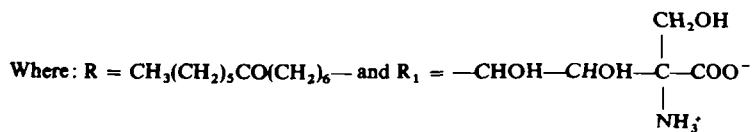
Synthesis of **12** was performed by acylation of 1-morpholinocyclodecene[7] (**15**) with heptanoyl chloride in the presence of Et_3N to give intermediate **16** subsequently hydrolysed to the cyclic-β-diketone (**17**).[8] Alkaline hydrolysis of **17** afforded **14** which was esterified with CH_2N_2 to give **12**.

The adjacent hydroxymethyl and secondary hydroxyl groups in **4** probably have *trans* configurations because N-acetylthermozymocidin did not give ketals with acetone nor acetals with benzaldehyde even under forcing conditions. The relative configurations of the asymmetric carbon atoms, and especially of those bearing secondary hydroxyl groups in **1** could not be resolved by NMR investigation of γ-lactones **2**, **4** and **5**, it has been demonstrated[9] that stereoisomeric γ-lactones do not show any significant differentiation in the coupling of their protons. Work to obtain the relative stereochemistry of **1** and X-ray analysis are in progress.

EXPERIMENTAL

Microanalyses were performed on a Perkin–Elmer 240 Elemental Analyser IR spectra were measured with a Perkin–Elmer 257 spectrophotometer. NMR spectra were recorded with a Perkin–Elmer R 10 spectrometer with TMS as internal reference: values are reported in δ/p.p.m. M.ps were determined on a Büchi apparatus and are uncorrected. Mass spectrometric investigations were conducted on a LKB 9000 (70 eV) GLC mass spectrometer system.

Cultural conditions and isolation of thermozymocidin (**1**). The antibiotic was produced by submerged culture at 43° in three stages: 750 ml flask with 100 ml (40 hr), 10 l fermentor with 5 l (40 hr), 20 l fermentor with 12 l. For first and second stages the following medium was used: 20 g glucose, 3 g corn steep, 2 g yeast extract, 0·5 g $MgSO_4$ $7H_2O$, 1000 ml water, pH 6·5–7. The same medium, without corn steep, was used for the third stage. 10 % Amount of inoculum showed it to be satisfactory. For both 10 and 20 l fermentors, aeration of 0·6 l/l/m and agitation of 350 rpm were employed.

The fermentation was interrupted after 90 hr; the pH was ≃ 7 and total antibiotic concentration ≃ 50 mg/l. The mycelium was separated by centrifugation, washed with 0·2 % NH_3 (3 × 1000 ml) and MeOH extracted (3 × 1200 ml). The supernatant broth together with MeOH phase obtained from mycelium extraction were charged into a column of Amberlite XAD-2 (8 × 42 cm) eluting with acetone–water (2/8) containing 0·2 % NH_3 (10 l), then with 80 % MeOH (50 ml fractions). Fractions 65–160 of eluate shoped by TLC (isoamyl acetate/MeOH/HCOOH/H_2O 65/25/5/5; detection: 0·3 % ninhydrin in n-BuOH/AcOH 98/2) to contain **1** were collected and concentered to one fifth original volume. After 12 hr at 5° the precipitate was collected by filtration, washed with acetone (20 ml), EtOAc (20 ml) and crystallized from EtOH/H_2O (1·28 g), m.p. 170–172°; $[\alpha]_D^{24°}$ + 4° (c, 1 % DMSO); ν_{max} (KBr) 3375, 3240, 3150, 1710, 1665, 1605, 1570, 1530, 1470, 1410, 965 cm^{-1}. (Found: C, 62·73; H, 9·76; N, 3·51. $C_{21}H_{39}NO_6$ requires C, 62·81; H, 9·79; N, 3·49 %).

Thermozymocidin lactone hydrochloride (**2**). 0·35 ml of a 44 % methanolic HCl solution were added to a suspension of 0·1 g of **1** in 25 ml MeOH. After 20 hr at room temp. the resulting solution was evaporated to dryness. Crystallization of residue from ether yielded **2** (90 mg), m.p. 158–164°; $[\alpha]_D^{25°}$ + 33° (c, 1 % MeOH); ν_{max} (nujol) 3320, 2855, 2500, 1785, 1710, 965 cm^{-1}; m/e (%) 383 (2), 256 (41), 200 (46), 113 (24), 102 (100). (Found: C, 60·10; H, 9·15; N, 3·39. $C_{21}H_{38}NO_5Cl$ requires C, 60·05; H, 9·12; N, 3·33 %).

Dihydrothermozymocidin (**3**). A solution of 0·2 g of **1** in 100 ml of MeOH were hydrogenated at room temp and ambient pressure in the presence of 0·2 g 10% Pd/C. After filtration and evaporation to dryness under vacuum, the residue was crystallized from EtOH–H_2O yielding 182 mg, m.p 166–168°, $[\alpha]_D^{25°}$ − 4° (c, 1 % DMSO); ν_{max} (KBr) 3370, 3160, 3230, 1710, 1670, 1604, 1570, 1530, 1415 cm^{-1}. (Found: C, 62·55; H, 10·42; N, 3·46. $C_{21}H_{41}NO_6$ requires C, 62·49; H, 10·24; N, 3·47 %).

Thermozymocidin N-acetyl-γ-lactone (**4**). 20 ml Ac_2O were added to 200 mg of **1** in MeOH (60 ml). After 24 hr at room temp, evaporation to dryness yielded a residue which was chromatographed on silica-gel 0·05–0·2 mm Merck (R = 100) eluting with EtOAc/hexane 8/2. The evaporation of fractions containing pure **4** afforded a residue which was crystallized from EtOH/H_2O to give 156 mg, m.p. 101–102°; $[\alpha]_D^{25°}$ + 32° (c, 1 % $CHCl_3$); NMR ($CDCl_3$) 6·57 (1H, b.s., —NHAc), 5·3–5·7 (2H, m, —CH=CH—), 4·5–4·7 (2H, m, —CHOH— and —CH(O—)—), 3·86 (2H, s, —CH_2OH), 2·1 (3H, s, C$\underline{H}_3$CONH—); ν_{max} (nujol) 3510,

3300, 1760, 1710, 1650, 1565 and 965 cm^{-1}. (Found: C, 64·78; H, 9·33; N, 3·20. $C_{23}H_{39}NO_6$ requires C, 64·91; H, 9·24; N, 3·29%).

Thermozymocidin triacetyl-γ-lactone (5). 5 ml Ac_2O were added to 400 mg of 1 in pyridine (50 ml). After 24 hr at room temp excess anhydride was decomposed with 10 ml H_2O and stirring for 30 min. Evaporation to dryness yielded a residue which was chromatographed on silica-gel 0·05–0·2 mm Merck (R = 60) eluting with EtOAc/hexane 7/3 affording 440 mg of the oily compound 5, b.p. 95–98° at 10^{-4} mm Hg; $[\alpha]_D^{24°}$ + 57° (c, 1% CHCl₃); NMR (CDCl₃) 6·49 (1H, b.s., —NHAc), 5·75 (1H, d, J = 4·5 Hz, —CHOAc—), 5·3–5·7 (2H, m, —CH═CH—), 4·72 (1H, 6 lines, —CH₂—CH(O—)—CHOAc—), 4·5 (2H, s, —CH₂OAc), 2·01, 2·03 and 2·07 (3H, s, CH₃CO—); ν_{max} (nujol) 1785, 1755, 1712, 1690, 1530 and 965 cm^{-1}. (Found: C, 63·56; H, 8·71; N, 2·80. $C_{27}H_{43}NO_8$ requires: C, 63·63; H, 8·51; N, 2·75%).

Diastereoisomeric mixture of diols (6). 300 mg OsO_4 in pyridine (3 ml) were added to 500 mg of 5 in pyridine (30 ml). After 60 min at room temp 600 mg $NaHSO_3$ in water (20 ml) were added, the solution was stirred for 20 min, diluted with water (200 ml) and acidified to pH 3. $CHCl_3$ extraction (3 × 100 ml) and evaporation of extracts afforded a residue which was chromatographed on silica-gel 0·05–0·2 mm Merck (R = 100) eluting with EtOAc. 480 mg of 6 were obtained and sublimed at 110°/10^{-4} mm Hg; ν_{max} (nujol) 3450, 3300, 1785, 1755, 1710, 1690, 1530 cm^{-1}. (Found: C, 59·60; H, 8·59; N, 2·61. $C_{27}H_{45}NO_{10}$ requires C, 59·65; H, 8·34; N, 2·58%).

Compounds 7 and 8. 3 ml of 0·2 M $NaIO_4$ were added to the solution of 230 mg of 6 in dioxane (40 ml). After stirring for 60 min at room temp, the mixture was filtered and the filtrate evaporated to dryness giving a residue which was chromatographed on silica-gel 0·05–0·2 mm Merck (40 g) eluting with EtOAc/n-heptane 1/9 (10 ml fractions). Fractions 7–9 eluted 89 mg of unstable aldehyde 8 (characterized as acid 9). Further elution with EtOAc (80 ml) gave 8 mg of 6. Aldehyde 7 (98 mg) was then eluted with amyl acetate/MeOH/H₂O/HCOOH 65/25/5/5 (100 ml) and purified by solvent evaporation, dissolving the residue in CHCl₃, filtering insolubles and crystallising from ether. Pure 7 (46 mg) had m.p. 122–124°; $[\alpha]_D^{25°}$ − 58° (c, 1% CHCl₃); ν_{max} 1795, 1760, 1695 cm^{-1}; NMR (CDCl₃) 9·78 (1H, t, J = 1 Hz, —CHO), 7·04 (1H, s, —NHAc), 4·88–5·16 (2H, m, —CHOAc— and —CH(O—)—), 2·02 (3H, s, CH₃COO—), 2·09 (6H, s, CH₃CO—). (Found: C, 49·59; H, 5·60; N, 4·32. $C_{13}H_{17}NO_8$ requires C, 49·52; H, 5·44; N, 4·44%).

8-Oxotetradecanoic acid (9). (a) 85 mg $AgNO_3$ in water (1 ml) and 60 mg KOH in water (1 ml) were added to 35 mg of crude aldehyde 8 in dioxane (30 ml). After 2 hr stirring at room temp in the dark the mixture was filtered and the filtrate, diluted with 100 ml of water, adjusted at pH 10 with $NaHCO_3$ and $CHCl_3$ extracted (3 × 25 ml). The aqueous phase was acidified to pH 3 (dilute H_2SO_4) and $CHCl_3$ extracted (3 × 50 ml). Compound 9 (35 mg) obtained by evaporation of organic extracts was crystallized from EtOAc, m.p. 67–68°; ν_{max} (CHCl₃) 1705 cm^{-1}. (Found: C, 69·26; H, 10·70. $C_{14}H_{26}O_3$ requires C, 69·38; H, 10·81%).

(b) 6·44 g of $CdCl_2$ were added to Grignard reagent prepared from 10·88 g hexyl bromide in ether (230 ml) and 1·59 g magnesium in ether (10 ml). After 45 min at reflux (stirring), benzene (45 ml) was added and ether was distilled. A solution of 11·52 g ω-carbethoxysuberoyl chloride in benzene (14 ml) was added and, after 1 hr at reflux the suspension was treated with 14 ml of 20% H_2SO_4 and 45 g crushed ice. The benzene phase was water washed (20 ml), then with 5% K_2CO_3 (20 ml) and sat. NaCl aq (20 ml). The residue after evaporation (13·05 g) was saponified by 4 hr reflux with 1 N KOH (100 ml) and MeOH (180 ml). After acidification with 1 N HCl (indicator Congo red) and $CHCl_3$ extraction (3 × 100 ml), solvent evaporation yielded 12 g crude 8-oxotetradecanoic acid which crystallized to give 8·012 g pure material, m.p. 67–68°.

11-Oxoheptanoic acid (12). (a) 125 mg 3 in 38 ml of AcOH was treated with a mixture obtained by dissolving 225 mg of CrO_3 in water (2 drops) and diluting with 5 ml AcOH. After 22 hr at room temp (stirring), 0·5 ml of isopropanol were added. Evaporation yielded a residue which was taken in 5 ml of H_2O and EtOAc extracted (3 × 20 ml). The residue of the evaporation (70 mg) was treated with excess CH_2N_2 and chromatographed on silica-gel 0·05–0·2 mm Merck (R = 200) eluting with n-heptane/EtOAc 98/2 (5 ml fractions). Fractions 8–10 eluted 22 mg of pure 12, m.p. 71–72°; ν_{max} 1708 cm^{-1}. (Found: C, 71·80; H, 11·55. $C_{17}H_{32}O_3$ requires C, 71·78; H, 11·34%).

(b) Heptanoyl chloride (0·74 g) and Et₃N (0·7 g) were added to 1·08 g of 1-morpholinocyclodecene in CHCl₃ (4 ml). After 14 hr stirring at room temp CHCl₃ (1·3 ml) and 6 N HCl (1·75 ml) were added and the mixture stirred for 40 hr. Evaporation to dryness gave a residue which was dissolved in H_2O and n-hexane extracted (3 × 50 ml). The collected organic extracts were washed with dil. $NaHCO_3$ aq and water then dried (Na_2SO_4). After evaporation to dryness the residue (1·28 g) was chromatographed on silica-gel 0·05–0·2 mm Merck (90 g) eluting with n-hexane/EtOAc 9/1 (100 ml fractions). Fractions 5–12 gave 0·25 g of 2-pentyl-1,3-cyclodecandion with m.p. 40–42°; ν_{max} (CHCl₃) 1698 cm^{-1}. (Found: C, 76·84; H, 11·65.

$C_{17}H_{30}O_2$ requires C, 76·64; H, 11·35%). A solution of 0·25 g of 2-pentyl-1,3-cyclodecandion in EtOH (20 ml) and 0·88 ml of 40% NaOH was refluxed for 2 hr then concentered to one fifth original volume, acidified to ph 3 with dil. HCl and ether extracted. 11-Oxoheptadecanoic acid thus obtained (220 mg) crystallized from EtOAc m.p. 71–72° v_{max} 1705 cm^{-1}. (Found: C, 71·72; H, 11·63. $C_{17}H_{32}O_3$ requires C, 71·78; H, 11·34%).

Isolation of formaldehyde in the periodic oxidation of (1). The solution obtained by warming 30 mg of 1 and 15 ml of 0·2 M NaHCO$_3$ was added at 25° to 78 mg of NaIO$_4$ in H$_2$O (2 ml). After 10 min stirring, 84 mg of dimedone in EtOH (2 ml) were added. After 1 hr at room temp, the mixture was acidified to pH 4 (20% H$_2$SO$_4$) and after 2 hr a precipitate was filtered, washed with 6 ml 10% AcOH aq, then with water until neutral, dissolved in 5 ml of EtOAc and filtered from insoluble material. EtOAc was evaporated and the residue crystallised from EtOH/H$_2$O obtaining 15 mg of formaldehyde-dimedone adduct, m.p. 188–189°. (Found: C, 69·88; H, 8·32. $C_{17}H_{24}O_4$ requires C, 69·83; H, 8·27%).

REFERENCES

[1] R. Craveri, P. L. Manachini and A. Craveri, *Ann. Micr.* **14**, 13 (1964); R. Craveri and C. Colla, *Ibid.* **16**, 97 (1966); A. Guicciardi, P. L. Manachini and R. Craveri, *Atti 14° Congr. Naz. Microb.* Messina (1967); R. Craveri, A. Craveri and A. Guicciardi, *Ann. Micr.* **17**, 1 (1967); R. Craveri and F. Aragozzini, *Atti 15° Congr. Naz. Microb.* Torino (1969); F. Aragozzini, P. Toppino and B. Rindone, *Ann. Micr.* **20**, 44 (1970); C. Merendi, F. Aragozzini and P. L. Manachini, *Ibid.* **20**, 23 (1970); R. Craveri, P. L. Manachini and F. Aragozzini, *Ital. Pat.* N° 22113 A/71, 23/3/71.

[2] R. Craveri, P. L. Manachini and F. Aragozzini, *Experientia* (in press)

[3] F. Aragozzini, P. L. Manachini, R. Craveri, B. Rindone and C. Scolastico, *IV International Fermentation Symposium*, 19–25 March 1972, Kyoto, Japan; *Experientia* (in press)

[4] F. Weygand and F. Mayer, *Chem. Ber.* **101**, 2065 (1968)

[5] P. Pfander and T. Wieland, *Liebigs Annalen Chem.* **700**, 126 (1966)

[6] J. Cason and F. S. Prout, *Org. Syn.* **28**, 75 (1948)

[7] S. Hunig, H. J. Buysch, H. Hoch and W. Lendle, *Chem. Ber.* **100**, 3996 (1967)

[8] J. S. V. Hunter and R. J. Light, *Biochem.* **9**, 4283 (1970)

[9] D. Savostianoff and M. Pfan, *Bull. Soc. Chim. Fr.* 4162 (1967)