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

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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, 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. Thermozymocidin 1, the antibiotic isolated, is a white crystalline substance with molecular formula \( \text{C}_{21}\text{H}_{39}\text{NO}_6 \), m.p. 170–172°, \( m/e \) 383. It is almost insoluble in water, slightly soluble in lower alcohols, CHCl₃, pyridine, and DMSO and soluble in \( \text{N NaOH}, \text{N HCl} \) or conc. HCOOH and AcOH.

\( \text{HCl} \) 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 for some α-amino-γ-lactones. Since the characteristic fragmentation of such compounds 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.

\[
\begin{align*}
\text{m/e} & \quad \% \\
383 & \quad 2 \quad \text{Parent peak} \\
256 & \quad 41 \quad \left[\text{OC(CH}_2\text{)}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{-R}\right]^* \quad (\beta \text{ cleavage}) \\
200 & \quad 46 \quad \left[\text{OC(CH}_2\text{)}_3\text{CH}_2\text{CH}_2\text{-CH}-\text{R}\right]^* \quad (\gamma \text{ cleavage}) \\
113 & \quad 24 \quad \left[\text{OC(CH}_2\text{)}_3\text{CH}_2\text{-H}_2\text{N}\right]^* \quad (\alpha \text{ cleavage}) \\
102 & \quad 100 \quad (2) \rightarrow \text{CO}_2 + \text{CH}_3(\text{CH}_2)_3\text{CO(CHOH)}_2\text{CH}==\text{CHCH}_2 + \text{CHOH} \\
\end{align*}
\]

\[ \text{H}_2\text{N} \quad \text{CH}_2\text{OH} \]

\[ \begin{align*}
\text{HOCH} & \quad \text{C} \quad \text{CO} \\
\text{NH}_2 & \quad \text{CH}_3\text{OH} \\
\end{align*} \]

\( * \text{ R = CH}_2\text{OH} \)

**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 ± 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 Ac₂O 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 Ac₂O 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 dimeredone derivative after sodium periodate oxidation.

In order to localize the position of the double bond in 1, O₃O₄ 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 Ag₂O. 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 from 1-bromohexane and o-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 CH₂N₂ 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.
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1. \( R - \text{(CH}_2\text{)}_n - \text{R} \)

2. \( R_2 = H, \ X = \text{NH}_3\text{HCl} \)
3. \( R_2 = H, \ X = \text{NHAc} \)
4. \( R_2 = \text{Ac}, \ X = \text{NHAc} \)

5. \( R - \text{(CH}_2\text{)}_3 - \text{R}_1 \)

6. \( \text{R} - \text{OCH}_2 \text{CH(OAc)} - \text{R} \)

7. \( \text{R} - \text{OCH}_2 \text{CH(OAc)} - \text{R} \)

8. \( Y = \text{CHO} \)
9. \( Y = \text{COOH} \)
10. \( Y = \text{COOCH}_3 \)

11. \( \text{R} - \text{OCH}_2 \text{CH(OAc)} - \text{R} \)

12. \( \text{R} - \text{OCH}_2 \text{CH(OAc)} - \text{R} \)

13. \( \text{R} - \text{CHO} \)
14. \( \text{R} - \text{COOH} \)

15. \( \text{R} - \text{COOCH}_3 \)

16. \( \text{R} - \text{COOCH}_3 \)

17. \( \text{R} - \text{COOCH}_3 \)

Where: \( R = \text{CH}_3\text{(CH}_2\text{)}_3\text{CO(CH}_2\text{)}_6\) and \( R_1 = \text{-CHOH-CHOH-C-COO}^- \text{NH}_3\)
Synthesis of 12 was performed by acylation of 1-morpholinocyclodecene\(^7\) (15) with heptanoyl chloride in the presence of \(\text{Et}_3\text{N}\) to give intermediate 16 subsequently hydrolysed to the cyclic-\(\beta\)-diketone (17).\(^8\) Alkaline hydrolysis of 17 afforded 14 which was esterified with \(\text{CH}_3\text{N}_2\) to give 12.

The adjacent hydroxymethyl and secondary hydroxyl groups in 4 probably have trans configurations because \(\text{N-acetylthermozymocidin}\) did not give ketals with acetone nor acetics with benzoaldehyde 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 \(\gamma\)-lactones 2, 4 and 5, it has been demonstrated\(^9\) that stereoisomeric \(\gamma\)-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 \(\delta\)/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\(^\circ\) in three stages: 750 ml flask with 100 ml (40 hr), 10 1 fermentor with 5 I (40 hr), 20 1 fermentor with 121. For first and second stages the following medium was used: 20 g glucose, 3 g corn steep, 2 g yeast extract, 0.5 g \(\text{MgSO}_4\) \(\times\) \(\text{H}_2\text{O}\), 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 201 fermentors, aeration of 0.6-1/l/m and agitation of 350 rpm were employed.

The fermentation was interrupted after 90 hr; the pH was \(\approx 7\) and total antibiotic concentration \(\approx 50\) mg/l. The mycelium was separated by centrifugation, washed with 0.2% \(\text{NH}_3\) (3 \(\times\) 1000 ml) and \(\text{MeOH}\) extracted (3 \(\times\) 1200 ml). The supernatant broth together with \(\text{MeOH}\) phase obtained from mycelium extraction were charged into a column of Amberlite XAD-2 (8 \(\times\) 42 cm) eluting with acetone–water (2/8) containing 0.2% \(\text{NH}_3\) (101), then with 80% \(\text{MeOH}\) (50 ml fractions). Fractions 65-160 of eluate shopped by TLC (isomethyl acetate/\(\text{MeOH}/\text{HCOOH}/\text{H}_2\text{O} 65/25/5/5\); detection: 0.3% ninhydrin in \(\text{n-BuOH}/\text{AcOH} 98/2\) to contain 1 were collected and concentrated to one fifth original volume. After 12 hr at 5\(^\circ\) the precipitate was collected by filtration, washed with \(\text{AcOEt} (20\text{ml})\) and crystallized from \(\text{EtOH}/\text{H}_2\text{O}\) \(1.28\), \(m.p. 170-172\^\circ\); \([\alpha]_D^{20} + 4\^\circ\) (c, \(1\)%, DMSO); \(v,\_\_\text{max} (\text{KBr}) 3375, 3240, 3150, 1710, 1660, 1570, 1530, 1470, 1410, 965 \text{cm}^{-1}\). (Found: C, 62.73; H, 9.76; N, 3.51. \(\text{C}_{21}\text{H}_{29}\text{NO}_6\) requires C, 62.81; H, 9.79; N, 3.49%).

Thermozymocidin lactone hydrochloride (2). 0.35 ml of a 44% methanolic \(\text{HCl}\) solution were added to a suspension of 0.1 g of 1 in 25 ml \(\text{MeOH}\). 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\^\circ\;\text{C}; \([\alpha]_D^{20} + 33\^\circ\) (c, \(1\)% \(\text{MeOH}\)); \(v,\_\_\text{max} (\text{nujol}) 3320, 2855, 2500, 1785, 1710, 965 \text{cm}^{-1}\); \(m/e (\%)\) 383 (2), 256 (41), 200 (46), 113 (24), 102 (100). (Found: C, 60.10; H, 9.15; N, 3.39. \(\text{C}_{21}\text{H}_{38}\text{NO}_6\) requires C, 60.05; H, 9.12; N, 3.33%).

Dihydrothermozymocidin (3). A solution of 0.2 g of 1 in \(100\) ml of \(\text{MeOH}\) were hydrogenated at room temp and ambient pressure in the presence of 0.2 g 10% \(\text{Pd/C}\). After filtration and evaporation to dryness under vacuum, the residue was crystallized from \(\text{EtOH}/\text{H}_2\text{O}\) yielding 182 mg, m.p 166-168\^\circ\, \([\alpha]_D^{20} + 4\^\circ\) (c, \(1\)% DMSO); \(v,\_\_\text{max} (\text{KBr}) 3370, 3160, 3230, 1710, 1670, 1604, 1570, 1530, 1470, 1410, 965 \text{cm}^{-1}\). (Found: C, 62.73; H, 9.76; N, 3.51. \(\text{C}_{21}\text{H}_{38}\text{NO}_6\) requires C, 62.81; H, 9.79; N, 3.49%).

Thermozymocidin N-acetyl-\(\gamma\)-lactone (4). 20 ml \(\text{AcO}_2\) were added to 200 mg of 1 in \(\text{MeOH}\) (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 \(\text{EtOAc}/\text{hexane} 8/2\). The evaporation of fractions containing pure 4 afforded a residue which was crystallized from \(\text{EtOH}/\text{H}_2\text{O}\) to give 156 mg, m.p. 101-102\^\circ\;\text{C}; \([\alpha]_D^{20} + 32\^\circ\) (c, \(1\)% \(\text{CHCl}_3\)); NMR (CDCl\(_3\)) 6.57 (1H, b.s., -\(\text{NHAc}\)), 5.3-5.7 (2H, m, -\(\text{CH}=\text{CH}-\)), 4.5-4.7 (2H, m, -\(\text{CHOH}-\) and -\(\text{CH(OH)}-\)); 3.86 (2H, s, -\(\text{CH}_2\text{OH}\)), 2.1 (3H, s, \(\text{CH}_3\text{CONH}\)); \(v,\_\_\text{max} (\text{nujol}) 3510,
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3300, 1760, 1710, 1565 and 965 cm⁻¹. (Found: C, 64.78; H, 9.33; N, 3.20. C₁₃H₁₉NO₆ requires C, 64.91; H, 9.24; N, 3.29%).

Thermozymocidin triacetyl-γ-lactone (5). 5 ml Ac₂O were added to 400 mg of 1 in pyridine (50 ml). After 24 hr at room temp excess anhydride was decomposed with 10 ml H₂O 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; δ (CDCl₃, NMR) 3.74 (1H, s, -NH₂), 4.28 (1H, s, -NH₃), 5.51-5.71 (2H, m, -CH₃, -CH₂), 4.29 (2H, s, CH₂COO⁻), 4.04 (6H, s, CH₃CO-); ν (cm⁻¹) 3300, 1712, 1690, 1530 and 965 cm⁻¹. (Found: C, 63.56; H, 8.71; N, 2.80. C₁₃H₁₉NO₆ requires C, 63.56; H, 8.51; N, 2.75%).

Diastereoisomeric mixture of diols (6). 3C₀mg 0-5 in pyridine (3 ml) were added to 500 mg of 5 in pyridine (30 ml). After 60 min at room temp 600 mg NaHSO₃ in water (20 ml) were added, the solution was stirred for 20 min, diluted with water (200 ml) and acidified to pH 3. CH₂Cl₂ extraction (3 x 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; δ (CDCl₃, NMR) 7.85 (1H, s, -NH₂), 4.28 (1H, s, -NH₃), 5.51-5.71 (2H, m, -CH₃, -CH₂), 4.29 (2H, s, CH₂COO⁻), 4.04 (6H, s, CH₃CO-); ν (cm⁻¹) 3300, 1712, 1690, 1530 cm⁻¹. (Found: C, 59.60; H, 8.59; N, 2.61. C₁₃H₁₉NO₆ requires C, 59.65; H, 8.34; N, 2.58%).

Compounds 7 and 8. 3 ml of 0.2 M NaO₃ in pyridine (3 ml) were added to the solution of 230 mg of 6 in dioxane (40 ml). After stirring for 30 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 fractions) and purified by solvent evaporation, dissolving the residue in CH₂Cl₂, filtering insolubles and crystallising from ether. Pure 7 (46 mg) had m.p. 122-124°; δ (CDCl₃, NMR) 7.85 (1H, s, -NH₂), 4.28 (1H, s, -NH₃), 5.51-5.71 (2H, m, -CH₃, -CH₂), 4.29 (2H, s, CH₂COO⁻), 4.04 (6H, s, CH₃CO-); ν (cm⁻¹) 1795, 1760, 1695 cm⁻¹. (Found: C, 49.59; H, 5.60; N, 4.32. C₁₃H₁₉NO₈ requires C, 49.52; H, 5.44; N, 4.44%).

8-Oxotetradecanoic acid (9). (a) 85 mg AgNO₃ 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 NaHCO₃ and CH₂Cl₂ extracted (3 x 25 ml). The aqueous phase was adjusted at pH 3 (dilute H₂SO₄) and CH₂Cl₂ extracted (3 x 50 ml). Compound 9 (35 mg) obtained by evaporation of organic extracts was crystallized from CH₂Cl₂/m.p. 77-78°; ν (CH₂Cl₂) 1705 cm⁻¹. (Found: C, 69.26; H, 11.50. C₁₉H₂₆O₂ requires C, 69.38; H, 11.81%).

(b) 6-44 g of CdCl₂ were added to Grignard reagent prepared from 10-88 g hexyl bromide in ether (230 ml) and 1-59 g magnesium in ether (100 ml). After 45 min at reflux (stirring), benzene (45 ml) was added and ether was distilled. A solution of 11-52 g o-carbethoxysuberoyl chloride in benzene (14 ml) was added and, after 1 hr at reflux the suspension was treated with 14 ml of 20% H₂SO₄ and 45g crushed ice. The benzene phase was water washed (20 ml), then with 5 % K₂CO₃ (20 ml) and sat. NaCl aq (20 ml). The residue after evaporation (1105 g) was dissolved by 4 hr reflux with 1 N KOH (100 ml) and MeOH (180 ml). After acidification with 1 N HCl (indicator Congo red) and CH₂Cl₂ extraction (3 x 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-Oxohexadecanoic acid (12). (a) 125 mg 3 in 38 ml of AcOH was treated with a mixture obtained by dissolving 225 mg of CrO₃ in water (2 drops) and diluting with 5 ml AcOH. After 2 hr at room temp (stirring), benzene (45 ml) was added and ether was distilled. A solution of 11-52 g o-carbethoxysuberoyl chloride in benzene (14 ml) was added and, after 1 hr at reflux the suspension was treated with 14 ml of 20% H₂SO₄ and 45g crushed ice. The benzene phase was water washed (20 ml), then with 5 % K₂CO₃ (20 ml) and sat. NaCl aq (20 ml). The residue after evaporation (1105 g) was dissolved by 4 hr reflux with 1 N KOH (100 ml) and MeOH (180 ml). After acidification with 1 N HCl (indicator Congo red) and CH₂Cl₂ extraction (3 x 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-Oxohexadecanoic acid (12). (b) 644 g of CdCl₂ were added to Grignard reagent prepared from 10-88 g hexyl bromide in ether (230 ml) and 1-59 g magnesium in ether (100 ml). After 45 min at reflux (stirring), benzene (45 ml) was added and ether was distilled. A solution of 11-52 g o-carbethoxysuberoyl chloride in benzene (14 ml) was added and, after 1 hr at reflux the suspension was treated with 14 ml of 20% H₂SO₄ and 45g crushed ice. The benzene phase was water washed (20 ml), then with 5 % K₂CO₃ (20 ml) and sat. NaCl aq (20 ml). The residue after evaporation (1105 g) was dissolved by 4 hr reflux with 1 N KOH (100 ml) and MeOH (180 ml). After acidification with 1 N HCl (indicator Congo red) and CH₂Cl₂ extraction (3 x 100 ml), solvent evaporation yielded 12 g crude 8-oxotetradecanoic acid which crystallized to give 8.012 g pure material, m.p. 67-68°.
C$_{17}$H$_{30}$O$_2$ requires C, 76.64; H, 11.35%. A solution of 0.25 g of 2-pentyl-1,3-cyclodecanone in EtOH (20 ml) and 0.88 ml of 40% NaOH was refluxed for 2 hr then concentrated to one fifth original volume, acidified to pH 3 with dil. HCl and ether extracted. 11-Oxooctadecanoic acid thus obtained (220 mg) crystallized from EtOAc m.p. 71-72° $\nu_{\text{max}}$ 1705 cm$^{-1}$. (Found: C, 71.72; H, 11.63. C$_{17}$H$_{32}$O$_2$ 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 dimeredone 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-dimeredone adduct, m.p. 188-189°. (Found: C, 69.88; H, 8.32. C$_{17}$H$_{34}$O$_4$ requires C, 69.83; H, 8.27%).

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REFERENCES
2 R. Craveri, P. L. Manachini and F. Aragozzini, Experientia (in press)
5 P. Pfander and T. Wieland, Liebigs Annalen Chem. 700, 126 (1966)
6 J. Cason and F. S. Prout, Org. Syn. 28, 75 (1948)
8 J. S. V. Hunter and R. J. Light, Biochem. 9, 4283 (1970)