TRYPTOPHAN-DEHYDROBUTYRINE DIKETOPIPERAZINE, A METABOLITE OF *STREPTOMYCES SPECTABILIS*

KATSUMI KAKINUMA and KENNETH L. RINEHART, Jr.

School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801, U.S.A.

(Received for publication August 17, 1974)

A new metabolite, tryptophan-dehydrobutyrine diketopiperazine (TDD) was isolated from *Streptomyces spectabilis*, the organism producing the streptovaricin antibiotics. The structure was assigned from spectral properties and degradation products.

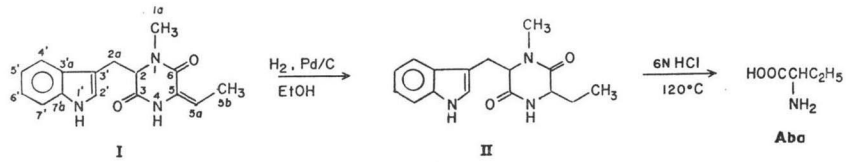
The biological activities of the streptovaricins, members of the ansamycin[1] class of antibiotics, are of continuing interest and we are now investigating the minor components of the streptovaricin complex. In the course of these studies we recently isolated a compound to which we here assign the structure tryptophan-dehydrobutyrine diketopiperazine (TDD, **I**).

Streptovaricin complex, prepared by the fermentation of *Streptomyces spectabilis*, was chromatographed repeatedly on silica gel to give a fraction containing a mixture of more polar streptovaricins[2] and a colorless ultraviolet-absorbing component which was chromatographed repeatedly over silica gel columns to give pale yellow, crystalline needles (TDD) whose high resolution mass spectrum indicated the molecular formula $C_{16}H_{17}N_3O_2$. TDD shows weak activity against RNA directed DNA polymerase (reverse transcriptase)—30 % inhibition of RLV reverse transcriptase at 200 μg/ml[2]—but it does not have significant *in vitro* antibacterial activity or inhibitory activity toward *Escherichia coli* DNA-dependent RNA polymerase.

Hydrogenation of TDD over palladium charcoal in ethanol gave dihydro TDD (**II**), whose molecular formula was assigned as $C_{16}H_{19}N_3O_2$ by high resolution mass spectrometry. Hydrolysis of **II** in 6 N hydrochloric acid at 120°C gave α-aminobutyric acid (Aba), identified by comparison with an authentic sample on thin-layer chromatographs employing a ninhydrin assay. Other products of the hydrolysis were apparently decomposed under these conditions and could not be detected on tlc.

The carbon skeleton of Aba can be detected in **II** from the proton magnetic resonance (pmr) spectrum (C_5D_5N) of **II** (Table 1),* which contains a methyl triplet at 0.58 ppm and non-equivalent methylene protons in multiplets at 0.58 and 1.56 ppm, both coupled to a proton at 3.90 ppm in addition to being coupled to the methyl group.

Fig. 1. Tryptophan-dehydrobutyrine diketopiperazine (TDD, **I**) and compounds derived from it —tryptophan-butyrine diketopiperazine (**II**), and α-aminobutyric acid (Aba, butyrine)



I II

* The spectral pattern is clearer after-addition of deuterium oxide.

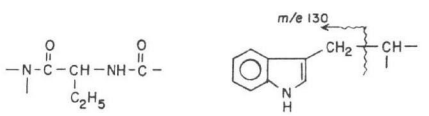
Table 1. Proton and carbon magnetic resonance absorptions of TDD (I) and dihydro-TDD (II)

Position[c]	Group	Pmr (I)[a]		Pmr (II)[b]		Cmr (I)[a]
		δ, [d]m[e]	J, Hz	δ, [d]m[e]	J, Hz	δ, [d]m[f]
1	N	—	—	—	—	—
1a	CH_3	3.04s	—	3.10s	—	32.8q
2	CH	4.25tb	3	4.43t	4	63.3d
2a	CH_2	{3.26dd 3.56dd	15,5} 15,3}	{3.53dd 3.87dd	15,5} 15,3}	27.7t
3	C=O	—	—	—	—	166.9s
4	NH	9.05sb*[g]	—	11.87sb[g]	—	—
5	-C=[h]	—	—	3.90[h]	5[f]	126.4s*
5a	-CH=[i]	5.51q	6	{0.58sx 1.56sx} i	6[g] 6	113.0d
5b	CH_3	1.00d	6	0.58t	6	9.9q
6	C=O	—	—	—	—	160.5s
1′	NH	8.19sb*[g]	—	9.15sb[g]	—	—
2′	=CH	6.77s	—	7.39s	—	124.7d
3′	=C-	—	—	—	—	107.6s
3′a	=C-	—	—	—	—	127.5s*
4′	=CH	7.28d	7	7.55d	7	118.4d**
5′	=CH	{7.09qn	7}	{7.20qn	6}	122.0d
6′	=CH					119.4d**
7′	=CH	7.62d	7	8.11d	8	111.0d
7′a	=C-	—	—	—	—	136.4s

[a]$CDCl_3$ solution, I=TDD (tryptophan-dehydrobutyrine diketopiperazine). [b]C_5D_5N solution, II=dihydro-TDD (Try-Aba diketopiperazine). [c]See Fig. 1 for numbering scheme. [d]Ppm from TMS. Signals in the same column marked * or ** may be interchanged. [e]Multiplicity: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, m=multiplet, b=broad, qn=quintet, sx=sextet. [f]Multiplicity in off-resonance proton-decoupled spectrum, same abbreviations as Footnote e. [g]Disappears on D_2O exchange (TFA catalyzed). [h]CH in II. [i]CH_2 in II.

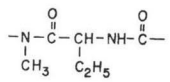
Compound II is neutral and does not give amino or carboxyl tests. Thus, both the nitrogen and carbonyl groups of Aba are apparently present in amides in II. This conclusion is in agreement with the infrared spectrum of II, which contains amide absorption at 3410, 3230, 1680 and 1650 cm^{-1} (NH and C=O). An amide NH proton is observed in the pmr spectrum at 11.87 ppm, exchangeable with deuterium oxide. Thus, the partial structure **a** is assigned to II.

A second structural unit found in II is a 3-alkylindole nucleus, **b**. This can be identified from the pmr spectrum (Table 1), with 5 aromatic protons in a pattern nearly identical to those for 3-methylindole,[3a] indoleacetic acid,[3b] and tryptophan,[4] as well as from the ultraviolet spectrum, with maxima at 220 nm (log ε 4.38), 273 nm (log ε 3.53), 282 nm (log ε 3.54), and 290 nm (log ε 3.50), like those of 3-methylindole with maxima at 222 nm (log ε 4.51), 275 nm (log ε 3.73), 281 nm (log ε 3.76) and 290 nm (log ε 3.69).[5] 3-Alkylindole pmr and ultraviolet spectral characteristics are also shown by TDD.

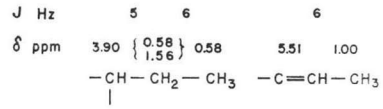


a **b**

The mass spectra of TDD and dihydro-TDD both contain an intense ion at *m/e* 130 characteristic of the unit **b**,[6] as well as its fragment ions at *m/e* 103 and 77.



a′



$\mathbf{c}_{II}$ $\mathbf{c}_{I}$

The NH proton of **b** is observed in the pmr spectrum of dihydro TDD at 9.15 ppm and is exchangeable with deuterium oxide. The methylene protons of **b** are found in dihydro TDD at 3.53 and 3.87 ppm (Table 1) and form the AB portion of an ABX multiplet, with the X proton being found as a broad triplet at 4.43 ppm. The remaining unit of dihydro TDD is a methyl group ($C_{16}H_{19}N_3O_2-C_5H_7N_2O_2-C_{10}H_9N=CH_3$) which appears at 3.10 ppm in its pmr spectrum. This chemical shift value is only appropriate for location of the methyl on nitrogen (of the free bonds in **a** and **b**), as in **a′**. Combination of partial structures **a′** and **b** can only give the diketopiperazine **II**.

Comparison of the pmr spectra (Table 1) of TDD and dihydro TDD reveals that the ethyl group of **II** has been formed by hydrogenation of an ethylidene group in TDD, as summarized in $\mathbf{c}_{II}$ and $\mathbf{c}_{I}$. Thus, the structure of TDD, **I**, follows directly from that of **II**, and **I** is seen to be the diketopiperazine formed from tryptophan and dehydro-α-aminobutyric acid (dehydrobutyrine). The infrared spectrum (KBr) of **I** is much like that of **II** except that the carbonyl absorptions are more widely spaced—at 1685 and 1635 cm^{-1}. Presumably the latter is due to the unsaturated amide. Although dehydrobutyrine is unusual it is by no means unknown, being found in the peptide antibiotics stendomycin,[7] nisin,[8] and subtilin.[9]

Although the discussion above leaves no doubt regarding the structure of **I**, a few points warrant comment. First, the proton spectra of **I** and **II** are quite sensitive to solvent changes. Thus, chemical shifts for protons of the aromatic rings differ considerably in pmr spectra of **I** ($CDCl_3$) and **II** (C_5D_5N; *cf.* Table 1).

The carbon magnetic resonance (cmr) absorptions of **I** have been assigned and are in general agreement with structure **I**. First, carbon atoms were grouped according to the number of hydrogens attached (indicated by the off-resonance proton decoupled spectra), then assigned according to standard chemical shift data,[10] as shown in Table 1. The cmr spectrum of 3-methylindole[10b] was especially useful in assigning the aromatic portion of the spectrum of **I**, its chemical shifts being as follows: C-2, 122.7 ppm; C-3, 111.4; C-3a, 129.2; C-4, 119.4; C-5, 122.3; C-6, 119.6; C-7, 111.7; C-7a, 137.3. The close agreement of the 3-methylindole spectrum with that of the aromatic portion of TDD is further evidence for the structure assigned (**I**). The two amide carbonyl carbons were assigned by considering the effects of both α, β-unsaturation and N-methylation. Thus, a carbonyl carbon shows an upfield shift on introduction of a conjugated double bond (*cf.* propionamide carbonyl at 177.2 ppm *vs.* methacrylamide at 170.3 ppm) and methyl substitution on an amide nitrogen also causes a slight upfield shift of the amide carbon (*cf.* acetamide at 172.7 ppm *vs.* N-methylacetamide at 171.6 ppm). Therefore, the C-3 carbonyl could be assigned to the signal at 166.9 ppm and C-6 to the signal at 160.5 ppm.

Although compound **I** shows no antibacterial activity, several groups of diketopiperazine antibiotics have been reported, including the sporidesmins,[11] aranotins,[12] gliotoxins,[13] and

albonoursin.[14] The latter compound (albonoursin), which contains two unsaturated amino acid units, was recently reported to have antitumor activity *in vivo*.[15]

Experimental Section*

Isolation of I. Impure streptovaricin A (9.7 g) prepared from streptovaricin complex (Upjohn, Lot No. 11560-4) by silica gel column chromatography employing benzene-acetone as eluant was rechromatographed over 400 g of silica gel (Brinkmann) in a column 50×600 mm employing chloroform-methanol (97:3) as eluant to give 2.06 g of a fraction containing several components and 3.4 g of streptovaricin A. The former fraction was further chromatographed on 100 g of Bio-Sil A (silicic acid) in a column 32×400 mm employing chloroform-methanol (98:2) as eluant. All fractions were examined by thin-layer chromatography (tlc) on Bio-Sil A (silicic acid) using chloroform-methanol (95:5) as solvent and an ultraviolet lamp as detector. Appropriate fractions were then combined and evaporated to dryness to give 1.05 g of impure **I**, which was further purified on 50 g of Bio-Sil A (silicic acid) in a column 30×400 mm employing chloroform-methanol (97:3) as eluant. Again, fractions were examined by tlc and appropriate fractions were combined. Evaporation gave 730 mg of **I**, which was crystallized from chloroform-carbon tetrachloride and recrystallized from acetone-cyclohexane to give 515 mg of slightly yellowish needles, soluble in acetone, chloroform, methanol, and ethanol; mp 121~123°C, $[\alpha]_D^{24.5}$ +10.0° (*c* 1.1, 95% EtOH), uv_{max} (95% EtOH) 220 nm (ε 34,900), 260 sh (ε 8470), 283 (ε 5780), 290 (ε 4830), ir (KBr) 3300, 3230 (NH), 3100, 1700, 1645 (amide), 1600 (aromatic), 1400, 1130, 1110 and 740 cm^{-1} (aromatic).

Anal. Calcd. for $C_{16}H_{17}N_3O_2$: mol wt, 283.1320. Found: mol wt, 283.1303 (HRMS).

Hydrogenation of I. A sample (100 mg) of **I** was hydrogenated over 30 mg of 5% palladium-charcoal in 50 ml of ethanol at atmospheric pressure. After 5 hours, hydrogenup take had ceased and the reaction mixture was filtered through Celite, The filtrate was evaporated to dryness to give 90 mg of hydrogenated product which was chromatographed over 10 g of Bio-Sil A (silicic acid) in a column 10×250 mm, employing chloroform-methanol as eluant. Two fractions were isolated, *ca.* 10 mg of an oil followed by 55 mg of a solid. The solid fraction was crystallized from chloroform-carbon tetrachloride to give 43 mg of **II**; colorless needles, mp 126~128°C, ir (KBr): 3400, 3260, 1680, 1650 and 735 cm^{-1}.

Anal. Calcd. for $C_{16}H_{19}N_3O_2$: mol wt, 285.1477. Found: mol wt, 285.1479 (HRMS).

Acidic Hydrolysis of II. A sample (1.5 mg) of **II** was hydrolyzed with 2 ml of 6 N hydrochloric acid at 120°C for 16 hours in a sealed tube. The resulting hydrolyzate was diluted with *ca.* 5 ml of water and evaporated to dryness *in vacuo* and this procedure was repeated several times to remove hydrochloric acid. Finally, a small amount of water was added to the dried material and the solution was examined by tlc employing a ninhydrin spray assay. α-Aminobutyric acid (**III**) in the hydrolyzate had R_f values identical to those of authentic racemic α-aminobutyric acid (Nutritional Biochemical Corporation) on Eastman Chromagram plates employing 1-butanol-acetic acid-water (4:1:2) and 1-butanol saturated with water as solvents.

Acknowledgments

This work was supported by Contract NIH-NCI-C-72-3208 from the Division of Cancer Treatment,

* Melting points were determined on a Kofler hot stage and are uncorrected. Proton magnetic resonance spectra were obtained by R. L. THRIFT and associates on Varian A-60 and HR-220 spectrometers; carbon magnetic resonance spectra were recorded by S. K. SILBER on a Varian XL-100 spectrometer operated in the FOURIER transform mode with Digilab computer. Pmr and cmr chemical shifts are reported as ppm relative to tetramethylsilane as internal standard. High resolution mass spectra were determined by J.C. COOK, Jr., on a Varian MAT 731 spectrometer.

National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare. The high resolution mass spectrometer was provided by grants from the National Cancer Institute (CA 11388) and the National Institute of General Medical Sciences (GM 16864). The ^{13}C nmr spectrometer was purchased with an instrumentation grant from the National Science Foundation. We also thank The Upjohn Co., Kalamazoo, Mich., for the streptovaricin sample, Dr. L.H. LI, The Upjohn Co., for the RLV reverse transcriptase assay, and Dr. F. REUSSER, The Upjohn Co., for antibacterial and *E. coli* RNA polymerase assays.

References

1) (a) A recent review: RINEHART, K. L., Jr.: Antibiotics with ansa rings. Accts. Chem. Res. 5: 57~64, 1972
(b) PRELOG, V. & W. OPPOLZER: Ansamycine, eine neuartige Klasse von mikrobiellen Stoffwechselprodukten. Helv. Chim. Acta 56: 2279~2287, 1973
(c) WEHRLI, W. & M. STAEHELIN: Actions of the rifamycins. Bacteriol. Rev. 35: 290~309, 1971
2) RINEHART, K. L., Jr.; F.J. ANTOSZ, K. SASAKI, P. K. MARTIN, M. L. MAHESHWARI, F. REUSSER, L. H. LI, D. MORAN & P. F. WILEY: Relative biological activities of individual streptovaricins and streptovaricin acetates. Biochemistry 13: 861~867, 1974
3) (a) "The Sadtler Standard Spectra", Sadtler Research Laboratories, Inc., Philadelphia, Pa.; Spectrum No. 1198. (b) Spectrum No. 9665
4) "High Resolution NMR Spectra Catalog," Vol. 2, Varian Associates, Palo Alto, Calif., 1963; Spectrum No. 582
5) PHILLIPS, J.P.; J.C. DACONS & R. G. RICE, Ed.: "Organic electronic spectral data", Vol. 7, p. 212, Wiley-Interscience, New York, N.Y., 1971
6) PORTER, Q.N. & J. BALDAS: "Mass spectrometry of heterocyclic compounds." Wiley-Interscience, New York, N.Y., 1971
7) BODANSZKY, M.; J. IZDEBSKI & I. MURAMATSU: The structure of the peptide antibiotic stendomycin. J. Amer. Chem. Soc. 91: 2351~2358, 1969
8) GROSS, E. & J.L. MORELL: The structure of nisin. J. Amer. Chem. Soc. 93: 4634~4635, 1971
9) GROSS, E.; H.H. KILTZ & E. NEBELIN: Subtilin. VI. Die Struktur des Subtilins. HOPPE-SEYLER's Z. Physiol. Chem. 354: 810~812, 1973
10) (a) JOHNSON, L. F. & W. C. JANKOWSKI: "Carbon-13 nmr spectra." Wiley-Interscience, New York, N.Y., 1972
(b) STOTHERS, J.B.: "Carbon-13 nmr spectroscopy." Academic Press, New York, N.Y., 1972
(c) LEVV, G.C. & G.L. NELSON: "Carbon-13 nuclear magnetic resonance for organic chemists." Wiley-Interscience, New York, N.Y., 1972
11) FRANCIS, E.; R. RAHMAN, S. SAFE & A. TAYLOR: Sporidesmins. XII. Isolation and structure of sporidesmin G, a naturally-occurring 3,6-epidithiopiperazine-2,5-dione. J. Chem. Soc. Perkin I, 1972: 470~472, 1972
12) NAGARAJAN, R.; L.L. HUCKSTEP, D.H. LIVELY, D. C. DELONG, M. M. MARSH & N. NEUSS: Aranotin and related metabolites from *Arachiotus aureus*. I. Determination of structure. J. Amer. Chem. Soc. 90: 2980~2982, 1968
13) BEECHAM, A.F.; J. FRIDRICHSONS & A. MCL. MATHIESON: Structure and absolute configuration of gliotoxin and the absolute configuration of sporidesmin. Tetrahedron Letters 1966: 3131~3138, 1966
14) BROWN, R.; C. KELLY & S. E. WIBERLEY: The production of 3-benzylidene-6-isobutylidene-2, 5-dioxopiperazine by a variant of *Streptomyces noursei*. J. Org. Chem. 30: 277~280, 1965
15) FUKUSHIMA, K.; K. YAZAWA & T. ARAI: Biological activities of albonoursin. J. Antibiotics 26: 175~176, 1973