TIRANDALYDIGIN, A NOVEL TETRAMIC ACID OF THE TIRANDAMYCIN-STREPTOLYDIGIN TYPE

II. ISOLATION AND STRUCTURAL CHARACTERIZATION

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(Received for publication May 30, 1987)

Tirandalydigin a structurally unique hybrid of the tirandamycin-streptolydigin families of tetramic acid antibiotics has been isolated from the fermentation beers of *Streptomyces* sp. AB-1006A-9. The structure of this anti-anaerobic antibiotic has been characterized based upon NMR, UV and mass spectrometric data.

In the course of screening for microorganisms producing novel selective activity against obligate anaerobes, a streptomycete was discovered which produced an activity with a biological spectrum similar to that of the tetramic acid class of antibiotics. However, dereplication studies employing resistant strain bioactivity spectrum and HPLC-diode array UV analysis distinguished this activity from tetramic acid standards. Preliminary isolation on a small scale revealed a compound with a ^{1}H NMR spectrum similar to that of streptolydigin, but having a molecular weight less than that of any reported streptolydigin analog.[1] The producing organism was fermented on a larger scale in order to isolate enough material for detailed spectroscopic evaluation. The subsequent isolation and structural characterization are outlined in this paper. Antibiotic production and microbiological data are covered in the companion paper.[2]

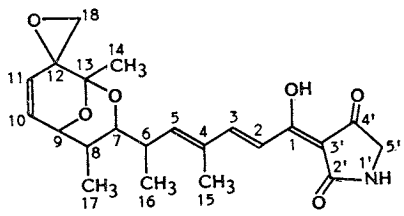
Isolation

The whole beer was adjusted to pH 3.0 by the addition of mineral acid. The activity was extracted into methylene chloride. This was concentrated and backwashed with 0.05 M sodium bicarbonate solution (pH 6.0). The methylene chloride was then dried over sodium sulfate and concentrated. The active concentrate was digested in methanol and washed several times with hexane. The active principle from the methanol was chromatographed on Sephadex LH-20 in an organic partition system (chloroform - methanol - *n*-heptane). Further purification was achieved employing an Ito Coil planet centrifuge (CPC).[3] Two combinations of solvents were used in varying proportions; methanol - water - chloroform - carbon tetrachloride and hexane - ethyl acetate - methanol - water. A portion of the active compound was associated with a paramagnetic metal ion which necessitated a final clean up on LiCroprep RP-8 (acetonitrile - methanol - water) and the subsequent conversion to the sodium salt. Activity was monitored by disc diffusion bioassay on agar plates seeded with *Bacteroides fragilis*.

Characterization and Structure Determination

The antibiotic, tirandalydigin (**1**), is soluble in all common organic solvents both as the free acid and sodium salt. The free acid is also soluble in alkaline water. The antibiotic has an optical rotation $[\alpha]_D^{26}$ $-4.0°$ (*c* 0.50, methanol).

A fast atom bombardment mass spectra (FAB-MS) (positive ion mode) experiment gave a weak



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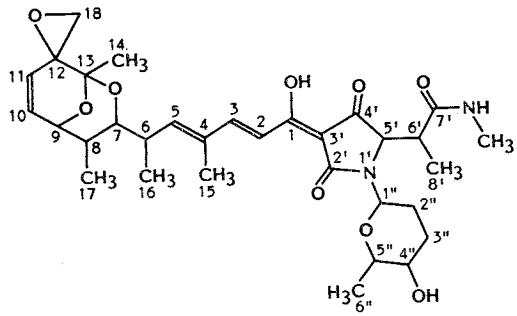

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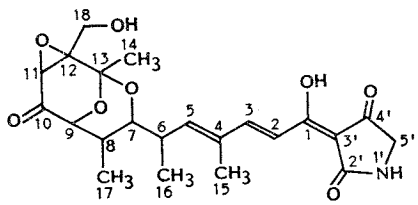

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parent peak, but a strong $(M+Na)^+$ at *m/z* 424. In FAB-MS (negative ion mode) the strong parent peak *m/z* 400.1767 (peak matched) was observed, which corresponded to a formula $C_{22}H_{26}NO_6$ (calcd 400.1760) and was assigned to the deprotonated molecular ion.

In acidic solution tirandalydigin exhibited UV adsorption maxima at 234, 352 and 364 nm ($E^{1\%}_{1cm}$ 142, 610 and 570). In basic solution the maxima occurred at 253, 287 and 328 nm ($E^{1\%}_{1cm}$

Fig. 1. UV spectrum of tirandalydigin Na salt.

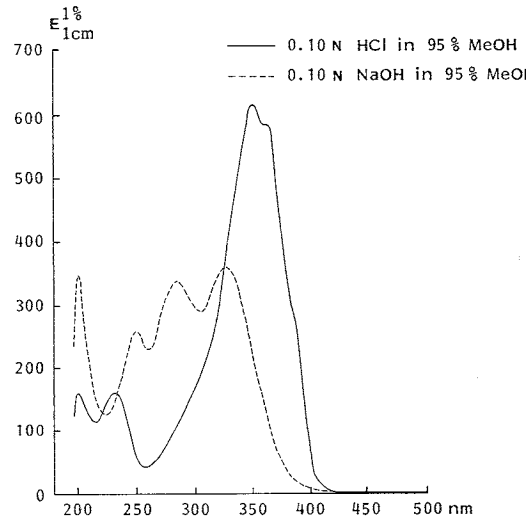


Fig. 2. IR spectrum of tirandalydigin Na salt (KBr).

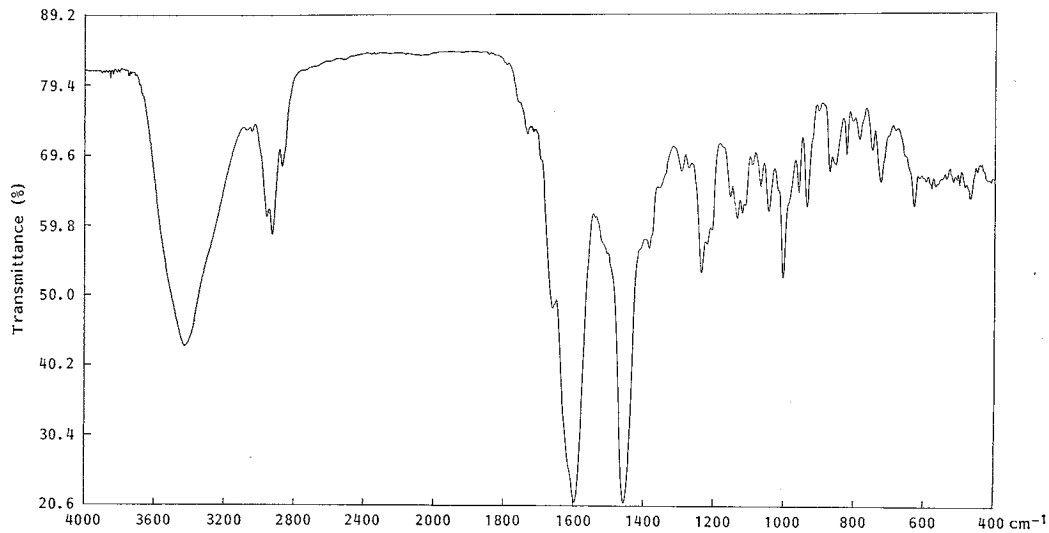
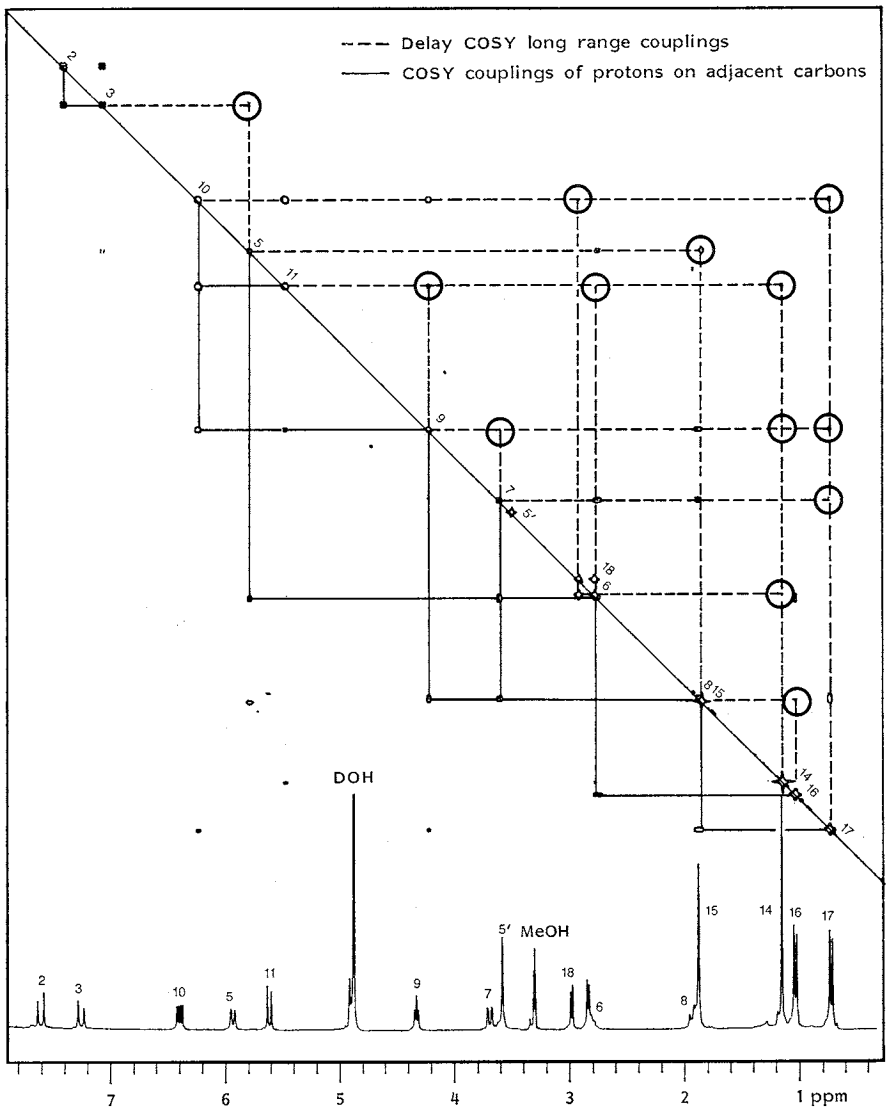


Fig. 3. ^{1}H NMR COSY and delay COSY data for tirandalydigin in CD_3OD.



230, 314 and 346). The UV of the sodium salt in methanol was characterized by similar maxima; 254, 289 and 332 nm ($E^{1\%}_{1cm}$ 204, 287 and 353) (Fig. 1). This UV spectrum is essentially identical to that described for tirandamycin A.[4] The hypsochromic-hypochromic shift exhibited by the sodium salt is characteristic of the dienoyltetramic acid (enol anion) chromophore found in the tirandamycin-streptolydigin families of antibiotics.[5~7]

The ^{1}H NMR exhibited a doublet at δ 5.94 (J=10.2 Hz, 5-H) in addition to a pair of doublets at δ 7.26 (J=15.3 Hz, 3-H) and δ 7.61 (J=15.3 Hz, 2-H). A broadened methyl singlet was found at δ 1.88 (15-H_3). A delay correlation spectroscopy (COSY) experiment demonstrated a long range allylic type coupling of the 15-H_3 protons to the 5-H proton and a similar coupling between the 3-H and 5-H protons (Fig. 3). In addition, a fully coupled heteronuclear (^{1}H-^{13}C) delay COSY correlation (FUCOUP) experiment demonstrated a coupling between the quaternary olefinic C-4 carbon

Fig. 4. Fully coupled heteronuclear ^{1}H-^{13}C delay COSY correlation experiment on tirandalydigin in DMSO-d_6. Cross-peaks are numbered corresponding to protons and carbon signals are labeled with carbon numbers.

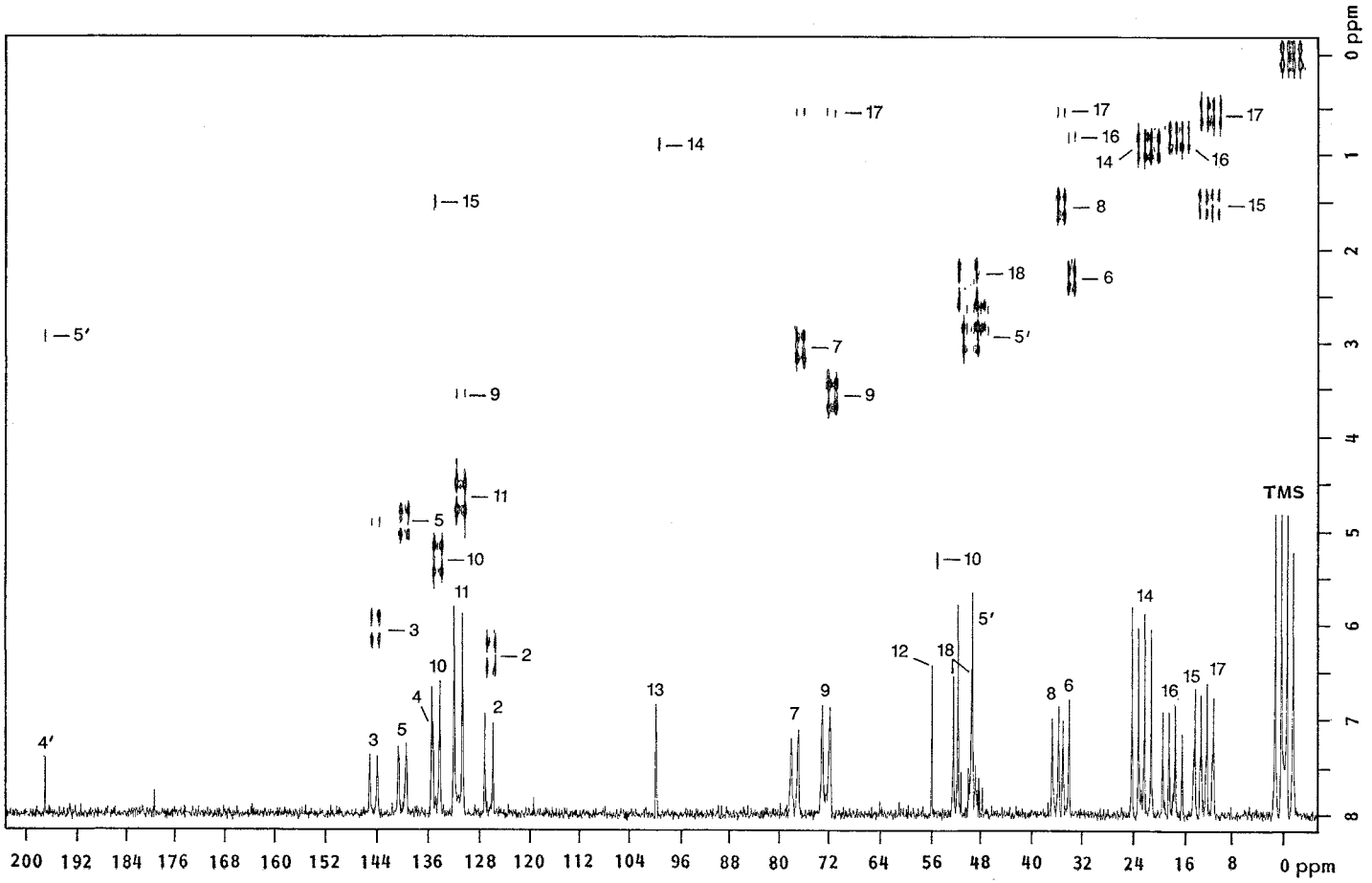
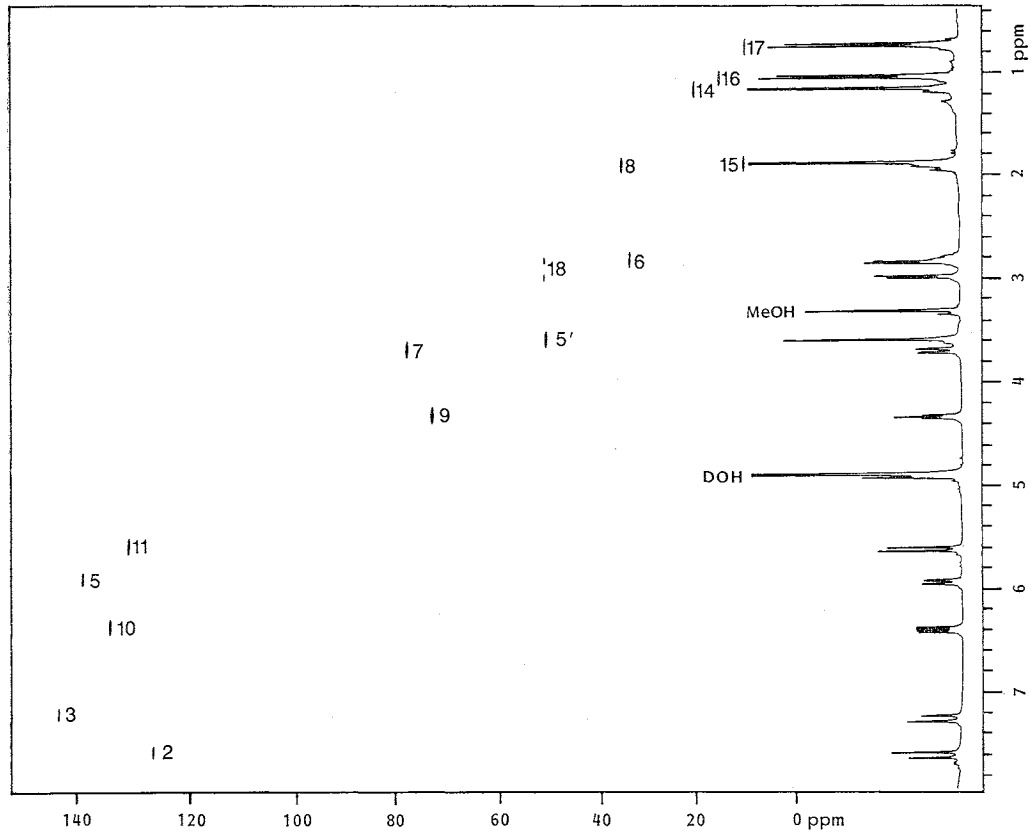


Fig. 5. ^{1}H-^{13}C Chemical shift correlation map (CSCM) for tirandalydigin in CD_3OD.



(135.5 ppm) and the protons of the methyl group it bears (Fig. 4). In this experiment a three bond coupling was also seen between the C-3 carbon (144.9 ppm) and the 5-H proton. These data are consistent with the presence of a dienoic acid type chromophore; indicating a *trans* substitution of the 2, 3 double bond with no adjacent protons and a positioning of the remaining olefinic proton on carbon 5 (140.3 ppm).

A ^{13}C NMR spectrum exhibited three carbonyl type sp^2 carbon signals at 179.9, 186.1 and 197.2 ppm (C-2′, C-1 and C-4′) and a high field quaternary olefinic signal at 104.2 ppm (C-3′). The carbon signal at 50.9 ppm (C-5′) was implicated as a methylene in a distortionless enhancement by polarization transfer (DEPT) ^{13}C NMR experiment and by ^{1}H-^{13}C chemical shift correlation mapping (CSCM) it corresponded to a two proton broadened singlet at δ 3.58 (5′-H_2) (Fig. 5). Single frequency decoupling experiments in dry DMSO showed this proton signal to be coupled to an NH proton signal at δ 6.48 (1′-H). The 4′-carbon showed a strong coupling to the C-5′ protons in the FUCOUP experiment. These data indicate the presence of the complete dienoyltetramic acid chromophore, unsubstituted at the 1′-N and C-5′ positions. A comparison with a sample of tirandamycin B (**3**) from these laboratories under similar conditions showed an exact one to one proton-carbon chemical shift and coupling constant correspondence for this portion of the molecule (Table 1).

Other features of the ^{1}H and ^{13}C NMR which identify tirandalydigin with the streptolydigin class of antibiotics were prominent. Other than carbon C-5′, the only methylene carbon indicated by

Table 1. ^{13}C NMR assignments for streptolydigin, tirandalydigin and tirandamycin (δ, ppm).

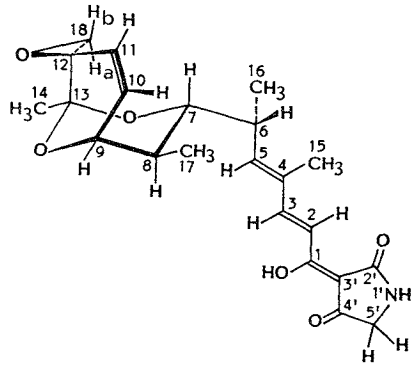
Carbon No.	Tirandamycin B[a]	Tirandalydigin[a]	Streptolydigin[b]
C-2′	179.8 (Q)	179.9 (Q)	174.7 (Q)
C-3′	104.2 (Q)	104.2 (Q)	99.7 (Q)
C-4′	197.3 (Q)	197.2 (Q)	193.5 (Q)
C-5′	50.9 ($COCH_2N$)	50.9 ($COCH_2N$)	63.0 (COCHN)
C-1	186.1 (Q)	186.1 (Q)	173.3 (Q)
C-2	127.1 (CH=)	126.6 (CH=)	116.0 (CH=)
C-3	144.6 (CH=)	144.9 (CH=)	150.3 (CH=)
C-4	136.4 (Q)	135.5 (Q)	133.9 (Q)
C-5	138.6 (CH=)	140.3 (CH=)	145.9 (CH=)
C-6	35.2 (CH)	34.8 (CH)	34.1 (CH)
C-7	78.3 (CHO)	77.8 (CHO)	75.9 (CHO)
C-8	35.5 (CH)	36.5 (CH)	35.2 (CH)
C-9	80.1 (COCHO)	72.9 (CHO)	71.3 (CHO)
C-10	203.5 (Q)	135.1 (CH=)	133.6 (CH=)
C-11	61.0 (CHO)	131.5 (CH=)	130.4 (CH=)
C-12	57.8 (Q)	56.1 (Q)	55.0 (Q)
C-13	97.1 (Q)	100.1 (Q)	98.8 (Q)
C-14	23.7 (CH_3)	22.7 (CH_3)	22.2 (CH_3)
C-15	12.9 (CH_3)	12.9 (CH_3)	12.2 (CH_3)
C-16	17.6 (CH_3)	17.8 (CH_3)	17.1 (CH_3)
C-17	11.7 (CH_3)	12.7 (CH_3)	12.5 (CH_3)
C-18	58.2 (CH_2OH)	51.4 (CH_2O)	50.5 (CH_2O)

[a] Sodium salt in CD_3OD.
[b] Free acid in $CDCl_3$.[8]
Q: Quaternary.

Table 2. ^1H NMR NOE relationships determined by the CAMEL technique for tirandalydigin in CD_3OD.[11]

Proton No.	To proton No.
2	15
3	5
5	3
6	7, 15, 16, 17
7	6, 16, 17
8	9, 17
9	8, 10, 17
10	9, 11, 17
11	10, 18b
14	18a
15	2
16	6, 7
17	6, 7, 8, 9, 10
18a	14, 18b
18b	11, 18a

Fig. 6. The relative stereochemical configuration of tirandalydigin as determined by ^1H NMR NOE relationships.



DEPT ^{13}C NMR was that at 51.4 ppm (C-18). CSCM experiments showed that this carbon is attached to two protons resonating as a pair of doublets at δ 2.84 (J=5.2 Hz, 18-H_b) and δ 2.98 (J=5.2 Hz, 18-H_a). A quaternary carbon signal was seen at 56.1 ppm (C-12). These signals are assigned to an α,α di-substituted epoxide ring. In the ^1H NMR, two olefinic protons appear at δ 5.62 (J=10.2 Hz, 11-H) and δ 6.40 (J=5.0 and 10.2 Hz, 10-H) as a doublet and doublet of doublets, respectively. The

FUCOUP experiment showed a coupling of the quaternary epoxide carbon (C-12) to the olefinic proton 10-H and in addition, delay COSY experiments showed long range couplings between the olefinic and epoxide protons (10-H→18-H_a, 11-H→18-H_b). A quaternary carbon signal at 100.1 ppm (C-13) was shown by the FUCOUP experiment to be coupled to the protons of a methyl singlet at δ 1.15 (14-H_3). A series of long range couplings of these same methyl protons was seen by delay COSY to the 11-H, 18-H_b and 9-H protons. These data establish the most distinctive portion of the streptolydigin dioxabicyclic ring system with its unique double bond and spiro-epoxide. The remainder of this system is quite clearly delineated by the combination of the FUCOUP experiment with an overlay of long and short range COSY experiments as shown in Figs. 3 and 4. Methyl doublets appear at δ 0.73 (J=7.1 Hz, 17-H_3) and δ 1.04 (J=6.8 Hz, 16-H_3) each coupled to separate single proton multiplets at δ 1.91 (8-H) and δ 2.80 (6-H), respectively. A triplet at δ 4.33 (J=5.0 Hz, 9-H) and a doublet of doublets at δ 3.70 (J=10.6 and 2.1 Hz, 7-H) represent the protons of the oxygen bearing carbons. The FUCOUP experiment showed a coupling of the C-17 methyl protons with carbons 7, 8 and 9 and also a coupling of carbon 6 with the protons of the methyl it bears (16-H_3). A comparison of carbon and proton data for C-5 through C-18 with that published for streptolydigin (**2**) suggest these portions of the two compounds to be identical.[8,9]

Stereochemical Considerations

The complete stereochemical assignment of tirandamycin and streptolydigin has been reported by DUCHAMP *et al.*[10] In a measurement of two-dimensional ^{1}H nuclear Overhauser effects (NOE's) in a rotating frame (a CAMEL experiment), spacial relationships were established for protons on carbons 2~18 for tirandalydigin (Table 2).[11] As illustrated in Fig. 6 these data indicate a molecule with the same relative stereochemistry as that reported for streptolydigin. A comparison with a sample of streptolydigin from these laboratories demonstrated an identical set of NOE's. In Fig. 6, tirandalydigin is depicted with the same absolute configuration as established for tirandamycin and streptolydigin.[10] Although a postulated common biosynthesis would suggest this to be the case, we have no evidence to exclude the alternate absolute configuration.

Experimental

Spectroscopy

Using TMS as an internal standard, NMR spectra were measured on samples with a General Electric GN300, GN500 or Bruker AM500 MHz spectrometer. ^{1}H NMR COSY data were acquired at 300.1 MHz in CD_3OD. ^{13}C NMR and DEPT ^{13}C NMR data were measured at 75.5 MHz in CD_3OD. CSCM data were acquired on a sample in CD_3OD at spectrometer frequency settings of 75.5 MHz and 300.1 MHz. Long range ^{1}H-^{13}C correlations were determined FUCOUP experiment employing spectrometer frequencies of 125.8 MHz and 500.1 MHz. The sample was in DMSO-d_6. The CAMEL experiment was done in CD_3OD at a spectrometer frequency setting of 500.12 MHz. UV spectra were measured on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, and IR spectra on a Perkin-Elmer 683 dual beam dispersive instrument. Optical rotation was measured in a 10-cm tube on a Perkin-Elmer model 241 polarimeter. Mass spectra were measured on a Kratos MS-50 spectrometer in the FAB mode.

HPLC Analysis

Concentrated CH_2Cl_2 extract of initial fermentation beer was chromatographed against standards of tirandamycin A, tirandamycin B and streptolydigin. A 15-cm 7 μm Adsorbosphere HS column was used with a mobile phase consisting of CH_3CN - 0.1% H_3PO_4 (1 : 1). The flow rate was 1 ml/

minute. An LKB 2140 Rapid Spectral Detector in the Isogram mode was used for detection (continuous 190~370 nm UV monitoring of component peaks, 1s.-int./2s.). Co-injection of sample and standards resulted in the following retention times; tirandamycin B 5.1 minutes, tirandamycin A 13.1 minutes, unknown (tirandalydigin) 13.6 minutes and streptolydigin 17.5 minutes. The UV scan of the unknown peak clearly indicated a tetramic acid like spectrum with a different retention time than the standards used.

Isolation

The fermentation broth of *Streptomyces* sp. AB-1006A-9 was adjusted to pH 3.0 using 6 N HCl. Eighty liters of whole beer were extracted with two 40-liter portions of CH_2Cl_2. The CH_2Cl_2 extracts were combined, concentrated to 4 liters, and backwashed with a half portion of 0.05 M sodium bicarbonate solution (pH 6.0). The CH_2Cl_2 was dried over sodium sulfate and concentrated to an oil. The oil was taken up in MeOH (1,000 ml) and extracted with three equal portions of hexane (saturated with MeOH). The activity remained in the MeOH layer. The active MeOH layer was concentrated and charged onto a Sephadex LH-20 column (5.5×100 cm) in a solvent system consisting of $CHCl_3$ - MeOH - *n*-heptane (1 : 1 : 1).

Fractions with substantial activity against *B. fragilis* were combined and concentrated. The active concentrate was chromatographed on an Ito multi-layer CPC in three portions. The following conditions were used: Solvent system MeOH - H_2O - $CHCl_3$ - CCl_4 (lower phase stationary 4 : 2 : 2 : 3), No. 14 Ito Coil with tail as inlet, flow rate, 4 ml/minute, 800 rpm, 85~90% stationary retention, 10 ml fractions. The bulk of the activity remained in the stationary phase and was rechromatographed on the CPC under the following conditions: Solvent system MeOH - H_2O - $CHCl_3$ - CCl_4 (lower phase stationary 5 : 2 : 2 : 3), tail inlet, 4 ml/minute, 800 rpm, 85~90% stationary retention, 12~14 ml fractions. The activity divided up into two areas, a large portions being found in fractions 80~160 and another remaining in the stationary phase. The ^1H NMR's of the activities were identical except that the activity in the stationary was associated with a paramagnetic metal ion. The activity from fractions 80~160 was chromatographed on the CPC under the following conditions: Solvent system hexane - EtOAc - MeOH - H_2O (2 : 3 : 4 : 1), tail inlet, 4 ml/minute, 800 rpm, 85~90% stationary retention, 15 ml fractions. The majority of the activity was concentrated from fractions 68~83. In a similar manner the stationary activity was chromatographed by CPC employing the system: Hexane - EtOAc - MeOH - H_2O (2 : 3 : 3 : 2). The subsequent majority of activity was found in fractions 61~86. The activity from fractions 61~86 was chromatographed on Merck LiCroprep RP-8 (1×25 cm Lobar) in a solvent system consisting of CH_3CN - MeOH - H_2O (8 : 1 : 8) under 0.35 kg/cm^2 pressure. The active fractions were combined and the concentrate was compared by ^1H NMR to that in fractions 68~83 after conversion of both to the sodium salt. The activities were shown to be identical samples of tirandalydigin (135 mg total).

Conversion to the Sodium Salt

Samples of tirandalydigin and tirandamycin B were converted to the sodium salt form. The samples were taken up in a small amount of MeOH and water was added to create a suspension. The MeOH was removed under vacuum and Bio Rex 70 (Na^+) was added to the suspension until the pH reached 10.5 (the suspension dissolved). The solution was filtered and the supernate was freeze dried.

Acknowledgments

The authors wish to express their gratitude to Dr. ALEX BUKO of Abbott Laboratories Mass Spectroscopy group, for providing valuable data and Lyle Coen for HPLC analysis.

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