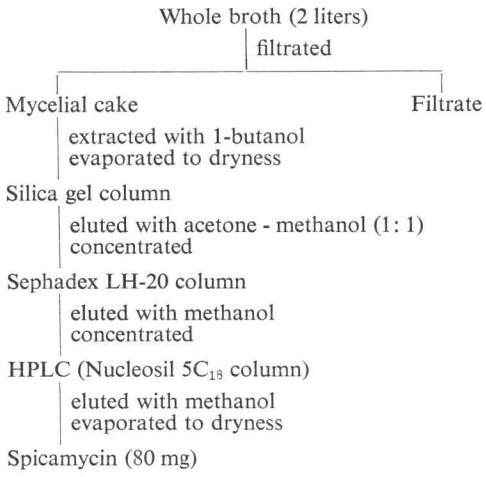
STUDIES ON THE DIFFERENTIATION INDUCERS OF MYELOID LEUKEMIC CELLS III.* SPICAMYCIN, A NEW INDUCER OF DIFFERENTIATION OF HL-60 HUMAN PROMYELOCYTIC LEUKEMIA CELLS

Sir:

In the course of our screening program for inducers of differentiation of myeloid leukemic cells,[1,2] we have recently isolated an active substance from the culture broth of a streptomycete. This antibiotic "spicamycin" is a potent inducer of differentiation of human promyelocytic leukemia cells (HL-60).[3]

The spicamycin producing organism, identified as a *Streptomyces alanosinicus* was cultivated on a rotary shaker at 37°C for 4 days in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of 2.5% glucose, 1.5% soybean meal, 0.2% dry yeast, and 0.4% calcium carbonate. The active material was isolated by the scheme shown in Fig. 1, and further purification was achieved by semi-preparative HPLC (Nucleosil $5C_{18}$) with methanol.

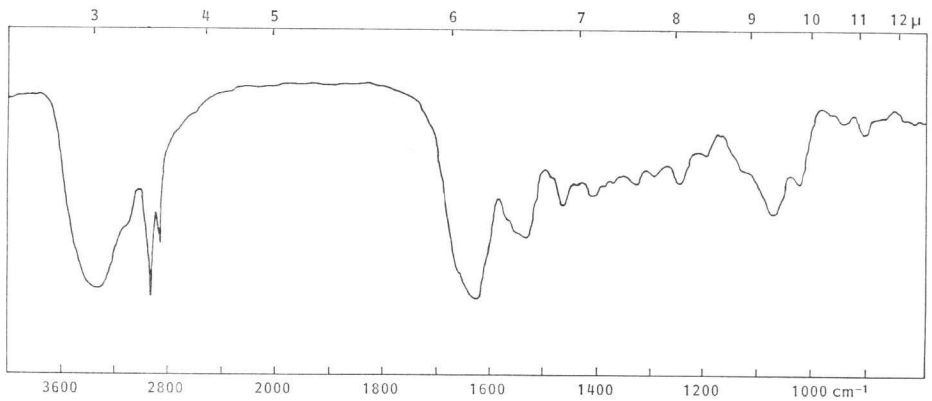
Fig. 1. Isolation and purification of spicamycin.



Spicamycin is a colorless powder soluble in methanol, 1-butanol, pyridine, and dimethylsulfoxide, but insoluble in ethyl ether, *n*-hexane, chloroform, ethyl acetate, and acetone. It is also insoluble in water or 0.1 N HCl, but is soluble in 0.1 N NaOH. The melting point of spicamycin is 215~220°C with decomposition. The UV spectra showed maxima at 264 nm ($E^{1\%}_{1cm}$ 257) in methanol, 273 nm ($E^{1\%}_{1cm}$ 258) in 0.01 N HCl - methanol, and 272 nm ($E^{1\%}_{1cm}$ 226) in 0.01 N NaOH - methanol. The IR spectrum and the ^1H NMR spectrum are shown in Fig. 2 and Fig. 3, respectively. The field desorption mass spectrometry revealed molecular ion peaks at *m/z* 644 and 658 $(M+Na)^+$, thereby suggestive that spicamycin is a mixture of closely related compounds. They are difficultly separable by chromatographic procedures. The elemental analysis were as follows: C 57.37, H 8.26, N 15.74, O 18.63.

On hydrolysis with 1 N HCl at 100°C for 1 hour, spicamycin was degraded into three components (Fig. 4). One was a rather insoluble acidic material, and the other two products were bases which could be separated by Toyopearl HW40-water column chromatography. One of the bases

Fig. 2. IR spectrum of spicamycin (KBr).



* For part II of this series, see reference 2.

Fig. 3. 400 MHz ^{1}H NMR spectrum of spicamycin in methanol-d_4.

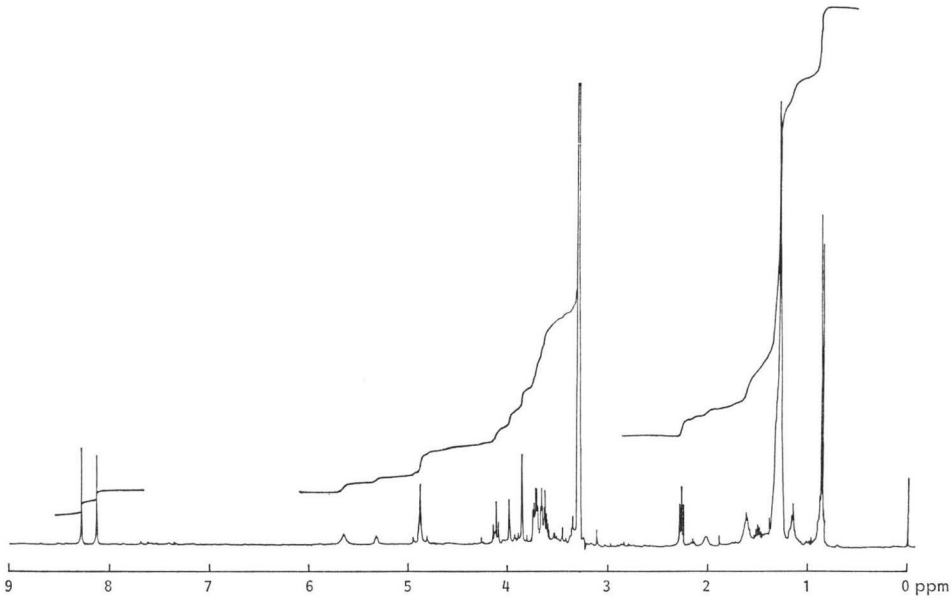
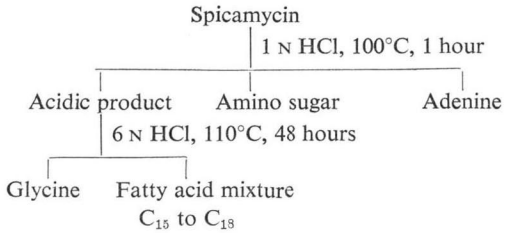


Fig. 4. Products from hydrolysis of spicamycin.



was identified as adenine by direct comparison with an authentic sample. This moiety accounts for the UV spectrum of spicamycin. The second base gave characteristic reactions common to amino sugars. It reduced Fehling's reagent and gave a positive ninhydrin reaction.

The insoluble acidic product was hydrolyzed with 6 N HCl at 110°C for 48 hours to yield an amino acid (identified as glycine) and a fatty acid fraction which was revealed to be a mixture of closely related acids by GC-MS analysis after methylation with diazomethane (Table 1). The major fatty-acid component, representing about 40% of the total, was identified as *iso*-palmitic acid. Since the other three moieties of the spicamycin molecule *i. e.* adenine, glycine, and an amino sugar were devoid of related compounds, spicamycin appears to be a family of unusual nucleoside substances with the variety of fatty acids.

Table 1. GC-MS analysis of the methyl esters of fatty acids from the hydrolysate of spicamycin.

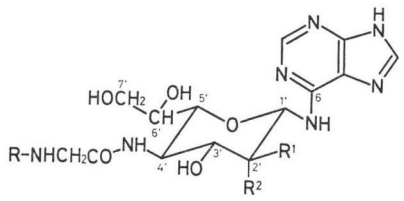
Fatty acids	R. T.*	Approximate mol%
iso-C_{15}	0.65	6
iso-C_{16}	0.90	40
n-C_{16}	1.00	11
iso-C_{17}	1.27	31
n-C_{17}	1.33	2
iso-C_{18}	1.80	8
n-C_{18}	2.04	2

* Retention time of the methyl esters of fatty acids relative to that of methyl palmitate on a 1.5% Silicone OV-1 column at 180°C.

In the ^{13}C NMR spectrum of spicamycin in pyridine-d_5, the signals are classified into the following four groups: δ 154.0 (s), 153.0 (d), 152.6 (s), 140.6 (d), and 119.6 (s) due to the adenine residue; 80.1 (d), 79.1 (d), 74.0 (d), 73.6 (d), 71.5 (d), 64.2 (t), and 51.2 (d) ascribed to the aminoheptose moiety; 174.1 (s) and 43.8 (t) originating from the glycine group; and 171.7 (s), 39.2 (t), 36.3 (t), 29.6~30.1 (t), 27.6 (d), 26.0 (t), and 22.7 (q) due to the *iso*-type fatty acid residue.

The ^{1}H NMR spectrum of spicamycin in methanol-d_4 showed two aromatic protons (δ 8.28, 8.12) due to the adenine nucleus, an anomeric proton (δ 5.67), five oxymethine or aminomethine protons (δ 4.14, 4.00, 3.76, 3.69, 3.67),

Fig. 5. Structure of spicamycin.



$R = \begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH(CH_2)_nCO-$ Spicamycin $R^1=H,\ R^2=OH$

$CH_3CH_2CH_2(CH_2)_nCO-$ Septacidin $R^1=OH,\ R^2=H$

$n=8\sim14$

and a hydroxymethyl group (δ 3.75, 3.63) ascribed to the aminoheptose moiety, an isolated methylene group (δ 3.90, 3.85) due to the glycine moiety, and 12~13 methylene groups (δ 2.28, 1.63, 1.26~1.37, 1.16), methine proton (δ 1.52), and two methyl protons (δ 0.88) assignable to the *iso*-type fatty acid moiety.

Acetylation of spicamycin with acetic anhydride in pyridine produced a tetraacetate. The ^{1}H NMR spectrum of the aminoheptose moiety of spicamycin tetraacetate displayed the following resonances: δ 6.75 (4′-NH, J=8.4), 6.10 (1′-H, broad), 5.53 (2′-H, J=3.0), 5.26 (3′-H, J=10.6, 3.5), 5.15 (6′-H, J=7.2, 3.5, 2.3), 4.43 (7′-Ha, J=12.0, 3.5), 4.31 (7′-Hb, J=12.0, 7.2), 4.28 (4′-H, J=10.6, 10.5, 8.4), and 3.95 (5′-H, J=10.5, 2.3). These signals indicate that the aminoheptose moiety is a 4-aminoheptose with axial configurations for 3′-H, 4′-H, and 5′-H, and an equatorial configuration for 2′-H. Because the broad signal at δ 6.10 sharpened on irradiation of NH at δ 4.31, it is possible that the aminoheptose moiety binds to the 6-amino group on the adenine residue. The nuclear Overhauser effects (NOE) were observed between 1′-H and 3′-H, and between 1′-H and 5′-H. This indicates that the anomeric proton has an axial configuration.

It is concluded that the structure of spicamycin is as shown in Fig. 5 with the uncertainty about the stereochemistry at 6′ position and the absolute configuration. This structure is quite similar to that of septacidin,[4,5] an antitumor and antifungal antibiotic. Spicamycin is probably the 2′-epimer of septacidin (Fig. 5).

The effect of spicamycin on the induction of phagocytic activity of HL-60 cells is summarized in Table 2. Spicamycin at 2.5~640 ng/ml induced marked differentiation of the cells.

Spicamycin showed antitumor activity (T/C 140~154%) against P388 leukemia in mice when administered (i.p.) at 0.25~2.0 mg/kg/day dosages (Table 3). The LD_{50} in mice was approximately 40 mg/kg (i.p.).

Table 2. Effect of spicamycin on the growth and induction of phagocytic activity of HL-60 cells.

Dose (ng/ml)	Phagocytic cells (%)	Number of cells (cells/ml)
0	3	1.2×10^6
0.6	16	9.5×10^5
2.5	51	5.9×10^5
10	58	3.4×10^5
40	72	1.7×10^5
160	71	6.0×10^4
640	61	2.5×10^4

HL-60 cells at 5×10^5 cells/ml were incubated with various concentrations of spicamycin for 48 hours.

Table 3. Antitumor activity of spicamycin against P388 mouse leukemia.

Dose* (mg/kg/day)	T/C (%)	Toxicity
0.125	129	0 / 6
0.25	140	0 / 6
0.5	153	0 / 6
1.0	154	0 / 6
2.0	154	3 / 6

* Treatment schedule: days 1~9 (i.p.).

Acknowledgments

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