stirred on a steam bath for 5 hr. Tlc analysis of the solution showed the presence of four major orange-yellow spots: R_f 0.23 (II), R_f 0.35 (III), R_f 0.52 (I), and R_f 0.62 (IV) in the approximate ratio 2:1:3.5:1.5, besides other colored by-products at R_f <0.1. After cooling, the solution was worked up by extracting the rifamycins twice with $CHCl_3$. The organic layer was evaporated to dryness under vacuum. The crude residue taken up in CCl_4 gave, after evaporation to a small volume, a crystalline crop (19.6 g) consisting mainly of I–III. The mother liquor showed the presence of I and IV, besides other degradation products (R_f <0.1).

III was recovered by column chromatography using a $CHCl_3$ solution of the crystalline crop on a pH 6.0 buffered (McIlvaine) silica gel column, by stepwise elution with $CHCl_3$ containing 2 and 4% MeOH. The selected fractions were collected and evaporated to dryness. The residue, crystallized from $CHCl_3$ and recrystallized from acetone, gave 0.7 g of pure (tlc) III: mp 158–160° dec; pK_a = 2.3, pK_{MCS} = 6.7.

The CCl_4 mother liquor was evaporated to dryness. The residue (12 g) was taken up in $CHCl_3$ and passed through a silica gel column using mixtures of $CHCl_3$ with 1, 2, and 3% MeOH as eluent. The first colored eluate, containing IV, was collected and evaporated to dryness. After crystallization of the residue from EtOH–H_2O, 1.7 g of pure (tlc) IV was obtained: mp 166–170° dec; pK_a = 2.5, pK_{MCS} = 6.8.

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Structure of Sangivamycin[1]

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Sangivamycin is an antitumor substance and a metabolite of a streptomycete culture. It has the molecular formula $C_{12}H_{15}N_5O_5$ and shows spectral properties similar to those of nucleosides. The presence of a pentose residue was established indirectly by periodate oxidation and hydrolysis. The compound also has an aromatic amino group and a carboxamide group. Some of the similarities in properties to toyocamycin led to the hypothesis that sangivamycin may be closely related to this compound. This hypothesis is shown to be correct in that sangivamycin has a carboxamide group and toyocamycin has a nitrile group on the same carbon skeleton. This relationship is established by three different methods. A brief study of the biological activity of some of the derivatives of sangivamycin is described.

Sangivamycin is an antitumor substance produced by an unidentified species of *Streptomyces*.[2] It shows significant activity against leukemia L1210 in mice and is strongly cytotoxic toward HeLa cells grown in cell culture. It has very slight antibacterial or antifungal activity. The compound is currently under clinical trials. Only the method of isolation from the broth and some preliminary characterizations have appeared so far.[2] Degradation reactions which led to the elucidation of its structure are presented here.

Sangivamycin is a colorless crystalline solid of low solubility in water and common organic solvents. It is weakly basic and forms salts such as hydrochloride, sulfate, and picrate. Titration of the hydrochloride shows that it is a monoacidic base with a pk_a of 3.4 and an equivalent weight of 310 (Figure 1A). The molecular formula, $C_{12}H_{17}N_5O_6$, proposed earlier appears to be that of the monohydrate of the compound. The formula $C_{12}H_{15}N_5O_5$ is now considered correct for sangivamycin as is shown by the analysis of several derivatives.

The spectral properties of the compound have been briefly described earlier. The uv spectrum has maxima at 228 mμ (ε 9250) and at 278 mμ (ε 14,500). In acid solution the major maximum is shifted to 272 mμ. The ir spectrum has prominent peaks to suggest the presence of amino and/or hydroxyl groups (2.90 and 3.05 μ) and either a conjugated carbonyl, an amide, or a C=N system (6.10 μ).

The nmr spectrum of sangivamycin run in trifluoroacetic acid shows the following characteristics: two broad based singlets at τ 1.42 and 1.53 equal to two to four protons, a doublet at τ 3.59 and 3.68 equal to one proton, and the rest, several broad peaks in the region τ 4.87–5.75. The spectrum shows some resemblance to the spectra of purine nucleosides.

In harmony with this observed similarity, sangivamycin forms a tetraacetyl derivative. Its nmr spectrum indicates clearly that one of the acetyl groups (τ 7.57, 3 protons) is different from the other three (τ 7.93, 3 protons) which appear to be due to three O-acetyl groups.

Further support for the possible presence of a sugar is provided by the fact that sangivamycin reacts with 1 mole of periodate. No formaldehyde or formic acid is formed and the main product retains the original carbon skeleton. In spite of the foregoing suggestive evidence for the nucleosidic nature of the compound, direct acid hydrolysis of sangivamycin failed to yield a sugar component even under relatively drastic conditions. However, the periodate-reaction product underwent smooth hydrolysis to yield a crystalline aglycone of composition $C_7H_6N_4O_2$ together with NH_4Cl. The loss of a $C_5H_8O_4$ residue thus established the existence of a pentose residue in sangivamycin.

Sangivamycin is readily deaminated when treated with sodium nitrite in acetic acid. The oxydesamino compound thus formed is a much weaker base than sangivamycin. Its uv maximum at 268 mμ is unchanged when acidified. These properties indicate that the amino group is of aromatic type and that it is part of the chromophore. The broad signal in the nmr spectrum at τ 1.42 (or 1.53) is in agreement with this. Also, the acetyl signal (τ 7.57, 3 protons) in tetraacetyl-

(1) Research supported by Contract No. PH43-64-50, with the National Institutes of Health, Public Health Service, Department of Health, Education and Welfare. This paper was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965. Sangivamycin is also known as BA-90912.

(2) K. V. Rao and D. W. Renn, *Antimicrobial Agents Chemotherapy*, 77 (1963).

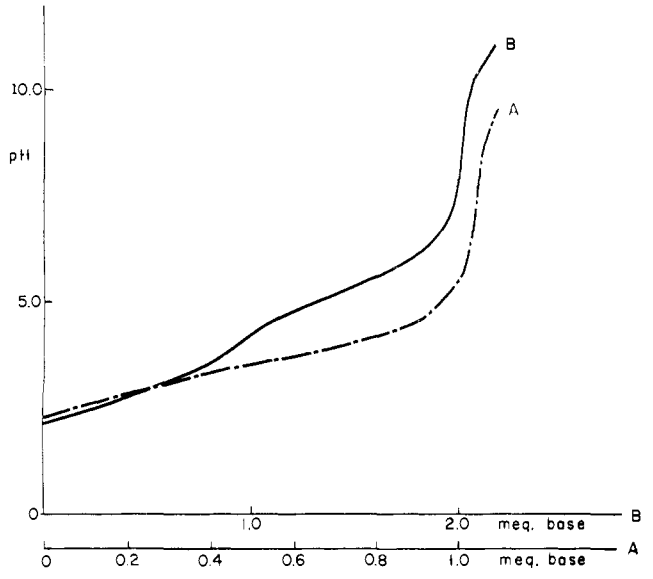


Figure 1.—Titration curves of sangivamycin hydrochloride (A) and desamidosangivamycin hydrochloride (B).

sangivamycin can be assigned to the aromatic acetamido group.

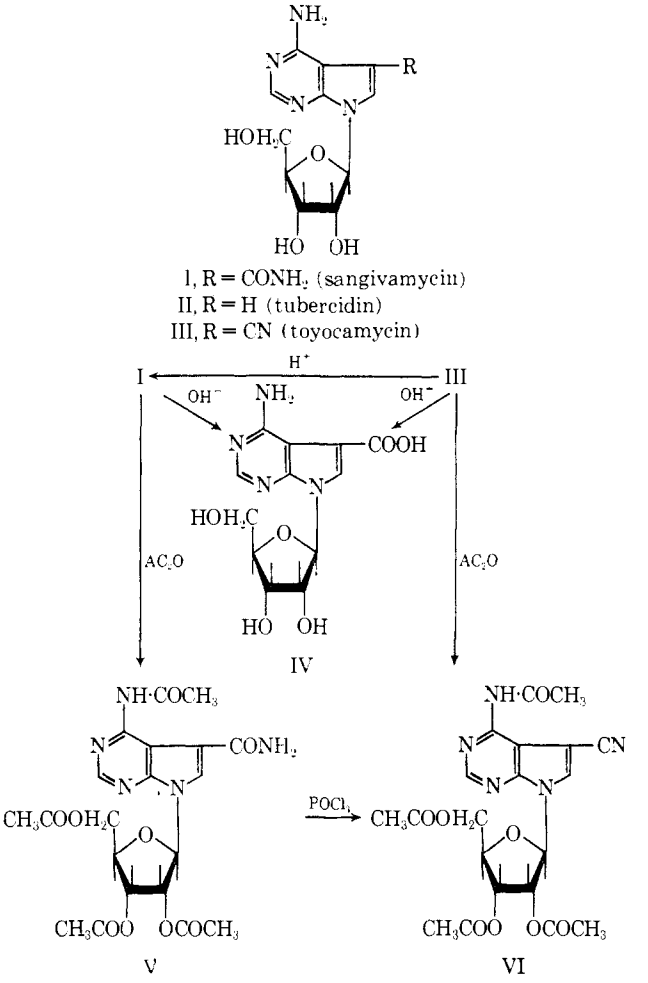
Sangivamycin is rather stable to acid. It resists boiling in 3 *N* HCl for 1 hr but, on prolonged heating, forms NH_4Cl and a crystalline solid, $C_{12}H_{14}N_4O_6$. The same compound, desamidosangivamycin is also obtained by alkaline hydrolysis. Its uv properties are very similar to those of sangivamycin. Titration of its hydrochloride shows two regions of equilibrium (pK_a = 2.9 and 5.6) (Figure 1B). This as well as the ir spectral peak at 5.80 μ suggest that desamidosangivamycin has a carboxyl group. It can be esterified to a monomethyl ester, $C_{13}H_{16}N_4O_6$, which shows a sharp peak at 5.85 μ. It also has a methoxyl peak at τ 6.00.

From the foregoing discussion it is evident that sangivamycin has a pentose residue, an aromatic amino group, and a carboxamide group. It has two protons (τ 1.42 and 1.53) as part of the heterocyclic system. Of the many possibilities, a pyrrolo[2,3-*d*]pyrimidine skeleton present in other actinomycete metabolites such as tubercidin[3] and toyocamycin[4] appeared probable. A comparison of the uv spectra of sangivamycin and toyocamycin showed that they are very similar including the spectral shift in acid medium. Their respective elementary compositions suggested that sangivamycin and toyocamycin may have the same carbon skeleton with the former having a carboxamide while the latter has a nitrile group.

That the above hypothesis is correct is shown in several ways. First, alkaline hydrolysis of toyocamycin yields an acid which is identical with desamidosangivamycin. Next, the tetraacetyl derivative of sangivamycin can be dehydrated by means of $POCl_3$ to the tetraacetyl derivative of toyocamycin. The acetates were compared in the form of their crystalline picrate salts. In the ir both compounds show the characteristic sharp peak at 2230 cm^{-1} (CN). Finally, by selective acid hydrolysis of toyocamycin in 2 *N* HCl at 100°, sangivamycin itself is isolated from the products as its hydrochloride. These reactions are shown in Chart I. Sangivamycin can thus be assigned structure I.

CHART I

TRANSFORMATIONS OF SANGIVAMYCIN AND TOYOCAMYCIN



In view of the structural similarity between tubercidin (II), toyocamycin (III), and sangivamycin (I), it is of interest to examine their biological activities. Although all three exhibit a high degree of cytotoxicity toward HeLa cells *in vitro*, only sangivamycin shows significant activity against leukemia L1210 in mice. Because of this, a brief study was made of the available derivatives against L1210 in mice. The structural changes cover three areas: the amide group, the amino group, and the pentose residue.

Hydrolysis of sangivamycin to desamidosangivamycin (IV) leads to loss of activity. Formation of a methyl ester restores activity slightly. Restoration of activity is much higher in compounds with nitrogen substitution. For instance, the *N*-methylamide, the hydroxamic acid, and the hydrazide of desamidosangivamycin all show significant activity. Among these, the hydrazide appears to be the most active.

Deamination of sangivamycin to the oxydesamino derivative leads to loss of activity. Also, acetylation of the amino group as well as of the hydroxyl groups of the pentose brings about inactivation. Likewise, the periodate-oxidation product is also inactive. Table I shows the approximate dose ranges and the corresponding increase in survival times obtained with

(3) Y. Mizuno, M. Ikehara, K. A. Watanabe, S. Suzuki, and T. Itoh, *J. Org. Chem.*, **28**, 3329 (1963).

(4) K. Okhuma, *J. Antibiot.* (Tokyo), **A14**, 343 (1961).

TABLE I

ANTILEUKEMIC ACTIVITY OF SANGIVAMYCIN AND ITS DERIVATIVES

Compound	Dose, mg/kg	Extension of survival time, %
Sangivamycin	0.6	150
	0.4	160
	0.27	133
Desamido	20	98
Methyl ester of desamido	3	132
	2	132
	1.33	139
	0.88	138
N-Methyl	2.4	149
	1.8	145
	1.2	144
	1.0	128
Hydroxamic acid of desamido	6	144
	4	127
	2.7	119
Hydrazide of desamido	2.5	134
	2.0	162
	1.5	149
	1.0	141
Oxydesamino	20	102
Tetraacetyl	20	100
Periodate-oxidation product	20	98

some of these derivatives in leukemia L1210 carried out according to the protocols of the Cancer Chemotherapy National Service Center. A compound is considered active if the survival time is 125% or higher of that of untreated animals.

Experimental Section

Melting points are corrected and were determined using a Fisher-Johns apparatus.

Sangivamycin was recovered from the broth by adsorption on Darco G-60 (1.5%) followed by several elutions with 0.05 *N* MeOH–HCl. The combined eluates were concentrated without neutralization and set aside in the refrigerator. The crude crystalline solid of sangivamycin hydrochloride which separated out was filtered and recrystallized from hot H_2O. It separated as colorless long needles, mp 250–252°. *Anal.* Calcd for $C_{12}H_{15}N_5O_5 \cdot HCl \cdot {}^1/_2H_2O$: C, 39.75; H, 5.00; N, 19.30; Cl, 9.78. Found: C, 40.14; H, 5.30; N, 19.26; Cl, 9.94.

Tetraacetylsangivamycin.—A mixture of sangivamycin (1 g), Ac_2O (10 ml), and pyridine (2 ml) was heated at 100° for 2 hr. The cooled solution was diluted (H_2O) and extracted ($CHCl_3$) after 30 min. The solvent extract was washed ($NaHCO_3$–H_2O) and concentrated to dryness. The acetate crystallized from EtOH as colorless prisms, mp 153–155°. *Anal.* Calcd for $C_{20}H_{23}N_5O_9 \cdot 0.5H_2O$: C, 49.39; H, 4.93; N, 14.41; acetyl, 35.40. Found: C, 49.06; H, 5.21; N, 14.39; acetyl, 34.58.

Oxydesaminosangivamycin.—A solution of sangivamycin (0.5 g) in AcOH (50 ml) was cooled to 5° and treated with $NaNO_2$ (1 g in 10 ml). After 30 min at 5°, the blue solution was heated at 80° for 30 min and then concentrated to dryness. The residue was crystallized from MeOH–H_2O. The product crystallized as colorless needles, mp 290–292°. *Anal.* Calcd for $C_{12}H_{14}N_4O_6$: C, 46.45; H, 4.55; N, 18.06. Found: C, 46.25; H, 4.68; N, 18.07.

Desamidosangivamycin.—Sangivamycin (2 g) was boiled under reflux with 2 *N* NaOH (200 ml) for 3 hr. The cooled solution was diluted (H_2O) and passed through a column of Amberlite IR-C50 in H^+ form. The effluent and wash were concentrated to dryness and the residue was crystallized as a hydrochloride from MeOH. The hydrochloride crystallized as colorless long rectangular plates, mp 236–238°. *Anal.* Calcd for $C_{12}H_{14}N_4O_6 \cdot HCl$: C, 41.50; H, 4.34; N, 16.12; Cl, 10.15. Found: C, 41.00; H, 4.52; N, 15.89; Cl, 10.18.

Desamidosangivamycin Methyl Ester.—Desamidosangivamycin (1 g) was boiled under reflux with 10% H_2SO_4 in MeOH (100 ml) for about 24 hr. The cooled solution was diluted with water (100 ml) and passed through a column of Dowex-1 in acetate form. The effluent and wash were concentrated to dryness and the solid crystallized from MeOH. The ester separated as colorless prisms, mp 216–218°. *Anal.* Calcd for $C_{13}H_{16}N_4O_6$: C, 48.15; H, 4.97; N, 17.28; OCH_3 (1), 9.60. Found: C, 48.10; H, 5.07; N, 17.11; OCH_3, 9.69.

Periodate Oxidation of Sangivamycin and Hydrolysis.—Sangivamycin (1 g) was dissolved in 1 *N* HCl (30 ml) and treated with 0.2 *M* aqueous H_5IO_6 (30 ml). After 20 hr the crystalline solid was separated, dissolved in H_2O (20 ml), and treated with a slight excess of $NaHCO_3$. The crystalline base was separated and recrystallized from H_2O. The product was a colorless crystalline solid, mp 170° dec. *Anal.* Calcd for $C_{12}H_{13}N_5O_5 \cdot 3H_2O$: C, 39.89; H, 5.30; N, 19.36. Found: C, 39.79; H, 5.15; N, 19.39.

A portion of the oxidation product (0.5 g) was boiled under reflux with 6 *N* HCl for 6 hr. The dark colored mixture was concentrated to dryness and the residue was crystallized twice from MeOH. The product appeared as colorless needles, mp 285–289°. *Anal.* Calcd for $C_7H_6N_3O_2 \cdot HCl$: C, 39.17; H, 3.58; N, 26.00. Found: C, 38.80; H, 3.90; N, 25.54.

Conversion of Tetraacetylsangivamycin to Tetraacetyltoyocamycin.—A solution of tetraacetylsangivamycin (0.2 g) in $CHCl_3$ (25 ml) was boiled under reflux with $POCl_3$ (0.3 ml) for 3 hr. The mixture was concentrated to a small volume and diluted with H_2O. After 30 min it was neutralized and extracted ($CHCl_3$). The extract was concentrated and the residue was converted to a picrate, which crystallized from Me_2CO–MeOH as yellow long needles, mp 160–162°. Mixture melting point with the picrate of tetraacetyltoyocamycin was undepressed.

Conversion of Toyocamycin into Sangivamycin.—A solution of toyocamycin (0.2 g) in 2 *N* HCl (5 ml) was heated at 100° for about 4 hr. It was cooled in an ice bath and the solid which separated out was filtered and crystallized from MeOH. It separated as colorless long needles, mp 250–252°, alone or in admixture with sangivamycin hydrochloride.

N-Methylsangivamycin.—To a suspension of desamidosangivamycin methyl ester (0.5 g) in MeOH (15 ml) was added 40% aqueous $MeNH_2$ (10 ml). The mixture was warmed to obtain a clear solution and let stand at room temperature for 24 hr. The solution was concentrated to dryness and the solid crystallized as its hydrochloride from MeOH. The compound is a colorless crystalline solid, mp 242–246°. *Anal.* Calcd for $C_{13}H_{17}N_5O_5 \cdot HCl$: C, 42.80; H, 5.48; N, 19.20; Cl, 9.70. Found: C, 42.76; H, 5.24; N, 19.11; Cl, 9.29.

Hydroxamic Acid of Desamidosangivamycin.—A mixture of sangivamycin (1 g), $HONH_3{}^+Cl^-$ (1 g), and pyridine (10 ml) was boiled under reflux for 6 hr. The cooled mixture was concentrated to dryness. It was then dissolved (H_2O) and passed through a column of Dowex-1 in acetate form (50 ml of the resin). After washing (H_2O), elution was carried out with 2% AcOH. The eluate was concentrated to dryness and the solid crystallized from MeOH as its hydrochloride. The compound is a colorless crystalline solid, mp 216–218°. It gives a blue color with $FeCl_3$. *Anal.* Calcd for $C_{12}H_{15}N_5O_6 \cdot HCl$: C, 40.00; H, 4.44; Cl, 9.78. Found: C, 40.19; H, 4.59; Cl, 9.29.

Desamidosangivamycin Hydrazide.—A mixture of desamidosangivamycin methyl ester (0.5 g), MeOH (5 ml), and hydrazine (2 ml) was warmed until a clear solution was obtained. After 24 hr at room temperature, ether (25 ml) was added and the solid was filtered off. It was recrystallized from MeOH–H_2O. The compound is a colorless crystalline solid, mp 238–240°. *Anal.* Calcd for $C_{12}H_{16}N_6O_5$: C, 44.44; H, 4.97; N, 25.92. Found: C, 44.29; H, 5.22; N, 25.56.

Alternatively, a mixture of sangivamycin (1 g), *n*-BuOH (25 ml), and hydrazine (4 ml) was boiled under reflux for 20 hr. The solution was cooled and diluted (Et_2O), and the solid was filtered. It was crystallized from MeOH–H_2O. Paper chromatography and the ir spectrum of the sample are identical with those of the sample described above.