92. The Structure of the Antibiotic Hedamycin
I. Chemical, Physical and Spectral Properties[1])

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Summary

Interpretation of the chemical and spectral (IR., UV., ^{1}H- and ^{13}C-NMR.) properties of the antitumor antibiotic hedamycin ($C_{41}H_{50}N_2O_{11}$) suggests that the molecule contains a methyl substituted 1-hydroxyanthraquinone nucleus, an α,β-unsaturated ketone, two sugar-like tetrahydropyran rings (**4** and **8**) and an aliphatic chain **2**, presumably with an epoxy group (see the *Scheme*).

Introduction. – Hedamycin is a yellow, crystalline metabolite, which can be isolated from cultures of *Streptomyces griseoruber* [1]. It is a potent antibacterial agent and has antitumor activity [2]. Thus, the growth of a large number of bacteria is strongly inhibited, whereas the effect with yeasts and protozoa is not so pronounced. The compound proved to be highly cytotoxic (HeLa cells) and was able to induce bacteriophage λ production in a lysogenic strain of *E. coli*. Its effectiveness on transplanted tumors of rats was shown to be 6 to 20 times that of the related antibiotics iyomycin B_1 and rubiflavin. Furthermore, hedamycin inhibits the synthesis of bacterial RNA and DNA [3] [4]. Detailed studies have revealed that hedamycin – like rubiflavin – binds strongly to DNA, stabilizing the double helix. From these observations a mechanism of action for the *in vivo* effect against bacteria was suggested [3].

Schmitz et al., who isolated hedamycin [1], carried out a preliminary investigation of the physical and spectral properties, which mainly served to prove that hedamycin was a new antibiotic, different from related compounds that were known at that time (pluramycin A, iyomycin B_1, rubiflavin). A more recent study on the classification of antibiotics according to their chemical structures [5] grouped hedamycin together with the griseophagins, indomycins, iyomycins, kidamycin, neopluramycin, pluramycins, rubiflavin and tumimycin.

[1]) Presented in part at the fall meeting on the Swiss Chemical Society in Aarau, October 4, 1975.

General characterization of hedamycin. – Hedamycin is *soluble* in chloroform, pyridine, ethyl acetate, benzene and slightly soluble or *insoluble* in water, ethanol, acetone, ether and petroleum ether. It readily dissolves in dilute aqueous acids. For TLC. analysis only one solvent system so far proved to be useful: chloroform/triethylamine 4:1 (Rf = 0.65).

Crystallization of hedamycin is possible from chloroform/ether (yellow silky needles), aqueous pyridine (rosettes), methanol (needles), chloroform/acetone (needles, acetone is strongly retained by the crystals as revealed by NMR. spectroscopy), chloroform/2-propanol and other solvents. Large crystals, however, could never be obtained. The m. p. is 243–245° (dec.) [1]. When the sample begins to melt, rapid decomposition takes place, apparently with the evolution of some gaseous products, to give a brown gum.

Elemental analyses gave correct results for $C_{41}H_{50}N_2O_{11}$. *C*-Methyl and *N*-methyl determinations carried out at *Bernhardt*'s gave 5.7 and 2.6 respectively. More recent

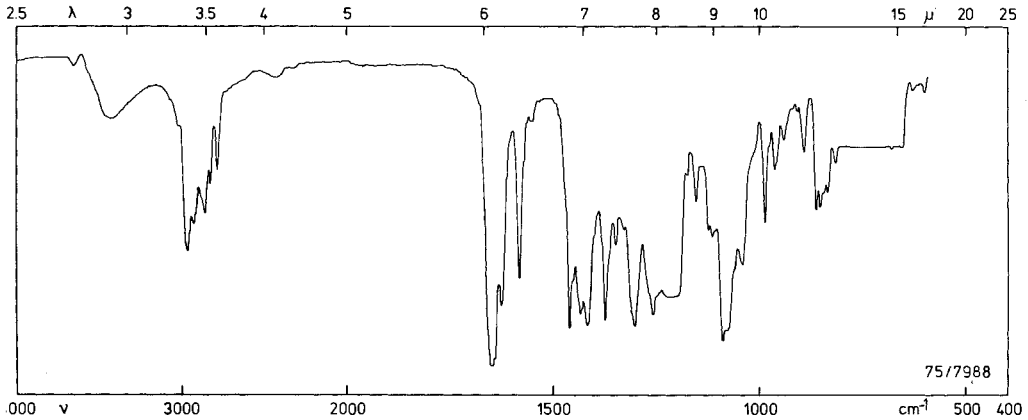

Fig. 1. *IR. spectrum of hedamycin in $CHCl_3$*

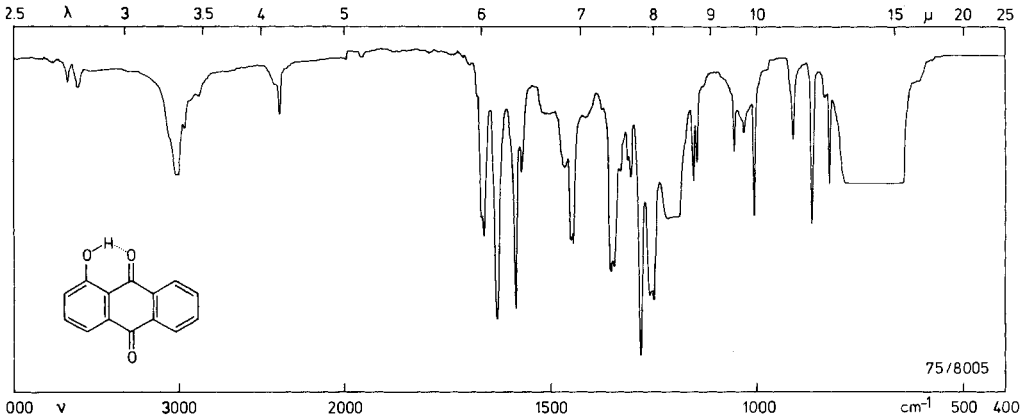

Fig. 2. *IR. spectrum of 1-hydroxyanthraquinone in $CHCl_3$*

values obtained with the same analytically pure sample but determined at *Midwest Microlabs* are 7.5/7.6 for *C*-methyl, 0.99/0.89 for *O*-methyl, 2.8/2.8 for *N*-methyl and 3.7/4.6 for active hydrogen atoms.

UV. spectra of hedamycin were extensively measured by *Schmitz* [1] and in our laboratories. The spectra resemble those of a 1-hydroxyanthraquinone derivative, the alizarine 2-methyl ether. Hedamycin has a strong absoprtion at 245 nm, a shoulder at 260 to 265 nm and a weak band around 430 nm [1]. In dilute acid the spectrum undergoes only minor changes, whereas in 0.01 N NaOH the absorptions are at 255, 332 and 535 nm. Alkaline solutions of hedamycin thus are purple.

Hedamycin is able to form *colored complexes* with many metal ions. The color produced is reddish brown with Fe^{3+}, purple with Mg^{2+}, violet with Ba^{2+} and Co^{2+}, a dull red with Cu^{2+} and a brownish violet to blue with Ni^{2+}.

The main features of the *IR.-spectrum* (in chloroform) are (*Fig. 1*): a broad band at about 3500 cm^{-1} indicating hydroxyl groups; five strong absorptions between 2970 and 2790 cm^{-1} corresponding to aliphatic C–H vibrations, including $N–CH_3$ groups (eventually $O–CH_3$); a strong, relatively broad band at 1650 cm^{-1} and a sharp band at 1625 cm^{-1}, both corresponding to carbonyl groups. Comparison with the IR. spectrum of 1-hydroxyanthraquinone (*Fig. 2*) where the carbonyl band at 1665 cm^{-1} is weaker than the one at 1630 cm^{-1}, suggests that hedamycin might contain an additional carbonyl group with an absorption around 1650 cm^{-1}.

A *mass spectrum* of hedamycin was reported by *Schmitz* [1] with M^+ at *m/e* 748, corresponding to the formula $C_{41}H_{52}N_2O_{11}$ (which was also consistent with the elemental analyses). We measured both, the parent compound (M^+ at *m/e* 746) and its tris(trimethylsilyl) derivative (M^+ at m/e 962). These values support the formula $C_{41}H_{50}N_2O_{11}$. According to this, the molecule contains 18 equivalents of unsaturation (= rings or double bonds).

Due to the thermal lability of the compound, it has not been possible to determine the exact mass of the molecular ion, which would have given a confirmation of the molecular formula. Neither peak matching nor the photoplate method[2]) were successful. As regular mass spectrometry is very difficult with hedamycin and leads to irreproducible results, a solution to this problem might come from the use of field desorption mass spectrometry [6].

^{1}H-NMR. spectra have been recorded in $CDCl_3$ (*cf.* also [1]), C_5D_5N and CF_3COOD. The spectrum in pyridine (*Fig. 3*) is much better resolved than the one in chloroform (*Fig. 4*), where many signals overlap. The main features of the spectra are as follows[3]):

At about 14 ppm a broad signal can be seen corresponding to one proton which readily exchanges with D_2O. This corresponds most certainly to the hydroxyl proton of the 1-hydroxyanthraquinone, as this latter compound shows a similar signal at 12.6 ppm. Three one-proton singlets can be discerned in the region of the aromatic hydrogen atoms; the two at 8.81 (8.33) and 8.02 (8.00) ppm are slightly broad, the one at 6.60 (6.46) is very sharp. Signals corresponding to six *C*-methyl groups can be

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[2]) This was kindly done in Dr. *H. Lichti*'s laboratory, *Sandoz AG*, Basel.

[3]) Chemical shifts in δ-values determined at 270 MHz in C_5D_5N; values for $CDCl_3$ (from 90 MHz FT measurements) given in parentheses.

found, three of them as singlets at 2.93 (2.99), 1.97 (1.96) and 0.81 (0.71) ppm, three as doublets at 1.68 (1.51, $J=6$ Hz), 1.54 (1.43, $J=6$ Hz) and 1.14 (1.44 $J=5$ Hz) ppm. The methyl at about 3 ppm could be an *O*-methyl at relatively high field (normal

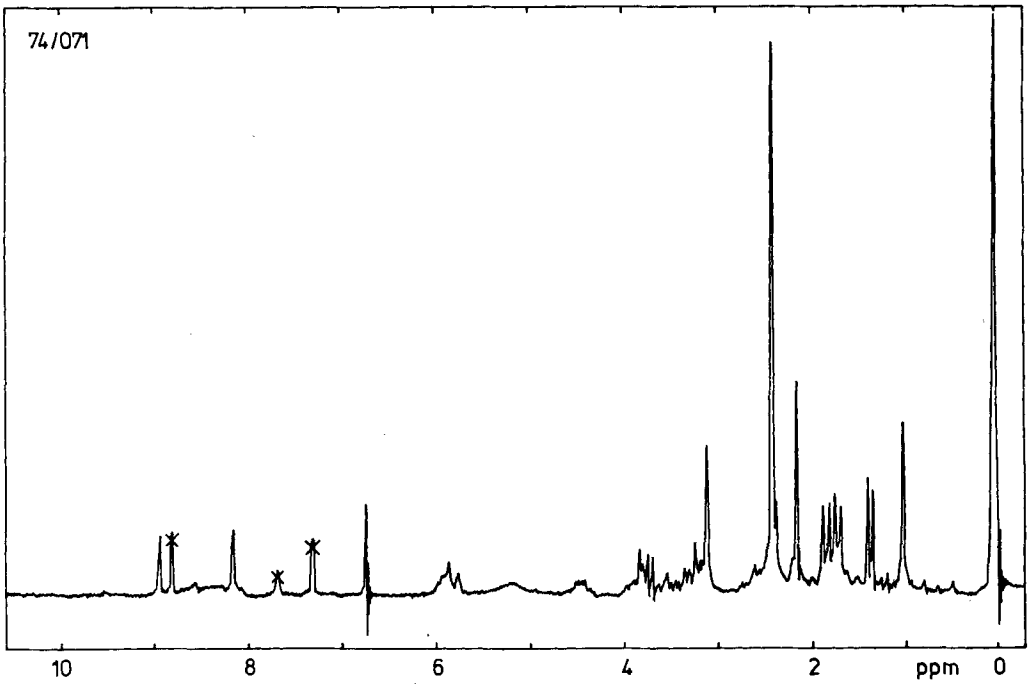


Fig. 3. *100-MHz-^{1}H-NMR. spectrum of hedamycin in C_5D_5N*

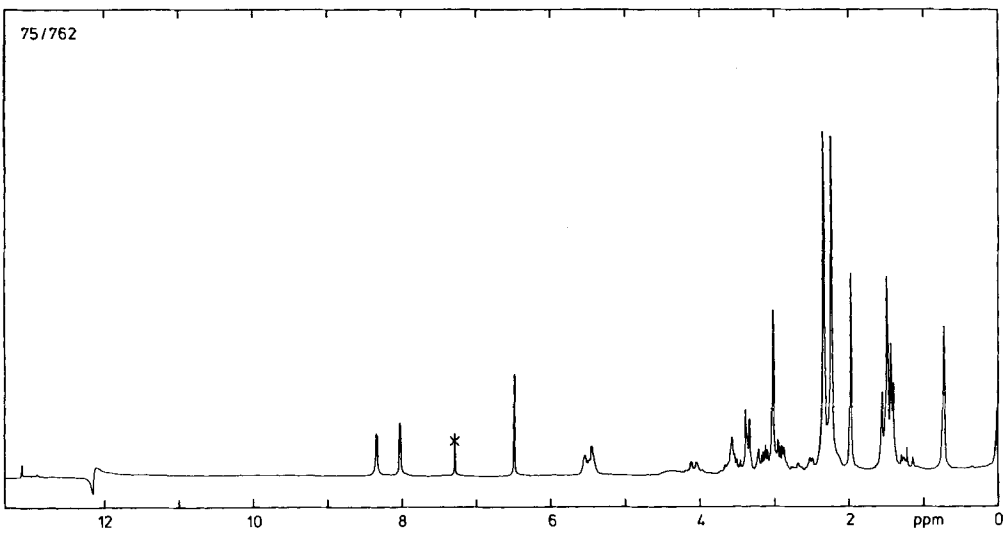


Fig. 4. *90-MHz-^{1}H-FT-NMR. spectrum of hedamycin in $CDCl_3$*

range 3.3–3.8 ppm) or an aromatic methyl group with resonance at very low field (normally around 2.3 ppm). The signal at 2 ppm, although well in the range of *O*-acetyl resonances, cannot belong to such a group, since the molecule does not contain any ester linkages as can be seen in the IR. The spectrum further contains two strong singlets at 2.27 and 2.26 (2.32 and 2.22) ppm, each corresponding to two *N*-methyl groups as deduced from their δ-values and the ~0.8 ppm downfield shift experienced when the spectrum is recorded in CF_3COOD. Since hedamycin is not stable in trifluoroacetic acid, this experiment was repeated using $CDCl_3$ as solvent containing a trace of CF_3COOD. Again the two singlets were shifted downfield (to 2.84 ppm). The rest of the spectrum underwent only minor changes with the exception of the methyl signal at 0.7 ppm, which was shifted to 1.23 ppm. This suggests that this methyl group must be very close to one of the two dimethylamino groups. The chemical shifts of these dimethylamino groups indicate that they are bonded to aliphatic systems, as those bonded to aromatic rings have their resonances at lower field. The detection of four $N\text{–}CH_3$ groups in the NMR. spectrum is apparently in contradiction with the microanalytical *N*- and *O*-methyl determinations which suggested four such groups, three bonded to N and one bonded to O. But, assuming that partial cleavage of the dimethylamino groups can occur under the conditions of the $O\text{–}CH_3$ determination, the two results are in agreement with each other. These findings now rule out the possibility of an *O*-methyl group for the resonance at 3 ppm which therefore most probably belongs to an aromatic methyl group. In the region of 3 to 5 ppm the spectra show a broad signal which must correspond to OH groups; yet, since this part of the spectrum is already rich in resonances, it is very difficult to determine quantitatively the changes in the spectrum which occur upon addition of D_2O.

Chemical reactions. – The following *classification tests* were carried out: Heating hedamycin with $Na_2S_2O_4$ and alkali gave a red color, which is a reaction typical for anthraquinones [7]. With phosphoric acid [9] no furfurals could be generated from hedamycin, whereas sucrose and galactosamine hydrochloride give positive results. Thus, hedamycin may only contain carbohydrate derivatives which are not readily dehydrated to furfurals. A test with periodic acid [8] did not give an immediate precipitate of white silver iodate (sucrose and glycerol react instantaneously). After about 10 minutes, however, a fine precipitate could be detected, which after removal of the yellow supernatant and washing with water proved to be white. The conclusion is, that probably a slow oxidation took place; but a *cis*-1,2-diol group can definitely be ruled out.

Mild treatment with *dilute acid* did not lead to any defined degradation products but rather to unspecific decomposition (*cf.* also [1]). The conclusion is that hedamycin does not contain any simple glycosidic linkages, which, upon hydrolysis, liberate carbohydrates and aglycones.

Treatment with *alkali* leads to very rapid decomposition; again, no defined products could be isolated.

Hydrogenation resulted in the uptake of one mol H_2, but the product was rapidly reoxidized in the atmosphere to hedamycin. Other reductions were tried (Zn/HCl, Zn/HOAc, $NaBH_4$/MeOH, zinc dust distillation), all of which failed to give a clean product.

Attempts to *oxidize* hedamycin with conc. HNO_3, $KMnO_4$/acetone, O_2/NaOH or *N*-chlorosuccinimide again only gave intractable products.

Acetylation has been tried many times, but with little success. Already *Schmitz et al.* reported [1] that several products were obtained, but were not crystalline and readily decomposed so that no characterization was possible. We were able to isolate a compound which, according to its NMR. spectrum, was a diacetate. This was confirmed by mass spectrometry, which showed M^+ at *m/e* 830. The compound was thought to be crystalline, but a close examination revealed that it was amorphous. Further attempts were made using acetic acid/trifluoroacetic anhydride [9], acetyl chloride/pyridine and acetic anhydride/pyridine. In this latter case TLC. revealed the formation of mainly two rather unpolar products. After prep. TLC. and elution from the silica gel the acetylation products had completely decomposed to polar products.

Schmitz et al. also tried to *benzoylate* hedamycin [1] and obtained two products in which, apparently, the phenolic hydroxyl group was esterified; detailed characterization was, however, not given.

Reaction with p-*bromobenzoyl chloride* in pyridine yielded several products. Prep. TLC. was used to isolate the least polar of these. As was observed with the acetates, more polar products were formed during chromatography. The compound isolated after three successive chromatographies was not yet pure and could not be crystallized. A NMR. spectrum suggested that four *p*-bromobenzoyl groups had been introduced into hedamycin.

Reaction with anhydrous *formic acid* produced after neutralization a base in which the original chromophore had survived. The compound could be crystallized from methanol; upon drying, however, it became amorphous.

Methylation of hedamycin was also tried. With diazomethane in ether there was no discernible reaction. CH_3I/acetone/K_2CO_3 seemed to methylate the phenol, but the product was not pure and did not crystallize. Solutions of hedamycin were treated with methyl iodide at room temperature: amorphous precipitates were deposited slowly.

The following *heavy atom derivatives* were prepared but did not crystallize: hydrobromide, mercuri-chloride, methiodide, mercuri-iodide of the methiodide. None of the colored complexes mentioned above was suitable and the addition of $AgBF_4$ in chloroform to a solution of hedamycin resulted in an amorphous precipitate.

^{13}C-NMR. spectra. –As it was not possible to get welldefined degradation products, derivatives or crystals suitable for an x-ray analysis, further spectroscopic data were collected.

^{13}C-NMR. spectra have been recorded under various conditions, at 20 and 22.63 MHz.

Proton noise decoupled spectra. A spectrum covering the range from 0 to 400 ppm[4]) showed that hedamycin contains no resonances beyond 200 ppm thus ruling out 'normal' ketones (as was already observed in the IR.). Due to the large size of the molecule it is highly unlikely that a downfield carbon atom was lost in this spectrum as a consequence of an extremely long spin-lattice relaxation time, especially as a long pulse repetition time, 15.5 s, was used.

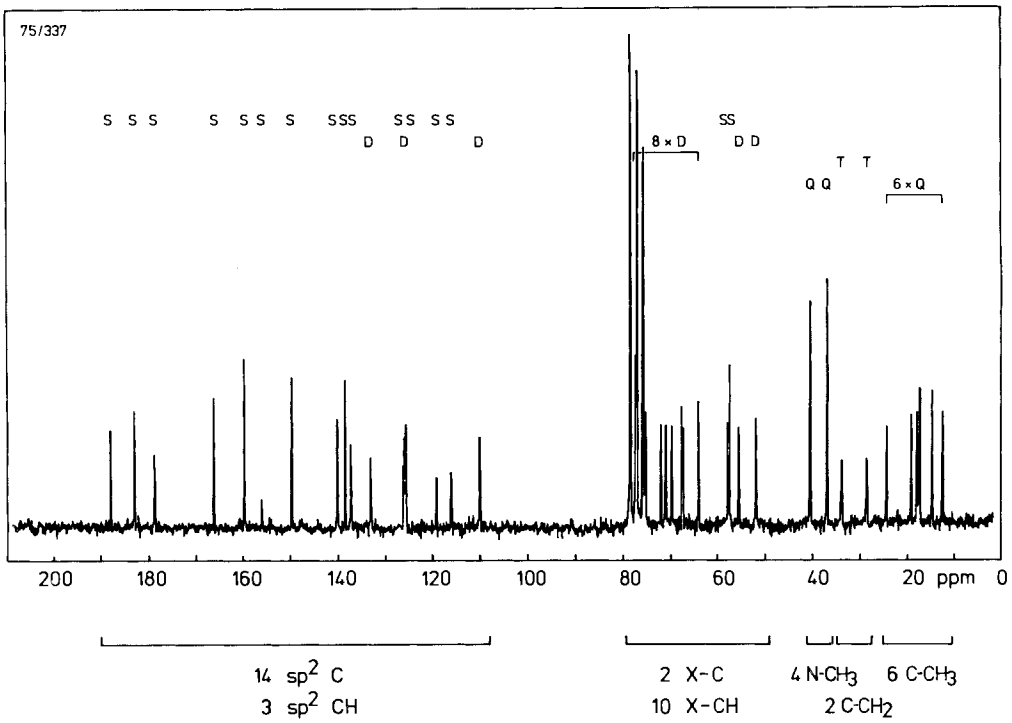


Fig. 5. *Proton noise decoupled 22.63-MHz-^{13}C-NMR. spectrum of hedamycin in $CDCl_3$*

The ^{13}C-NMR. spectrum in deuteriochloroform (*Fig. 5*) is clearly divided into two parts, an upfield part, showing 22 lines between about 12 and 77 ppm and a downfield part with 17 signals from 110 to 188 ppm.

One of the 17 signals in the *downfield region* is very small, suggesting that the corresponding carbon atom has a relatively long spin-lattice relaxation time. The cluster at about 126 ppm, which is not always resolved into three lines has clearly been shown in off-resonance decoupled spectra to correspond to three carbon atoms.

The fact that the spectrum shows no resonances *between 77 and 110 ppm* (except the solvent lines) indicates that hedamycin contains no glycosidic carbon atoms, as these resonances should be around 90 to 100 ppm [10]. The same conclusion was already drawn from the degradation experiments with dilute acid.

In the *upfield region* two of the 22 observed lines (at 36.8 and 40.4 ppm) are much taller than the others, indicating that they might correspond to two carbon atoms each. In low resolution spectra the signal at 77.3 ppm was usually covered by the center line of the deuteriochloroform resonance, and the two lines at 67.2 and 67.4 ppm appeared as one broad singlet. When the spectrum is recorded in deuteriopyridine, eight clearly separated lines can be seen between 60 and 80 ppm. This was also confirmed in high resolution spectra in deuteriochloroform.

Off-resonance decoupled spectra. They were recorded under various conditions: decoupler at high or low field, single frequency or modulated with narrow bandwidth

[4]) All chemical shifts are given as δ_C-values in ppm from internal TMS.

(500 Hz) noise. The multiplicities obtained from these experiments are indicated in *Fig. 5*. The difficulty of distinguishing doublets and quartets (the outer lines of which usually can hardly be seen in crowded areas of the spectrum) can be overcome by bearing in mind that usually no methyl resonances are found beyond about 47 ppm [10] except for methyl groups at oxygen or quaternary nitrogen atoms, both of which are not present in hedamycin.

Spectra measured in solutions containing Fe(acac)$_3$. The addition of a paramagnetic relaxation reagent such as tris(acetylacetonato)iron (Fe(acac)$_3$) results in more equalized peak intensities in ^{13}C-NMR. spectra by quenching the nuclear *Overhauser* effect and general shortening of spin-lattice relaxation times [11]. This technique was used to confirm the number of carbon atoms corresponding to the sometimes unresolved clusters and the tall lines at 36.8 and 40.4 ppm. Again the findings described above were confirmed. In addition, any carbon resonance that might not have been observed so far because of extremely long spin-lattice relaxation-time should now be observable, but no additional lines appeared.

Attempts to record off-resonance decoupled spectra of these solutions gave unexpected results: the quaternary carbon atoms could be seen as sharp singlets, whereas all the other resonances were very broad and almost indistinguishable from the background. This observation confirmed the number of quaternary carbon atoms in hedamycin, and initiated a study on the chemical suppression of long-range ^{13}C,^{1}H-coupling in ^{13}C-NMR. spectra [12].

Discussion. From all the above data and typical carbon chemical shift values [10] [13] the following conclusions can be drawn: Hedamycin contains six *C*-methyl groups (12 to 24 ppm), only two *C*-methylenes (28.3 and 33.7 ppm), two dimethylamino groups (36.8 and 40.4 ppm), definitely *no O*-methyl groupes (would appear between 50 and 60 ppm)[5]), 2 quaternary carbon atoms and 10 CH's bonded to an electronwithdrawing group such as O or N (50 to 80 ppm) and, finally, 17 sp^2 carbon atoms, 14 of them quaternary, three as CH's. If all the protons thus accounted for are added up, one gets 47. The three remaining ones must correspond to three hydroxyl groups. Although this figure is contradictory to the microanalytical determination of active hydrogen atoms (3.7/4.6), it is believed to be a more reliable index.

Assuming an anthraquinone unit in the molecule, this consumes 14 sp^2 carbon atoms and 11 equivalents of unsaturation. The remaining three sp^2 carbon atoms could perhaps form an α,β-unsaturated carbonyl system (in agreement with IR. data), probably contained in a ring. There are still four equivalents of unsaturation that have not yet been accounted for and which must be rings since no more sp^2 carbon atoms are left over.

^{1}H-NMR. studies at 270 MHz. – Since the ^{1}H-NMR. spectrum showed nicely separated signals when recorded in deuteriopyridine, proton-proton decoupling experiments seemed to be feasible. Better results should be obtained, however, if these experiments were carried out in an instrument with extremely high magnetic field strength. At 270 MHz[6]) the spectrum was indeed spread out and 16 different signals or groups of signals could be irradiated. These decoupling experiments are sum-

[5]) No explanation has been found so far for the discrepancy between the number of methyl groups deduced from the spectra and the results of the microanalytical determinations.

marized in *Fig. 6*. From the sequences of protons coupled to each other, which were thus obtained, four structural fragments could be deduced[7]).

Fragment A (**1**). The coupling constant of < 3 Hz suggests that free rotation around the corresponding C–C bond is not possible, whereas the 6 Hz couplings probably correspond to open aliphatic chains where typically ~ 7 Hz is encountered [14]. These facts led to the proposal of structure **2** for fragment A (coupling constants in epoxides are in the range of 1.5 to 5 Hz [15]).

Fragment B (**3**). Here again free rotation seems to be restricted as indicated by coupling constants of 9 Hz and more.

In addition, the coupling constant of 12 Hz might rather arise from geminal than from vicinal coupling. Structure **4** with one carbon atom less than **3** is therefore proposed. The observed coupling constants are in reasonable agreement with the values expected in cyclohexane chair forms, which usually are 10–13 Hz for J_{aa}, and 2–5 Hz for J_{ae} and J_{ee} [16]. Note that X at C(6) of this fragment cannot be an oxygen or a nitrogen atom, as hedamycin contains no glycosidic linkages. But C(6) could be

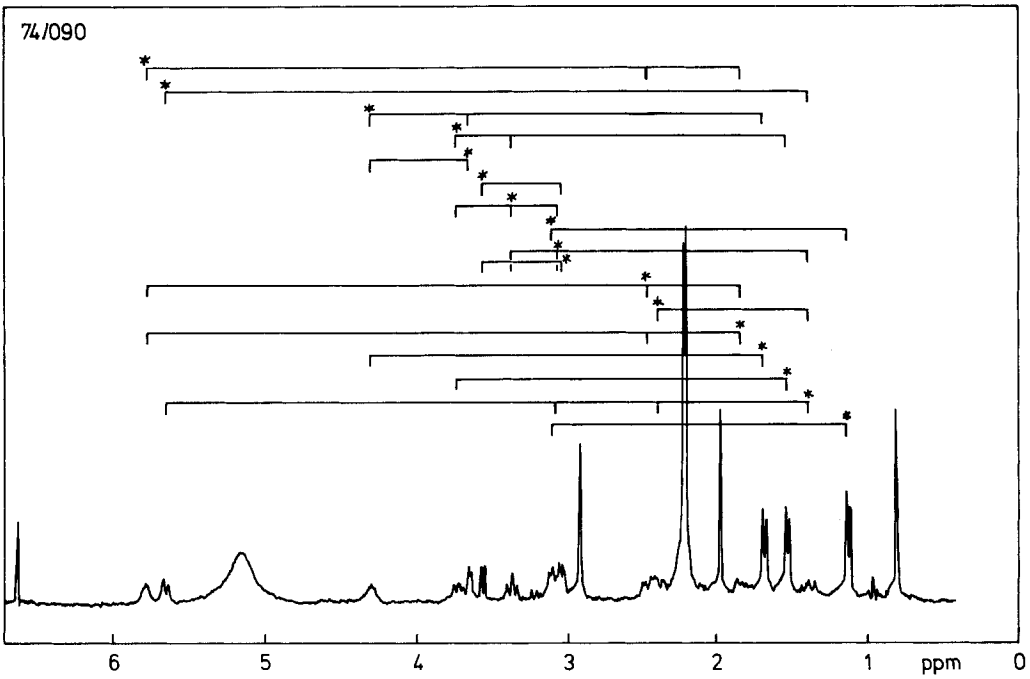
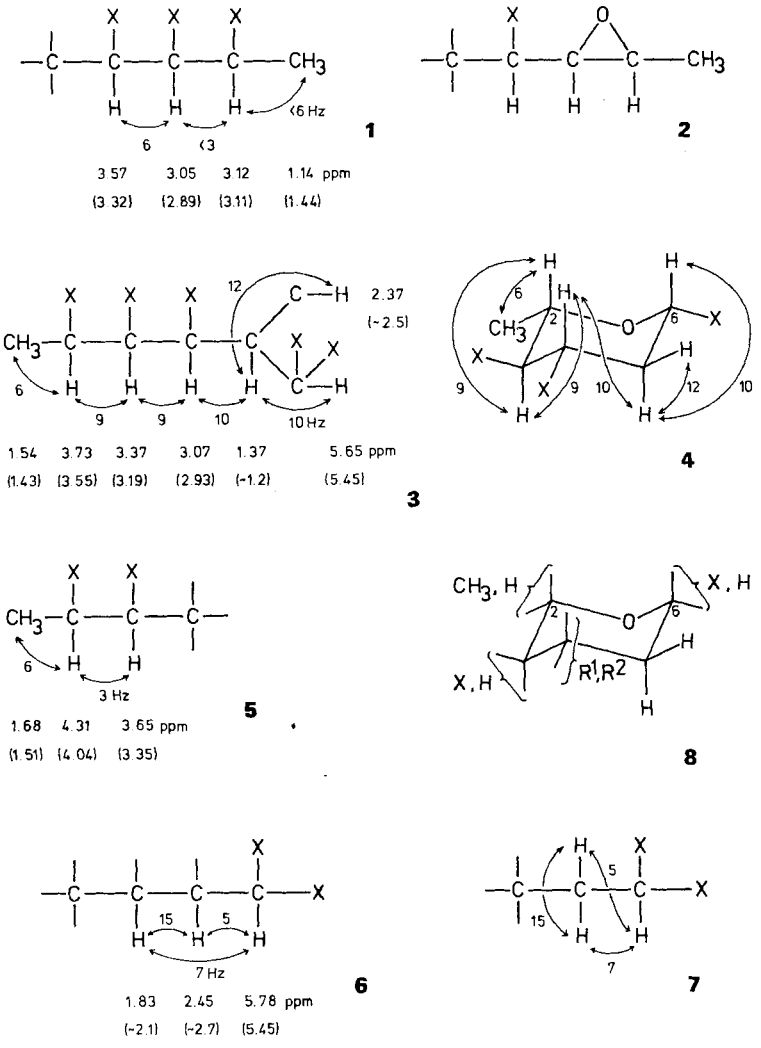


Fig. 6. *270-MHz-^{1}H-NMR. spectrum of hedamycin in C_5D_5N* (asterisks indicate points of irradiation for double resonance experiments; brackets link signals of protons coupling with each other)

[6]) 270-MHz experiments were kindly carried out by Dr. *E. P. Krebs*, The University of Chicago.

[7]) In the formulae the figures near hydrogen atoms are chemical shift values in C_5D_5N (for easier comparison with the literature, values for $CDCl_3$ are given in parentheses). The figures near arrows are coupling constants; the values derived from these 270-MHz measurements are only accurate to about $\pm$ 0.5–1 Hz. X denotes a deshielding substituent.

Scheme



the point where this fragment is attached to the aromatic system in hedamycin, which would also explain the chemical shift of the C(6) proton. The other two X's can, of course, be O or $N(CH_3)_2$.

Fragment C(**5**). Again, the 3 Hz coupling suggests that this fragment is part of a sterically fixed, more or less rigid system.

Fragment D(**6**). The 15 Hz coupling is most certainly a geminal coupling, and therefore fragment D is better represented by structure **7**.

Fragments C and D would together constitute another sugar-like structure(**8**). In this system, however, the observed coupling constants are not in good agreement with the predicted values, at least not for the chair form shown. Again, X at C(6) of

this fragment cannot be O or N, but might be the aromatic nucleus. R^1 and R^2 cannot both be groups bonded through aliphatic carbon atoms at the same time, as C(4) would then have a δ_C-value around 40 ppm. The singlet observed at highest field in the ^{13}C-NMR. spectrum is, however, at 57.3 ppm. Thus, one of the two R's is probably an electron withdrawing group, X.

Conclusion. – The chemical and spectral data of hedamycin show that the molecule contains the following structural elements: a methyl substituted 1-hydroxyanthraquinone, an α,β-unsaturated ketone and the partial structures **2**, **4** and **8**, probably as substituents of the aromatic nucleus. In addition, it has to contain two *C*-methyl groups, two dimethylamino and two hydroxyl groups, two ether oxygen atoms and two rings.

We thank Dr. *I. Hooper*, *Bristol Laboratories*, Syracuse, for a generous gift of hedamycin. One of us (*U.S.*) greatfully acknowledges the receipt of a *Reichstein Postdoctoral Fellowship* 1973–1974.

Experimental part. – The following apparatus were used to record the spectra discussed in this communication: *Beckmann* UV. spectrophotometers models DK 2, and 25; *Perkin-Elmer* IR. grating spectrometer, model 125; *Bruker* WH 90-NMR. spectrometer for ^{1}H- and ^{13}C-FT-NMR. measurements at 90 and 22.63 MHz, respectively; *JEOL* Minimar for 100-MHz-^{1}H-NMR. spectra; *Varian* CFT 20 for 20-MHz-^{13}C-NMR. spectra.

Microanalyses were carried out by the following laboratories: University of Sussex, *Alfred Bernhardt*, or *Midwest Microlabs*.

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