CHEMISTRY OF THE ACTINOMYCINS

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The actinomycins are yellow-red crystalline antibiotics that are produced by various species of *Streptomyces*. *Streptomyces* is a genus of soil microbes that, on the basis of its morphology, has a position between the fungi and the bacteria.
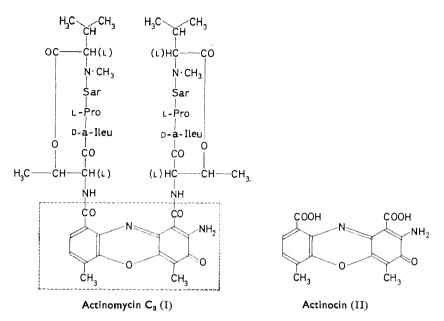
The actinomycins are highly toxic and cannot, therefore, be used in the treatment of infectious diseases. They are, however, of interest because in small, non-toxic dosages they have an anti-cancer effect in laboratory animals, and in man a favourable effect on Hodgkin's disease, a cancer-like disease of the lymphatic system. Moreover, it has been observed that actinomycins are able to stop the development of various human tumours, and to increase the effect of radiotherapy in the treatment of some forms of tumours[1, 2].

During the last few years we have been investigating the structure of the actinomycins. In the course of these studies, we have been able to clarify the constitution of some of them, and to find a method for their synthesis. A brief account of this work[3] will be given here.

Strains of *Streptomyces* able to synthesize actinomycins usually produce several actinomycins simultaneously. These mixtures of actinomycins form mixed crystals, and cannot be separated by fractional crystallization. In the early investigations, these mixtures were believed to be homogeneous substances. Methods for the separation of such actinomycin mixtures by counter-current distribution or partition chromatography were first developed in our Institute[4]. By means of these methods, it was possible to separate small quantities of actinomycins and to identify them by their R_F values. Moreover, it was then possible to obtain homogeneous actinomycins in larger quantities, so that thereafter all experiments designed to clarify the structure of the actinomycins were on a sure basis. In the course of our investigations, we have isolated from different strains of *Streptomyces* nine crystalline actinomycins, and the total number has been raised to twenty-four by other workers.

The first chemical investigations carried out with mixtures of actinomycins by Todd, Johnson and co-workers[5], as well as by the author and co-workers[6], showed that the molecule of the actinomycins consists of a polypeptide moiety and a part responsible for the yellow-red colour of the compounds. Thus the actinomycins are representatives of a new class of natural products which can be named " chromopeptides ".
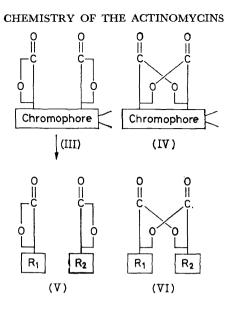
When homogeneous actinomycins were at our disposal, we continued our work on their structure and decided to concentrate initially on one of the actinomycins, namely actinomycin C_3. By hydrolysis of actinomycin C_3 with dilute and with concentrated hydrochloric acid, or with dilute sodium hydroxide, we obtained a number of degradation products, the structure of which enabled us to propose[7] for actinomycin C_3 the formula (I).

Actinomycin C₃ (I) Actinocin (II)

The dotted line in formula (I) circumscribes the chromophore of actinomycin C_3 responsible for its yellow-red colour. This chromophore is the residue of the amino-phenoxazone-dicarboxylic acid (II) which we have named " actinocin ".
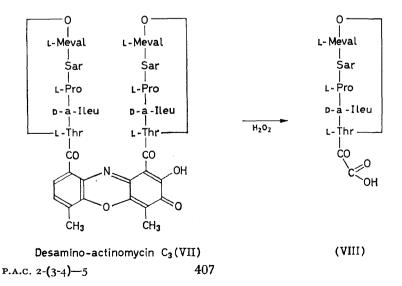
In the molecule of actinomycin C_3 (I), this actinocin (II) is linked by amide bonds derived from its two carboxyl groups to two identical peptide lactone groups. It is remarkable that these peptide groups contain four methylamino-acids, namely two molecules of sarcosine and two molecules of N-methyl-valine. It is also remarkable that the two molecules of allo-isoleucine between the threonine and the proline have the " unnatural " D-configuration.
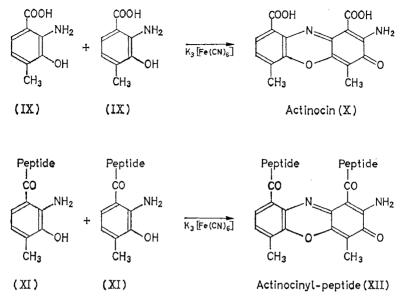
Until recently, it could not be decided with certainty whether the carboxyl groups of the two N-methyl-valine residues at the end of each of the peptide chains were esterified with the threonine of the same peptide chain, as in formula (I), or with the hydroxyl group of the threonine of the other peptide chain. This alternative is demonstrated by the two simplified formulae (III) and (IV). To decide between these two formulae, we tried to split the chromophore into two parts without opening the lactone rings. If structure (III) were correct, such a degradation would give two fission products (V) each containing one of the peptide lactone rings of the original actinomycins. The groupings R_1 and R_2 linked to the peptide lactone groups represent fragments of the chromophore. The isolation of only one of these fission products would suffice to establish the correctness of the structure (III). On the other hand, if the actinomycin were correctly represented by (IV), the envisaged fission of the chromophore would give a monocyclic degradation product (VI), in which the ring would contain two peptide units and two lactone groups.
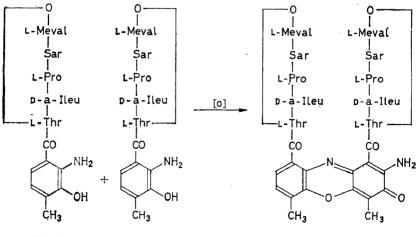
(III)   (IV)

(V)   (VI)

A fission of the chromophore which did not harm the peptide groups was achieved by oxidation of desamino-actinomycin C_3 (VII), a derivative which can be obtained in nearly quantitative yield by mild hydrolysis of actinomycin C_3 (I) with dilute hydrochloric acid. Treatment of desamino-actinomycin C_3 (VII) with hydrogen peroxide in acetic acid yielded a colourless crystalline degradation product which was shown to have the structure (VIII). In other words, the degradation product contains one intact peptide lactone group of the actinomycin C_3, thus proving that actinomycin C_3 has two peptide lactone groups[8], as shown in formula (VII).

The structure of the actinomycin C_3, in particular the fact that it contains two identical peptide groups, raises the question of how the actinomycins are synthesized in the cell.

Desamino-actinomycin C_3(VII)   (VIII)

(IX)　　　　(IX)　　　　　　　　　Actinocin (X)

(XI)　　　　(XI)　　　　　　Actinocinyl-peptide (XII)

We arrived at a hypothesis for this biosynthesis from the results of experiments on the synthesis of the chromophore of actinomycin C_3. This chromophore can be produced[9] easily by oxidative condensation of two molecules of 3-hydroxy-4-methylanthranilic acid (IX). A similar condensation is possible when the acid (IX) is linked *via* its carboxyl group to the amino group of an amino-acid, dipeptide, tripeptide, or tetrapeptide, as is indicated by the general formulae (XI) and (XII). In this way, we were able to synthesize a number of actinocinyl-peptides of the general formula (XII) from precursors similar to (XI). The observation that these condensations can be carried out in water at pH 7·8 with air as an oxidant, led us to the assumption[7] that the actinomycins are synthesized *in vivo* in a
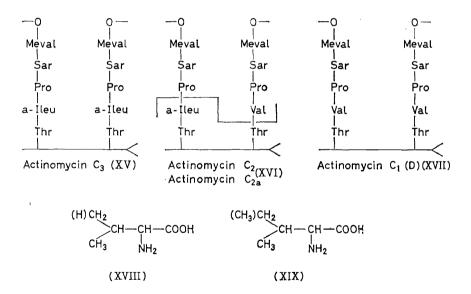

(XIII) Precursor (XIII)　　　　　　　Actinomycin C_3 (XIV)

similar way. Actinomycin C_3 would then be built up by the oxidative condensation of two molecules of a precursor (XIII). On this hypothesis, from two precursors of type (XIII) differing only in the structure of their peptide groups, the cell should be able to synthesize four different actinomycins: two with identical and two with different peptide groups. How far this prediction is in accordance with the experimental findings will be discussed later.

After the structure of actinomycin C_3 had been determined, we turned our attention to the question of how the other actinomycins differ from actinomycin C_3. As has already been mentioned, strains of *Streptomyces* able to synthesize actinomycins usually produce a mixture of different actinomycins. An investigation of the actinomycin mixtures of twenty-one strains showed us that these strains can be classified into three groups according to the composition of their actinomycin mixtures. One of these groups produces an actinomycin mixture, designated with the letter C, that contains the actinomycins C_3 and C_2 as main components and actinomycin C_1 in smaller amounts.

By means of the same degradation methods as those[10] employed for the investigation of actinomycin C_3, we were also able to establish the structures of actinomycins C_1 and C_2 [11]. They have the same chromophore as actinomycin C_3, and differ from it only slightly in the structure of their peptide

Actinomycin C_3 (XV)

Actinomycin C_2 (XVI)
Actinomycin C_{2a}

Actinomycin C_1 (D)(XVII)

(XVIII)

(XIX)

groups. For discussion of these differences it will be convenient to simplify the structural formulae in the manner which is illustrated in the case of actinomycin C_3 (I) by formula (XV). The chromophore is symbolized by a horizontal line, and the amino and carbonyl group associated with it by two short diagonal dashes. The roman capitals indicating the configuration of the amino- and methylamino-acids are omitted, and the lactone bond between the N-methyl-valine and the threonine is represented by a short dash.

Actinomycin C_1 (XVII) differs from actinomycin C_3 (XV) only in that it contains two molecules of valine (XVIII) instead of two molecules of allo-isoleucine (XIX). In this connection it should be noted that, on the basis of degradation experiments, Johnson and Bullock[12] concluded that actinomycin D (described for the first time in 1954 by Waksman and Vining[13]) also has the constitution (XVII). This proves unequivocally that actinomycin D is identical with our actinomycin C_1, which was first described in 1952. Moreover, one of the three groups of actinomycin-producing strains mentioned above is characterized by the fact that it produces an actinomycin mixture containing predominantly actinomycin C_1.

Actinomycin C_2 (XVI) holds an intermediate position between actinomycin C_1 and C_3. One of its peptide groups has the same structure as the peptide groups of actinomycin C_3 (XV) and the other as the peptide groups of actinomycin C_1 (XVII).

At first we could not eliminate the possibility that actinomycin C_2 (XVI) is a mixture of two isomers, one having the valine and allo-isoleucine in the positions shown in formula (XVI), and the other having the positions of these units interchanged as indicated by the serpentine line of formula (XVI). Recently, however, we have been able to show by degradation experiments (not discussed here) that actinomycin C_2 is homogeneous and not a mixture of two isomers.

Precursor (XX)   Precursor (XXI)
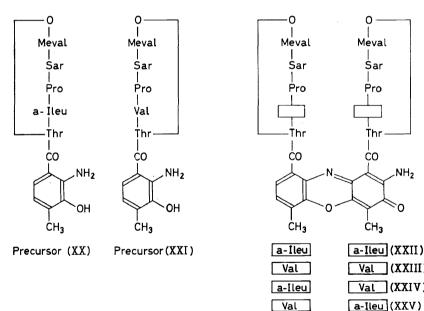
a-Ileu	a-Ileu (XXII)
Val	Val (XXIII)
a-Ileu	Val (XXIV)
Val	a-Ileu (XXV)

According to our hypothesis of the biosynthesis of the actinomycins, those strains of *Streptomyces* which produce actinomycins C_1, C_2 and C_3 simultaneously, would need two precursors for this synthesis. One of them (XXI) has D-valine, and the other (XX) has D-allo-isoleucine, in the peptide group. Oxidative condensation of two molecules of the precursor (XXI) would give actinomycin C_1 (XXIII). Analogous condensation of two molecules

of the precursor (XX) would give actinomycin C_3 (XXII). On the other hand, if one molecule of the precursor (XX) is condensed with one molecule of the precursor (XXI), the formation of two isomers is to be expected, one that has the valine in the left-hand peptide group and the allo-isoleucine in the right-hand peptide group as in the formula (XXV), and the other with the positions of these two acids reversed as in formula (XXIV). One* of these two isomers is actinomycin C_2.
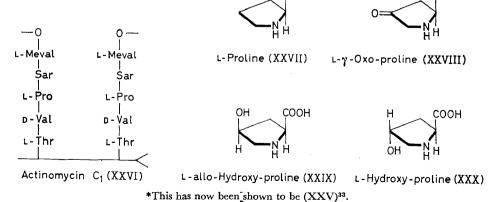
An indication of the existence of the second isomer is given by an actinomycin which we have isolated from *Streptomyces* cultures producing actinomycins C_1, C_2 and C_3. This new compound, which we have named[14] actinomycin C_{2a}, and which differs only slightly in its R_F value from actinomycin C_2, contains the same amino and methylamino acids as actinomycin C_2. Up to now we have obtained only small quantities of this new actinomycin, so that we have not as yet been able to determine the exact structure of its peptide groups. But, in all probability, actinomycin C_{2a} is the isomer of actinomycin C_2 whose existence is to be expected on the basis of our hypothesis.

As already mentioned, all strains of *Streptomyces* investigated in our Institute can be classified into three groups according to the composition of their actinomycin complexes. It will be recalled that one of these groups produces a mixture of the actinomycins C_1, C_2 and C_3, and another group a mixture containing mainly actinomycin C_1. The third group synthesizes an actinomycin mixture that we have designated with the letter X[4, 15]. This mixture consists of a main component, named actinomycin X_2, and a number of other actinomycins, among them actinomycin C_1. We have shown that all components of the actinomycin mixture X have the same chromophore as actinomycins C_1, C_2 and C_3. Consequently, they differ from each other, and from the C actinomycins, only in the structure of their peptide units.

Actinomycin X_2, the main component of the actinomycin complex X, is characterized by the fact that it contains one molecule of L-γ-oxo-proline[16] (XXVIII). Two other components contain L-hydroxy-proline (XXX) or L-allo-hydroxy-proline (XXIX). The γ-oxo-proline (XXVIII) is a derivative of proline that was unknown before 1956. It was first synthesized by

—O
|
L-Meval
|
Sar
|
L-Pro
|
D-Val
|
L-Thr
|

O —
|
L-Meval
|
Sar
|
L-Pro
|
D-Val
|
L-Thr
|

Actinomycin C_1 (XXVI)

L-Proline (XXVII)

L-γ-Oxo-proline (XXVIII)

L-allo-Hydroxy-proline (XXIX)

L-Hydroxy-proline (XXX)

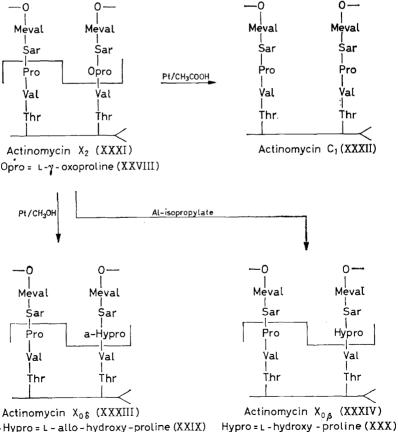*This has now been shown to be (XXV)[33].

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Kuhn and Osswald[17]. These authors showed that γ-oxo-proline—in the form of carbethoxy-oxo-proline-ethylester—is reduced to proline by catalytic hydrogenation in acetic acid, and to allo-hydroxy-proline by catalytic hydrogenation in methanol. If, on the other hand, the reduction is carried out with aluminium iso-propylate, a mixture of the hydroxy-proline and allo-hydroxy-proline derivatives is formed.

We have found that actinomycin X_2 differs from actinomycin C_1 (XXVI) in that it contains one molecule of L-γ-oxo-proline (XXVIII) in place of one molecule of L-proline (XXVII). Actinomycin $X_{0\beta}$[18], another component of the actinomycin complex X, differs from actinomycin C_1 in that it contains one molecule of L-hydroxy-proline (XXX) in place of one molecule of proline (XXVII).

Finally, actinomycin $X_{0\delta}$[19], which can also be a component of the actinomycin complex X[20], differs from actinomycin $X_{0\beta}$ only in that it contains one molecule of L-allo-hydroxy-proline (XXIX) in place of one molecule of L-hydroxy-proline (XXX).

The close structural relationship of the actinomycins X_2, $X_{0\beta}$, $X_{0\delta}$ and C_1 was disclosed by reduction studies. Catalytic hydrogenation in acetic
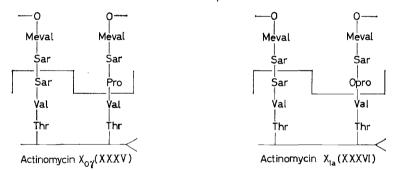

Actinomycin X_2 (XXXI)
Opro = L-γ-oxoproline (XXVIII)

Actinomycin C_1 (XXXII)

Actinomycin $X_{0\delta}$ (XXXIII)
a-Hypro = L-allo-hydroxy-proline (XXIX)

Actinomycin $X_{0\beta}$ (XXXIV)
Hypro = L-hydroxy-proline (XXX)

acid over a platinum catalyst reduces actinomycin X_2 to actinomycin C_1 (XXXII), thus proving that actinomycin X_2 differs from actinomycin C_1 only in that it contains one molecule of L-γ-oxo-proline in place of one molecule of L-proline[20]. Actinomycin X_2 can thus be given the formula (XXXI).

Reduction by means of aluminium iso-propylate converted the actinomycin X_2 (XXXI) into actinomycin $X_{0\beta}$, whereas catalytic hydrogenation of actinomycin X_2 in methanol gave actinomycin $X_{0\delta}$. On the basis of these results the formulae (XXXIV) and (XXXIII) can be attributed to the actinomycins $X_{0\beta}$ and $X_{0\delta}$ respectively[20]. The serpentine line in these formulae as well as in (XXXI) indicates that it is still uncertain whether the proline is a part of the left-hand or of the right-hand peptide group.
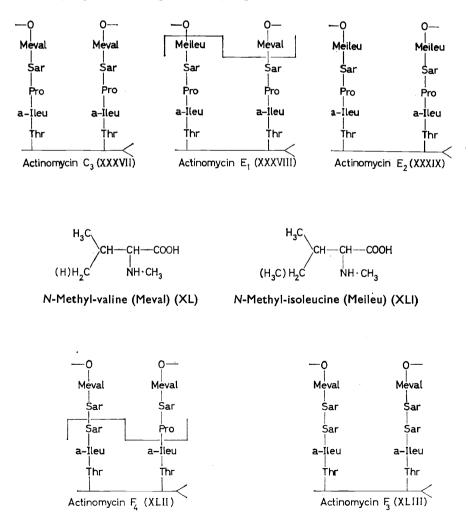
According to the formulae (XXXIII) and (XXXIV), actinomycin $X_{0\beta}$ is a stereoisomer of actinomycin $X_{0\delta}$, differing from it only in the configuration of the hydroxyl-substituted carbon atom of the L-hydroxy-proline. This slight distinction is sufficient to cause a marked difference in the solubility of the two stereoisomers, and also in their antibiotic activity. For instance, actinomycin $X_{0\delta}$ is about three times as soluble as actinomycin $X_{0\beta}$ in methanol containing 5 per cent water, and it is about ten times as active against *Bacillus subtilis* as actinomycin $X_{0\beta}$.

Actinomycin $X_{0\gamma}$ (XXXV)   Actinomycin X_{1a} (XXXVI)

We have found a new variation of the peptide moiety in two other components of the actinomycin complex X; we have named these[20] actinomycin $X_{0\gamma}$ and actinomycin X_{1a}. A comparison of their formulae ((XXXV), (XXXVI)) with that of actinomycin C_1 (XXXII) shows that actinomycin $X_{0\gamma}$ (XXXV) can be considered as a derivative of actinomycin C_1, one molecule of sarcosine being substituted for one molecule of proline. Likewise, actinomycin X_{1a} (XXXVI) may be regarded as being a derivative of actinomycin C_1, one molecule of sarcosine and one molecule of γ-oxo-proline being substituted for the two molecules of proline. The serpentine line in the formulae again indicates that the positions of the proline and sarcosine, or of the oxo-proline and sarcosine, have not been ascertained and could in fact be the reverse of that shown. At this point it must be mentioned that Johnson and Mauger[21] have investigated an actinomycin, designated with the number III, and shown it to have the same amino- and methylamino-acids as actinomycin $X_{0\gamma}$. Whether our actinomycin $X_{0\gamma}$ is identical with actinomycin III, or whether it is an isomer, remains to be established.

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After the actinomycins had been shown to be chromopeptides, the question arose whether the actinomycin-producing organisms could be stimulated to incorporate into the actinomycin molecule amino- or methyl-amino-acids that had been added to the culture broth. That this is indeed the case was demonstrated for the first time by Schmidt-Kastner[22]. After addition of either sarcosine or DL-isoleucine to the culture broth of strains producing actinomycin C_1, C_2 and C_3, he was able to isolate from the cultures a number of new actinomycins in addition to those normally formed.

In co-operation with Schmidt-Kastner, we have made quantitative determinations of the amino- and methylamino-acids of the new actino-mycins. Moreover, we have identified in their peptide groups those methylamino-acids that are esterified with the threonine residues. We cannot yet give an exact proof of any sequence of the amino-acids of the new

—O	O—	—O	O—	—O	O—
Meval	Meval	Meileu	Meval	Meileu	Meileu
Sar	Sar	Sar	Sar	Sar	Sar
Pro	Pro	Pro	Pro	Pro	Pro
a–Ileu	a–Ileu	a–Ileu	a–Ileu	a–Ileu	a–Ileu
Thr	Thr	Thr	Thr	Thr	Thr

Actinomycin C_3 (XXXVII)   Actinomycin E_1 (XXXVIII)   Actinomycin E_2 (XXXIX)

N-Methyl-valine (Meval) (XL)

N-Methyl-isoleucine (Meileu) (XLI)

—O	O—	—O	O—
Meval	Meval	Meval	Meval
Sar	Sar	Sar	Sar
Sar	Pro	Sar	Sar
a–Ileu	a–Ileu	a–Ileu	a–Ileu
Thr	Thr	Thr	Thr

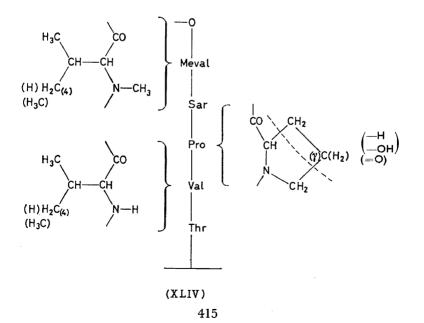Actinomycin F_4 (XLII)   Actinomycin F_3 (XLIII)

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actinomycins produced by the so-called "directed biosynthesis". If, however, it is assumed that the "directed biosynthesis" is controlled by a tendency to make the structural differences between the actinomycins of the "directed biosynthesis" and the "normal" actinomycins as slight as possible, we arrive at the formulae (XXXVIII)–(XLIII).

The actinomycins E_1 (XXXVIII) and E_2 (XXXIX) are formed when DL-isoleucine is added to the culture broth of strains normally producing mixtures of the actinomycins C_1, C_2 and C_3. As the formulae show, the addition of DL-isoleucine induces the strain to vary the molecule of actinomycin C_3 by replacing one or both molecules of N-methyl-valine (XL) by N-methyl-isoleucine (XLI).

A second group of new actinomycins, named actinomycins F_1, F_2, F_3, F_4 and F_5, was formed when sarcosine was added to the culture broth of strains "normally" producing the actinomycins C_1, C_2 and C_3. The formulae of actinomycin F_3 and F_4 are given by (XLIII) and (XLII) respectively. A comparison with the formula of actinomycin C_3 shows that the presence of sarcosine induces the cell to incorporate into the peptide groups one or two molecules of sarcosine instead of proline. In the same way the peptide groups of the actinomycin C_1 and C_2 can be varied by the cell.

Summarizing the above results on the structural differences of the peptide moiety of the actinomycins, one arrives at the following conclusions. In all actinomycins that we have investigated, the two carboxyl groups of the chromophore, which in all actinomycins has the same structure, are bonded to the amino group of two molecules of threonine. Variations in the structure of the peptide groups have been found in three constituents of these peptide groups, as is shown by (XLIV). The latter is the simplified formula of one of the two peptide groups of the actinomycin C_1. These groups may be regarded as the "basic structure" of an actinomycin peptide group.
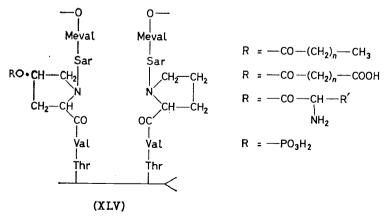

(XLIV)

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This " basic structure " can be varied at the valine and at the N-methyl-valine positions by substitution of a methyl group for a hydrogen atom at $C_{(4)}$. By this substitution the valine is converted into allo-isoleucine and the N-methyl-valine to N-methyl-isoleucine; variations of this type are possible in either or both of the peptide groups.

The proline residue can be varied firstly by the substitution of a hydroxyl group for one of the two hydrogen atoms at the γ-carbon atom, and secondly by the substitution of a carbonyl oxygen atom for the two hydrogen atoms at the γ-carbon atom of the proline. Finally, one or two molecules of sarcosine can be substituted for molecules of proline; in other words, the $-CH_2-CH_2-$ group of the proline—see dashed line in formula (XLIV)—can be replaced by two hydrogen atoms.

Obviously there exist still other variations of the peptide groups. Recently Keller-Schierlein[23] and co-workers have isolated five actinomycins, named actinomycin Z_1, Z_2, Z_3, Z_4 and Z_5, that all contain N-methyl-alanine. Some actinomycins similar to these have been isolated in our laboratory. Moreover, Katz and Goss[24] have described actinomycins containing pipecolic acid. These actinomycins were produced by the addition of pipecolic acid to the culture broth.

In view of the fact that, up to now, twenty-four different actinomycins have been isolated from *Streptomyces* cultures, it will naturally be asked whether there are marked differences in the cytostatic activity of these actinomycins. From animal experiments it was discovered that there are indeed such differences, although they do not appear to be significant. For this reason, we have attempted to obtain derivatives of actinomycins in the hope of finding one which has a better carcinolytic activity than the actinomycins.
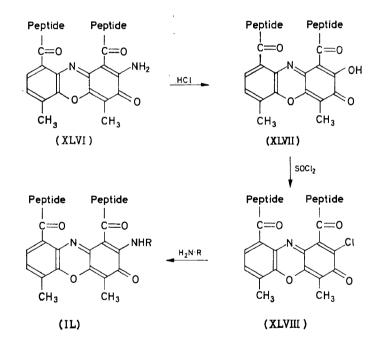
Derivatives of the actinomycins may be obtained in two ways: firstly, by modifying the peptide part, and secondly, by modifying the chromophore. Thus far, we have obtained derivatives in which the peptide part is modified only from the actinomycins $X_{0\beta}$ and $X_{0\delta}$, which, as already mentioned, contain hydroxy-proline or allo-hydroxy-proline. Both these actinomycins possess a free hydroxyl group (in either the hydroxy-proline or allo-hydroxy-proline unit) which can be esterified with acids. On the assumption that

$$R = -CO-(CH_2)_n-CH_3$$

$$R = -CO-(CH_2)_n-COOH$$

$$R = -CO-CH-R'$$
$$\quad\quad\quad\quad NH_2$$

$$R = -PO_3H_2$$

(XLV)

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the hydroxy-proline or allo-hydroxy-proline is located in that peptide group which is bonded to the benzoid ring of the chromophore, the esters of actinomycin $X_{0\beta}$ and actinomycin $X_{0\delta}$ may be represented by the formula (XLV), in which R represents the acid residue. With acetic acid and palmitic acid, we obtained the acetates and palmitates of actinomycins $X_{0\beta}$ and $X_{0\delta}$. These derivatives have less antibiotic and cytostatic activity than the parent actinomycins. Esterification was also effected with dicarboxylic acids, amino-acids and phosphoric acid. The esters thus obtained have a greater solubility in water than the actinomycins used as starting material, but their cytostatic activity has still to be determined.
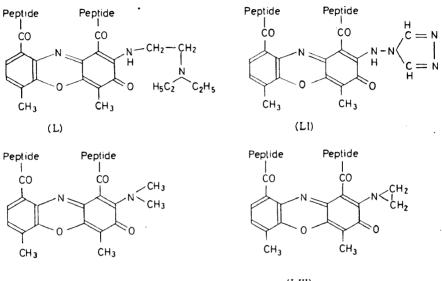
Except for a few cases still under investigation, the only modifications of the chromophore that we have been able to effect are associated with the amino group. The derivatives which we have obtained were prepared in a manner which is summarized by the formulae (XLVI)–(XLVIII).

If an actinomycin (XLVI) is treated under mild conditions (four hours at 30°C) with 10 per cent hydrochloric acid, one molecule of ammonia is split off and a desamino-actinomycin (XLVII) is formed in almost quantitative yield[25]. During this process the peptide groups remain intact. In contrast to the actinomycins, the desamino-actinomycins have no toxic, antibiotic or cytostatic activity.

We have found that chlorine may be substituted for the hydroxyl group of the chromophore of the desamino-actinomycins (XLVII) by treatment with thionyl chloride. In this way crystalline antibiotically inactive chloro-actinomycins (XLVIII) are obtained[26]. If a chloro-actinomycin is treated with ammonia, the original actinomycin is recovered[26].

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The chloro-actinomycins can be treated with primary and secondary amines instead of ammonia[26] to yield actinomycin derivatives in which one (IL) or both of the hydrogen atoms of the amino group of the chromophore have been replaced by alkyl groups or other residues. In this way we have prepared a number of derivatives whose biological activity is still under investigation[27]; (L)–(LIII) are the formulae of four such derivatives.
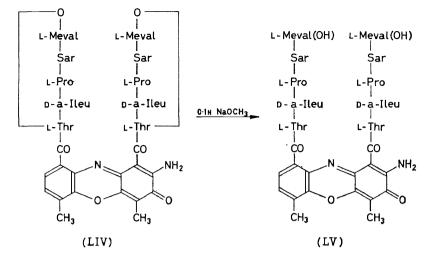

(L)

(LI)

(LII)

(LIII)

The dimethyl compound (LII) has only low antibiotic and carcinolytic activity. The N-diethylamino-ethyl derivative (L) also has less antibiotic and cytostatic activity than actinomycin C_3, but whether its therapeutic index is better than that of actinomycin C_3 remains to be determined. The same is true for the triazolyl derivative (LI). The most promising of the four derivatives seems to be N-dimethylene-actinomycin (LIII). In tumour experiments, its therapeutic index is better than that of the actinomycin C_3, a result that has encouraged us to make this derivative available for clinical trials. Its antibiotic activity is less than that of the actinomycin C_3, however.

The modifications of the peptide groups and the chromophore described above are limited by the fact that all the reactions involved must be carried out under conditions which do not destroy the peptide groups or the chromophore. From the very beginning it was obvious that this limitation would be overcome if the actinomycins and their derivatives could be prepared by synthesis. During the last three years we have been attempting to achieve this, and very recently we have been successful in synthesizing actinomycin C_3.
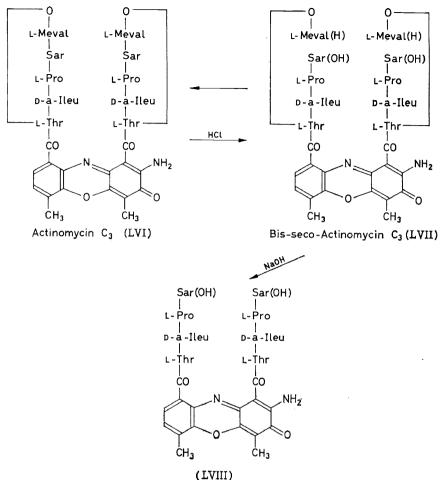
Our first attempt to synthesize actinomycin C_3 was based on the fact that the lactone groups of the actinomycin C_3 (LIV) are hydrolyzed by dilute sodium methoxide. The resulting actinomycin C_3 acid can be given the formula (LV). From our work on the synthesis of actinocinyl-peptides,

(LIV)   (LV)

we had formed the opinion that it would not be too difficult to synthesize the actinomycin C_3 acid (LV), which is an actinocinyl-bis-pentapeptide. Our plan, therefore, was to carry out such a synthesis, and then to cyclize the two peptide chains of the actinomycin C_3 acid by esterification of the terminal carboxyl groups with the hydroxyl groups of the threonine residues. As this cyclization was expected to be the main difficulty in the synthesis, we decided to carry out an initial study with the actinomycin C_3 acid prepared from actinomycin C_3, and not with the precious synthetic material. However, experiments to prepare sufficient starting material for this purpose showed that the opening of the lactone rings was accompanied by side reactions, and that the resultant by-products could not be separated easily.
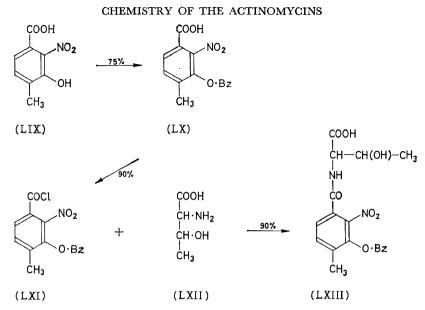
While these experiments were under way, Dr Sunderkötter in our laboratory made an observation which opened up a new route for the synthesis of actinomycin C_3. By treating actinomycin C_3 (LVI) with concentrated hydrochloric acid for four hours at 40°C, Sunderkötter obtained a degradation product which he found to have the formula (LVII)[29]. This product we have named bis-seco-actinomycin C_3. Its formation shows that concentrated hydrochloric acid attacks the peptide chains of the actinomycin preferentially at the bond between sarcosine and the N-methyl-valine. On mild treatment with sodium hydroxide, the bis-seco-actinomycin C_3 is converted into the actinocinyl-peptide (LVIII), which was synthesized in our laboratory by Lackner.

Bis-seco-actinomycin C_3 (LVII), contains the tetrapeptide L-threonyl-D-allo-isoleucyl-L-prolyl-sarcosine, in which the amino group is blocked by a carboxyl group of the chromophore but in which the carboxyl group is free. In addition to this tetrapeptide, the bis-seco-actinomycin C_3 contains N-methyl-valine with the methylamino group free but with the carboxyl group esterified with the hydroxyl group of the threonine residue. In other words, the molecule of bis-seco-actinomycin C_3 contains the two reactive groups necessary for a peptide synthesis.

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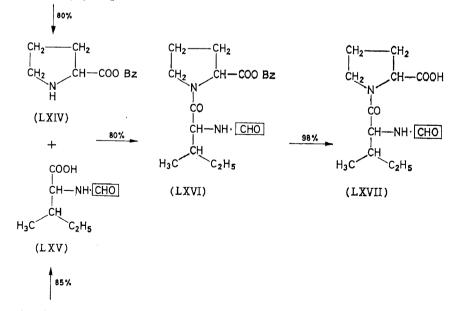Actinomycin C₃ (LVI) → Bis-seco-Actinomycin C₃ (LVII)

(LVIII)

It was to be expected, therefore, that reagents producing peptide bonds would make it possible to couple the carboxyl group of the sarcosine with the methylamino group of the N-methyl-valine, and thus to regenerate the original actinomycin that was used to prepare the bis-seco-actinomycin. As Dr Ohly and Dr Sunderkötter in our laboratory have found[30], this reaction can be carried out either with cyclohexyl-carbodiimide, following Sheehan's method, or with ethyl chloroformate. By means of these reagents, they obtained crystalline actinomycin C₃ (LVI) in a yield of about 2·5 per cent from bis-seco-actinomycin C₃ (LVII).

In this way a partial synthesis of actinomycin C₃ had been achieved. To develop this partial synthesis into a total synthesis, we had to synthesize the bis-seco-actinomycin C₃. This problem was solved in our Institute by Lackner[31]. The separate steps of this synthesis are illustrated by the formulae (LIX)–(LXXIV). 2-Nitro-3-hydroxy-4-methyl-benzoic acid (LIX) was benzylated at the hydroxyl group to give the compound (LX), which was chlorinated by means of thionyl chloride to give the chloride

(LIX)   (LX)

(LXI)   (LXII)   (LXIII)
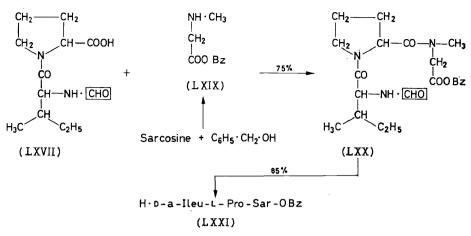
(LXI).   Coupling of this chloride with L-threonine (LXII) in dilute sodium
hydroxide gave the compound (LXIII).   The carboxyl group of the com-
pound (LXIII) was then coupled with the amino group of the benzyl
ester (LXXI) of the tripeptide D-allo-isoleucyl-L-prolyl-sarcosine.   The
synthesis of this tripeptide-benzyl ester is illustrated by the formulae (LXIV)–
(LXXI).   L-Proline was esterified with benzyl alcohol to give L-proline-

L-Proline + $C_6H_5 \cdot CH_2 \cdot OH$

(LXIV)

(LXVI)   (LXVII)

(LXV)

D-allo-Iso-leucine + HCOOH

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CH₂——CH₂
| |
CH₂   CH—COOH
   \\ /
    N
    |
    CO
    |
    CH—NH · [CHO]
    |
    CH
   / \\
H₃C   C₂H₅

(LXVII)

+

NH · CH₃
|
CH₂
|
COO Bz

(L XIX)

↑

Sarcosine + C₆H₅ · CH₂ · OH

——75%——→

CH₂——CH₂
| |
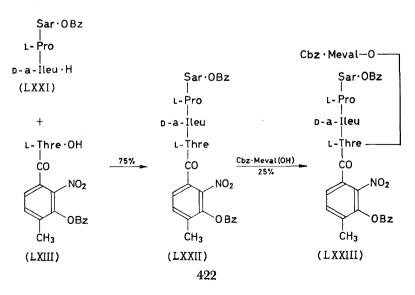CH₂   CH—CO—N—CH₃
   \\ / |
    N   CH₂
    | |
    CO   COO Bz
    |
    CH—NH · [CHO]
    |
    CH
   / \\
H₃C   C₂H₅

(LXX)

——85%——→

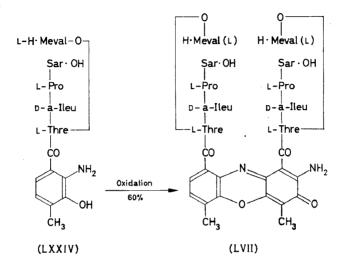H · D - a - Ileu - L - Pro - Sar - OBz
(LXXI)

benzylester (LXIV), and D-allo-isoleucine was formylated to give formyl-D-allo-isoleucine (LXV). Coupling of the compound (LXIV) with the compound (LXV) by means of dicyclohexyl-carbodiimide yielded the compound (LXVI). The benzyl group was removed from the carboxyl group of this compound by catalytic hydrogenolysis, and the resulting formyl-D-allo-isoleucyl-proline (LXVII) was coupled with sarcosine benzylester (LXIX) (which was obtained from sarcosine by esterification with benzyl alcohol) to give the tripeptide derivative (LXX). Mild hydrolysis of this compound by means of hydrochloric acid in benzyl alcohol removed the formyl group from the amino group of the D-allo-isoleucine residue, thus giving D-allo-isoleucyl-L-prolyl-sarcosine benzylester (LXXI).

This tripeptide ester (LXXI) was then coupled with the L-threonine derivative (LXIII) to give the compound (LXXII). The next step was the esterification of the hydroxyl group of the threonine residue in compound (LXXII) with N-carbobenzoxy-methyl-valine, a reaction that could not be

Sar · OBz
|
L - Pro
|
D - a - Ileu · H

(LXXI)

+

L - Thre · OH
|
CO
   \\
    [ring] NO₂
    |
    OBz
    |
    CH₃

(LXIII)

——75%——→

Sar · OBz
|
L - Pro
|
D - a - Ileu
|
L - Thre
|
CO
   \\
    [ring] NO₂
    |
    OBz
    |
    CH₃

(LXXII)

——Cbz·Meval(OH)——→
        25%

Cbz · Meval—O ——┐
                      |
Sar · OBz |
| |
L - Pro |
| |
D - a - Ileu |
| |
L - Thre ————————————┘
|
CO
   \\
    [ring] NO₂
    |
    OBz
    |
    CH₃

(LXXIII)

accomplished by conventional methods of esterification. The desired result was finally achieved by using the N,N-carbonyl-diimide reagent first introduced for esterification by Staab[32]. The compound (LXXIII) thus obtained was hydrogenated in order to reduce the nitro group and to split off the two benzyl groups and the carbobenzoxy group. The reduction

(LXXIV)   (LVII)

product (LXXIV) thus formed was then oxidized by means of potassium ferricyanide in phosphate buffer at pH 7·3. In this way we obtained a mixture of different products from which we were able to separate by means of partition chromatography a compound (LVII) that had the same R_F value, the same spectrum, the same functional groups, and the same amino- and methylamino-acid content as the bis-seco-actinomycin prepared from actinomycin C_3. The best proof of the identity of the two compounds, however, was the fact that, by treatment with ethyl chloroformate, the synthetic compound (LVII) was converted into a crystalline, yellow-red, antibiotically active compound that was identical in every respect with actinomycin C_3 (LVI).

There is no doubt that the other actinomycins can be synthesized in the same way as actinomycin C_3. Moreover, it should now be possible to synthesize actinomycins and chromopeptides having chromophores and peptide parts different from those of the actinomycins we have at present. Perhaps among such compounds one will be found that has a better cytostatic activity than the actinomycins and actinomycin derivatives known at the present time.

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