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THE ORTHOSOMYCINS, A NEW FAMILY OF ANTIBIOTICS

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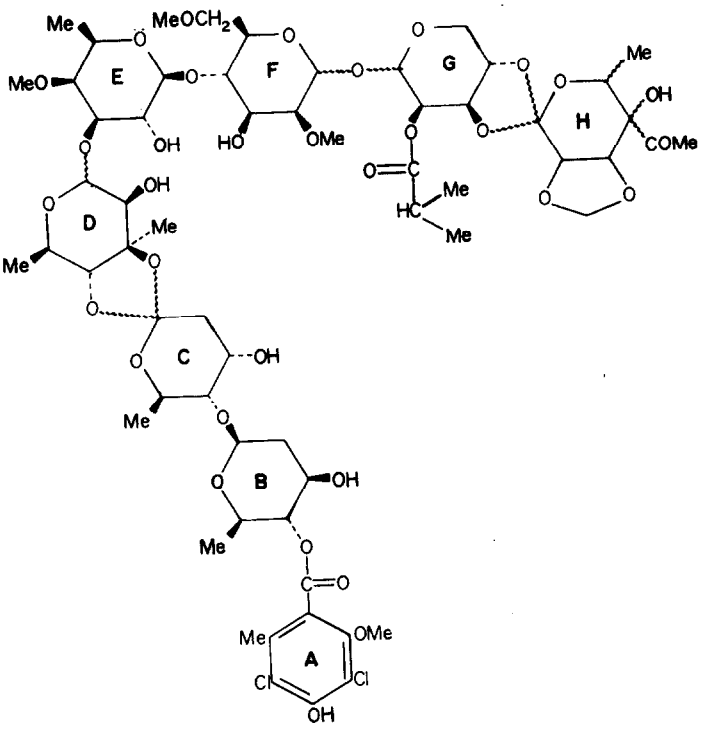
Abstract—A new family of antibiotics, the orthosomycins, which contain in their structures one or more orthoester linkages associated with carbohydrate residues, is described. Examples of orthosomycin antibiotics include flambamycin (1), the everninomicins -B(2a), -C(2b), -D(2c) and -2(3), hygromycin B(4a), the destomycins -A(4b), -B(5) and -C(4c), the antibiotics SS-56-C(4d), A-396-I(4e) and, more recently, the avilamycins -A(49a) and -C(49b). The structural elucidation of these orthosomycin antibiotics is discussed, with emphasis on structural similarities and differences, together with a commentary on their biological activities and structure-activity relationships.

CONTENTS

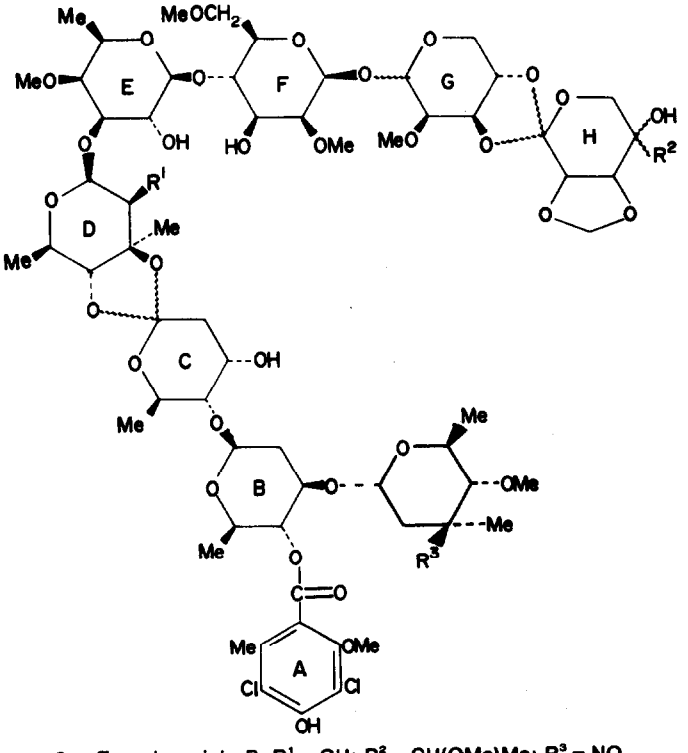
INTRODUCTION

In this Report the name *orthosomycins* is proposed for a new family of antibiotics which are characterised by the presence, in their structure, of one or more orthoester linkages which are associated with carbohydrate residues. The natural occurrence of orthoesters is rare, but within recent years several examples of antibiotics possessing this common structural feature have been described. It now seems desirable to group them together under the generic name of orthosomycins. These antibiotics include flambamycin (1),[1-3] the everninomicins-B(2a),[4] -C(2b),[5] -D(2c)[6] and -2(3),[7] hygromycin B(4a),[8] the destomycins -A(4b),[9,10] -B(5)[10] and -C(4c),[11] and the antibiotics SS-56-C(4d)[12] and A-396-I(4e).[12,13]

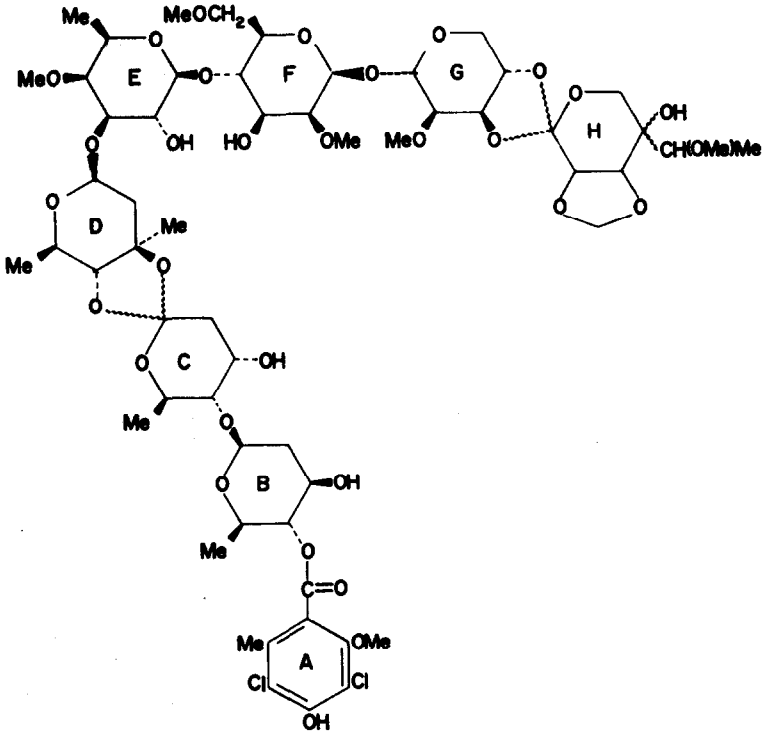
For the purposes of this Report, the orthosomycin group of antibiotics can, at this time, be conveniently divided into two distinct series on the basis of additional structural features, namely (a) those which are esters of dichlorisoeverninic acid (6) (for example flambamycin and the everninomicins), and



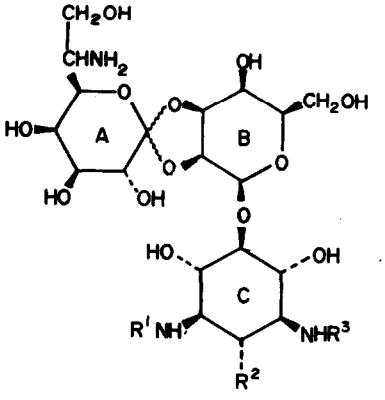
1 Flambamycin



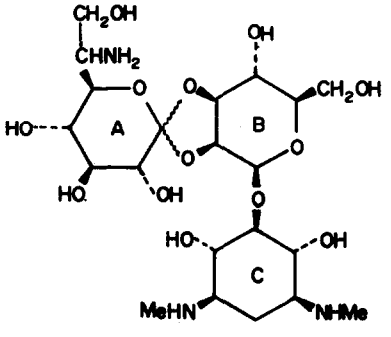
2a: Everninomicin-B, $R^1 = OH$; $R^2 = CH(OMe)Me$; $R^3 = NO_2$
2b: Everninomicin-C, $R^1 = R^2 = H$; $R^3 = NO_2$
2c: Everninomicin-D, $R^1 = H$; $R^2 = CH(OMe)Me$; $R^3 = NO_2$
2d: $R^1 = H$; $R^2 = CH(OMe)Me$; $R^3 = NO$
2e: $R^1 = H$; $R^2 = CH(OMe)Me$; $R^3 = NHOH$
2f: $R^1 = OH$; $R^2 = CH(OMe)Me$; $R^3 = NO$
2g: $R^1 = OH$; $R^2 = CH(OMe)Me$; $R^3 = NHOH$
2h: $R^1 = R^2 = H$; $R^3 = NO$
2i: $R^1 = R^2 = H$; $R^3 = NHOH$



3: Everninomicin-2



4a: Hygromycin B, $R^1 = R^2 = H$; $R^3 = Me$
4b: Destomycin A, $R^1 = Me$; $R^2 = R^3 = H$
4c: Destomycin C, $R^1 = R^3 = Me$; $R^2 = H$
4d: SS-56-C, $R^1 = R^3 = H$; $R^2 = OH$
4e: A-396-I (=SS-56-D), $R^1 = R^2 = R^3 = H$



5: Destomycin B

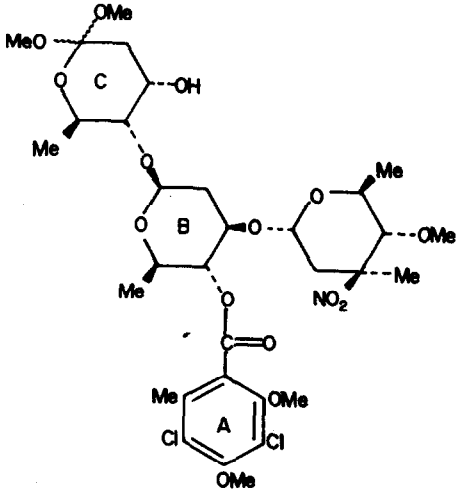
(b) *Everninomicin*-B (**2a**)

The degradation studies leading to the elucidation of the structure **2a** of everninomicin-B by the Schering group[4,25] were essentially similar to those described above for everninomicin-D, namely the identification of products obtained from the acidic hydrolysis and methanolysis of the antibiotic, and salient points from the results of these studies with everninomicin-B are summarised as follows.

(i) Aqueous acidic hydrolysis of everninomicin-B gave a heptasaccharide (everheptose-B) whose constitution (**17a**) was established by its fission, using diazomethane, to the δ-lactone (**18a**) (previously obtained from everninomicin-D) and a tetrasaccharide (evertetrose-B). The constitution **14d** of evertetrose-B was determined by (a) its acidic hydrolysis which gave evertriose (**12**) (previously obtained from everninomicin-D) and a new monosaccharide, D-evalose (**8b**), and (b) the acidic hydrolysis of evertetrose-B permethyl ether which established the mode of linkage between D-evalose (**8b**) and evertriose (**12**) thus leading to the assignment of structure **14d** for evertetrose-B, and hence everheptose-B could be represented by **17b**.

(ii) Mild acidic hydrolysis of everninomicin-B gave everninomicin-B_1 (**21b**) [compare the similar formation of everninomicin-D_1 (**21a**) from everninomicin-D (**2c**)]. Treatment of everninomicin-B_1 (**21b**) with diazomethane resulted in cleavage of the molecule with the formation of the δ-lactone (**18a**) and olgose-B [compare the similar cleavage with everninomicin-D_1 (**21a**)]. The constitution **22c** of olgose-B was deduced from the results of its acidic hydrolysis [which, *inter alia*, gave evertetrose-B (**14d**) and the ester (**23a**)] using arguments similarly advanced to explain the results from the acidic hydrolysis of olgose (**22a**) which gave the corresponding hydrolysis products evertetrose (**14a**) and the ester (**23a**).

(iii) Methylation of everninomicin-B with diazomethane followed by mild acidic methanolysis of the resulting (phenolic) monomethyl ether gave, *inter alia*, the orthoesters **25** and **26**. The isolation of these orthoesters (**25**) and (**26**) is noteworthy. Under different conditions the corresponding normal esters **19a** and **23a** were obtained from the solvolytic (acid methanolysis) fission of the intermonosaccharide orthoester linkages which were shown, from related studies (see section a above), to be present in everninomicin-D. Since the presence of an orthoester linkage, associated with residues G and H had already been established in olgose-B (**22c**), the isolation of the ester **25** also suggested the presence of a second orthoester linkage associated with the carbohydrate residues C and D in everninomicin-B (**2a**) [corresponding with the orthoester linkage similarly situated in everninomicin-D (**2c**)]. Further evidence for the presence and mode of linkage of the C–D orthoester grouping in everninomicin-B (**2a**) was subsequently obtained[4] from the products isolated from the reaction of the permethyl ether of everninomicin-B_1 (**21b**) with diazomethane, which gave the δ-lactone monomethyl ether (**18b**) and the partially-methylated derivative (**22d**) of olgose B, where the C-3 and C-4 OH groups of the D-evalose (**8b**) residue D were free in **22d**. Thus it was concluded[4] that the two OH groups located at positions C-3 and C-4 of the D-evalose (**8b**) residue D of everninomicin-B (**2a**) were involved in an orthoester linkage with the δ-lactone (**18a**), or its hydroxy-acid equivalent (**19b**), so leading to the constitution **2a** for everninomicin-B. As in the case of the conversion of everninomicin-D (**2c**) to everninomicin-D_1 (**21a**), the



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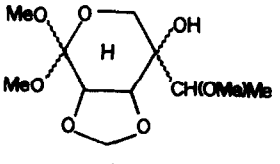

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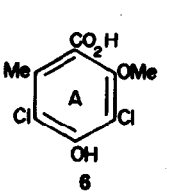
(i) A monosaccharide (evermicose) which was identified[18] as 2-deoxy-D-evalose (**8a**).

(ii) A non-reducing disaccharide component (everninose) which on acidic hydrolysis gave 2,6-di-O-methyl-D-mannose (**9**) and 2-O-methyl-L-lyxose (**10a**), thus leading to the constitution **11** for everninose.[19]

(iii) A non-reducing trisaccharide (evertriose) which was assigned the constitution **12** on the basis of its acidic hydrolysis to everninose (**11**) and 4-O-methyl-D-fucose (**13**)(= D-curacose), coupled with mass spectral evidence and results from permethylation studies.[20]

(iv) A non-reducing tetrasaccharide (evertetrose) which on further acidic hydrolysis yielded evertriose (**12**) and 2-deoxy-D-evalose (**8a**). The glycosidic linkage of the 2-deoxy-D-evalose (**8a**) residue D with the C-3 OH group of the 4-O-methyl-D-fucose (**13**) residue E of evertrose (**12**) was established[21] from the results of the permethylation of evertetrose, with mass spectral analysis and subsequent acidic hydrolysis of the resulting evertetrose penta- and hexamethyl ethers, leading to the constitution **14a** for evertetrose.[21]



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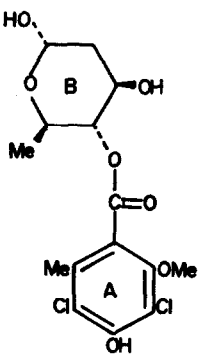
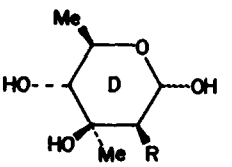
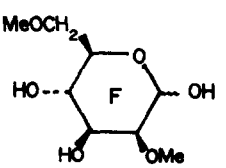
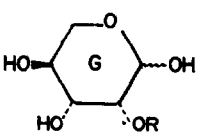


7: Curacin

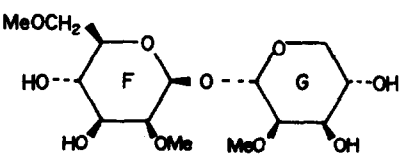


8a: 2-Deoxy-D-evalose, R = H
8b: D-Evalose, R = OH

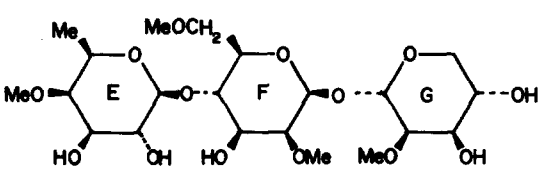




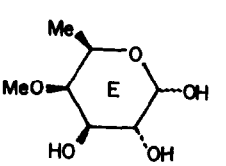
9: 2,6-Di-O-methyl-D-mannose **10a**: 2-O-Methyl-L-lyxose, R = Me
10b: L-Lyxose, R = H



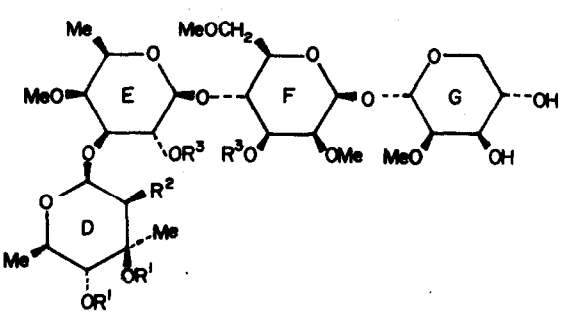
11: Everninose



12: Evertriose



13: 4-O-Methyl-D-fucose

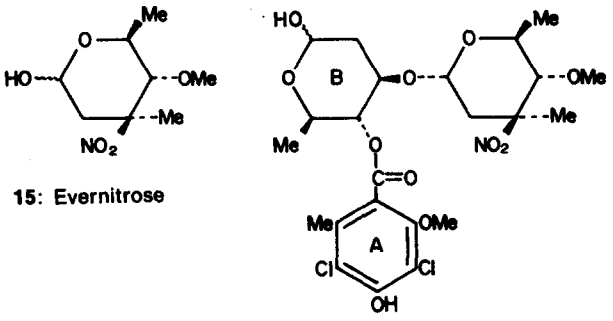


14a: Evertetrose, $R^1 = R^2 = R^3 = H$
14b: $R^1 = R^3 = Me$; $R^2 = H$
14c: $R^1 = R^2 = H$; $R^3 = Me$
14d: Evertetrose-B, $R^1 = R^3 = H$; $R^2 = OH$

(v) A disaccharide component (everninonitrose), which on acidic hydrolysis gave curacin (7) and a nitro-sugar (evernitrose), whose structure **15** and stereochemistry was established[22] from X-ray analysis, since a previous[23] structural assignment for evernitrose, based on NMR evidence, was incorrect. This result, coupled with further NMR and mass spectral studies established the constitution **16** for everninonitrose. It should be noted that evernitrose (**15**) was the first naturally occurring nitro-sugar to be isolated, and its revised[22] stereochemistry at C-3, indicated in **15**, has been included in all appropriate structures in this Report, whereas the original papers[23] indicate the evernitrose residue to possess the epimeric configuration at C-3.

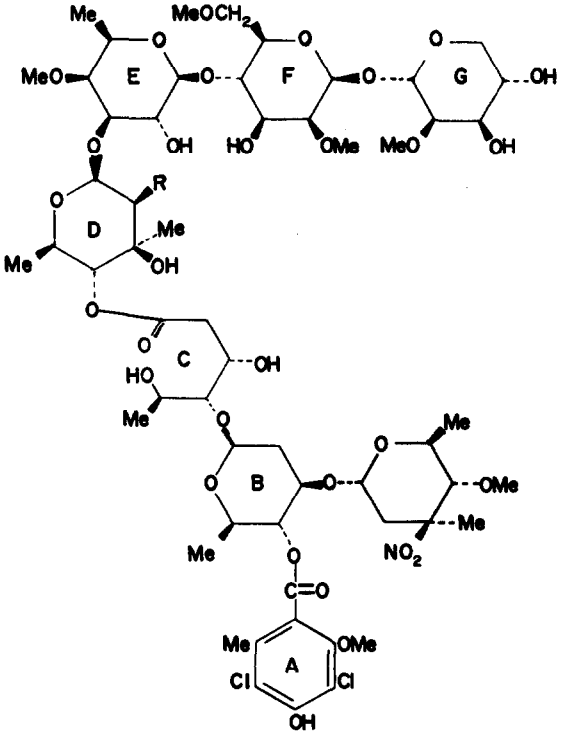
(vi) A heptasaccharide component (everheptose), the constitution **17a** of which was assigned from the following evidence.[24]

(a) Treatment of everheptose (**17a**) with diazomethane gave evertetrose (**14a**) and a δ-lactone which was readily converted to the corresponding ring-opened methyl ester on acidic methanolysis. On the basis of UV, IR, NMR and mass spectral properties, the structure **18a** was assigned to the δ-lactone and structure **19a** to the corresponding ring-opened methyl ester.



15: Evernitrose

16: Everninonitrose

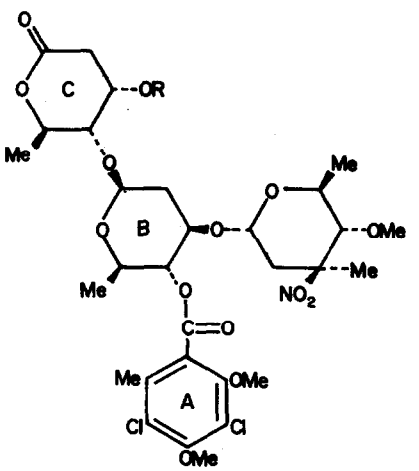


17a: Everheptose, R = H

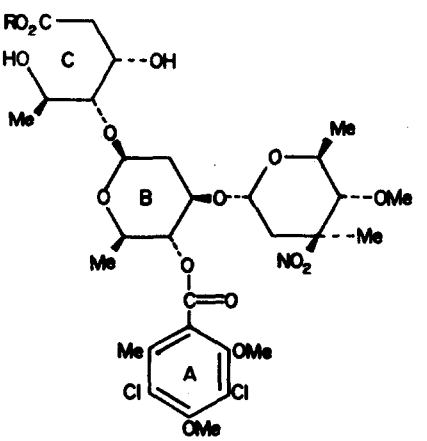
17b: Everheptose-B, R = OH

(b) Information regarding the mode of linkage of the δ-lactone (**18a**) (or its open chain hydroxy-acid equivalent **19b**) to evertetrose (**14a**) to form everheptose (**17a**) was determined[24] from the structural elucidation of the related ester (**20**), another product obtained[24] from the acidic hydrolysis of everninomicin-D. This ester (**20**) was cleaved on treatment with diazomethane to the δ-lactone (**18a**) and 2-deoxy-D-evalose (**8a**). This result, coupled with NMR and mass spectral studies with the tetra-acetate derived from **20**, established the structure of **20** and hence the linkage of the 2-deoxy-D-evalose (**8a**) residue D to the δ-lactone (**18a**) in the ester (**20**). It thus followed that the mode of linkage of the δ-lactone (**18a**) [or its hydroxy-acid equivalent (**19b**)] with evertetrose (**14a**) to form everheptose (**17a**) must be via esterification [by the hydroxy-acid (**19b**)] of the C-4 OH group of the 2-deoxy-D-evalose (**8a**) residue D of evertetrose (**14a**), thus leading to the constitution **17a** for everheptose.

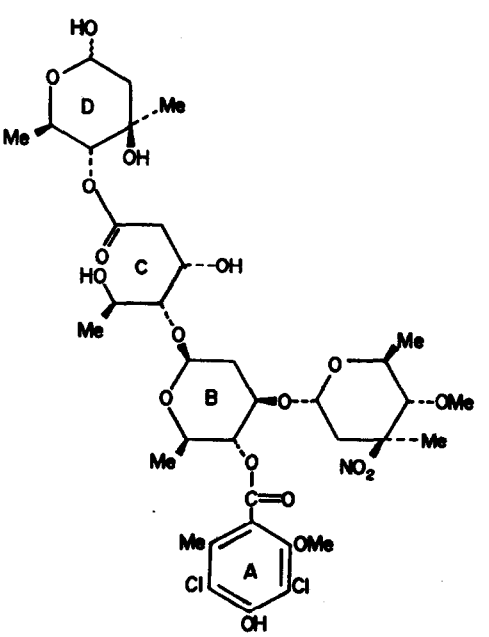
The cleavage of the C–D ester-groupings in everheptose (**17a**) and the related ester (**20**) (and also with everninomicin-D_1—see below), by diazomethane to give the δ-lactone (**18a**) is noteworthy in this structural investigation, and the mechanism of this cleavage is, at present, uncertain. One proposal[24] is that the stability of everheptose (**17a**) (and of the ester **20**) is due to the presence of a free phenolic OH group in the



18a: R = H
18b: R = Me



19a: R = Me
19b: R = H



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dichloroisoeverninoyl residue A, which may be involved in H-bonding in the molecule and when methylated cleavage of the C–D ester grouping could occur by solvolysis to give the δ-lactone (**18a**). An alternative explanation for this C–D ester cleavage may lie in the possible presence of trace quantities of base in this reaction, perhaps introduced during the generation of diazomethane, and be a result of the relative stability of the aromatic (dichloroisoeverninoyl) A–B ester group over that of the C–D ester grouping in everheptose (**17a**) (and in the ester **20**) towards base hydrolysis, under the conditions of the reaction. It is of interest that treatment of everninomicin-D (**2c**) with diazomethane gave, as expected, the corresponding (phenolic) mono-methyl ether.[6]

(vii) Mild acidic hydrolysis of everninomicin-D gave everninomicin-D_1 which differed in its empirical formula from that of the parent antibiotic by the addition of one molecule of water. The structure **21a** of everninomicin-D_1, and hence of everninomicin-D (**2c**), was established[6] by the following sequence of reactions.

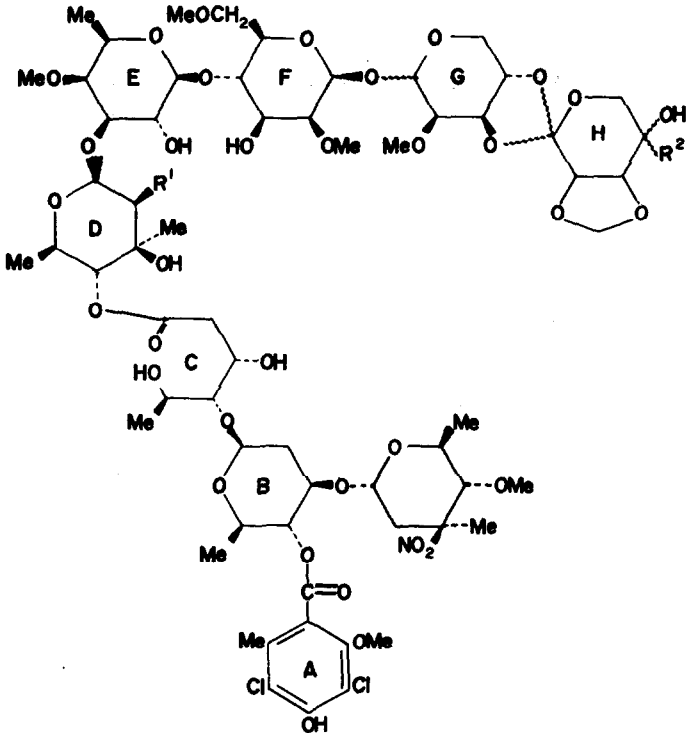
(a) Treatment of everninomicin-D_1 with diazomethane gave the δ-lactone (**18a**) and a new oligosaccharide-olgose (**22a**). Acidic methanolysis of olgose yielded evertetrose (**14a**) and an ester (**23a**), which was oxidised by sodium metaperiodate to the keto-ester (**24**). The latter compound (**24**) on heating with sulphuric acid gave formaldehyde, indicative of the presence of the methylenedioxy group.

(b) The mode of linkage of the evertetrose (**14a**) and the ester (**23a**) residues to form olgose (**22a**) was obtained from the results of acidic methanolysis of olgose permethyl ether (**22b**) which yielded evertetrose tetramethyl ether (**14b**) (where the C-3 and C-4 OH groups were free in the 2-O-methyl-L-lyxose **10a** residue G in **14b**) and the ester (**23b**) which bears a direct structural relationship to the ester (**23a**) obtained from the acidic methanolysis of olgose (**22a**) itself. Thus, the primary OH function and ester group in **23a** must be involved in linkage with the C-3 and C-4 OH groups present in the 2-O-methyl-L-lyxose (**10a**) residue G in evertetrose (**14a**) to give olgose (**22a**), and to explain the properties of olgose, linkage by means of an orthoester grouping involving these functional groups was proposed, leading to the constitution **22a** for olgose and **22b** for olgose permethyl ether. Thus, the formation of evertetrose (**14a**) and the ester (**23a**) from the acidic methanolysis of olgose (**22a**) can be readily explained by the solvolytic fission of the orthoester group in olgose (**22a**). Further support for the presence of an orthoester function in olgose (**22a**) was obtained from its ^{13}C NMR spectrum which possessed a signal at δ 119.8 ppm which was assigned to the orthoester C atom.

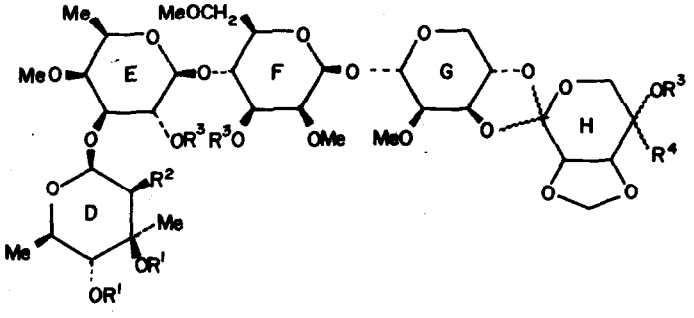
(c) Acidic hydrolysis of everninomicin-D_1 (**21a**) gave everheptose (**17a**), and from the foregoing results in sections (a) and (b) above, which showed a close correspondence of the chemical properties of everinomicin-D_1 (**21a**) with those already described for everheptose (**17a**) (see section vi), especially those involving reaction with diazomethane, it was proposed[6] that everninomicin-D_1 could be represented by the constitution **21a**.

(d) Acidic methanolysis of everninomicin-D permethyl ether gave a mixture of products from which was isolated, *inter alia*, the δ-lactone monomethyl ether (**18b**). The formation of this δ-lactone (**18b**) suggested that the OH group located in the β-position to the lactone CO function in (**18a**) (or in the open-chain hydroxy-acid equivalent **19b**) was free in everninomicin-D. A second product isolated from the acidic methanolysis of everninomicin-D permethyl ether was evertetrose dimethyl ether (**14c**) where the C-3 and C-4 OH groups present in the 2-deoxy-D-evalose residue (D) and 2-O-methyl-L-lyxose residue (G) were free in **14c**. The isolation of the evertetrose dimethyl ether (**14c**) was significant since it had already been established that the C-3 and C-4 OH groups present in the 2-O-methyl-L-lyxose (**10a**) residue G of evertetrose (**14a**) were involved in orthoester formation in olgose (**22a**) (section b above). Thus it was concluded that the OH groups located at C-3 and C-4 of the 2-deoxy-D-evalose (**8a**) residue D of evertetrose (**14a**) were also involved in an orthoester linkage with the δ-lactone (**18a**), or its hydroxy-acid equivalent (**19b**), in everninomicin-D.

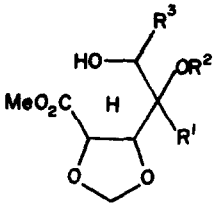
Based on these observations, the constitution **2c** was proposed for everninomicin-D by the Schering group.[6] The presence of two orthoester linkages in everninomicin-D (**2c**) was confirmed by examination of its ^{13}C NMR spectrum which showed the presence of two orthoester C atoms at δ 119.6 and δ 120.0 ppm. Thus, the conversion of everninomicin-D (**2c**) to everninomicin-D_1 (**21a**) could be explained in terms of a selective hydrolytic fission of the orthoester linkage associated with residues C and D in everninomicin-D (**2c**); whereas acidic hydrolysis under more vigorous conditions resulted in hydrolysis of *both* the C–D and G–H orthoester linkages in everninomicin-D (**2a**) to yield everheptose (**17a**) and the δ-lactone (**18a**) (or its hydroxy-acid equivalent). This increase in sensitivity of the central C–D orthoester linkage towards acidic hydrolysis, as illustrated by the formation of everninomicin-D_1 (**21a**) from everninomicin-D (**2a**), is also observed with other orthosomycin antibiotics of this type.

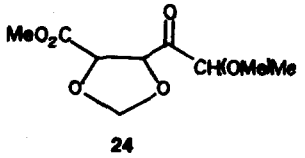


21a: Everninomicin-D$_1$, R^1 = H; R^2 = CH(OMe)Me
21b: Everninomicin-B$_1$, R^1 = OH; R^2 = CH(OMe)Me
21c: Everninomicin-C$_1$, R^1 = R^2 = H



22a: Olgose, R^1 = R^2 = R^3 = H; R^4 = CH(OMe)Me
22b: R^1 = R^3 = Me; R^2 = H; R^4 = CH(OMe)Me
22c: Olgose-B, R^1 = R^3 = H; R^2 = OH; R^4 = CH(OMe)Me
22d: R^1 = H; R^2 = OMe; R^3 = Me; R^4 = CH(OMe)Me
22e: Olgose-C, R^1 = R^2 = R^3 = R^4 = H





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23a: R^1 = CH(OMe)Me; R^2 = R^3 = H
23b: R^1 = CH(OMe)Me; R^2 = Me; R^3 = H
23c: R^1 = R^2 = R^3 = H
23d: R^1 = R^3 = H; R^2 = Me
23e: Methyl eurekanate, R^1 = COMe; R^2 = H; R^3 = Me

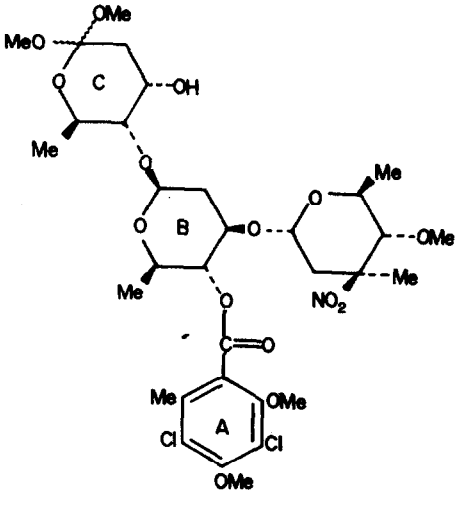
(b) *Everninomicin*-B (**2a**)

The degradation studies leading to the elucidation of the structure **2a** of everninomicin-B by the Schering group[4,25] were essentially similar to those described above for everninomicin-D, namely the identification of products obtained from the acidic hydrolysis and methanolysis of the antibiotic, and salient points from the results of these studies with everninomicin-B are summarised as follows.

(i) Aqueous acidic hydrolysis of everninomicin-B gave a heptasaccharide (everheptose-B) whose constitution (**17a**) was established by its fission, using diazomethane, to the δ-lactone (**18a**) (previously obtained from everninomicin-D) and a tetrasaccharide (evertetrose-B). The constitution **14d** of evertetrose-B was determined by (a) its acidic hydrolysis which gave evertriose (**12**) (previously obtained from everninomicin-D) and a new monosaccharide, D-evalose (**8b**), and (b) the acidic hydrolysis of evertetrose-B permethyl ether which established the mode of linkage between D-evalose (**8b**) and evertriose (**12**) thus leading to the assignment of structure **14d** for evertetrose-B, and hence everheptose-B could be represented by **17b**.

(ii) Mild acidic hydrolysis of everninomicin-B gave everninomicin-B_1 (**21b**) [compare the similar formation of everninomicin-D_1 (**21a**) from everninomicin-D (**2c**)]. Treatment of everninomicin-B_1 (**21b**) with diazomethane resulted in cleavage of the molecule with the formation of the δ-lactone (**18a**) and olgose-B [compare the similar cleavage with everninomicin-D_1 (**21a**)]. The constitution **22c** of olgose-B was deduced from the results of its acidic hydrolysis [which, *inter alia*, gave evertetrose-B (**14d**) and the ester (**23a**)] using arguments similarly advanced to explain the results from the acidic hydrolysis of olgose (**22a**) which gave the corresponding hydrolysis products evertetrose (**14a**) and the ester (**23a**).

(iii) Methylation of everninomicin-B with diazomethane followed by mild acidic methanolysis of the resulting (phenolic) monomethyl ether gave, *inter alia*, the orthoesters **25** and **26**. The isolation of these orthoesters (**25**) and (**26**) is noteworthy. Under different conditions the corresponding normal esters **19a** and **23a** were obtained from the solvolytic (acid methanolysis) fission of the intermonosaccharide orthoester linkages which were shown, from related studies (see section a above), to be present in everninomicin-D. Since the presence of an orthoester linkage, associated with residues G and H had already been established in olgose-B (**22c**), the isolation of the ester **25** also suggested the presence of a second orthoester linkage associated with the carbohydrate residues C and D in everninomicin-B (**2a**) [corresponding with the orthoester linkage similarly situated in everninomicin-D (**2c**)]. Further evidence for the presence and mode of linkage of the C–D orthoester grouping in everninomicin-B (**2a**) was subsequently obtained[4] from the products isolated from the reaction of the permethyl ether of everninomicin-B_1 (**21b**) with diazomethane, which gave the δ-lactone monomethyl ether (**18b**) and the partially-methylated derivative (**22d**) of olgose B, where the C-3 and C-4 OH groups of the D-evalose (**8b**) residue D were free in **22d**. Thus it was concluded[4] that the two OH groups located at positions C-3 and C-4 of the D-evalose (**8b**) residue D of everninomicin-B (**2a**) were involved in an orthoester linkage with the δ-lactone (**18a**), or its hydroxy-acid equivalent (**19b**), so leading to the constitution **2a** for everninomicin-B. As in the case of the conversion of everninomicin-D (**2c**) to everninomicin-D_1 (**21a**), the



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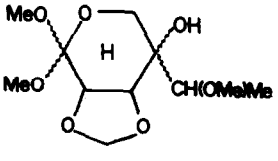

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formation of everninomicin-B_1 (**21b**) by mild acidic hydrolysis of everninomicin-B (**2a**) can also be explained in terms of the selective hydrolytic fission of the central orthoester linkage associated with residues C and D in everninomicin-B (**2a**). Acidic hydrolysis of everninomicin-B (**2a**) under more vigorous conditions resulted in hydrolysis of both the orthoester linkages associated with residues C, D and G, H of everninomicin-B (**2a**) with the formation of everheptose-B (**17b**) and the δ-lactone (**18a**) (or its hydroxy-acid equivalent).

The constitution **2a** of everninomicin-B thus differs from that (**2c**) of everninomicin-D in only one respect in that the 2-deoxy-D-evalose (**8a**) residue D in everninomicin-D (**2c**) is replaced by a D-evalose (**8b**) residue D in everninomicin-B (**2a**); in all other respects the two structures, **2a** and **2c**, are identical.

(c) *Everninomicin*-C (**2b**)

The constitution **2b** of everninomicin-C was also determined by the Schering group[5] using methods similar to those described above for the elucidation of the structures of everninomicin-B (**2a**) and everninomicin-D (**2c**), and which resulted in the formation and isolation of corresponding degradation products from everninomicin-C. Relevant points from this structural investigation[5] are as follows.

(i) Mild aqueous acidic hydrolysis of everninomicin-C gave everninomicin-C_1 (**21c**) which on treatment with diazomethane gave the δ-lactone (**18a**) and olgose-C [compare similar cleavage of everninomicin-B_1 (**21b**) and everninomicin-D_1 (**21a**) with diazomethane to give the δ-lactone (**18a**) and olgose-B (**22c**) or olgose (**22a**) respectively]. Acidic methanolysis of olgose-C resulted in the formation of evertetrose (**14a**) and the ester (**23c**), thus leading to the constitution **22e** for olgose-C.

(ii) The union of the δ-lactone (**18a**) with olgose-C (**22e**) by the formation of an orthoester linkage between these two residues (**18a** and **22e**) to form everninomicin-C was deduced[5] in the same manner as already described for everninomicin-D. Acidic hydrolysis of the permethyl ether of everninomicin-C gave a mixture of products from which was isolated the δ-lactone monomethyl ether (**18b**), evertetrose dimethyl ether (**14c**) and the ester (**23d**). Since the orthoester mode of linkage between the 2-O-methyl-L-lyxose (**10a**) residue G of evertetrose (**14a**) and the ester (**23c**) residue H had been established in olgose-C (**22e**) it followed that the C-3 and C-4 OH groups present in the 2-deoxy-D-evalose (**8a**) residue D of evertetrose (**14a**) were also involved in orthoester linkage with the δ-lactone (**18a**) (or its hydroxy-acid equivalent), thus leading to the constitution **2b** for everninomicin-C.

The structure **2b** of everninomicin-C differs from that (**2c**) of everninomicin-D only in the nature of the terminal residue H of the two antibiotics where the grouping –CH(OMe)Me present in everninomicin-D (**2c**) has been replaced by hydrogen in everninomicin-C (**2b**); in all other respects the structures of the two antibiotics are identical.

(d) *Everninomicin*-2 (**3**)

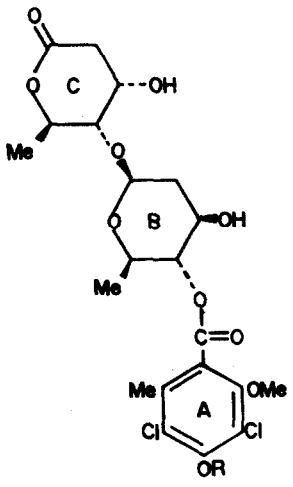
The degradative methods employed by the Schering group[7] in their determination of the structure of everninomicin-2 were again similar to those which had been employed with everninomicin-B (**2a**), -C (**2b**) and -D (**2c**), and corresponding degradation products were obtained from everninomicin-2 (**3**). The salient features of this work are outlined below.

(i) Treatment of everninomicin-2 with diazomethane gave a (phenolic) monomethyl ether, which on acidic methanolysis yielded, *inter alia*, evertetrose (**14a**), the ester (**23a**) and a δ-lactone, (**27a**) derived from flambalactone (**27b**), a product obtained from the acidic methanolysis of flambamycin (**1**) (see below).

(ii) The ^{13}C NMR spectrum of everninomicin-2 contained signals at δ 120.5 and δ 119.7 ppm which indicated the presence of two orthoester C atoms [the ^{13}C NMR spectrum of everninomicin-D (**2**) possessed corresponding signals at δ 120.0 and 119.6 ppm] in the molecule. On the basis of these results, the constitution **3** was proposed[7] for everninomicin-2.

Everninomicin-2 (**3**), in contrast to everninomicin-B (**2a**), -C (**2b**) and -D (**2c**), does not contain the evernitrose (**15**) residue linked glycosidically to the curacin residue A–B present in **2a**, **2b** and **2c**. In this respect everninomicin-2 (**3**) bears a closer structural resemblance to flambamycin (**1**) (see below) than the other related everninomicin antibiotics (**2a**, **2b** and **2c**).

The conversion of everninomicin-D (**2c**) to everninomicin-2 (**3**) has been reported.[7] This transformation required the selective hydrolysis of the evernitrose (**15**) glycosidic bond in everninomicin-D (**2c**), whilst preserving other glycosidic bonds and labile orthoester linkages which are present in the antibiotic molecule **2c**. It was considered[7] that a likely route for this structural modification of everninomicin-D (**2a**) was via the corresponding nitroso analogue (**2d**). The latter compound (**2d**) could be converted to the corresponding nitrene[7] (using triethyl phosphite or triphenylphosphine) followed by bond migration to an



27a: R = Me
27b: Flambalactone, R = H

enamine,[7] which should enable the specific hydrolysis of the required glycosidic bond to yield everninomicin-2. That this premise proved to be correct was shown by the results from the following sequence of reactions.[7] Reduction of everninomicin-D (**2c**), using aluminium amalgam in aqueous ethanol, yielded the corresponding hydroxylamino compound (**2e**), which was oxidised (sodium hypobromite) to the nitroso-derivative (**2d**). Treatment of a benzene solution of the nitroso compound (**2d**) with triphenylphosphine and subsequent purification of the reaction product by chromatography on silica gel gave everninomicin-2 (**3**) (in an overall yield of 30% from everninomicin-D) which was identical with a naturally-occurring sample. The effect of these structural modifications (**2d**, **2e**), of everninomicin-D (**2c**) on antibacterial activity will be discussed in a later section of this Report.

(e) *Flambamycin* (**1**)

As in the case of the everninomicins, the constitution **1** of flambamycin was obtained from the isolation, identification and sequential analysis of the products obtained from classical chemical degradation procedures (acidic hydrolyses, acidic methanolyses coupled with permethylation studies) with the antibiotic, in association with extensive physico-chemical measurements employing IR, UV, ^{1}H and ^{13}C NMR spectroscopy and low and high resolution mass spectrometry.[3] It should be noted carefully at this stage that the structure previously assigned[2] to flambamycin is incorrect and that subsequent investigations[3] have led to the revised structure **1** of the antibiotic, and it is this structure **1** that is included in this Report. For detailed arguments concerning the structural and stereochemical assignments to the antibiotic and its degradation products, the original papers should be consulted, as indicated in the following sequence of reactions.

(i) Acidic hydrolysis[26] of flambamycin gave curacin (**7**), 3,5-dihydroxy-γ-caprolactone (**28**), D-evalose (**8b**), 4-O-methyl-D-fucose (**13**), 2,6-di-O-methyl-D-mannose (**9**), L-lyxose (**10b**), and a disaccharide, flambabiose (**29**) which was derived from the monosaccharide units 2,6-di-O-methyl-D-mannose (**9**) and L-lyxose (**10b**).

(ii) The identification of the intermonosaccharide linkages associated with the above units was obtained from the acidic methanolysis of flambamycin permethyl ether which yielded, *inter alia*, the following significant products;

(a) 2-O-methyl-D-evalose methyl glycoside which suggested that the C-2 OH group of the D-evalose (**8b**) residue D was free in flambamycin, and that the C-3 and C-4 OH groups were involved in linkage in flambamycin;

(b) 2,4-di-O-methyl-D-fucose methyl glycoside† which indicated that the C-3 OH group of the 4-O-methyl-D-fucose (**13**) residue E was involved in linkage in flambamycin whereas the C-2 OH group was free;

†Wrongly identified as 3,4-di-O-methyl-D-fucose methyl glycoside in Ref. 2, thus leading to an incorrect structure for flambamycin.

(c) 2,3,6-tri-O-methyl-D-mannose methyl glycoside which indicated that the C-4 OH group of the 2,6-di-O-methyl -D-mannose (**9**) residue F was involved in linkage in flambamycin, whereas the C-3 OH group was free;

(d) 2-O-methyl-L-lyxose methyl glycoside, which suggested that the C-3 and C-4 OH groups of the L-lyxose (**10b**) residue G were involved in linkage in flambamycin. The isolation and identification of flambabiose (**29**) had previously established the location of the glycosidic linkage between the mannose (**9**) and lyxose (**10b**) residues F and G in flambabiose (**29**).

(iii) Mild acidic hydrolysis of flambamycin gave[3,26] a mixture of tri- and tetrasaccharides, separation of which yielded, *inter alia*, the following products:

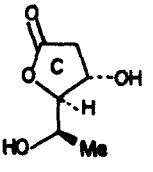
(a) flambatriose (**30a**) (compare evertriose **12**), whose identity was established[3,26] by aqueous acidic hydrolysis to give the constituent monosaccharide residues E (**13**), F (**9**) and G (**10b**);

(b) flambatetrose (**31a**), a non-reducing tetrasaccharide which was similarly shown to be derived from the monosaccharide units D (**8b**), E (**13**), F (**9**) and G (**10b**);

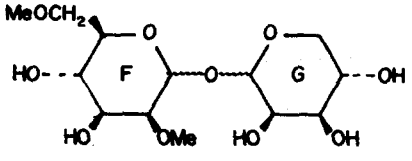
(c) flambatetrose isobutyrate (**31b**). The ^{13}C NMR spectrum of this degradation product (**31b**) indicated the presence of an isobutyrate grouping, and its location on C-2 of the lyxose residue G of the molecule **31b** was established[3,27] by comparison of its mass spectrum with that of its hexa-acetate and with that of flambatetrose hepta-acetate.

Thus the isolation of 2-O-methyl-L-lyxose methyl glycoside from the acidic methanolysis of flambamycin permethyl ether (section ii above) was due to cleavage of the 2-isobutyroyl group present in the L-lyxose residue G of flambamycin (**1**) with subsequent O-methylation under the conditions of permethylation of flambamycin (**1**).

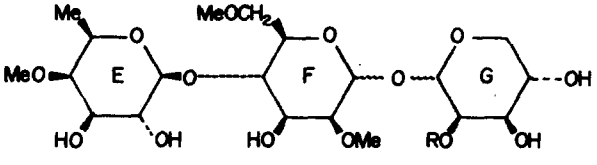
(iv) Mild acidic methanolysis of flambamycin yielded[3,27] a complex mixture of products from which was isolated curacin methyl glycoside, methyl D-evalopyranoside, flambatriose (**30a**), flambatriose isobutyrate (**30b**), flambatetrose (**31a**), flambatetrose isobutyrate (**31b**), flambalactone (**27b**) and the esters methyl flambate (**32a**) and methyl eurekanate (**23e**). The isolation of curacin methyl glycoside established the presence of a terminal curacin (**7**) residue A–B in flambamycin which was linked to the rest of the antibiotic molecule through the glycosidic oxygen of the 2-deoxyrhamnose residue B present in the



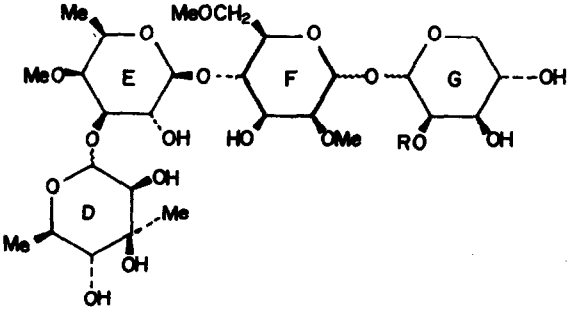
28:: 3,5-Dihydroxy-γ-caprolactone



29: Flambabiose



30a: Flambatriose, R = H
30b: Flambatriose isobutyrate, R = $COCHMe_2$



31a: Flambatetrose, R = H
31b: Flambatetrose isobutyrate, R = $COCHMe_2$

curacin modecule (7). The structure **27b** of flambalactone was obtained[3,27] from spectroscopic evidence in association with its empirical relationship to curacin (7) and 3,5-dihydroxy-γ-caprolactone (**28**) and its facile conversion, using methanolic hydrogen chloride, to methyl flambate (**32a**).

The isolation and identification[3,28] of methyl eurekanate (**23e**) from the acidic methanolysis of flambamycin deserves special mention in view of its close structural relationship with the ester (**23a**) obtained from everninomicin-B (**2a**), -D (**2c**) and -2 (**3**), and with the ester (**23c**) obtained from everninomicin-C (**2b**). The presence of an ester grouping in methyl eurekanate was indicated from spectroscopic studies and supported by its acidic hydrolysis to the corresponding acid (eurekanic acid) and its acidic ethanolysis to the corresponding ethyl ester (ethyl eurekanate). Vigorous acidic hydrolysis of methyl eurekanate gave formaldehyde (which was also similarly obtained in initial studies with flambamycin) indicating the presence of a methylenedioxy group in the molecule **23e**. Periodate oxidation gave acetaldehyde due to the cleavage of the α-glycol system [$CH_3CH(OH)$–C–OH] present in the molecule. The structure **23e** assigned to methyl eurekanate was fully supported by ^1H, ^{13}C NMR and mass spectral studies.[3,28]

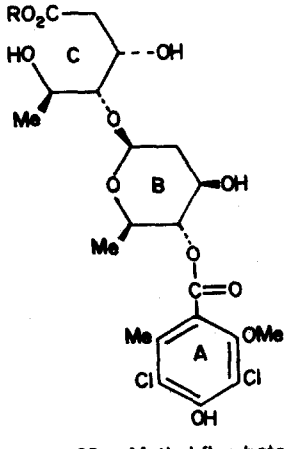
(v) The mode of incorporation of methyl eurekanate (**23e**) in the flambamycin molecule was deduced from the results[3,29] of the alkaline hydrolysis of the antibiotic. It is important to point out that in this aspect of the structural investigation of flambamycin, the normal working-up procedures after alkaline hydrolysis involved "neutralisation" with acid, and the presence of a very slight excess of acid resulted in the acidic hydrolysis of acid-labile groups during isolation procedures.†

Prolonged alkaline hydrolysis of flambamycin with subsequent acidification yielded dichloroisoeverninic acid (**6**), whereas one of the products obtained[3,29] from the alkaline hydrolysis of the antibiotic under milder conditions, with subsequent "neutralisation" with hydrochloric acid, was flambeurekanose (**33a**). The structure **33a** of flambeurekanose was deduced from the identity of the products obtained from its acidic methanolysis, which yielded flambatetrose (**31a**) and methyl eurekanate (**23e**). The combination of flambatetrose (**31a**) with methyl eurekanate (**23e**) [or its free acid equivalent] to form flambeurekanose (**33a**) by means of an orthoester linkage between the C-3 and C-4 OH groups of the L-lyxose residue G of flambatetrose (**31a**) and the primary OH and ester groups of methyl eurekanate (**23e**) was indicated[3,29] by:

(a) the demonstration that the C-3 and C-4 OH groups of the L-lyxose residue G are involved in linkage in flambamycin [section ii (d) above];

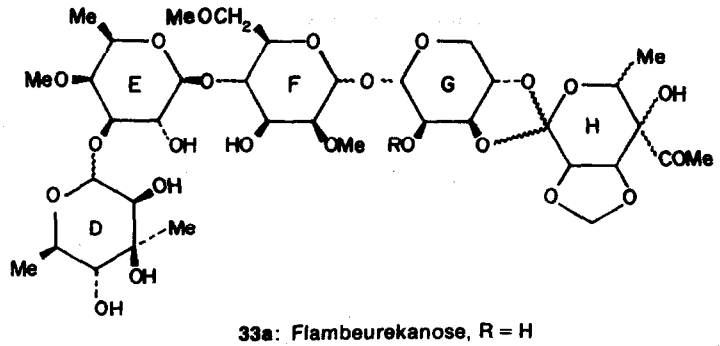
(b) flambatetrose (**31a**) forms a hepta-acetate whereas, under similar conditions, flambeurekanose (**33a**) forms a penta-acetate;

(c) a comparison of the ^{13}C NMR spectrum of flambeurekanose (**33a**) and methyl eurekanate (**23e**) demonstrated that the acetyl grouping present in methyl eurekanate (**23e**) residue H was retained in flambeurekanose, whereas the methyl ester grouping (δ 171.7 ppm) in methyl eurekanate (**23e**) was absent in flambeurekanose (**33a**) and was replaced by a corresponding signal at δ 119.8 ppm, indicative of the presence of an orthoester C atom.



32a: Methyl flambate, R = Me
32b: Flambic acid, R = H

†When suitable precautions were taken to avoid the use of acid in neutralisation processes, different but related products were obtained (see section (vii) below).



33a: Flambeurekanose, R = H
33b: R = $COCHMe_2$

On the basis of these results, the constitution **33a** was assigned to flambeurekanose, and the presence of an orthoester grouping in the molecule **33a** would thus account for its stability towards alkaline hydrolysis but its facile cleavage by acidic methanolysis to give flambatetrose (**31a**) and methyl eurekanate (**23e**). It is important to point out the close structural similarities between flambeurekanose (**33a**) and olgose (**22a**), olgose-B (**22c**) and olgose-C (**22e**) which were obtained from everninomicin-D (**2a**), -B (**2b**), and -C (**2c**) respectively. It is also important to note that at this stage of the structural investigation of flambamycin that the presence of an isobutyroyloxy group on position C-2 of the lyxose residue G of flambatetrose isobutyrate (**31b**) and flambatriose isobutyrate (**30b**) had been firmly established. This isobutyroyloxy grouping was thus similarly located in the flambamycin molecule, and had been removed during the alkaline hydrolysis of flambamycin, giving flambeurekanose (**33a**) and *not* the corresponding "flambeurekanose isobutyrate" (**33b**).

(vi) Consideration of the following empirical relation between "flambic acid"† (**32b**) and "flambeurekanose isobutyrate"† (**33b**) provided information regarding the mode of linkage between these two residues (**32b** and **33b**) in the flambamycin molecule (**1**), thus:

"flambic acid" (**32b**) + "flambeurekanose isobutyrate" (**33b**)

$$C_{21}H_{28}Cl_2O_{11} \qquad C_{40}H_{64}O_{24}$$

$$\xrightarrow[-2H_2O]{} \text{flambamycin (1)}$$

$$C_{61}H_{88}Cl_2O_{33}$$

The removal of two molecules of water from "flambic acid" (**32b**) and "flambeurekanose isobutyrate" (**33b**) to form flambamycin (**1**) must involve the formation of an orthoester linkage between the carboxyl and OH groups of "flambic acid" (**32b**) and two appropriately-placed OH groups present in "flambeurekanose isobutyrate" (**33b**). Of the six available OH groups present in "flambeurekanose isobutyrate" (**33b**), only those located on C-2, C-3 and C-4 of the terminal D-evalose residue D of (**33b**) are sterically suitable for involvement in orthoester formation. The isolation of methyl 2-O-methyl-D-evalopyranoside from the acidic methanolysis of flambamycin permethyl ether suggested that the C-3 and C-4 OH groups of the D-evalose (**8b**) residue D of **33b** were involved in linkage in flambamycin [section ii (a) above]. Further support for the presence of orthoester groupings in flambamycin (**1**) was obtained from the ^{13}C NMR spectrum of the antibiotic which showed the presence of *two* orthoester groupings with signals at δ 120.9 and δ 119.8 ppm. The signal at δ 119.8 ppm had already been assigned to the orthoester C atom between the G and H residues of flambeurekanose (**33a**) (section v above) and thus the signal at δ 120.9 ppm was assigned to the second orthoester C atom derived from the "flambic acid" (**32b**) residue A–B–C, thus leading to the constitution **1** for flambamycin. The signals at δ 120.9 and δ 119.8 ppm assigned to the orthoester C atoms present in flambamycin (**1**) correspond well with those obtained (δ 120.0, 119.6 ppm) with everninomicin-D (**2c**) and those obtained (δ 120.5, 119.7 ppm) with everninomicin-2 (**3**).

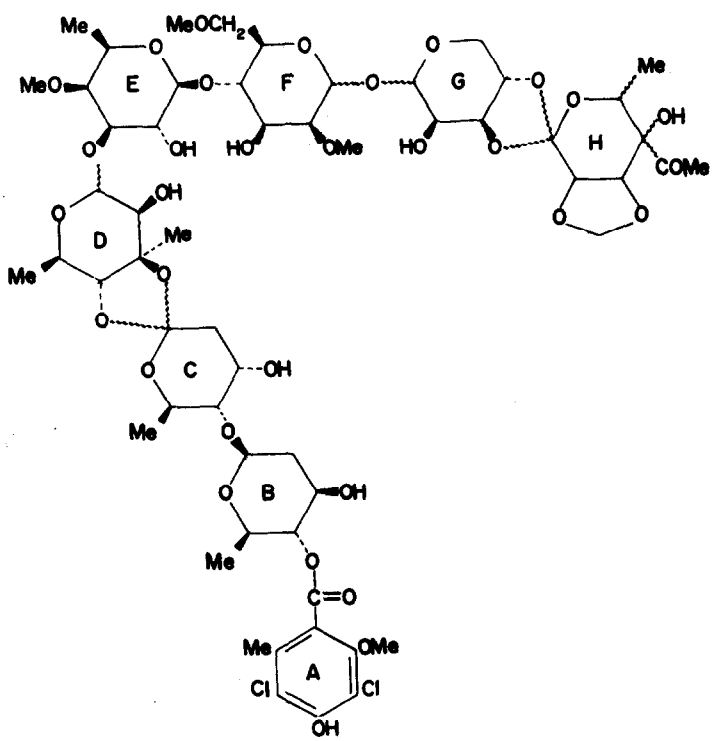
†When names are given in inverted commas, they refer only to constitutional formulae and not to isolated products.

(iii) Further evidence for the structure 1 proposed for flambamycin was obtained from the results of the following transformations:[3]

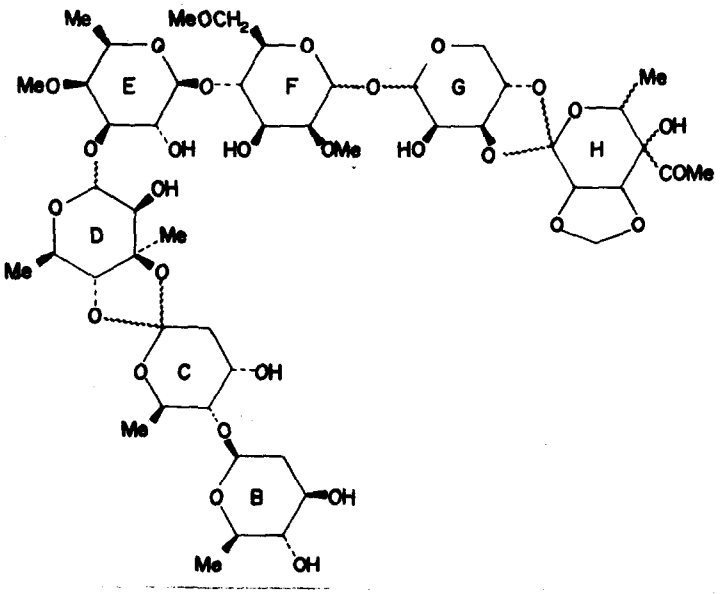
(a) hydrolysis of flambamycin (1) with a methanolic solution of potassium carbonate followed by treatment of the potassium salt with carbon dioxide gave *des*-isobutyroyl-flambamycin (34), whereas hydrolysis using aqueous sodium hydroxide, with similar work-up procedures, resulted in hydrolysis of both ester groupings giving *des*-dichloroisoeverninoyl-*des*-isobutyroylflambamycin (35), where both orthoester groupings have remained intact;

(b) mild acidic hydrolysis of flambamycin (1) using acidic resin resulted in the selective cleavage of the C–D orthoester grouping in the antibiotic (1) with the formation of flambeurekanose flambate isobutyrate (36a). A similar selective cleavage of the C–D orthoester grouping occurred with *des*-isobutyroylflambamycin (34), using identical hydrolytic conditions, and yielded flambeurekanose flambate (36b). This relative ease of cleavage of the C–D orthoester grouping over that of the G–H orthoester grouping present in flambamycin (1) and its derivative (34) is also reflected in the mass spectrum of flambamycin (1) by its more facile cleavage on electron impact.[3] It also has its parallel in the everninomicin series where, as it has already been pointed out, mild acidic hydrolysis of everninomicin-B (2a), -C (2b) and -D (2c) also resulted in the selective cleavage of the C–D orthoester grouping present in these antibiotics (2a, 2b and 2c), yielding everninomicin-B_1 (21b), -C_1 (21c) and -D_1 (21a) respectively; in each of these products (21b, 21c and 21a), the G–H orthoester grouping has remained intact.

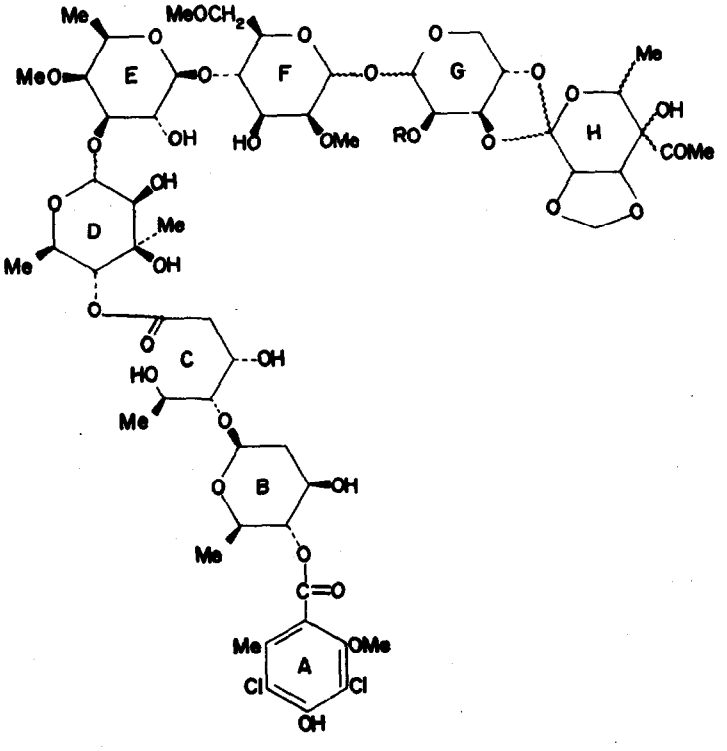
In the selective hydrolytic cleavages (1 → 36a, 34 → 36b, 2a → 21b, 2b → 21c and 2c → 21a) mentioned in the preceding paragraph, the hydrolysis of the C–D orthoester linkage in these compounds is regio-specific, since in the derived esters (36a, 36b, 21b, 21c and 21a) the ester groupings are located at the secondary C-4 position of the D-evalose (8b) or 2-deoxy-D-evalose (8a) residues D present in the derivatives 36a, 36b, 21b, 21c and 21a. The relative stabilities of the C–D and G–H orthoester groupings in flambamycin (1) and in the everninomicins-B (2a), -C (2b) and -D (2c) towards acidic hydrolysis is not, at present, understood, but the stereochemistry of the hydrolytic reactions of these orthoester groups present in the orthosomycins may well be influenced by certain aspects of stereoelectronic control, characteristic of the hydrolysis of orthoesters.[30,31]



34: Des-isobutyroyl flambamycin



35: Des-dichloroisoeverninoyl-des-isobutyroyl flambamycin



36a: Flambeurekanose flambate isobutyrate, R = $COCHMe_2$
36b: Flambeurekanose flambate, R = H

(f) *Avilamycin*

The structure of avilamycin has not yet been described, but results from initial degradation studies[15] with the antibiotic has firmly established that it possesses a number of structural features which are also present in this class of orthosomycin antibiotics, exemplified by flambamycin (**1**) and the everninomicins (**2a–2c** and **3**).

Acidic hydrolysis of avilamycin yielded[15] curacin (**7**), 2,6-di-O-methyl-D-mannose (**9**), 4,O-methyl-D-fucose (**13**), L-lyxose (**10b**) and 3,5-dihydroxy-γ-caprolactone (**28**). Thus, the residues A–B (**7**), C (**28**), E

(13), F (9) and G (10b) have been shown[15] to be present in the antibiotic molecule, and these residues are also present in, and characteristic of this class of orthosomycin antibiotics. It will be of considerable interest to see whether avilamycin can be correctly described as an orthosomycin antibiotic when its constitution has been fully elucidated.†

(g) *Curamycin*

The constitution of curamycin has not been reported, but as in the case of avilamycin, results from initial degradation studies[14] has also established that this antibiotic possesses structural features which are present in this class of orthosomycin antibiotics.

Acidic hydrolysis of curamycin yielded[14] curacin (7) and further acidic hydrolysis of the aqueous hydrolysate gave a mixture of sugars from which was isolated[14] 2,6-di-O-methyl-D-mannose (9) (= D-curamicose), 4-O-methyl-D-fucose (13) (= D-curacose) and L-lyxose (10b). Thus in the case of curamycin, the residues A–B (7), E (13), F (9) and G (10b) have been shown to occur in the antibiotic molecule and which are also present in flambamycin (1) and the everninomicins (2a–2c and 3). Further studies should confirm whether curamycin belongs to this class of orthosomycin antibiotics.

(h) *Sporocuracin A and Sporocuracin B*

A recent Japanese patent[32] has described the isolation of Sporocuracin-A and Sporocuracin-B from cultures of *Streptosporangium vulgare* var. *eborea* A-11166 (FERM-P 2494). These two antibiotics, which are active against Gram-positive bacteria, possess the same empirical formula ($C_{63}H_{94}Cl_2O_{35}$) but differ in m.p. and optical rotatory properties.[32] Sporacuracin-A and -B are stated[32] to possess a curacin (7) residue in their structure, but no further information regarding their constitution has, so far, been reported, and thus it is not known, at present, whether sporacuracin-A and -B can be correctly described as orthosomycin antibiotics. However, their reported empirical formula[32] ($C_{63}H_{94}Cl_2O_{35}$) bears an obvious relation to those of flambamycin[2,3] (1) ($C_{61}H_{88}Cl_2O_{33}$) and avilamycin[15] ($C_{63}H_{91}Cl_2O_{35}$), which suggests that these compounds may also be related in structure.

(iii) *Structural similarities and differences*

An examination of the structures of flambamycin (1), everninomicin-B (2a), everninomicin-C (2b), everninomicin-D (2c) and everninomicin-2 (3) reveals a number of sequential structural features which are common to these antibiotics, and which can be summarised as follows:

(a) a terminal curacin (7) residue A–B which is derived from 3,5-dichloroisoeverninic acid (6), residue A, and a 2-deoxy-D-rhamnose residue B;

(b) a residue C derived from 3,4,5-trihydroxyhexanoic acid and which is associated by means of an orthoester linkage with a D-evalose (8b) or 2-deoxy-D-evalose (8a) residue D;

(c) a trisaccharide sequence E–F–G which is comprised of monosaccharide units derived from 4-O-methyl-D-fucose (13) (residue E), 2,6-di-O-methyl-D-mannose (9) (residue F) and L-lyxose (10b) (residue G);

(d) a terminal residue H derived from 2,3-di-O-methylene-4,5-dihydroxyhexanoic acid or 2,3-di-O-methylene-4,5-dihydroxypentanoic acid. The terminal residue H is joined by means of an orthoester linkage with the L-lyxose (10b) residue G.

Despite these close similarities in the structures of flambamycin (1) and the everninomicins (2a, 2b, 2c and 3), there are, nevertheless, important structural features which are different in these antibiotics. The most striking of these is that everninomicin-B (2a), everninomicin-C (2b) and everninomicin-D (2c) each contain a nitro sugar residue, evernitrose (15), which is linked glycosidically to the 2-deoxy-D-rhamnose residue B of these orthosomycins (2a, 2b and 2c) whereas in flambamycin (1) and everninomicin-2 (3) this nitro sugar (15) is absent. Whether the presence of evernitrose (15) is necessary for biological activity will be discussed in a later section of this Report, but its presence and isolation from the everninomicins-B (2a), -C (2b) and -D (2c) was the first example of a naturally-occurring nitro sugar to be reported.[22,23] It has already been pointed out in this Report that the correct stereochemistry at C-3 of evernitrose (15) has recently been established[22] by X-ray crystallography.

Other structural differences between flambamycin (1) and the everninomicins (2a–2c and 3) reside in the L-lyxose residue G present in these antibiotics. In flambamycin (1) this residue is derived from

†See Addendum to this Report.

2-isobutyroyloxy-L-lyxose, whereas in the everninomicins (**2a**, **2b** **2c** and **3**) the L-lyxose residue G occurs as its 2-O-methyl ether (**10a**). In addition, the different substitution patterns which occur at C-4 of residue H in flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) may well be related to a common biogenetic pathway for these antibiotics.

Finally, a common feature of the chemical properties of flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) which has emerged from the degradation studies leading to their structural elucidation was the ease of selective fission of their central C–D orthoester linkages, which readily occurred under the conditions of mild acidic hydrolysis whilst leaving their terminal G–H orthoester linkages intact. This unique chemical property has already been discussed in section [vii (b)] under flambamycin, but the presence of the central C–D orthoester linkage and its facile selective conversion to a normal ester grouping by mild acidic hydrolysis, as indicated by the established transformations (**1**→**36a**, **2a**→**21b**, **2b**→**21c** and **2c**→**21a**), may well be related to the antimicrobial properties of these orthomycin antibiotics (**1**, **2a–2c** and **3**).

The effect of the structural modifications (**1**→**36a**, **2a**→**21b**, **2b**→**21c** and **2c**→**21a**) of flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) on antibacterial activity will be discussed in a later section of this Report, but it is important to note at this point that the stability of various preparations of these orthosomycins may also be related to this ease of selective fission of their central C–D orthoester linkages. For example, stability studies with everninomicin-D (**2c**) *using bio-assay methods* (*in vitro* activity against *S.aureus*) have shown[16] that a rapid deterioration in antibacterial activity occurred when methanolic solutions of the antibiotic were heated at 100° in the presence of various buffers ranging from pH 6.0 to pH 2.2, whereas similar preparations of the antibiotic between pH 7.0 and pH 10.0 were stable at 100° for 25 min. In addition, suspensions of purified micronised everninomicin-D (**2c**) in water slowly lost activity on heating over a period of 6 weeks, the rate depending on the temperature employed.[17] Solutions of everninomicin-D (**2c**) in acetic acid also lost antibacterial activity within a few days[17] and solutions of everninomicin-D in chloroform or methanol slowly lost activity on standing at room temperature.[17]

2. ORTHOSOMYCINS WHICH CONTAIN AN AMINOCYLITOL RESIDUE

This group of orthosomycin antibiotics which contain an orthoester linkage associated with carbohydrate residues are also characterised by the presence of an aminocyclitol residue, derived from D-streptamine, in their structures. Examples of orthosomycin antibiotics of this type are hygromycin-B(**4a**),[8] the destomycins-A(**4b**),[9,10] -B(**5**)[10] and -C(**4c**)[11] and the antibiotics SS-56-C(**4d**)[12] and A-396-I(**4e**).[12,13]

(i) *Occurrence and isolation*

Hygromycin-B(**4a**) occurs with hygromycin in extracts of cultures of *Streptomyces hygroscopicus*, and was separated from the antibiotic mixture by repeated chromatography on cation exchange resin, followed by crystallisation of the benzenesulphonate salt.[33,34] The destomycins A(**4b**), -B(**5**) and -C(**4c**) were obtained as a mixture from the culture broth of *Streptomyces rimofaciens* ATCC 21066 and were separated by chromatography of the mixture on Dowex resin giving destomycin A as the major component, and destomycin-B and -C as the minor components.[11,35] The antibiotic A-396-I(**4e**) was isolated from cultures of *Streptoverticillium eurocidicus*[36] together with a second antibiotic (A-396-II) which was identified as hygromycin B(**4a**) on the basis of analytical data, chromatographic and optical rotatory properties.[36] The antibiotic SS-56-C(**4d**) was obtained from cultures of *Streptomyces eurocidicus* SS-56 together with A-396-I(**4c**) (=SS-56-D).[12] In addition, two biologically-inactive related compounds (SS-56-A and SS-56-B), which may be regarded as biogenetic intermediates, were also isolated. The antibiotic AB-74 which may be related to, or identical with, destomycin-C(**4c**) was isolated[37] from cultures of *Streptomyces aquacanus* A 14317 together with hygromycin-B(**4a**) and the structurally-unrelated[38] neomycins-A, -B and -C.

3. DEGRADATION STUDIES LEADING TO ELUCIDATION OF STRUCTURE

The constitutions of hygromycin-B(**4a**), the destomycins-A(**4b**), -B(**5**) and -C(**4c**), and the antibiotics A-396-I(**4e**) and SS-56-C(**4d**) were established from degradation studies which, as in the case of flambamycin and everninomicins, employed the classical techniques of acidic hydrolysis and acidic methanolysis coupled with comparative optical rotatory dispersion, IR, UV, NMR, mass spectra, and chromatographic data. For detailed arguments concerning the structural and stereochemical assignments to

these orthosomycin antibiotics, the original papers should be consulted but where necessary, relevant points have been included in the following summary of structural studies with this series of orthosomycin antibiotics, in order to emphasize structural similarities and conclusions.

(a) *Hygromycin-B* (**4a**)

Initial structural studies[33,34] with hygromycin-B by the Lilly group of workers were concerned with the purification of the antibiotic together with the identification of functional groups in the molecule. Subsequent investigations[8,34] of the products from the acidic hydrolysis, under varying conditions, of hygromycin-B established:

(i) the presence of an N-methyl-2-deoxystreptamine (**37a**) (hyosamine) and a D-talose (**38a**) residue;[8,34]

(ii) the formation of a pseudo-disaccharide (hygromycin-B_2) (**39a**) which was shown to be derived from N-methyl-2-deoxystreptamine (**37a**) and D-talose (**38a**);[8,34]

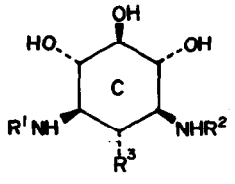
(iii) the presence of destomic acid (**40a**), a degradation product similarly obtained[8] and identified from destomycin-A (see section b below).

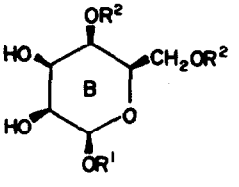
The presence of an orthoester grouping in hygromycin-B (**4a**), associated with the destomic acid (**40a**) and D-talose (**38a**) residues A and B was first suggested[8] by the formation of a crystalline undeca-acetate of hygromycin-B. Of these eleven acetyl groups, three were identified as N-acetyl groups, leaving eight O-acetyl groups present in the undeca-acetate. A consideration of the empirical formula, $C_{20}H_{37}NO_{13}$, of hygromycin-B(**4a**) showed that of the thirteen O atoms present in the antibiotic, five O atoms are therefore not associated with acetyl groups in the undeca-acetate [that is, are not acylable as OH groups under the acetylation conditions employed (pyridine–acetic anhydride)].

Of these five O atoms, one is associated with the pyranose ring of the D-talose (**38a**) residue B, and one with its glycosidic linkage to the N-methyl-2-deoxystreptamine (**37a**) (hyosamine) residue C in hygromycin B_2(**39a**), thus leaving *three* O atoms unaccounted for in the empirical formula of hygromycin-B. This led to the conclusion[8] that these three O atoms were involved in orthoester linkage of the destomic acid (**40a**) residue A to the D-talose (**38a**) residue B in hygromycin-B (**4a**). The presence of an orthoester C atom in the antibiotic **4a** was also indicated[8] from its ^{13}C NMR spectrum which possessed a signal at δ120.6 ppm (corrected from carbon disulphide to tetramethylsilane as chemical shift reference), and a comparison[8] of the ^{13}C NMR spectrum of hygromycin-B (**4a**) with that of hygromycin-B_2 (**39a**) and hyosamine (**37a**) led to the assignment of the constitution **4a** for hygromycin-B.

A further comparison[8] of the ^{13}C NMR spectrum of hygromycin-B(**4a**) with that of destomycin-A (**4b**) [see section (b) below] showed that although the two antibiotics possessed a close structural relationship, there were some differences in their spectra which were also reflected in their CD and ORD properties. These differences may be interpreted in terms of the different locations of an N-Me group on the aminocyclitol residue C, present in hygromycin-B(**4a**) and destomycin-A(**4b**) [see action (b) below] and indicated in their structures **4a** and **4b**.

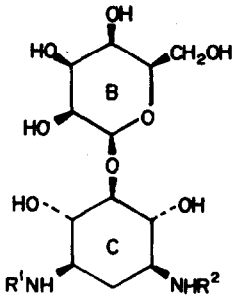
Although hygromycin-B(**4a**) occurs with hygromycin in cultures of *Streptomyces hygroscopicus*, the results from initial degradation studies[39] with hygromycin have already established that the two antibiotics bear no structural resemblance to each other.



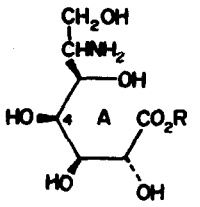


37a: Hyosamine, $R^1 = R^3 = H$; $R^2 = Me$
37b: $R^1 = R^2 = Me$; $R^3 = H$
37c: $R^1 = R^2 = R^3 = H$
37d: $R^1 = R^2 = H$; $R^3 = OH$

38a: D-talose, $R^1 = R^2 = H$
38b: $R^1 = R^2 = Me$
38c: $R^1 = Me$; $R^2 = H$



39a: Hygromycin B_2, $R^1 = H$; $R^2 = Me$
39b: $R^1 = Me$; $R^2 = H$



40a: Destomic acid, R = H
40b: R = Me

(b) *Destomycin-A* (**4b**)

This antibiotic was obtained[35] as the major component of the mixture of destomycins-A, -B and -C from the culture broth of *Streptomyces rimofaciens*. Aqueous acidic hydrolysis of destomycin-A(**4b**) gave the following products:

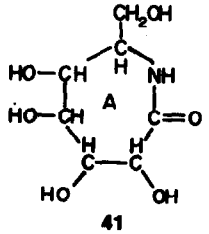
(i) a non-reducing basic glycoside (**39b**), which on further acidic hydrolysis yielded an aminocyclitol, identified as an N-methyl-2-deoxystreptamine, and D-talose (**38a**).[9,10,35,40]

(ii) a polyhydroxyamino acid named destomic acid (**40a**).[10,40]

The basic glycoside (**39b**) was thus derived from an N-methyl-2-deoxystreptamine and D-talose (**38a**) and in view of its non-reducing character, the glycosidic attachment between these two hydrolysis products must involve the anomeric C atom of D-talose (**38a**) with either C-4, C-5 or C-6 of the N-methyl-2-deoxystreptamine residue.[40] The latter aminocyclitol differed from the isomeric hyosamine (**37a**), which had been previously similarly obtained from hygromycin-B(**4a**) [see section (a) above] in that hyosamine (**37a**) was laevorotatory whereas the aminocyclitol obtained from destomycin-A(**4b**) was dextrorotatory.[40]

To distinguish between the linkage of D-talose (**38a**) with either C-4, C-5 or C-6 of the N-methyl-2-deoxystreptamine residue, the N,N'-diacetyl derivative of the basic glycoside (**39b**) was subjected to periodate oxidation which yielded formic acid (but no formaldehyde) together with *unchanged* N-methyl-2-deoxystreptamine. This result established that the position of linkage of the D-talose (**38a**) residue was at C-5 of the N-methyl-2-deoxystreptamine residue, and thus the constitutions **39b** was assigned[40] to the basic glycoside. The identity of destomic acid (**40a**) was established[41] as 2,3,4,5,7-pentahydroxy-6-aminoheptanoic acid by its conversion to the corresponding lactam (**41**), coupled with degradation studies. The position of the amino group in destomic acid (**40a**) was determined[41] by oxidation of **41** to D,L-serine.

The mode of linkage of the destomic acid (**40a**) residue A to the basic glycoside (**39b**) residue B–C to give destomycin-A(**4b**) was elucidated by the isolation and identification[9] of methyl 4,6-di-O-methyl-D-talopyranoside (**38b**) which was obtained[9] from the acidic methanolysis of tri-N-acetyldestomycin A permethyl ether. The isolation of this important degradation product (**38b**) indicated that the OH groups located at C-2 and C-3 of the D-talose (**38a**) residue B were involved in orthoester linkage with the destomic acid (**40a**) residue A, thus leading to the constitution **4b** for destomycin-A.[9] The presence of this orthoester linkage in destomycin-A (**4b**) was subsequently confirmed from its ^{13}C NMR spectrum[11] which possessed a signal at δ 121.2 ppm and which was assigned[11] to the orthoester carbon atom of the destomic acid residue A of the antibiotic (**4b**).



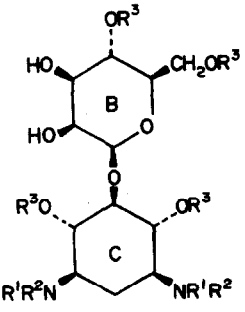
41

It should be noted that hygromycin-B(**4a**) and destomycin-A(**4b**) are diastereoisomers and differ only in the relative configuration of their respective N-methyl-2-deoxystreptamine residues C, as represented in structures **4a** and **4b** respectively. This property is reflected in differences in the optical rotations of hygromycin-B (**4a**) and destomycin-A (**4b**), [hygromycin-B (**4a**) has $[\alpha]_D^{26}+39.8$ (H_2O),[8] whereas destomycin-A (**4b**) has $[\alpha]_D^{22}+7°$ (H_2O)[35]] and of their degradation products, namely hygromycin-B_2 (**39a**), $[\alpha]_D^{26}+39.8°$ (H_2O),[8] obtained from hygromycin-B (**4a**), and the basic glycoside (**39b**), $[\alpha]_D^{22}-17.5°$ (H_2O),[40] obtained from destomycin-A (**4b**).

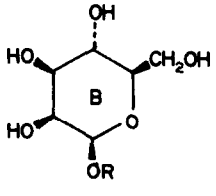
(c) *Destomycin-B* (**5**)

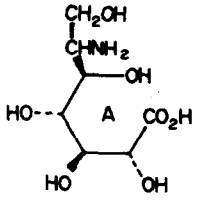
This antibiotic is present as a minor component of the mixture of destomycins,[11,35] and results from initial physico-chemical studies[35] together with its antibacterial properties[35] suggested that destomycin-B (**5**) was structurally-related to destomycin A(**4b**). This relationship was further emphasised by the identification of the products obtained from the acidic hydrolysis, under varying conditions, of destomycin-B(**5**), namely N,N′-dimethyl-2-deoxy-streptamine (**37b**), a basic non-reducing glycoside (**42a**) derived from N,N′-dimethyl-2-deoxy-streptamine (**37b**) and D-mannose (**43a**), and a polyhydroxyamino-acid which was shown[10] to be *epi*-destomic acid (**44**), where the configuration at C-4 of destomic acid (**40a**) is reversed. The mode of linkage of epi-destomic acid (**44**) to the D-mannose (**43a**) residue A of the basic glycoside (**42a**) to give destomycin-B(**5**) was indicated from the results of the acidic hydrolysis of the permethyl ether of tri-N-acetyldestomycin B which, *inter alia*, gave a neutral compound, presumably (**42b**), which on acidic methanolysis yielded methyl 4,6-di-O-methyl-α-D-mannopyranoside (**45**). As in the case of the corresponding degradation product (**38b**) from destomycin-A (**4b**) (section b above), the isolation of this compound **45** indicated that the OH groups located at C-2 and C-3 of the D-mannose (**43a**) residue B were involved in orthoester linkage with the *epi*-destomic acid (**44**) residue A in destomycin-B(**5**). It is of interest to note the change of anomeric configuration which occurred on acidic methanolysis of the β-anomer (**42b**), giving the α-mannopyranoside (**45**).

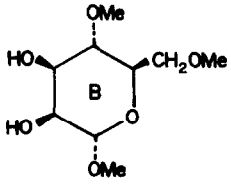
The presence of an orthoester linkage in the antibiotic **5** was subsequently confirmed[11] from its ^{13}C NMR spectrum, which possessed a signal at δ 121.7 ppm, which was assigned to the orthoester C atom of the *epi*-destomic acid residue A of destomycin-B(**5**).



42a: R^1 = Me; R^2 = R^3 = H
42b: R^1 = R^3 = Me; R^2 = COMe







43a: D-mannose, R = H
43b: R = Me

44: epi-Destomic acid

45

(d) *Destomycin-C* (**4c**)

This antibiotic is also a minor constituent[11] of the mixture of destomycins, obtained from culture filtrates of *Streptomyces rimofaciens*. The structure **4c** of destomycin-C was elucidated[11] from comparison of its ^{13}C NMR spectrum with that of destomycin-A (**4b**) and destomycin-B (**5**), together with ^1H NMR and mass spectral studies. The presence of a D-talose (**38a**) residue B in destomycin-C (**4c**) was confirmed by the isolation of methyl α-talopyranoside from the acidic methanolysis of destomycin-C (**4c**). In addition, permethylation of the tri-N-acetyl derivative of destomycin-C (**4c**) gave[11] tri-N-acetyl-mono-N-methyl-octa-O-methyldestomycin-C which was identical with that similarly obtained[11] from tri-N-acetyl-destomycin-A, thus confirming the constitution **4c** of destomycin-C.

Destomycin-C(**4c**) is thus a configurational isomer of destomycin-B(**5**) where the D-mannose (**43a**) residue B in destomycin-B(**5**) is replaced by a D-talose (**38a**) residue B in destomycin-C(**4c**).

(e) *Antibiotic A-396-I* (**4e**).

The antibiotics A-396-I and A-396-II were isolated[42] from cultures of *Streptoverticillium eurocidus* A-396. Both antibiotics possessed similar physicochemical and biological properties to those of hygromycin-B (**4a**) and the destomycins-A (**4b**), -B(**5**) and -C(**4c**). In addition, A-396-II possessed tlc and optical properties identical with those of hygromycin-B (**4a**), and on the basis of this preliminary investigation,[42] it was proposed[13,42] that A-396-I was structurally related to hygromycin-B(**4a**) and the destomycins (**4b**, **5** and **4c**), whereas A-396-II was identical with hygromycin-B (**4a**).

Subsequent acidic hydrolysis[13] of A-396-I (**4e**) confirmed the presence of 2-deoxystreptamine (**37c**), D-talose (**38a**) and destomic acid (**40a**) residues in the antibiotic **4e**, a result which further emphasised its structural relationship with hygromycin-B (**4a**). A comparison of the ^1H NMR and mass spectra of the two antibiotics (**4e** and **4a**), and of their per-acetylated derivatives, established the constitution **4e** for A-396-I, which differs from that of hygromycin-B (**4a**) in that the latter antibiotic **4a** contains an N-methyldeoxystreptamine (**37a**) residue C, whereas a deoxystreptamine (**37c**) residue C is present in A-396-I (**4e**).

(f) *Antibiotic SS-56-C* (**4d**)

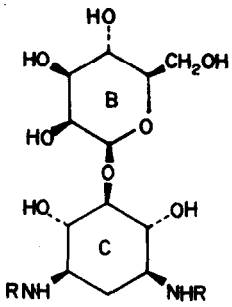
The antibiotic SS-56-C (**4d**) was isolated[12] from cultures of *Streptomyces eurocidicus* SS-56, together with a second antibiotic (SS-56-D), the main component, and two bio-inactive compounds, SS-56-A and SS-56-B. the antibiotic SS-56-D was shown[12] to be identical with antibiotic A-396-I (**4e**) on the basis of comparative physico-chemical and mass spectral studies. The structure **4d** of SS-56-C (and that of SS-56-A and SS-56-B) was elucidated[12] from degradative evidence, coupled with IR, UV, ^1H NMR and mass spectral studies and ORD, tlc and glc data, in comparison with those similarly obtained from hygromycin-B (**4a**) and destomycin-A (**4b**).

The presence of a streptamine (**37d**) residue C in SS-56-C (**4d**) was indicated[12] from the results of ^1H NMR and mass spectral studies with the antibiotic (**4d**), and this was confirmed from degradation studies, when acidic hydrolysis of SS-56-C yielded streptamine (**37d**), destomic acid (**40a**) and D-talose (**38a**). Acidic methanolysis of SS-56-C (**4d**) gave, correspondingly, streptamine (**37d**), destomic acid methyl ester (**40b**) and methyl-D-talopyranoside (**38c**). No definite evidence was obtained regarding the stereochemistry of the linkages of the three structural residues destomic acid (**40a**) (residue A), D-talose (**38a**) (residue B) and streptamine (**37d**) (residue C) in SS-56-C (**4d**), but in view of its close resemblance to the antibiotic A-396-I (**4e**) in ^1H NMR and mass spectral properties, and optical and biological properties in addition to its simultaneous production with A-396-I (**4e**) in cultures of *Streptomyces eurocidus*, the structure **4d** was assigned[12] to SS-56-C.

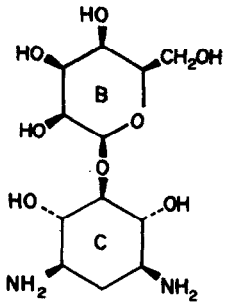
The structures of the antibacterially-inactive SS-56-A (**46a**) and SS-56-B (**47**) are of particular interest in view of their relationship to the antibiotic A-396-I (**4e**) and the other members of this group of orthosomycin antibiotics. ^1H NMR and mass spectral studies[12] showed that the structures of SS-56-A and SS-56-B were closely related and contained 2-deoxy-streptamine (**37c**) and hexoglycosyl residues. Chemical degradation studies allowed a structural distinction to be made between SS-56-A(**46a**) and SS-56-B (**47**) since acidic methanolysis of the former compound (**46a**) gave 2-deoxystreptamine (**37c**) and methyl D-mannopyranoside (**43b**), whereas acidic methanolysis of SS-56-B (**47**) yielded 2-deoxystreptamine (**37c**) and methyl D-talopyranoside (**38c**). In addition, mild acidic hydrolysis of the antibiotic A-396-I (**4e**) gave SS-56-B (**47**), and on the basis of these results, the structure **46a** was assigned to SS-56-A and structure **47** to SS-56-B.

It has already been pointed out that the compounds SS-56-A (**46a**) and SS-56-B (**47**) and the antibiotics SS-56-C (**4d**) and A-396-I (**4e**) are present in the culture fluid of *Streptomyces eurocidicus* SS-56. These compounds (**46a**, **47**, **4d** and **4e**) were isolated using conditions which excluded the presence of acid, that is conditions which would not cause the cleavage of the orthoester groups present in the antibiotics SS-56-C (**4d**) and A-396-I (**4e**). It is thus very likely that SS-56-A (**46a**) and SS-56-B (**47**) are not merely artifacts which have been produced during the isolation procedures, but that SS-56-B (**47**) is a true biogenetic precursor of the antibiotic A-396-I (**4e**).[12] If this is indeed the case, then it poses the interesting question whether the compound SS-56-A (**46a**) might also be a precursor to this group of orthosomycin antibiotics, since the D-mannose (**43a**) residue B which is present in SS-56-A (**46a**) could, theoretically, be subjected to similar orthoesterification by a destomic acid (**40a**) residue A to give a new compound which would be structurally-related to this group of orthosomycin antibiotics. Such a compound would possess a D-mannose (**43a**) residue B instead of a D-talose (**38a**) residue B, and it would thus be of considerable interest if such an antibiotic could be found in Nature.

This expectation[12] was subsequently shown to be correct by the isolation[11,35] of destomycin-B (**5**) from cultures of *Streptomyces rimofaciens*, and which contains an epi-destomic acid (**44**) residue A which is attached by means of an orthoester linkage to a β-D-mannosyl-N-N′-dimethyl-2-deoxystreptamine (**46b**) residue B–C derived from SS-56-A (**46a**).



46a: SS-56-A, R = H
46b: R = Me



47: SS-56-B

(g) *Antibiotic AB-74*

This antibiotic has been recently isolated[37] from cultures of *Streptomyces aquacanus* A-14317, together with the neomycins-A, -B and -C, and hygromycin-B (**4a**). Acidic hydrolysis of AB-74 gave destomic acid (**40a**), D-talose (**38a**) and N,N′-dimethyl-2-deoxystreptamine (**37b**). These residues (**40a**, **38a** and **37b**) also occur in destomycin-C (**4c**) and a close structural relationship of AB-74 with destomycin-C (**4c**) was also indicated by the similarity[37] of their ^1H and ^{13}C NMR spectra. The only measured differences between AB-74 and destomycin C (**4c**) were found in their optical properties, where AB-74 possessed $[\alpha]_D + 18.4°$ (H_2O) and destomycin-C (**4c**) had $[\alpha]_D + 9°$ (H_2O). The identity (or non-identity) of AB-74 with destomycin-C (**4c**) requires further clarification.

(iii) *Structural similarities and differences*

This group of orthosomycin antibiotics, exemplified by hygromycin-B (**4a**), the destomycins-A (**4b**) -B (**5**) and -C (**4c**) and the antibiotics SS-56-C (**4d**) and A-396-I (**4e**), possess a number of common structural features which can be summarised as follows:

(a) a terminal residue A derived from destomic acid (**40a**) or its 4-epimer (**44**) (epi-destomic acid) which is associated, by means of an orthoester linkage, with

(b) a residue B derived from D-talose (**38a**) or D-mannose (**43a**), which is linked glycosidically to

(c) a terminal residue C derived from D-streptamine (**37**, $R^3 = OH$) or a 2-deoxy-D-streptamine, for example **37** ($R^3 = H$).

This group of orthosomycins (**4a–4e** and **5**) thus bear a close structural relationship with each other, and accordingly, their chemical and antibacterial (*vide infra*) properties are correspondingly very similar. Minor structural differences between these antibiotics reside in configurational differences of their residues A, B and C, and also in the extent of N-methylation and C-hydroxylation of the terminal streptamine or 2-deoxystreptamine residue C. Thus, hygromycin-B (**4a**) contains an N-methyl-2-deoxystreptamine (**37a**) residue C, associated with a D-talose (**38a**) residue and a destomic acid (**40a**)

Table 1. *In vitro* antibacterial activities of everninomicin-D (2a) and flambamycin (1)

Organism	Minimum inhibitory concentration (MIC) (μg/ml)	
	Everninomicin-D[16,44]	Flambamycin[1]
Staphylococcous aureus	0.03	1.2
Sarcina lutea	0.24	0.2
Streptococcus pyogenes	0.01	0.25
Streptococcus faecalis	0.06	2
Bacillus subtilis	0.12	15
Diplococcus pneumoniae	0.15	0.1
Mycobacterium smegmatis	7.5	> 150
Escherichia coli	—	> 150
Neisseria meningitidis	—	0.6
Neisseria gonorrhoea	—	1.25

residue A whereas the isomeric destomycin-A (4b) differs from hygromycin-B (4a) only in the relative configuration of its N-methyl-2-deoxystreptamine residue C, as illustrated in a comparison of the structures 4a and 4b of the two antibiotics. Destomycin-C (4c) possesses an N,N'-dimethyl-2-deoxystreptamine (37b) residue C associated with D-talose (38a) and destomic acid (40a) residues B and A, and destomycin-B (5) differs from destomycin-C (4c) only in respect of the residues B and A which are associated with D-mannose (43a) (residue B) and epidestomic acid (44) (residue A) in destomycin-B (5). The antibiotics SS-56-C (4d) and A-396-I (4e) differ only in the constitution of the residue C present in each antibiotic (4d and 4e), where a 2-deoxystreptamine (37c) residue is present in A-396-I (4e) but is replaced by a streptamine (37d) residue in SS-56-C (4d).

4. BIOLOGICAL ACTIVITY OF THE ORTHOSOMYCINS

(I) *Orthosomycins which are esters of dichloroisoeverninic acid*

Reports[16,17,43,44] concerning the antibacterial properties of the everninomicins have, as yet, been mainly confined to the activity of everninomicin-D (2c) since this antibiotic is the major component present in the mixture of everninomicins produced by cultures of *Micromonospora carbonacea*. Everninomicin-D (2c) is highly active *in vitro* against a variety of Gram-positive bacteria (Table 1), including penicillin-resistant strains, but inactive against Gram-negative organisms, e.g. *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* and *Salmonella schotlmulleri*.[16,43] Serum-binding studies[16] (by bioassay, using *Staphylococcus aureus* or *Streptococcus pyogenes*) indicated that in the presence of 50% human serum, everninomicin-D (2c) was 94% bound. Acute toxicity studies[16,43] in mice showed that the antibiotic (2c) possessed a low order of toxicity (LD_{50} > 3000 mg/kg) by the oral, subcutaneous and intraperitoneal routes of administration, but showed a higher degree of toxicity (LD_{50} 125 mg/kg) by the intravenous route. *In vivo* studies[16,43] in mice infected with *Staphylococcus, Streptococcus* and *Diplococcus* showed that subcutaneous doses of 1–4 mg/kg of everninomicin-D (2c) protected mice against these infections. The results from the toxicity studies in mice where LD_{50} levels were only obtained from the intravenous route indicated that everninomicin-D (2c) was poorly absorbed in this species, despite its *in vivo* activity by the subcutaneous route.

The results of pharmacokinetic and clinical tolerance studies with everninomicin-D (2c) have also been reported.[43] Examination of blood-levels in dogs showed a dose/response relationship with little evidence of accumulation, and decay curves after intravenous administration of 20 mg/kg indicated a half-life of 90 min. The high serum levels obtained after intravenous administration (as compared with those obtained after intramuscular dosing) indicated that, as in mice, absorption was poor in dogs, but the levels obtained were sufficient to exert an antibacterial effect against Gram-positive bacteria.[43] The urinary and biliary routes of excretion were found[43] to be significant in dogs, dosed i.v. and i.m. with everninomicin-D (4c), and tissue distribution studies[43] revealed the presence of the antibiotic 2c in 17 tissues. The absence of everninomicin-D (2c) in cerebrospinal fluid indicated that the antibiotic did not pass the blood-brain barrier.[43]

Clinical tolerance studies with everninomicin-D (2c) in twelve subjects using intramuscular doses of 0.75, 1.0, 1.5 and 2.0 mg/kg have also been described.[43] Tissue tolerance studies indicated a possible allergic response to the antibiotic at the site of injection since primary irritation was unlikely.[43] The results from the determination of blood and urine levels after intramuscular dosing were erratic and also demonstrated poor absorption.[43] Tolerance to oral dosing was also investigated, and administration of

Table 2. *In vitro* antimicrobial activities of destomycin-A (**4b**), destomycin-B (**5**), SS-56-C (**4d**) and A-396-I (**4e**)

Organism	Minimum inhibitory concentration (MIC) (μg/ml)			
	Destomycin-A[35]	Destomycin-B[35]	SS-56-C[12]	A-396-I[12,36]
Staphylococcus aureus	80	40	50	25
Sarcina lutea	40	40	25	12.5
Bacillus subtilis	20	20	3.125	1.56
Myocobacterium smegmatis	5	40	25	25
Mycobacterium tuberculosis $H_{37}R\mu$	—	—	—	1.25
Klebsiella pneumoniae	40	40	50	12.5
Escherichia coli	40	40	100	50
Proteus vulgaris	—	—	100	100
Pseudomonas aeruginosa	—	—	50	50
Salmonella typhosa	40	20	—	25
Candida albicans	—	—	—	>50
Fusarium oxysporium	—	—	12.5	12.5
Cladosporium herbarum	1.56	1.56	3.1	—
Trichomonas vaginalis	—	—	—	20

everninomicin-D (**2c**) in single doses up to 2 mg/kg produced mild gastro-intestinal disturbances and anorexia.[43] No significant levels were found in the blood and urine after oral dosing, and the antibiotic (**2c**) was excreted in the faeces.[43]

Activity studies with everninomicin-B (**2a**) have recently been briefly described.[44,45] This antibiotic (**2a**) is reported to possess *in vitro* activity against a variety of Gram-positive bacteria and *Neisseria*, with MIC values ranging from 0.002 to 4.0 μg/ml,[44] and showed no cross-resistance with other antibiotics. The *in vitro* activity of everninomicin-B (**2a**) against *Mycoplasma* and the anaerobes *Bacteroides*, *Clostridium* and *Peptostreptococcus* has also been reported[45] and in the latter context, everninomicin-B possessed a similar order of potency as that of clindamycin.[45] Everninomicin-B was inactive against Gram-negative bacteria and was weakly tuberculostatic.[44] Single intravenous doses of up to 200 mg/kg of everninomicin-B are reported to be well-tolerated in dogs.[45]

The possible mode of action of everninomicin-B (**2a**) has also been examined and preliminary studies with *B.subtilis* have indicated a site of action at the cell membrane, leading to inhibition of metabolic uptake and DNA replication.[46]

Flambamycin (**1**) also exhibits good *in vitro* activity against a variety of Gram-positive bacteria and *Neisseria* (Table 1), but it is inactive against Gram-negative bacilli, yeasts, and filamentous fungi.[1] *In vivo* studies in mice have shown that the antibiotic (**1**) possesses good therapeutic activity against staphylococcal, streptococcal and meningococcal infections, when administered by the subcutaneous route.[1] Thus flambamycin (**1**), in common with everninomicin-D (**2c**), is active *in vivo* only by parental administration since it is poorly absorbed by the oral route and not assimilated through the intestinal tract. Acute toxicity studies in mice have shown that flambamycin (**1**) is virtually non-toxic orally, and possesses low toxicity (LD_{50} 2,500 mg/kg) by the subcutaneous route.[1]

(ii) *Orthosomycins which contain an aminocyclitol residue*

Hygromycin-B (**4a**),[33] the destomycins-A (**4b**),[35] -B (**5**)[35] and -C (**4c**),[11] and the antibiotics SS-56-C (**4d**)[12], A-396-I (**4e**)[12,36,42] and AB-74[37] all possess a similar order of weak but significant *in vitro* activity against both Gram-positive and Gram-negative bacteria, and against a number of fungi (available details are summarised in Table 2). In addition, *in vitro* antiprotozoal activity (against *Trichomonas vaginalis*) has been reported[42] for the antibiotic A-396-I (**4e**). Anthelmintic activity has also been reported for hygromycin-B against *Ascaris* in pigs,[33] and for destomycin-C against roundworms in fowls.[11] Insecticidal activity (against *Muscaria domestica*) has also been claimed[35] for destomycin-A (**4b**).

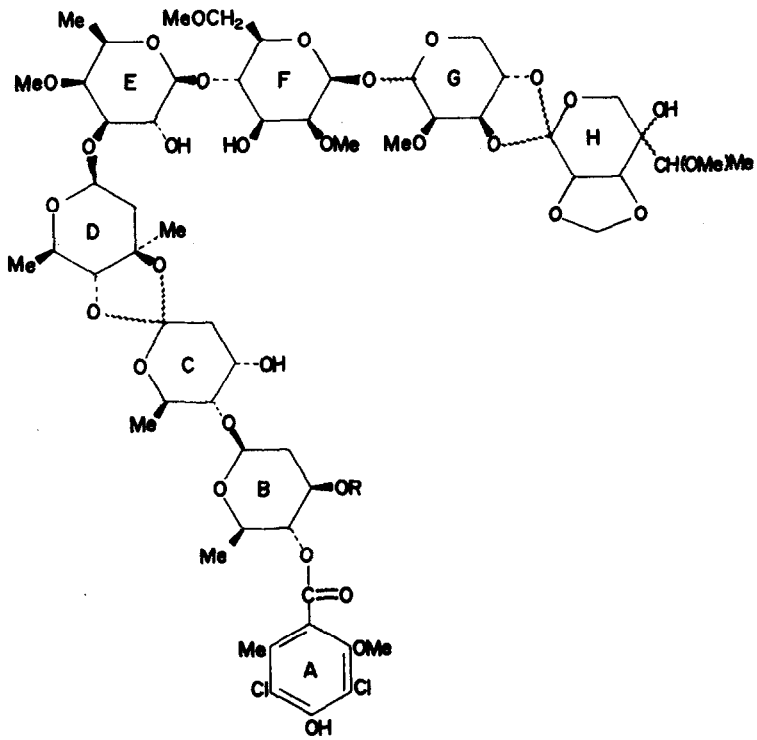
5. EFFECT OF STRUCTURAL MODIFICATION ON BIOLOGICAL ACTIVITY

(i) *Orthosomycins which are esters of dichloroisoeverninic acid*

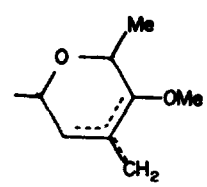
The levels of antibacterial activity which have been found for flambamycin (**1**), and the everninomicins -B (**2a**), -C (**2b**), -D (**2c**) and -2 (**3**), suggests that the presence of the evernitrose residue (**15**) present in the everninomicins -B (**2a**), -C (**2b**) and -D (**2c**) is not, *per se*, essential for antibacterial activity, and chemical modifications of this residue has produced some interesting results.

For example, examination of the antibacterial activity of the intermediate everninomicin derivatives obtained during the conversion of everninomicin-D (**2c**) to everninomicin-2 (**3**), namely nitrosoeverninomicin-D (**2d**) and hydroxylamino-everninomicin-D (**2e**) has shown that these compounds (**2d**, **2e**), possess similar levels of *in vitro* and *in vivo* antibacterial activity against Gram-positive organisms as the parent antibiotics (**2c** and **3**). Also in the case of the intermediate hydroxylaminoeverninomicin-D (**2e**), this modification of the parent antibiotic (**2c**) led to superior serum levels in the dog after intramuscular administration.[7] Further chemical modification of the evernitrose residue (**15**) present in everninomicin-D (**2c**) produced a similar trend in antibacterial activity. For example, electrochemical reduction[47] of the tetraethylammonium salt of everninomicin-D (**2c**) gave a mixture of everninomicin-D_3 (**48a**) and everninomicin-D_7 (**48b**). These compounds, (**48a** and **48b**), possessed similar levels of *in vitro* and *in vivo* antibacterial activity against Gram-positive organisms as the parent antibiotic (**2c**), and also produced superior serum levels in the dog.[47]

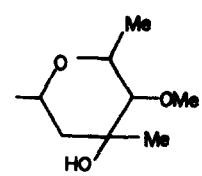
Similar antibacterial properties have also been claimed[7] for corresponding derivatives of everninomicin-B (**2a**) and -C (**2b**), for example nitroso- and hydroxylaminoeverninomicin-B, (**2f** and **2g**), and nitroso- and hydroxylaminoeverninomicin-C, (**2h** and **2i**).



48a: Everninomicin-D_3, R =



48b: Everninomicin-D_7, R =



A further structural modification of everninomicin-D (**2c**), described[17] in the initial studies with the antibiotic, was the formation of a methyl ether by treatment of everninomicin-D (**2c**) with diazomethane. The identity of this ether was not firmly established, but it is likely that this reaction resulted in the methylation of the phenolic OH function of the dichloroisoeverninoyl residue A of the antibiotic (**2c**). This methyl ether was found[17] to be inactive *in vitro* against *Staphylococcus aureus.*

An examination[48] of the *in vitro* antibacterial activity of the various transformation and degradation products of flambamycin obtained during the structural studies with the antibiotic has also produced useful information regarding structure-activity relationships. One important result that emerged from these activity studies[48] was that the presence of the C–D orthoester linkage in flambamycin (**1**) was essential for antibacterial activity, since its conversion to the normal ester function in flambeurekanose flambate isobutyrate (**36a**) was accompanied by a considerable loss in antibacterial activity. Whether this structural requirement is necessary for the antibacterial activity of other orthosomycin antibiotics of this type (e.g. the everninomicins) is not known, since the activities of everninomicin-B_1 (**21b**) -C_1 (**21c**) and -D_1 (**21a**) have not been reported.

Other factors regarding structure-activity requirements which have emerged[48] as a result of the degradation studies with flambamycin are as follows:

(a) Removal of the isobutyroyl group from the L-lyxose residue G of flambamycin (**1**) to give des-isobutyroylflambamycin (**34**) resulted in a considerable loss of *in vitro* activity against, e.g. *S. aureus.*[48] Thus, suitable derivatisation of the hydroxyl group present on C-2 of the L-lyxose residue G of flambamycin (**1**) appears necessary for antibacterial activity, and it is of interest to note in this connection that the hydroxyl group located on C-2 of the L-lyxose residue in everninomicin-B (**2a**), -C (**2b**), -D (**2c**) and -2 (**3**) is present as its methyl ether.

(b) Other major degradation products of flambamycin, for example des-dichloroisoeverninoyl-des-isobutyroyl flambamycin (**35**), flambeurekanose flambate (**36b**), flambeurekanose (**33a**) and flambalactone (**27b**), were found[48] to be devoid of antibacterial activity. These results suggest that the antibacterial activity of flambamycin (**1**) is concerned with the antibiotic molecule as a whole and not with any particular sequence of carbohydrate residues present in the antibiotic.

A comparison of the constitutions of flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) in relation to their antibacterial activity does, however, allow certain conclusions to be drawn concerning possible areas which might be explored for structure-activity relationships. These are as follows:

(a) since the replacement of the evernitrose residue (**15**) in everninomicin-B (**2a**), -C (**2b**) and -D (**2c**) by hydrogen in flambamycin (**1**) and everninomicin -2 (**3**) does not markedly effect antibacterial activity, this suggests that the hydroxyl function located at C-3 of the 2-deoxyrhamnose residue B of flambamycin (**1**) and everninomicin-2 (**3**) might be replaced by other suitable groups, including aminoglycoside residues;

(b) the replacement of the 2-OH group present in the D-evalose residue D in flambamycin (**1**), and everninomicin-B (**2a**) by hydrogen in everninomicin-C (**2b**), -D (**2c**) and -2 (**3**) without loss of antibacterial activity indicates a second area for possible structural modification, including the replacement of the D-evalose or 2-deoxy-D-evalose residue D by alternative sugar residues, *provided that* the C–D orthoester linkage and D–E glycoside linkage can be maintained using appropriately-located OH groups;

(c) further derivatisation of the 2-OH group present in the L-lyxose residue G of flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) is also a possible area for structural modification, as discussed previously;

(d) the differing but biogenetically-related substitution patterns at C-4 of residue H in flambamycin (**1**) and the everninomicins (**2a–2c** and **3**) suggests another area for structural modification, although it has not been established whether the presence of the G–H orthoester linkage and/or the presence of a 2,3-methylenedioxy group in residue H in these antibiotics (**1**, **2a–2c** and **3**) is necessary for antibacterial activity.

It will thus be of considerable interest if further examples of naturally occurring orthosomycin antibiotic of this type, which are modified in the areas (a)–(d) described above are discovered.

(ii) *Orthosomycins which contain an aminocyclitol residue*

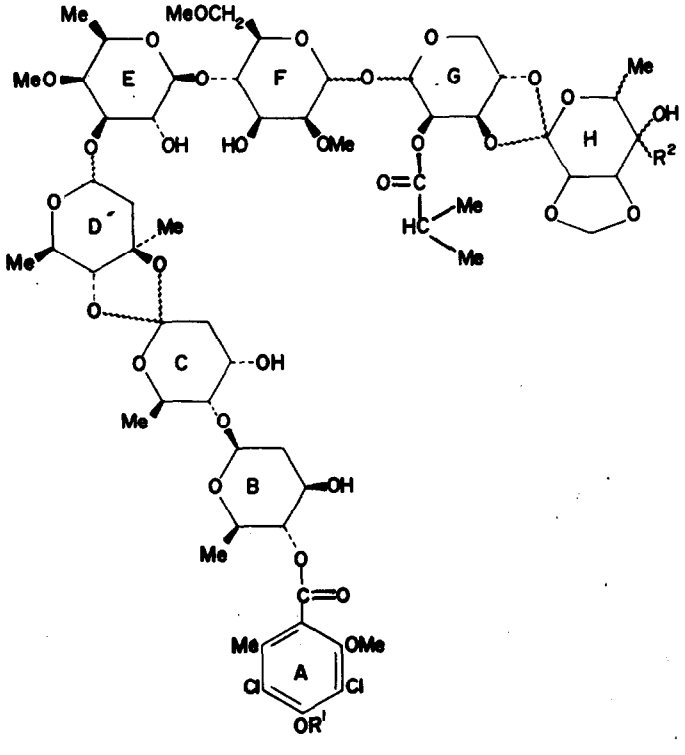
Apart from the structural studies already described in this Report, chemical modification of this class of orthosomycin antibiotics has not been reported, and thus it is difficult at this stage to identify and comment upon the effects of their structural modification on antibacterial activity. However, as in the

case of flambamycin and the everninomicins described above, an examination of the structures of hygromycin-B (**4a**), the destomycins-A (**4b**), -B (**5**) and -C (**4c**), and the antibiotics SS-56-C (**4d**) and A-396-I (**4e**) allows some observations to be made regarding structure-activity requirements, and these can be summarised as follows:

(a) the deoxystreptamine residue C present in these orthosomycin antibiotics can be variously N-methylated, as in hygromycin-B (**4a**) and the destomycins (**4b**, **4c** and **5**), without loss of antibacterial activity:

(b) the D-talose (**38a**) residue B present in **4a–4e** can be replaced by a D-mannose (**43a**) residue B, as in **5**, without loss of antibacterial activity;

(c) a common structural feature of this group of orthosomycin antibiotics is the presence of a destomic acid (**40a**) residue A, which is linked by means of an orthoester grouping to either a D-talose (**38a**) or a D-mannose (**43a**) residue B. The presence of this A–B orthoester linkage appears to be essential for antibacterial activity, since the compounds SS-56-A (**46a**) and SS-56-B (**47**), which do not possess this structural feature, are inactive. Also, in this context, it is of interest to note here that results from stability studies[35] (using bioassay methods) with destomycin-A (**4b**) have indicated that the antibacterial activity of the antibiotic decreased slightly on prolonged incubation in aqueous acidic solution (pH2.0) at 37°C, but the antibiotic was stable in aqueous solution (pH3.8–8.2) at 37°C for one month, and was also unaffected after heating in 5% aqueous ammonia at 100°C for 30 minutes.[35] The reduction in antibacterial activity observed on prolonged incubation of an aqueous acidic solution (pH2.0) of destomycin-A (**4b**) may well be due to the slow acidic hydrolysis of the A–B orthoester linkage in **4b** (to a normal ester linkage?) with the ultimate formation of the inactive hydrolysis products destomic acid (**40a**) and the basic glycoside (**39b**). Related to this proposal is the observation[8,34] that mild acidic hydrolysis of hygromycin-B (**4a**) resulted in the formation of the hydrolysis products hygromycin-B_2 (**39a**) and N-methyl-2-deoxystreptamine (hyosamine; **37a**) which lacked antibacterial activity.[8,34]



49a: Avilamycin A, $R^1 = H$; $R^2 = COMe$
49b: Avilamycin C, $R^1 = H$; $R^2 = CH(OH)Me$
49c: $R^1 = Me$; $R^2 = COMe$
49d: $R^1 = Me$; $R^2 = CH(OH)Me$

ADDENDUM

After the manuscript of this Report was completed, Professor W. Keller-Schierlein kindly made available details of the structures of avilamycin-A (**49a**) and avilamycin-C (**49b**) which are to be reported.[49,50] These orthosomycin antibiotics (**49a** and **49b**), together with avilamycin-B, were obtained[49] from cultures of the avilamycin-producing organism *Streptomyces viridochromogenes* ETH 23575, and were separated from the crude extract by chromatography on silica, when avilamycin-A and avilamycin-C were isolated in crystalline form.[49] The close structural relationship of avilamycin-A (**49a**) with avilamycin-C (**49b**) was demonstrated by the ready conversion[49] of the (phenolic) methyl ether (**49c**) of avilamycin-A to the corresponding methyl ether (**49d**) of avilamycin-C on treatment with sodium borohydride, when the methyl ketone group associated with residue H in the former compound (**49c**) was reduced to a methylcarbinol group in the latter compound (**49d**).

A comparison[50] of the ^{13}C NMR spectra of flambamycin (**1**), avilamycin-A (**49a**) and avilamycin-C (**49b**) with those of their corresponding per-acetates established the close structural relationships of these orthosomycin antibiotics. Thus, avilamycin-A (**49a**) and -C (**49b**) contain only very minor differences in their constitution from that of flambamycin (**1**). These differences are associated with residue D in **1**, **49a** and **49b**, where the D-evalose residue present in flambamycin (**1**) is replaced by a 2-deoxy-D-evalose residue in avilamycin-A (**49a**) and -C(**49b**), and with residue H, where the methyl ketone grouping in flambamycin (**1**) and avilamycin-A (**49a**) is replaced by a methylcarbinol grouping in avilamycin-C (**49b**).

Avilamycin-A (**49a**) and avilamycin-C (**49b**) can now be correctly described as members of the orthosomycin family of antibiotics which are esters of dichloroisoeverninic acid (**6**) [see previous section 1(f) above].

Information regarding the results of further studies with curamycin has also become available.[51] The presence of two orthoester carbon atoms in the antibiotic molecule has been demonstrated from its ^{13}C NMR spectrum and degradation experiments[51] with curamycin suggest that a methyl flambalactone (compare structure **27b**) residue is present in the antibiotic together with a ψ-olgose residue. The structure of the latter degradation product has yet to be fully elucidated. These results lend further support to the proposal that curamycin is a member of the orthosomycin family of antibiotics.

In addition the structure (**22a**) of olgose has been confirmed by single crystal X-ray analysis,[52] and from the results of this study the stereochemistry of this degradation product (from everninomicin-D) especially that associated with residue H in (**22a**), has now been fully established.[52]

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