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CURAMYCIN—I

ISOLATION AND CHARACTERIZATION OF SOME HYDROLYSIS PRODUCTS

OSCAR L. GALMARINI and VENANCIO DEULOFEU
Laboratorios de Investigación, E. R. Squibb & Sons, Argentina S.A.,
Martinez, Pcia. Buenos Aires, Argentina

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Abstract From cultures of *Streptomyces cura-coi*, a chlorine containing glycosidic antibiotic, named curamycin, has been isolated. Curamycin on acid hydrolysis yields a crystalline product, curacin, and a mixture of monosaccharides. Curacin, which contains all of the chlorine of curamycin, is the ester of dichloroisoeverninic acid (3,5-dichloro-4-hydroxy-6-methoxy-2-methyl-benzoic acid) with a di-desoxyaldose $C_6H_{12}O_4$, whose structure has not as yet been established. Three other monosaccharides are present, two of which could be identified as L-lyxose and 4-O-methyl-D-fucose.

FROM the culture broth of a new Streptomyces species, *S. curacoi*, isolated from a soil sample collected in the Province of La Pampa, Argentina, a chlorine containing antibiotic, named by us curamycin, has been isolated in crystalline form. Analysis revealed the composition $C_{53\ 55}H_{82\ 86}Cl_2O_{32\cdot 33}$. It gave a strong positive Molisch test for carbohydrates, the Fehling reaction being negative.

Curamycin inhibits the growth of *S. aureus* in a concentration of 0·125 mg/ml and was found to be stable at pH 7, much less so at pH 9·5 and very unstable at pH 2·0.

Curamycin, which is insoluble in sodium carbonate solution, contains one phenolic group that is responsible for its ready solubility in 0·1 N sodium hydroxide. It does not give a color reaction with ferric chloride, but gives a positive test with Millon's reagent. With diazomethane, a monomethyl curamycin is formed which is insoluble in strong alkalies and gives a negative test with Millon's reagent. The activity against *S. aureus* has also been lost.

When curamycin is heated at 100° with 0·1 N hydrochloric acid for 30 min, a product, representing 26 per cent in weight of the antibiotic, precipitates in crystalline condition, while a partially hydrolyzed mixture of carbohydrates remains in solution.

The crystalline product, which has been named curacin, contains all the chlorine and the phenolic group of curamycin and has the composition $C_{15}H_{16}Cl_2O_7$. Curacin has a $pK^{\bullet}_{MCS}$ 7·55 and is soluble in sodium hydrogen carbonate. When curacin is treated with diazomethane, a mono methylcuracin is produced which fails to give the Millon reaction and is insoluble in 0·1 N sodium hydroxide.

Curacin has been found to be an ester of an aromatic acid $C_9H_8Cl_2O_4$, identified as dichloroisoeverninic acid (I), with a carbohydrate-like substance, which is responsible for the positive Molisch, Fehling and iodoform tests. The reaction with tetrazolium salts is negative.

The ester linkage of dichloroisoeverninic acid to the carbohydrate is very sensitive to alkali. Treatment of curacin with aqueous 0·1 N sodium hydroxide at 100° for 5 min or for 24 hr at room temperature hydrolyzes the ester and dichloroisoeverninic acid can be obtained in quantitative yield by acidification and extraction with ether.

The aqueous solution containing the carbohydrate failed to yield any recognizable products.

This hydrolysis can be followed very easily by U.V.-spectrophotometry, because curacin in 0·1 N sodium hydroxide, exhibits in its U.V.-spectrum an intense peak at 294 mμ (ε 7800), which after 24 hr at room temperature has shifted to 300 mμ (ε 4700), the absorption characteristic of dichloroisoeverninic acid under the same conditions. (λ_{max} 300 mμ; ε 4750) (Fig. 1).

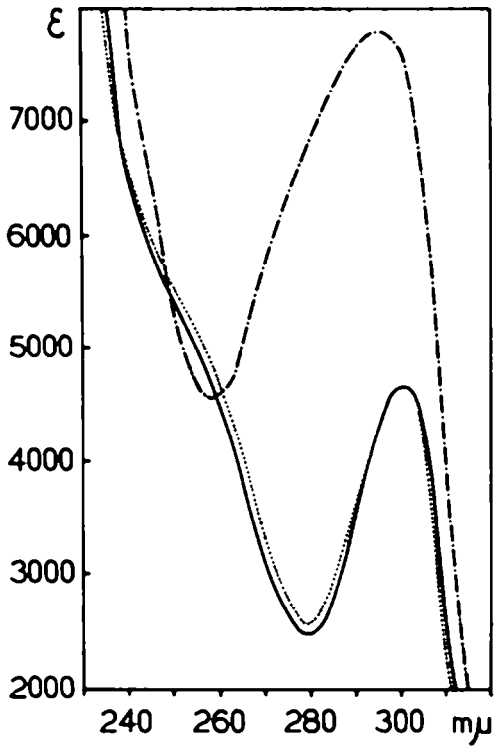


FIG. 1. U.V.-Spectra in 0·1 N sodium hydroxide.
– · – · – Curacin (After 6 min)
. . . . . Curacin (After 24 hr)
—— — Dichloroisoeverninic acid.

It is interesting to note that the hydroxamic acid test for esters is negative with curacin and methyl-curacin, but this seems to be the rule with heavily substituted benzoic acids.[1]

Curamycin in 96 per cent ethanol shows λ_{max} 284 mμ ($E^{1\%}_{1cm}$ 9·9) and from this figure and the identity of absorption of curacin in the same solvent (λ_{max} 284 mμ, ε 7400) (Fig. 2), a molecular weight of about 1360 can be calculated for the antibiotic, in fair agreement with that obtained from analytical and degradation data. From the U.V. spectra of curamycin and curacin it is evident that the dichloroisoeverninic acid is the only chromophoric group present.

If from curacin, $C_{15}H_{18}Cl_2O_7$, the dichloroisoeverninic acid residue, $C_9H_6Cl_2O_3$, is

[1] F. M. Dean, J. C. Roberts and A. Robertson, *J. Chem. Soc.* 1432 (1954).

deducted, there remains a residue $C_6H_{12}O_4$, that corresponds to the carbohydrate component. This moiety has not yet been isolated either as such or in form of a derivative, as it is decomposed during the alkaline hydrolysis. The formula $C_6H_{12}O_4$ and the positive iodoform test indicate that the carbohydrate-like compound is a di-desoxyaldohexose containing the grouping (CH_3-CHOH—). That it is an aldose follows from the fact that curacin contains a carbonyl group (semicarbazone, *p*-tosyl-hydrazone and dinitrophenylhydrazone) which can be titrated as an aldehyde according to Willstätter-Schüdel.

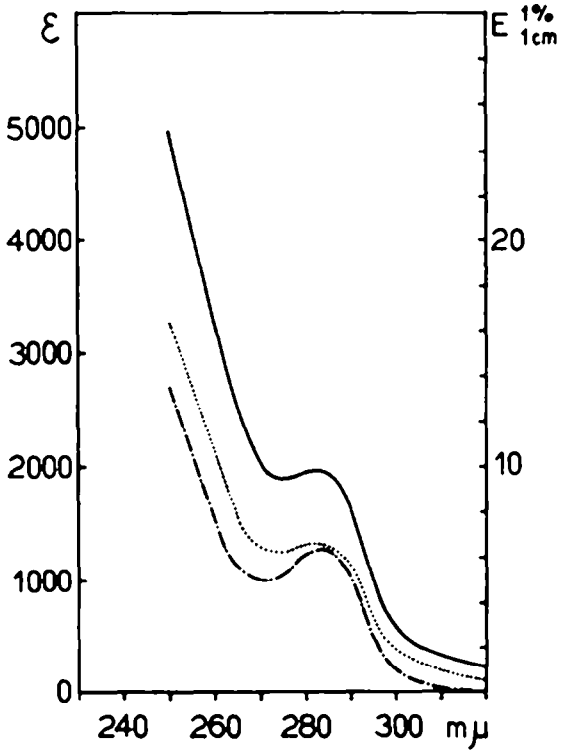


FIG. 2. U.V.-Spectra in 96% ethanol.
———— Curamycin (calc. $E^{1\%}_{1cm}$)
..... Curacin
-·-·-·-·-·- Dichloroisoeverninic acid.

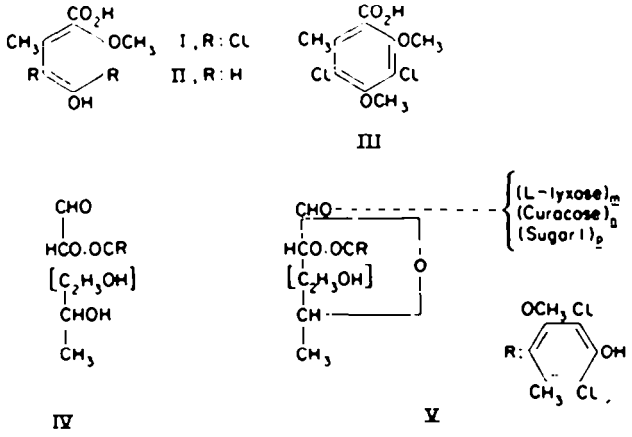
This carbonyl group can be reduced with sodium borohydride. In the resulting dihydrocuracin the ester linkage is more stable than in curacin. It is necessary to boil dihydrocuracin with 0·1 N sodium hydroxide for 100 min, to effect complete hydrolysis, whereas 5 min boiling suffices in the case of curacin.

Curacin on acetylation yields a tri-acetyl derivative. Since one of the acetyl groups must reside on the phenolic group of the dichloroisoeverninic acid residue, two acylatable hydroxyl groups must be present in the carbohydrate compound. One of these must be hemiacetalic because triacetylcuracin does not give an immediate positive Fehling reaction. This is also in agreement with the fact that curacin and

methyl-curacin, in contradiction to dihydrocuracin and triacetylcuracin, show mutarotation.

Since neither curacin nor dihydrocuracin consume periodic acid under the usual conditions of oxidation, it is clear that the two non-phenolic hydroxyls present in curacin are not located at vicinal carbon atoms. Furthermore structures in which the carbon atom next to the carbonyl contains a free hydroxyl group are excluded by these results.

In the solid state curacin seems to have at least in part an aldehyde structure as its IR-spectrum in potassium bromide shows a band at 1730 cm $^{-1}$ (ester carbonyl) as well as one at 1709 cm $^{-1}$ that can only be attributed to an aldehydo group.



That the aromatic acid, $C_9H_8Cl_2O_4$, obtained from curacin, was dichloroisoeverninic acid followed from its conversion by catalytic reduction, with Raney-Nickel as catalyst, to isoeverninic acid (II), which was identified by direct comparison with an authentic sample. Dichloroisoeverninic acid could also be obtained by treatment of curamycin with alkali.

Alkaline hydrolysis of methyl-curacin and methyl-curamycin furnished dichloro-O-dimethylorsellinic acid (III), which could be also prepared by the action of diazomethane on dichloro-isoeverninic acid and subsequent saponification.

Dichloroisoeverninic acid has not been found in nature in the free state. A pattern of substitution similar to that of dichloroisoeverninic acid is found in one of the benzene rings of some natural occurring depsidones, such as the chlorine containing compounds from lichens gangaleodine, diploicine[2] and vicanicin[3] and in the antibiotics of the nidulin group.[1] In both cases, the carboxyl contributes to the formation of a depside and the *ortho* hydroxyl participates in ether formation with a phenyl group, instead of with methyl as in isoeverninic acid.

From these data, a preliminary partial structure for curacin (IV) can be deduced. It is based in the reasonable hypothesis that the aldehydo group, by enhancing the electrophilic activity of the carbonyl of the dichloroisoeverninic acid residue, facilitates the hydrolysis of curacin by alkali and that the absence of this group in dihydrocuracin

[1] T. J. Nolan and J. Keane, *Proc. Roy. Soc. Dublin* **22**, 199 (1940); V. E. Davison, J. Keane and T. J. Nolan, *Ibid.* **23**, 143 (1943); T. J. Nolan, J. Algar, E. McCann, W. A. Manahan and N. Nolan, *Ibid.* **24**, 319 (1948).

[2] S. Neelakantan, T. R. Seshadri and S. S. Subramanian, *Tetrahedron Letters* No. 9, 1 (1959).

is cause of its greater resistance to hydrolysis. The grouping (C_2H_3OH) may or may not be branched, the only condition being that the two remaining hydroxyls cannot be located at neighboring carbon atoms and that the terminal methyl adjoins a carbon carrying a hydroxyl group (iodoform test). Hemiacetal formation between the carbonyl and one of the hydroxyls as exemplified in V will explain the mutarrotation.

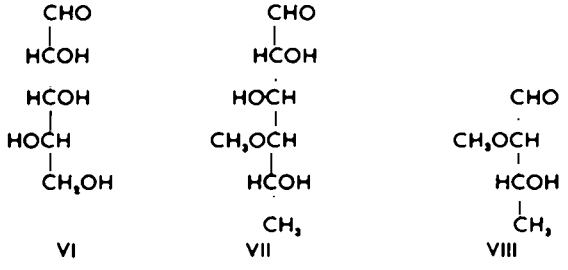
It is doubtful that curacin contains a 2-deoxy aldose sugar, because the Dische and the xanthydrol reactions are negative, although the possibility that this is due to constitutional features in the molecule cannot be dismissed.

When the material remaining in solution after the separation of curacin from the hydrolysis of curamycin was subjected to further treatment with 0·1 N hydrochloric acid and then to paper chromatography, the presence of three different substances which reacted with the usual carbohydrate reagents could be demonstrated. Preparative separation of the three components could be effected by chromatography on a cellulose column.

The substance first eluted from the column could not be crystallized, either as such or in form of derivatives and was set aside for future work. As it gives some carbohydrate reactions, it is provisionally designated sugar 1. The component in the following eluate crystallized readily and was found to be the unknown 4-O-methyl-D-fucose (VII) named by us D-curacose.

The sugar eluted with the last fractions was only obtained as a syrup. It was identified as L-lyxose (VI) by the observation that it gave an osazone m.p. 163°, identical with L-xylosazone, which when mixed with D-xylosazone melted at 205°, the m.p. reported for DL-xylosazone. The *p*-bromophenylhydrazone had the same m.p. and rotation, except for the opposite sign, as the derivative from D-lyxose. The behavior of the sugar on ionophoresis was identical to that of D-lyxose and different from the other pentoses.

This is the first time that L-lyxose (VI) has been found in a natural product, although other sugars configurationally analogous to it have been isolated from certain antibiotics. Streptose, oxystreptose and novobiose have three asymmetric carbon atoms corresponding sterically to the three central carbon atoms of L-lyxose.



The sugar $C_7H_{14}O_5$ having intermediate mobility contained one C-methyl and one methoxy group. When it was treated with hydrobromic acid according to Hough *et al.*[4] and the reaction products were submitted to paper chromatography, a faint spot with an R_f identical to that of L-fucose was observed. When larger amounts of curacose became available a fully methylated derivative could be prepared. This crystalline compound was found identical, except for the sign of rotation, with α-methyl-2,3,4-O-trimethyl-L-fucoside, prepared from L-fucose in the same manner.

[4] L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.* 1702 (1950).

This confirmed that D-curacose was an O-methyl-D-fucose. The known 2- and 3-O-methyl ethers of D- and L-fucose differ from curacose in their m.p.s and rotation (Table 1) and behavior on paper chromatography. D-Curacose is not 5-O-methyl-D-fucose, because the methylated methyl glycoside prepared from it has a pyranose structure, and it follows that it must be 4-O-methyl-D-fucose (VII).

Another methyl ether of D-fucose found in nature is the well known digitalose (3-O-methyl-D-fucose), but the 4-methyl-ether has never been isolated before. It is interesting also to note that the methylation of the 4-hydroxyl group could not be effected by Gardiner and Percival[5] by incomplete methylation of methyl-α-L-fucopyranoside.

When curacose is oxidized with an excess of sodium periodate at pH 3·6, the consumption of the reagent is completed in 2 hr with 2 moles of periodic acid reduced and one mole of formic acid produced. This seems to be a case similar to the oxidation of L-fucose described by Hough *et al.*[6] who found that the sugar consumed rapidly 3 moles of the reagent, forming a rather stable formyl ester of lactaldehyde. In our case a 3-formyl ester of 2-O-methyl-D-threose (VIII) should be formed, which explains why only 1 mole of formic acid is detected.

Curacose is dextrorotatory and does not mutarotate. It is interesting that all the known ethers of D-fucose are dextrorotatory (Table 1).

TABLE 1. MONO-O-METHYL ETHERS OF D- AND L-FUCOSE

Substances	M.p.	$[\alpha]_D$ (H_2O)	Reference
2-O-Methyl-D-fucose	155°; 161°	+73° → +87°	*a*
2-O-Methyl-L-fucose	151°	−87·2°	*b*
3-O-Methyl-D-fucose (D-digitalose)	119°	+106°	*c*
3-O-Methyl-L-fucose	109°; 110°	−97°	*b*
4-O-Methyl-D-fucose (D-curacose)	131°; 132°	+82°	

a H. B. MacPhillamy and R. C. Elderfield, *J. Org. Chem.* **4**, 150 (1939).
b J. G. Gardiner and E. Percival, *J. Chem. Soc.* 1414 (1958).
c J. D. Lamb and S. Smith, *J. Chem. Soc.* 442 (1936).

It is evident that in curamycin, the curacin moiety (IV) is linked glycosidically to an oligosaccharide of a molecular weight about 1000, formed by several molecules of L-lyxose, D-curacose and sugar 1. It does not reduce Fehling reagent and hence cannot contain a free carbonyl group. Schematically curamycin structure can be represented shown in V, where *m*, *n* and *p* are the number of molecules of each individual sugar present in the oligosaccharide.

EXPERIMENTAL

M.p.s are not corrected. pK^{*}_{MCS} were determined in methylcellosolve-water (80:20 by weight).

Streptomyces cura-coi was grown in a medium containing 3% (by weight) of cotton seed meal, 5% corn starch, 0·015% protein hydrolyzate, 0·5% sodium chloride, 0·2% calcium carbonate and water added to complete 100 parts in volume.

Preparation of curamycin

Ten liters of harvested broth, with a content of 155 mg of curamycin per liter (based on biological potency), were adjusted to pH 9·5 with 40% sodium hydroxide solution, 900 g filter aid added, the

[5] J. G. Gardiner and E. Percival, *J. Chem. Soc.* 1416 (1958).
[6] L. Hough, T. J. Taylor, G. H. S. Thomas and B. M. Woods, *J. Chem. Soc.* 1213 (1958).

mycelium filtered and the cake washed with 1 liter of water three times. The filtrate and washings were adjusted to pH 7·0 with 10% hydrochloric acid and extracted twice with 2500 ml of ethyl acetate, the activity passing into the solvent. The extracts were concentrated in vacuum to 500 ml, the concentrate was washed with 250 ml water. After addition of 5 g of decolorizing charcoal added to the ethyl acetate layer and filtration, the solution was concentrated to 30 ml. On standing overnight at 5°, the curamycin precipitated in crystalline form (930 mg, m.p. 196–198°). It was recrystallized by dissolving it in a minimum amount of boiling ethanol (98%) and adding after cooling four volumes of petroleum ether (b.p. 69–72°) and one volume of absolute ethyl ether (m.p. 198°). For analysis the material was recrystallized several times from isopropyl alcohol and then from ethyl acetate. Needles, m.p. 198°, $[\alpha]_D$ −5·3° (*c*, 1; chloroform); 5·0° (*c*, 1·0; pyridine); no mutarrotation. λ_{max} 284 mμ, $E^{1\%}_{1cm}$ 9·9. pK^{*}_{MCS} 7·48 (Found: C, 48·85, 49·12; H, 6·45, 6·49; Cl, 4·67, 5·05; OCH_3, 8·85; (C) CH_3, 9·38. $C_{53}H_{83}Cl_2O_{23}$ (1301) requires C, 48·89; H, 6·30; Cl, 5·45; $4OCH_3$, 9·53; 8 (C)CH_3, 9·22. $C_{54}H_{86}Cl_2O_{23}$(1345) requires C, 48·89; H, 6·30; Cl, 5·45; $4OCH_3$, 9·53; 8(C) CH_3, 9·22 C_{53}-$H_{86}Cl_2O_{23}$ (1345) requires C, 49·06; H, 6·38; Cl, 5·27; $4OCH_3$, 8·99; 8 (C)CH_3, 8·90%).

Curamycin is easily soluble in acetone, chloroform, acetic acid and pyridine; less so in methanol, ethanol, isopropyl alcohol and butyl acetate; almost insoluble in water, ether and benzene. Curamycin gives a positive Molisch, anthrone and furfural reactions; Fehling and Tollens tests are negative. It gives a positive Millon's test for phenols, although other phenolic reactions are negative (Folin, ferric chloride). A positive iodoform test is obtained.

Methyl curamycin

Three hundred milligrams of curamycin were dissolved in a mixture of 7 ml ethanol and 7 ml ethyl acetate. An excess of diazomethane in ether was added and the solution left at 5° for 72 hr. The solvents were then evaporated to dryness and the solid, partially crystalline, residue, was recrystallized from 5 ml ethanol. 283 mg of *methyl curamycin* were collected by filtration. Needles, m.p. 224°, unchanged by further crystallization. $(\alpha)_D$ −6·6 (*c*, 1·21, pyridine). The substance was purified for analysis by repeated recrystallization from benzene. (Found: C, 49·97, 50·37; H, 6·45, 6·34; Cl, 5·50, 5·33; OCH_3, 11·59. $C_{54}H_{84}Cl_2O_{23}$ (1315) requires: C, 49·26; H, 6·38; Cl, 5·39; $5OCH_3$, 11·78. $C_{55}H_{88}Cl_2O_{23}$ (1359) requires: C, 49·44; H, 6·47; Cl, 5·22; $5OCH_3$, 11·40%).

Methyl curamycin is easily soluble in acetone, chloroform, acetic acid, pyridine and ether and less soluble in methanol, ethanol, with a low solubility in benzene. It gives the same reactions as curamycin, except for the Millon's reaction, which is negative.

Mild acid hydrolysis of curamycin

(a) *Curacin.* One gram of curamycin was suspended in 100 ml 0·1 N hydrochloric acid and the solution was heated in a boiling water bath, with good agitation, for 30 min. The solid dissolved slowly and when the heating was finished, a clear, light yellow solution was obtained. It was filtered hot to eliminate a very small amount of insoluble dark products. On cooling a crystalline precipitate began to appear that after standing overnight at 5° was filtered. Yield: 267 mg, m.p. 138–140°. Recrystallized several times from chloroform, irregular plates, m.p. 145°, $[\alpha]_D$ +41·3 → +21·3 (20 hr) (*c*, 1·0, pyridine). pK^{*}_{MCS} 7·55. (Found: C, 45·77, 45·78; H, 5·15, 4·85; Cl, 17·93, 18·28; OCH_3, 8·13; (C)CH_3, 10·87; CHO, 0·96. $C_{18}H_{31}Cl_3O_7$ requires: C, 45·33; H, 4·53; Cl, 17·88; $1OCH_3$, 7·83; 2 (C)CH_3, 7·55%).

Curacin is very soluble in methanol, ethanol and acetone; less so in chloroform and benzene, sparingly soluble in ethyl ether and water. It is soluble in sodium hydrogen carbonate solution. It gives a positive Molisch, Tollens, Fehling, Millon and Folin (phenol) reaction and a positive iodoform test. The ferric chloride test is negative.

(b) *Carbohydrate fraction.* The mother liquors from the filtration of curacin, which give a strong Molisch and Fehling test, were treated with Amberlite IR 4B until the solution was free of chlorine ions, concentrated in vacuum to a small volume and freeze dried. 658 mg of a vitreous yellow product were obtained and employed in the work described further below.

Methylcuracin

500 mg of curacin were suspended in 10 ml ethyl ether and treated with an excess of diazomethane in ether. After 24 hr the clear solution was evaporated and a fresh solution of diazomethane added. The glassy residue obtained on evaporation crystallized by dissolving it in 3 ml of dry benzene

(m.p. 118–119°). For analysis it was recrystallized first from benzene and then from water, m.p. 118–119°: $(\alpha)_D$ +56° → +45° (5 hr) (*c*, 1·0 pyridine). λ_{max} 284 mμ (ε 570) in ethanol. (Found: C, 48·15; H, 5·20; Cl, 17·52; OCH_3, 15·12. $C_{13}H_{16}Cl_2O_7$ requires: C, 48·60; H, 5·08; Cl, 17·97; $2OCH_3$, 15·77%).

Methyl curacin is very soluble in ether, chloroform; less in methanol, ethanol, benzene; soluble in boiling water, poorly soluble in cold water. Millon and Folin reactions are negative. The Molisch, Fehling and Tollens tests are positive. It behaves as a neutral product, consuming no alkali when titrated in 50% ethanol. It is insoluble in 0·1 N sodium hydroxide.

Curacin semicarbazone

One hundred milligrams of curacin were suspended in 5 ml of a water solution containing 500 mg semicarbazide hydrochloride and 750 mg sodium acetate. By heating at 80°, curacin dissolves in about 15 min. On cooling a crystalline precipitate appeared that was filtered and recrystallized from ethanol (prisms, m.p. 168°). It is soluble in saturated sodium hydrogen carbonate solution. (Found: C, 44·13, 44·08; H, 4·87, 4·88; Cl, 16·13; N, 9·37, 9·47. $C_{14}H_{21}Cl_2N_3O_7$ requires: C, 43·83; H, 4·79; Cl, 16·21; N, 9·58%).

Curacin p-*tosylhydrazone*

Five hundred milligrams of curacin and 500 mg of *p*-tosylhydrazide were suspended in 30 ml ethanol and refluxed for 30 min. A clear solution was obtained that on cooling yielded long needles, that were filtered and recrystallized from methanol, m.p. 176°. It is soluble in 0·1 sodium hydroxide, insoluble in sodium hydrogen carbonate and sodium carbonate. (Found: C, 47·72; N, 4·83; Cl, 12·85; N, 5·59; S, 6·08. $C_{21}H_{24}Cl_2N_2O_9S$ requires: C, 48·05; H, 4·91; Cl, 12·93; N, 5·10; S, 5·84%.)

Curacin dinitrophenylhydrazide

Twenty milligrams of curacin were treated with the usual acidic dinitrophenyl hydrazine–ethanol reagent and heated in a boiling water bath for 2 min. On cooling, a gummy precipitate was formed, which when separated and treated with 50% ethanol, solidified. On recrystallization from 70% ethanol yellow needles were obtained, m.p. 202–203°. Soluble in 0·1 N sodium hydroxide, insoluble in sodium carbonate. (Found: C, 44·63; H, 3·97; N, 10·53. $C_{21}H_{22}Cl_2N_4O_{10}$ requires: C, 44·90; H, 3·90; N, 9·98%).

Dihydrocuracin

Two hundred milligrams of curacin were dissolved in 20 ml of water containing 200 mg of sodium borohydride. After 20 min the solution was acidified to pH 2 with 5 N hydrochloric acid and extracted four times with 10 ml of ethyl ether. The ether was well washed and evaporated to dryness, giving 170 mg of a vitreous solid. It was dissolved in boiling butyl ether and on standing at room temperature and scratching, irregular prisms were collected. After recrystallization from hot butyl ether, the m.p. was 90–93°. $[\alpha]_D^{28}$ +17·6 (*c*, 1·0 pyridine, no mutarrotation). λ_{max} 284 mμ (ε 1330). (Found: C, 47·17; H, 5·34; Cl, 18·19. $C_{13}H_{20}Cl_2O_7$ requires: C, 46·99; H, 5·25; Cl, 18·53%.)

Triacetylcuracin

A solution of 800 mg of curacin in 40 ml pyridine and 32 ml acetic anhydride was boiled for 5 min. After cooling the solution was poured into 300 ml of ice-water. The resulting gummy precipitate solidified on addition of fresh cold water. The solid was then collected and well dried, (560 mg). On digestion with a few ml of dry ether it crystallized. On three recrystallizations from the minimum amount of boiling ethanol 400 mg of small short prisms were obtained, m.p. 193–194°. $[\alpha]_D$ +15·8 (*c*, 1·0 pyridine). No change in rotation was detected in 24 hr. λ_{max} 284 mμ (ε 905) in ethanol. (Found: C, 49·71; H, 4·71; Cl, 13·77; CH_3CO, 26·46. $C_{18}H_{18}Cl_2O_7(COCH_3)_3$ requires: C, 49·70; H, 4·14; Cl, 14·00; $3CH_3CO$, 25·44).

Alkaline hydrolysis of curacin. Dichloroisoeverninic acid (I)

One hundred milligrams of curacin were dissolved in 6 ml of 0·1 N sodium hydroxide solution and left for 24 hr at room temperature. After standing, the clear brown yellow solution was acidified to pH 2 with 2 N hydrochloric acid and extracted 3 times with 3 ml of ethyl ether. On evaporation

of the ether a crystalline residue was obtained which when recrystallized from boiling water (Darco) gave 30 mg of prisms, m.p. 129–130°, unchanged by further crystallization. λ_{max} 284 mμ (ε 1270) in ethanol. $pK^{\bullet}_{MCS}$ 5·47; 9·51. (Found: C, 43·30; H, 3·36; Cl, 28·58; OCH_3, 12·60. $C_9H_8Cl_2O_4$ requires: C, 43·15; H, 3·26; Cl, 28·37; 10 CH_3, 12·35%.)

The brown solution remaining after the extraction of dichloroisoeverninic acid gave a strong Fehling reaction. The same results were obtained by boiling the alkaline solution of curacin for 5 min under nitrogen.

Alkaline hydrolysis of dihydrocuracin

A solution of 13 mg of hydrocuracin in 2·6 ml of 0·1 N sodium hydroxide was boiled under nitrogen. Hydrocuracin gradually disappeared and after 100 min the only acidic compound detected was dichloroisoeverninic acid.

The solution was then acidified to pH 2 with hydrochloric acid and the dichloroisoeverninic acid extracted with ether and identified in the usual way (m.p. 129°). The aqueous solution gave a negative Fehling and Molisch reaction. No effort was made to characterize the fraction remaining in solution.

For the control of the hydrolysis, descending chromatography on Whatman No. 1 was employed. The mobile system was the upper phase of a mixture of benzene:acetic acid:water (2:2:1). For development a mixture (1:1) of 1% solutions of potassium ferricyanide and ferric chloride was used.

Isoeverninic acid (II)

One hundred and sixty milligrams of dichloroisoeverninic acid were dissolved in 12 ml of methanolic 1 N potassium hydroxide solution, 5 ml of the usual Raney nickel suspension added and the solution was shaken with hydrogen at 5 atm pressure for 24 hr. After filtering the catalyst, the solution was evaporated to dryness, the residue dissolved in water, acidified to pH 2 and extracted with ether. The ether was washed with water and after drying gave on evaporation 120 mg of small prisms, which after recrystallization from water (Darco), melted at 174–176°. This m.p. was not depressed on admixture of a sample of isoeverninic acid with m.p. 176° prepared by oxydation of acetylisoeverninic aldehyde by an adaptation of the method of Robertson and Stephenson for everninic acid.[7]

Dichloro-O-dimethylorsellinic acid (O-*methyl-dichloroisoeverninic acid*) (III)

Twenty milligrams of the acid melting 129° were dissolved in 4 ml of ethyl ether and an excess of ethereal solution of diazomethane was added. After 48 hr standing in the cold, the ether was evaporated, and the residue refluxed for 1·5 hr in a solution of 2 ml of ethanol and 1·6 ml sodium hydroxide. The alcohol was evaporated in vacuum and the aqueous solution washed with 3 ml ethyl ether, acidified to pH 2 with 20% hydrochloric acid and cooled. White prisms formed which were collected (14 mg), and recrystallized from petroleum ether (b.p. 69–72°) gave a m.p. 135° (Nolan and Murphy).[8] give m.p. 135–136°). $pK^{\bullet}_{MCS}$ 4·91. (Found: C, 45·52; H, 4·02; Cl, 26·48; $C_{10}H_{10}Cl_2O_4$ requires: C, 45·28; H, 3·77; Cl, 26·78%).

Dichloro-O-dimethylorsellinic acid

(a) *From methyl curamycin.* Fifty milligrams of methyl curamycin were suspended in 5 ml 0·1 N hydrochloric acid and heated in a boiling water bath for 30 min. The antibiotic dissolves slowly and a gummy precipitate appeared. After cooling, the water solution was decanted and the precipitate dissolved in ether. The ethereal solution was washed with water, dried and evaporated to dryness. The amorphous residue was heated at 100° for 5 min, with 0·1 N sodium hydroxide. The solution was cooled, acidified with hydrochloric acid and extracted with ethyl ether. On evaporation the ethereal extract gave a solid residue that was recrystallized from petroleum ether boiling 69–72°; it was obtained as long prisms, m.p. 133–134°. It gave no depression in the m.p. when mixed with the acid obtained by methylation of dichloroisoeverninic acid.

(b) *From methyl curacin.* Methyl dichloroisoeverninic acid was obtained by a similar alkaline hydrolysis of methyl curacin, m.p. 135°.

[7] A. Robertson and R. J. Stephenson, *J. Chem. Soc.* 1388 (1932).
[8] T. J. Nolan and D. Murphy, *Proc. Roy. Soc. Dublin* **22**, 315 (1940).

Acid hydrolysis of the carbohydrate fraction

The 658 mg of freeze-dried carbohydrate containing fraction obtained by mild acid hydrolysis of 1 g of curamycin were dissolved in 140 ml of 0·1 N hydrochloric acid and heated in a boiling water bath for 3 hr. The chlorine ions were eliminated by treating batchwise with Amberlite IR4B, the chlorine free solution was concentrated to about 40 ml and finally freeze dried. The material so obtained showed three different spots in papergrams, with R_f's 0·28, 0·52 and 0·59 (descending chromatography, Whatman No. 1, n-butanol:ethanol:water (4:1:5); upper phase was used as mobile phase).

Column chromatography of the hydrolysis products

A column of 18 × 300 mm packed with Whatman Standard Grade Cellulose powder equilibrated with solvent (n-butanol:water, 40:6) was used. Crude hydrolysate (490 mg) in 6 ml of solvent was placed on the top of the column and chromatography carried out as usually. Fractions of about 3 ml were collected and analyzed for the presence of aniline phtalate positive substances. Tubes containing fractions with the same R_f were pooled. As expected, the substance with R_f 0·59 was eluted first, followed by the one with R_f 0·52. Finally that of R_f 0·28 was obtained.

The solutions were evaporated in vacuum, while water was added to eliminate the n-butanol, and then freeze-dried. The amounts recovered were 141 (R_f 0·59); 152 (0·52) and 123 mg (0·28).

Identification of the substance with R_f 0·28 as L-*lyxose* (VI)

The freeze-dried solid was a water clear syrup that could not be crystallized. It reduced Fehling solution and gave a positive Bial test. After several chromatographic trials failed to separate D-lyxose and D-xylose, it was found that ionophoresis with 220 V on Whatman No. 1 impregnated with borate buffer at pH 9·6 during 4 hr, gave a spot (aniline phtalate) that migrated like D-lyxose and was different from the remaining pentoses.[9]

The phenylosazone was prepared in the usual way and melted at 162 163° $[\alpha]_D^{25}$ + 9·0° → 41·2° (24 hr) (*c*, 0·5 pyridine:ethanol, 1:1). Mixed with a sample of L-xylosazone prepared from L-xylose, with m.p. 182 and $[\alpha]_D^{25}$ - 8·5° → 42·0° no depression was observed. In mixture with D-xylosazone of m.p. 162°, the m.p. was of 204–205°. Zerner and Waltuch[10] reported a m.p. 205° for DL-xylosazone.

The *p*-bromophenylhydrazone had m.p. 157–158°, $[\alpha]_D^{25}$ 30·1° → −10·0° (40 hr) in pyridine. Levene and Timpson[11] give m.p. 155 157° and $[\alpha]_D$ + 34·5° → +10·0° (115 hr) for the D-isomer.

The *p*-nitrophenylhydrazone melted at 171° in agreement with Levene and La Forge[12] who gave 169° for D-lyxose *p*-nitrophenylhydrazone.

Identification of the substance with R_f 0·52 as 4-O-*methyl*-D-*fucose* (D-*curacose*)

The freeze-dried material corresponding to the second fraction was crystallized by dissolving in the minimal amount of isopropanol and slowly evaporating at room temperature until crystals appear. The suspension was cooled at 5° overnight and crystallization increased. The crystals were filtered and washed well with cool isopropanol, m.p. 120–122°. On several recrystallizations from ethyl acetate long prisms, m.p. 131–132°, were obtained. $[\alpha]_D^{25}$ + 82°. No mutarrotation was observed (*c*, 1% water). (Found: C, 46·91; H, 7·89; OCH_3, 17·21. $C_7H_{14}O_6$ requires: C, 47·19; H, 7·86; OCH_3, 17·41.)

D-*Curacose* p-*tosylhydrazone*

Fifty milligrams of curacose and 50 mg of the hydrazide were dissolved in 3 ml of methanol and refluxed for 30 min. On cooling, crystals appeared which were filtered and recrystallized three times from acetonitrile. Long prisms melting at 134° were obtained $[\alpha]_D^{23}$ −16·0° → 3·3° (*c*, 1% pyridine). (Found: C, 48·20; H, 6·34; N, 8·24; S, 9·58; OCH_3, 8·98. $C_{14}H_{21}N_2O_6S$ requires: C, 48·55; H, 6·36; N, 8·09; S, 9·24; 10 CH_3, 8·95%).

Periodate oxidation of curacose

Curacose (8·795 mg) was dissolved in 25 ml of water, 2·5 ml of 0·3 M of sodium metaperiodate added, the volume was brought to 50 ml with water and the solution was allowed to stand at room

[9] R. Consden and W. M. Stanier, *Nature, Lond.* **169**, 783 (1952); M. J. Crumpton, *Ibid.* **180**, 606 (1957).
[10] E. Zerner and R. Waltuch, *Monatsch. Chem.* **34**, 1639 (1913).
[11] P. A. Levene and R. S. Timpson, *J. Biol. Chem.* **115**, 731 (1936).
[12] P. A. Levene and F. B. La Forge, *J. Biol Chem.* **18** 319 (1914).

temperature. Aliquots were taken from time to time and the consumed *meta*-periodate and formic acid produced determined. After 120 min, 1·94 moles of periodate were consumed per mole of sugar and 1·10 moles of formic acid were titrated. A stable state was already reached at that time, because after 240 min the figures were 1·93 moles periodate and 1·18 moles formic acid.

α-Methyl 2,3,4-tri-O-methyl-D-fucopyranoside (methyl-2-3-di-O-methyl-D-curacoside)

A solution of 160 mg of curacose in 9 ml absolute methanol containing 1% hydrochloric acid was refluxed for 6 hr. After elimination of the acid with silver carbonate, the clear filtered solution was evaporated to dryness and the partly crystalline residue was dissolved in 5·5 ml of dimethylformamide and 1·6 ml of methyl iodide. Silver oxide (1·6 g) was added slowly with vigorous stirring, that was continued for 40 hr.[13] The insoluble was separated and washed with dimethylformamide (2 × 2 ml) and chloroform (4 × 4 ml). The washings and the original solution were pooled and washed with 10 ml of 10% sodium cyanide solution in water. The aqueous washing was extracted six times with 5 ml chloroform which was added to the organic phase. Evaporation of the combined extracts yielded 165 mg of a syrup. This material, partly crystalline, well dried, was dissolved in 2 ml of warm petroleum ether (40–60°). On standing at 5° for several days, crystals appeared. They were filtered (50 mg) and recrystallized from petroleum ether several times. Short prisms, m.p. 96–97°; $[\alpha]_D^{25}$ +206° (*c*, 1·0 water).

When mixed with a α-methyl-2,3,4-tri-O-methyl-D-fucoside, m.p. 97°, $[\alpha]_D$ +211° (*c*, 1·0; H_2O), prepared from D-fucose by the method described by Gardiner and Percival[4] for the L-isomer, the product showed no melting point depression. When mixed with the same amount of the L-isomer (m.p. 97°, $[\alpha]_D$ −202°[14] a sharp m.p. 69–70° was observed.

Paper chromatography of mono-O-methyl-fucoses

On descending paper chromatography, employing Whatman No. 1, D-curacose had, with the n-butanol–ethanol-water (4:1:5), mobile phase already described, R_f 0·52 and R_g 0·60, while 2-O-methyl-L-fucose had 0·54 and 0·63 and 3-O-methyl-L-fucose, 0·48 and 0·56. Gardiner and Percival[4] found with the same system 0·56 and 0·60 for the 2-O-methyl isomer and for the 3-O-methyl isomer 0·48 and 0·56.

When benzene-n-butanol-pyridine-water (1:5:3:3) was used as mobile phase the following R_f and R_g were found: D-curacose: 0·56 and 0·66; 2-O-methyl-L-fucose: 0·62 and 0·72; 3-O-methyl-L-fucose: 0·56 and 0·66. Gardiner and Percival[3] found R_f 0·63 for the 2-O-methyl and 0·56 for the 3-O-methyl-L-fucose.

Sugar 1

This amorphous solid gave a positive Molisch reaction and a weak positive Fehling test. Bial, tetrazolium and iodoform reactions were negative. With Dische's reagent for 2-deoxy pentoses, the reaction was a typical (brown-yellow color).

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[13] R. Kuhn, H. Trischmann and I. Löw, *Angew. Chem.* 67, 32 (1955).
[14] O. Th. Schmidt, W. Mayer and A. Distelmayer, *Liebigs Ann.* 555, 26 (1944).