457. *The Structure and Reactions of a Polyacetylenic Glycoside.*

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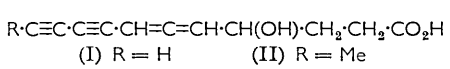
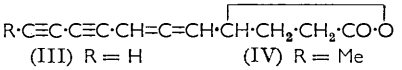
The polyacetylenic metabolites of Basidiomycete B-841 comprise, not only the free hydroxy-acids and lactones (I)—(IV), but also a glycoside fraction of which the main component is the β-D-xylopyranoside of nemotinic acid. Both this xyloside and its hydrogenation product are hydrolysed by alkali, as well as by acid; the course of these reactions is elucidated and a probable configuration assigned to $C_{(4)}$ of nemotinic acid.

In 1950, Kavanagh, Hervey, and Robbins [1] observed that only a part of the antibiotic activity in cultures of *Agrocybe dura* could be extracted by organic solvents, whilst a further part, apparently due to the same compound, could be extracted after the culture medium had been boiled. Later a similar phenomenon was observed with the antibiotics from *Polyporus biformis*.[2] In each case the extractable antibiotics are simple polyacetylenes with alcoholic hydroxyl groups.

In experiments on the polyacetylenes produced by Basidiomycete B-841 it was the usual practice to extract aqueous culture media with organic solvents and to resolve the resulting mixture by counter-current distribution in various solvent systems. In this way it proved possible to isolate and characterise the hydroxy-acids (I) (nemotinic acid, the main component) and (II) (odyssic acid) and the corresponding lactones (III) (nemotin) and (IV) (odyssin).[3] During this work it became apparent that an important fraction of the polyacetylenes in the medium was not very readily extracted by organic solvents, and that some of the extracted material was far more hydrophilic than the pure substances



(I) R = H (II) R = Me



(III) R = H (IV) R = Me

(I)—(IV). Remembering the observations cited above, we suspected that the B-841 culture medium also contained conjugates, probably of (I) and/or (II), with some more polar entity. Since other work on the fungus involved repeated assays of the total polyacetylene content, a method for determining this and also the proportion present in conjugated forms was devised and is described in the Experimental part. In general only about 40—60% of the total polyacetylenes produced by B-841 was in the form of free acids or lactones (I)—(IV).

The conjugate material was most simply isolated by repeated extraction of the culture medium with ethyl acetate, until the extracts contained negligible amounts of polyacetylenes; where the yield was not important the first few extracts were discarded, the remainder containing almost exclusively the conjugate material. Otherwise the extracts were combined and subjected to counter-current distribution, first between benzene and

[1] Kavanagh, Hervey, and Robbins, *Proc. Nat. Acad. Sci., U.S.A.*, 1950, **36**, 102.
[2] Kavanagh, Hervey, and Robbins, *Proc. Nat. Acad. Sci., U.S.A.*, 1947, **33**, 176; Anchel and Cohen, *J. Biol. Chem.*, 1954, **208**, 319.
[3] Bu'Lock, Jones, and Leeming, *J.*, 1955, 4270; 1957, 1097.

water to remove the free compounds (I)—(IV), then between ethyl acetate and water to give substantially pure conjugate. Further purification, if required, was effected by redistribution between ether and water. On such rigorous purification the conjugate material was found to comprise two compounds; the greater part of the major constituent could be obtained pure, as shown by the shape of the distribution curve (based on spectroscopic assay), and the uniformity, through the relevant fractions of the distribution, of ultraviolet absorption spectra and, with radioactive material, of specific activity. This component proved to be a conjugate of the C_{11} compound (I). The minor component was never completely resolved, but showed the spectroscopic and partition characteristics which could be predicted for a similar derivative of the C_{12} compound (II); it was not investigated further. Much of the work on the conjugate was carried out with less pure material containing a small proportion (<5%) of the suspected homologue.

The ultraviolet absorption spectrum of the conjugate was like that of the ene-diynes (I)—(IV), and the infrared spectrum revealed the presence of allene, carboxyl, and ethynyl groups, the latter being more clearly detectable after treatment with diazomethane. Evaporation of solutions gave an insoluble brown resin, and the conjugate was therefore manipulated by the general techniques used for the compounds (I)—(IV) which avoid the isolation of solid material.[3] In this way it was found that the conjugate behaved as a monobasic acid, with pK_a similar to that of acid (I); the equivalent weight, and comparison of the observed $E^{1\%}_{1\,cm.}$ values with the ε values for corresponding peaks in the absorption spectrum of acid (I), indicated a molecular weight of *ca.* 325 [for (I), $M = 190$]. With a platinum catalyst 6 mol. of hydrogen were rapidly taken up and after hydrolysis of the hydrogenation product γ-undecanolactone was isolated and characterised as the optically inactive derivative 4-oxoundecanamide.[3] The reactions of the conjugate with alkali (see below) confirmed, what was apparent from the above data, that the conjugate contained combined acid (I) and that the attached polar group was combined with the hydroxyl group of this acid.

The conjugate contained no nitrogen, phosphorus, or sulphur, and the attached polar group was therefore thought likely to be of carbohydrate nature. Moreover, when B-841 was supplied with [1-^{14}C]acetate and an excess of unlabelled glucose the radioactivity of the conjugate was due entirely to the combined C_{11} group. Unlike nemotinic acid the conjugate reacted with periodate, and also gave a positive Molisch test for carbohydrate. By acid or alkaline hydrolysis of the hydrogenation product, or by direct treatment with alkali, D-xylose was split off; this was identified by paper chromatography, determination of optical rotation, and conversion into its *p*-nitrophenylhydrazone, osazone, and osotriazole. The hydrogenation product did not reduce Fehling's solution (compounds with free ethynyl groups react anomalously with this reagent) so that the xylose was combined as a xyloside. The molecular weight, periodate uptake, and a pentose determination[4] showed that only 1 mol. of xylose was combined in the xyloside, and the rate of the periodate oxidation,[5] with formation of only 1 mol. of formic acid, showed the compound to be a D-xylopyranoside of nemotinic acid.

The molecular rotations of the xyloside and of nemotinic acid, and of some corresponding derivatives, are indicated in Chart 1. Because of the unsaturated nature of the asymmetric centres no significant comparisons can be made between the rotations of the two allenes, but this factor does not operate in the corresponding hydrogenation products. Now the molecular rotations of the methyl α- and β-D-xylopyranoside are +253° and −108° (*i.e.*, $M_\alpha + M_\beta = +144°$) and the α-D-xylopyranosides are the more dextrorotatory; therefore the observed rotations of the hydrogenated xyloside and of (+)-4-hydroxyundecanoic acid, from (I), with a difference of −93°, imply that the natural product is the β-isomer.

The difference in sign of rotation between samples of γ-undecanolactone derived from

[4] Mejbaum, *Z. physiol. Chem.*, 1939, **117**, 258.
[5] Jackson and Hudson, *J. Amer. Chem. Soc.*, 1937, **59**, 994.

acid (I) and from its xyloside (see Chart 1) was surprising since it seemed unlikely that the two metabolites should have different configurations at $C_{(4)}$. The explanation lies in the mechanism of hydrolysis of the hydrogenated xyloside, which is anomalous since it can be effected with equal ease by acid or by alkali. To explain this we suppose that in either case the sugar residue is displaced by an intramolecular nucleophilic attack by the carboxyl

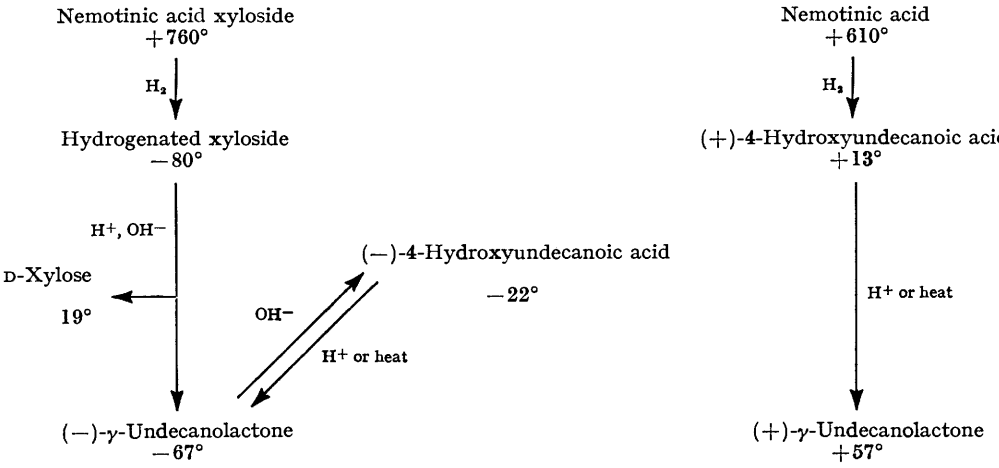
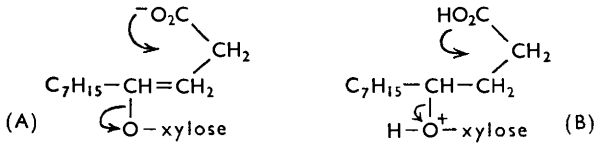
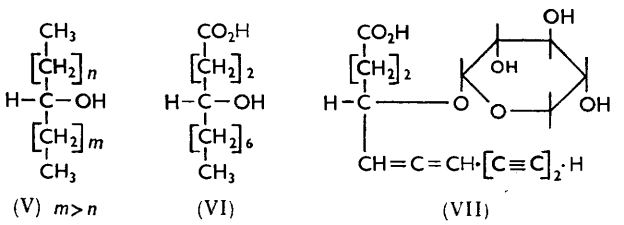


Chart 1. *Molecular rotations* $[M]_D$ *observed in ethanol at* 20°.

group (possibly as in A—B), necessarily involving inversion of configuration at $C_{(4)}$, and giving D-xylose and (−)-γ-undecanolactone, the latter subsequently undergoing normal hydrolysis under alkaline conditions.



Thus the configuration of $C_{(4)}$ in both nemotinic acid and the xyloside is that found in (+)-4-hydroxyundecanoic acid. It is known that alcohols of the series (V), where $m > n$, are dextrorotatory for the D line; [6] by analogy, since introduction of the carboxyl group is unlikely to affect the sign of rotation,[7] our (+)-hydroxyundecanoic acid should have the configuration (VI). The xyloside of nemotinic acid therefore has structure (VII), in which the configuration of the allene group remains unknown.



The xyloside of nemotinic acid exemplifies a new type of natural polyacetylene, though it is probably not unique. In B-841 only part of the polyacetylenes produced is converted into xylosides, and their formation can hardly be considered as a detoxication since both

[6] Klyne, " Progress in Stereochemistry," Butterworth, London, 1954, p. 205.
[7] Marker, *J. Amer. Chem. Soc.*, 1936, **58**, 976.

glycosides and aglycones pass entirely into the aqueous medium. The process of xyloside formation must be relatively specific since the xyloside (VII) is not accompanied by glycosides with other sugar residues even when a large excess of glucose is available. The route by which the xylose of this material (VII) is formed is known; [8] it does not appear to be of importance in hexose utilisation for respiration, but the formation of xylose may be an alternative to the synthesis of ascorbic acid. Simple glycosides are not common metabolites of micro-organisms; further, simple xylosides are relatively rare even in plants. On the other hand, xylose is very common in vegetable polysaccharides, such as the xylans with chains of β-D-xylopyranosyl units, and since Basidiomycete B-841 is a wood-destroying fungus it probably contains appropriate glycosidases which might in other circumstances direct the synthesis of material (VII).

The naturally occurring allenes are all unstable to alkali, with which they react in a variety of ways. Nemotinic acid (I) reacts relatively slowly, giving the isomeric acid (VIII), whereas nemotin (III) reacts rapidly, the lactone group participating, to give nemotin A (IX). This difference can be explained in terms of the electron-attracting

(VIII) $H\cdot[C\equiv C]_3\cdot CH_2\cdot CH(OH)\cdot CH_2\cdot CH_2\cdot CO_2H$ $\qquad$ $H\cdot[C\equiv C]_3\cdot CH=CH\cdot CH_2\cdot CH_2\cdot CO_2H$ (IX)

$H\cdot[C\equiv C]_3\cdot CH_2\cdot CH(OR)\cdot CH_2\cdot CH_2\cdot CO_2H$ (X) (R = β-D-xylopyranosyl)

powers of the lactone-carbonyl group.[9] The xyloside (VII) reacted relatively slowly with dilute ethanolic alkali, giving as the main product (*ca.* 80%) a hydrophilic triyne acid which is presumably (X), the reaction therefore being analogous to that of the aglycone (I). However, with dilute alkali in aqueous solution, though the reaction was still relatively slow, only about 35% of the isomer (X) was formed, the other products being nemotin A (IX) (60%) and free xylose. This formation of nemotin A appears analogous to the isomerisation of the lactone (III), but in this case the driving force of the reaction can hardly be an electron-attracting electromeric effect of the eliminated group, and should be ascribed instead to an entropy effect, increased solvation in the transition state. This would explain the reduced importance of the elimination reaction in ethanol, which is less powerfully solvating than water. The question of the importance of this effect in isomerisations of (I) and (III) therefore arises. For the lactone (III) the effect might be important, but it is the same as that of the electromeric effect of the carbonyl group. For the hydroxy-acid (I) the effect should be less than in the xyloside (VII); in fact the reaction of acid (I) with alkali regularly gives a small yield of the elimination product (IX), an observation originally ascribed to the possible presence [9] of traces of lactone (III) but which seems as likely to be due to a competing reaction such as that of the xyloside (VII).

However, it is also possible that the formation of nemotin A by the action of alkali on the xyloside (VII) may have another explanation, *viz.*, the intermediate formation of a stereoisomer of nemotin (III), by an intramolecular displacement such as occurs with the hydrogenated xyloside, followed by rapid alkali-catalysed isomerisation of the product.

Experimental

Replacement cultures of B-841 were grown as described previously [10] and supplied with 4% aqueous glucose or 1·5% aqueous ethanol as replacement medium. The general experimental methods were those described earlier; [3] evaporation was under reduced pressure in a stream of air-free nitrogen. Optical rotations were determined for the sodium D line in ethanol at 20°.

Isolation of Nemotinic Acid β-D-*Xylopyranoside* (VII).—The following account of the working-up of some ^{14}C-labelled material is typical. Three B-841 cultures were each supplied

[8] Bu'Lock, Gregory, and Hay, *Experientia*, 1959, **15**, 420.
[9] Bu'Lock, Jones, Leeming, and Thompson, *J.*, 1956, 3767.
[10] Bu'Lock and Leadbeater, *Biochem. J.*, 1956, **62**, 476.

with 750 ml. of 1·5% aqueous ethanol containing 150 μc of sodium [1-^{14}C]acetate (2·7 mg.), and after 9 days the medium was collected and extracted 7 times with 0·1 vol. of ethyl acetate. The combined extract, containing 1·7 g. of polyacetylenes [as (I)], was evaporated over water (320 ml.) and the aqueous residue put into the first 8 tubes of a counter-current-distribution apparatus. Distribution between benzene and water (40/40 ml., 60 tubes, 60 transfers) removed free polyacetylenes (I)—(IV), and the material remaining in the first aqueous phases was then distributed between ethyl acetate and water (40/40 ml., 40 tubes, 70 transfers). The yield of *xyloside* (VII) was *ca.* 1·1 g., stored in 1100 ml. of ethyl acetate. When the product was purified by further distribution between ether and water a small fraction contaminated with the xyloside of acid (II), showing an ultraviolet absorption maximum at slightly longer wavelengths, could be discarded.

Properties of the Xyloside (VII) [Determined by the methods used for (I)—(IV) [3]].—Found: equiv., 325 ± 10. $C_{16}H_{18}O_7$ requires equiv., 322. pK_a (in water) 4·4. $[\alpha]_D^{20}$ +237° (*c* 0·1). Partition coefficients, EtOAc–H_2O 2·0, Et_2O–H_2O 0·14. $\lambda_{max.}$ 211, 237·5, 250, 264, and 279 mμ ($10^{-3}\varepsilon$ 22, 4·5, 8·3, 12·1, 9·9 respectively).

Like acid (I), the xyloside (VII) gave an unstable brown precipitate of a copper acetylide with Fehling's solution, but, unlike this acid, it gave a positive Molisch test and contained 1 mol. of pentose according to a pentose determination by the orcinol–ferric chloride method.[4] For periodate oxidation, a solution of the xyloside (VII) (80 mg.) in water (90 ml.) was treated with sodium metaperiodate (428 mg. in 10 ml. of water) in the dark at room temperature, and at intervals 5 ml. samples were removed and treated with excess of sodium hydrogen carbonate. 5% Aqueous potassium iodide was added, immediately followed by 1·0 ml. of 0·1N-sodium arsenite, after which the excess of arsenite was titrated with 10^{-2}M-iodine in 1·5% aqueous potassium iodide. Samples were also titrated with 0·01N-sodium hydroxide (Methyl Red) to measure the formic acid produced. With nemotinic acid there was no reaction; with the xyloside (VII) reaction was 50% complete after 2—3 hr. and on completion 2·0 mol. of periodate had been consumed and 1·0 mol. of formic acid liberated.

Hydrogenation of the Xyloside (VII) *and Hydrolysis.*—When the xyloside (VII) (720 mg.) in ethyl acetate was hydrogenated over Adams platinum catalyst (500 mg.), 6 mol. were taken up; the solution was filtered and evaporated, giving the xyloside of (+)-4-hydroxyundecanoic acid as a syrup, $[\alpha]_D^{20}$ −25° (*c* 0·92), which contained carbohydrate (Molisch test) but reduced Fehling's solution only on prolonged heating.

The hydrogenation product [from 720 mg. of acid (VII)] was heated under reflux with 0·5N-hydrochloric acid in 1 : 1 ethanol–water (20 ml.) for 1 hr.; the solution was then extracted with ether (2 × 20 ml.), and the ether extracts were washed with water. Evaporation of the combined ether extracts and distillation of the residue at 180°/15 mm. gave (−)-γ-undecanolactone, $[\alpha]_D^{20}$ −36·5° (*c* 0·95), converted into 4-oxoundecanamide, m. p. and mixed m. p. 129—131°, as previously described for the (+)-isomer.[3] The aqueous extracts and washings were combined, treated with excess of silver carbonate, filtered, and evaporated; the residue was taken up in hot ethanol, filtered through charcoal, and evaporated, giving D-xylose as a colourless syrup (61%), identified by paper chromatography in butan-1-ol–acetic acid–water and collidine–water and by conversion into the osazone, m. p. and mixed m. p. 158—160°, osotriazole, m. p. and mixed m. p. 86·5—87°, and *p*-nitrophenylhydrazone, m. p. and mixed m. p. 151—153°.

In another experiment, hydrogenated xyloside (VII) (200 mg.) was heated under reflux with a 10% solution of sodium hydroxide in 1 : 4 ethanol–water (20 ml.), then acidified with 10% sulphuric acid, and extracted with ether; evaporation of the ether extracts gave an oil which was taken up in hot ethanol, filtered through charcoal, and evaporated, giving (−)-4-hydroxyundecanoic acid as an oil, $[\alpha]_D^{20}$ −11° (*c* 0·8), which on distillation afforded (−)-γ-undecanolactone, $[\alpha]_D^{20}$ −37·5° (*c* 0·8).

Hydrogenation of nemotinic acid and evaporation of the filtered reaction mixture gave (+)-hydroxyundecanoic acid, $[\alpha]_D^{20}$ +6·5° (*c* 1·0), affording the (+)-lactone, $[\alpha]_D^{20}$ +31° (*c* 0·95), on distillation.[3]

Reaction of the Xyloside (VII) *with Alkali.*—(a) *In aqueous solution.* To a solution of the xyloside (VII) (14 mg.) in water (10 ml.), 2—3 drops of aqueous 10% sodium hydroxide were added and after about 12 min. (when the absorption spectrum showed no further change) the solution was acidified with 2N-sulphuric acid and subjected to counter-current distribution (10 transfers) between benzene and water. The first two benzene fractions were evaporated over water, and the aqueous solutions extracted with ether. The ultraviolet spectrum of this

ether extract was that of nemotin A (IX) (*ca.* 60%); the extract was evaporated and the residue immediately taken up in carbon disulphide for measurement of the infrared spectrum, which confirmed the identification. The first two aqueous fractions were repeatedly shaken with ether, which extracted the triyne xyloside (X), with partition coefficient ether–water *ca.* 0·15, with the ultraviolet absorption spectrum of isonemotinic acid (VIII), and giving a positive Molisch test.

(b) *In ethanol.* A similar reaction in absolute ethanol was complete after about 30 min.; the solution was then acidified and diluted with water, and the ethanol was evaporated. The aqueous solution was extracted with ether and the ether extracts were washed with water. Spectroscopic examination of the combined aqueous extracts showed the presence of acid (X) (*ca.* 80%), and examination of the combined ether extracts revealed the free acid (IX) (*ca.* 15%).

Estimation of Free and Combined Polyacetylenes in B-841 Culture Media.—The proportion of the total polyacetylenes in B-841 culture media combined as xylosides normally varies between 35% and 65%, and the partition coefficients of the free and the combined forms are different, so that the total concentration bears no simple relation to the amount extracted by an aliquot part of organic solvent for spectrophotometric assay. The lactones are completely extracted by equilibration of an aqueous solution with an equal volume of ethyl acetate, and the hydroxy-acids are almost completely extracted, whereas only about one-half of the xylosides is extracted. The concentration of free and combined polyacetylenes in a sample of medium, expressed as an equivalent amount of nemotinic acid (I), can therefore be determined by spectroscopic assay of two successive extracts of the same sample, with equal volumes of ethyl acetate and correction for the volume changes in the first extraction.

If ΔD_1 and ΔD_2 are the first and the second measurement of the optical density difference between 263 mμ (maximum) and 271 mμ (minimum), measured in 1 cm. cells. then since ΔD for a 1% solution of acid (I) is 538, the first extract has a concentration of $\Delta D_1/53{\cdot}8$ mg./ml. In the first extraction 10 ml. of aqueous medium and 5 ml. of ethyl acetate give 4·1 ml. of extract; the amount removed from the aqueous phase is $4{\cdot}1\Delta D_1/53{\cdot}8$ mg. This comprises all the lactones and hydroxy-acids and part of the xylosides. In a second similar extraction, with no further volume change, the amount removed is $5\Delta D_2/53{\cdot}8$ mg. and this is one-half of the xylosides remaining after the first extraction. The total concentration of the original aqueous solution is therefore $(4{\cdot}1\Delta D_1 + 10\Delta D_2)/53{\cdot}8$ mg./ml. $= 7{\cdot}6\Delta D_1 + 18{\cdot}6\Delta D_2$ μg./ml., of which $37{\cdot}2\Delta D_2$ μg./ml. is present as xylosides.

This method gave results which agreed closely with the composition of synthetic mixtures (I)—(VII) and gave consistent results with culture media prepared in a variety of ways; but in media from cultures severely affected by toxic substances or undergoing degeneration it gave anomalous results unless the aqueous sample was brought to pH 5 with acetate buffer before extraction.

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