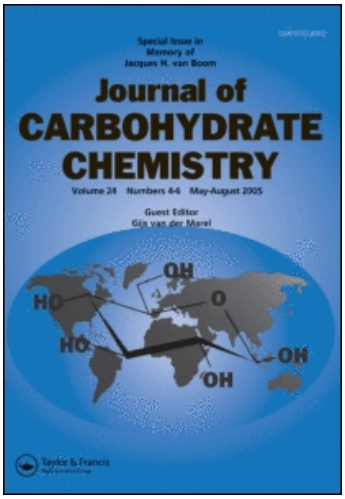

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^{1}H AND ^{31}P NMR Spectroscopy of Agrocinopine

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^{1}H AND ^{31}P NMR SPECTROSCOPY OF AGROCINOPINE

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ABSTRACT

The ^{1}H NMR data of agrocinopine in D_2O solution as extracted from standard 2D NMR experiments, along with 1D ^{31}P and ^{13}C NMR experiments allow to support the trisaccharide structure originally proposed on basis of comparative ^{13}C NMR measurements.

INTRODUCTION

The structure of agrocinopine has been proposed by Ryder et al.[1]. In a previous report[2] we have mentioned that the proposed structure of agrocinopine, a F4→A2 phosphodiester of sucrose and L-arabinose calculated as 554.4 dalton, was not compatible with our mass determination by size-exclusion chromatography, which indicate a molecular mass between vitamin B12 (1355.4) and stachiose (666.6), closer to vitamin B12. We have suggested that

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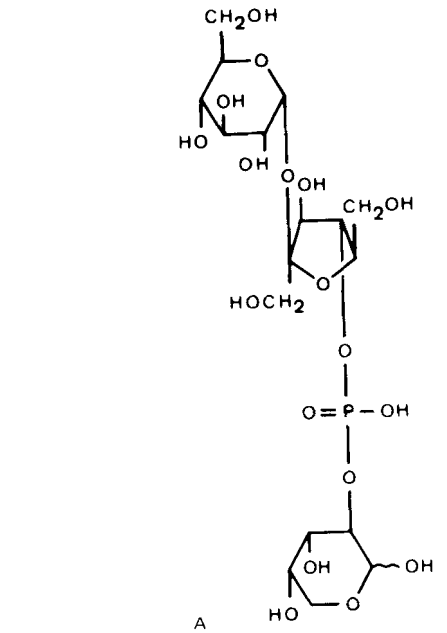
agrocinopine was a hexasaccharide consisting of two identical trisaccharides, i.e. a dimer of the structure proposed by Ryder et al.[1]. The hexasaccharide hypothesis was supported by stoichiometric proportions in the ^{31}P NMR spectrum. The structure of the trisaccharide (a phosphodiester from the C2-hydroxylarabinose to the C4-hydroxyl of the fructose moiety in sucrose; Fig. 1a), was evident from our first ^{1}H NMR spectrum. Further experiments were carried out to test whether the molecule was more complex.

The hypothetical hexasaccharide is built up by a phosphodiester linkage on the anomeric places of arabinose (Fig. 1b). The chromatographic and electrophoretic behaviour can indeed be very similar to that of the trisaccharide. Since such an ester is a labile bond and the hypothetical dimer can hydrolyse into inorganic phosphate and the trisaccharide, an unambiguous determination of the molecular mass would be decisive. This was however not possible, but other experiments did not confirm the hexasaccharide hypothesis. We present here original spectroscopic data, complete assignment by ^{1}H NMR spectroscopy at 500 MHz, and by ^{31}P NMR spectroscopy at 202.5 MHz. The basic trisaccharide structure of agrocinopine could be systematically unravelled by 1D and 2D ^{1}H NMR experiments as well as by ^{31}P NMR measurements. The structural conclusions were verified by ^{13}C NMR. A ^{13}C NMR study on a stronger spectrometer allowed to see an ascertaining fine structure not mentioned yet.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of agrocinopine at 500 MHz is displayed in Fig. 2a. Although it is impossible, even at 500 MHz, to analyse the spectrum completely by one dimensional techniques, the COSY 90 experiment (see Fig. 2b) gave the necessary information for the proton assignments (Table 1).

The solvent peak was small enough to certify that, except for anomeric protons discussed here, none were hidden. At least



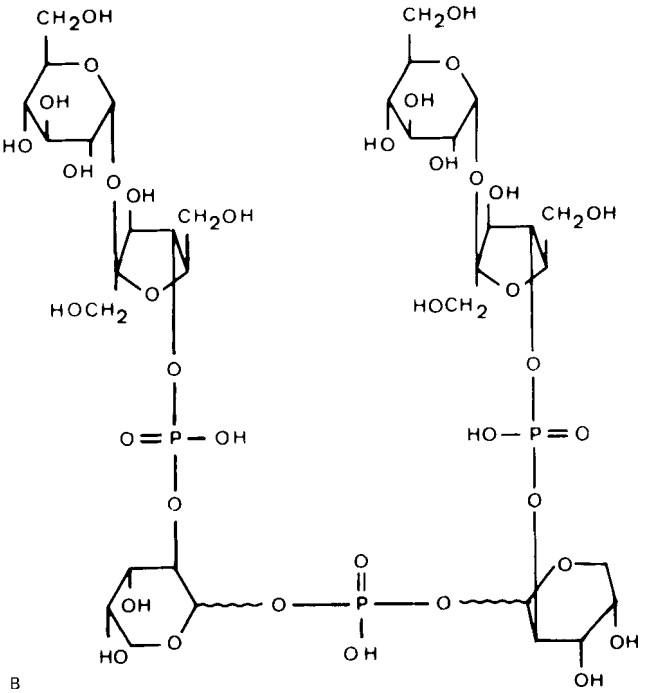


FIG 1. Structure formulas.
a. Agrocinopine.
b. Hexasaccharide hypothesis.

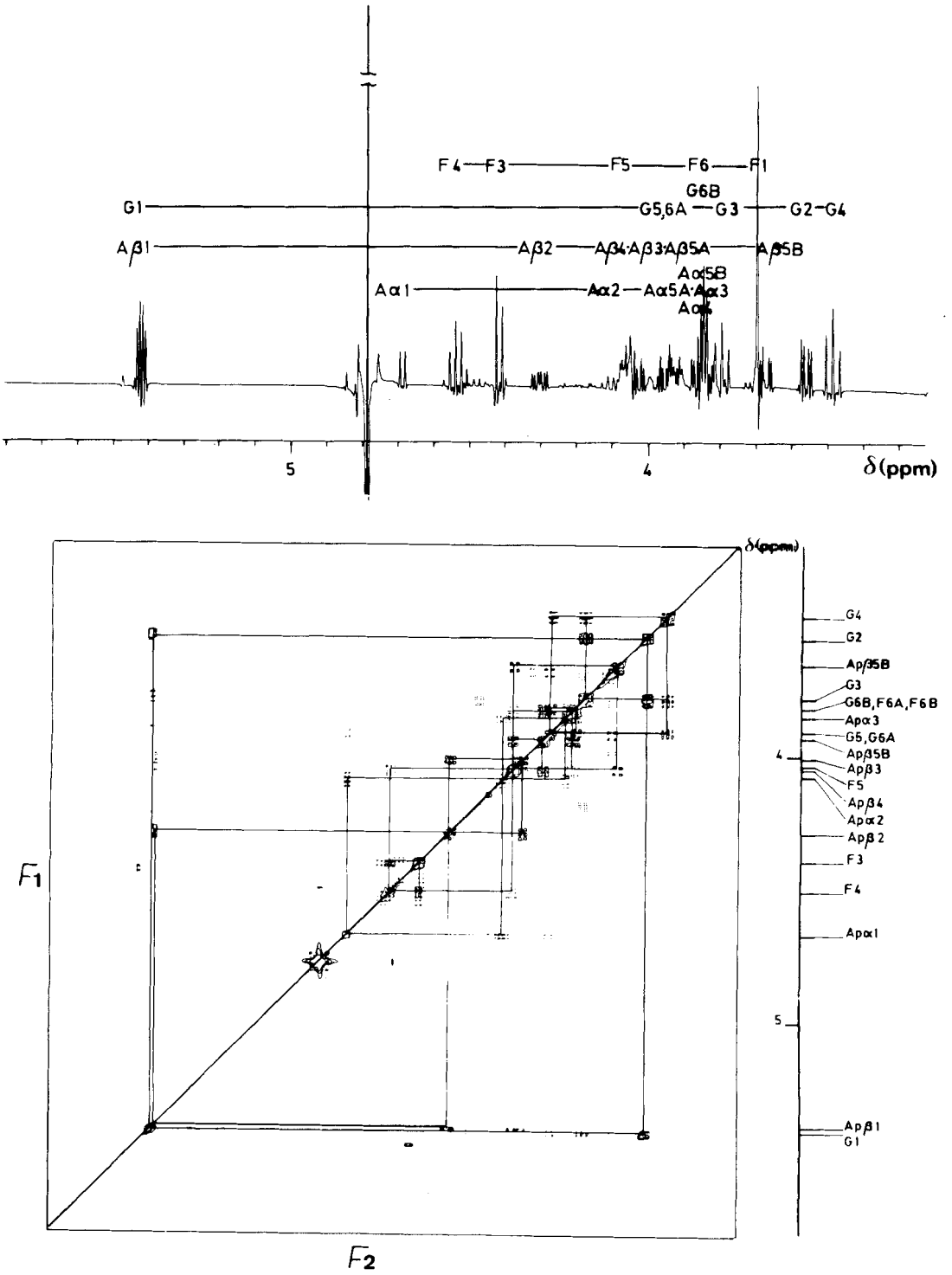


FIG. 2.

a. ^{1}H-NMR spectrum of agrocinopine (D_2O solution, TSP internal) at 500 MHz (LB = -4, GB = 0.09).

b. The COSY 90 experiment on a D_2O solution of agrocinopine (for the annotation of the protons, see text).

TABLE 1. ^{1}H NMR data of agrocinopine in D_2O solution (TSP internal standard)

Chemical shifts	H-1A	H-1B	H-2	H-3	H-4	H-5A	H-5B	H-6A	H-6B
α-D-glucopyranosyl	5.43	--	3.57	3.82	3.50	3.97	--	3.95	3.87
β-D-fructofuranosyl	3.71	3.71	--	4.43	4.55	4.07	--	3.87	3.87
β-L-arabinopyranose (60%)	5.43	--	4.32	4.02	4.07	3.96	3.68	--	--
α-L-arabinopyranose (25%)	4.70	--	4.12	3.87	3.85	3.95	3.83	--	--
?-L-arabinofuranose (10%)	5.43	--	--	--	--	--	--	--	--

Coupling constants	$^{3}J(1,2)$ $^{2}J(1A,1B)$	$^{3}J(2,3)$	$^{3}J(3,4)$	$^{3}J(4,5A)$	$^{3}J(4,5B)$	$^{2}J(5A,5B)$	$^{3}J(5,6A)$	$^{3}J(5,6B)$	$^{2}J(6A,6B)$	$^{3}J(P,H)$
α-D-glucopyranosyl	3.6	9.2	9.4	9.0	--	--	--	2.5	11.5	--
β-D-fructofuranosyl	--	--	7.5	7.5	--	--	--	--	--	7.5
β-L-arabinopyranose (60%)	3.0	8.0	3.4	2.5	--	11.5	--	--	--	7.5
α-L-arabinopyranose (25%)	6.7	7.0	--	--	--	--	--	--	--	7.5
?-L-arabinofuranose (10%)	3.0	--	--	--	--	--	--	--	--	--

three doublets are centered at 5.43 ppm at room temperature. Upon heating to 40°C, three well-separated doublets were evident and the J-coupling measured. The most intense doublet is H-1 of an α-D-glycopyranosyl moiety and two other doublets, each showing a splitting of about 3 Hz, integrate for 60% and 10% of the most intense doublet. At δ 4.70, we found another doublet with a coupling constant of 6.7 Hz, integrating for about 25% of the anomeric α-D-glycopyranosyl doublet.

In the COSY 90 experiment (see Fig. 2b; the resonances of the D-glucopyranosyl moiety are indicated by G, those of the D-fructofuranosyl moiety by F), the connectivities starting from the most intense doublet at δ 5.43 allow to assign six more protons, pointing to an aldohexose, because of the presence of an anomeric proton. Except for H-5 and the two protons of the exocyclic methylenehydroxyl function, the other resonances of this aldohexose are well isolated. From the vicinal coupling constants between these four protons (which determine the sugar configuration) we can safely say that the present aldohexose has the gluco configuration[5,6]. This sugar moiety is α-D-glucopyranose.

More resonances of the same intensity as those assigned to the α-glycopyranosyl part are present. As no further anomeric or glycosidic proton is found in the anomeric region of the spectrum, a ketose comes into mind. Starting from the doublet at δ 4.43, the COSY 90 experiment shows connectivities between four more protons. The nature of the doublet structure of the resonance at δ 4.43 suggests that it is a proton on a carbon in α place of a quaternary carbon. Between H-3, H-4, and H-5 a coupling constant of 7.5 Hz is found. These data, together with the presence of a singlet at δ 3.71 integrating for two protons, suggests the presence of a β-D-fructofuranosyl part[7]. The structure of the cross peak in the high resolution COSY 90 spectrum (Fig. 3) suggests that the two resonances for the protons on C-6 of this β-D-fructofuranosyl part are very close. The complexity

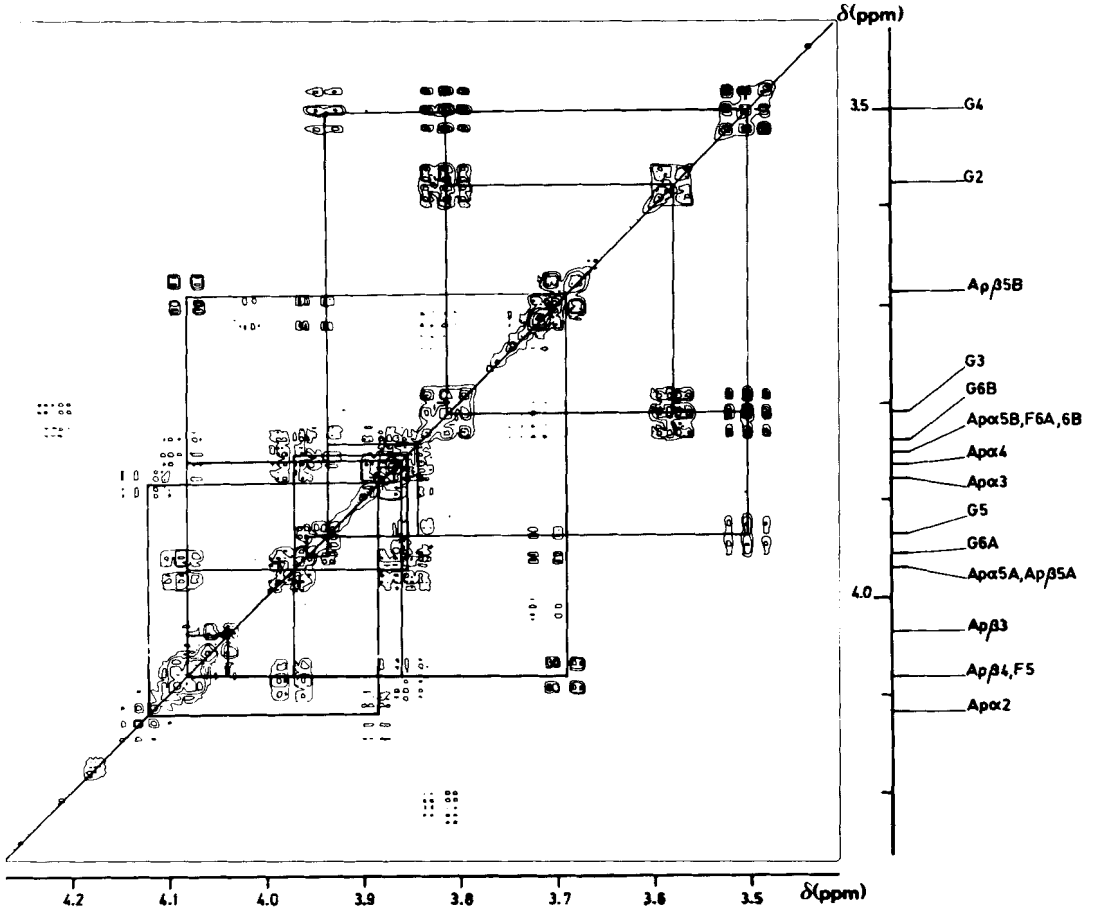


FIG. 3. High resolution COSY 90 experiment on a solution of agrocinopine in D_2O (for the annotations of the protons, see text).

of the spectrum does not allow a valid assignment of the structure of the spin system formed by these protons. Since the integrations of the resonances from the α-D-glucopyranosyl and the β-D-fructofuranosyl moieties are the same, and only one of the possible anomeric forms is present, we may accept that both moieties are linked to a sucrose unit on their anomeric positions. If this were not the case, the D-fructose moiety should

occur mainly as the β-D-fructopyranose form[7]. Moreover, except for H-4 of the β-D-fructopyranosyl part, the present proposal agrees completely with the data for sucrose in D_2O solution as described earlier[8].

The expected doublet(s) for H-4 in the β-fructofuranosyl part is further split by 7.5 Hz. Moreover, for this resonance at least two discernible quadruplets are present. Inspection of the projection of the 2D J-resolved spectrum shows three projected peaks. This observation allows two conclusions : (i) the origin of the further splitting of H-4, as well as the reason for its position at a lower field than expected[8], must be due to a phosphate link at C-4, and (ii) the three observable resonances for H-4 must be explained by the fact that it is linked to different other sugar units.

Knowing that the remaining doublets in the anomeric region integrate for about 60% and 10% at δ 5.43 and for 25% at δ 4.71 vs the integration patterns of the protons on sucrose, two possibilities can be suggested : either there is a very complex molecule with high proportions of sucrose linked by several other units, or these proportions are anomers of a third sugar.

Taking the complexity of the spectrum into consideration, the COSY 90 experiment allows to trace the connectivities of the chemical shifts of the two major anomers of the third sugar. Only from the major anomer could the coupling constants measured with high certainty in order to identify the third sugar moeity. We propose the β-L-arabinopyranose form. Indeed, the COSY connectivity (see Fig. 3; the different isomers of L-arabinose are indicated as follows : Apβ, the β-L-pyranose modification; Apα, its α anomer; Afβ, the β-L-furanose modification; and Afα, its α anomer) suggests only 6 protons, pointing to an aldopentose.

One of these coupling constants of H-2 of about 8 Hz is the result of its coupling with H-3, suggesting a quasi-antiperiplanar arrangement of H-2 and H-3. The other coupling constant of about 8 Hz is due to a phosphate link in this place. H-3

shows up as an isolated doublet of doublets pattern at δ 4.02, with J = 8.0 Hz and J = 3.4 Hz, pointing to an equatorial placement of H-4. Because of the complexity of the spectrum, the pattern of H-5A is hidden, but H-5B can unambiguously be measured. We find coupling constants of 2.5 Hz and 11.5 Hz, confirming the axial position of the OH group on C-4. These data suggest a β-L-arabinopyranose moeity[8]. The coupling constant point to an equilibrium 1C $\rightleftarrows$ C1 where the 1C conformation is more pronounced than for free β-L-arabinopyranose in D_2O[9].

Although some of the chemical shifts of the second and third anomer of L-arabinose can be assigned, it is not possible to obtain useful J-coupling for identification. However, we want to point out that also an indication is found from the pattern of H-2 of the second major anomer of L-arabinose that the phosphate bond occurs in this position.

We have compared the ^{1}H NMR chemical shifts of L-arabinose in D_2O solution with the present data[10]. For the two major anomers (thus both the pyranose forms) H-1 and H-3 show a slight downfield shift of 0.1 ppm, while H-2 shows an important downfield shift of 0.4 ppm in both forms. This finding, in combination with the fact that H-2 is more split, allows us to suggest that sucrose is linked to the C-2 place of L-arabinose on its C-4 place of the fructofuranosyl part. Since the anomeric position of L-arabinose remains free, four isomers are indeed expected.

In order to verify our proposal concerning the phosphate bond, we have run a ^{31}P NMR spectrum at 202.5 MHz. This spectrum (Fig. 4), and the ^{1}H-decoupled spectrum were both run. In the decoupled spectrum 5 peaks of different intensity are observed. The integration of the downfield peak is half of the sum of the other peaks. From the intensity of the 4 upfield peaks, we find we find again the proportion of the four L-arabinose anomers as measured in the anomeric region of the ^{1}H NMR spectrum. When comparing the spectrum with the ^{1}H-decoupled spectrum, the latter

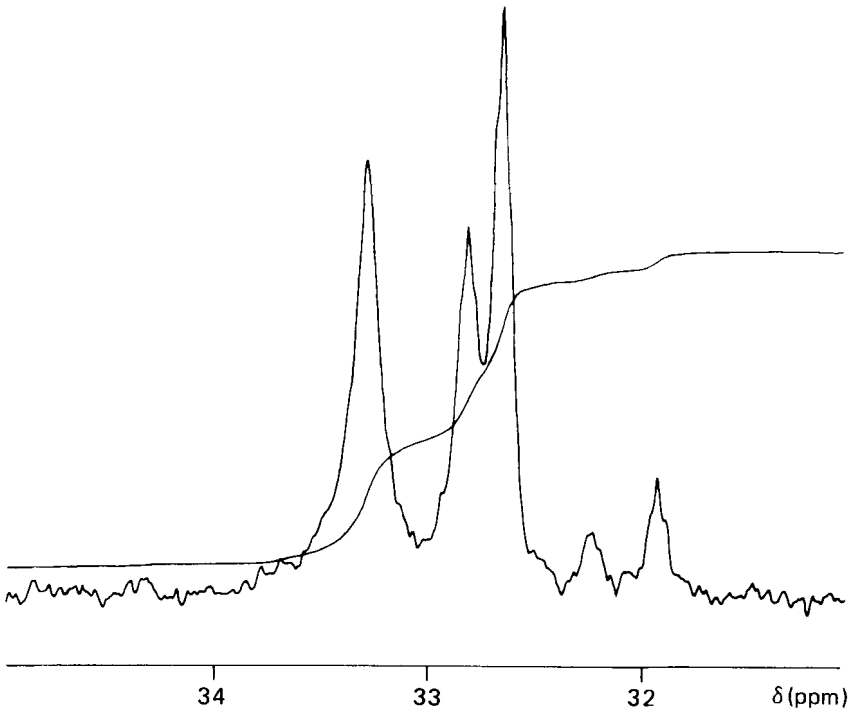


FIG. 4. Proton-decoupled ^{31}P-NMR spectrum of agrocinopine.

shows a narrowing in the four peaks obeying the proportion of L-arabinose, but a more striking narrowing occurs in the downfield peak. We can safely say that the four upfield peaks belong to the phosphate linking of sucrose with the four L-arabinose isomers, while the other peak at the lowest field should be Pi. Because of the stoichiometry it can be suggested that the phosphate originally linked the two trisaccharides; but, because of the treatment of the solution, hydrolyzed to Pi. Therefore, we considered it important to test whether the original compound is indeed a hexasaccharide.

A simple chemical test could show us the answer. If the structure is the hexasaccharide as drawn in fig. 1b, then we expect the compound to be nonreducible to the arabitol derivative

by sodium borohydride, and should be readily split by mild ammonolysis into free inorganic phosphate and the trisaccharide shown in fig. 1a. The dimer to monomer conversion must show a least a longer retention time in size exclusion chromatography.

If the structure is indeed the trisaccharide (fig. 1a) the molecule should be smoothly reduced by sodium borohydride and only split by mild ammonolysis after that reduction because of the presence of a newly generated vicinal hydroxyl group adjacent to the phosphodiester linkage. Since intact ^{32}P-labelled agrocinopine is actively secreted by crown gall cells, and not a single ^{32}P product is displayed when undamaged calluses were used in a ^{32}P-labelled MS medium[1], no other purification steps are needed than gel permeation to obtain the single ^{32}P-labelled agrocinopine (fig. 5a, lane 1).

The reaction product of the reduction of agrocinopine with excess sodium borohydride during 30 minutes at room temperature (fig. 5a, lane 3) is different from the original form as judged by its electrophoretic mobility at pH 5.0. The mixture of native and reduced agrocinopine gives a double band (fig. 5a, lane 2). The size exclusion chromatograms of both native and reduced agrocinopine are identical and no ^{32}P inorganic phosphate was found in the reaction mixtures after reduction. The reduced form was readily hydrolysed to a phosphomonoester by mild ammonolysis (0.5 M NH_4OH, 1 hour, 60°C); agrocinopine was left intact by the same treatment with ammonia and no inorganic ^{32}P was detected in either reaction mixture. The optical rotation of this phosphomonoester was found to be negative and of the expected sign for L-arabitol-2-phosphate. Thus, our results definitively confirmed the trisaccharide structure proposed originally[2] (fig. 5).

In order to confirm our finding we have run a ^{13}C NMR spectrum at 125.75 MHz. The ^{1}H noise-decoupled spectrum of the proposed structure was compared to that of sucrose and 2-O-phos-

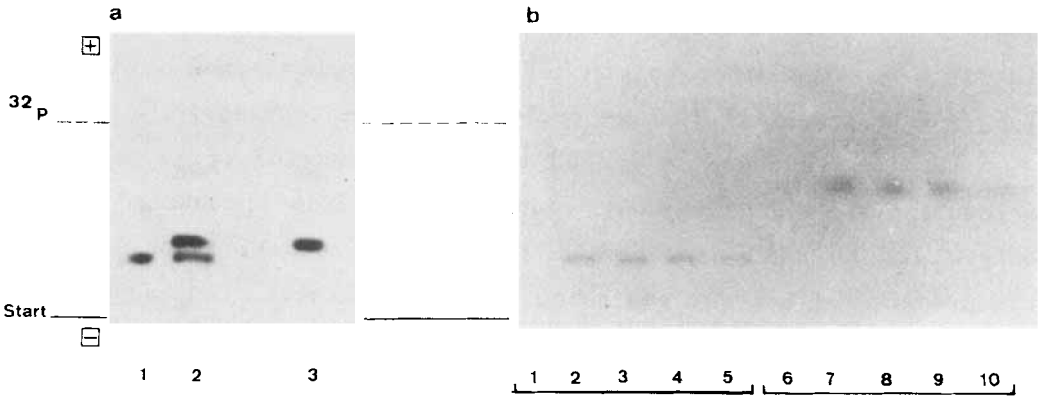


FIG. 5. Reduction and ammonolysis of ^{32}P-agrocinopine analyzed by thin-layer electrophoresis at pH 5.0, 0.05 M citrate, 1 hour, 400 V, and autoradiography.

a. Lane 1, original agrocinopine (1 µl) as isolated from gel permeation; lane 2, mixture of samples of lanes 1 and 3 (agrocinopine and reduced agrocinopine); lane 3, 2-µl sample of the reaction mixture of agrocinopine at the same concentration as in lane 1, in 0.01 M phosphate buffer, pH 5.8, and 0.1 mg sodium borohydride after 30 minutes at room temperature.

b. Reaction products of ammonolysis of original agrocinopine and its reduced form, as displayed by size exclusion chromatography; Bio-Gel P2 superfine column (bed volume ~ 80 ml) at elution volumes 22, 24, 26, 28, and 30 ml for agrocinopine, lanes 1, 2, 3, 4, and 5; and at the elution volumes 40, 42, 44, 46, and 48 ml for the reduced agrocinopine, lanes 6, 7, 8, 9, and 10.

phate-L-arabinose[11]. The spectra are identical, except for C-4 of the β-D-fructofuranosyl moiety of sucrose and C-2 in the several anomers of L-arabinose, which are not only split due to a ^{31}P-^{13}C coupling, but also show the expected downfield shift. With a high resolution spectrometer, operating at a carbon frequency of 125.75 MHz, we see clearly that, in accord with the four anomers of L-arabinose, the C-4 resonance of sucrose and the C-2 resonance of L-arabinose show four resonances, each occurring as doublets due to the phosphate linkage.

EXPERIMENTAL

Isolation and purification of agrocinopine. A fully grown suspension of tobacco crown gall W38C58 tumour cells is filtered through a loose cotton gauze and the filtrate (3 liters) is phenolised. The aqueous phase is extracted with ether and concentrated to a final volume of 150 ml. This aliquot is cleared by a 70% ethanol precipitation at -20°C. The procedures are repeated twice, and the final sample (50 ml) is loaded on a 5 x 100 cm column Bio-Gel P2 with 1 mg blue dextran marker. The column is eluted with 0.01 M triethylammonium bicarbonate (2 ml. min^{-1}). The fractions containing the blue dextran are pooled and freeze-dried. This sample (25 mg) was further purified by DEAE-Sephadex A25 chromatography (column : 40 cm, 12 mm diameter) with a linear gradient of 0.01 M (150 ml) -0.2 M (150 ml) triethylammonium bicarbonate (pH 8.5) (1 $ml.min^{-1}$) as the elution buffer. Agrocinopine, eluting at 0.12 M buffer, is detected by addition to the original sample of ^{32}P-agrocinopine (0.8×10^{6} cpm) prepared as described previously[1]. Radioactive fractions are pooled and twice redissolved and freeze-dried. Agrocinopine is further purified by chromatographing on a Varian 5000 liquid chromatography system. A first separation was performed on a 4.6 mm x 25 cm 10 µ anion exchange (partisil-SAX) column. The column was run at a flow of 0.5 $ml.min^{-1}$ isocratically in 0.01 M KH_2PO_4, pH 4.3, absorbance monitored at 195 nm, and RI detected with attenuation factor 124x. ^{32}P is monitored in each 0.5-ml fraction by counting the Čerenkov effect in a liquid scintillation counter. Retention time between 12 to 16 minutes. Subsequently, a single component eluted from a 4.6 mm x 25 cm lichrosorb-NH_2 10 µ direct phase HPLC column isocratically run with a flow of 1 $ml.min^{-1}$ aqueous 67% acetonitrile, 0.002 M phosphate buffer pH 4.9. Retention time between 20 to 22 minutes. The radioactive fractions were pooled and desalted on Sephadex G10; the salt peak is monitored by conductivity and cut off over 100 µS.

Agrocinopine is freeze-dried three times in deuterium oxide 99.96 atom % D prior to one freeze-drying in deuterium oxide 99.996 atom % D. For NMR study the sample is taken up in 0.5 ml fresh deuterium oxide 99.996 atom % D (Aldrich 19,170-1), and sealed in a dry glove box.

We note here that the inaccurate estimation of the molecular mass in our previous work[1] is due to non-ideal size exclusion chromatography. At low ionic strength (0.01 M triethylammonium bicarbonate) electrostatic effects dominate and anionic solutes are excluded from the gel permeation pores much earlier, thus permeate at higher hydrodynamic volumes than in pure size exclusion retention mechanism[3,4]. By running at a low pH (0.16 M acetic acid) we expected to suppress these effects, but for agrocinopine the P2 Bio gel patterns at both conditions were the same. This, together with the artifact of an exact stoichiometric amount of inorganic phosphate toward sucrose phosphoarabinose (1:2, w:w) present in our preparative batch, made us consider it important to test whether the original compound was a hexasaccharide.

Size exclusion chromatography. A sample of ^{32}P-agrocinopine (10^4 cpm) is mixed with the molecular weight markers blue dextran, vitamin B12 (1355.4 dalton), stachiose (666.6 dalton), and inorganic ^{32}P phosphate. Samples placed on a Bio-Gel P2 superfine column (bed volume ~ 80 ml, internal column diameter 18 mm) was run at a flow of 0.5 ml.min^{-1} in a 0.01 M triethylammonium bicarbonate (pH 8.5). An identical column was also used under similar conditions except for 0.16 M acetic acid. The elution pattern of both separations were identical : elution volume (Ve) of agrocinopine, 24 ml; interstitial volume (Vz) or blue dextran, 13 ml; volume of mobile phase (Vm) or ^{32}P inorganic phosphate, 50 ml; elution volumes of vitamin B12, 19 ml; stachiose, 38 ml; and L(-)arabitol-2-phosphate, 42 ml.

Reduction and ammonolysis. [^{32}P]-agrocinopine is isolated by gel permeation in 0.01 M triethylammonium bicarbonate pH 8.5 from the aqueous phase of 100 mg W38C58 cells grown during 7 days in 200 µl MS medium supplemented with 10 µCi ^{32}P at pH 5.8 (for details, see ref. 1).

The pooled agrocinopine fractions are freeze-dried and taken up in 100 µl 0.01 M sodium phosphate, pH 5.8. Excess sodium borohydride (0.1 mg) is added to half of the agrocinopine sample and the reaction mixture is subjected to gel permeation after 30 minutes at room temperature. The radioactive fractions are pooled and freeze-dried. Hydrolysis is performed by taking up in 0.5 ml 0.5 M ammoniumhydroxide and heating under argon in a sealed glass tube for 1 h at 60°C.

NMR spectroscopy. In order to avoid complex pulse sequences for suppressing the water peak, the sample was lyophilized several times from D_2O, until a remaining water peak was found at δ 4.79, which did not disturb the spectrum. The 500 MHz ^{1}H NMR spectra were recorded at 298 K for a 0.1 M solution in $^{2}H_2O$ on a Bruker AM 500 spectrometer (Vrije Universiteit Brussel) operating in the Fourier transform mode. The chemical shifts (δ) in ppm were determined relative to TSP as internal standard. Typical acquisition parameters were : sweep width 1300 Hz, and a 90° flip angle corresponding to 11.0 µs. The resolution was 0.1 Hz per point, and the relaxation delay 2 s.

For the COSY 90 experiment (absolute value) the following pulse sequence was used : RD - 90° - t_1 - 90°- t_2 - FID; 256 increments were accumulated in t_1 and 2 K data points in t_2. Quadrature detection was used; the FID was multiplied by a sine bell function and t_1 was zero filled to 512 w. The sweep was ± 650 Hz in t_1 and 1300 Hz in t_2; the relaxation delay (RD) was 1.5 s.

The ^{31} P spectra (proton-decoupled) were recorded at 298 K on the same spectrometer, operating at 202.4 MHz. Typical ^{31} P

parameters were : sweep width 10000 Hz, a 90° flip angle corresponding to 19 μs, a resolution of 4.8 Hz per point, and a relaxation delay of 2.5 s. The gated noise-decoupled ^{13}C NMR spectra were recorded 298 K on the same spectrometer, operating at 125.75 MHz. Typical ^{13}C parameters were : sweep width 25000 Hz, a 90° flip angle corresponding to 11 μs (a 5 mm selective ^{13}C probe head was used), and a resolution of 0.9 Hz per point.

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