GLYSPERIN, A NEW ANTIBIOTIC COMPLEX OF BACTERIAL ORIGIN

II. STRUCTURES OF GLYSPERINS A, B AND C

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Structures of glysperins A, B and C were determined on the basis of chemical degradation studies in conjunction with spectroscopic analyses. Glysperin A consisted of L-alanine, p-hydroxybenzoic acid, a C11-alkyl tetramine and four sugar moieties, three of which were identified as d-ribose, d-galactose and 2,4-diamino-2,4,6-trideoxy-d-galactose. The fourth sugar was a novel exoenose, 6-deoxy-d-xylo-hex-5-enose. Structural difference between glysperins A and B resided solely in the terminal polyamine moiety which was spermidine in glysperin B. Glysperin C contained d-glucose in place of the exoenohexose moiety of glysperin A. Glysperins A, B and C are, in some respects, structurally related to the glycocinnamoylspermidine antibiotics, LL-BM 123 β, γ1 and γ2.

Glysperin* is a complex of basic water-soluble antibiotics produced by strains of Bacillus cereus. In the preceding paper, characteristics of the producing strain, fermentation and isolation methods, as well as the chemical and biological properties of the antibiotics were described13. This paper presents evidence to assign the following structures to glysperins A (Ia), B (Ib) and C (Ic).

![Chemical Structures]

General Structural Characteristics

Glysperin components showed positive responses to ninhydrin, anthrone and ELSON-MORGAN reagents but were negative to SAKAGUCHI and ferric chloride reactions. The molecular formulae of

* This antibiotic was formally called Bu-2349.
C_{44}H_{75}N_{7}O_{18}, C_{40}H_{40}N_{8}O_{18} and C_{44}H_{77}N_{7}O_{18} were assigned to Ia, Ib and Ic, respectively, based on microanalyses and 13C-NMR (CMR) spectra. The three components showed similar UV spectra, exhibiting a single maximum at 247 nm which did not shift in acidic or alkaline solution. The IR spectra indicated the presence of multi-hydroxyl (3,400 and 1,040 cm\(^{-1}\)) and amide groups (1,630 and 1,560 cm\(^{-1}\)). The proton NMR (PMR) spectrum of Ia showed, in common with that of Ib and Ic, two doublet methyl groups (\(\delta\) in ppm, 1.28 and 1.49), multiple methylene protons (1.6-2.4) and 4 aromatic protons (AB-quartet, 7.12 and 7.71). The PMR spectra of Ia and Ib indicated 6 protons in the anomeric and/or olefinic region (4.8 ~ 6.0 ppm), while the PMR of Ic contained 4 protons in that field. The CMR spectra also reflected this difference: Ia and Ib exhibited two sp\(^{2}\) carbons (155.8, s and 98.0, t) in addition to the 6 sp\(^{2}\) carbons also present in Ic. On the other hand, the CMR of Ic showed two sp\(^{3}\) carbons (at around 60 ~ 80 ppm) not present in the spectra of Ia and Ib.

**Catalytic Hydrogenation of Ia and Ib**

The catalytic hydrogenation of Ia in ethanol afforded dihydroglysperin A (IIa, C\(_{44}H_{77}N_{7}O_{18}\)). The UV and IR spectra of IIa were very similar to those of Ia. The PMR spectrum of IIa differed from Ia in the presence of an additional methyl group (\(\delta\) 1.33, d) and the absence of two protons in the olefinic region (\(\delta\) 4.8 ~ 6.0). This suggested that a CH\(_{2}\)=C(H) group in Ia had been reduced to a CH\(_{2}\)-CH(H) group in IIa. Dihydroglysperin B (IIb) was obtained similarly from Ib. Further structural studies were carried out mainly on IIa and IIb because the degradation products obtained from their acid hydrolysis were more stable than those from Ia and Ib.

**Acid Hydrolysis of IIa and IIb (Chart 1)**

Upon mild acid hydrolysis (0.1 N H\(_{2}\)SO\(_{4}\) in MeOH, 27°C, 16 hours), IIa was cleaved into two fragments, a UV-absorbing basic compound (IIIA) and a tetrasaccharide (IV). Further methanolysis of IV in 0.5 N H\(_{2}\)SO\(_{4}\) at 27°C afforded methyl glycosides of a pentose (V) and a trisaccharide fragment (VI). The pentose was identified as methyl riboside by TLC and GLC (as a trimethylsilyl derivative). The \(\alpha\)-configuration was assigned from the optical rotational value of its crystalline tosylhydrazone. Further hydrolysis of VI in 0.5 N H\(_{2}\)SO\(_{4}\) at 70°C afforded another neutral sugar (VII) and a disaccharide (VIII)
which was presumed to contain a basic sugar moiety. The disaccharide resisted further methanolysis but liberated L-alanine (IX) on vigorous aqueous acid hydrolysis. N-Acetylation of VIII followed by heating with methanolic hydrogen chloride afforded methyl d-galactoside (X) which was identified by TLC, GLC and specific rotation. The presumed basic sugar moiety was not isolated in the above acid hydrolysis studies.

On treatment with 0.1 N H2SO4 in methanol, IIb was hydrolyzed to yield a new UV-absorbing fragment (IIIb) and tetrasaccharide IV, the latter being identical with that obtained from IIa. Thus, the structural difference between Ia and Ib was found to reside only in the chromophoric aglycone moiety.

**Structures of IIIa and IIIb**

The basic aglycone IIIa was obtained as a crystalline hydrochloride (C18H22N2O2.3HCl) and gave a positive reaction with ferric chloride. The UV spectrum of IIIa exhibited a maximum at 252 nm in water and in 0.1 N HCl, and the absorption underwent a bathochromic shift to 289 nm in 0.1 N NaOH. As shown in Fig. 1, the PMR spectrum of IIIa displayed 10 higher-field and 12 lower-field methylene protons along with four aromatic (AB quartet) protons. Acid hydrolysis of IIIa in 6 N HCl yielded a UV-absorbing acidic compound (XI, C7H6O3) and a strongly basic amine (XIIa, C11H18N4). From its UV (λmax 252 nm) and IR spectra, XI was proved to be p-hydroxybenzoic acid. The tetrahydrochloride of XIIa was isolated as fine needles and showed physicochemical properties similar to those of spermidine or spermine. The PMR spectrum of XIIa indicated an asymmetric structure and the presence of six C–CH2–N and five C–CH2–C groups. The mass spectrum (MS) of XIIa included the molecular ion at m/z 216 and prominent fragment ions (m/z 172, 158, 101, 87) produced by β-cleavages. These data suggested XIIa to be 1,13-diamino-4,9-diazatridecane, a novel compound, and the structure was proven by the following synthesis: utilizing the method of Kuttan2, two molecules of 4-aminoo-
butyric acid were coupled to obtain 4-(4'-aminobutyramido)butyramide. Reaction of the amide with
N-hydroxysuccinimide ester of 3-benzyloxycarbonylamidopropionic acid, followed by deblocking, af-
forded 4-[4'-(3''-aminopropionamido)butyramido]butyramide. Reduction of the triamide with borane
in THF gave 1,13-diamino-4,9-diazatridecane. The synthetic compound was identical with XIIa
in all respects. The MS of IIIa showed, in addition to the M+ ion at m/z 336, abundant ion peaks
at m/z 278 (M+–NH2(CH2)3) and m/z 207 (M+–NH2(CH2)4NH(CH3)3), indicating that the terminal
propylamine group of XIIa was acylated with XI (Fig. 2).

Fig. 2. Mass spectrum of IIIa.

The physico-chemical properties of IIIb (C14H23N3O2), the aglycone of Ib, were similar to those of
IIla. Upon acid hydrolysis, IIIb yielded XI and spermidine (XIIb). The MS and CMR of IIIb indicated
that XI was also linked to the aminopropyl site of XIIb.

The structures of XIIa and XIIb assigned above were supported by microbiological transformation
using spermidine oxidase5). Upon incubation with the enzyme preparation of Serratia marcescens,
IIIa was converted into two ninhydrin-positive, UV-absorbing fragments. One was identified as IIIb
and the other (IIIc, m/z 194 in MS) afforded XI and 1,3-diaminopropane on acid hydrolysis. IIIb
was converted to IIIc by similar enzymatic reaction. Consequently, the structures of IIIa, IIlb and
IIIc are as shown below:

Structure of VII

The neutral sugar fragment, VII (C7H14O5), was separated by silica gel chromatography into two
anomers, VIIa and VIIb. Both anomers exhibited one C–CH3 group in their PMR spectrum and an
(M+1)+ ion peak at m/z 179, indicating that they were methyl glycosides of 6-deoxyhexose. The
magnitude of coupling constants observed for the anomeric protons (J1–2=2.0 Hz for VIIa and 4.6 Hz
for VIIb) and for the C2 proton (J4–5=3.3 Hz for VIIa and 4.0 Hz for VIIb) suggested an ido or a talo
pyranoside configuration for VII. Eventually, VIIa was identified as methyl 6-deoxy-β-L-idopyranoside6)
by direct comparison with an authentic sample, and hence VIIb was the α-anomer.

Since VII was not formed when Ia was hydrolyzed, VII arises by catalytic hydrogenation of the
CH2=CH< group of Ia. Thus the antecedent structure for VII in Ia was concluded to be 6-deoxy-D-
xylo-hex-5-enose (XIII). It has been reported4) that catalytic reduction of 6-deoxy-α-D-xylo-hex-5-
epiopyranoside gave 6-deoxy-α-L-idose predominantly along with a small amount of 6-deoxy-D-glucose,
while reduction of the β-anomer of the enopyranoside afforded 6-deoxy-D-glucose as a major product5).
In fact, the acid hydrolyzate of VI was found to contain a small amount of another sugar which was identified as an anomeric mixture of methyl 6-deoxy-β-glucopyranoside (XIV). The structures of VII, XIII and XIV are:

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\text{Structures of VIII}
\]

As described earlier the disaccharide fragment, VIII (C\(_{16}H\_31N_3O_9\)), contains L-alanine (IX) and D-galactose (X). The presence of a diaminohexose moiety (XV) in VIII was presumed from the analytical and spectral data. Heating VIII with saturated Ba(OH)\(_2\) afforded the dealanyl derivative, XVI (Chart 2). This was converted to its N-acetyl derivative, XVII, which was treated in acetate buffer (pH 4.6) with excess periodate. The oxidation product was heated with benzyl alcohol containing 2% hydrogen chloride and then acetylated in pyridine to obtain benzyl N,O-acetyl-glycoside (XVIII). Chromatographic purification of XVIII gave a homogeneous anomer (XVIIIa, (M+1)+: m/z 379, M\(_0\): +488) which was identified as benzyl 2,4-diacetamido-3-O-acetyl-2,4,6-trideoxy-α-D-galactopyranoside\(^{59}\).

Therefore, XV was determined to be 2,4-diamino-2,4,6-trideoxy-β-galactose. The MS of XVI exhibited an intense fragment ion at m/z 145, suggesting that XV was linked glycosidically to X in XVI. Since XVII consumed 1 molar equivalent of periodate, the site of glycosidic linkage should be either the C-2 or C-4 hydroxyl group of X. It was concluded to be C-4 because no axial O-acetyl group was observed in the PMR spectrum of the N,O-peracetyl derivative of XVI and because the C-2 proton showed a downfield shift on acetylation. The α-glycosidic configuration of XV was assigned from the PMR (anomeric proton: \(\delta 4.89, d, J=3.5\) Hz) and the CMR (anomeric carbon: \(\delta 99.0\)) spectra of XVI, and from the modified HUDSON's rule\(^{57}\) (calculated M\(_0\) for XV: +559). The alanine-containing disaccharide fragment (VIII) showed an amide carbonyl absorption in the IR spectrum. The PMR spectrum of VIII indi-
cated a lower-field shift of the methine proton at C-2 than observed in XVI. Thus the site of acylation with L-alanine was determined to be the C-2 amino group of XV.

Structures of Ia and Ib

Treatment of Ia with acidic water (pH 4.0) or 50% acetic acid at 27°C gave rise to another UV-absorbing fragment (XIX) which afforded IIIa and V upon acid methanolysis. An α-furanoside structure was assigned to V based on the PMR spectrum (anomeric proton at δ 5.78, J=4.0 Hz), specific rotation (M₆: +445) and periodate oxidation (1 mole consumed) of XIX.

![Figure 3](https://example.com/figure3)

The sequence of four sugar moieties in IV was deduced to be XV→X→VII→V by the MS analysis of tetrasaccharide IV and its dealanyl derivative as shown in Fig. 3.

Thus, structures Ia and Ib shown previously were assigned to glysperins A and B, respectively.
Structure of Ic

Upon hydrolysis in methanolic 0.1 N \( \text{H}_{2}\text{SO}_4 \), Ic afforded IIIa and a new tetrasaccharide fragment (IVc). Further degradation of IVc by refluxing with 0.5 N \( \text{H}_{2}\text{SO}_4 \) in methanol gave V, VIII and another sugar XXII. XXII was identified as methyl D-glucoside by its TLC, GLC and optical rotational values. An \( \alpha \)-pyranoside configuration was indicated by the PMR (anomeric proton: \( \delta \) 5.02, \( J = 3.5 \) Hz) and CMR (anomeric carbon: \( \delta \) 99.3) spectra of IVc. Thus the structure of Ic, as previously shown, had D-glucose in place of the unsaturated sugar moiety of Ia.

Discussion

Glysperins A, B and C are a new class of antibiotic with a glycobenzoyl-polyamine structure and several unusual constituents. The occurrence of 2,4-diamino-2,4,6-trideoxy-D-galactose was recently reported in the polysaccharide of *Streptococcus pneumoniae* and *Shigella sonnei*). Glysperins A and B contained 6-deoxy-D-xylo-hex-5-enose, which is the first reported instance of its occurrence in nature although its synthesis has been reported). The polyamine component of gysperins A and C, 1,13-diamino-4,9-diazatridecane, is a hitherto undescribed compound.

Glysperins are, in some respects, structurally related to the glycocinnamoylspermidine antibiotics, LL-BM 123 \( \beta \) and \( \gamma \), which are produced by *Nocardia species*). However, the two groups of antibiotics differ in both the chromophore and sugar components. Glysperins do not contain ureido and guanidino groups which are present in the LL-BM 123 antibiotics.

Experimental

UV spectra were determined on a Shimadzu UV-200 spectrophotometer and IR spectra on a JASCO IRA-1 spectrometer. Optical rotational values were measured by use of a JASCO DIP-140 automatic polarimeter. PMR spectra were recorded on a JEOL C-60-HL, a Hitachi 90 MHz or Varian HR-220 apparatus using tetramethylsilane (TMS) as internal or external (in case of D\( _2\)O) references. CMR spectra were determined on a Varian FT-80A apparatus operated in FOURIER transform system. Mass spectra were measured on a Hitachi RMU-6MG mass spectrometer modified with an in-beam/direct inlet ion source. Thin-layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60 F\( _254 \)) using the solvent systems shown below.

<table>
<thead>
<tr>
<th>System No.</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-102</td>
<td>MeOH - 10% AcONH(_4) (1:1)</td>
</tr>
<tr>
<td>S-114</td>
<td>MeOAc - n-PrOH - 28% ( \text{NH}_4)OH (45: 105: 60)</td>
</tr>
<tr>
<td>S-117</td>
<td>CHCl(_3) - MeOH - 28% ( \text{NH}_4)OH (1: 3: 2)</td>
</tr>
<tr>
<td>SD-108</td>
<td>CHCl(_3) - MeOH (93: 7)</td>
</tr>
<tr>
<td>SD-109</td>
<td>CHCl(_3) - n-PrOH - 28% ( \text{NH}_4)OH (45: 105: 25)</td>
</tr>
<tr>
<td>SD-110</td>
<td>CHCl(_3) - EtOH (9: 1)</td>
</tr>
</tbody>
</table>

Hydrogenation of gysperin A (Ia)—Isolation of IIa

A solution of Ia (1.3 g) in 100 ml of 50\% aqueous EtOH was hydrogenated over PtO\(_2\) (560 mg) at 41 p.s.i. in a Parr apparatus for 18 hours. The catalyst was removed by filtration and the filtrate was evaporated in vacuo. The residual solid was loaded on a column of Amberlite CG-50 (NH\(_4\)^+), \( \phi \) 1.8 x 40 cm) which was developed successively with water, 0.5 N \( \text{NH}_4\)OH and 1.0 N \( \text{NH}_4\)OH. The ninhydrin-positive fractions eluted with 1.0 N \( \text{NH}_4\)OH were pooled, concentrated in vacuo and lyophilized to afford 798 mg of IIa as a white amorphous powder, m.p. 143-149°C (dec.). TLC: Rf 0.21 (S-102) and 0.32 (S-117). \( [\alpha]_D^{25} +135^\circ \) (c 0.5, H\(_2\)O), \( \lambda_{\text{max}}^{\text{DMSO}} 247 \text{ nm (c 14,000).} \) PMR \( \delta_{\text{DMSO}}^\text{D} \) in ppm: 1.26 (3H, d, \( J = 6.5 \) Hz), 1.33 (3H, d, \( J = 6.5 \) Hz), 1.50 (3H, d, \( J = 7.0 \) Hz), 5.83 (1H, d, \( J = 4.0 \) Hz), 7.20 (2H, d, \( J = 9.0 \) Hz), 7.77 (2H, d, \( J = 9.0 \) Hz), etc. Anal. Calcd. for C\(_{14}\)H\(_{17}\)N\(_4\)O\(_5\) \( 2\)H\(_2\)CO\(_2\): C, 50.36; H, 7.47; N, 9.04. Found: C, 50.11; H, 7.62; N, 9.03.
Acid hydrolysis of IIIa—Isolation of IIIa and IV

A solution of IIIa (550 mg) in 50 ml of 0.1 N H₂SO₄-MeOH was stirred at 27°C for 16 hours. The resulting solution was neutralized with Amberlite IR-45 (OH⁻) and then evaporated in vacuo to dryness. The residue was chromatographed on a column of Amberlite CG-50 (NH₄⁺, φ 1.8 x 45 cm) which was developed with water, 0.05 N NH₄OH and 1.0 N NH₄OH, successively. The elution was followed by ninhydrin and anthrone tests. Concentration of anthrone-positive fractions eluted by water gave 240 mg of white solid which contained a small amount of V and, for the most part, inorganic salts. Anthrone and ninhydrin-positive fractions from the 0.05 N NH₄OH eluate afforded crude IV (324 mg). The ninhydrin-positive eluate with 1.0 N NH₄OH gave IIIa (165 mg) after evaporation in vacuo.

Crude IV (320 mg) was purified by silica-gel chromatography (φ 1.6 x 50 cm) using the solvent system, CHCl₃ - n-PrOH - 28% NH₄OH (5: 8: 2). Elution was monitored by the DAP test (diphenylamine - aniline - acetone - 80% H₃PO₄) and appropriate fractions were evaporated in vacuo. A small amount of trisaccharide VI (58 mg) was eluted first, followed by the major tetrasaccharide IV (252 mg).

IIIa: Crystallized as colorless needles from aqueous EtOH containing dil. HCl, m.p. 255.5-256°C. TLC: Rf 0.34 (S-102) and 0.15 (S-114). λₘ₅₈₀ or 0.1N HCl (252 nm (ε 13,900)) and λₘ₅₅₆ N₄O₂ 289 nm (ε 20,400). PMR δIr in ppm: 1.52-4.4 (10H, m), 2.73-3.6 (12H, m), 6.85 (2H, d, J=9.0 Hz) and 7.55 (2H, d, J=9.0 Hz). MS: m/z 336 (M)+, 278, 249, 221, 207, 186, 178, 141, 121, 101, etc. Anal. Calcd. for C₅₈H₇₄N₄O₁₂•3HCl: C, 48.49; H, 7.91; N, 12.57; Cl, 23.86. Found: C, 48.48; H, 7.96; N, 12.46; Cl, 23.97.

IV: White amorphous powder. TLC: Rf 0.23 (S-114) and 0.13 (SD-109). [α]D+94° (c 0.3, H₂O). PMR δIr in ppm: 1.24 (3H, d, J=6.5 Hz), 1.33 (3H, d, J=6.5 Hz), 1.44 (3H, d, J=7.0 Hz), 3.29 (1H, s), 3.40 (3H, s), 4.59 (1H, d, J=7.0 Hz) and 4.8-5.0 (3H, m). MS: m/z 688 (M+1)+, 524, 378, 216, etc.

Acid hydrolysis of IIb—Isolation of IIb

Hydrogenation of IIb (45 mg) in the same manner as above afforded 35 mg of IIb. TLC: Rf 0.29 (S-102) and 0.45 (S-117). λₘ₅₈₀ 247 nm (ε 14,500).

IIb (31 mg) was hydrolyzed with 0.1 N H₂SO₄ - MeOH and the hydrolyzate was chromatographed by the procedure described above to isolate IIb (7 mg) and IV (10 mg).

IIb: White powder. TLC: Rf 0.58 (S-102) and 0.28 (S-114). λₘ₅₈₀ or 0.1N HCl (252 nm (ε 12,400)) and λₘ₅₅₆ N₄O₂ 289 nm (ε 18,500). PMR δIr in ppm: 1.4-2? (6H, m), 2.63-3.6 (8H, m), 6.64 (2H, d, J=9.0 Hz) and 7.55 (2H, d, J=9.0 Hz). MS: m/z 265 (M)+.

Methanolyis of IV—Isolation of VI and V

Saccharide IV (550 mg) in 50 ml of 0.5 N H₂SO₄ - MeOH was allowed to stand at 27°C for 16 hours. The reaction mixture was neutralized with Amberlite IR-45 (OH⁻) and evaporated to leave an amorphous solid which was chromatographed on a column of Amberlite CG-50 (NH₄⁺, φ 1.2 x 30 cm). Development of the column with water gave a mixture of V and inorganic salts (292 mg); subsequent development with 0.05 N NH₄OH yielded trisaccharide VI (303 mg). VI: Amorphous solid. TLC: Rf 0.32+0.39 (S-114) and 0.17+0.22 (SD-109). MS: m/z 556 (M+1)+, 555 (M)+, 538, 524, 512, 378, 216, etc.

The powder containing V (260 mg) was chromatographed on a column of Sephadex LH-20 (φ 2.0 x 75 cm) which was developed with 50% aqueous MeOH. The DAP-positive fractions were concentrated in vacuo and lyophilized to afford 136 mg of V as an oily solid. TLC: Rf 0.45 (S-114). [α]D+88° (c 0.3, H₂O). GLC of the TMS-derivative (OV-17, 3 %, temperature programing 5°C/min. from 150°C): Rt 3.7 (major) and 3.9 (minor).

V (100 mg) was hydrolyzed with 0.2 N HCl (20 ml) to yield 82 mg of free sugar. Part of the sugar (55 mg) and p-toluenesulfonflylhydrazide (160 mg) in 10 ml of methanol was heated under reflux for 30 minutes. The yellow precipitate deposited upon cooling was crystallized from a mixture of MeOH and EtOH to give 55 mg of tosylhydrazone. M.p. 116°C. [α]D+15°+14.5° (c 0.49, pyridine). The IR spectrum was identical with that of d-ribose tosylhydrazone.
Methanolysis of VI—Isolation of VIIa, VIIb, VIII and XIV

A solution of VI (440 mg) in 40 ml of 0.5 N H₂SO₄ - MeOH was heated under reflux for one hour. After being neutralized with Amberlite IR-45 (OH⁻), the solution was evaporated to give an amorphous solid which was applied to a column of Amberlite CG-50 (NH₄⁺, 5.0 x 30 cm). Development of the column with water afforded neutral sugar VII (anomeric mixture, 84 mg); VIII (285 mg) was then eluted with 0.05 N NH₄OH. Sugar VII (80 mg) was chromatographed on silica-gel (Φ 1.8 x 46 cm) using CHCl₃ - MeOH (98:2) as eluant. The course of elution was monitored by a DAP test and TLC (SD-108); appropriate fractions were combined and concentrated to give VIIb (18 mg), VIIa (20 mg) and a trace amount of XIV (2 mg).

VIIa: Hygroscopic white solid. TLC: Rf 0.57 (S-114) and 0.10 (SD-108). [α]D⁰ +44° (c 0.19, H₂O). MS: m/z 179 (M+1)⁺, 147, 129, 116, 100, 74, 60, etc. PMR δ⁰ in ppm: 1.31 (3H, d, J=6.7 Hz), 3.51 (3H, s), 3.4-3.8 (2H, m), 3.99 (1H, t, J=5.0 Hz), 4.10 (1H, d-q, J=6.7 & 3.3 Hz) and 4.71 (1H, d, J=2.0 Hz). Identified as methyl 6-deoxy-β-L-idopyranoside by direct comparison with an authentic specimen.

VIIb: TLC: Rf 0.57 (S-114) and 0.15 (SD-108). [α]D³-87° (c 0.4, H₂O). PMR δ⁰ in ppm: 1.26 (3H, d, J=6.7 Hz), 3.44 (3H, s), 3.3-3.9 (3H, m), 4.21 (1H, d-q, J=6.7 & 4.0 Hz) and 4.58 (1H, d, J=4.6 Hz).

XIV: TLC: Rf 0.42 (S-114) and 0.06 (SD-108). Identified as methyl 6-deoxy-α-D-glucoside by TLC and GLC (TMS derivative) comparison with an authentic sample.

XVIII: White amorphous powder. TLC: Rf 0.36 (S-114). pKa': 7.3 and 8.3 (titration equivalent; 410). IR νKBO 1680 and 1570 cm⁻¹. MS: m/z 410 (M⁻, 1)+, 366, 305, 276, 249, 216, etc. PMR δ⁰ in ppm: 1.23 (3H, d, J=6.5 Hz), 1.38 (3H, d, J=7.0 Hz), 3.13 (1H, m), 3.41 (3H, s), 4.86 (1H, d, J=1.5 Hz) and 4.90 (1H, d, J=3.5 Hz).

Acid hydrolysis of VIII—Isolation of IX

A solution of VIII (340 mg) in 6 N HCl (17 ml) was heated at 110°C for 16 hours in a sealed tube. The hydrolyzate was chromatographed on Dowex 50W x 4 (H+, 0.1 x 32 cm) which was developed with an increasing concentration of HCl. Ninhydrin-positive fractions eluted with 0.1 N HCl were concentrated to afford 87 mg of IX hydrochloride.

IX HCl: Crystalline solid. M.p. 206-207°C (dec.). TLC: Rf 0.81 (S-102) and 0.26 (S-114). [α]D² +6° (c 2.0, 5 N HCl). Identified as L-alanine by IR spectrum.

Acid methanolysis of N-diacetyl VIII—Isolation of X

N-Diacetyl-VIII (130 mg) was hydrolyzed by refluxing with 1.8 N methanolic hydrogen chloride. The hydrolyzate was concentrated and passed through a column of Amberlite CG-50 (NH₄⁺) to remove basic products. The fractions containing neutral sugar were concentrated in vacuo and the residue was fractionated on a column of Sephadex LH-20 (Φ 2.0 x 66 cm) with 50% aqueous MeOH. The DAP-positive eluate was concentrated to give 27 mg of IX hydrochloride.

X: TLC: Rf 0.30 (S-114). [α]D²⁻+95° (c 0.2, H₂O). GLC of TMS derivative (OV-17, 3%, temperature programing at 5°C/min. from 150°C). Rt 6.32 (minor), 7.00 (major) and 7.38 (minor). Identified as methyl D-galactoside.

Hydrolysis of IIIa and IIIb—Isolation of XI, XIIa and XIIb

A solution of IIIa hydrochloride (450 mg) in 50 ml of 6 N HCl was heated at 120°C for 16 hours in a sealed tube. The reaction mixture was cooled and extracted with two 50-ml portions of ethyl ether. The extracts were combined, evaporated and lyophilized to give 71 mg of XI. The aqueous layer was decolorized with active carbon, concentrated to 5 ml and then diluted with EtOH to deposit colorless needles of XI hydrochloride (322 mg).

XI: δH₂O 252 nm (ε 12,600) and δmax NaOH 280 nm (ε 15,100). Anal. Calcd. for C₉H₆O₅·NaOH: C, 60.81; H, 4.38. Found: C, 60.61; H, 4.26. Identified as p-hydroxybenzoic acid by IR spectrum.

N-Diacetyl-VIII (130 mg) was hydrolyzed by refluxing with 1.8 N methanolic hydrogen chloride. The hydrolyzate was concentrated and passed through a column of Amberlite CG-50 (NH₄⁺) to remove basic products. The fractions containing neutral sugar were concentrated in vacuo and the residue was fractionated on a column of Sephadex LH-20 (Φ 2.0 x 66 cm) with 50% aqueous MeOH. The DAP-positive eluate was concentrated to give 27 mg of syrupy X.

X: TLC: Rf 0.30 (S-114). [α]D²⁻+95° (c 0.2, H₂O). GLC of TMS derivative (OV-17, 3%, temperature programing at 5°C/min. from 150°C). Rt 6.32 (minor), 7.00 (major) and 7.38 (minor). Identified as methyl D-galactoside.

Hydrolysis of IIIa and IIIb—Isolation of XI, XIIa and XIIb

A solution of IIIa hydrochloride (450 mg) in 50 ml of 6 N HCl was heated at 120°C for 16 hours in a sealed tube. The reaction mixture was cooled and extracted with two 50-ml portions of ethyl ether. The extracts were combined, evaporated and lyophilized to give 71 mg of XI. The aqueous layer was decolorized with active carbon, concentrated to 5 ml and then diluted with EtOH to deposit colorless needles of XIa hydrochloride (322 mg).

XI: δH₂O 252 nm (ε 12,600) and δmax NaOH 280 nm (ε 15,100). Anal. Calcd. for C₉H₆O₅·NaOH: C, 60.81; H, 4.38. Found: C, 60.61; H, 4.26. Identified as p-hydroxybenzoic acid by IR spectrum.
XIIa: Fine needles, m.p. >300°C. TLC: Rf 0.08 (S-102). MS: m/z 216 (M+), 186, 172, 158, 84, 72, etc. PMR \( \delta_{\text{TMS}} \) in ppm: 1.5-2.5 (10H, m) and 2.6-3.5 (12H, m). Anal. Calcd. for C<sub>11</sub>H<sub>28</sub>N<sub>4</sub>·4HCl: C, 36.48; H, 8.90; N, 15.47; Cl, 39.15. Found: C, 36.43; H, 9.14; N, 15.37; Cl, 38.94.

IIIb (10 mg) was hydrolyzed with 6 N HCl at 120°C for 16 hours and the hydrolyzate worked up as above to yield XI (2 mg) and XIIb hydrochloride (3 mg). XIIb: TLC: Rf 0.11 (S-102). Identified as spermidine hydrochloride by IR spectrum and TLC.

Preparation of 1,13-diamino-4,9-diazatridecane

4-Benzoyloxycarbonyl(Cbz)-aminobutyric acid (7.12 g) and N-hydroxysuccinimide (NOS, 3.45 g) were condensed in the presence of dicyclohexylcarbodiimide (6.19 g) in 120 ml of ethyl acetate at 5°C to yield 4-Cbz-aminobutyryl-NOS (12.12 g).

An ethanol solution of the active ester (6.0 g) was poured into 15 N NH<sub>3</sub>OH at -3°C to deposit 2.89 g of 4-Cbz-aminobutyramide as colorless needles, m.p. 132-135°C. Hydrogenolysis of the amide (2.48 g) in the presence 10% palladium on charcoal in 75% aqueous EtOH gave a quantitative yield of deblocked amide which was cooled with 6 g of 4-Cbz-aminobutyryl-NOS to afford 4-(4'-Cbz-aminobutyramido)butyramide, 2.14 g. M. p. 172-173°C. This compound was hydrogenated with 10% palladium on charcoal to yield free diamide. The amide (1.03 g) was treated with 3-Cbz-aminopropionyl-NOS (1.76 g) in 50 ml of dimethylformamide at 25°C. The reaction solution was concentrated to 10 ml \textit{in vacuo} and diluted with 40 ml of 0.5 N NaOH to precipitate 4-[4'-3'-Cbz-aminopropionamido]-butyramido]butyramide. Yield 821 mg. M. p. 206-207°C. Anal. Calcd. for C<sub>19</sub>H<sub>29</sub>N<sub>4</sub>O<sub>5</sub>: C, 58.15; H, 7.19; N, 14.28. Found: C, 57.76; H, 7.18; N, 14.17. Deblocking of the triamide (704 mg) by hydrogenation in 80% aqueous MeOH gave aminotriamide quantitatively. This was refluxed with 40 ml of borane in tetrahydrofuran (1 mole/liter solution) for 26 hours. The amine produced was chromatographed on a column of Dowex 50W x 4 (H+, 0.1 x 18 cm) with increasing concentration of HCl. Appropriate fractions were concentrated to afford 159 mg of 1,13-diamino-4,9-diazatridecane. Fine needles from aqueous EtOH. M. p. >300°C. TLC: Rf 0.08 (S-102). Anal. Calcd for C<sub>11</sub>H<sub>28</sub>N<sub>4</sub>·4HCl: C, 36.48; H, 8.90; N, 15.47; Cl, 39.15. Found: C, 36.51; H, 8.98; N, 15.24; Cl, 38.81. Identified as XIIa.

Enzymatic degradation of IIIa

A mixture of IIIa hydrochloride (20 mg) and an enzyme preparation from Serratia marcescens IAM-1223 (40 mg) in 20 ml of M/15 phosphate buffer (pH 7.0) was incubated at 37°C for 4 days. After removal of insolubles by filtration, the filtrate was applied to a column of Amberlite CG-50 (NH<sub>4</sub>+, 0.1 x 25 cm) which was eluted successively with water (300 ml), N/20 NH<sub>4</sub>OH (200 ml), N/2 NH<sub>4</sub>OH (200 ml) and N NH<sub>4</sub>OH (200 ml). UV-absorbing fractions eluted first with N/20 NH<sub>4</sub>OH were concentrated to afford 2 mg of IIIc. A subsequent UV-absorbing fraction eluted with N/2 NH<sub>4</sub>OH gave 11.5 mg of white powder which was identified as IIIb obtained from Ib. IIIc: TLC: Rf 0.68 (S-102) and 0.41 (S-114). \( \lambda_{\text{max}}^o \text{ or } \Phi^o \text{ HCl} 252 \text{ nm (e 7,800) and } \lambda_{\text{max}}^{\text{NaOH}} 289 \text{ nm (e 10,500). MS: m/z 194 (M+)}, 178, 164, 150, 121, 73, etc.

Alkaline hydrolysis of VIII—Isolation of XVI

VIII (383 mg) was refluxed with 38 ml of saturated barium hydroxide solution for 16 hours. The solution was adjusted to pH 7.0 with an addition of solid carbon dioxide and the mixture filtered. The filtrate was concentrated to a sticky residue which was chromatographed on a column of Amberlite CG-50 (NH<sub>4</sub>+, 1.8 x 60 cm). Elution with water afforded IX (82 mg); subsequent elution with 0.1 N NH<sub>4</sub>OH gave 240 mg of XVI.

XVI: White amorphous powder. TLC: Rf 0.36 (S-114). pKa': 6.6 and 8.5 in water (titration equivalent; 340). MS: m/z 339 (M+1)+, 307, 276, 265, 235, 145, etc. PMR \( \delta_{\text{TMS}} \) in ppm: 1.29 (3H, d, J=6.5 Hz), 2.94 (1H, d-d, J=10.5 & 3.5 Hz), 3.19 (1H, d-d, J=2.0 & 4.0 Hz), 3.42 (3H, s), 4.84 (1H, d, J=1.5 Hz) and 4.89 (1H, d, J=3.5 Hz).

XVII (di-N-acetyl XVI): Obtained by acetylation of XVI in anhydrous MeOH. TLC: Rf 0.41 (S-114). MS: m/z 423 (M+1)+, 405, 391, 373, 306, 277, 229, 211, 156, 99, etc. PMR \( \delta_{\text{TMS}} \) in ppm: 1.10 (3H, d, J=6.5 Hz), 2.08 (3H, s), 2.11 (3H, s), 3.41 (3H, s), and 4.81 (2H, m). Anal. Calcd for C<sub>17</sub>H<sub>35</sub>N<sub>10</sub>O<sub>2</sub>·2H<sub>2</sub>O: C, 45.02; H, 7.56; N, 6.18. Found: C, 45.28; H, 7.34; N, 5.87.
Periodate oxidation of XVII—Isolation of XVIIIa

A solution of XVII (420 mg) and NaIO₄ (340 mg, 1.6 equivalent) in 100 ml of 0.1 M sodium acetate buffer (pH 4.6) was stirred for 24 hours at 20–25°C. After excess periodate was decomposed with ethylene glycol (30 mg), the solution was acidified to pH 3.0 and allowed to stand for 2 hours at 25°C. The mixture was filtered, neutralized with Amberlite IR-45 (OH⁻) and evaporated to leave a sticky solid which was applied on a column of Diaion HP-20 (φ 1.0 x 45 cm). After inorganic materials were washed out with water, the fractions containing the sugar fragments were eluted with 50% MeOH. Concentration of the fractions gave 315 mg of amorphous solid. The sugar was heated at 72–75°C for 2 hours with 7.8 ml of benzyl alcohol containing 2% hydrogen chloride. Addition of Et₂O to the mixture gave 277 mg of benzyl glycoside as a white powder.

A solution of glycose (80 mg) in acetic anhydride (1 ml) and pyridine (2 ml) was kept at 20–25°C for 72 hours. Evaporation of the mixture afforded a syrupy residue which was taken up in CHCl₃ (50 ml). The solution was washed with water, dried and concentrated to yield 84 mg of white powder. This was chromatographed on silica gel in a 1.0 x 50 cm column using CHCl₃ with increasing proportion of EtOH as eluants. The major sugar fragment (30 mg) eluted with CHCl₃ - EtOH (99: 1) was further purified by preparative TLC developed with CHCl₃ - EtOH (9: 1). The appropriate sugar zone (Rf 0.34–0.42) was extracted with CHCl₃ and the extract concentrated in vacuo to afford 11 mg of XVIIIa. TLC: Rf 0.40 (SD-110). [α]⁺/° '129° (c 0.14, CHCl₃). MS: m/z 379 (M+1)+, 361, 335, 319, 270, 227, 199, etc. PMR ⁶°H in ppm: 1.12 (3H, d, J=6.5 Hz), 1.92 (3H, s), 1.99 (3H, s), 2.08 (3H, s), 4.0–4.5 (3H, m), 4.5–4.7 (2H, m), 4.88 (1H, d, J=3.5 Hz), 5.14 (1H, d, J=4.0 & 12.0 Hz), 5.77 (1H, NH) and 6.19 (1H, NH). Identified as benzyl 2,4-diacetamido-3-O-acetyl-2,4,6-trideoxy-a-D-galactopyranoside.

Mild hydrolysis of la—Isolation of XIX

la (1.10 g) in 110 ml of 50% aqueous acetic acid was incubated at 27°C for 15 hours. The resulting mixture was evaporated to leave a sticky solid which was chromatographed on a column of Amberlite XT-2 (φ 2.0 x 64 cm) developed with water. Saccharide fragments were eluted first and the fractions containing XIX were evaporated in vacuo to yield a syrupy residue (540 mg). A portion of the residue (110 mg) was purified by silica gel chromatography (φ 1.0 x 23 cm) developed with CHCl₃ - MeOH - 28% NH₄OH (1: 2: 1). The recovered XIX was further fractionated on a column of Sephadex LH-20 (φ 1.0 x 25 cm) using 50% aqueous methanol. Concentration of UV-absorbing fractions afforded 63 mg of homogeneous XIX as colorless syrup. TLC: Rf 0.15 (S-114). [α]⁺/° +95° (c 0.5, H₂O). ⁶°H in ppm: 1.3–2.2 (10H, m), 2.6–3.6 (12H, m), 3.70 (2H, d, J=6.0 Hz), 4.0–4.5 (3H, m), 5.78 (1H, d, J=4.0 Hz), 7.13 (2H, d, J=9.0 Hz) and 7.71 (2H, d, J=9.0 Hz).

Isolation of XX and XXI

A mixture of la (1.02 g), acetic anhydride (0.57 ml) and anhydrous methanol (50 ml) was stirred for 3 hours. The solution was evaporated to yield 950 mg of N-pentaacetyl-la. To a dimethylformamide solution (30 ml) of the acetate (610 mg), 1.32 g of sodium hydride was added and the mixture was stirred at 48–52°C for one hour under a nitrogen atmosphere. Methyl iodine (24 g) was added dropwise to the solution at 0°C and the stirring was continued overnight at room temperature. The insolubles were removed by filtration and the solution was evaporated to dryness under reduced pressure. The residue was taken up in CHCl₃, washed with water and concentrated to obtain 660 mg of N-acetyl-N,O-permethyl-la as a sticky solid. TLC: Rf 0.78 (S-114). This solid (370 mg) was hydrolyzed with 50% aqueous acetic acid (37 ml) at 27°C. The hydrolyzate was chromatographed on a column of silica gel (φ 2.0 x 40 cm) developed with CHCl₃ - MeOH (95: 5). The UV-absorbing fractions were combined and concentrated to yield XX as a syrup (124 mg).

XX: TLC: Rf 0.51 (S-114). ²H in ppm: 3.89 (1H, d-d, J=4.0 & 6.0 Hz), 4.1–4.4 (2H, m), 5.73 (1H, d, J=4.0 Hz). XX (60 mg) was acetylated with acetic anhydride (3 ml) and pyridine (6 ml) at room temperature to afford 59 mg of XXI as an oil. XXI: TLC: Rf 0.67 (S-114). ²H in ppm: 3.98 (1H, d-d, J=4.0 & 6.2 Hz), 4.27 (1H, q, J=2.8 Hz), 5.30 (1H, d-d, J=2.8 & 6.2 Hz).
Acid methanolysis of Ic—Isolation of IVc and XXII

Ic (820 mg) was hydrolyzed with 0.1 N H₂SO₄ - MeOH at 27°C for 16 hours and the hydrolyzate was fractionated by a procedure similar to that used for the methanolyzate of Ia. Tetrasaccharide IVc (356 mg) was obtained after chromatographic purification, together with 202 mg of chromophore fragment IIIa.

IVc: TLC: Rf 0.22 (S-114) and 0.03 (SD-109). [α]D +135° (c 0.5, H₂O). PMR δ7.30 in ppm: 1.23 (3H, d, J=6.5 Hz), 1.34 (3H, d, J=7.0 Hz), 3.14 (1H, m), 3.40 (3H, s) and 5.02 (1H, d, J=3.5 Hz). Anal. Calcd. for C₂₇H₄₇N₂O₁₆·H₂O: C, 44.93; H, 7.12; N, 5.82. Found: C, 44.90; H, 7.12; N, 5.67.

A solution of IVc (240 mg) in 24 ml of 0.5 N H₂SO₄ - MeOH was heated under reflux for 30 minutes. The reaction mixture was neutralized with Amberlite IR-45 (OH⁻) and evaporated to dryness in vacuo. The residue was charged on a column of Amberlite CG-50 (NH₄⁺, ~ 2.0 x 30 cm) which was developed with water followed by 0.1 N NH₄OH. A mixture of neutral sugars (116 mg) was recovered from the eluate with water and disaccharide VIII (142 mg) from the eluate with 0.1 N NH₄OH. The neutral sugars were separated on a silica gel column (φ 1.0 x 30 cm) developed with MeOAc - n-PrOH - 28% NH₄OH (45: 105: 60) to yield XXII (42 mg) and V (35 mg).

XXII: TLC: Rf 0.33 (S-114). GLC of TMS derivative (OV-17, 3%, temperature programing of 5°C/min. from 150°C): Rt 8.6. Identity with methyl D-glucoside was confirmed by direct comparison.

Periodate consumption

Periodate oxidation was carried out by the conventional procedure for the following N-acetyl derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Periodate consumption (mole)</th>
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<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>Penta-N-acetyl-Ia</td>
<td>0.35</td>
</tr>
<tr>
<td>Penta-N-acetyl-IIa</td>
<td>0.35</td>
</tr>
<tr>
<td>Di-N-acetyl-IV</td>
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</tr>
<tr>
<td>Di-N-acetyl-VI</td>
<td>0.60</td>
</tr>
<tr>
<td>Di-N-acetyl-VIII</td>
<td>0.05</td>
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<tr>
<td>XVII</td>
<td>0.25</td>
</tr>
<tr>
<td>Tri-N-acetyl-XIX</td>
<td>0.07</td>
</tr>
</tbody>
</table>

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References


