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Synthesis of *C*-(β-D-glycosyl) analogues of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) as potential inhibitors of CMP-Kdo synthetase

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ABSTRACT

Eight *C*-(β-D-glycosyl) analogues (**14**–**21**) of Kdo (3-deoxy-D-*manno*-2-octulosonic acid) were obtained from isopropylidene-protected ester precursors and tested *in vitro* for their inhibitory activity on CMP-Kdo synthetase. None had more than minor inhibitory activity.

INTRODUCTION

The synthesis of several analogues of the 2-deoxy analogue* of 3-deoxy-D-*manno*-2-octulosonic acid (β-Kdo, R = OH in formula **1**) modified at the carboxyl group has been reported[1]. These compounds were investigated for inhibitory activity on the enzyme CMP-Kdo synthetase (CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase; EC 2.7.7.38), a key enzyme in the biosynthetic incorporation of Kdo into the lipopolysaccharide (LPS) of Gram-negative bacteria. The parent compound, "2-deoxy-β-Kdo" (**1**) is a potent inhibitor of CMP-Kdo synthetase[2,3].

The present study reports the synthesis and inhibitory activity of Kdo analogues (Table I) having a substituent attached via a carbon–carbon bond to C-2 to afford β-*C*-glycosyl compounds. Only **2**, (ref. 1) **3**–**4a**, (ref. 4) and **4b** (ref. 5) from this class of compounds have been reported previously. Compounds **4a** and **4b** were furthermore prepared as product analogues of CMP-Kdo; in comparison to **2** and **3**, they have sterically more-demanding substituents. The purpose of the present study was to investigate whether it would be possible to replace H-2 of compound **1** with small substituents of varying polarity and retain, or even enhance, the inhibitory activity.

* Removal of the anomeric oxygen atom renders these products anhydroaldonic acids, and they are so named in the Experimental section.

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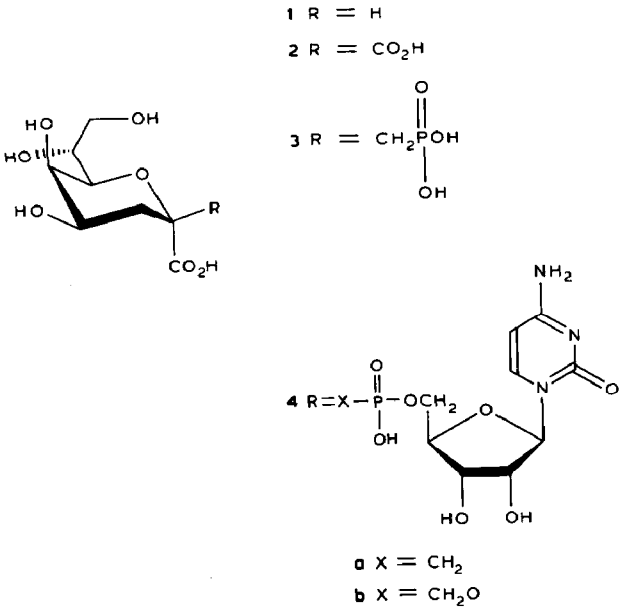

RESULTS AND DISCUSSION

The compounds tested, **14–21**, were obtained by deprotection of the isopropylidene-protected precursors **6–13** (Table I). The latter were obtained when different electrophiles were treated with the enolate formed from **5**. These enolate reactions, except for those giving **10** and **12**, have been reported elsewhere, and were shown to be stereoselective, with the biologically more-interesting *β*-*C*-glycosyl product predominating[4,6]. The nitrile **13** was obtained from the methyl ester analogue of **5**. The phosphonate **12** was obtained by using diethyl phosphonomethyltriflate[7] as the electrophile. This is a more straightforward method than one reported previously, which used four synthetic steps to reach the corresponding dimethyl phosphonate[4]. The phosphate **22** was synthesized from the hydroxymethyl derivative **6**, which was first treated with phosphorus oxychloride to give a dichlorophosphate. Methanolysis of the latter gave **22**. In an attempt to deprotect this compound, as described for **12** (see later), the deprotected product appeared to be labile under the acidic isolation conditions used, and could not be isolated.

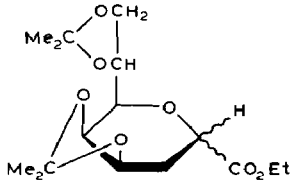

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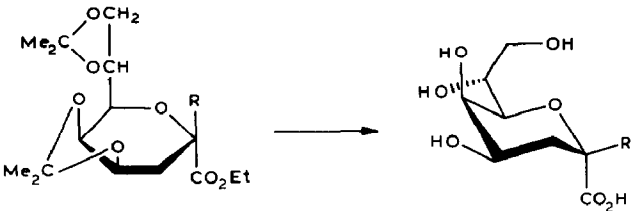
TABLE I

Chemical yields in the deprotection reactions



6–13 14–21

R in the precursors	*R in the deprotected compounds*[a,b]	*Yield %*
6 CH_2OH	**14** CH_2OH	62
7 CH_3	**15** CH_3	88
8 benzyl	**16** benzyl	68
9 propargyl	**17** propargyl	100
10 CH_2CO_2-octyl	**18** CH_2CO_2H	100
11 $CH_2CH_2CO_2Et$	**19** $CH_2CH_2CO_2H$	67
12 $CH_2PO(OEt)_2$	**20** $CH_2PO(OH)_2$	73
13[c] CN	**21** CN	75

[a] The purity of the deprotected compounds was checked by analytical l.c.[16] before they were biochemically tested. [b] The inhibitory activity for all compounds was < 15% inhibition at equimolar concentration of inhibitor and Kdo. [c] The methyl ester was used.

The deprotection of **6–11** followed the same general route. First the isopropylidene groups were removed by treatment with trifluoroacetic acid in ethanol, followed by base-promoted hydrolysis of the ester groups. The resulting sodium carboxylates were treated with Dowex H^+ ion-exchange resin to give the free acids. Treatment of compound **12** with bromotrimethylsilane in chloroform gave the disilyl ester of the phosphonic acid, which, after removal of chloroform and addition of water, afforded the phosphonic acid, which catalyzed the hydrolysis of the isopropylidene groups. Base-promoted hydrolysis of the carboxylic ester group concluded the deprotection.

The nitrile group of compound **13** proved to be incompatible with the hydrolytic conditions used. It was possible, however[8], to cleave the methyl ester with sodium cyanide in Me_2SO.

N.m.r. and conformation. — The conformations in solution of the methyl β-glycoside of Kdo and of **1** have been studied previously[9,10]. The ring in these compounds was found to adopt the $^5C_2$-conformation, in which H-4 and the carboxylic group have an axial and the C-7–C-8 exocyclic side-chain has an equatorial orientation.

The assigned ^{1}H-n.m.r. shifts, $J_{H,H}$ coupling constants, and the assigned ^{13}C-n.m.r. shifts for compounds **14–21** are listed in Tables II–IV. In all compounds, the coupling constants between H-4 and each of the two deoxy protons range between 12.2–12.4 and 4.6–4.9 Hz, respectively, reflecting a diaxial and an axial–equatorial relationship. In

compounds **14** and **20**, a W-coupling between H-3*e* and H-5 may also be observed. These data indicate the same $^5C_2$ conformation as found in the methyl β-glycoside of Kdo and of **1**; consequently the conformation is not altered by the 2-substituent. This result has been confirmed by determination of the $^3J_{H,C}$ coupling-constants between the ^{13}C-labeled hydroxymethyl group and the deoxy protons in the labeled compounds **14** and its 2-epimer. In ^{13}C-labeled **14**, these coupling constants are 3.9 and <1 Hz, respectively, reflecting an equatorial–axial and a diequatorial relationship. In the ^{13}C-labeled 2-epimer, the corresponding coupling constants are 7.9 and 3.3 Hz, respectively, thus reflecting a diaxial and an equatorial–axial relationship. In addition, the $^3J_{H,C}$ coupling-constant between H-3a and the 2-methyl carbon in compound **15** was

TABLE II

^1H-N.m.r. chemical shifts (δ) for compounds **14–21**

Compound	*Chemical shifts (δ)*									
R-	*H-3a*	*H-3e*	*H-4*	*H-5*	*H-6*	*H-7*	*H-8(1)*	*H-8(2)*	*H-9(1)*	*H-9(2)*
14 CH_2OH	1.72	2.17	3.77	3.96	3.55	[a]	3.72	[a]	3.60	3.66
15 CH_3	1.63	2.30	3.71	3.93	3.37	3.81	3.73	3.84	1.33[b]	
16 $CH_2C_6H_5$	1.82	2.23	3.73	3.94	3.42	3.87	3.69	3.76	2.99	
17 $CH_2C{\equiv}CH$[c]	1.72	2.34	3.76	3.95	3.51	3.84	3.72	3.86	2.53	2.58
18 CH_2CO_2H	1.84	2.31	3.76	3.92	3.50	[d]	3.71	[d]	2.54	2.63
19 $CH_2CH_2CO_2H$[e]	1.69	2.31	[f]	3.94	3.50	[f]	3.71	[f]	1.92	
20 $CH_2PO(OH)_2$	1.90	2.40	[g]	3.97	3.59	[g]	3.71	3.86	2.30–2.41	
21 CN	2.25	2.55	3.77	3.97	3.62	3.84	3.73	3.82		

[a] H-7 and H-8(2) 3.83–3.87. [b] Methyl. [c] H-11, 2.37. [d] H-7 and H-8(2) 3.82–3.86. [e] Determined from a 200-MHz n.m.r. spectrum; H-10(1) and H-10(2) were not determined. [f] Not determined. [g] H-4 and H-7 3.78–3.85.

TABLE III

Coupling constants for compounds **14–21**

Compound	*Coupling constants (Hz)*[a]							
R-	$J_{3a,3e}$	$J_{3a,4}$	$J_{3e,4}$	$J_{4,5}$	$J_{6,7}$	$J_{7,8(1)}$	$J_{7,8(2)}$	$J_{8(1),8(2)}$
14 CH_2OH[b,c]	−12.8	12.3	4.8	2.1	8.0	7.5	[d]	−11.9
15 CH_3	−12.8	12.3	4.8	3.1	9.0	1.8	4.7	−11.8
16 $CH_2C_6H_5$	−12.9	12.3	4.6	3.1	9.0	2.4	4.7	−12.2
17 $CH_2C{\equiv}CH$	−12.7	12.2	4.8	2.9	8.9	2.0	4.4	−11.7
18 CH_2CO_2H[e]	−12.7	12.3	4.8	2.8	8.7	3.8	[d]	−13.6
19 $CH_2CH_2CO_2H$[f]	−12.7	12.1	4.5	2.8	9.2	4.3	[d]	[d]
20 $CH_2PO(OH)_2$[g]	−13.0	12.4	4.9	2.1	8.5	5.0	3.0	−11.6
21 CN	−12.8	12.0	4.8	2.9	8.9	5.9	2.5	−12.3

[a] $J_{5,6}$ Coupling constants were not determined. [b] $J_{3,5}$ 1.0; determined from a 90-MHz n.m.r. spectrum. [c] $J_{9(1),9(2)}$ −11.8. [d] Not determined. [e] $J_{9(1),9(2)}$ −13.5. [f] Determined from a 200-MHz n.m.r. spectrum; coupling constants involving H-9 and H−10 were not determined. [g] $J_{3,5}$ 0.7; determined from a 90-MHz n.m.r. spectrum.

TABLE IV

^{13}C-N.m.r. chemical shifts for compounds **14–21**

Compound	*Chemical shifts (δ)*										
R-	*C–1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*C–10*	*C–11*
14 CH_2OH	178.72	83.75	31.36	68.39	66.81	74.90	69.98	65.00	68.39		
15 CH_3	181.69	80.65	36.58	68.44	66.59	75.27	69.98	64.95	27.41		
16 $CH_2C_6H_5$[a]	176.71	82.35	35.09	67.94	66.64	75.87	70.08	65.00	46.65		
17 $CH_2C{\equiv}CH$	178.86	81.85[b]	34.89	68.44	66.69	75.52	69.93	65.15	30.80	80.70[b]	72.88
18 CH_2CO_2H	179.91	80.88	34.98	68.33	67.04	75.33	70.13	64.95	47.46	176.89[b]	
19 $CH_2CH_2CO_2H$	178.36[b]	80.60	34.94[b]	67.94	66.74	75.62	70.18	64.75	35.23[b]	29.15	176.91[b]
20 $CH_2PO(OH)_2$[c]	175.77	78.13	35.60	67.45	66.65	75.98	70.09	64.61	38.81		
21 CN	170.73	75.32	34.24	66.84	66.34	75.82	70.03	64.45	119.88		

[a] Phenyl carbons C–1 136.04; C-2, C-3 129.15, 131.15; C-4 128.21. [b] Might be interchanged. [c] $J_{1,P} = 4.5$; $J_{2,P} = 2.9$; $J_{3,P} = 4.6$; $J_{4,P} = 2.6$; $J_{9,P} = 122$; δ^{31}P 22.4.

determined to be 4.0 Hz, which does agree with an axial–equatorial relationship and is consistent with a $^5C_2$-conformation.

In compound **20**, a W-coupling is observed between phosphorus and C-4 of the pyranose ring. This might be explained by hydrogen-bond formation between the phosphonate and the ring oxygen.

A thorough ^{1}H- and ^{13}C-n.m.r. examination of the protected compounds will be published separately[11].

Biochemistry. — Compounds **14–21** were tested for their inhibitory activity in a combined CMP-Kdo synthetase (CKS) and Kdo-Lipid A transferase (CMP-Kdo:Lipid A Kdo transferase, KLT) enzyme assay[12]. CMP-Kdo synthetase catalyzes the formation of the nucleotide derivative CMP-Kdo from Kdo and CTP (cytidine triphosphate)[13]. There is good evidence that this formation proceeds *via* a nucleophilic-displacement mechanism in which the anomeric hydroxyl group of β-Kdo acts as the nucleophile[14].

Subsequently, CMP-Kdo serves as a substrate for a series of Kdo-Lipid A transferases which are able to incorporate Kdo into the developing lipopolysaccharide, which later is positioned in the outer membrane of Gram-negative bacteria[15]. The main purpose of this work was to establish whether any of the compounds tested had an inhibitory activity matching that of **1**. Our results show that this was not the case; all compounds displayed markedly low activity (< 15% inhibition, footnote *b* in Table I).

Obviously there appears to be a close fit for the potent inhibitor **1** in the active site of the enzyme since a change from hydrogen in **1** to even such a small group as methyl in compound **15** results in almost complete loss of inhibitory activity. Our findings could be rationalized through the hypothesis proposed[2] in explaining the great difference in inhibitory activity between the methyl β-glycoside of Kdo and **1**, suggesting that Kdo binds to the active site together with CTP. If so, **1** and CTP can readily coexist in the active site, but when substituents are introduced a negative steric interaction could develop between the supposed inhibitor and CTP, resulting in an unfavourable thermo-

dynamic equilibrium. Conformational factors appear to be irrelevant, as our data show that the 2-substituent does not alter the conformation of the pyranose ring (see earlier). Interestingly, compound **14**, which structurally is closely related to Kdo, including the possibility of reacting with CTP, exhibits only low inhibitory acitity. Even if **14** is considered a false substrate, this should reasonably be shown as a depletion in the incorporation of Kdo into the lipid A precursor in the CKS-KLT enzyme assay.

EXPERIMENTAL

General procedures. — High-resolution mass spectroscopy (f.a.b.) was performed on a Jeol DX-303 instrument. For the protected compound **10**, ^{1}H- and ^{13}C-n.m.r. spectroscopy was performed on a Jeol FX 90 Q instrument; $CDCl_3$ was used as reference (δ_H 7.25 and δ_C 77.10 p.p.m.) and coupling constants were measured in Hz. For the deprotected compounds **14–21**, ^{1}H-n.m.r. spectra were recorded at 22° with a Bruker AM-500 spectrometer with a sweep width of 5000 Hz, 32 K datapoints, and a recycle time of 3.3 s. ^{13}C-N.m.r. spectra were recorded at 22° with a Jeol FX 90 Q spectrometer using a sweep width of 4600 Hz, 16 K datapoints, and a recycle time of 3.0 s. Chemical shifts were referenced using tetramethylsilane (Me_4Si) and *tert*-butanol (δ_H 1.23 and δ_C 30.6 p.p.m.) as internal standards in $CDCl_3$ and D_2O, respectively. The carbon–proton coupling constants for compound [$^{13}CH_2OH$]-**14** and its 2-epimer were determined from the proton coupled ^{13}C-n.m.r. spectra, and for compound **15** the corresponding coupling-constant was determined from a ^{13}C-n.m.r. spectrum using proton–carbon selective decoupling centered at the chemical shift of H-3*e* at 2.30 p.p.m.

T.l.c. was performed on Merck silica gel 60 F_{254} aluminium sheets and spots were detected by u.v. light and/or charring with sulphuric acid. Preparative chromatography was performed on Merck silica gel (0.040–0.063 mm) and Bio-gel P-2, the latter in a 81 × 2.5-cm solumn, equipped with a Gilson minipump 2 (speed 30 mL/h) and a multireference Optilab detector 902 B with a 1-mm cell. The purity of the deprotected compounds was tested by analytical l.c.[16]

General procedures for enolate formation. — *Ethyl 2,6-anhydro-3-deoxy-4,5:7,8-di-*O*-isopropylidene-2-octyloxycarbonylmethyl-*D-glycero-D-talo-*octonate* (**10**). Octyl 2-bromoacetate (0.45 g, 1.79 mmol) dissolved in anhydrous THF (0.5 mL) was added to the enolate (prepared from 0.54 g of **5**; 1.63 mmol) in anhydrous THF (15 mL) at −75°. The mixture was stirred for 2 h before 1 mL of a saturated NH_4Cl solution was added. The mixture was allowed to reach room temperature and dried (Na_2SO_4). Evaporation gave a syrup that was purified on a silica gel column (1:2 EtOAc–pentane) giving 0.31 g (38%) of pure **10**; n.m.r. ($CDCl_3$): ^{1}H, δ 0.70–1.80 [m, 30 H, C*Me*$_2$, CH_2CH_3, $(CH_2)_6CH_3$], 1.95 [dd, 1 H, $^2J_{3(1),3(2)}$ −15.7, $^3J_{3(1),4}$ 3.1, H-3(1)], 2.22 [dd, 1 H, $^3J_{3(2),4}$ 3.2, H-3(2)], 2.91 [d, 1 H, $^2J_{9(1),9(2)}$ −15.3, H-9(1)], 3.29 [d, 1 H, H-9(2)], 3.51 (dd, 1 H, $^3J_{5,6}$ 1.5, H-6), 3.90–4.40 [m, 8 H, H-5, H-7, H-8(1), H-8(2), O*CH*$_2$], and 4.58 (ddd, 1 H, H-4); ^{13}C, δ 14.07 (CH_2CH_3), 22.63, 24.93, 25.22, 25.82, 26.22, 27.12, 28.56, 29.21, 30.01, 30.31, 31.81 (C*Me*$_2$, C-2′–C-8′ in octyl ester, C-3), 44.62 (C-9), 61.32 (O*CH*$_2$), 64.66 (C-1′ in octyl ester), 66.85 (C-8), 70.69 (C-4), 71.79 (C-5), 73.48, 73.68 (C-6, C-7), 75.62 (C-2), 109.32 (*C*Me$_2$), 169.89, and 172.83 (carbonyls).

Anal. Calc. for $C_{26}H_{44}O_9$: C, 62.4; H, 8.9. Found: C, 62.5; H, 8.9.

*Ethyl 2,6-anhydro-3-deoxy-2-diethoxyphosphorylmethyl-4,5:7,8-di-*O*-isopropylidene-*D-glycero-D-talo-*octonate* (**12**). Diethyl phosphonomethyltriflate[7] (1.16 g, 3.86 mmol) was added to the enolate (prepared from 1.02 g of **5**; 3.09 mmol) in anhydrous THF (15 mL) at −75°. The mixture was stirred for 10 min before addition of a saturated NH_4Cl solution (1 mL). The mixture was allowed to reach room temperature and dried (Na_2SO_4). Evaporated gave a syrup that was purified on a silica gel column (1:1 EtOAc–ether) giving 0.75 g (50%) of pure **12**; *m/z* (f.a.b.): calc. for $C_{21}H_{38}O_{10}P$ $(M + H)^+$: 481.2203. found: 481.2176.

*Ethyl 2,6-anhydro-3-deoxy-4,5:7,8-di-*O*-isopropylidene-2-dimethoxyphosphoryloxymethyl-*D-glycero-D-talo-*octonate* (**22**). Phosphorus oxychloride (0.51 g, 3.33 mmol) and Et_3N (1.67 mL, 12.00 mmol) were dissolved in 20 mL of MeCN. A solution of **6** (0.24 g, 0.67 mmol) in 1 mL of MeCN was slowly added. The mixture was stirred under nitrogen for 1 h, cooled in an ice bath, and then MeOH (0.50 mL, 12.34 mmol) and 4-dimethylaminopyridine (0.05 g, 0.41 mmol) were slowly added. The mixture was filtered twice and extracted with ether. The ether phase was dried (Na_2SO_4) and evaporated to a syrup that was chromatographed on silica gel (9:1 ether–hexane) giving 0.25 g (80%) of pure **22**; *m/z* (f.a.b.): calc. for $C_{19}H_{34}O_{11}P$ $(M + H)^+$: 469.1849. Found: 469.1823.

General procedures for deprotection (**14–21**). — Compounds **6–11** (0.05–0.50 g) were dissolved in a mixture of EtOH (4 mL) and CF_3CO_2H (1 mL) and stirred under nitrogen for ~3h. The acid was removed by repeated evaporation *in vacuo* together with EtOH. Absence of the isopropylidene groups was confirmed by ^{1}H-n.m.r. spectroscopy before the residue was dissolved in 2–3 mL of 5M NaOH and stirred overnight. Dowex H^+ ion-exchange resin was then added. The mixture was stirred until neutral pH was reached, filtered, and the filtrate evaporated and the residue chromatographed on a Bio-gel P-2 column (1% AcOH).

*2,6-Anhydro-3-deoxy-2-hydroxymethyl-*D-glycero-D-talo-*octonic acid* (**14**). Compound **6** (0.30 g, 0.83 mmol), treated as just described, gave 0.13 g (62%) of pure **14**.

Anal. Calc. for $C_9H_{19}NO_8 \cdot H_2O$ (ammonium salt of **14**): C, 38.43; H, 7.29; N, 4.98. Found: C, 38.3; H, 7.1; N, 5.0.

*2,6-Anhydro-3-deoxy-2-methyl-*D-glycero-D-talo-*octonic acid* (**15**). Compound **7** (0.46 g, 1.34 mmol) treated as just described gave 0.27 g (88%) of pure **15**; *m/z* (f.a.b.): calc. for $C_9H_{15}O_7$ $(M - H)^-$: 235.0818. Found: 235.0798.

*2,6-Anhydro-2-benzyl-3-deoxy-*D-glycero-D-talo-*octonic acid* (**16**). Compound **8** (0.12 g, 0.29 mmol) treated as just described gave 0.06 g (68%) of pure **16**; *m/z* (f.a.b.): calc. for $C_{15}H_{19}O_7$ $(M - H)^-$: 311.1131. Found: 311.1099.

*2,6-Anhydro-3-deoxy-2-propargyl-*D-glycero-D-talo-*octonic acid* (**17**). Compound **9** (0.14 g, 0.38 mmol) treated as already described gave 0.10 g (100%) of **17**; *m/z* (f.a.b.): calc. for $C_{11}H_{17}O_7$ $(M + H)^+$: 261.0974. Found: 261.0985.

*2,6-Anhydro-2-carboxymethyl-3-deoxy-*D-glycero-D-talo-*octonic acid* (**18**). Compound **10** (0.07 g, 0.14 mmol), treated as already described, gave 0.04 g (100%) of pure **18**; *m/z* (f.a.b.): calc. for $C_{10}H_{15}O_9$ $(M - H)^-$: 279.0716. Found: 279.0713.

2,6-Anhydro-2-(2-carboxyethyl)-3-deoxy-D-glycero-D-talo-*octonic acid* (**19**). Compound **11** (0.09 g, 0.22 mmol), treated as already described, gave 0.04 g (67%) of **19**; *m/z* (f.a.b.): calc. for $C_{11}H_{17}O_9$ $(M - H)^-$: 293.0873. Found: 293.0841.

2,6-Anhydro-3-deoxy-2-phosphonomethyl-D-glycero-D-talo-*octonic acid* (**20**). — Compound **12** (0.22 g, 0.46 mmol) and $BrSiMe_3$ (155 μL, 1.15 mmol) were stirred in dry $CDCl_3$ (5 mL) for 2 h (the reaction was monitored by ^{1}H-n.m.r.), and then concentrated. The residue was dissolved in 5M NaOH (5 mL). The mixture was stirred overnight, neutralized with Dowex-H^+ ion-exchange resin, filtered, and the filtrate evaporated. The residue was chromatographed on a Bio-gel P-2 column, giving 0.11 g (73%) of pure **20**; *m/z* (f.a.b.): calc. for $C_9H_{18}O_{10}$ $(M + H)^+$: 317.0638. Found: 317.0653.

2,6-Anhydro-2-cyano-3-deoxy-D-glycero-D-talo-*octonic acid* (**21**). — Compound **13** (0.05 g, 0.16 mmol) and NaCN (0.02 g, 0.31 mmol) were dissolved in Me_2SO (3 mL) and stirred for 24 h at 50°. The mixture was evaporated, MeOH (4 mL) and CF_3CO_2H (1.5 mL) were added, and the mixture was stirred overnight. The solution was deionized with Dowex H^+ ion-exchange resin and then evaporated. The residue was purified on a Bio-gel P-2 column (1% AcOH) giving 0.03 g (75%) of pure **21**; *m/z* (f.a.b.): calc. for $C_9H_{14}NO_7$ $(M - H)^-$: 246.0614. Found: 246.0584.

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