Synthesis of a Simplified Transition-State Analogue in an Attempt to Obtain an Inhibitor of CMP-KDO Synthetase

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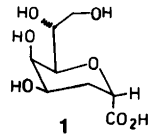
The thioglycoside **5** of 3-deoxy-β-D-*manno*-2-octulosonic acid (β-KDO) has been synthesized in an attempt to make a simplified transition-state analogue inhibitor of the enzyme CMP-KDO synthetase (3-deoxy-D-*manno*-octulosonate cytidylyltransferase). Compound **5** was tested *in vitro* and was found to have no inhibitory activity.

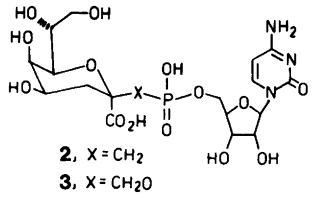
A new[1] approach to the task of discovering agents active against Gram-negative bacteria[2] has developed over the last decade to include the design of compounds which inhibit the incorporation of 2-deoxy-D-*manno*-2-octulosonic acid (KDO) into the lipopolysaccharide (LPS) of Gram-negative bacteria. KDO is essential for the bacterial cell growth and mutants lacking this function in the LPS are not viable.[3] There are several biosynthetic steps involved,[4] all of which could serve as targets. One attempt, for example, was made to inhibit the enzyme arabinose 5-phosphate isomerase,[5] which is involved in the biosynthesis of KDO, but more interest has recently been focused on CMP-KDO synthetase (CTP:CMP-3 deoxy-D-*manno*-octulosonate cytidylyltransferase; EC 2.7.7.38).[6] This enzyme (CKS) activates KDO by catalyzing the formation of the nucleotide derivative CMP-KDO (cytidine 5′-monophosphate KDO) from KDO and CTP (cytidine triphosphate) before KDO is transferred to the core region of LPS via a series of events.

Claesson and co-workers reported the substrate analogue **1**[7] which was shown to be a potent inhibitor of CKS, and later work by two different groups has concentrated on modifications of this compound, with the aim of facilitating penetration through the bacterial cell wall.[8–10] The penetration problem is, of course, of prime importance in the development of CKS inhibitors with antibacterial activity, but regardless of this, attempts have been made to find better inhibitors (mostly substrate analogues) of the enzyme *in vitro*. Variations of the carboxylate group of **1**,[11] as well as β-*C*-glycosides based on **1**,[12,13] proved to be inferior inhibitors. The same was also true for the two product analogues, **2**[13] and **3**.[14]

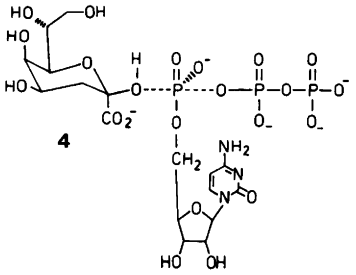
It has been argued that transition-state or multisubstrate analogues would be stronger enzyme inhibitors compared with simple substrate analogue.[15] One of the problems of applying this approach is that the detailed molecular mechanism of the formation of CMP-KDO is not known. A sequential mechanism has been proposed,[16] which suggests that KDO and CTP bind in an ordered or random sequence to the active site of the enzyme and then undergo an S_N2-like substitution reaction via the hypothetical transition-state structure **4**. This structure can be divided into



1



2, X = CH_2
3, X = CH_2O



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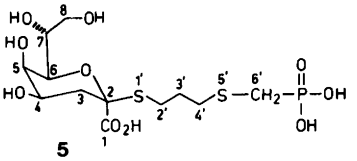

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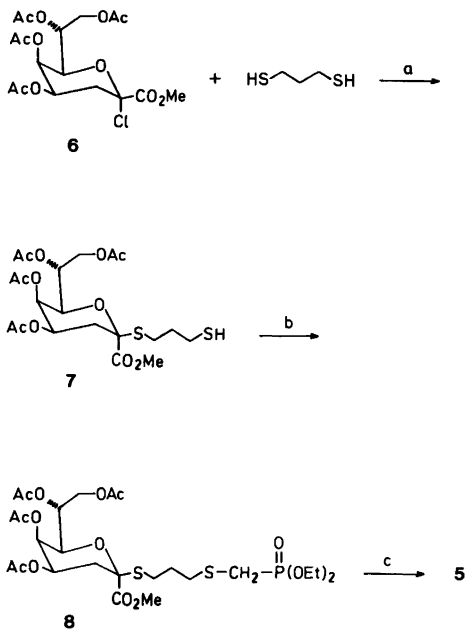

a: TMG, CH_3CN

b: TMG, $F_3CSOCH_2P(OEt)_2$, CH_3CN

c: 1. $BrSI(CH_3)_3/CDCl_3$ 2. NaOMe/MeOH 3. $NaOH/H_2O$

Scheme 1.

three parts; a KDO part, a cytidine part and a pyrophosphate part. The β-pyranose configuration of KDO in CMP-KDO has been determined by ^{13}C NMR spectroscopy[17] and mechanistically it appears most reasonable that KDO also has the β-configuration in the hypothetical transition-state structure. ^{31}P and ^{13}C NMR studies with ^{18}O-labelled KDO are also consistent with a nucleophilic displacement mechanism, supporting the transition-state model **4**[16] (disregarding absolute configuration).

As a compromise between structural design and synthetic possibilities we have synthesized a simplified transition-state analogue **5**, in which the cytidine part is omitted, but which takes into account and mimics the KDO and the pyrophosphate parts. It has been reported,[13,14] that product analogues, i.e. compounds with an intact cytidine moiety, but lacking pyrophosphate or pyrophosphate-mimicking groups, are poor inhibitors.

Chemistry

Compound **5** was synthesized according to Scheme 1. Compound **7** was obtained from the reaction of **6**[18] with 1,3-propanedithiol in the presence of tetramethylguanidine (TMG) with scrupulous exclusion of oxygen in order to avoid disulfide formation. Attempts to use Et_3N as the base resulted in an inconveniently slow reaction rate. When all of **6** had been consumed, the reaction mixture was acidified with acetic acid and filtered through silica gel before further purification. If these precautions were not taken, an insoluble precipitate was obtained almost immediately when the reaction mixture was concentrated. Under the same general conditions, the thiolate of **7** was allowed to react with diethyl phosphonomethyltriflate, which is a good alkylating agent,[19] to give **8**. This compound was then deprotected in three steps and **5** was isolated. In the first step the phosphonate was transformed into its silyl ester analogue by treatment with bromotrimethylsilane in chloroform. Subsequent treatment with sodium methoxide in methanol gave the fully deacetylated phosphonic acid, which finally was hydrolyzed with NaOH. Neutralization of the sodium carboxylate with Dowex H^+ ion exchange resin gave **5**, which was immediately transformed into its diammonium salt by addition of aqueous ammonia.

^1H and ^{13}C NMR spectra confirm the structures of **5**, **7** and **8**. With compound **8** a downfield shift is found in the ^{13}C NMR spectrum for C4′ in the side chain and a small split due to its spin–spin coupling with the ^{31}P nucleus, in accordance with expectations. The thiol proton of compound **7** is observed in the ^1H NMR spectrum as a triplet (^3J = 8.0 Hz); this is a good indication that no disulfide bond has been obtained. A broadened singlet in the ^{31}P NMR spectrum for compound **8** contrasts the sharp singlet found for the more hydrophilic compound **5**.

The determination of the β-configuration in compound **5** was based on the assumption that the reaction between **6** and thiolates proceeds by inversion of configuration, which has been observed in similar reactions.[20] During the course of other studies in our laboratory a phenylthio analogue of **7** was synthesized under the same general conditions. After incorporation of isopropylidene protecting groups, a comparison of ^1H NMR spectra of this compound[21] and of similar compounds of known β-configuration[22] indicated that it had the same configuration. In general, the 3-deoxy proton signals appear at quite different chemical shifts in the isopropylidene protected α-form of *C*-glycosides of KDO, in contrast with the signals for the β-form. Compounds in which a carbonyl or cyano group is linked to the anomeric carbon do not conform to this rule.[22,23]

Biochemical result

The inhibitory activity of compound **5** was determined in a combined CKS and KDO-lipid A transferase (KLT; CMP-KDO:lipid A KDO transferase) enzyme assay,[24] the latter enzyme being responsible for the transfer of KDO to a lipid A precursor.[25] The inhibitory activity was found to be zero at equal substrate and inhibitor concentrations. Speculations about the cause of this negative finding might of course involve the far-reaching simplification in our design but could also include the uncertainty of the enzyme mechanism, especially concerning random or ordered binding.

Experimental

High resolution mass spectrometry (FAB MS) was performed on a Jeol DX-303 and NMR spectroscopy on a Jeol FX90Q instrument. In ^1H and ^{13}C NMR spectroscopy $CDCl_3$ was used as the reference for compounds **7** and **8** (δ_H 7.25 and δ_C 77.10 ppm) and *tert*-BuOH (δ_H 1.23 and δ_C 30.60 ppm) for compound **5**. In ^{31}P NMR spectroscopy, an ampoule containing H_3PO_4 (10 %) was used as an external reference (0 ppm). Coupling constants were measured in Hz. GC analyses were performed at 250 °C by means of a Carlo Erba Strumentazione GC 6000 Vega Series equipped with a 25 m SE 52 capillary column. TLC was performed on Merck silica gel 60 F_{254} aluminium sheets and spots were detected by UV light and/or charring with sulfuric acid. Preparative chromatography was performed on Merck silica gel (0.040–0.063 mm). All water used for preparative purposes was distilled twice and stored under nitrogen.

*Methyl 4,5,7,8-tetra-*O*-acetyl-2,3-dideoxy-2-(6'-diethoxyphosphoryl-1',5'-dithiahexyl)-*β*-*D*-manno-octulosonate* (**8**). A solution of 1,3-propanedithiol (0.69 ml, 6.85 mmol) and TMG (0.21 ml, 1.64 mmol) in 20 ml of acetonitrile was stirred under a nitrogen atmosphere for 10 min. Compound **6** (0.06 g, 1.37 mmol) dissolved in 2 ml of acetonitrile was then added. After 1 h, compound **6** could not be detected by TLC and 0.5 ml of acetic acid were added. The mixture was filtered through a short column of silica gel which was washed with diethyl ether. After being concentrated the filtrate was chromatographed on silica gel (diethyl ether/pentane 5:1), resulting in 0.43 g (61 %) of **7** (98 % pure according to GC): ^1H NMR ($CDCl_3$) δ 1.33 (t, 1 H, ^3J = 8.0, H5'), 1.50–2.10 (m, 15 H, acetyls, H-H'3', $H3_{ax}$), 2.21–2.80 (m, 5 H, H-H'2', H-H'4', $H3_{eq}$), 3.74 (s, 3 H, methyl ester), 3.81–5.19 (m, 6 H, H4-8, H'8); ^{13}C NMR ($CDCl_3$) δ 20.72, 10.87 (acetyls), 23.41, 27.65, 32.53, 33.33 (C2', C3', C4', C3), 53.12 (methyl ester), 62.34, 63.94, 67.18, 67.83, 71.92 (C4–C8), 84.08 (C2), 168.53, 169.73, 169.87, 170.52, 170.72 (carbonyls).

Compound **7** (0.11 g, 0.22 mmol) and diethyl phosphonomethyltriflate[19] (0.07 g, 0.22 mmol) were dissolved in 10 ml of acetonitrile under nitrogen and TMG (0.03 ml, 0.24 mmol) was added. The mixture was stirred for 30 min; the reaction was then complete according to TLC. After concentration the mixture was chromatographed on silica gel (first diethyl ether and then acetone) resulting in 0.12 g (49 % from **6**) of pure **8**: ^1H NMR ($CDCl_3$) δ 1.32 (t, 6 H, ^3J = 7.1, ethyl ester methyls), 1.65–2.27 (m, 15 H, acetyls, H-H'3', $H3_{ax}$), 2.30–2.90 (m, 7 H, H-H'2', H-H'4', H-H'6', $H3_{eq}$), 3.79 (s, 3 H, methyl ester), 3.85–5.35 (m, 10 H, H4–8, H'8, ethyl ester methylenes); ^{13}C NMR ($CDCl_3$) δ 16.61 (d, $^3J_{C-P}$ = 5.6, ethyl ester methyls), 20.82, 20.92 (acetyls), 25.26 (d, $^1J_{C-P}$ = 150.5, C6'), 28.00, 28.74 (C2', C3'), 32.31 (d, $^3J_{C-P}$ = 3.4, C4'), 32.58 (C3), 53.17 (methyl ester), 62.79 (d, $^2J_{C-P}$ = 6.7, ethyl ester methylenes), 63.34, 64.04, 67.23, 72.02 (C4–C8), 84.08 (C2), 168.63, 169.77, 169.97, 170.57, 170.22 (carbonyls); ^{31}P NMR ($CDCl_3$) δ 24.24 (s, br); FAB-MS, $(M+H)^+$ at *m/z* 661.1721 (Calcd. 661.1754).

*Diammonium 2,3-dideoxy-2-(6'-phosphono-1',5'-dithiahexyl)-*β*-*D*-manno-octulosonate* [**5**-$(NH_3)_2$]. Compound **8** (0.08 g, 0.12 mmol) was dissolved in 2 ml of deuteriochloroform and bromotrimethylsilane (0.11 ml, 0.85 mmol) was added. The reaction mixture was stirred under nitrogen overnight. The absence of ethyl ester groups was confirmed by ^1H NMR spectroscopy. The mixture was concentrated and dissolved in 1 ml of dry methanol, concentrated again and dried *in vacuo*. To this intermediate was added NaOMe (0.42 mmol), from a solution of 5 mg Na ml^{-1} MeOH. The solution was stirred under nitrogen for 3 h and then 1 ml of H_2O was added, before concentration. The residue was dissolved in 2.5 ml of 1.25 M NaOH and this solution was stirred overnight and then eluted with H_2O through a column of Dowex H^+ ion exchange resin. The eluate was concentrated and redissolved in H_2O three times before the mixture was finally concentrated and dried *in vacuo*. The absence of acetic acid was confirmed by ^1H NMR spectroscopy. The product was dissolved in 2 ml of H_2O and 0.5 ml of concentated NH_3, then concentrated and dried *in vacuo*, resulting in 0.04 g (67 %) of **5**-$(NH_3)_2$: ^1H NMR (D_2O) δ 1.65–2.05 (m, 3 H, H-H'3', $H3_{ax}$), 2.30–2.90 (m, 7 H, H-H'2', H-H'4', H-H'6', $H3_{eq}$), 3.30–4.05 (m, 6 H, H4-8, H'8); ^{13}C NMR (D_2O) δ 29.23 (d, $^1J_{C-P}$ = 133.7, C6'), 29.15, 29.45 (C2', C3'), 33.12 (d, $^3J_{C-P}$ = 7.9, C4'), 35.98 (C3), 65.10, 66.09, 68.24, 69.58, 75.92 (C4–8), 87.33 (C2), 175.37 (carbonyl); ^{31}P NMR (D_2O) δ 16.67 (s); FAB-MS, $(M-H)^+$ at *m/z* 421.0392 (Calcd. 421.0362).

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