Excretion of a Phosphorus-Containing Carbohydrate by *Streptomyces* sp. A50

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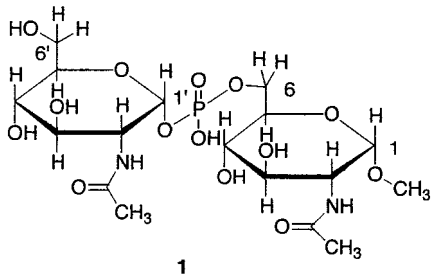
A new phosphorus-containing compound (**1**) was detected by ^{31}P NMR spectroscopy in *Streptomyces* sp. A50. Compound **1**, 1(α)-*O*-methyl-2-(*N*-acetyl)glucoseamine-6-*O*-phosphate-1(α)-2-(*N*-acetyl)glucosamine, exhibited a pK_a value around zero. The compound was found both in the extracellular culture broth and in the cells. While very low concentrations of **1** were found in the culture broth of other species of *Streptomyces*, its presence in high concentrations was specific to *Streptomyces* sp. A50. The highly acidic compound was isolated from the broth, and its structure was elucidated by a combination of 1D-, 2D-homonuclear, and inverse heteronuclear NMR techniques and mass spectroscopy.

We have previously shown that *Streptomyces* sp. A50 exhibits the unusual capacity to convert up to 30% of the inorganic phosphate (Pi) present in the growth medium into polyphosphate during the stationary phase of growth.[1] ^{31}P NMR analysis demonstrated that polyphosphate was accumulated inside the cells during the first 2 or 3 days of growth on minimal media and was subsequently released into the broth. During this study, ^{31}P NMR analysis revealed the presence of another phosphorus-containing compound (**1**), resonating at δ −1.07 ppm, which did not correspond to any known material. During growth on minimal medium, a portion of **1** was also found in the culture broth. In this report, we present the molecular structure of **1** using both NMR and MS analysis. Both NMR and biochemical analysis were used to study the cellular distribution of this compound.



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Results and Discussion

The structure of compound **1** was established on the basis of the analysis of data from ^{1}H, ^{13}C, COSY, TOCSY, ^{1}H−^{31}P HSQC, ^{1}H−^{13}C HMQC, and HMBC experiments. The ^{1}H−^{31}P HSQC spectrum revealed two H−P correlations of protons at δ_H = 5.55 and 4.17 (2H) with a phosphorus signal at δ_P = −1.07, suggesting a phosphodiester molecule**,** with the phosphorus atom bonded to an anomeric and a methyleneoxy group of two different sugar units. The COSY, TOCSY, and HMQC experiments were used to assign the proton and carbon spin systems of the two amino sugar units, while correlations from the HMBC experiment assisted in assembly of the complete structure of **1** (see Table 1 in Supporting Information). The structure was found to consist of two *N*-acetyl glucose amine residues linked through a phosphodiester bridge in a α-1,6 linkage. An O-methyl residue substitutes the anomeric position of one of the sugar residues. Negative-ion FABMS, using glycerol as the matrix, gave a weak [M − H]$^-$ quasi-molecular ion at *m/z* 517, corresponding to the molecular formula $C_{17}H_{31}N_2C_{14}P$. A high-resolution mass spectrum could not be obtained for this compound because its spectrum disappeared upon the addition of the reference material. The pK_a of compound **1** was measured and found to be near zero with δ_b = −1.07 ± 0.01 (δ_a could not be determined confidently). The pK_a of **1** is very acidic, and therefore the ^{31}P chemical shift of the compound in the pH interval of 5−8 can be used as an internal reference.

Compound **1** was detected in the culture broth after 2 days of growth in a monosodium glutamate (MSG) medium and after 4 days of growth in a tryptic soya broth (TSB) medium, i.e., at a time period prior to the appearance of polyphosphate in the medium. Moreover, after 3 days of growth in MSG and 1 day of starvation for Pi, intracellular polyphosphate was completely depleted, while **1** was detected at original levels. This result suggests that the release of **1** is not related to polyphosphate metabolism. It is most likely that the presence of **1** in sp. A50 culture broth results from cell wall turnover. Cell wall turnover is a known phenomenon in many bacterial species[2] in which cell wall bound autolysins hydrolyze peptidoglycans at specific bonds. In Gram-positive cells, soluble cell wall teichoic and teichuronic acid fragments can frequently be recovered from the supernatant broth.[3] When different strains of *Streptomyces* were grown under the same conditions as *Streptomyces* sp. A50, a ^{31}P NMR signal, similar to that of **1**, was detected in the culture media of these strains but in concentrations 5−10 times lower than the concentration of the same peak in sp. A50 broth. Most of compound **1** was found in the soluble fraction obtained after removal of the cell walls, while the concentration of **1** in the insoluble cell wall fragments or in the protoplasts was essentially negligible. Although a polymer based on this dimer is known to form *Staphylococcus lactis 2102*[4] teichoic acid, this dimer form has never been found in nature before.

The location of **1**, in the intact cells (see Figure 1 in Supporting Information), was studied by determining the effect of the paramagnetic Mn^{2+} ion on the NMR relaxation times of the phosphorus signal resonating at −1.07 ppm. When $MnCl_2 \cdot 6H_2O$ (0.02−0.1 mM) was added to cells resuspended in fresh medium, the height of the peak of compound **1** was decreased with a concomitant increase in

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line width. Addition of EDTA caused the peak to become narrow again. In contrast, the peak associated with intracellular polyphosphate remained unchanged during both procedures, indicating that Mn^{2+} ions did not penetrate into the cell during the measurements. In cells that were incubated with Mn^{2+} for 8 h without bubbling of oxygen, signals from both polyphosphate and compound **1** disappeared, indicating penetration of Mn^{2+} during this period. After addition of EDTA (to a final concentration of 14 mM) the signal of compound **1** was again detected, in contrast to the signal of polyphsophate, which did not recover. These data show that compound **1** is associated with the cell wall and is most likely present on the external surface. The longitudinal and transverse relaxation times of compound **1** both in the cells and in the broth medium and after the additions of $MnCl_2 \cdot 6H_2O$ and EDTA were measured (see Table 2, Supporting Information). The T_2 relaxation time of the cell associated with compound **1** varied from 0.45 ms in the Mn^{2+}-containing medium to 23 ms in EDTA-containing medium. Even in the presence of EDTA the ratio of T_1/T_2 remained significantly larger than unity. There are three reasons for a large value of T_1/T_2: (a) effect of paramagnetic ions;[5,6] (b) diamagnetic systems with slow rotation, $\omega_0\tau_{rot} \gg 1$;[7] (c) systems where chemical exchange is taking place. In the present system all three mechanisms are involved. This indicates that the compound has slow rotation, most probably due to its binding to the cell wall. This binding can be either covalent binding that retains the identity of **1** so that its chemical shift is identical to that in the broth or noncovalent binding which is strong enough so it does not disappear after washing with fresh medium. The relaxation times of **1** in the broth exhibit exceptional sensitivity to paramagnetic ions, varying between 6 ms in the normal broth medium that contains paramagnetic ions to 130 ms in the same broth medium after treating with CHELEX 100 and further addition of 40 mM EDTA. The fact that the ratio T_1/T_2 remained larger than unity in the presence of EDTA and that T_2 increased when measured by a fast CPMG sequence points to the possibility of some exchange process. The exact nature of this mechanism is now under investigation.

Experimental Section

General Experimental Procedures. ^{31}P NMR spectra of the cell suspensions were acquired at 145.2 MHz on a Bruker AMX-360 WB NMR spectrometer with a 20 mm dedicated phosphorus probe at 298 K. The samples with the cell suspension were bubbled with oxygen during the NMR measurement. ^1H, ^{13}C, and ^{31}P NMR data of the purified compound, dissolved in D_2O, was acquired at 500.13 (^1H), 125.76 (^{13}C), and 202.46 (^{31}P) MHz on a Bruker ARX 500 NMR spectrometer with a 5 mm inverse probe at 298 K. Data on the supernatant was acquired on both instruments with a 10 mm broadband probe. The ^{31}P T_1 and T_2 relaxation times of compound **1** in the supernatant were acquired at 294.5 K. Calibration of the phosphorus chemical shifts was carried out relative to a capillary reference of EDTMP, purchased from Monsanto, relative to GPC ($\delta_{GPC} = 0.49$ ppm). For the investigation of the binding of **1** to the cell wall $MnCl_2 \cdot 6H_2O$ and EDTA were added to the cell suspension to final concentrations of 0.04 and 18 mM, respectively. To ensure that in the cell suspension experiments the signal of **1** originates from the cells and not from the medium, before each measurement the cell suspension was washed either by fresh medium I, which did not contain the Pi, or by fresh medium II, which did not contain Pi, $FeSO_4$, and the other trace elements. To remove divalent ions from the culture broth, it was treated with CHELEX 100, and then EDTA was added to a final concentration of 40 mM. The pK_a value of compound **1** was obtained from a titration curve over the pH range 7.0−0.3. The phosphorus chemical shifts (δ_P), as a function of pH, were fitted to yield the pK_a, chemical shift of the acidic form (δ_a) and the chemical shift of the basic form (δ_b), according to the equation $pH = (\delta_a \times 10^{(pKa-\delta)} + \delta_b)/(10^{(pKa-\delta)} + 1)$. Mass spectra were recorded on a Fisons VG AutoSpecQ M 250 instrument. Both positive- and negative-ion modes were explored, using different compounds (aminopropanediol, glycerol triethanolamine, and dithiothritol/dithioerythriol) as matrix. An interpretable spectrum could be obtained only in the negative-ion mode using glycerol as matrix.

Biological Material. *Streptomyces* sp. A50 was one of the strains isolated in our laboratory during a large screening program to isolate specific enzyme inhibitors of microbial origin.[8] Typically, this strain was grown at 30 °C on a rotary shaker at 250 rpm, in a chemically defined medium (MSG) containing per liter of distilled water: 2.5 g of monosodium glutamate, 10 g of glycerol, 1 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 mL of 1% solution of $FeSO_4 \cdot 7H_2O$, and 2 mL of a trace metal solution consisting of $CuSO_4 \cdot 5H_2O$ (0.1%), $ZnSO_4$ (0.1%), and $MnSO_4$ (0.1%). An aliquot of 10−50 μL of spores from a frozen stock containing 20% glycerol was inoculated into 50 mL of MSG medium and incubated for 3 days as described above. The resulting seed culture was inoculated (5−20% volume) into shake flasks and incubated for 2−4 days. Samples were taken at various times, and culture broth was obtained after centrifugation at 10 000*g* for 15 min at 5 °C. For preparation of protoplasts, an inoculum of 10−50 μL of spores of *Streptomyces* sp. A50 was grown in 25 mL of TSB (3% Tryptic Soya Broth, Biolife) for 24 hours**,** at 30 °C on a rotary shaker at 250 rpm. The resulting culture was digested by lysozyme according to Hopwood et al.[9] Undigested cells, whole protoplasts, and insoluble cell wall fragments were fractionated into pellets by successive centrifugations (at 500 rpm for 10 min, at 2000 and 15 000 rpm for 60 min, respectively). The resulting supernatant contained the soluble wall fragments.

Isolation Procedure. Culture broth (800 mL) was loaded on a 30 mL DEAE shepharose column preequilibrated with buffer (MOPS 0.01 M NaCl 0.25%, pH 7.2) and eluted with a gradient of 0.25 to 3% NaCl. The presence of compound **1** was detected by ^{31}P NMR spectroscopy. Fractions containing the compound were concentrated and subjected to Sephadex G-25 chromatography (twice). When the compound was purified from cell wall digested fragments, only Sephadex G-25 chromatography was used. Fractions **1** were lyophilized for further studies. A total of 0.5−1.0 mg of **1** was isolated from 25 mL of MSG growth medium after 4 days of growth.

1(α)-*O*-Methyl-2-(*N*-acetyl)glucoseamine-6-*O*-phosphate-1(α)-2-(*N*-acetyl)glucosamine (1): amorphous white solid; ^1H NMR (D_2O, 500 MHz) δ 5.00 (s, H-1), 4.04 (m, H-2), 3.86 (t, H-3), 3.63 (t, H-4), 3.94 (m H-5), 4.17 (brd, H-6), 3.79 (s, O-CH_3′), 5.55 (brd, H-1′), 4.04 (brd, H-2′), 3.86 (t, H-3′), 3.76 (t, H-4′), 3.77 (t, H-5′), 4.10 (dd, H-6′), 2.20 (s, Ac-CH_3); ^{13}C NMR (D_2O, 125 MHz) δ 95.8 (d, C-1), 52.2 (d, C-2), 69.3 (d, C-3), 68.1 (d, C-4), 69.5 (dd, C-5, $J_{C-P} = 7.0$ Hz), 63.0 (dd, C-6, $J_{C-P} = 6.0$ Hz), 57.9 (q, O-CH_3′), 92.6 (dd, C-1′, $J_{C-P} = 4.5$ Hz), 52.4 (dd, C-2′, $J_{C-P} = 7.0$ Hz), 69.6 (d, C-3′), 67.9 (d, C-4′), 63.8 (C-5′), 70.4 (d, C-6′), 173.1 (s, Ac-CO), 20.6 (q, Ac-CH_3); FABMS (negative, glycerol) *m/z* (rel intensity) 517 ($[M - H]^-$, 1), 451 (10), 442 (7), 417 (17), 325 (100), 298 (35), 219 (33), 205 (25), 127 (45).

Supporting Information Available: ^{31}P NMR spectrum of a suspension of *Strptomyces* sp. A50 cells in low-Pi medium (Figure 1). ^{31}P chemical shifts of compound **1** vs the pH (Figure 2). NMR data and assignments of compound **1** in D_2O (Table 1). Longitudinal and transverse relaxation times of compound **1** in the cells and in the broth medium and after the addition of ETDA and Mn^{2+} (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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