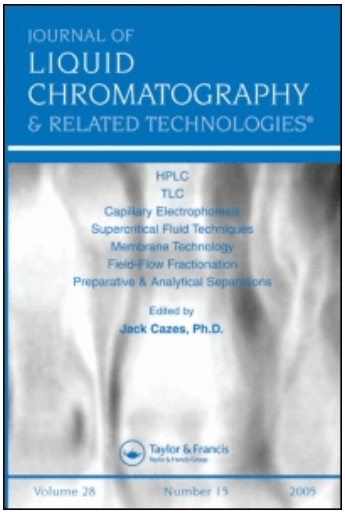


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# Ion-Pair Reversed-Phase HPLC Identification of Sugar Nucleotides in Cell Free Extracts of *Streptomyces Griseus*

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# ION-PAIR REVERSED-PHASE HPLC IDENTIFICATION OF SUGAR NUCLEOTIDES IN CELL FREE EXTRACTS OF *STREPTOMYCES GRISEUS*

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## ABSTRACT

2,6-Dideoxysugars, biosynthetically derived from glucose through sugar nucleotide intermediates, are important structural components of antibiotics. A HPLC method was developed for the detection of ADPG, UDPG, CDPG, GDPG and/or dTDPG in cell free extracts of *Streptomyces griseus*. The resolution of these sugar nucleotides and fourteen additional related mono-, di- and triphosphoribo-nucleotides was achieved by gradient elution, ion-pair reversed-phase chromatography (RP-IPC) over an ODS-C18 column. Cell free extracts contained UDPG, GDPG and UDP, thus implicating the two sugar nucleotides in chromomycin-$A_3$ biosynthesis.

## INTRODUCTION

The Aureolic acids are a family of antitumor antibiotics produced by Streptomycetes. Chromomycin $A_3$ is an antineoplastic

member of the aureolic acids produced by strains of *Streptomyces griseus*. The structure of chromomycin $A_3$ is composed of chromomycinone, a highly functionalized aglycone which is substituted at position 2 and 6 with tri- and disaccharide moieties comprised of 2,6-dideoxysugars. Biosynthetic studies of chromomycin $A_3$ conducted in our laboratory have indicated that glucose is the major precursor in the biogenesis of all five dideoxysugars [1]. The mechanism of dideoxy-sugar formation in bacteria often involves sugar nucleotides such as UDPG, or related cytidine, adenosine, guanosine, or thymidine sugar nucleotides as key intermediates. In an investigation of the biosynthetic potential of our *Streptomyces griseus* culture producing chromomycin $A_3$ [1], we sought to determine whether sugar nucleotides such as these could be directly detected in cell-free extracts of the producing culture, as a means of guiding additional work concerned with the enzymology of sugar biotransformations.



Liquid chromatography (LC) (including ion-pairing reversed-phase HPLC) has been used in the analysis of nucleotides, nucleosides and their bases in biological samples, usually derived from mammalian preparations [2-8]. GDP-Mannose, GDP-fucose, UDPG and CDPG each have been resolved by strong-anion-exchange (SAX) chromatography [9-11]. UDPG and UDP-galactose have been separated by lectin affinity HPLC as well [12]. The separation of major purine bases, their nucleotides and nucleosides has also been achieved by glass column HPLC with reversed-phase particle packings [13]. However,

the simultaneous HPLC resolution of sugar nucleotides of relevance to deoxysugar biogenesis has not been reported. Since in our own experience, sugar nucleotides like TDPG, ADPG and GDPG were inseparable by SAX chromatography we established an ion-pair, reversed-phase, high-performance liquid chromatographic (RP-HPLC) method for the complete resolution of this family of biosynthetically related compounds in cell free extracts of *Streptomyces griseus*. The analytical separation was obtained using a 5-μm Whatman Partisil ODS-5 C18 column at room temperature under gradient conditions, with quantitation by UV detection at 262 nm.



## EXPERIMENTAL

### Reagents and Chemicals

All nucleotides were of the highest purity available and were purchased from Sigma. Tetrabutylammonium hydroxide (TBAH) (40% aqueous solution) was also purchased from Sigma (St.Louis, MO, U.S.A.). HPLC grade monopotassium hydrogen phosphate and orthophosphoric acid (85%) were both from Fisher Scientific (Fair Lawn, New Jersey, U.S.A.). Acetonitrile (HPLC grade) was from E. M. Science (Gibbstown, NJ, U.S.A.) and was filtered through type HV 0.45-μm Millipore membranes (Bedford, MA, U.S.A.) before use. Stock solutions of sugar nucleotides (1 mg/ml) were prepared in ultrapure water (E. M. Science, NJ, U.S.A.) and filtered using a 0.2 μm filter (Gelman Science, Ann Arbor, MI, U.S.A.), and kept frozen at -20°C. Standards were routinely used to calibrate HPLC analyses.

## Instrumentation

The LC system consisted of a Rheodyne injector type 7125 with a 100-µl loop connected to a Model LC-6A HPLC pump, a SPD-6AV Module UV-VIS detector, a CR-501 Chromatopac recording integrator and a SCL-6B system controller, all from Shimadzu Co. (Osaka, Japan). The analytical column (250 mm x 4.6 mm I.D.) was packed with 5-µm partisil ODS-C18 (Whatman Inc., Clifton, New Jersey, U.S.A.), and preceded by a guard column of the same composition (Alltech Inc., Deerfield, IL, U.S.A.).



## Chromatographic Conditions

For solvent system 1, the mobile phase consisted of two solvents: A, pH 5.3, 15 mM $KH_2PO_4$, containing 10 mM TBAH; and B, pH 5.3, 35 mM $KH_2PO_4$ with 10 mM TBAH in 30% (v/v) acetonitrile. The mobile phase was prepared in double distilled water, degassed by stirring under house vacuum for 2-3 h, filtered through a 0.22-µm Millipore filter membrane, then degassed again before use. The separation was obtained at a flow rate of 1 ml/min using a concave gradient ranging from 5% to 100% of solution B over a period of 58 min (TABLE 1) while eluting peaks were monitored at 262 nm. Alternatively for those nucleotides not well resolved, the separation was also obtained using solvent system 2 at a flow rate of 1.2 ml/min using a gradient ranging from 0% to 33.3% of solution B over a period of 30 min.

TABLE 1

**HPLC Gradient Used in Nucleotide Resolution**

| Time min | % Solvent Composition A | B | Gradient Curve |
|---|---|---|---|
| Initial | 95 | 5 | - |
| 12 | 95 | 5 | 04 |
| 20 | 69 | 31 | 04 |
| 28 | 52 | 48 | 06 |
| 35 | 45 | 55 | 04 |
| 45 | 40 | 60 | 04 |
| 48 | 15 | 85 | 06 |
| 52 | 0 | 100 | - |
| 58 | 95 | 5 | - |

A: 10 mM Tetrabutylammonium hydroxide, (TBAH), 15 mM $KH_2PO_4$, pH 5.3
B: 30% $CH_3CN$, 10 mM TBAH, 35 mM $KH_2PO_4$, pH 5.3.Flow rate: 1 ml /min.



## Growth of *Streptomyces griseus*

A slant of *Streptomyces griseus* (ATCC-13273) was used to inoculate 25 ml of culture medium in 125 ml DeLong flasks [1]. The composition of the medium was (% w/v) dextrose 2.5% stage I, and 5% in stage II, 0.3% sodium chloride, 0.3% calcium carbonate, 1.5% soybean meal. Cultures were incubated at 27 °C while shaking at 250 RPM on

G25 Gyrotory shakers from New Brunswick Scientific Co. After 72 h, stage I cultures were used to inoculate (10 % by volume) stage II cultures which were incubated as before. Stage II cultures (24 h, 70 h, 120 h and 168 h) were harvested by filtration through cheese cloth and subsequent centrifugation of the filtrate in a Sorvall RC-5 Superspeed refrigerated centrifuge at 13,200 x g for 10 min. Cell pellets were washed twice with 30 ml of chilled pH 6, 0.12 M $KH_2PO_4$ buffer, and centrifuged at 13,200 x g for 10 min each time. Pellets were suspended in cold pH 6, 0.12 M phosphate buffer in $H_2O$ and in 50% MeOH:$H_2O$ to a final concentration of 0.5 g cells (wet weight) per ml. Cell free preparations were made by passing cell suspensions twice through a French Press at 17,000 psi, followed by centrifugation at 50,000 x g for 50 min. Supernatants were filtered through a 0.4-μm filter (Gelman, Acrodisc 13) and 20 μl samples were injected for HPLC nucleotide analyses.



## UDPG-Pyrophosphorylase Activity

UDPG-Pyrophosphorylase activity in *Streptomyces griseus* was determined by the modified method of Franke and Sussman [14] in which UDPG synthesis from UTP and glucose-1-phosphate is measured. The reaction mixture contained in a volume of 910 ml: 1.4 mmoles of Glucose-1-phosphate; 0.7 mmole of UTP; 1 mmole of $MgCl_2$; 1.2 mmoles of $NAD^+$; 340 mmoles of Tris/HCl pH 8.0 buffer; 80 mg (25-50 munits) of UDPG dehydrogenase. Reactions were incubated at room temperature for 1 min while the absorption at 340 nm was

recorded. One unit of enzyme activity reduces 2.0 mmoles of $NAD^+$ per min under these conditions.

## Calibration, Reproducibility and Sugar Nucleotide Recovery

Calibration graphs were prepared using standard solutions of sugar nucleotides, and HPLC-UV quantitation was based on peak areas. Linearity in detector response was observed between 0.1 μg-2.5 μg for each compound. Least squares regression lines intercepted near zero with correlation coefficients greater than 0.98. In general, the measurement range of this method is 0.04 aufs (absorbance unit full scale) with a signal-to-noise ratio no less than 2.5/1. Under these conditions, the detection limit of this assay is 0.1 μg (approximately 8 mM) for dTDPG, UDPG, ADPG, GDPG and CDPG. The retention volumes of analytes and the void volume (2.0 mL) are listed in TABLE 2. Recoveries of standards of dTDPG, UDPG, ADPG, GDPG and CDPG were determined by preparing 320 mM solutions of these sugar nucleotides in fresh, 72 h cell free extracts, and by injecting spiked extracts for HPLC analyses.

Confirmation of peaks in *Streptomyces griseus* cell free extracts was done by co-injections with standard samples.

## RESULTS AND DISCUSSION

A reversed phase, concave gradient, ion-pairing HPLC method was designed to assay sugar nucleotides in *Streptomyces griseus* cell free extracts. Sugar nucleotides of interest were well resolved by this

TABLE 2

Retention Volumes of Nucleotides by Ion-Pairing RP-HPLC, Solvent System 1, (Void Volume= 2.0 ml)

| Compound | Mean Retention Volume[a] (ml) |
|---|---|
| CMP | 13.8±0.3 |
| UMP | 16.4±0.4 |
| GMP | 22.0±0.3 |
| dTMP | 27.0±0.2 |
| AMP | 30.2±0.1 |
| CDP | 32.0±0.1 |
| UDP | 34.3±0.1 |
| GDP | 35.0±0.2 |
| dTDP | 38.5±0.5 |
| ADP | 40.8±1.0 |
| CTP | 42.9±0.2 |
| UTP | 45.0±0.1 |
| GTP | 45.5±0.1 |
| dTTP | 54.1±1.0 |
| ATP | 58.2±1.2 |
| CDPG | 25.9±0.3 |
| UDPG | 28.2±0.1 |
| GDPG | 30.3±0.2 |
| dTDPG | 33.1±0.1 |
| ADPG | 35.8±0.1 |

[a]Mean of at least 3 replicates ± SD.

**TABLE 3**

**Retention Volumes of Various Nucleotides Using Solvent System 2**

| Compound | Retention Volume (ml) |
|---|---|
| UTP | 21.0 |
| UDP | 14.3 |
| UDPG | 10.5 |
| CDPG | 9.2 |
| GDPG | 12.8 |
| GTP | 20.0 |
| dTDP | 16.4 |
| dTDPG | 13.2 |
| AMP | 11.8 |
| ADPG | 15.2 |



method, and the system could be used to resolve twenty nucleotides (TABLE 2). A second system was established to resolve those nucleotides with very similar retention volumes (TABLE 3). HPLC resolution of a mixture of standards of CMP, UMP, GMP, CDPG, UDPG, GDPG, dTDPG, ADPG, dTDP, ADP and dTTP is shown, for example, in FIGURE 1.

Our strategy was to assay *Streptomyces griseus* cell free extracts for sugar nucleotides, to confirm their presence by co-injection with standards, and to confirm the existence of compounds by the presence

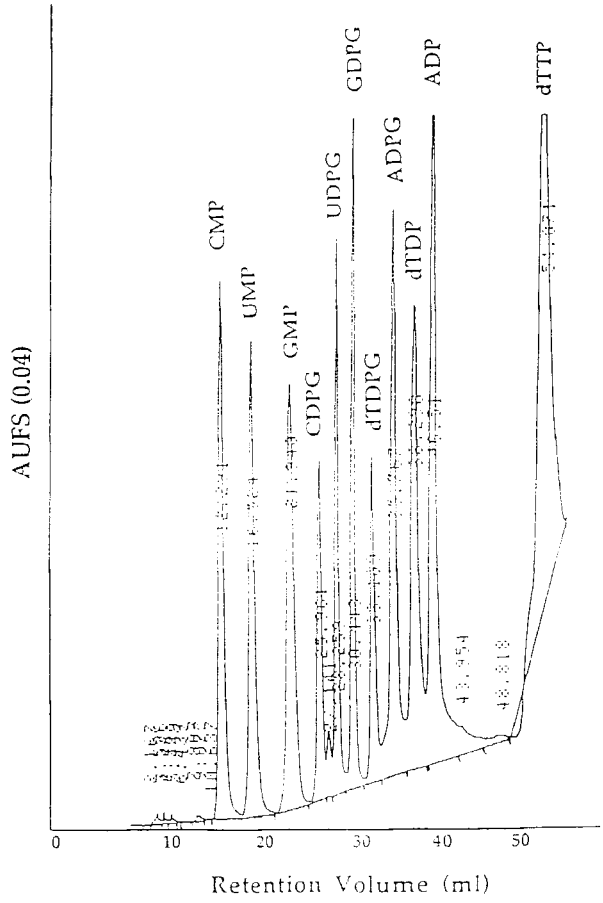


**FIGURE 1.**

RP-HPLC Chromatogram produced by injecting a mixture containing 1.5 mg of nucleotide standards using solvent system 1 (TABLE 1).

of enzyme activities that form them. While the use of enzyme assays alone has been used to detect the presence of sugar nucleotide biosynthetic enzymes, this combination LC and enzyme approach has apparently never been used. Recoveries of the five sugar nucleotides from spiked extract samples ranged between 97% and 100%. This result indicates that the sugar nucleotides are not subject to enzymatic or other decomposition in cell free extracts. Antibiotic biosynthesis typically begins at 48 h with this culture [1], and cell samples were taken periodically at 24 h intervals beginning at 24 h and ending at 168 h of growth. Representative chromatograms of cell-free-extracts of stage II *Streptomyces griseus* cultures harvested at 70 h and 120 h are shown in FIGURE 2. None of the peaks eluting in the 70 h extract were prominent, although relatively small peaks with *Rv* for UDPG and GDPG were observed. However, peaks for UDPG (90 mM), GDPG (40 mM) and UDP (37 mM) were observed in the 120 h cell extract. None of the other peaks observed in these extracts corresponded to known nucleotides evaluated in this study. Peaks, such as that at $R_v$ 14.3 in the 120 h sample, (FIGURE 2) could be putative deoxysugar nucleotide intermediates important in chromomycin $A_3$ biogenesis, and for which no standards are available.

Using HPLC, the time-course for formation of UDPG was evaluated from 24 h to 168 h. Over this time period, UDPG begins to increase in concentration at 70 hours, and climaxes between 120 to 140 hours (FIGURE 3). Although nucleotide identities in extracts were confirmed by coinjections with authentic standards and with the

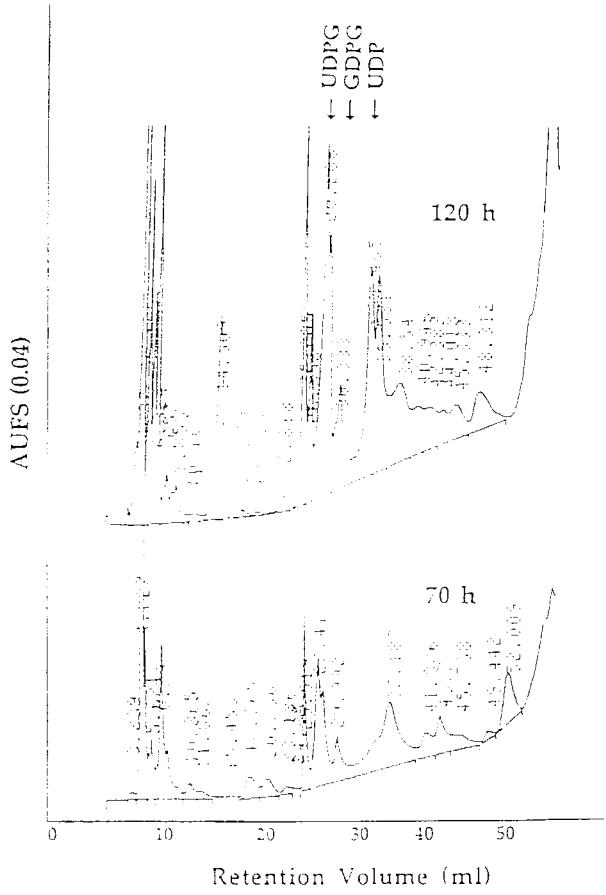


**FIGURE 2.**

Chromatograms of 70 h and 120 h *Streptomyces griseus* cell-free extracts.

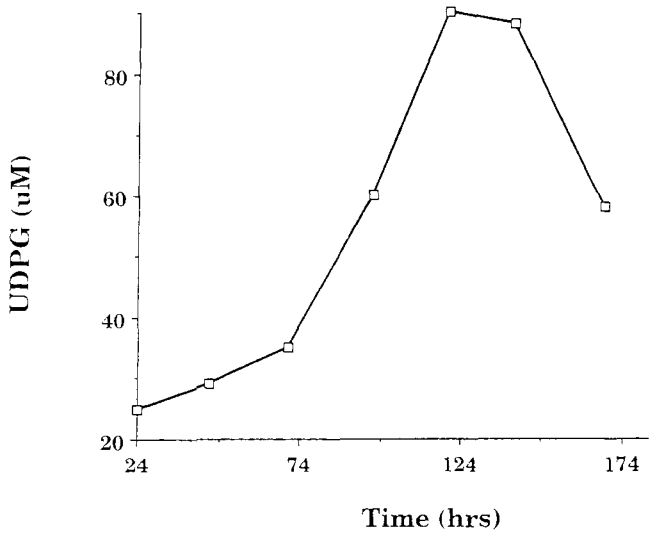


**FIGURE 3.**

Concentration of UDPG in cell free extracts *vs* fermentation time as determined by HPLC analysis.



second HPLC system (TABLE 3), the presence of imperfectly resolved unknown analytes appearubg as shoulders on peaks for UDPG, and GDPG and an overlapping peak with UDP (FIGURE 3) precluded accurate quantitative analyses of the nucleotides, or their ready characterization by isolation, chemical or spectral means.

The results suggested that UDPG was the active sugar nucleotide intermediate involved in the conversion of glucose to 2,6-dideoxy sugars in the biosynthesis of aureolic acid[1]. Cell free extracts of *S.griseus* were evaluated for UDPG pyrophosphorylase enzyme activities to confirm that the enzyme system necessary for the

formation of UDPG from UTP and glucose-1-phosphate was present. Enzyme activity *vs* time (0.022 units/mg protein at maximum) increased in essentially the same manner as UDPG by HPLC.

This HPLC ion-pairing, reversed-phase gradient approach affords a rapid means of assessing cell free extracts of antibiotic-producing Streptomycetes for the possible presence of sugar nucleotides relevant to antibiotic biosynthesis. Results from chromatographic analyses provide information crucial to the specific nature of enzymes such as UDPG pyrophosphorylase involved in sugar activation and metabolism.



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