Enzymological Characterization of the *Pasteurella multocida* Hyaluronic Acid Synthase†

Paul L. DeAngelis

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, Oklahoma 73190

Received January 22, 1996; Revised Manuscript Received May 6, 1996⊗

ABSTRACT: Hyaluronic acid (HA), a linear polysaccharide composed of alternating glucuronic acid and *N*-acetylglucosamine residues, is an essential molecule of higher vertebrates. The fowl cholera pathogen *Pasteurella multocida* Carter Type A also produces HA in the form of an extracellular capsule in order to evade host defenses. HA synthase activity could be obtained from cell-free membrane preparations of *P. multocida*. The enzyme utilized UDP-sugar precursors of HA in the presence of Mg^{2+} or Mn^{2+} at neutral pH. Mn^{2+} at 1 mM stimulated ∼2-fold more incorporation than Mg^{2+} at 10 mM. On the other hand, the analogous enzyme from group A *Streptococcus*, HasA, is stimulated more by Mg^{2+} than Mn^{2+}. The apparent Michaelis constants, K_M, of the *P. multocida* HA synthase for UDP-*N*-acetylglucosamine and UDP-glucuronic acid were estimated to be ∼75 and ∼20 μM, respectively, in the presence of Mg^{2+}, which suggests that the substrates are bound with 2−3-fold higher affinity than by the HasA enzyme. The rate enhancement observed with Mn^{2+} is apparently not due to better binding of the sugar nucleotide precursors complexed to Mn ion because the K_M value, a measure of substrate affinity, increases by 25−50% in comparison to Mg^{2+}. In summary, the HA synthase from *P. multocida*, a Gram-negative bacterium, has kinetic optima distinct from those of HasA, the analog from the Gram-positive group A *Streptococcus*.

The polysaccharide hyaluronic acid (HA)[1] or hyaluronan is an essential component of higher animals that serves both structural and recognition roles [reviewed in Laurent and Fraser (1992)]. In mammals and birds, HA is present in large quantities in the skin, the joint synovial fluid, and the vitreous humor of eye. Certain pathogenic bacteria, namely, Gram-positive group A and C *Streptococcus* and Gram-negative *Pasteurella multocida* Carter Type A, produce extracellular capsules containing HA with the same chemical structure as the HA molecule found in their vertebrate hosts (Kass & Seastone, 1944; Carter & Annau, 1953). This clever strategy of "molecular mimicry" foils attempts to mount a strong antibody response to the capsular polysaccharide (Quinn & Singh, 1957). In contrast, capsular polysaccharides with different structures produced by other bacteria are often quite antigenic. The HA capsule also apparently helps the pathogens evade host defenses including phagocytosis (Harmon *et al*., 1991; Wessels *et al*., 1991).

Streptococcal bacteria have been used as the model organism for the study of HA biosynthesis for over 35 years. This system yielded seminal information about the HAS enzyme including its subcellular localization in the membrane, utilization of UDP-sugar precursors, and the requirement for Mg^{2+} (Markovitz *et al*., 1959); these important observations also seem to hold true for cell-free extracts containing vertebrate HAS activity (Appel *et al*., 1979). Recently, the group A streptococcal HAS has been identified and the gene molecularly cloned (DeAngelis *et al*., 1993a,b). A single membrane protein, HasA, migrating at 42 kDa by SDS−polyacrylamide gel electrophoresis, appears to transfer both GlcA and GlcNAc from uridine diphosphate donors to the growing polymer chain (DeAngelis *et al*., 1993b; DeAngelis & Weigel, 1994).

The capsule of *P. multocida* was long suspected of containing HA (Carter & Annau, 1953), and the identity of the polysaccharide has been rigorously confirmed (Rosner *et al*., 1992). No report, however, has described the preparation of biosynthetically active cell-free extracts or otherwise characterized the HAS activity from *P. multocida*. Here I demonstrate that the *P. multocida* HA synthase present in membrane preparations has some interesting enzymological differences from the streptococcal HasA protein.

EXPERIMENTAL PROCEDURES

Materials and Bacteria. Protein was determined by the Coomassie dye-binding assay utilizing a bovine serum albumin standard (Bradford, 1976). *P. multocida* P-1059 (American Type Culture Collection 15742), a highly virulent turkey strain isolated by K. L. Heddleston that forms very mucoid colonies, was maintained on brain/heart infusion medium under aerobic conditions at 37 °C. An acapsular mutant of P-1059 which formed smaller, "drier" colonies, named TnA, was generated by a newly described Tn*916* insertional mutagenesis method for *P. multocida* (DeAngelis, 1996).

Membrane Preparation. Total membranes from *P. multocida* were prepared by a modification of the method for

† This work was supported by National Research Initiative Grant for Sustaining Animal Health and Well-Being from the U.S. Department of Agriculture (94-37204-0929).

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: HA, hyaluronic acid or hyaluronan; HAS, hyaluronic acid synthase; UDP, uridine diphosphate; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; SDS, sodium dodecyl sulfate; Tris, tris-[hydroxymethyl]aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; K_M, Michaelis constant, PCR, polymerase chain reaction; Tn, transposon; *v*, enzyme velocity; V_{max}, maximal enzyme velocity; [S], substrate concentration.

S0006-2960(96)00154-7 CCC: $12.00

producing HA synthase from *Escherichia coli* with recombinant plasmids containing *hasA* (DeAngelis *et al*., 1994). Cells were grown with vigorous shaking to mid-log phase (0.4−0.8 A_{600}) and then ovine testicular hyaluronidase (Sigma Type V, 20 units/mL final) was added to remove the capsule. After 40 min, the cells were chilled on ice and harvested by centrifugation (2000*g* for 15 min). The cells were washed twice with PBS by repeated suspension and centrifugation, and the cell pellet could be stored at −80 °C. All of the following steps were performed on ice unless noted otherwise. The cells were resuspended by pipetting in $^1/_{400}$ the original culture volume of 20% sucrose and 30 mM Tris, pH 8.0, containing the protease inhibitors pepstatin and leupeptin. Cell lysis was carried out by using lysozyme digestion (addition of $^1/_{10}$ the suspended volume of 4 mg/mL enzyme in 0.1 M EDTA, 40-min incubation) followed by ultrasonic disruption (power setting 3, three cycles of 30 s on/off; Heat Systems W-380 with microprobe). Before the ultrasonication step, sodium thioglycolate was added to the mixture (0.1 mM final concentration) following the addition of phenylmethanesulfonyl fluoride. In all the remaining manipulations, the PBS also contained freshly added thioglycolate at the same concentration. The lysate was treated with DNase and RNase (1 μg/mL each, 10 min at 4 °C) and the cellular debris was removed by low-speed centrifugation (10000*g* for 5 min). The supernatant fraction was diluted 6-fold with PBS and the membrane fraction was harvested by ultracentrifugation (100000*g* for 1 h). The pellet was washed twice by repeated suspension in PBS containing 10 mM $MgCl_2$ followed by ultracentrifugation. For generating membrane preparations used in metal specificity studies, $MgCl_2$ was omitted and replaced with 0.2 mM EDTA during the wash steps. Membrane preparations were suspended in 50 mM Tris, pH 7, and 0.1 mM thioglycolate, at a concentration of 1−3 mg/mL protein and stored at −80 °C.

HA Synthase Assay. HA synthase activity was routinely detected by incorporation of the radiolabel derived from the sugar nucleotide precursor UDP-[^{14}C]GlcA (0.27 Ci/mmol, ICN), into higher molecular weight products. The various assay buffers, described in the figure legends, also contained 0.3 mM DTT. Assays (100 μL final volume) were initiated by addition of membranes to the reaction mixture and incubation at 37 °C. After 1 h, the reactions were terminated by addition of SDS (2% final) and mixing. For the kinetic studies, the product and precursors were separated by descending paper chromatography (Whatman 3M with 65:35 ethanol/1 M ammonium acetate, pH 5.5). The HA polysaccharide at the origin of the paper chromatogram was eluted with water before liquid scintillation counting. The assays were typically performed under conditions in which no more than 5% of the precursors were consumed by limiting amounts of enzyme.

Controls to verify incorporation into authentic HA included omission of the required second sugar nucleotide precursor or digestion using the specific hyaluronidase from *Streptomyces hyalurolyticus*. Gel-filtration chromatography with Sephacryl S-200 (Pharmacia) in PBS was used to assess the molecular weight of the radiolabeled polymer formed *in vitro* under optimized assay conditions. These samples were treated as for paper chromatography except that, after termination, they were heated at 95 °C for 2 min and clarified by centrifugation (15000*g* for 7 min) before application to the column.

EDTA (0.2 mM) was used to chelate any metal ions present in assay mixtures to verify metal dependence of the HAS activity. Various divalent metals, including Mg, Mn, Cu, Co, and Ni, were tested as their chloride salts. The K_M values of the substrates were estimated by titration of one sugar nucleotide concentration while holding the other radiolabeled precursor at a constant and saturating concentration. For these studies, UDP-[^{3}H]GlcNAc (30 Ci/mmol, NEN) was employed as well as the UDP-[^{14}C]GlcA precursor.

RESULTS

Membrane Preparation. *P. multocida* cells produce a readily visible extracellular HA capsule, and since the streptococcal HasA is a putative transmembrane protein, membrane preparations of the fowl cholera pathogen were tested. In early trials, crude membrane fractions derived from ultrasonication alone possessed very low levels of UDP-GlcNAc-dependent UDP-[^{14}C]GlcA incorporation into HA [~0.2 pmol of GlcA transfer (μg of proteins)$^{-1}$ h^{-1}] when assayed under conditions similar to those for measuring streptococcal HAS activity. The enzyme from *E. coli* with the recombinant *hasA* plasmid was also recalcitrant to isolation at first (unpublished observations; DeAngelis & Weigel, 1994). These results were in contrast to the easily detectable amounts obtained from *Streptococcus* by similar methods.

An alternative preparation protocol using ice-cold lysozyme treatment in the presence of protease inhibitors in conjunction with ultrasonication allowed the substantial recovery of HAS activity from both species of Gram-negative bacteria. Specific activities of 5−10 pmol of GlcA transfer (μg of protein)$^{-1}$ h^{-1} were routinely obtained for crude membranes of wild-type *P. multocida* with the new method. In the absence of UDP-GlcNAc, virtually no radioactivity (<1% of identical assay with both sugar precursors) from UDP-[^{14}C]GlcA was incorporated into higher molecular weight material. Membranes prepared from the acapsular mutant, TnA, possessed no detectable HAS activity when supplemented with both sugar nucleotide precursors (data not shown). Gel-filtration analysis using a Sephacryl S-200 column indicates that the molecular mass of the majority of the ^{14}C-labeled product synthesized *in vitro* is $\geq 8 \times 10^4$ Da since the material elutes in the void volume (data not shown); such a value corresponds to a HA molecule composed of at least 400 monomers. This product is sensitive to *Streptomyces* hyaluronidase digestion but resistant to Pronase treatment (data not shown).

Analysis of Enzyme Requirements. The parameters of the HAS assay were varied to maximize incorporation of UDP-sugars into polysaccharide by *P. multocida* membranes. Streptococcal HasA requires Mg^{2+} and therefore this metal ion was included in the initial assays of *P. multocida* membranes. The *P. multocida* HAS was relatively active from pH 6.5 to 8.6 in Tris-type buffers with an optimum at pH 7 (Figure 1). The HAS activity was linear with respect to the incubation time at neutral pH for at least 1 h (data not shown). The *P. multocida* enzyme was apparently less active at higher ionic strengths because the addition of 100 mM NaCl to the reaction containing 50 mM Tris, pH 7, and 20 mM $MgCl_2$ reduced sugar incorporation by ~50%.

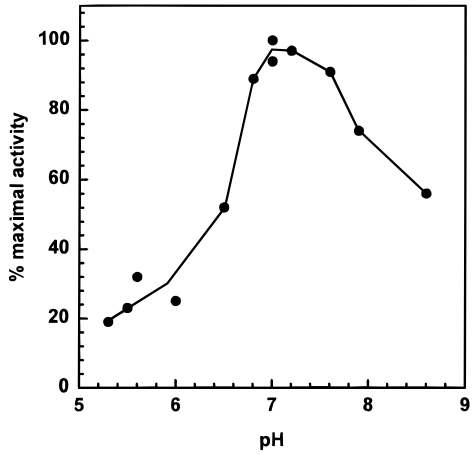

FIGURE 1: pH dependence of *P. multocida* HAS activity. The incorporation of [^{14}C]GlcA into HA polysaccharide catalyzed by membranes (38 μg of protein) was measured in reactions buffered at various pH values (50 mM Tris/2-(*N*-(morpholino)ethanesulfonic acid, bis-Tris/HCl, or Tris/HCl; no major buffer ion-specific effects were noted). The incubation mixture also contained 20 mM $MgCl_2$, 120 μM UDP-GlcA (4.5×10^4 dpm/assay), and 300 μM UDP-GlcNAc. The incorporation of the assay using the optimal buffer, pH 7 Tris, was set to 100% activity. A broad pH optimum around neutrality was observed.

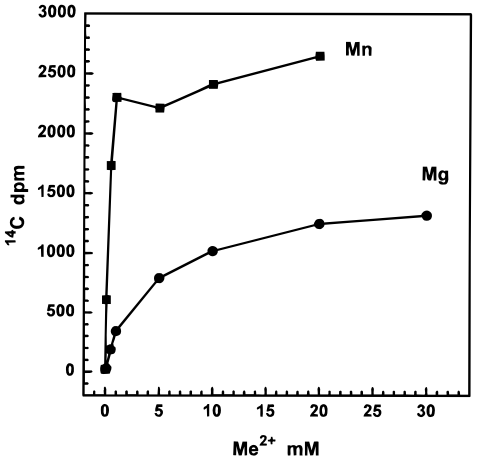

FIGURE 2: Metal dependence of HAS activity. The production of HA was measured in the presence of increasing concentrations of Mg (circles) or Mn (squares) ion. The membranes (46 μg of protein), prewashed with EDTA as described under Membrane Preparation, were incubated in a mixture of the metal ion in 50 mM Tris, pH 7, 120 μM UDP-GlcA (4.5×10^4 dpm/assay), and 300 μM UDP-GlcNAc for 1 h. The background with no metal present (22 dpm) was subtracted from each point. Mn is more effective than Mg.

The metal ion specificity of the *P. multocida* HAS was assessed at pH 7 (Figure 2). Under metal-free conditions in the presence of EDTA, no incorporation of radiolabeled precursor into polysaccharide was detectable (<0.5% of maximal signal). Mn^{2+} gave the highest incorporation rates at the lowest ion concentrations for the tested metals (Mg, Mn, Co, Cu, and Ni). Mg^{2+} gave about 50% of the Mn^{2+} stimulation but at 10-fold higher concentrations. Co^{2+} or Ni^{2+} at 10 mM supported lower levels of activity (20% or 9%, respectively, of 1 mM Mn^{2+} assays), but membranes supplied with 10 mM Cu^{2+} were inactive (data not shown). Indeed, mixing 10 mM Cu^{2+} and 20 mM Mg^{2+} with the membrane preparation resulted in almost no incorporation of label into polysaccharide (<0.8% of Mg only value).

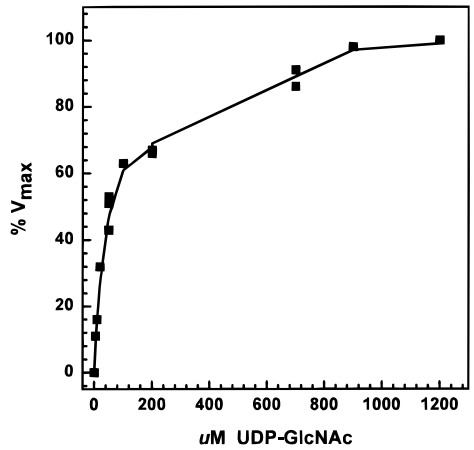

FIGURE 3: HAS activity dependence on UDP-GlcNAc concentration. Membranes (20 μg of protein) were incubated with increasing concentrations of UDP-GlcNAc in buffer containing 50 mM Tris, pH 7, 20 mM $MgCl_2$, and 800 μM UDP-GlcA (1.4×10^5 dpm of ^{14}C) for 1 h. The background radioactivity (identical assay but no added UPD-GlcNAc) was subtracted from each point. The highest specific incorporation rate into HA (average ~780 dpm/h) in the titration was defined as V_{max} for normalization to 100%.

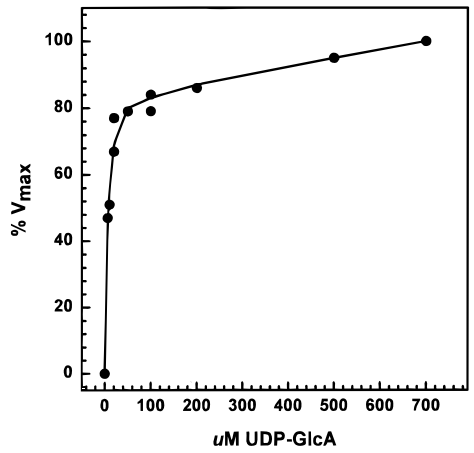

FIGURE 4: HAS activity dependence on UDP-GlcA concentration. In experiments parallel to those described in Figure 3, increasing amounts of UDP-GlcA were incubated with 1 mM UDP-GlcNAc (2.7×10^5 dpm of ^{3}H) under the same general buffer and assay conditions. The background radioactivity (assay with no added UDP-GlcA) was subtracted from each point. The data is presented as in Figure 3. Specific incorporation at V_{max} averaged ~730 dpm/h.

Kinetic Analysis. Initial characterization of the *P. multocida* HAS was performed in the presence of Mg^{2+}. The binding affinity of the enzyme for its sugar nucleotide precursors was assessed by measuring the apparent K_M value. Incorporation of [^{14}C]GlcA or [^{3}H]GlcNAc into polysaccharide was monitored at varied concentrations of UDP-GlcNAc or UDP-GlcA, respectively (Figures 3 and 4). In Mg^{2+}-containing buffers, the apparent K_M values of ~20 μM for UDP-GlcA and ~75 μM for UDP-GlcNAc were determined utilizing Hanes−Woolf plots ([S]/v *versus* [S]) of the titration data (Figure 5). The V_{max} values for both sugars were the same because the slopes, corresponding to $1/V_{max}$, of the Hanes−Woolf plots were equivalent. In comparison to results from assays with Mg^{2+}, the K_M value for UDP-GlcNAc was increased by about 25−50% to ~105 μM and the V_{max} increased by a factor of 2−3-fold in the presence of Mn^{2+} (Table 1).

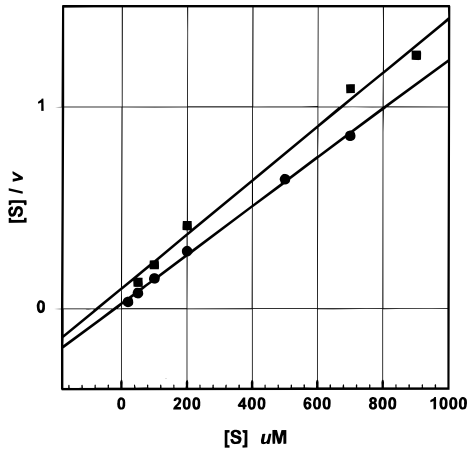

FIGURE 5: Hanes−Woolf plot estimation of V_{max} and K_M. The specific incorporation data used to generate Figures 3 (UDP-GlcNAc, squares) and 4 (UDP-GlcA, circles) were graphed as [S]/v *versus* [S]. The parallel slopes, which correspond to $1/V_{max}$, indicate that the maximal velocities for the sugar nucleotide precursors were equivalent. The *x*-axis intercept, which signifies $-K_M$, yielded K_M values of 75 and 20 μM for UDP-GlcNAc and UDP-GlcA, respectively.

Table 1: Metal Dependence of V_{max} and K_M[a]

membrane wash	assay ion	K_M (μM)	V_{max} (pmol/h)
Mg	Mg	75 ± 5	114 ± 36
EDTA	Mg	55 ± 25	98 ± 1
EDTA	Mn	105 ± 5	380 ± 70

[a] Further kinetic analyses of radiolabeled GlcA incorporation as a function of UDP-GlcNAc concentration were performed in the presence of Mg or Mn ion, and the data were analyzed by Hanes−Woolf plots. The membranes were washed with buffers containing either $MgCl_2$ or EDTA as described under Membrane Preparation and assayed as in Figure 3 but containing the indicated metal ion at 20 mM. Mn increased V_{max}, but the K_M value for UDP-GlcNAc was slightly elevated.

DISCUSSION

The HA capsules of the pathogens *P. multocida* and *S. pyogenes* are putative virulence factors that aid the evasion of host defenses. The HA synthase enzyme from either bacterial source utilizes UDP-sugars, but they possess somewhat different kinetic optima with respect to pH and metal ion dependence and K_M values. Both enzymes are most active at pH 7; however, the *P. multocida* HAS functions better on the alkaline side of this pH optimum up to at least pH 8.6. On the other hand, the *S. pyogenes* HAS reportedly displays more activity at slightly acidic pH and is relatively inactive above pH 7.4 (Stoolmiller & Dorfman, 1969). The *P. multocida* enzymes utilizes Mn^{2+} more efficiently than Mg^{2+} under the *in vitro* assay conditions, but the identity of the physiological metal cofactor in the bacterial cell is unknown. In comparison, in previous studies with the streptococcal enzyme (Markovitz *et al*., 1959; Stoolmiller & Dorfman, 1969), Mg^{2+} was much better than Mn^{2+} but the albeit smaller effect of Mn^{2+} was maximal at ~10-fold lower concentrations than the optimal Mg^{2+} concentration. The *P. multocida* HAS apparently binds the UDP-sugars more tightly than streptococcal HasA. The measured K_M values for the *P. multocida* HAS in crude membranes are about 2−3-fold lower for each substrate than those obtained from the HAS found in streptococcal membranes: 50 or 39 μM for UDP-GlcA and 500 or 150 μM for UDP-GlcNAc [Stoolmiller and Dorfman (1969) or van de Rijn and Drake (1992), respectively, for each value].

By kinetic analyses, the V_{max} of the *P. multocida* HAS was 2−3-fold higher in the presence of Mn^{2+} than Mg^{2+}, but the UDP-GlcNAc K_M value was increased slightly in assays with the former ion. This observation of apparent lowered affinity suggests that the increased polymerization rate was *not* due to better binding of the Mn^{2+} ion/sugar nucleotide complex to the enzyme active site(s). Therefore it is possible that Mn^{2+} enhances some other reaction step, alters another site/structure of the enzyme, or modifies the phospholipid membrane environment.

Elucidation of the complete primary structure of the *P. multocida* HAS would allow comparison with the streptococcal HasA enzyme prototype. Sequence similarities may yield insight on the enzyme features critical for HA biosynthesis such as the catalytic residues or substrate binding sites. It will also be interesting to see if the *P. multocida* sequence(s) belong to a new apparent class of polysaccharide synthases, which includes chitin synthases, with sequence similarity to HasA (DeAngelis *et al*., 1994).

ACKNOWLEDGMENT

I thank Dr. Paul H. Weigel for helpful discussions.

REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Carter, G. R., & Annau, E. (1953) *Am. J. Vet. Res. 14*, 475−478.
DeAngelis, P. L. (1996) *Microb. Pathog.* (submitted for publication).
DeAngelis, P. L., & Weigel, P. H. (1994) *Biochemistry 33*, 9033−9039.
DeAngelis, P. L., Papaconstantinou, J., & Weigel, P. H. (1993a) *J. Biol. Chem. 268*, 14568−14571.
DeAngelis, P. L., Papaconstantinou, J., & Weigel, P. H. (1993b) *J. Biol. Chem. 268*, 19181−19184.
DeAngelis, P. L., Yang, N., & Weigel, P. H. (1994) *Biochem. Biophys. Res. Commun. 199*, 1−10.
Harmon, B. G., Glisson, J. R., Latimer, K. S., Steffens, W. L., & Nunnally, J. C. (1991) *Am. J. Vet. Res. 52*, 1507−1511.
Kass, E. H., & Seastone, C. V. (1944) *J. Exp. Med. 79*, 319−330.
Laurent, T. C., & Fraser, J. R. E. (1992) *FASEB J. 6*, 2397−2404.
Markovitz, A., Cifonelli, J. A., & Dorfman, A. (1959) *J. Biol. Chem. 234*, 2343−2350.
Quinn, A. W., & Sing, K. P. (1957) *Proc. Soc. Exp. Biol. Med. 95*, 290−294.
Rosner, H., Grimmecke, H.-D., Knirel, Y. A., & Shashkov, A. S. (1992) *Carbohydr. Res. 223*, 329−333.
Stoolmiller, A. C., & Dorfman, A. (1969) *J. Biol. Chem. 244*, 236−246.
van de Rijn, I., & Drake, R. R. (1992) *J. Biol. Chem. 267*, 24302−24306.
Wessels, M. R., Moses, A. E., Goldberg, J. B., & DiCesare, T. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8317−8321.

BI960154K