Streptomycin Biosynthesis and Metabolism

ENZYMATIC PHOSPHORYLATION OF DIHYDROSTREPTOBIOSAMINE MOIETIES OF DIHYDROSTREPTOMYCIN-(STREPTIDINO)PHOSPHATE AND DIHYDROSTREPTOMYCIN BY STREPTOMYCIN EXTRACTS*

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MARGARET S. WALKER AND JAMES B. WALKER

From the Department of Biology, Rice University, Houston, Texas 77001

SUMMARY

Extracts of streptomycin-producing strains of Streptomyces phosphorylate dihydrostreptomycin-(streptidino)phosphate with adenosine 5'-triphosphate as the phosphoryl donor. The second phosphate is esterified with the dihydrostreptobiosamine moiety. Streptomycin-(streptidino)phosphate was not phosphorylated under similar conditions. Because of its lability, the new enzyme that is responsible for the phosphorylation could not be separated from the previously described streptomycin streptidinokinase, present in streptomycin producers, which phosphorylates the streptidine moieties of both streptomycin and dihydrostreptomycin. However, extracts of a strain which lacks streptidinokinase, Streptomyces griseus ATCC 10971, phosphorylated the dihydrostreptobiosamine moieties of both dihydrostreptomycin and dihydrostreptomycin-(streptidino)phosphate; streptomycin was not phosphorylated. The kinase is an idiosyncratic enzyme, not detectable during the early rapid growth phase of these Streptomyces. The observation that streptomycin-(streptidino)phosphate was phosphorylated only after reduction with borohydride to the dihydrostreptose moiety suggests that the second phosphate is esterified with the 3-hydroxyethyl group of the dihydrostreptose moiety of dihydrostreptomycin-(streptidino)phosphate. Fresh extracts of streptomycin producers can synthesize the diphosphorylated derivative of dihydrostreptomycin from dihydrostreptomycin via either of the two monophosphorylated dihydrostreptomycin derivatives.

During the course of studies on the biosynthesis and metabolism of the streptomycin family of antibiotics (cf. Reference 1), we observed that freshly prepared extracts of Streptomyces bikiniensis ATCC 11062 catalyzed the formation of a new compound when incubated with ATP, Mg2+, and [3H]dihydrostreptomycin-(streptidino)phosphate. The purpose of this paper is to describe some of the characteristics of this new compound and to report the substrate specificity and biological distribution of the enzyme that catalyzes this reaction. The structure of dihydrostreptomycin is given in Fig. 1.

MATERIALS AND METHODS

Cultures of Streptomyces strains and Bacillus subtilis ATCC 6633 came from the American Type Culture Collection. Purified alkaline phosphatases from Escherichia coli (type III) and calf intestinal mucosa (type VII) came from Sigma. 3H-Dihydrostreptomycin (730 Ci per mole), labeled at position 3' of the dihydrostreptose moiety, and ATP-γ-32P (620 Ci per mole on receipt) came from Amersham-Searle, Des Plaines, Illinois. (8,14C)-ATP (33 Ci per mole) came from Schwarz BioResearch.

Cultures of Streptomyces were grown from slant moclcula on 2% peptone-0.2% yeast extract-tap water in 2-liter Erlenmeyer flasks, 500 ml per flask, at 25° on a rotary shaker for 3 days. Mycelia were harvested by suction filtration on Buchner funnels, pressed dry with paper towels, and stored frozen. As a source of streptomycin streptidinokinase for studies with H-labeled compounds, 48-hour dialyzed lysozyme extracts of frozen pads were used (2). 3H-Dihydrostreptomycin-(streptidino)phosphate was prepared by incubating the following at 37° for 1 hour: 3H-dihydrostreptomycin (60,000 cpm/5 μl), 0.5 ml; 0.5 M Tris, pH 9.0, containing 0.04 M MgCl2, 0.5 ml; 0.036 M ATP, pH 7, 0.5 ml; and 48-hour dialyzed lysozyme extract of S. bikiniensis, 1.0 ml. Labeled product was isolated from the mixture by adsorption on a Bio-Rex 70 (NH4) carboxylic acid resin column, 100 to 200 mesh, and elution with 0.8 M ammonium formate (3); dihydrostreptomycin remained on the column (3). More highly purified streptomycin streptidinokinase preparations were required for synthesis of dihydrostreptomycin-(streptidino-32P)phosphate and streptomycin-(streptidino-32P)phosphate, with ATP-γ-32P as the phosphate donor; such enzyme preparations were obtained from Sephadex G-100 columns (4). The 32P-phosphorylated derivatives were prepared by the sealed up procedure described elsewhere (4). The same general enzyme purification procedure also yielded a preparation containing streptomycin-(streptidino)phosphate phosphatase activity, which was eluted earlier than the kinase from the Sephadex column, with the bulk of the protein.

Formation of the new compounds, PD8MP1 and PDSM, was

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* The abbreviations used are: PDSMP, diphosphorylated derivative of dihydrostreptomycin, believed to be N-methyl-D-glucosamine(α-1→2)-L-dihydrostreptose 3-phosphate (α-1→4)
catalyzed by "fresh" Streptomyces extracts prepared as follows: a frozen mycelial pad (approximately 8 g) was minced with a chilled beaker for a total of 8 min, in 30 set bursts, with intervals for retooling. The sonic extract was centrifuged at 30,000 × g for 20 min at 4°, as needed; aliquots were diluted with water as indicated and the remainder was rapidly refrozen, since the enzyme is extremely labile, even at 4°, although stable in the frozen state and fairly stable in 30% glycerol. The term "fresh extract" means a freshly made sonic extract or, more usually, a freshly thawed extract prepared as described above.

Labeled PDSMP was synthesized by incubating the following reaction mixture, or a scaled up version, for 30 min at 37°: 3H-dihydrostreptomycin (60,000 cpm/5 μl), 5 μl; 0.5 mM glycylglycine, pH 8.0, containing 0.04 mM MgCl₂, 5 μl; 0.036 μM ATP, pH 7.5, 5 μl; and fresh extract of S. griseus (usually diluted 20- to 50-fold), 10 μl. Labeled PDSM was synthesized by substituting a fresh extract of S. griseus ATCC 10971 for S. griseus extract in the above mixture. To prepare larger amounts of nonlabeled PDSMP and PDSM for antibiotic assays and phosphate determinations, the same relative proportions were used, except that solutions of nonlabeled dihydrostreptomycin or streptomycin, 2 mg per ml, were substituted for labeled compound, and the incubation time was lengthened.

Early in our study, 3H-PDSM was isolated, at low temperature, by adsorption on a Pasteur pipette column containing Dowex 50-X8 (H⁺), 200 to 400 mesh; the column was washed with 5 ml of water and then with 5 ml of 1.0 N HCl. Fractions containing 3H-PDSM were eluted with 2.5 N HCl and rapidly evaporated in dishes in a vacuum over NaOH pellets. Rapid handling at low temperature minimized degradation by acid.

Larger quantities of PDSMP have subsequently been isolated by passing the incubation mixture through a Dowex 1-X8(C₂) column, 200 to 400 mesh; the water wash was then applied to a Bio-Rex 70 (NH₄⁺) column as described elsewhere (3), and washed through with water. PDSMP was not adsorbed by either resin, but it was separated from ATP and certain other phosphorylated compounds, and from other dihydrostreptomycin derivatives. Isolation of PDSM was performed similarly, except that it was eluted from the Bio-Rex 70 (NH₄⁺) column with 0.3 M ammonium formate, following a wash with 0.1 M ammonium formate. Dihydrostreptomyein-streptidino phosphate (eluted with 0.8 M ammonium formate) and dihydrostreptomyein (eluted with 2 M ammonium formate) remained on the column (3). Column fractions were evaporated to dryness in a vacuum over CaCl₂. Ammonium formate was removed in a vacuum over concentrated H₂SO₄ in Petri plates, to which glass wool had been added to increase the surface area. For certain studies, when most of the 3H-dihydrostreptomycin had been enzymatically converted to 3H-PDSM, separation involved only the Dowex 1 column step.

Antibiotic assays were performed by paper disc assay (3), before and after incubation of PDSMP and PDSM (or fractions which would have contained the corresponding streptomycin derivatives) with E. coli alkaline phosphatase, with B. subtilis as the test organism and dihydrostreptomycin as the standard. The molar ratio of dihydrostreptomycin equivalents to phosphate content (5) was then determined.

Separations of labeled compounds were performed on Whatman No. 1 filter paper by: (a) ascending paper chromatography, with 24 cm travel, developed with 80% phenol 20% H₂O, ammonia atmosphere (provided by adding 1 ml of concentrated NH₄OH to inside of cylinder); and (b) high voltage paper electrophoresis with a refrigerated Savant horizontal plate apparatus. The effective path was 46 cm, 30 volts per cm. For separations at pH 10.4, glycine-NaOH buffer was used, and for pH 3.6, ammonium formate buffer, both at an ionic strength of 0.2. For both types of separations, strips were cut at 1-mm intervals, put in bottles containing Liquidfluor, and counted with a liquid scintillation system. In the figures, gross counts per min are given, uncorrected for background.

RESULTS

Detection of Reaction between ATP and Dihydrostreptomycin-streptidino (streptidine) phosphate—When 3H-dihydrostreptomycin-streptidino (streptidine) phosphate was incubated with Mg²⁺, ATP, and a fresh extract of S. griseus, a new labeled compound (Compound PDSM) was formed, as detected by paper chromatography (Fig. 2A). The same compound, PDSM, was also formed when nonphosphorylated 3H-dihydrostreptomycin was a substrate (Fig. 2B). The fact that the same compound was formed from either phosphorylated or nonphosphorylated dihydrostreptomycin could be explained by our previously reported finding that these extracts contain an active kinase which rapidly phosphorylates the streptidine moieties of both streptomycin and dihydrostreptomycin with ATP as the phosphate donor (4). The enzyme appeared to be specific for ATP; no activity was detected with UTP, GTP, or CTP.

Biological Distribution of Reaction—To obtain clues concerning the metabolic significance of this reaction, extracts of other Streptomyces were assayed. A number of streptomycin-producing strains tested had high activity, e.g., S. griseus strains ATCC...
FIG. 2. Formation of new compound, PDSMP, catalyzed by fresh extract of S. bikiniensis. Compounds were separated by paper chromatography. A, substrates: ATP and 3H-dihydrostreptomycin-(streptidino)phosphate (DSMP), 20-fold diluted extract; dashed curve, 0 time control. (Control without ATP was completely dephosphorylated to dihydrostreptomycin.) B, substrates: ATP and 3H-dihydrostreptomycin (DSM), 20-fold diluted extract; dashed curve, ATP omitted.

FIG. 3. Formation of PDSMP and another new compound, PDSM, catalyzed by fresh extract of S. griseus ATCC 10971. A, substrates: ATP and 3H-dihydrostreptomycin-(streptidino)phosphate (DSMP), 5-fold diluted extract; dashed curve, 0 time control. B, substrates: ATP and 3H-dihydrostreptomycin (DSM), 5-fold diluted extract; dashed curve, ATP omitted.

FIG. 4. Formation of PDSMP from 3H-PDSM and ATP, catalyzed by dialyzed extract of S. bikiniensis. This extract had streptomycin streptidinokinase activity, but was unable to form PDSMP from dihydrostreptomycin-(streptidino)phosphate; dashed curve, extract omitted.

Stability of Enzyme—In contrast to streptomycin streptidinokinase (4, 6), which is a relatively stable enzyme, the enzyme or enzymes catalyzing the synthesis of PDSMP and PDSM are quite unstable. Initially high activity in freshly prepared or freshly thawed extracts declines to an undetectable level after several hours at either 2° or room temperature. This lability has hindered attempts at enzyme purification. However, experiments with S. bikiniensis enzyme could often be carried out with a 50-fold dilution of the original supernatant solution from a 50% (w/v) sonic extract of a frozen mycelial pad. These diluted enzyme preparations were markedly stabilized by glycerol and could be kept overnight in 30% glycerol at 4° with significant retention of activity. The enzyme is stable to freezing, either in the intact mycelial pads or in extracts; extracts can be stored for months in the frozen state.

Evidence for Presence of Two Monoesterified Phosphate Groups in PDSMP—High voltage paper electrophoresis proved to be...
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Fig. 5. Action of various purified phosphomonoesterases on \(^\text{3H-PDSMP}\). Reaction mixtures were incubated 165 min; compounds were separated by high voltage paper electrophoresis at pH 10.4. Origin indicated by vertical arrows. Picric acid migrated 17 cm. A, standards, showing mobilities of \(^\text{3H-dihydrostreptomycin-(streptidino)phosphate (DSMP)}\) and \(^\text{3H-dihydrostreptomycin (DSM)}\). B, conversion of \(^\text{3H-PDSMP}\) by \(^{E.\ coli}\) alkaline phosphatase (+E. coli Pase) to compound migrating to DSM position. Note that intervals between \(^\text{PDSMP}\) and \(^\text{DSMP}\) and between \(^\text{PSMP}\) and \(^\text{DSM}\) are approximately equal, consistent with one phosphate difference in each case. C, conversion of \(^\text{3H-PDSMP}\) by \(^\text{streptomycin-(streptidino)phosphate phosphatase (+SMP Pase)}\) preparation to compound migrating to DSM position. D, conversion of \(^\text{3H-PDSMP}\) by calf mucosa alkaline phosphatase (+Calf M. Pase), first into compound migrating to DSMP position, which in turn is converted into compound migrating to DSM position (order of conversion determined by separate time course experiments).

Fig. 6. Experiment showing that the compound formed by action of \(^{E.\ coli}\) alkaline phosphatase on \(^\text{3H-PDSMP}\) can be reconverted to \(^\text{PDSMP}\). Compounds were separated by high voltage paper electrophoresis at pH 10.4. Picric acid migrated 18 cm. A, curves showing preparation of substrate for subsequent conversions. B, conversion of dephosphorylated \(^\text{3H-PDSMP}\) back to \(^\text{PDSMP}\) following incubation with ATP and fresh extract of \(^S.\ bikiniensis\), diluted 20-fold. Note occurrence of trace of intermediate compound. C, conversion of dephosphorylated \(^\text{3H-PDSMP}\) to compound migrating to dihydrostreptomycin-(streptidino)phosphate position, when incubated with ATP and dialyzed extract of \(^S.\ bikiniensis\). (In this and subsequent figures, when calibration marks appear on right axis, these are for the dashed curve, and each mark has the same value as a mark on left axis.)

Evidence for Presence of Dihydrostreptomycin Moiety in PDSMP and PDSM—It was apparent from the mobilities at pH 10.4 that the streptidine moiety was present in \(^\text{PDSM}\) and \(^\text{PDSMP}\), but, since a crude nondialyzed extract was used in their preparation, other modifications of the dihydrostreptomycin moiety, in addition to phosphorylation, might have occurred. Neither PDSMP nor PDSM had antibiotic activity against \(^B.\ subtilis\). However, when either PDSMP or PDSM was incubated with \(^{E.\ coli}\) alkaline phosphatase, the resulting product had antibiotic activity similar to that of dihydrostreptomycin standard. This indicated that all three moieties of dihydrostreptomycin (Fig. 1) were present in essentially unmodified form. Furthermore, the molar ratio of antibiotic activity (as dihydrostreptomycin) to phosphate was found to be 1:1.1 for PDSM and 1:1.9 for PDSMP.

When PDSMP was treated with \(^{E.\ coli}\) alkaline phosphatase, the dephosphorylated product (Fig. 6A) could be (a) reconverted to PDSMP when incubated with a fresh nondialyzed \(^S.\ bikiniensis\) extract and ATP (Fig. 6B), or (b) converted to a compound...
with the mobility of dihydrostreptomycin-(streptidine)phosphate when incubated with a dialyzed extract of *S. bikiniensis* and ATP (Fig. 8C). Again these results suggest that the major portion of the dihydrostreptomycin moiety is conserved in PDSMP.

**Evidence Concerning Location of Phosphate Groups in PDSMP and PDSM.**—In the formation of PDSMP, ATP appeared to donate phosphoryl groups rather than adeny1 moieties, since (a) the ultraviolet absorption spectrum of PDSMP preparations showed no significant absorption at 260 μm; (b) label from 14C-<sup>3</sup>HCl at 50° for 3 hours and then evaporated to dryness in a vacuum; this procedure hydrolyzes linkage with streptidine. Labeled hydrolysate product (solid curve) has mobility different from intact PDSMP (dashed curve). B, labeled compound formed following incubation of mild acid hydrolysate of *H*-PDSMP with *E. coli* alkaline phosphatase (*E. coli* Pase). Note that dephosphorylated hydrolysate product does not migrate to dihydrostreptomycin (DSM) position, as occurs when nonhydrolyzed PDSMP is treated with alkaline phosphatase.

The simplest interpretation of the experimental results described up to this point was that PDSMP was a diphosphorylated dihydrostreptomycin with at least one phosphate group esterified with the streptidine moiety, and PDSM was a monophosphorylated dihydrostreptomycin. The next step was to determine whether the second phosphate group in PDSMP was located on the dihydrostreptobiosamine or the streptidine moiety. Mild acid treatment, which hydrolyzes the linkage between the streptidine moiety of dihydrostreptomycin and the dihydrostreptobiosamine portion (7, 8), was found to degrade PDSMP in such a way that the labeled dihydrostreptopeptidase moiety and phosphate were still joined (Fig. 7). The *R*<sub>f</sub> value of this new phosphorylated compound (Fig. 7A) was 0.46 on paper chromatograms developed with ammoniacal phenol. Subsequent treatment of the labeled hydrolysate product with *E. coli* alkaline phosphatase gave a labeled compound that migrated similarly to a compound with no net charge at pH 10.4 (Fig. 7B). This compound, presumably dihydrostrephtobiosamine, was not rephosphorylated on incubation with ATP and fresh *S. bikiniensis* extract. Its *R*<sub>f</sub> value on ammoniacal phenol paper chromatograms was 0.90. These results showed that the second phosphate in PDSMP is esterified to the dihydrostreptobiosamine moiety of dihydrostreptomycin. Similar results were obtained by mild acid hydrolysis of PDSM (Fig. 8).

PDSM and dihydrostreptomycin-(streptidine)phosphate, which have approximately the same electrophoretic mobilities, can be distinguished by electrophoresis following mild acid hydrolysis, as shown in Fig. 9. Phosphate remained with the labeled hydrolysate product in the case of PDSM, but not in the case of dihydrostreptomycin-(streptidine)phosphate. Electrophoresis here was performed at pH 3.6 to illustrate mobilities at acid pH. Similar separations were obtained at pH 10.4. These two compounds can also be distinguished by (a) their elution behavior following adsorption on a Bio-Rex 70 column ("Materials and Methods"), and (b) the fact that incubation with a streptomycin streptidinekinase preparation converts PDSM, but not dihydrostreptomycin-(streptidine)phosphate, to PDSMP (Fig. 4).

Attempts to determine the position of the phosphate on the dihydrostreptobiosamine moiety by further acid hydrolysis or...
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The unfractionated reaction mixture was incubated with sodium borohydride, label appeared in PDSMP fresh and 11. Inactivity of streptomycin-(streptidino-32P)phosphate was not caused by an inhibitory component since, following hydrolysis, gave a labeled compound which migrated identically with streptidine phosphate (2, 6).

Enzyme 1 is streptomycin streptidinokinase, which was previously shown to phosphorylate free streptidine (6), streptomycin, and dihydrostreptomycin (4), and now has been found to phosphorylate PDSMP to give PDSMP (Fig. 4). This enzyme might function to keep intermediates in the biosynthesis of streptomycin (1, 2) in a phosphorylated form, and perhaps is involved, in conjunction with a specific phosphatase, in a cellular regulatory mechanism (3). Streptomycin-(streptidino)phosphate accumulates in culture media of streptomycin producers under certain conditions (3, 10, 11) and indirect evidence from periodate oxidation (11) and enzyme substrate specificity (2, 4) suggest that phosphate is esterified at position 6 of streptidine.

Enzyme 2 is streptomycin-(streptidino)phosphate phosphatase, from streptomycin-producing Streptomyces, which hydrolyzes phosphate esterified with the streptidine moieties of streptomycin, dihydrostreptomycin, and PDSMP (Fig. 5C); this enzyme has not yet been extensively purified or fully characterized. Alkaline phosphatases from a number of sources will also hydrolyze PDSMP and PDSM from Bio-Rex 70 columns (cf. "Materials and Methods").

Enzyme 3 is the new kinase reported in this paper, which assay here was for antibiotic activity against B. subtilis, following E. coli alkaline phosphatase treatment of the appropriate pooled fractions (corresponding to PDSMP and PDSM) from Bio-Rex 70 columns (cf. "Materials and Methods").

Discussion

The enzymatic reactions described in this paper are summarized in Fig. 12.

Enzyme 1 is streptomycin streptidinokinase, which was previously shown to phosphorylate free streptidine (6), streptomycin, and dihydrostreptomycin (4), and now has been found to phosphorylate PDSMP to give PDSMP (Fig. 4). This enzyme might function to keep intermediates in the biosynthesis of streptomycin (1, 2) in a phosphorylated form, and perhaps is involved, in conjunction with a specific phosphatase, in a cellular regulatory mechanism (3). Streptomycin-(streptidino)phosphate accumulates in culture media of streptomycin producers under certain conditions (3, 10, 11) and indirect evidence from periodate oxidation (11) and enzyme substrate specificity (2, 4) suggests that phosphate is esterified at position 6 of streptidine.

Enzyme 2 is streptomycin-(streptidino)phosphate phosphatase, from streptomycin-producing Streptomyces, which hydrolyzes phosphate esterified with the streptidine moieties of streptomycin, dihydrostreptomycin, and PDSMP (Fig. 5C); this enzyme has not yet been extensively purified or fully characterized. Alkaline phosphatase from a number of sources will also hydrolyze this linkage, but more slowly (Fig. 5, B and D). It is not known whether the phosphatase activity from S. griseus described by Nomi et al. (12) is caused by a specific or nonspecific phosphatase. This is an important point, since this enzyme might be the final enzyme involved in streptomycin biosynthesis (3, 4, 11) and perhaps participates in the regulation of macromolecular syntheses (5).

Enzyme 3 is the new kinase reported in this paper, which
phosphorylates both dihydrostreptomycin- (streptidine)phosphate to give PDSMP (Figs. 2A and 3A) and dihydrostreptomycin to give PDSM (Fig. 3B). The phosphate was shown to be esterified with the dihydrostreptobiosamine moiety of these compounds (Figs. 7 and 8); the streptidine moiety appears to be required for activity since dihydrostreptobiosamine was not a substrate. Since neither streptomycin nor streptomycin-(streptidine)phosphate was phosphorylated by this enzyme (Figs. 10 and 11), it is tentatively suggested that the 3-hydroxymethyl group of the dihydrostreptose moiety is phosphorylated. The possibility remains, however, that an extra ring structure permitted in streptomycin but not in dihydrostreptomycin (13) might hinder phosphorylation of another group in the molecule by this kinase. Nevertheless, the simplest interpretation of our data is that PDSMP is N-methyl-D-glucosamine(α-1 → 2)-D-hydrostreptose 3'-phosphate (α-1 → 4)streptidine 6-phosphate (cf. Fig. 1).

Enzyme 4 represents various nonspecific phosphatases (Fig. 5, B and D); no evidence has yet been obtained for a phosphatase specific for this linkage. This linkage appears to be more readily attacked by nonspecific phosphatases than is phosphate esterified with the streptidine moiety.

Three different kinases are now known for the streptomycin family of antibiotics: (a) streptomycin streptidinokinase (4, 6); (b) streptomycin N-methyl-D-glucosaminokinase, present in bacteria containing certain streptomycin resistance factors, which phosphorylates position 3 of that moiety (14); and (c) the enzyme described in this paper, tentatively given the trivial name, dihydrostreptomycin dihydrostreptosokinase. In addition, an enzyme present in certain resistant bacteria, which adenylates position 3 of the N-methyl-D-glucosamine moiety of streptomycin, has been reported (15, 16); another resistance factor codes for phosphorylation of kanamycin (17). Other phosphorylated derivatives of interest include those of neomycin (18) and lincomycin (19). It is tempting to speculate that certain antibiotic resistance factors now present in enteric bacteria might have originated in organisms which produce these antibiotics.

Phosphorylated lincomycin has been shown to be antibiotically active in animal systems, presumably after dephosphorylation by nonspecific phosphatases (19, 20). We have suggested that streptomycin-(streptidine)phosphate might likewise be a useful antibiotic in vivo (3). By the same reasoning, PDSM and PDSMP might offer useful therapeutic advantages, being slowly converted to active antibiotic in vivo, but this remains to be established.

The function of PDSM and PDSMP in the metabolism of streptomycin is not known. These compounds might be detoxification products of this potent inhibitor of protein biosynthesis, or perhaps they are derivatives related to biosynthesis of streptomycin or its function in the producing organism, e.g. in regulation of macromolecular syntheses, or as components of cell walls (21) or membranes. Further work on these problems is planned.

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Margaret S. Walker and James B. Walker


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