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Bioconversion of *N*-Butylglucamine to 6-Deoxy-6-butylamino Sorbose by *Gluconobacter oxydans*

Bryan H. Landis,* Joseph K. McLaughlin, Robert Heeren, Roy W. Grabner, and Ping T. Wang

Bioprocess and Formulation Technology, Pharmacia, 700 Chesterfield Parkway North, St. Louis, Missouri 63198, U.S.A.

Abstract:

***Gluconobacter oxydans* has the unique ability to regioselectively and rapidly oxidize sorbitol and other erythro saccharides. In this report a new process is described by which *N*-butylglucamine is regioselectively oxidized by the organism. A large-scale process is described by which *N*-butylglucamine can be converted to an intermediate (6-deoxy-6-butylaminosorbose) which can be readily converted to *N*-butyldeoxynojirimycin by catalytic hydrogenation. The primary process variables of temperature, pH, and added acids and salts were investigated in laboratory bioreactors. Since degradation of the sorbose product was rapid above room temperature, significant enhancement of the selectivity was achieved by lowering the temperature at which the bioconversion was run. The optimum temperature for this conversion was 12−15 °C. The pH maximum of the bioconversion was 5.5−6.0. However, the small gain in rate relative to pH 5.0 was at least offset by the increase in degradation of the product at the higher pH. Nitrate salts of *N*-butylglucamine could replace chloride salts, but sulfate, acetate, and phosphate salts could not. Sulfate in particular led to inhibition of the conversion, while phosphate and acetate led to increased degradation. At temperatures in the range of 12−15 °C, pH of around 5.0 and substrate concentrations of 0.2 M, *Gluconobacter oxydans* catalyzed bioconversion to 6-deoxy-6-butylaminosorbose with yields approaching 95%. These conditions were used to scale this process to 5500-L scale.**

Introduction

N-Butyldeoxynojirimycin (*N*-butyl DNJ) is an inhibitor of glycosidase activity,[1] and as such was clinically evaluated by Pharmacia R&D as a potential therapeutic agent against retroviral infections, in particular acquired immune deficiency syndrome (AIDS). The activity of this compound was determined in vitro to prevent infection of H9 cells by the AIDS virus,[2] and these results were promising enough to encourage Pharmacia to pursue the development of processes to make this compound in quantity for clinical trials and commercial production. At least four processes were given extensive consideration, three of which combined bioprocess technology with chemical synthesis. A novel, high-efficiency process based on the selective microbial oxidation of *N*-butylglucamine to an intermediate suitable for reduction to *N*-butyldeoxynojirimycin was demonstrated on a laboratory scale and selected as the best available technology for scale-up. A broad range of operating conditions was evaluated to demonstrate the technical feasibility and performance.

This report describes in detail five primary bioconversion parameters: temperature, pH, *N*-butylglucamine (NBG) concentration, addends, and type of acid (counterion). Stabilization of the oxidized product by the butyl group was found to arise from increasing the pK_a of the amino nitrogen (NBG vs glucamine) and providing steric crowding, lessening the ability of nucleophilic nitrogen to interact with the carbonyl carbon of the oxidized product.

The microorganism used for the bioconversion was *Gluconobacter oxydans*, a member of the family *Acetobacteraceae*.[3] This microorganism is used industrially to prepare sorbose from sorbitol (glucitol) and dihydroxyacetone from glycerol. *G. oxydans* has also been used to prepare deoxynojirimycin[4] (DNJ) by first inactivating the basic amino nitrogen, performing the bioconversion with *G. oxydans*, then removing the inactivating group and reducing the unalkylated aminosorbose. All of these bioconversions are catalyzed by membrane-bound polyol dehydrogenases (a portion of these dehydrogenases may be soluble), one of which is sorbitol dehydrogenase. Sorbitol dehydrogenase is believed to be the enzyme responsible for catalyzing the bioconversion of *N*-butylglucamine to 6-deoxy-6-butylaminosorbose. Additional details and extensions may be found elsewhere.[5]

Experimental Section

***N*-Butylglucamine.** *N*-Butylglucamine (NBG) was obtained from Pharmacia, Skokie, IL, U.S.A. Material was prepared by reductive amination of glucose with *n*-butylamine. High-purity NBG was isolated from these reactions by crystallization. Bioconversion solutions were prepared by suspending NBG in water and adjusting the pH with an acid to below 6, simultaneously dissolving the NBG as the salt. If magnesium sulfate or other salts were to be added, it was added at this point, the pH was then adjusted to the target pH (usually 4.9 to 5.1), and the volume was adjusted to achieve the target concentration of NBG. For the pH-rate studies, the pH of a stock solution was adjusted to various

* To whom correspondence should be addressed. Telephone: 01 636-737-6760. Fax: 01 636 737 7281. E-mail: bryan.h.landis@pharmacia.com.

(1) Schweden, J.; Borgmann, C.; Legler, G.; Bause, E. *Arch. Biochem. Biophys.* **1986**, *248*, 335−340.

(2) Karpas, A.; Fleet, G. W. J.; Dwek, R. A.; Petursson, S.; Namgoong, S. K.; Ramsden, N. G.; Jacob, G. S.; Rademacher, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9229−9233.

(3) Krieg, N. R., Ed. *Bergey's Manual of Systematic Bacteriology*; Williams & Wilkins: Baltimore/London, 1984; Vol. 1, p 275.

(4) Kinast, G.; Schedel, M. U.S. Patent 4,246,345, 1981; Kinast, G.; Schedel, M. U.S. Patent 4,266,025, 1981; Schroeder, T.; Stube, M. U.S. Patent 4,806,650, 1989; Kinast, G.; Schedel, M.; Koebernick, W. U.S. Patent 4,405,714, 1983.

(5) Grabner, R. W.; Landis, B. H.; Wang, P. T.; Scaros, M.; Prunier, M. U.S. Patent 5,916,784, 1999.

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target pH values, and 50 mL aliquots were removed for the individual tests.

***Gluconobacter oxydans* Cells.** *Gluconobacter oxydans* (DSM 2003 or ATCC 621) cells were prepared using either batch fermentation or chemostat fermentation. In either case, the protocols were defined to ensure full differentiation and activity of the *Gluconobacter* cells.[6] The cells were cultivated in a liquid medium containing 24 g/L yeast extract (Red Star Specialty Products, Milwaukee, WI), 60−120 g/L sorbitol (Roquette, Gurnee, IL), 3 g/L succinic acid (Mallinckrodt Inc., Chesterfield, MO), and 0.5 g/L Ucon LB625 antifoam (Union Carbide Chemicals & Plastics, S. Charleston, WV). Initial pH was adjusted to 6.3 with sodium hydroxide. The three-stage fermentation batch process for production of *G. oxydans* was initiated by inoculating 1 mL stock culture into 0.5 L growth medium in a 2.8 L Fernbach. The culture was incubated in a shaker at 30 °C and 200 rpm for 24 h. The primary seed was then transferred to a secondary fermenter at either 10- or 800-L scale for 12 to 24 h. Production fermentation was carried out in a 40000-L fermenter. The final cell concentration at harvest was measured by optical density at 550 nm and was always greater than 6. The cells were isolated by centrifugation and washed with chilled 20 mM $MgSO_4$. These were used immediately or stored frozen as cell pastes or 40% suspensions in 20 mM $MgSO_4$ at −30 °C. For short periods of time (2 or 3 h), cells were also stored refrigerated.

Shake Flask Experiments. The substrate (NBG) solutions (if necessary) were incubated at the desired temperature in baffled 500-mL shake flasks. *G. oxydans* cells were added, either freshly prepared or thawed (or stored refrigerated), as a cell paste or 40% cell suspension. The target was 40 g of *G. oxydans* (wet) cell paste (approximately 8 g/L dry cell weight) per liter of NBG solution. No aeration was supplied other than that achieved by rotation at 100−120 rpm. The working volumes for shake flasks were typically 50 mL. The shake flask experiments were limited to 30 °C and ambient room temperature, generally 22−23 °C. Because of these relatively higher temperatures, degradation (vide infra) of the 6-deoxy-6-butylaminosorbose (DBS) was substantial.

Spinner Flask Experiments. Spinner flasks were used to perform bioconversions below room temperature. The solutions were incubated at the desired temperature in a 500-mL spinner flask. *G. oxydans* cells were added, either freshly prepared or thawed (or stored refrigerated), as a cell paste or 40% cell suspension. The target was 40 g of *G. oxydans* (wet) cell paste (approximately 8 g/L dry cell weight) per liter of NBG solution. The suspension was aerated at 1 vvm (vol/vol/min) by means of a gas dispersion stone and the solution agitated. The working volumes were typically 200 mL. Antifoam was added to control foaming once cells were added.

Analytical. Samples were clarified and analyzed by ion-pairing chromatography, with pulsed amperometric (PAD) detection, IEX HPLC, or GC after reduction with $NaBH_4$ and acetylation with acetic anhydride. This last method gave substantial reduction of the DBS back to NBG. If excessive sodium borohydride were used, little of the *N*-butyldeoxynojirimycin was observed. The procedure used 50 μL of sample, 50 μL of 2% NH_4OH (v/v from concentrated) to make the pH approximately 9, and 200 μL of aqueous $NaBH_4$ (5 mg/mL). After reduction for 1 h at room temperature, excess borohydride was destroyed with glacial acetic acid. After evaporation of the water, acetylation was accomplished with acetic anhydride and either pyridine or triethylamine. While the GC method gave the best resolution of products from starting materials, it suffered from variable efficiency of the chemical reduction step and could not detect the DBS directly. The IEX HPLC gave the least resolution. Overall the ion-pairing chromatography with PAD gave the best results.

Preparation of *N*-Butyldeoxynojirimycin. In the following example, the bioconversion product (6-deoxy-6-butylaminosorbose) was catalytically hydrogenated to *N*-butyl-DNJ. In a manner similar to the above, NBG solutions were prepared and bioconverted, except that the concentration of NBG was varied from 20 to 80 g/L. After 48 h, the percent conversions were all approximately 90%. The cells from each were removed by centrifugation, and aliquots of the supernatants were combined and filtered to remove all debris. This clear, light-yellow solution was decolorized with activated charcoal and placed in a Parr hydrogenator with 4% palladium on charcoal catalyst and 50 psig hydrogen at ambient temperature. After 2 h, 20 min the reduction was complete (approximately stoichiometric consumption of hydrogen), and the catalyst was filtered off. The filtrate was applied to a column of cation-exchange resin (Dowex 50 mm × 8 mm, 200 mesh), washed with water and absolute methanol; the product was then eluted in batch mode with methanol:ammonium hydroxide (3:1). The eluate was evaporated to a thick oil in vacuo and recrystallized from methanol/acetone. Yield from starting NBG to *N*-butyl-DNJ was 53%. GC of the crude oil indicated 88% purity for the *N*-butyl-DNJ. After one crystallization the purity was 99.5% by GC.

Results and Discussion

The initial phase of this project was to evaluate experimentally the concept of a simple, novel process to produce *N*-butyl-DNJ. *G. oxydans* cells had been shown to catalyze conversion of NBG to a material which could be converted to *N*-butyl-DNJ by reduction. Although the yields were low (<5%), these experiments indicated the feasibility of this bioconversion.

Three aspects of the NBG bioconversion needed to be optimized. The first was percent conversion. The percent conversion was the percent of the starting NBG which disappeared during the bioconversion reaction. The second aspect was selectivity. Selectivity was the proportion of the NBG which was converted and was recovered as DBS. Since this impacted yield, maximizing selectivity was important. The third was the rate of conversion. Early rates of conversion were on the order of a few percent per day, and the target was to complete the conversion (97%) in 24 h.

Conversion. The original conditions tried for the conversion of NBG were based on the literature.[6] It was originally believed that oxalate or succinate was required for effective

(6) White, S. A.; Claus, G. W. *J. Bacteriology* **1982**, *150*, 934−943.

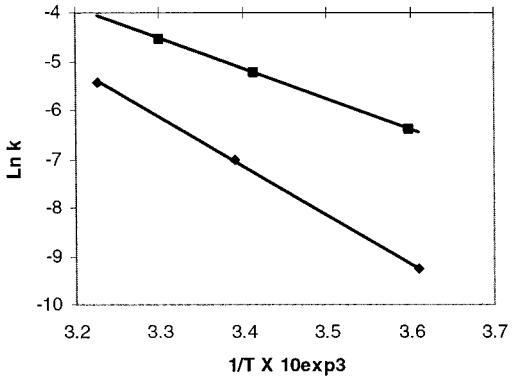

Figure 1. **Arrhenius plots for bioconversion of NBG to DBS and degradation of DBS; (■) bioconversion, (◆) degradation.**

Table 1. **Effect of acid used to titrate NBG to pH 5 on the conversion to DBS at 30 °C**

acid	% conversion
oxalic acid	30
succinic acid	60
ascorbic acid	<10
hydrochloric acid	>90

conversion of NBG. When oxalic acid was used to titrate the NBG free base to pH 5 for the bioconversion, the percent conversions at 30 °C were low (Table 1). In addition, other organic di- and triprotic acids gave poor percent conversions. However, addition of magnesium salts along with any of these organic acids improved the percent conversion. This suggested that these organic acids might have been chelating a metal ion essential for the bioconversion. When hydrochloric acid was substituted for the organic acids, in the presence of 20 mM magnesium sulfate, the conversion proceeded to at least 90%. This dramatic improvement indicated complete conversion was possible, and after removal of the magnesium salts as well and lowering the temperature (see below), conversions approaching 100% were attained.

Selectivity. The selectivities, however, were lower than desired. It was observed during the bioconversions that the absorbance at 280 nm increased dramatically. It was believed that this UV absorbance increase was due to degradation (via piperidinose intermediates) to a pyridine derivative. Since the degradation reaction was not an enzyme-catalyzed reaction, and was independent of cells, it was proposed that its temperature-dependence would be greater than that of the biochemical oxidation. In Figure 1 are superimposed Arrhenius plots for the degradation and bioconversion reactions. The temperature dependence of the degradation was steep, with an energy of activation of about 20 kcal/mol. The temperature dependence of the bioconversion was clearly much shallower than that of the degradation. This indicated that a significant lessening of the degradation could be realized by lowering the temperature a few degrees without greatly diminishing the rate of conversion. When shake-flask experiments were performed at room temperature rather than 30 °C, the selectivity increased from less than 70% to about 85−90%. At lower temperatures, the selectivity increased a little more. The temperature giving the best balance of rate

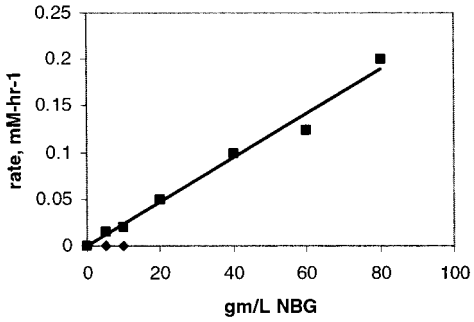

Figure 2. **Rate of bioconversion of NBG to DBS as a function of NBG concentration.**

Table 2. **Effect of counterion on selectivity of the bioconversion of NBG to DBS at room temperature**

counterion	% conversion	% selectivity[a]
chloride	>99	97
nitrate	>99	>99
sulfate	30	>99
phosphate	60	70
acetate	75	50

[a] Sum of furanose and piperidinose forms.

of conversion and selectivity was in the range of 12−15 °C. However, shake-flask experiments were still performed at room temperature.

The effect of other acids (counterions) was also examined at room temperature (Table 2). In these tests it was observed that titrating the NBG free base with nitric acid to pH 5 gave essentially the same results in terms of conversion and selectivity as using hydrochloric acid. However, titrating with sulfuric acid, while giving good selectivity, seemed to inhibit the bioconversion, the percent conversion proceeding to only a few percent. Phosphoric acid and acetic acid gave somewhat better conversions than sulfuric acid, but the selectivities were much diminished. With these two counterions the degradation increased, suggesting that they promoted formation of the piperidinose structure of DBS (vide infra).

Rate of Conversion. With the percent conversion and selectivity improved, it was possible to investigate the cause of the slow conversion rate. Relative to the published rates of conversion for sorbitol[6,7] and carbobenzyloxyglucamine, the rate of conversion of NBG was much slower. In Figure 2 are shown initial rates of conversion for NBG as a function of substrate concentration. In Figure 2 of ref 6, the profile for sorbitol had the appearance of an enzyme-catalyzed reaction, giving saturation at high substrate concentrations and half-maximal rate at about 0.1 M. The concentration dependence for NBG, on the other hand, was first order, with no apparent saturation. For simple enzyme-catalyzed reactions, the region where first-order kinetics is obeyed is where the substrate concentration is much less than the enzyme−substrate dissociation constant. This suggested that this dissociation constant was considerably increased for NBG relative to that for sorbitol, probably due to the positive charge on the amino group of NBG at pH 5. To give rates

(7) Yang, F.-L.; Wu, S.-H. *J. Chin. Chem. Soc.* **1999**, *46*, 707−714, reported K_m for sorbitol of 19 mM.

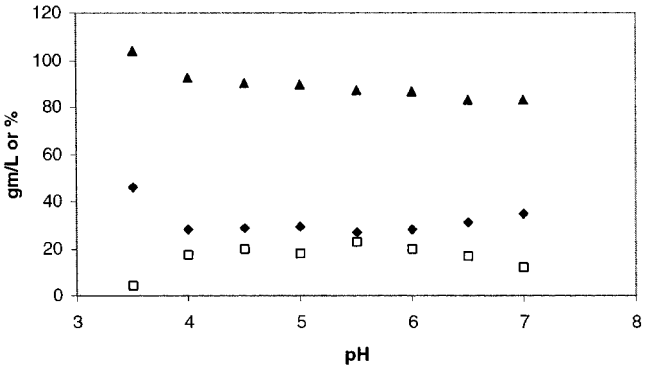

Figure 3. **Effect of pH on the bioconversion of NBG to DBS; 24 h incubation with *G. oxydans*; (□) DBS concentration; (▲) % selectivity; (◆) NBG concentration.**

sufficient to complete the bioconversion in 24 h, the levels of *G. oxydans* added to the shake flasks and bioreactors had to be severalfold higher than the levels achieved in a typical *G. oxydans* fermentation. A second contribution to increasing the rate was ensuring that fermentations to provide *G. oxydans* cell mass reached late log-phase growth. It had been noted by Claus and co-workers[6] that the membrane-bound sorbitol dehydrogenase increased at that point (due to membrane invagination). The qualification that cells be grown to late log phase was incorporated as part of the cell mass fermentation protocols.

pH Dependence. The pH dependence of the bioconversion is shown in Figure 3. The overall shape of the pH-rate profile was similar to that for sorbitol,[6,7] with a broad maximum centering on pH 5.5−6.0. Although the curves were similar, there was a fundamental difference between sorbitol and NBG. That is, the oxidized product for sorbitol (sorbose) was stable, whereas that for NBG had been shown to decompose. As the pH was increased there was a decrease in selectivity, most likely caused by increased degradation as the proportion of piperidinose form of DBS increased (see below). The selectivity decreased with increasing pH, the average linear decrease in selectivity being 3−4% per pH unit. Although there did appear to be some increase in rate to be gained by increasing the pH of the bioconversion somewhat above pH 5.0, this was offset by the increased degradation. While lowering the pH below 5.0 might have benefited the selectivity slightly, the rate decreased enough to render this insubstantial. For these reasons the pH was maintained at 5.0.

Structure of DBS. Structures for the bioconversion are shown in Figure 4. NBG was oxidized by *G. oxydans* to DBS (structure A). However, neither ^{13}C NMR nor infrared spectroscopy indicated the presence of significant amounts of carbonyl (data not shown). Rather, the molecule cyclized through the 5-hydroxyl to form the sorbofuranose, structure B. At alkaline pH, chromatographic evidence suggested the presence of a second structure, probably a piperidinose structure such as structure C. Since the DBS could be reduced at pH 5 by H_2 with Pd/C catalyst to *N*-butyl-DNJ, at least some portion of the DBS had to exist in this form, the reduction going to completion as the equilibria generated additional piperidinose. It was this piperidinose, however, which led to degradation, forming an aromatic product.

Effect of the Butyl Group on Stability. In Figure 5 are compared bioconversions of glucamine and NBG. As the bioconversion of NBG proceeded, the correctly oxidized product was obtained in high yield. As the bioconversion of glucamine proceeded, little, if any, of the desired oxidation product was obtained. After lyophilization and reconstitution of the oxidized products from each, the UV absorbance of the glucamine bioconversion was more than twelve times greater than that of NBG bioconversion. This suggested that the butyl group was stabilizing the oxidized product relative to the glucamine system.

The butyl group stabilized the DBS relative to aminosorbose in two ways. First, the butyl group made the amino nitrogen a stronger base. Titration curves for NBG, glucamine and DBS are shown in Figure 6. The butyl group increased the pK_a of the amino group from 9.23 ($K_a = 5.95 \pm 0.22 \times 10^{-10}$) to 9.59 ($K_a = 2.59 \pm 0.16 \times 10^{-10}$). Titration of isolated DBS indicated a pK_a of 8.16. While the pK_a of the 6-deoxy-6-aminosorbose could not be determined, it was not unreasonable to believe that a similar increase in the pK_a due to the butyl group occurred for the oxidized products as with the starting substrates, projecting a pK_a for the 6-deoxy-6-aminosorbose of about 7.8. Since the stronger base would be more highly protonated at the mildly acidic pH of the bioconversion, there would be less nucleophilic amine to compete with the hydroxyl for the carbonyl. This would shift the equilibria shown in Figure 4 more in the direction of the sorbofuranose for the DBS system than for the aminosorbose system. The other contribution from the butyl group was steric interference. The butyl group will provide some steric crowding around the nitrogen, with the result that attack at the carbonyl by the nitrogen will be inhibited. This would again shift the equilibria in favor of the sorbofuranose for the butylaminosorbose system relative to the aminosorbose system. By shifting the equilibria toward the stable sorbofuranose structure, less piperidinose would be formed, with the result that the degradation rate would be slowed. This in turn led to the bioconversion results shown in Figure 5.

In the literature there have been numerous attempts to quantify electronic and steric effects of substituents.[8,9] According to Fujita and co-workers,[10] equilibrium constants for reactions of amines (hydrogen bonding with $CHCl_3$, charge transfer with I_2 plus ester reactions) might be described by

$$\log K = \rho^* \sum \sigma^* + \sum a_i E_s^c(R_i) + c$$

where K was the equilibrium constant, σ^* was an electronic coefficient, E_s^c was the steric coefficient and ρ^* and a_i were constants of the particular reaction. For unsubstituted amines σ^* was 0.490, for *n*-butylamine it was −0.130; for unsubstituted amines E_s^c was 0.32, for *n*-butylamine it was −0.70.

(8) Gallo, R. *Progress in Physical Organic Chemistry*; John Wiley & Sons: New York, 1983; Vol. 14, pp 115−1163 and references therein.
(9) Taft, R. W., Jr. In *Steric Effects in Organic Chemistry*; Newman, M. S., Ed.; Wiley: New York, 1956; pp 556 and ff.
(10) (a) Takayama, C.; Fujita, T.; Nakajima, M. *J. Org. Chem.* **1979**, *44*, 2871−2879. (b) Bogatkow, S. V. et al. *Org. React. USSR* **1969**, *6*, 436, cited by Takayama in ref 10a.

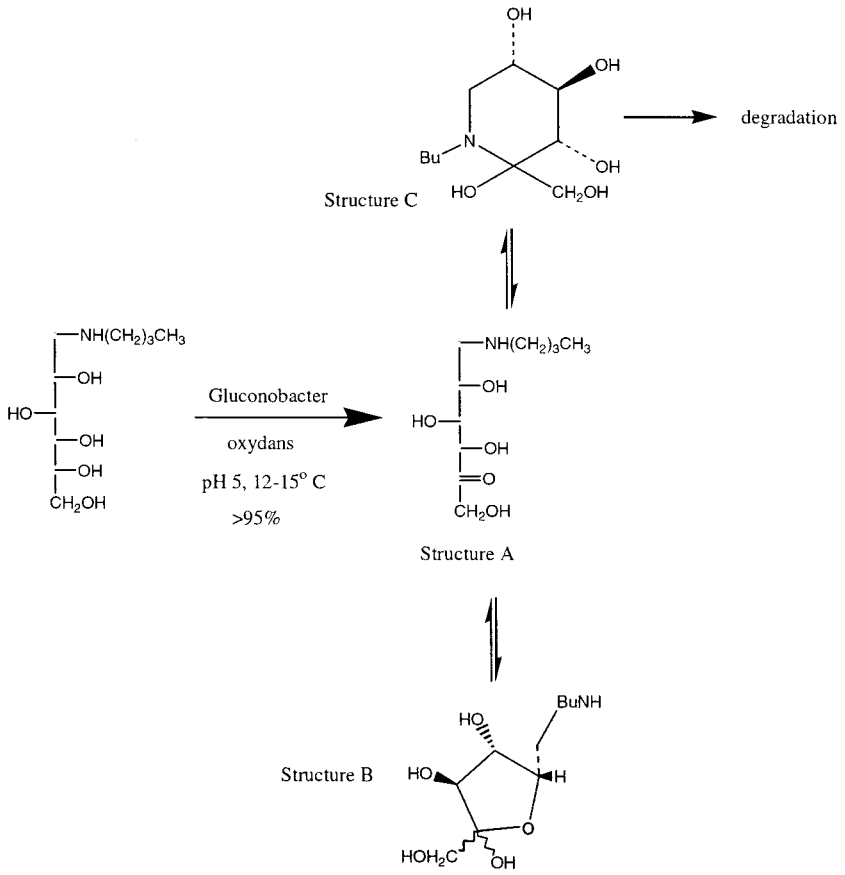

Figure 4. **Equilibria proposed for the bioconversion of NBG to DBS.**

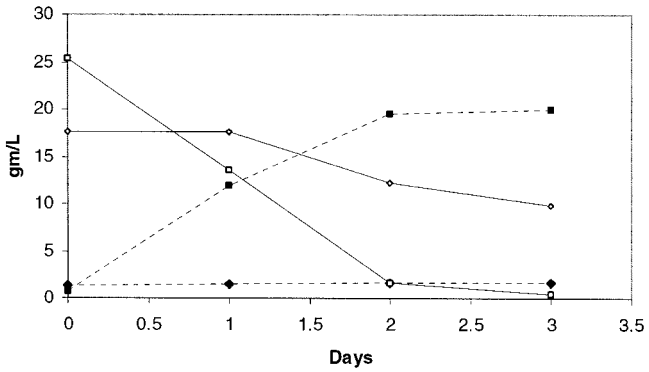

Figure 5. **Appearance of sorbose products as a function of time during bioconversion of the polyols at room temperature; (■) DBS concentration; (□) NBG concentration; (◇) glucamine concentration; (◆) aminosorbose concentration.**

Relative to the unsubstituted glucamine system, these coefficients predicted that the equilibrium constant for *n*-butylglucamine system should be 4-fold lower due to electronic effects (compare 2.3-fold for the change in pK_a) and 10-fold lower due to steric effects. Overall, this predicted 40−45-fold lower equilibrium constant for the *n*-butyl derivative than for the unsubstituted compound. This predicted large decrease in the equilibrium constant for attack of the basic amino nitrogen on the carbonyl carbon (C-5) was consistent with the increased stability of the bioconversion product obtained from NBG relative to that obtained from glucamine and the equilibria shown in Figure 4.

Scale-Up. The process was scaled up to produce material for clinical trials at a toll manufacturer, Abbott Laboratories,

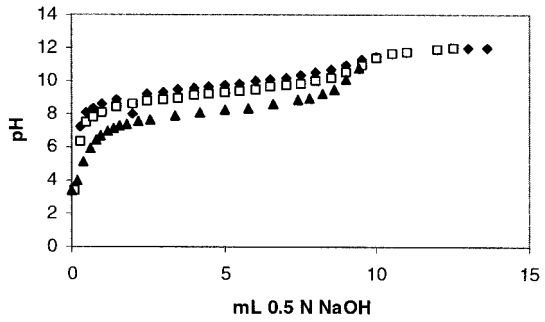

Figure 6. **Titration of NBG, DBS and glucamine; (◆) NBG titration; (□) glucamine titration; (▲) DBS concentration.**

Inc., North Chicago, Illinois. The scaled process utilized a 40000-L fermenter to produce the *G. oxydans* cells in batch fermentation. The cells were concentrated with BTUX 510 continuous discharge disc-stack centrifuge to 2000 L and washed with 40 000 L of 20 mM $MgSO_4$. The washed cells were reconcentrated and stored under refrigeration until used for the bioconversion. The bioconversion (Figure 7) was performed at approximately 5500-L scale at 220 g/L NBG, 14 g/L dry cell weight (dcw) *G. oxydans*. The bioconversion was completed in 48 h yielding over 700 kg of DBS.

Conclusions

By including one regioselective bioconversion step, it was possible to simplify synthesis of *N*-butyldeoxynojirimycin and utilize inexpensive starting materials. The key to implementing this technology, however, was the realization of the contribution of competing reactions and the relative

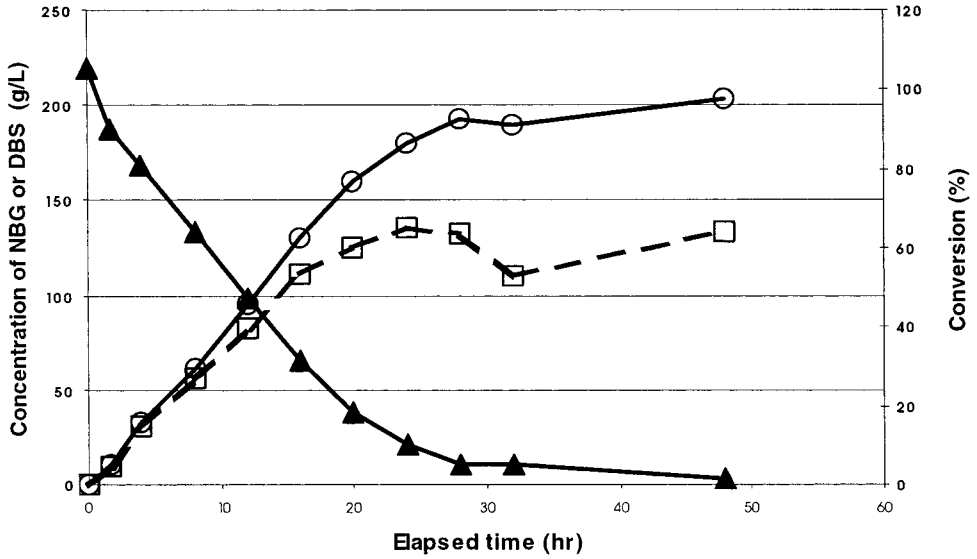

Figure 7. **Bioconversion of NBG to DBS at 5500-L scale, 15 °C, 220 g/L NBG; (○) % conversion; (□) DBS concentration; (▲) NBG concentration.**

energies of activation of the two reactions. Significant enhancement of the selectivity was therefore achieved by lowering the temperature at which the bioconversion was run. The optimum temperature for this system was 12−15 °C. The kinetics of the bioconversion appeared to be first-order, suggesting that the introduction of the charged butylammonium group (at pH 5) increased the K_m for this substrate. The pH maximum of the bioconversion rate was 5.5−6.0. However, the small gain in rate relative to pH 5.0 was at least off-set by the increase in degradation of the 6-deoxy-6-butylaminosorbose at the higher pH. It appeared that nitrate salts of *N*-butylglucamine could replace chloride salts, but sulfate, acetate, and phosphate salts could not.

Acknowledgment

J. Scanlon, who labored long and hard to optimize ion-pair analytical chromatography for this biooxidation, provided analytical support. ^{1}H NMR and its interpretation was accomplished by P. Finegan. Synthesis of *N*-butylglucamine was carried out by M. Scaros, M. Prunier and co-workers. The assistance of all of these individuals greatly facilitated this effort. We are grateful for their assistance.

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