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The formation of daptomycin by supplying decanoic acid to *Streptomyces roseosporus* cultures producing the antibiotic complex A21978C

F.M. Huber, R.L. Pieper and A.J. Tietz

Antibiotic Development Division, Eli Lilly and Company, Indianapolis, Indiana, U.S.A.

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Summary

Antibiotic substance A21978C is a complex of compounds having a common cyclic polypeptide nucleus and different fatty acid sidechains. Daptomycin is a semi-synthetic antimicrobial substance derived from the A21978C complex by a very elaborate chemical process. To obtain the daptomycin, the A21978C complex was first isolated from culture filtrates of *Streptomyces roseosporus* by several resin procedures. The resulting material was then 'blocked' and added to an *Actinoplanes* culture for deacylation. The protected A21978C nucleus was subsequently isolated by the same procedure as the parent complex and reacylated with the desired fatty acid (decanoic acid). The acylated compound was deblocked to yield daptomycin. This report describes the experimentation undertaken to establish a strategy to supply a very toxic precursor to cultures of *S. roseosporus* and, thereby, produce daptomycin biosynthetically.

Daptomycin; A21978C; Precursing

Introduction

The production of the antibiotic complex A21978C, by *Streptomyces roseosporus* NRRL11379 was originally described by Hamill and Hoehn (1980) and shown to be

Correspondence to: F.M. Huber, Antibiotic Development Division, Eli Lilly and Company, Indianapolis, Indiana, U.S.A.

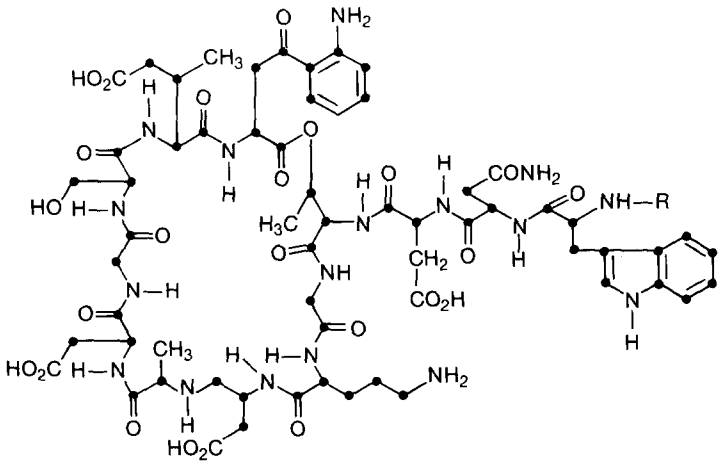

Fig. 1. Basic structure of the A21978C complex.

highly active against Gram-positive bacteria. This complex of compounds was demonstrated to have a common cyclic peptide nucleus (Fig. 1) with different fatty acid acyl groups attached by an *N*-acyl bond (Debono et al., 1980, 1984).

Subsequent to the discovery of the natural A21978C antibiotic complex, it was found that substitution of the naturally occurring fatty acid sidechains with decanoic acid resulted in a compound of superior biological activity (Fukuda et al., 1984; Counter et al., 1984). The latter substance was designated compound LY146032 and then later renamed daptomycin. In order to obtain daptomycin, two biological and three different recovery procedures were required. The processes involved culturing *S. roseosporus* in primary, secondary and tertiary inoculum development stages for 96 h and then in the producing stage for 140 h. The microorganism was separated from the soluble portion of the culture fluid by filtration. The antibiotic complex was adsorbed and eluted from a resin and concentrated. The free amino function on the complex was 'blocked' with di-*tert*-butyl dicarbonate and the mixture again concentrated. *Actinoplanes utahensis* was cultured for 120 h in primary and secondary inoculum development stages. The culture was further grown for 72 h in a stirred reactor and then the concentrated 'blocked' complex was added to the medium for deacylation. After 24 h the spent medium was filtered and the 'blocked' nucleus was adsorbed and eluted from a resin column. The eluate was concentrated, the 'blocked' nucleus was acylated with either the anhydride or halide of decanoic acid and then the protecting group was removed by hydrolysis. The final product was adsorbed and eluted from a resin column and subjected to final purification. In addition to low product concentration, the recovery yields were extremely low. If daptomycin could be made biosynthetically, most of the recovery procedures would be eliminated and overall process yields increased significantly. This report describes a study that resulted in both increased antibiotic concentrations and the biosynthetic production of daptomycin.

Materials and Methods

The organism used in this study was a mutant derived from *S. roseosporus* NRRL 11379 and designated A21978.65. The inoculum development stages prior to the final reactor have been previously described (Huber et al., 1987). The medium used for the production of the A21978C antibiotic complex contained soybean flour (2.0%, w/v), $FeSO_4 \cdot 7H_2O$ (0.06%), dextrose (0.75%), potato dextrin (3.0%, w/v), cane molasses (0.25%, w/v), SAG471 silicone antifoam (0.02%, w/v) and water. The medium was adjusted to pH 7.0 with NaOH and sterilized for 45 min at 121° C in a stirred reactor. The reactor was made of 304 stainless steel with an operating volume of 120 l. The reactor had four equally spaced vertical baffles, two impellers with six open turbine blades, and a single open-tube sparger. The reactor was inoculated with 400 ml of the secondary inoculum development stage. The culture was then agitated at 200–400 rpm, aerated a 1 v/v/m and maintained at 30° C.

Biological mass was estimated by mycelial volume and dry weight. Mycelial volume (MV) was determined by centrifuging a known amount of medium at $1200 \times g$ for 10 min and noting the volume of the insolubles in relationship to the total volume of the sample. The dry weight of the mass was estimated by transferring the pellet in distilled water to a tared aluminum weight boat and drying to constant weight at 29° C. Respiratory activity was estimated by analyzing the exit gas stream from the reactors by a Perkin Elmer mass spectrometer.

Glucose was determined by the Trinder method using glucose oxidase/peroxidase (Biodynamics/bmc, Indianapolis, IN). Total carbohydrate was estimated by the anthrone method (Morris, 1948). Inorganic phosphate and ammonia nitrogen were automatically determined by the 'Industrial Method No. 93-70W' (Technicon Industrial Systems, Tarrytown, New York).

The concentration of the various components of the A21978C complex were estimated by HPLC. The analytical HPLC column was from ES Industries (C18, 10 cm × 4.6 mm). The mobile phase consisted of 1% ammonium dihydrogen phosphate and acetonitrile (64 : 36, v/v).

Results and Discussion

The data presented in Fig. 2 and Table 1 are characteristic of the initial experiments with *S. roseosporus*. The A21978C complex increased with time, but no daptomycin was formed. Oxygen demand reached a maximum at 30 h and then decreased. It was possible that although there was a relatively high concentration of polymeric carbohydrate in the broth, some of that carbon might not be readily available to the microorganism. Thus, more dextrin 700 (1%) was added at 73 h and a slight increase in oxygen demand was observed. After 75 h the oxygen demand decayed further to a steady level at approximately 100 h. Much like the oxygen demand pattern, the mycelial volume also decreased significantly after a maximum had occurred early in the process. It was possible that only the cellular system for catabolizing carbohydrates was decaying. If that was true, the culture could still

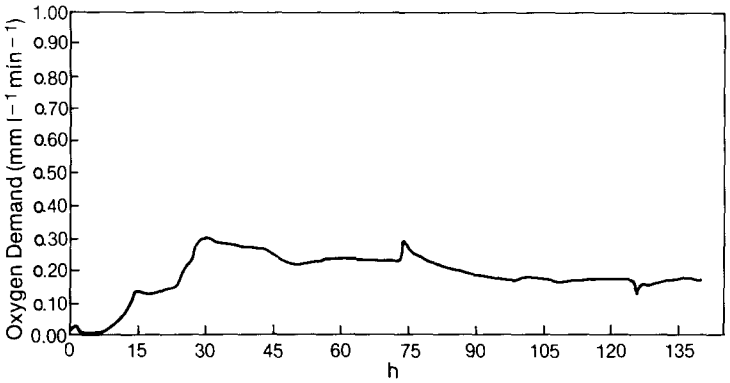

Fig. 2. Oxygen demand in original complex medium.

readily catabolize other substances (i.e. lipids, amino acids). If such a substance was toxic when in excess, it could be fed at a rate not to exceed the maximum rate for its oxidation. Under those conditions the toxic substance would never accumulate in the culture. Decanoic acid, a very toxic fatty acid, is the desired sidechain for the production of daptomycin. If decanoic acid was supplied to *S. roseosporus* so as not to accumulate, it might be possible to 'precurse' the A21978C complex and produce daptomycin biosynthetically. The latter achievement would save much processing chemistry. Some antibiotics have been successfully 'precursed' (Higuchi et al., 1946; Moyer and Coghill, 1947; Wolf and Arnstein, 1960).

Decanoic acid is a solid at the temperature of cultivation (30 °C). In order to conveniently add decanoic acid to the stirred reactor, a metabolizable solvent was sought. The original solution was a 5% solution of decanoic acid in an equal mixture of ethanol and water. The resulting solution was fed to the culture at 50 ml h^{-1} beginning at 40 h post inoculation. The information gained in these experiments is presented in Fig. 3 and Tables 2 and 3. The data clearly indicate that daptomycin could be produced biosynthetically. The concentration of daptomycin in the

TABLE 1

CHARACTERISTICS OF CULTURES OF *S. roseosporus* WITH 1% DEXTRIN 700 ADDED AT 73 h

h	pH	MV (%)	Total carbohydrate (mg ml^{-1})	Glucose (mg ml^{-1})	PO_4-P ($\mu g\ ml^{-1}$)	NH_3-N ($\mu g\ ml^{-1}$)	A21978C complex ($\mu g\ ml^{-1}$)
0	6.5	6.0	48	6	14.0	63	
18	5.5	8.7	44	5	2.0	10	
42	7.1	20.0	29	0	0.4	203	
66	6.6	18.7	24	0	11.0	146	293
90	6.0	13.3	29	0	11.0	104	347
114	5.9	10.6	25	0	5.0	100	387
138	5.9	11.3	13	0	3.0	100	458

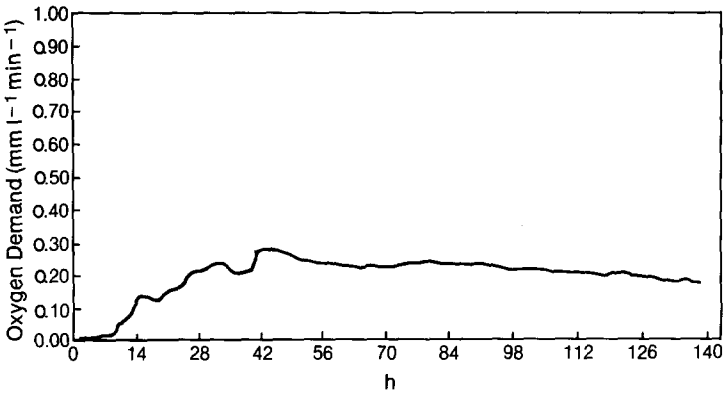

Fig. 3. Changes in oxygen consumption during the initial decanoic acid supplemented culture.

A21978C complex approximated 34%. The time the feed was initiated was clearly defined by increased oxygen demand. That the oxygen demand continued to decrease during the remainder of the process suggested that further increases in feed rates were necessary. The decrease in oxygen demand prior to the feed indicated

TABLE 2

MEDIUM CHARACTERISTICS DURING INITIAL DECANOIC ACID FED A21978C PROCESS

h	pH	MV (%)	Total CH_2O (mg ml^{-1})	Glucose (mg ml^{-1})	PO_4-P ($\mu g\ ml^{-1}$)	NH_3-N ($\mu g\ ml^{-1}$)	Total A21978C-like substance ($\mu g\ ml^{-1}$)
0	6.3	7	44	7	19	71	
16	5.9	10	40	5	3	32	
40	7.35	19	34	0	1	210	
64	6.9	12	29	1	11	204	200
88	6.3	11	27	3	10	156	306
112	6.2	10	24	6	4	137	399
136	6.0	8	30	9	4	142	364

TABLE 3

FACTOR COMPOSITION AND CYCLE TIME DURING INITIAL DECANOIC ACID FED PROCESS

h	Factors ($\mu g\ ml^{-1}$)				
	C_1	C_2	C_5	C_3	LY146032
64	60	71	0	33	36
88	73	105	16	40	72
112	82	133	22	54	108
136	72	109	19	42	122

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