KIBDELINS†, NOVEL GLYCOPEPTIDE ANTIBIOTICS

I. DISCOVERY, PRODUCTION, AND BIOLOGICAL EVALUATION

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A new subspecies of *Kibdelosporangium aridum* subsp. *largum* (SK&F AAD-609), was isolated and shown to produce novel glycopeptides related to aridicins, but containing a homologous series of glycolipids based on *N*-acylglucosamine. These compounds showed improvements over the aridicins in *in vitro* activity and were effective in mouse protection studies against a range of Gram-positive bacteria, including methicillin resistant staphylococci. Pharmacokinetic studies indicated that they have high serum concentrations and long-acting potential. The kibdelin complex modified rumen metabolism in a manner favorable for growth promotion.

Previously, novel glycopeptides, aridicins, were discovered as products of a new genus of *Actinomycetales*, *Kibdelosporangium aridum*[1,2]. Subsequently, other members of this genus were isolated and investigated for the production of cell wall-active antibiotics. A number of strains were shown to produce glycopeptide antibiotics[3]. In this paper we shall describe the discovery, production and biological evaluation of a new series of lipid containing glycopeptides from one of these strains[4,5].

Materials and Methods

Strain

SK&F AAD-609 was isolated from a desert soil sample collected in Pima County, Arizona. Stock cultures for taxonomy studies were grown on slants of thin potato - carrot agar or oatmeal agar (ISP 3). Morphological observations were made on plates of thin potato - carrot agar, oatmeal agar, water agar or soil extract agar. Aliquots for the taxonomic work were stored at −70°C while production lots were stored in the vapor phase of liquid nitrogen. The initial inoculum for kibdelins production was grown on slants of oatmeal agar medium (Difco ISP 3).

Chemotaxonomy, Physiological and Biochemical Characteristics

The physiological and biochemical tests used to characterize SK&F AAD-609 were those of GORDON[6,7] and GORDON and MIHM[8]. Inoculum for these tests was prepared by adding the contents of a frozen vial to a flask of glucose - yeast extract broth which was incubated at 28°C on a rotary shaker, at 250 rpm, for 3 to 5 days. The culture was harvested by centrifugation and washed three times with sterile, distilled water. All biochemical and physiological tests were incubated at 28°C. Readings of the results were made at various times up to 21 days for the plate media. Most of the tubed media were read at intervals up to 28 days. However, tests for decomposition of urea, allantoin and hippurate, as well as the tests for reduction of nitrates, were read after six weeks.

Whole-cell hydrolysates were analyzed by the method of LECHEVALIER[9]. Pure cell wall preparations were analyzed by the method of BECKER *et al.*[10]. Cell extracts were examined for mycolates and phospholipids by the methods of LECHEVALIER *et al.*[11,12].

† Kibdelins are originally designated as AAD-609 A, B, C, D and E.

Antimicrobial Activity

The minimum inhibitory concentrations (MICs, μg/ml) for aerobic bacteria were determined by microtiter broth dilutions tests using Dynatech MIC-2000 equipment. The growth medium was Trypticase soy broth, pH 7.0 and the inoculum size was approximately 10^5 cfu/ml. The microtiter plates were incubated at 37°C overnight. The MICs for *Clostridium difficile* were determined by agar dilution tests in Wilkins Chalgren agar. The inoculum was applied directly to the surface of the agar with a Steers replicator. The tests were incubated at 37°C for 48 hours in a Capco anaerobic chamber in an atmosphere of 88% N_2, 7% H_2 and 5% CO_2. Control compounds were commercial preparations.

In mouse protection studies, the growth from an 18 hours-Trypticase soy agar slant of *Staphylococcus aureus* HH 127 was diluted in 5% hog gastric mucin to a level of 3.0×10^7 cfu per ml. This inoculum, 0.5 ml/mouse, was injected intraperitoneally to produce a uniformly lethal mouse infection in 18~21 g Webster-derived CD1 male mice (Charles River Laboratories). The test antibiotics were administered subcutaneously at 1 and 5 hours after infection. The final percentage of survival for groups of 10 mice each, obtained after 3 days of observation, were used to estimate the 50% effective dose (ED_{50}, mg/kg) and the 50% lethal dose (LD_{50}, cfu/mouse) values.

Pharmacokinetic Studies

Strain CD-1 mice were obtained from Charles River Laboratories. They were fed mouse pellets from Ralston Purina and permitted free access to H_2O. Animals were fasted 18~24 hours before use. Mice were injected intravenously, *via* the tail vein and blood was obtained from pools of 8 to 10 mice at selected intervals. Serum was obtained after clot formation and retraction, and urine was collected from animals held in metabolism cages. All samples were held frozen until assayed. Antibiotic content of samples was evaluated using a disc-agar diffusion assay in penicillin-assay seed agar and employing a *Bacillus subtilis* ATCC 6633 spore suspension as the indicator. Serum samples were evaluated against standards prepared in mouse serum while urine was evaluated against standards prepared in 1% pH 6.0 phosphate buffer. Assays were incubated overnight at 30°C.

Time-serum concentration profiles obtained from mice dosed intravenously were analyzed on a HP-1000 computer using "Pharm" pharmacokinetics data analysis software. The terminal half-life of elimination was estimated by unweighted linear regression analysis. The peak serum concentration was taken from the time-serum concentration profiles. All compounds were administered at 20 mg per kilogram of body weight.

Growth Promotion Profile

Modification of the metabolism of microflora from rumenant and monogastric livestock was determined by methods previously described[13,14].

Results

Morphological and Cultural Characteristics

SK&F AAD-609 is a Gram-positive, non-acid-fast, filamentous organism that formed a mycelium differentiated into 1) a substrate mycelium that penetrated the agar, and 2) an aerial mycelium which originated from the substrate mycelium and bore chains of conidial and/or sporangium-like structures (Figs. 1 and 2). No motile elements were observed in either the aerial or substrate mycelium.

The substrate mycelium was well developed and exhibited a tendency to fragment without hyphal displacement. The long, moderately branching hyphae were septate and 0.5~1.0 μm in diameter. Present on the substrate hyphae were specialized structures which consisted of dichotomously branched, septate hyphae radiating from a common stalk. These structures were produced either deep in the agar or just below the surface of the agar and appeared to be "naked" sporangium-like structures analogous to the conidial structures which COUCH[15] described in the *Actinoplanaceae*. On many media SK&F AAD-609 produced characteristic crystals in the agar.

The aerial mycelium of SK&F AAD-609 produced straight or irregularly curved chains of rod

Fig. 1. Transmission electron micrograph of spore chains SK&F AAD-609 (3 week-old culture on thin-Pablum agar).
Bar: 1 μm.

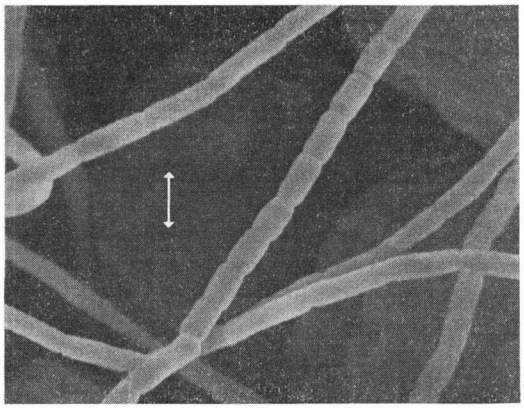
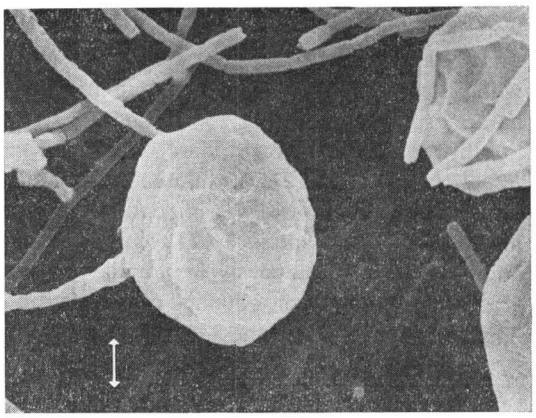


Fig. 2. Transmission electron micrograph of sporangium-like structures of strain SK&F AAD-609 (3 week-old culture on thin-Pablum agar).
Bar: 2 μm.



shaped, smooth-walled spores which tended to be irregular in length (0.5 μm $\times$ 0.8 $\sim$ 3.2 μm). These spore chains were usually very long with more than 50 spores per chain, but a few short chains of ten spores or less were also usually present. The spore chains were borne apically on the main thread or terminally on lateral branches. When placed on agar these spores germinated with the production of one or more germ tubes. On most media, the aerial mycelium of SK&F AAD-609 also produced sporangium-like structures. These were borne apically on branched or unbranched hyphae; they were also borne terminally on lateral branches of the main hyphal thread. Mature sporangium-like structures were usually round, approximately 12 $\sim$ 32 μm in diameter. Sporangium-like structures slightly flattened in one axis or very irregularly shaped were observed. The sporangium-like structures were surrounded by a well-defined wall and contained septate, branched hyphae embedded in an amorphous matrix. When placed on agar, these sporangium-like structures germinated directly with the production of one or more germ tubes.

Plates for the determination of cultural characteristics of SK&F AAD-609 were incubated at 28°C in closed petri dish cans and observed at 7, 17 and 21 days. The growth characteristics of SK&F AAD-609 on various media are presented in Table 1. On all media tested, the substrate mycelium of SK&F AAD-609 was off-white to grayish brown. The aerial mycelium was white to light gray and sparse to moderate in amount. No pigments, other than melanin or yellow-brown soluble pigments were produced.

Chemotaxonomy, Physiological and Biochemical Characteristics

Pure cell walls contained *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, galactose and a minor amount of arabinose and a trace of madurose. No mycolic acids of any type were present in the cell extracts. Phospholipids present were phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl inositol mannosides and diphosphatidyl glycerol. Thus SK&F AAD-609 has a type IV cell wall with a type A sugar pattern[16] plus traces of madurose and a phospholipid pattern of type PII[12]. SK&F AAD-609 did not grow under anaerobic conditions. Temperature range for growth was 15°C to 42°C with a trace of growth at 45°C. The following tests were positive: Hydrogen sulfide production; milk peptonization; melanin production; gelatin hydrolysis and

Table 1. Growth characteristics of SK&F AAD-609 on different media.

Medium		Growth and morphology
Yeast extract - malt extract agar (ISP 2)	G:	Good to excellent, raised, yellow-brown
	AM:	None to very sparse, white
	SP:	Yellow-brown
	SPL:	Sparse
	SC:	Sparse
	C:	Present
Oatmeal agar (ISP 3)	G:	Fair to good, slightly raised, off-white to pale yellow-brown
	AM:	Moderate, white
	SP:	Variably present, pale yellow-brown
	SPL:	Abundant
	SC:	Abundant
	C:	Present
Inorganic salts - starch agar (ISP 4)	G:	Good, slightly raised, off-white to pale yellow-brown
	AM:	None to sparse, white
	SP:	Variably present, pale yellow-brown
	SPL:	Moderate
	SC:	Moderate
	C:	Present
Glycerol - asparagine agar (ISP 5)	G:	Fair to good, raised, off-white to pale yellow-brown
	AM:	None to sparse, white
	SP:	Pale yellow-brown
	SPL:	None to abundant
	SC:	None to abundant
	C:	Present
Peptone - yeast extract - iron agar (ISP 6)	G:	Good, raised, grayish-brown
	AM:	None
	SP:	Brownish-black
	SPL:	None
	SC:	None
	C:	None detected
Thin potato - carrot agar	G:	Fair, slightly raised, off-white to pale yellow-brown
	AM:	Sparse to moderate, white to light gray
	SP:	Variably present, pale yellow-brown
	SPL:	Numerous
	SC:	Numerous
	C:	Variably present
Soil extract agar	G:	Fair, slightly raised, off-white to pale yellow-brown
	AM:	Sparse to moderate, white to light gray
	SP:	None
	SPL:	Moderate to abundant
	SC:	Moderate to abundant
	C:	None detected
Nutrient agar	G:	Fair, raised, grayish yellow-brown
	AM:	Sparse, white, sterile
	SP:	Yellow-brown
	SPL:	None
	SC:	None
	C:	None detected
Bennett agar	G:	Fair to good, raised, off-white to grayish yellow-brown
	AM:	None to sparse, white
	SP:	Grayish yellow-brown
	SPL:	None to moderate
	SC:	None to moderate
	C:	Variably present
Water agar	G:	Poor, relatively flat, translucent to off-white
	AM:	Sparse to moderate, white to light gray
	SP:	None
	SPL:	Moderate to numerous
	SC:	Moderate to numerous
	C:	None detected

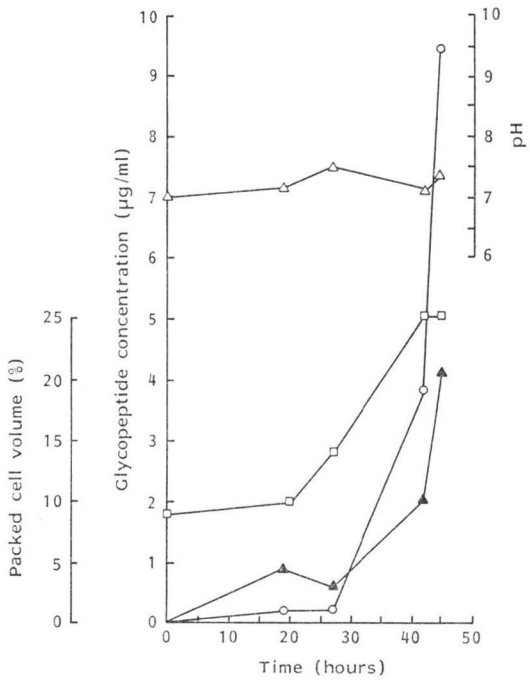
G=Growth of substrate mycelium; AM=aerial mycelium; SP=soluble pigment; SPL=sporangium-like structures; SC=spore chains; C=crystals.

liquefaction; hydrolysis of casein, L-tyrosine, hypoxanthine, guanine, elastin, urea, esculin and hippurate; catalase and phosphatase production. Results were negative for hydrolysis of starch, adenine, xanthine and cellulose (Avicel). Tests for allantoin decomposition were weakly positive. Nitrate reduction to nitrite was doubtful. No growth occurred in lysozyme broth. Growth in 2% NaCl was consistently positive; no growth occurred in 8% NaCl while growth in 3% to 7% NaCl was inconsistent.

Acid was produced from L-arabinose, D-cellobiose, dextrin, glucose, D-fructose, glycerol, glycogen, D-galactose, *iso*-inositol, lactose, D-mannitol, D-mannose, maltose, α-methyl-D-mannoside, melibiose, D-melezitose, raffinose, rhamnose, D-ribose, sucrose, trehalose and D-xylose. No acid was produced from dulcitol, *iso*-erythritol, inulin or L-sorbose and salicin was variable. Citrate, malate, succinate, oxalate, lactate, acetate, pyruvate, formate and propionate were utilized; benzoate and tartrate were not.

Fig. 3. Fermentation profile of kibdelins and aridicins production by strain SK&F AAD-609.

○ Total kibdelins concentration (μg/ml), ▲ total aridicins concentration (μg/ml), □ packed cell volume (%), △ pH.



Identification and Classification

A comparison of the description of SK&F AAD-609 with the actinomycete genera listed in BERGEY'S Manual of Determinative Bacteriology[17], the approved lists of bacterial names[18] and other recent taxonomic literature indicated that it did not belong to any of the genera described therein. SK&F AAD-609 belongs to the genus *Kibdelosporangium* described to accommodate the aridicin producer[1]. It was compared directly with the one species previously described in this genus, *K. aridum* (ATCC 39323) and found to differ from it only in minor morphological and chemotaxonomic characteristics.

Some of the differences between SK&F AAD-609 and *K. aridum* may be attributed to vigor, *i.e.*, the aerial mycelium of SK&F AAD-609 tended to be denser and the sporangium-like structures were usually larger than those of *K. aridum*. When grown in the dark SK&F AAD-609 frequently produced a pale gray aerial mycelium on thin potato-carrot agar, water agar and soil extract agar. This was never observed in *K. aridum* and the aerial mycelium of both cultures was white when grown in the light. The phospholipid pattern of the two cultures also differed slightly; phosphatidyl methylethanolamine was present in *K. aridum* but was not detected in SK&F AAD-609. The major components of the antibiotic complex produced by *K. aridum* (aridicins A, B and C) were also produced by SK&F AAD-609. In addition, SK&F AAD-609 produced a novel homologous series of glycopeptides which were not found in the broths from *K. aridum*.

The differences between SK&F AAD-609 and *K. aridum* were judged insufficient to warrant the

erection of a new species. SK&F AAD-609 was, therefore, designated a new subspecies of *K. aridum* for which we propose the name *Kibdelosporangium aridum* subsp. *largum* (largus, L. adj., abundant, plentiful, numerous). *K. aridum* subsp. *largum* (SK&F AAD-609) has been deposited in the American Type Culture Collection, as the subspecies type, under the accession number ATCC 39922.

Production of Kibdelins

An agar slant culture of SK&F AAD-609 was grown on medium ACL22 at 8°C for 14 days. The slant contents were dispersed and suspended in 10 ml of sterile distilled water and inoculated into 500 ml of seed medium 13H[1] contained in a 4-liter aspirator bottle. This seed culture was incubated at 28°C for 4 days on a reciprocal shaker at 250 rpm and 7.5-cm throw. The entire seed was transferred to 9.5 liters of medium 13H in a 14-liter New Brunswick Fermentor (M-19). The fermentor was controlled at 26°C for 3 days with agitation at 400 rpm and aeration at 4 liters/minute. The final seed was prepared by transferring 10 liters of the culture to 50 liters of medium 13H in a 75-liter Chemap fermentor. This was controlled at 26°C for 3 days, with agitation at 250 rpm and aeration at 25 liters/minute. This was used to inoculate 500 liters of production medium V-2[1] in a 750-liter ABEC fermentor. The production stage was maintained at 28°C with agitation at 150 rpm and aeration at 200 liters/minute. The production of kibdelins and aridicins was monitored carefully, by analytical HPLC[5], and the products were harvested at 45 hours (Fig. 3) by which time the kibdelins were the predominant components. Subsequent to this point the production of aridicins markedly surpassed the kibdelins by a factor of 40: 1. The early harvest facilitated isolation of the novel compounds from the known components.

Antibacterial Activity

The kibdelins had similar *in vitro* potency to vancomycin, and improved activity relative to aridicin A, against *Staphylococcus aureus* strains (Table 2). They were inferior to vancomycin against coagulase negative staphylococci, although approximately twice as active as aridicin A. In common with aridicin A these novel lipid-containing glycopeptides were particularly potent against enterococci and *Clos-*

Table 2. Antibacterial activity of kibdelins and comparative glycopeptides.

Test strains	MIC (μg/ml)					
	Vancomycin	Aridicin A	Kibdelin			
			A	B	C	D
Staphylococcus aureus HH127	1.6	3.1	1.6	1.6	1.6	1.6
S. aureus 910[a]	1.6	3.1	1.6	1.6	1.6	1.6
S. aureus 209P	0.8	0.8	0.4	0.8	0.8	0.8
S. aureus 674	1.6	3.1	0.8	0.8	1.6	1.6
S. aureus 675	3.1	12.5	6.3	6.3	6.3	6.3
S. epidermidis 2479	3.1	25	12.5	12.5	12.5	12.5
S. epidermidis 2683	3.1	50	25	25	25	25
S. epidermidis 651[a]	3.1	100	50	50	25	50
S. epidermidis 2265	1.6	50	25	25	25	50
Enterococcus faecalis 657	3.1	0.8	0.4	0.2	0.2	0.8
E. faecalis 34358	3.1	0.4	0.4	0.2	0.2	0.8
Escherichia coli 12140	>100	>100	>100	>100	>100	>100
Salmonella gallinarum BC-595	100	>100	>100	>100	>100	>100
Clostridium difficile	2	0.25	0.5	0.5	1	0.5

[a] Methicillin-resistant strains.

Table 3. Activity of kibdelins in mouse protection tests[a].

Compound	*S. aureus* HH127		*S. aureus*[b] 2620		*S. epidermidis* 2479		*S. epidermidis*[b] 651		*E. faecalis* 34358	
	MIC[c]	ED_{50}	MIC	ED_{50}	MIC	ED_{50}	MIC	ED_{50}	MIC	ED_{50}
Vancomycin	1.6	1.4	2	6.7	3.1	4.1	3.1	4.8	3.1	12.5
Aridicin A	3.1	2.2	4	29	25	8.2	100	>50	0.4	16
Kibdelin A	1.6	6.2	2	21.5	12.5	4.8	50	50	0.4	38
Kibdelin B	1.6	5.8	2	18	12.5	8.2	50	>50	0.2	50
Kibdelin C	1.6	8.5	4	8.4	12.5	22	25	50	0.2	>50
Kibdelin D	1.6	2.5	4	11.3	12.5	6.2	50	>50	0.8	35

[a] Mice dosed 1 and 5 hours post infection sc; ED_{50}, mg/kg.
[b] Methicillin-resistant strains.
[c] MIC, μg/ml.

tridium difficile (Table 2).

In mouse protection studies the four kibdelins were effective against methicillin-sensitive and methicillin-resistant staphylococci and against *Enterococcus faecalis* (Table 3). However, the improvements in *in vitro* activity over aridicin A were not reflected in marked differences in *in vivo* potency.

Table 4. Pharmacokinetics of kibdelin A in mice.

	Maximum serum concentration (μg/ml)	Elimination half-life (minutes)
Vancomycin	30	20
Kibdelin A	90	116
Aridicin A	121	226

Pharmacokinetics of Kibdelin A

Intravenous administration of kibdelin A to mice resulted in a very high peak serum concentration (90 μg/ml) and elimination half-life (116 minutes) compared to vancomycin (Table 4). The corresponding analog aridicin A, in which the glycolipid contains an acidic rather than neutral sugar, had a slightly greater serum concentration (121 μg/ml) and much longer half-life (226 minutes).

Evaluation of the Kibdelins Complex for Growth Promotion

In *in vitro* studies of the metabolism of the rumen and monogastric microflora the kibdelins complex was comparable to the aridicins and superior to antibiotics currently being used (Tables 5 and 6). The changes in rumen fermentation mediated by kibdelins indicated increased production of propionate, maintenance of butyrate and sparing of amino acids (Table 5). The kibdelins and the aridicins com-

Table 5. Effects of kibdelins and comparative compounds on the metabolism of rumen microflora *in vitro*.

Antibiotic	Changes in rumen metabolites (% of control value)						
	Concentration (ppm)	Propionate	Butyrate	Acetate	Propionate: total volatile fatty acids	Lysine	α-Amino nitrogen
Monensin	50	258	53	96	221	186	248
	5	208	61	101	189	144	185
Avoparcin	50	130	90	98	129	114	182
	5	116	82	94	123	150	120
Aridicins	50	146	96	97	139	120	214
	5	147	82	98	147	132	171
Kibdelins	25	151	101	103	146	138	161
	5	159	86	99	147	132	208

Table 6. Effects of kibdelins and comparative compounds on metabolism of monogastric (swine) microflora *in vitro*.

Antibiotics[a]	Changes in metabolites (% of control value)		
	Glucose	Volatile fatty acids	Lactic acid
Virginiamycin	180	63	44
Aridicins	180	274	22
Kibdelins	181	265	28

[a] All antibiotics were tested at 167 ppm.

plexes were more potent than avoparcin and showed a different profile to monensin, which depressed butyrate levels.

The response of the monogastric (swine) microflora to kibdelins showed a sparing of glucose, increase in volatile fatty acids and decreased production of lactic acid (Table 6).

Discussion

Thorough investigation of the morphology, chemotaxonomy and physiology of SK&F AAD-609, and comparison with known members of the *Actinomycetales*, confirmed it as a subspecies of the recently described genus *Kibdelosporangium*[1], designated *K. aridum* subsp. *largum*. This strain produced a greater variety of glycopeptides in which the acylglucuronic acid series of the aridicins were produced together with a related group of homologs, named kibdelins, containing acylglucosamine. The decrease in the negative charge of these compounds was reflected in slightly improved *in vitro* activity but no marked improvement in *in vivo* performance. The *in vivo* performance might be offset by the lower serum concentrations and shorter elimination rates as seen with kibdelin A compared with aridicin A. Also, the possibility that serum or tissue binding may be responsible is under investigation.

As observed for the other glycolipid containing glycopeptides, aridicins[1] and teicoplanin[19], the kibdelins showed enhanced *in vitro* activity in relation to vancomycin against enterococci and *Clostridium difficile*. Thus the acyl substituent seems to confer this improvement in spectrum over other glycopeptides. The improved potency did not result in increased efficacy in experimental infections possibly due to serum and protein binding.

The antibacterial activity and pharmacokinetic profile indicate that kibdelins may have potential for treatment of Gram-positive infections with once daily administration. They also have potential for growth promotion in rumenants, including dairy cattle, and monogastrics based on their modification of the metabolism of the microflora.

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