A54145 A NEW LIPOPEPTIDE ANTIBIOTIC COMPLEX: MICROBIOLOGICAL EVALUATION†

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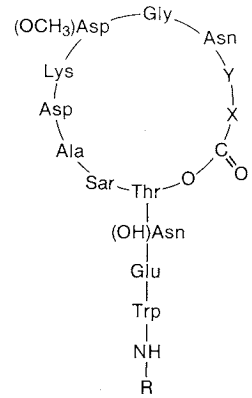
A54145 complex is made up of eight factors; A, A_1, B, B_1, C, D, E, and F which were active *in vitro* (MIC 0.25 ~ >32 μg/ml) against Gram-positive aerobic organisms. The complex, factors B and B_1 were found to be active against two strains of *Clostridium perfringens*. A calcium dependence study on some of the factors showed that their *in vitro* antibacterial activity was greatly enhanced by the presence of calcium (50 mg/liter) in the media. Resistance build-up was seen when *Staphylococcus* sp. and *Streptococcus* sp. were passed seven times in the presence of sublethal concentrations of A54145 antibiotics. This resistance disappeared immediately when the resistant organisms were passed in the absence of the antibiotics. Factor A was very effective against *Staphylococcus aureus* and *Streptococcus pyogenes* infections in mice (sc ED_{50}s of 3.3 ~ 2.4 mg/kg × 2, respectively). Factor B was more active against *S. pyogenes in vivo* (sc ED_{50}, 0.9 mg/kg × 2). Acute mouse toxicities were determined with these antibiotics. Semisynthetic derivatives were evaluated.

The lipopeptide antibiotics are a class of antibiotics which have a Gram-positive spectrum of activity. The activities of A21978C and its analogs which are members of this class were first reported in 1984 by COUNTER *et al.*[1]. Previous communications in this series have described the taxonomy and fermentation of culture A54145 (*Streptomyces fradiae* NRRL 18158, NRRL 18159, and NRRL 18160)[2]; the isolation, characterization[3] and structural elucidation[4] of eight acidic lipopeptide factors (A, A_1, B, B_1, C, D, E, and F) produced by the culture; the microbial deacylation of A54145 factors to provide individual A54145 core peptides[5] and the production of semisynthetic *N*-acyl A54145 analogs by means of the chemical reacylation of appropriately protected A54145 complex[5]. In this report we will discuss the *in vitro* and *in vivo* activity of A54145 factors and analogs made from these factors. The structure of A54145 complex antibiotics can be found in Fig. 1.

Fig. 1. Structure of A54145 complex antibiotics.



Factor	X	Y	R
A	Ile	Glu	8-Methylnonanoyl (iC_{10})
A_1	Ile	Glu	*n*-Decanoyl (nC_{10})
B	Ile	3-MethylGlu	*n*-Decanoyl (nC_{10})
B_1	Ile	3-MethylGlu	8-Methylnonanoyl (iC_{10})
C	Val	3-MethylGlu	8-Methyldecanoyl (aC_{11})
D	Ile	Glu	8-Methyldecanoyl (aC_{11})
E	Ile	3-MethylGlu	8-Methyldecanoyl (aC_{11})
F	Val	Glu	8-Methylnonanoyl (iC_{10})

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Materials and Methods

Antimicrobial Agents

A54145 factors and derivatives were prepared at The Lilly Research Laboratories, Indianapolis, IN, U.S.A.[3,5].

Bacteria

All bacterial strains used in this study were clinical isolates obtained from numerous sources of broad geographic distribution. Isolates were maintained frozen in liquid nitrogen or in −70°C electric freezers.

Growth Inhibition Activity

MICs were determined by an agar dilution method in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards[††]. Inocula were adjusted to yield approximately 10^{-5} cfu per spot. Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, MD.) was used for all of the aerobic organisms tested except for the calcium dependence studies. Schadler agar (Difco Laboratories, Detroit, MI.) was used for all of the anaerobic organisms. The MIC was considered to be the lowest concentration that prevented visible growth or less than three discrete colonies.

Mouse Protection Tests

Mouse protection tests were performed against one strain of *Staphylococcus aureus*, one stain of *Streptococcus pyogenes*, and one strain of *Streptococcus pneumoniae*. All strains were clinical isolates. Bacterial cells were counted and adjusted to yield an amount which would give 50 ~ 500 LD_{50}s in a 0.5-ml dose. The bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI.) and the inoculum was adjusted to the proper concentration with brain heart infusion broth or brain heart infusion broth with 5.0% mucin.

The mice used were 19 ~ 21 g random sex ICR mice (Harlan Industries, Cumberland, IN.). Mice (8 per group) were infected intraperitoneally with 0.5 ml of the bacterial suspension. Antibiotics were administered subcutaneously at 1 and 5 hours post-infection. The animals were observed for 7 days post-infection and the dead animals were removed from the cages and the date of death recorded. A control group of mice (five dilutions with 8 mice per dilution) were included with the test animals to titrate the LD_{50} of the infecting organism. An ED_{50} was calculated for each compound at 7 days after infection by the method of REED and MUENCH[6]. The LD_{50} determinations were done in 19 ~ 21 g random sex ICR mice. Mice (4 per group) were injected by the intraperitoneal route with the appropriate 2-fold dilution of the antibiotics. Each dilution was injected into a different group. These mice were then observed for 7 days. Deaths were recorded daily according to group. The LD_{50} was calculated according to the method of REED and MUENCH[6].

Biosynthesis of Peptidoglycan

Incorporation of radioactivity from [^{14}C]diaminopimelic acid into cell wall of *Bacillus megaterium* X67 (ATCC 8245) was measured exactly as described[7]. This organism was grown in cell wall synthesis medium (CWSM[8]) supplemented with 1.25 mM $CaCl_2$.

Formation of UDP-MurNAc-Pentapeptide

Incorporation of radioactivity from [^{14}C]diaminopimelic acid into uridine-diphospho-*N*-acetyl-muramyl-pentapeptide in *B. megaterium* was measured in CWSM supplemented with 1.25 mM $CaCl_2$ plus 100 μg/ml vancomycin. Labeled nucleotide-linked pentapeptide was detected chromatographically[7].

Results

In Vitro Activity

The *in vitro* activity of A54145 factors A, A_1, B, B_1, C, D, E, and F was compared against four *S.*

[††] National Comittee for Clinical Laboratory Standards. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A. National Comittee for Clinical Laboratory Standards, Villanova, 1985.

Table 1. Antimicrobial activity of A54145 factors.

Antibiotic	Agar dilution MICs (μg/ml)									
	S.a. X1.1	*S.a.* V41	*S.a.* X400	*S.a.* S13E	*S.e.* EPi.1	*S.e.* 222	*S.py.* C203	*S.pn.* Park	*Enterococcus* X66	*Enterococcus* 2041
A54145A	1.0	1.0	2.0	1.0	1.0	1.0	0.5	1.0	4.0	16
A54145A$_1$	4.0	8.0	8.0	4.0	—	4.0	1.0	2.0	4.0	32
A54145B	1.0	1.0	2.0	1.0	1.0	1.0	0.5	1.0	8.0	16
A54145B$_1$	2.0	2.0	2.0	2.0	1.0	1.0	0.5	2.0	8.0	32
A54145C	2.0	4.0	4.0	4.0	2.0	2.0	2.0	4.0	8.0	8.0
A54145D	2.0	2.0	4.0	4.0	4.0	1.0	0.5	4.0	16	32
A54145E	1.0	1.0	2.0	2.0	—	1.0	0.25	2.0	4.0	4.0
A54145F	16	16	16	16	1.0	8.0	2.0	32	128	>128

Abbreviations: *S.a.*, *Staphylococcus aureus*; *S.e.*, *Staphylococcus epidermidis*; *S.py.*, *Streptococcus pyogenes*; *S.pn.*, *Streptococcus pneumoniae*.

Table 2. Effect of nucleus variation on potency of A54145 derivatives.

A54145 Derivatives	Agar dilution MICs (μg/ml)									
	S.a. X1.1	*S.a.* V41	*S.a.* X400	*S.a.* S13E	*S.e.* EPi.1	*S.e.* 222	*S.py.* C203	*S.pn.* Park	*Enterococcus* X66	*Enterococcus* 2041
N-Trp-*n*-decanoyl-A54145A nucleus	8.0	8.0	8.0	8.0	4.0	4.0	2.0	8.0	64	64
N-Trp-*n*-decanoyl-A54145B nucleus	1.0	1.0	2.0	1.0	1.0	1.0	0.5	1.0	8.0	16
N-Trp-*n*-decanoyl-A54145F nucleus	16	16	16	16	16	16	4.0	32	128	128

Abbreviations: See Table 1.

Table 3. Effect of side chain variation on activity of A54145A semi-synthetic derivatives.

Derivatives[a] (R=)	Agar dilution MICs (μg/ml)									
	S.a. X1.1	*S.a.* V41	*S.a.* X400	*S.a.* S13E	*S.e.* EPi.1	*S.e.* 222	*S.py.* C203	*S.pn.* Park	*Enterococcus* X66	*Enterococcus* 2041
H	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
n-Hexanoyl	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
n-Nonanoyl	32	32	32	32	16	16	4.0	64	128	128
8-Methylnonanoyl	16	16	16	16	8.0	8.0	8.0	16	128	128
n-Decanoyl	8.0	8.0	8.0	8.0	4.0	4.0	2.0	8.0	64	64
n-Undecanoyl	2.0	4.0	4.0	4.0	2.0	2.0	0.5	2.0	16	32
n-Dodecanoyl	1.0	1.0	1.0	2.0	1.0	1.0	0.125	2.0	4.0	8.0
n-Tetradecanoyl	0.25	0.25	0.5	0.25	0.5	0.5	0.06	0.125	2.0	4.0

[a] See Fig. 1.
Abbreviations: See Table 1.

aureus, two *Staphylococcus epidermidis*, two *Streptococcus* strains, and two *Enterococcus* strains (Table 1). A54145 factors B, C, and E were the most active factors against all of the organisms including the *Enterococcus* strains. The effect of nucleus variation on the *in vitro* activity of A54145 factors was studied using the *N*-Trp-*n*-decanoyl derivatives (Table 2). Factor B was the most active followed by factors A and F in descending order of *in vitro* activity. The *in vitro* activity of eight semisynthetic derivatives of A54145A nucleus having the *N*-Trp-acyl substituents of varying chain lengths was evaluated (Table 3).

The A54145A nucleus (no *N*-Trp-acyl substituent) and the *N*-Trp-*n*-hexanoyl-A54145A nucleus were

Table 4. *In Vitro* activity of A54145 factors and derivatives against *Clostridium perfringens* strains.

Antibiotic	Agar dilution MICs (μg/ml)	
	C.p. ATCC 3624	*C.p.* NE
A54145A nucleus	>64	>64
N-Trp-*n*-hexanoyl-A54145A nucleus	>64	>64
N-Trp-8-methylnonanoyl-A54145A nucleus (A54145A)	32	16
N-Trp-*n*-decanoyl-A54145A nucleus (A54145A$_1$)	16	16
N-Trp-*n*-tetradecanoyl-A54145A nucleus	0.25	0.25
N-Trp-8-methylnonanoyl-A54145B nucleus (A54145B$_1$)	2.0	2.0
N-Trp-*n*-decanoyl-A54145B nucleus (A54145B)	1.0	1.0

Abbreviation: *C.p.*, *Clostridium perfringens*.

devoid of activity against Gram-positive aerobes *in vitro*. The *in vitro* activity of the factors increases with increasing chain length through *N*-Trp-*n*-tetradecanoyl-A54145A nucleus, which contains the longest acyl substituent tested. The branched chain derivative, 8-methylnonanoyl, was slightly less active than the *n*-decanoyl derivative.

The *in vitro* activity of A54145 factors A, B and B$_1$, as well as the A54145A nucleus and three A54145A substituted derivatives, was tested against *Clostridium perfringens*, an anaerobic organism that causes necrotic enteritis in chickens (Table 4). The same relationship of activity was observed against *C. perfringens* as was observed against the Gram-positive aerobes. The longer the chain length of the *N*-Trp-acyl substituent of the A54145 factors and derivatives the more potent the *in vitro* activity. The *N*-Trp-*n*-tetradecanoyl-A54145A nucleus was the most active followed by A54145 factors B and B$_1$ which were more active than the A54145A nucleus derivative tested. As with the Gram-positive aerobes tested *N*-Trp-*n*-decanoyl-A54145B nucleus was more potent than the comparable A nucleus derivatives.

Table 5. Effect of added calcium on MICs of A54145 factors.

Antibiotic	Agar dilution MICs (μg/ml)			
	E.f.		*S.a.*	
	w/o Ca^{++}	w/Ca^{++}	w/o Ca^{++}	w/Ca^{++}
A54145A	>176	21	>176	2
A54145B	—	—	109	<0.9

Abbreviations: *E.f.*, *Enterococcus faecalis* ATCC 29212; *S.a.*, *Staphyloccus aureus* FDA 209P.

Calcium Dependency Assay

Since this antibiotic is in the same chemical class as A21978C and it was previously reported[1,9] that A21978C had a calcium dependency for optimum activity, we evaluated the effect of calcium free and calcium substituted media on the MICs of A54145A and A54145B. Two organisms, *Enterococcus faecalis* (ATCC 29212) and *S. aureus* (FDA 209P) were used in the test which consisted of a broth dilution MIC determination in supplemented (50 mg Ca^{++}/liter) and unsupplemented Mueller-Hinton Broth (Difco Laboratories, Detroit, MI.) inoculated with 10^6 cfu of the test organism. MICs (Table 5) were judged from plotting growth curves provided by the MS-2 Research System (Abbott Laboratories, Chicago, Ill.). The results in Table 5 with the A54145 antibiotic show a potency increase from 8- to 100-fold in the presence of added calcium.

Resistance Development Studies

S. aureus (3055), *S. epidermidis* (ST-214) and *Streptococcus sanguis* (SS-910) were incubated in doubling dilutions of A54145A in cation-supplemented Mueller-Hinton broth. In the testing of *S. sanguis*

Table 6. *In Vitro* resistance development upon repeated subculturing of bacteria in medium containing A54145A[a].

Bacteria	MIC (μg/ml)[b]							
	Original	SC 1	SC 2	SC 3	SC 4	SC 5	SC 6	SC 7
S.a. 3055	0.5	1.0	4.0	8.0	8.0	16	16	16
S.e. ST-214	0.5	1.0	2.0	4.0	4.0	4.0	4.0	4.0
S.s. SS-910	1.0	2.0	4.0	4.0	4.0	4.0	4.0	4.0

[a] See Materials and Methods section.
[b] After subculture number (SC) in Trypticase-Soy broth containing A54145A.
Abbreviations: *S.a.*, *Staphylococcus aureus*; *S.e.*, *Staphylococcus epidermidis*; *S.s.*, *Streptococcus sanguis*.

Table 7. Change of MICs on subculture number (SC) in antibiotic-free Trypticase-Soy broth.

Bacteria	MIC (μg/ml)								
	Original	[a]	SC 1	SC 2	SC 3	SC 4	SC 5	SC 6	SC 7
S.a. 3055	0.5	16	16	16	8	16	8	8	2
S.e. ST-214	0.5	4	4	4	2	4	2	2	2
S.s. SS-910	1.0	4	2	2	2	2	2	2	2

[a] After seven subculturs in antibiotic.
Abbreviations: See Table 6.

the broth was supplemented additionally with laked rabbit blood and pyridoxal. Seven serial subcultures of these organisms were made in fresh antibiotic dilutions in order to determine the amount of resistance build-up to the antibiotic. The inoculum used for each subculture was from the well having the highest antibiotic concentration and containing good growth. The results from these experiments can be found in Table 6. These results show that with all three organisms the MICs rose at least 4-fold for *S. sanguis*, 8-fold for *S. epidermidis*, and 32-fold for *S. aureus* during the seven subcultures.

After the bacteria were passed for seven subcultures in antibiotic-containing media, those bacteria growing in the highest concentrations of antibiotic were subcultured in antibiotic-free medium to determine if the cultures would return to their original antibiotic MICs. Seven passages were made with each organism and MICs were determined after each passage. The results (Table 7) show that even though they do not return to their original MICs the MICs do decline to within 2- to 4-fold of the original MICs.

In Vivo Activity

The results of the acute toxicity (LD_{50}s) and efficacy (ED_{50}s) tests in mice on A54145 factors A and B and can be found in Table 8. A54145B was more active against *S. pyogenes* and more toxic in mice than A54145A. The effect of nucleus variation was evaluated in mice using both efficacy tests and acute toxicity tests. *S. pyogenes* was used as the challenge organism in the *in vivo* efficacy tests. The results found in Table 9 show that the *N*-Trp-*n*-decanoyl-A54145B nucleus (A54145B) was the most active and the most toxic. The effect of side chain variation was then tested using derivatives of the A54145A nucleus. The *N*-Trp-*n*-dodecanoyl-A54145A nucleus, the longest side chain tested, was the most active and the most toxic of the series (Table 10).

Mode of Action

A54145 inhibited biosynthesis of cell wall peptidoglycan with a dose response very similar to that of

Table 8. *In Vivo* efficacy and toxicity of A54145 factors in mice.

Antibiotic	ED_{50} (mg/kg × 2, sc)			LD_{50} (mg/kg × 1, ip)
	S.a.	*S.py.*	*S.pn.*	
A54145A	3.38	3.1	> 5.0	> 500
A54145B		0.94		28

Abbreviations: *S.a.*, *Staphylococcus aureus*; *S.py.*, *Streptococcus pyogenes*; *S.pn.*, *Streptococcus pneumoniae*.

Table 9. Effect of nucleus variation on *in vivo* efficacy and acute toxicity of A54145 factors in mice.

Derivative	ED_{50} (mg/kg × 2, sc) *S.p.*	LD_{50} (mg/kg × 1, ip)
N-Trp-*n*-decanoyl-A54145F nucleus	10.8	> 500
N-Trp-*n*-decanoyl-A54145A nucleus	5.0	> 500
N-Trp-*n*-decanoyl-A54145B nucleus (A54145B)	0.94	28

Abbreviation: *S.p.*, *Streptococcus pyogenes*.

Table 10. Effect of side chain variation on *in vivo* efficacy and toxicity in mice.

Derivative	ED_{50} (mg/kg × 2, sc) *S.p.*	LD_{50} (mg/kg × 1, ip)
N-Trp-*n*-nonanoyl-A54145A nucleus	10.6	> 500
N-Trp-8-methyldecanoyl-A54145A nucleus	3.6	> 500
N-Trp-*n*-decanoyl-A54145A nucleus	5.0	> 500
N-Trp-*n*-undecanoyl-A54145A nucleus	1.6	> 500
N-Trp-*n*-dodecanoyl-A54145A nucleus	1.2	321

Abbreviation: See Table 9.

Table 11. Effect of A54145 and daptomycin on cell wall biosynthesis in *Bacillus megaterium*[a].

Compound	Conc (μg/ml)	Radioactivity (dpm)	Inhibition (%)
None	—	38,305	0
$A54145A_1$	0.1	36,800	4
	1	23,269	39
	10	388	99
	100	219	99
Daptomycin	0.1	30,093	21
	1	22,333	42
	10	554	99
	100	200	99

[a] Incorporation of [^{14}C]diaminopimelic acid into peptidoglycan was measured as described in Materials and Methods.

Table 12. Effect of A54145 and daptomycin on the formation of UDP-MurNAc-[^{14}C]pentapeptide in *Bacillus megaterium*[a].

Inhibitor	Conc (μg/ml)	Radioactivity in pentapeptide (dpm)	Inhibition (%)
None	—	4,344	0
$A54145A_1$	5	4,707	< 0
	10	633	85
	20	473	89
Daptomycin	5	5,681	< 0
	10	1,404	68
	20	161	96

[a] UDP-MurNAc-pentapeptide was labeled with [^{14}C]diaminopimelic acid in the presence of 100 μg/ml vancomycin as described in Materials and Methods. In the absence of added vancomycin, there were 156 dpm associated with pentapeptide.

daptomycin (Table 11). In a separate experiment, A54145 failed to stimulate accumulation of the nucleotide-linked-sugar-pentapeptide precursor typical of inhibition by vancomycin (data not shown). The results in Table 12 demonstrate that like daptomycin, A54145 appears to inhibit the formation of the sugar-pentapeptide precursor. Ten μg/ml of either compound completely inhibited precursor formation suggesting that the target of A54145 may be identical to that of daptomycin[9].

Discussion

The chain length of the *N*-Trp-acyl substituent of A54145 factors and derivatives has a significant

effect on their biological properties. The A54145A nucleus (no *N*-Trp-acyl substituent) and *N*-Trp-*n*-hexanoyl-A54145A nucleus were devoid of activity against Gram-positive aerobes *in vitro*. The *in vitro* activity of the factors increases with increasing chain length through *N*-Trp-*n*-tetradecanoyl-A54145A nucleus, which contains the longest acyl substituent tested. The same relationship between acyl chain length and *in vitro* potency was seen vs. *C. perfringens*, an anaerobe. The same order of potency was observed in studies of the *in vivo* efficacy vs. *S. pyogenes* infections in mice and in mouse LD_{50}s.

In the comparison of A54145 derivatives containing the same *N*-Trp-acyl substituent, the A54145B nucleus derivative was more potent *in vitro* vs. Gram-positive aerobes than A54145A nucleus derivatives, which were more potent than the A54145F nucleus derivatives. This same order of potency was observed in studies of *in vivo* efficacy vs. *S. pyogenes* infections in mice. However, the A54145B nucleus-containing factor was also the most toxic of the three nuclei tested. The *in vitro* activity of A54145 factors appear to be calcium dependent.

A54145B caused resistance development in bacteria *in vitro* when the organisms were passed in subinhibitory concentrations of the antibiotic. The resistance was lost, however, after back passage of the resistant organism in antibiotic-free medium. A54145 appears to inhibit biosythesis of cell wall peptidoglycan through inhibition of sugar-pentapeptide precursor formation.

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