Aminimides: II. Antimicrobial Effect of Short Chain Fatty Acid Derivatives

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ABSTRACT

A new family of surfactants, aminimides, has been screened for *in vitro* antimicrobial activity. These compounds are active against both bacteria and yeast, activity being a function of chain length. Maximum activity for acetimide and acrylimide amine derivatives was extablished with chain lengths of C_{14}-C_{16}. Homologous compounds with lower or higher chain lengths were less active. While showing low antimicrobial activity against gram negative bacteria, mixtures containing C_{12} and C_{16} gave good activity against gram negative strains without losing gram positive activity. Aminimides gave low acute LD_{50}'s (200-400 mg/kg) when tested in mice by intraperitoneal injection.

INTRODUCTION

Surfactants, as a group of chemicals, have demonstrated antimicrobial activity (1). The present report deals with the antibacterial and antifungal properties of a new family of surfactants, amine acylimide aminimides. Aminimides represent a group of compounds with varied industrial application. The chemistry and potential uses of these chemicals have been reviewed recently (2). Previously our laboratory has reported on the structure-function activity of such surface active agents as soaps (3) and simple aliphatic amides and amines (4). A short communication summarizing the effects of acyl chain length on aminimide activity has been published (5). The present study is a continuing effort to screen aminimide surfactants for their antimicrobial properties. This paper is concerned with amimides in which the acyl portion of the imide is kept constant as either an acetimide or a methacrylimide group, and the chain length of the tertiary amine is a variable. The effect of varying the chain length of the amine of biological property of such short chain acylimide is presented.

MATERIALS AND METHODS

Compounds

All the aminimides used in these studies were obtained from the Ashland Chemical Company (Dublin, OH). For the most part, they were recrystallized 2-5 times and represent compounds of purity > 99%.

For screening purposes, the 0.2 g aminimide was dissolved in 0.5 ml absolute methanol. To this volume of alcohol 200 ml Trypticase soy broth (TSB, Baltimore Biological Laboratories, Baltimore, MD), was added aseptically. If the resulting solution was not clear, the suspension was heated carefully (ca. 60 c) to increase drug solubility. Routinely standard solutions or suspensions containing 1,000 μg/ml were diluted tenfold with additional broth. Because aminimides are heat labile, the tubes were not sterilized. Control experiments indicated differences in compound activity after sterilization and comparison with unheated controls.

Organisms

The microorganisms used for our screening studies were those frequently encountered in clinical specimens. In general, clinical isolates (CI) are more resistant to chemical inactivation than those registered with the American Type Culture Collection (ATCC). Clinical isolates were identified and made available to use by J.P. Truant, PhD (Advance Medical Laboratories, Pontiac, MI). The following gram negative organisms were used: *Escherichia coli* [EC] and *Pseudomonas aeruginosa* (ATCC-10145) [PA]. The gram positive organisms used were: *Streptococcus faecalis* (Group D) [SF]; *Streptococcus pyogenes* [SP]; *Staphylococcus aureus* [SA]; *Corynebacterium sp.* (ATCC-10700) [CSP]; and *Nocardia asteroides* (ATCC-3308) [NA]. *Candida albicans* [CA] and *Saccharomyces cerevisiae* [SC] were examples of yeast isolates.

Inoculum

A test inoculum consisted of 0.05 ml of an 18- to 24-hour TSB culture (ca. 10^8-10^9 organism/ml). The inoculum was aseptically delivered into dilutions of the compound, mixed well, and incubated at 36 C in a 5% CO_2:95% air. A tube of inoculated broth without drug, but containing 015 ml methanol, served as a positive control; also, an uninoculated set of drug solutions was incubated. After 18 hours of incubation, the minimal inhibitory concentration (MIC) of each compound against each organism was determined. In our study, the MIC was defined as the lowest concentration of compound at which no macroscopic evidence was observed when turbidity of the inoculated broth dilutions was compared with that of the control tubes.

In those cases in which the test compound itself caused turbidity so that the MIC could not be determined accurately a 0.015 ml sample of the well agitated broth in question was inoculated onto a Trypiticase soy agar plate containing 5 % defibrinated sheep blood, inoculated at 35 C, and examined after 24 and 48 hours for bactericidal end points.

It was found that turbidity owing to the compounds did

[a]Presented at AOCS Spring Meeting, Dallas, April, 1975.

TABLE I

Minimal Inhibitory Concentration (μg/ml) for Hexachlorophene from Replicate Experiments

	7/24/75	6/5/75	2/5/75	1/23/75	10/23/74	10/3/74	9/5/74	8/16/74	7/16/74	5/20/74
Escherichia coli	100	100	100	100	100	100	100	100	1,000	100
Pseudomonas aeruginosa	100	100	100	100	100	100	100	100	100	100
Streptococcus faecalis (Group D)	1	10	1	<1	1	10	10	1	10	10
Streptococcus pyrogenes	<1	1	<1	<1	<1	<1	< 1	<1	<1	<1
Staphylococcus aureus	<1	<1	<1	<1	<1	<1	< 1	<1	<1	<1
Corynebacterium sp.	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Nocardia asteroides	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Candida albicans	100	100	100	100	100	100	100	100	1,000	100
Saccharomyces cerevisiae	100	10	100	10	100	10	100	100	100	100

TABLE II

Minimal Inhibitory Concentration (μg/ml) of Acetamide Amines

Number	Compound[c]	Gram positive[a]		Gram negative[a]					Yeast[a]		Mouse Toxicity
		EC	PA	SF	SP	SA	CSP	NA	CA	SC	LD_{50} (mg/kg)
M-15	$CH_3C(=O)-NN^+(CH_3)_3$	NI[b]	NI	NI	NI	NI	NI	NI	NI	NI	---
M-70	$R\text{-}CH_2CH(OH)(CH_2)_9CH_3$	1,000	1,000	100	10	100	100	100	100	100	85(110)
M-71	$R\text{-}CH_2CH(OH)(CH_2)_{11}CH_3$	NI	NI	10	10	10	10	10	10	10	180
M-72	$R\text{-}CH_2CH(OH)(CH_2)_{13}CH_3$	NI	NI	100	10	10	10	10	10	10	165
M-24	$R\text{-}CH_2CH(OH)(CH_2)_{15}CH_3$	NI	NI	1	10	10	10	10	NI	NI	310

[a]EC = Escherichia *coli;* PA = *Pseudomonas acruginosa;* SF = *Streptococcus faecalis* Group D; SP = *Streptococcus pyrogenes;* SA = *staphylococcus aureus;* CSP = *Corynebacterium spo;* NA = *Nocandia asteroides;* CA = *Candida albicans;* SC = Saccharomyces cerevisae.

[b]NI = Not inhibitory at 1,000 μg/ml.

[c]Where R = $CH_3C(=O)-\overset{-}{N}\overset{CH_3}{\underset{CH_3}{N}}\pm$

not confuse the readings because most of the compounds were inhibitory at low concentrations where solubility was almost complete.

The pH of broth was monitored throughout the study by the use of an Accutint set (Anachemia, Montreal, Quebec, Canada) and was found to be within the range of 7.3±0.2. Also, at the concentration used, methanol was found not to be inhibitory, as demonstrated by controlled test experiments.

Hexachlorophene always was used as a chemical control in our screening experiments. This procedure served as a statistical control for the procedure. Repetitive MIC values obtained from tenfold dilution rarely deviated from single observation. Data accumulated over a 12-month period are present in Table I. Acute toxicity experiments were carried out on select aminimide derivatives. The compounds were suspended in 0.25% methylcellulose or saline and injected intraperitoneally into 20-22 gram male mice (CF-1). Animals in groups of 5 or 10 were observed for 72 hours after drug injection. The mode of dying and the number of survivors were recorded. The LD_{50} was calculated according to the method of Litchfield and Wilcoxon (6).

RESULTS

Five acetamide amines were tested for their *in vitro* antimicrobial activity. Acute toxicity testing was carried out only on selected derivatives and the results of these experiments are given in Table II. The acetamide trimethylamine, which is the simplest derivative, exhibited no activity. Where the chain length of the aryl portion of the acetimide amine was increased from C_{12} to C_{18}, activity against microorganisms was noted. In the homologous series, the antimicrobial properties increased with extended chain length up to C_{16}. Beyond this length the general activity of the compound generally decreased.

A larger number of methacrylimide amines, 10 compounds varying in alkyl chain length from C_3 to C_{18}, were examined for antimicrobial activity (Table III). As with the acetamide amines, chain lengths between C_{12} and C_{16} were active. Compounds (M-130 and M-131) representing chain length mixtures of C_8 to C_{11}, or C_{12} to C_{15}, respectively, were less active than those prepared from a single fatty acid.

Although mixed acyl aminimides were no more active than single chain compounds, it was of interest to determine the antimicrobial effects of specially rather than randomly mixed chain length aminimides. Consequently, mixtures of M-70 (C_{12}), which indicated slight gram negative activity, and M-72 (C_{16}) were screened for their antimicrobial activity. Ratios of M-70:M-72, varying from 4:1 (80%) to 1:3 (25%), were examined. The results of these studies can be found in Table IV. Maximum activity for mixture was achieved when a ratio of 3:1 (M-70:M-72) was tested. This ratio allowed good activity against gram negative organisms without substantial loss of gram positive activity.

LD_{50}'s of selected compounds were determined in mice. At high doses diarrhea and vomiting occurred. Some central nervous effects were noted in terms of agitation or clonic convulsions in some cases, followed by coma, marked depression, or lowered motor activity. These initial observations must be viewed as impressions rather than a descriptive toxicological report, because relatively few drugs and animals were studied. A more descriptive report is forthcoming.

The acetimide amine derivatives were, in general, more toxic than the methacrylimide amine. The toxicity of both groups of aminimides, however, were low. The first group LD_{50} ranged between 100-300 mg/kg; whereas, the methacrylimide amines had LD_{50} between 300-400 mg/kg.

Some preliminary data in our laboratory suggest that the LD_{50} varies markedly for mice of different strains. The reason for this discrepancy is not known.

DISCUSSION

In our first paper on the activity of acylimide amines, the chain length of acyl group was varied (5). Activity against microorganisms was, up to a point, dependent on

TABLE III

Structure-Function Activity of Methacrylamide Amines

Number	Compound[c]	Gram positive[a]		Gram negative[a]					Yeast[a]		Toxicity
		EC	PA	SF	SP	SA	CSP	NA	CA	SC	LD_{50} mg/kg)
M-59	$R{\cdot}CH_2CH{\cdot}CH_3$ (OH)	NI[b]	NI	NI	NI	NI	NI	NI	NI	NI	320
M-99	$R{\cdot}CH_2CH{\cdot}CH_2CH_3$ (OH)	NI	NI	NI	NI	NI	NI	NI	NI	NI	
M-21	$R{\cdot}CH_2CH(CH)_2)_5CH_3$ (OH)	NI	NI	NI	1,000	1,000	1,000	NI	NI	NI	
M-22	$R{\cdot}CH_2CH(CH_2)_7CH_3$ (OH)	NI	NI	1,000	10	1,000	100	1,000	1,000	1,000	
M-19	$R{\cdot}CH_2CH(CH_2)_9CH_3$ (OH)	NI	NI	100	10	100	100	100	100	100	322
M-20	$R{\cdot}CH_2CH(CH_2)_{11}CH_3$ (OH)	NI	NI	10	10	10	10	10	100	1	400 mg/kg
M-57	$R{\cdot}CH_2CH(CH_2)_{13}CH_3$ (OH)	NI	NI	1,000	1	100	1,000	100	1,000	100	
M-129	$R{\cdot}CH_2CH(CH_2)_{15}CH_3$ (OH)	NI	NI	NI	10	1,000	1,000	1,000	NI	1,000	
M-130	$R{\cdot}CH_2CH(CH_2)_{8\text{-}11}CH_3$ (OH)	NI	NI	100	10	100	10	100	100	100	
M-131	$R{\cdot}CH_2CH(CH_2)_{12\text{-}15}CH_3$ (OH)	NI	NI	100	10	10	10	10	NI	100	

[a]EC = *Escherichia coli;* PA = *Pseudomonas acruginosa;* SF = *Streptococeus faecalis* Group D; SP = *Streptococcus pyrogenes;* SA = *Staphylococcus aureus;* CSP = *Corynebacterium spa;* NA = *Nocardia asteroides;* CA = *Candida albicans;* SC = *Saccharomyces cerevisiae.*

[b]NI = Not inhibitory at 1,000 μg/ml.

[c]Where R = $CH_2=\underset{\displaystyle CH_3}{\overset{|}{C}}-\overset{\displaystyle O}{\overset{\|}{C}}-N^{-}N^{+}\begin{matrix}CH_3\\ \diagdown\\ CH_3\end{matrix}$

TABLE IV

Effect of Mixed Chain Length on Acetamide Amines

Compound mixture		Minimal inhibitory concentration (μg/ml)		
M-70 (C_{12}) (%)	M-71 (C_{16}) (%)	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus pyogenes*
100	---	250	100.0	25.0
80	20	500	10.0	2.5
75	25	250	10.0	2.5
60	40	500	10.0	2.5
50	50	1,000	5.0	2.5
25	75	NI	2.5	1.0
0	100	NI	2.5	1.0

increasing chain length. The structure of the amine group seemed to be less critical to compound activity, but this conclusion was not wholly acceptable. Early reports recognized the dependence of bactericidal activity of cationic agents upon chain length (7-10).

It was of interest, therefore, to determine whether long chain amine derivatives of short chain imides might fall into this same generalization. Our results, using a variety of amine derivatives of acetimide or methacrylimide, gave substance to Fergusons's Rule (11). Where chain length of the amine was studied as a variable, the most active compounds contained chain length of C_{14} or C_{16}. This was true, except where gram negative activity was considered. In this instance, the shorter chain (C_{12}) exhibited more gram negative activity than did the longer chain (C_{16}) derivative. Presumably, the longer chain derivatives cannot penetrate the lipopolysaccharide layer of gram negative organisms (12).

To take advantage of the gram negative activity of short chain derivatives and the greater activity of long chain derivatives, a mixture of the two was screened. Specific ratios of C_{12} and C_{16} were combined, and mixtures containing 60-80% C_{12} were active against gram negative as well as gram positive and yeast organisms. Such mixture of aminimide compounds represented a drug composition which was truly unique, a wide spectrum antimicrobial agent. Few therapeutic agents can claim action against gram-negative, gram positive and yeast organisms. In prelim-

inary experiments (J.J. Kabara, unpublished data, 1975) these compounds also were very active (MIC <10 μg/ml) against protozoa.

The length of the carbon chain also had an important influence upon the degree of surface activity of the compound. Increasing surface activity occurred as chain length increased over the range of C_6-C_{16}, with the maximum being reached at C_{14} or C_{16}. Above C_{16}, surface activity decreased (13). The importance of chain length in determining surface activity has been demonstrated with soaps (14), alcohols (14-18), and aliphatic acids (19). While all surface active agents that are efficient bactericides have been found to possess a marked ability to reduce surface tensions, the converse is not necessarily true. As an example, the nonionic agents are very surface active, but they have no effect on bacterial metabolism and are not bactericidal (20,21). Consequently, compounds that are surface active do not automatically qualify as bactericidal agents.

Because aminimide surfactants show good in vitro antimicrobial activity and remarkably low toxicity (200-400 mg/kg), current work in our laboratory is concerned with in vivo activity of select compounds, as well as continued in vitro screening of other aminimide derivatives.

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