# Antimicrobial Effects of $N^\alpha$-Palmitoyl-L-lysyl-L-lysine Ethyl Ester Dihydrochloride and Its Use To Extend the Shelf Life of Creamed Cottage Cheese

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The antimicrobial acyldipeptide $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1) was synthesized, and its activity was tested against spoilage organisms and pathogens sometimes found in dairy products. Antimicrobial activity was observed at <10 μg/mL in nutrient broth. R-1 concentrations of 500 μg/mL were required to inhibit spoilage organisms added to sterile reconstituted nonfat dried milk. A bacteriostatic effect was observed for *Staphylococcus aureus* in vanilla pudding held at 21 °C using R-1 at 1500 μg/g. The shelf-life of creamed cottage cheese stored at 7 °C was extended two–three-fold (to about 26 days) at an R-1 concentration of 750 μg/g. The shelf-life of creamed cottage cheese stored at 7 °C, inoculated with *Pseudomonas putrefaciens* or *Achromobacter pestifer* was extended two–three-fold over the control at an R-1 concentration of 1500 μg/g. At concentrations of 1500 μg/g in creamed cottage cheese, R-1 lent a bitter taste to the product. The inhibitor may have potential application as a sanitizing agent at a level of 50 μg/mL. Almost complete hydrolysis of the compound to lysine was effected with trypsin and pancreatin.

The broad spectrum antimicrobial effects of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1) have been known for some time (Vogler et al., 1964; Molin, 1964a–e; Dabrowska et al., 1976). Against many Gram-positive and Gram-negative organisms, this compound is significantly more effective than bacitracin, potassium benzylpenicillin, streptomycin, chloramphenicol, or oxytetracyclin (Molin, 1964c; Vogler et al., 1964). Molin (1964a) has also shown that R-1 effectively inhibits the growth of a number of fungi at a concentration much lower than that of potassium sorbate, which is a commonly used antifungal agent in foods. In addition, R-1 is relatively nontoxic, with an oral $LD_{50}$ in mice of 3500 mg/kg. The compound is also very digestible in the presence of proteolytic enzymes, yielding more than 90% of its lysine in guinea pig intestinal juice at 37 °C for 24 h (Vogler et al., 1964).

These properties present a promising prospect for the use of R-1 as a food preservative. It was the purpose of this work to investigate the potential application of this compound for use as a preservative in selected dairy products, with primary emphasis on creamed cottage cheese. Also, a preliminary study designed to test the sanitizing action of R-1 was initiated. In addition, the effect of various proteolytic enzymes on R-1 was determined.

## EXPERIMENTAL SECTION

**Analytical Procedures.** The NMR spectrum of R-1 was recorded using a Varian EM390 spectrometer employing tetramethylsilane as an internal standard. The mass spectrum of R-1 was prepared by using an AEI-MS9-DS50 spectrometer operated at 70 eV and having a source temperature of 160 °C. Optical rotations $[\alpha]_D$ were obtained with a Perkin-Elmer Model 241 polarimeter. The $R_f$ values from the thin-layer chromatograms were determined on precoated (0.25 mm) Brinkman thin-layer chromatography plates (silica gel G/UV$_{254}$). Shortwave ultraviolet light (mineralite UVS 11), iodine crystals, ninhydrin spray, and chlorine peptide spray (Stahl, 1969; Stewart and Young, 1969) were used as needed to detect

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the spots. Melting points were not corrected.

**Reagents.** Common laboratory chemicals used were all reagent grade or better. Distilled, deionized water was used throughout these studies. L-Lysine monohydrochloride (99+%), carbobenzoxy chloride (95%), triethylamine (TEA, 99%), $N,N'$-dicyclohexylcarbodiimide (DCC, 99%), and palladium on activated carbon (10% Pd) were all obtained from Aldrich Chemical Co., Milwaukee, WI. The TEA and DCC were redistilled prior to use. Palmitoyl chloride (99%) was purchased from Sigma Chemical Co., St. Louis, MO.

Nutrient broth and nutrient agar, plate count agar, and potato dextrose agar were products of Difco, Detroit, MI. Cottage cheese curd and dressing mix were obtained from the University of Wisconsin Dairy Plant. Cultures of *Flavobacterium aquatile* NRRL-B2157, *Achromobacter pestifer* NRRL-B4251, and *Pseudomonas viscosa* NRRL-2217 were kindly supplied by the United States Department of Agriculture, Northern Regional Research Center, Peoria, IL, whereas the remaining cultures belonged to the culture collection of the Department of Food Science, University of Wisconsin.

Enzymes used in the digestibility experiments included trypsin 4X, pancreatin (hog pancreas) from Nutritional Biochemicals Corp., Cleveland, OH, and trypsin (bovine pancreas), type III, Sigma Chemical Co., St. Louis, MO.

**Chemical Synthesis.** The complete scheme for the synthesis of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride is presented in Figure 1. This is essentially the procedure employed by Vogler et al. (1964). The following NMR and mass spectral data were obtained for the $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride: NMR (Me$_2$SO-$d_6$) δ (from Me$_4$Si) 0.87 (t, 3 H, $J$ = 4.5 Hz, palmitoyl CH$_3$), 1.03–1.83 [complex pattern, 41 H; includes broad peak centered at 1.28, 27 H, palmitoyl C$_4$–C$_{15}$ CH$_2$, ester CH$_3$; includes broad multiplet centered at 1.52, 14 H, palmitoyl C$_3$$CH_2$, lysine C$_3$–C$_5$$CH_2$], 2.21 (t, 2 H, $J$ = 6 Hz, palmitoyl C$_2$$CH_2$), 2.72 (broad multiplet, 4 H, lysine C$_6$$CH_2$), 4.08 (q, 2 H, $J$ = 7.5 Hz, ester CH$_2$), 4.25 (broad, 2 H, α protons), 7.20–8.02 (broad peak, 6 H, NH$_3$$^+$), 8.02 (d, 1 H, $J$ = 7.5 Hz, NH), 8.33 (d, 1 H, $J$ = 7.5 Hz, NH); mass spectrum (70 eV) $m/e$ (rel intensity) 540 (17.5), 494 (23.2), 482 (31.0), 367 (10.6), 340 (11.9), 238 (18.2), 175 (29.8), 158 (12.6), 84 (100), 72 (37.1).

**Antimicrobial Testing.** The antimicrobial effectiveness of R-1 was tested on a number of microorganisms with emphasis on psychrotrophic spoilage organisms and pa-

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$NH_2-LYS-OH \cdot HCl$

1) $CuCO_3$
2) CARBOBENZOXY CHLORIDE
3) EDTA, 2N NaOH

$\begin{array}{c} Z \\ | \\ NH_2-LYS-OH \end{array}$ (1)

PALMITOYL CHLORIDE

EtOH, $SOCl_2$

$\begin{array}{c} Z \\ | \\ PAL-LYS-OH \end{array}$ (3)

$\begin{array}{c} Z \\ | \\ NH_2-LYS-OEt \cdot HCl \end{array}$ (2)

DIMETHYLFORMAMIDE
DICYCLOHEXYLCARBODIIMIDE

$\begin{array}{ccc} Z & & Z \\ | & & | \\ PAL-LYS- & & LYS-OEt \end{array}$ (4)

$H_2$, Pd, $H^+$

$PAL-LYS-LYS-OEt \cdot 2HCl$ (5)

$$CH_3-(CH_2)_{14}-\overset{\overset{\displaystyle O}{||}}{C}-NH-CH-\overset{\overset{\displaystyle O}{||}}{C}-NH-CH-\overset{\overset{\displaystyle O}{||}}{C}-OCH_2CH_3$$
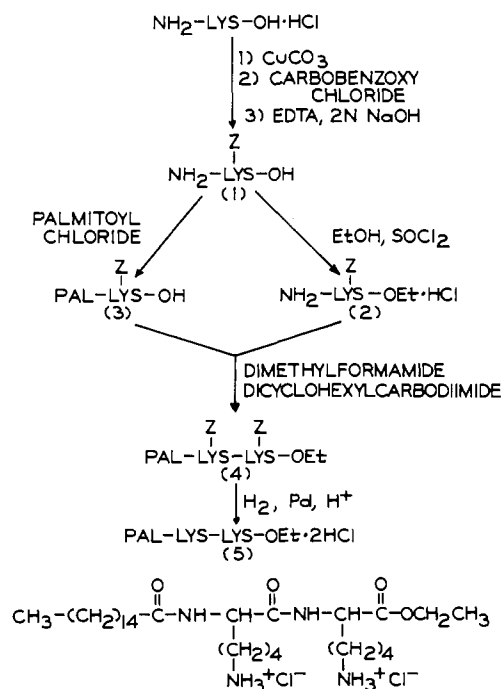$$\begin{array}{cc} (CH_2)_4 & (CH_2)_4 \\ | & | \\ NH_3^+Cl^- & NH_3^+Cl^- \end{array}$$

**Figure 1.** Reaction scheme for the synthesis of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1).

thogens sometimes found in dairy products. Initial assays were carried out in nutrient broth prior to testing in reconstituted nonfat dried milk, vanilla cream pudding, and creamed cottage cheese. A preliminary study was also performed to obtain some indication of the sanitizing activity of compound R-1.

The test bacteria consisted of freeze-dried or frozen specimens which were subcultured and maintained on plate count agar slants. Cell suspensions for the tests were prepared from agar slope cultures by serial subculture in nutrient broth. Inocula were taken from a 22–26-h-old culture in the logarithmic growth phase. The viable cell counts were obtained according to "Standard Methods for the Examination of Dairy Products" (Marth, 1978). Plates were incubated at 32 °C for 48 h for standard plate counts and at 21 °C for 3–5 days for yeast and mold counts. Samples were plated in duplicate, and average values are reported. Appropriate amounts of the inhibitor (R-1) were added to the test solutions in the form of either a powder or a liquid from a stock solution (10 000 μg/mL or 1000 μg/mL mixed in sterile water). Solutions of R-1, nutrient broth, nutrient agar, plate count agar, potato dextrose agar, phosphate buffer, and milk were sterilized by autoclaving at 121 °C for 15 min.

Specific testing procedures were as follows.

*Nutrient Broth.* Appropriate amounts of R-1 stock solution were added to 9 mL of sterile nutrient broth (for the 1-h exposure studies) or to 19 mL of sterile nutrient broth (for the continuous exposure studies) to provide the desired inhibitor concentrations. A control tube with no inhibitor added was included for each organism tested. The tubes were tempered at 21 °C, inoculated with 1.0 mL of the test organism, and incubated at 21 °C for the desired length of time at which point standard plate counts were obtained.

*Reconstituted Nonfat Dried Milk.* Samples of sterile reconstituted nonfat dried milk (149 mL) were prepared, and the appropriate amount of the R-1 stock solution was added to provide inhibitor concentrations of from 0 to 500 μg/mL. These samples were inoculated with 1.0 mL of a 1:100 dilution of the test organism and then incubated at

7 °C for 21 days or at 21 °C for 5 days. Standard plate counts were prepared every 1–3 days.

*Vanilla Cream Pudding.* Seventy grams of vanilla cream pudding, which was prepared from original ingredients according to a domestic recipe, was weighed into a beaker and 0.5 mL of a *Staphylococcus aureus* culture was added and mixed throughout the sample. Twenty grams of this mixture was added to each of three 4-oz paperboard cups with lids. Inhibitor powder (R-1) was added with mixing to two of these cups to provide concentrations of 750 or 1500 μg/g. Twenty grams of the pudding alone was added to a fourth cup, and all of the samples were incubated at 21 °C for 3 days. One-gram samples were taken at 4–24-h intervals and standard plate counts were performed by using the bag method.

*Creamed Cottage Cheese.* Cream dressing (36.5 g) was weighed into each of three separate beakers, whereas 83.5 g of cheese curd was weighed into each of three additional beakers. The R-1 powder (90 mg) was evenly mixed into one batch of cream dressing, whereas 180 mg of R-1 was mixed into a second batch of cream dressing. Curd was then added to each of the cream mixtures and stirred until well mixed. Each of these 120-g batches of creamed cottage cheese was equally divided into three 40-g samples, which were separately weighed into 5-oz paperboard cups with lids. A 0.2-mL aliquot of a 1:100 dilution of the test organism was added to each of the appropriate containers as indicated. The cottage cheese was incubated at 7 °C for 26 days. At 1–7-day intervals, 1.0-g samples were taken for making standard plate counts and yeast and mold counts by using the bag method. Additional samples were taken for pH measurements. At the end of 26 days, all samples were compared on the basis of their appearance and odor.

*Sanitizing Activity.* The Modified Cade and Halvorson method was used for studying the sanitizing activity of R-1 (Block, 1977). *Escherichia coli* ATCC 15222 and *Pseudomonas putrefaciens* were employed as the test organisms.

**Enzymic Proteolysis.** Ten milligrams of purified or crude enzyme in addition to 10 mg of R-1 was added to 10 mL of 0.05 M Tris buffer, pH 8, containing 0.2 mL of a 0.5 M solution of $CaCl_2$. The solutions were covered and gently stirred at 31 °C for 72 h. One-milliliter aliquots were periodically removed and evaporated to near dryness in vacuo in a rotary evaporator at 30 °C, and approximately 3 μL of the concentrated solution was spotted on silica gel G plates. The TLC plates were developed in butanol-acetic acid–water–pyridine (15:3:12:10, v/v) and spots were visualized after spraying the plates with ninhydrin solution.

RESULTS AND DISCUSSION

**Chemical Synthesis.** The compound R-1 was synthesized by using conventional peptide chemistry techniques. Purity of each of the compounds in the reaction scheme (Figure 1) was estimated to be ≥95% by using TLC in three different solvent systems. In general, the yields of product at each step of the synthesis were good with the exception of the ethyl ester of $N^\epsilon$-carbobenzoxylysine (2) which reflected the difficulty in synthesizing this compound. Melting points and optical rotations were in general agreement with those reported in the literature. The structure of R-1 was supported by the NMR and mass spectra. R-1 was too unstable in the hydrochloride form to provide a molecular ion in the mass spectrum. Loss of two HCl molecules corresponds to an ion of $m/e$ 540, whereas the ion fragment of $m/e$ 494 follows from the additional loss of the ethyl ester. The ion of $m/e$ 84 which is the base peak in the mass spectrum undoubtedly arises

Table I. Antimicrobial Effects of $N^{\alpha}$-Palmitoyl-L-lysyl-L-lysine Ethyl Ester Dihydrochloride (R-1) in Nutrient Broth (Exposure Time, 60 min)

| log reduction[a] | micrograms/milliliter | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Escherichia coli*[b] | *Alcaligenes faecalis*[b] | *Pseudomonas aeruginosa*[b] | *Pseudomonas viscosa*[b] | *Pseudomonas fragi*[b] | *Pseudomonas putrefaciens*[b] | *Pseudomonas putrefaciens*[c] |
| 1 | 1.0 | 44.0 | 7.0 | 4.5 | 2.0 | 2.0 | 6.0 |
| 2 | 3.0 | 81.0 | 11.0 | 10.0 | 3.5 | 3.0 | 7.5 |
| 3 | 6.5 | | 16.0 | 18.5 | 5.0 | 4.5 | 9.0 |
| 4 | 10.0 | | | | | 10.0 | 11.5 |
| 7 | | | | | | | ≤25.0 |

[a] Initial cell population ca. 10⁷/mL.    [b] 1 °C.    [c] 7 °C.

from the lysine residue since the mass spectrum of lysine ethyl ester also has the ion of $m/e$ 84 as its most prominent fragment (ASTM, 1969).

**Antimicrobial Activity of R-1 in Nutrient Broth.** Preliminary testing was carried out in nutrient broth to obtain comparative data with those of previous investigators (Vogler et al., 1964; Molin, 1964a-e; Dabrowska et al., 1976). These researchers generally reported inhibitory effects at from 5 to 50 μg/mL against a variety of Gram-positive and Gram-negative microorganisms with Molin (1964a) also reporting inhibitory effects against many yeasts and molds at 30–150 μg/mL. Results obtained from this investigation were in good agreement with those reported by the previous investigators. All but one of the ten organisms tested showed inhibitory effects at ≤10 μg/mL. The most resistant organism tested, *Alcaligenes faecalis*, underwent logarithmic death when incubated at 7 °C with no inhibitor added. This organism should therefore have little significance in the spoilage or pathogenicity of dairy products which are the primary concerns of this investigation. Psychrotrophic organisms in the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Escherichia* are known to be primary causes of spoilage in dairy products (Frazier, 1967; Marth, 1970). These organisms in addition to the pathogens *Staphyloccus aureus* and *Salmonella typhimurium*, which are also known to occur in dairy products, were the organisms of greatest interest in these studies.

It should be pointed out that the activity of R-1 has been shown to be quite different from that of any of its chemical constituents. Vogler et al. (1964) reported no activity of palmitic acid or the lysine ester against either Gram-positive or Gram-negative organisms. $N^{\alpha}$-Palmitoyllysine had some activity against some Gram-positive organisms, but at a reduced level compared to R-1, while having little or no activity against Gram-negative organisms. Some fatty acids possess antibacterial activity; however, in general this activity is limited to Gram-positive organisms (Nieman, 1954). Camien and Dunn (1957) have demonstrated antimicrobial activity of palmitic acid against several species of lactobacilli at from 0.1 to 420 μg/mL. Kato and Shibasaki (1975) have shown that a $C_{10}$ saturated fatty acid (capric acid) has antifungal activity against the molds *Aspergillus niger* (172 μg/mL) and *Penicillium citrinum* (172 μg/mL) as well as the yeasts *Saccharomyces cerevisiae* (86 μg/mL) and *Candida utilis* (172 μg/mL). On the other hand, the antimicrobial activity of palmitic acid against *Saccharomyces cerevisiae* and *Candida albicans* is evident at >1000 μg/mL as is the case for several other Gram-positive bacteria investigated (Kabara and Vrable, 1977). Molin (1964b) noted that both the fatty acid and the amino acid portion of R-1 are equally important in providing the antimicrobial properties of this compound. He noted that the dipeptide with two lysyl residues was more active than the corresponding compound with only one, and that differences in action between R-1 and sat-

urated fatty acids were also demonstrated by the fact that esters of fatty acids were inactive, whereas the activity of $N^{\alpha}$-palmitoyllysyllysine was strongly enhanced by esterification of the carboxyl group. Molin (1964d) also observed that when equimolar amounts of palmitic, linoleic, capric, or lauric acids were added to R-1 the antimicrobial activity was completely reversed. This again supports the view that the antimicrobial effects of R-1 are not a result of any activity exerted by palmitic acid.

The rate of death rather than complete death was measured in this investigation, following the method of Molin (1964a-e). Initial tests were performed using a 1-h exposure in nutrient broth at 21 °C. Concentrations of R-1 required to give logarithmic reduction in cell populations under these conditions are given in Table I. These data revealed the very high antimicrobial activity of R-1 under the given test conditions. In addition, exposure of *Pseudomonas putrefaciens* to R-1 at a lower temperature decreased the activity of R-1 at concentrations <10 μg/mL. A similar observation was made by Molin (1964c), who reported progressively decreasing activity of R-1 at 20 μg/mL with decreasing temperature until he found no activity at 6.5 °C for *Pseudomonas* sp. 128.

Molin (1964a) observed that when some bacteria were grown in the presence of R-1, a certain fraction of resistant cells frequently emerge which were capable of dividing at the same rate or faster than the initial population. To further investigate this phenomenon, a study was undertaken on a number of organisms, using a wide range of inhibitor concentrations. The results in Figure 2 indicate that within 24 h *Pseudomonas putrefaciens*, *Flavobacterium aquatile*, and *Staphylococcus aureus* were effectively reduced to a zero population at 5 μg/mL of R-1, whereas *Achromobacter pestifer* and *Salmonella typhimurium* required 50 μg/mL of R-1 to obtain the same results. Essentially no growth was evident in the totally inhibited cultures for 15–16 days at which time observations were ceased.

Several observations were made pertaining to the test organism's ability to adapt to the inhibitor. Not all of the organisms tested showed an ability to adapt; *F. aquatile* was unable to adapt even at 1 μg/mL. For those that did adapt, it appeared that there was some threshold level of the inhibitor above which the microorganisms could not adapt. Apparently, this effective concentration must be high enough to decrease the population level essentially to zero although both *S. aureus* and *S. typhimurium* were able to adapt to R-1 after being reduced to a zero population level. For unknown reasons, *S. aureus* was able to adapt to 10 μg/mL but not to 5 μg/mL of R-1. Although the probability of survival and adaptation decreased as the concentration of R-1 increased, there is always some possibility of survival which may have been observed. The length of time required for the organism to adapt varied with the organism (1–24 h for *P. putrefaciens*, 1–2 days for *S. typhimurium*, 2–4 days for *A. pestifer*, and 4–8 days
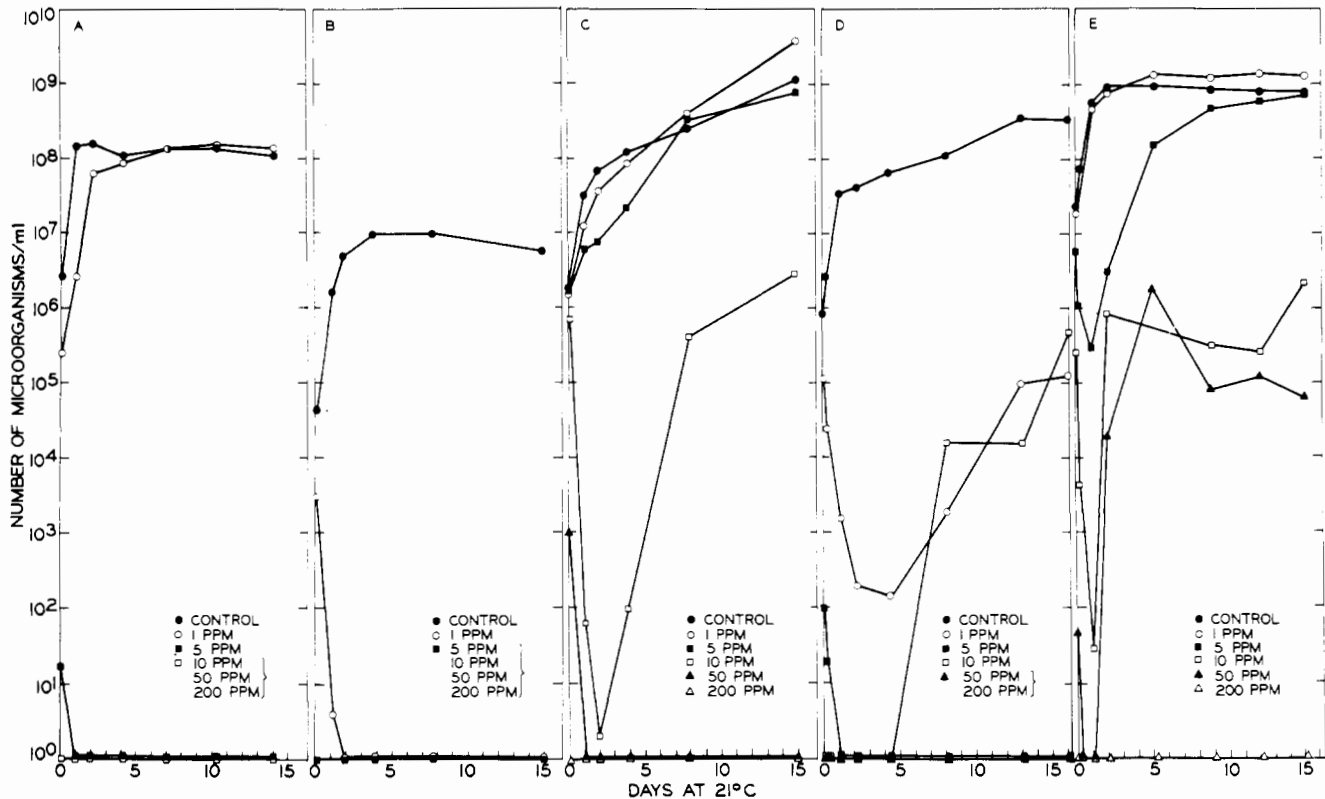
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**Figure 2.** Antimicrobial effects of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1) on selected psychrotrophic spoilage organisms and pathogens in nutrient broth at 21 °C: (A) *Pseudomonas putrefaciens*, (B) *Flavobacterium aquatile*, (C) *Achromobacter pestifer*, (D) *Staphylococcus aureus*, (E) *Salmonella typhimurium*. Ppm is micrograms/milliliter.

for *S. aureus*) and also was fairly constant for a given organism regardless of the inhibitor concentration. Also, if the organism was severely inhibited, but was still able to adapt, its rate of growth became constant at a population level several log cycles below the control level of growth (Figure 2C,D,E). In addition, low concentrations of the inhibitor may not only produce a decrease in population during the initial stages of exposure, but may actually result in a growth promoting effect after several days of continuous exposure (1 $\mu$g/mL for *A. pestifer* and *S. typhimurium*, Figure 2C,E).

**Antimicrobial Activity of R-1 in Dairy Products.** Sterile reconstituted nonfat dried milk inoculated with pure cultures of ca. $10^7$ organisms/mL was the first product tested. Inhibitor concentrations of 500 $\mu$g/mL were necessary to obtain a substantial inhibitory response, yet even this level of R-1 did not produce sufficient inhibition to prevent adaptation by the test organism. The 500 $\mu$g/mL is approximately 100-fold the concentration required to obtain a similar response in nutrient broth. This response might be expected in view of a previous observation that addition of 0.01% skim milk to a test medium reduced the inhibitory effect of R-1 (Molin, 1964c). Possibly the positively charged inhibitor is bound to the negatively charged milk proteins at the pH of milk.

There was some interest in testing R-1 in vanilla cream pudding because *S. aureus* has been known to grow in this type of product. When this occurs, food-borne illness is a likely result. The purpose of this experiment was to determine the efficacy of using R-1 in such a product as a means of reducing the likelihood of such a public health hazard from occurring. The results indicated that when the pudding was inoculated with ca. $10^6$ *S. aureus*/g and incubated at 21 °C a bacteriostatic effect could be maintained for 24 h by using R-1 at 1500 $\mu$g/g. Between 24 and 48 h the *S. aureus* population rose considerably; however,

the natural flora of the pudding in the absence of R-1 also rose to very high levels during this time and resulted in a product that would have been organoleptically unacceptable. While inhibiting an already high population of *S. aureus* from growing in pudding will not prevent food-borne disease from this organism, preventing a low population from growing, as would be expected to be present under a normal load of contaminating *S. aureus* in such a product, may prove to be beneficial in preventing staphylococcal food poisoning in these products.

Creamed cottage cheese is a rather perishable product subject to spoilage prior to consumption (Frazier, 1967). Psychrotrophic organisms most likely to cause defects in cottage cheese include organisms from the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, and *Escherichia* as well as a number of yeasts and molds (Marth, 1970). The purpose of this experiment was to determine if R-1 could be used to effectively improve the keeping quality of this product.

Results, given in Figure 3, are best examined by comparing samples which have been given similar microbiological treatments (1, 2, 3; 4, 5, 6; 7, 8, 9). It is assumed that quality defects can be detected at a total plate count of $10^6$ organisms/g or a yeast and mold count of $10^5$/g. The data indicate that the shelf-life of creamed cottage cheese held at 7 °C can be extended by 2.5–3-fold by using 1500 $\mu$g/g of R-1 based on the total plate count, or by about 2-fold based on the yeast and mold count.

This effect is most dramatically seen in comparing the samples in which no bacteria were added (1, 2, 3). Both 750 and 1500 $\mu$g/g of R-1 extended the shelf-life from 9.5 to >26 days based on total plate counts. From yeast and mold counts, the shelf-life was extended from 11.5 to 22.5 days for 750 $\mu$g/g or 24.5 days for 1500 $\mu$g/g of R-1. Samples inoculated with high numbers of *P. putrefaciens* had their shelf-life extended from 2.5 to 8.5 days in the
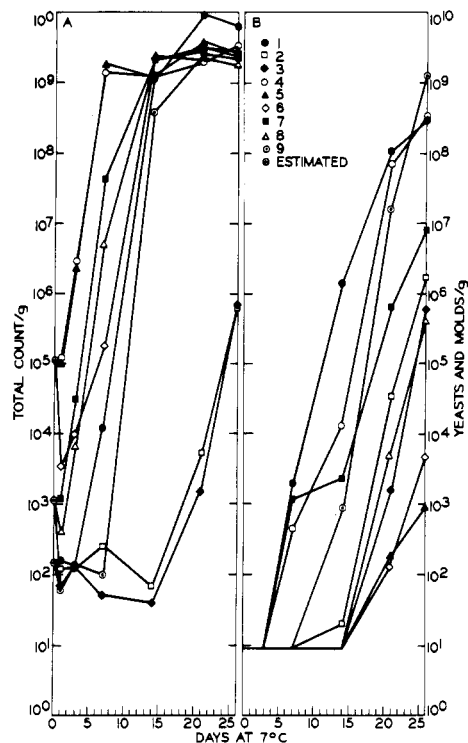
**Figure 3.** Antimicrobial effects of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1) in creamed cottage cheese held at 7 °C: (A) standard plate count, (B) yeast and mold count. Additions to creamed cottage cheese are as follows: (1) none, (2) 750 µg/g R-1, (3) 1500 µg/g R-1, (4) *Pseudomonas putrefaciens*, (5) *P. putrefaciens*, 750 µg/g R-1, (6) *P. putrefaciens*, 1500 µg/g R-1, (7) *Achromobacter pestifer*, (8) *A. pestifer*, 750 µg/g R-1, (9) *A. pestifer*, 1500 µg/g R-1.

**Table II.** Effect of $N^\alpha$-Palmitoyl-L-lysyl-L-lysine Ethyl Ester Dihydrochloride (R-1) on pH of Creamed Cottage Cheese Held at 7 °C

| sample no.[a] | days held at 7 °C | | | | |
|---|---|---|---|---|---|
| | 1 | 7 | 14 | 21 | 26 |
| 1 | 4.86 | 4.87 | 5.13 | 5.13 | 5.33 |
| 2 | 4.91 | 4.87 | 4.92 | 4.96 | 4.88 |
| 3 | 4.89 | 4.90 | 4.92 | 4.90 | 4.82 |
| 4 | 4.89 | 5.06 | 5.15 | 4.99 | 4.93 |
| 5 | 4.92 | 5.04 | 5.16 | 5.07 | 4.97 |
| 6 | 4.90 | 4.90 | 5.14 | 5.06 | 4.93 |
| 7 | 4.93 | 4.92 | 5.16 | 5.02 | 5.03 |
| 8 | 4.93 | 4.92 | 5.12 | 4.97 | 4.85 |
| 9 | 4.89 | 4.85 | 5.04 | 5.00 | 4.89 |

[a] Additions to creamed cottage cheese are as follows: (1) none, (2) 750 µg/g R-1, (3) 1500 µg/g R-1, (4) *Pseudomonas putrefaciens*, (5) *P. putrefaciens*, 750 µg/g R-1, (6) *P. putrefaciens*, 1500 µg/g R-1, (7) *Achromobacter pestifer*, (8) *A. pestifer*, 750 µg/g R-1, (9) *A. pestifer*, 1500 µg/g R-1.

presence of 1500 µg/g of R-1, whereas those containing 1500 µg/g of R-1 and inoculated with *A. pestifer* were acceptable for 11.5 days compared to 5 days for the control. These data indicate that using R-1 in cottage cheese samples containing a high initial bacterial load will not extend the shelf-life appreciably, but using R-1 in cottage cheese which has been prepared according to good manufacturing practices can have a very substantial effect in extending its shelf-life.

The data from the yeast and mold counts indicated that the growth of these organisms in samples containing either 750 or 1500 µg/g of R-1 was in general inhibited for an additional 2 weeks beyond those samples containing no inhibitor at all. The samples which showed the least amount of yeast and mold growth were those samples containing both the inhibitor and *P. putrefaciens*. The reason for this is unknown. It is unlikely that this response is simply the result of competitive inhibition since almost all of the samples had nearly the same total plate counts after the first 2 weeks of the test. It also seems unlikely that this response is the result of some natural inhibitor being produced by *P. putrefaciens* since the sample containing this organism with no R-1 added showed no signs of having such an inhibitory effect. There is apparently some type of synergistic reaction which takes place between R-1 and *P. putrefaciens* and/or its metabolites which serves to inhibit the yeasts and molds to a greater extent. Of still greater uncertainty is the cause for the response observed in sample 9. These yeasts and molds appear to have adapted more quickly to R-1 and actually experienced a growth-promoting effect in comparison to the other samples. An alternative explanation could be that these organisms were introduced into sample 9 as contaminants during testing and actually had a higher

resistance to R-1 initially as compared to those yeasts and molds naturally found present in the cottage cheese microflora of these samples.

Samples of cottage cheese containing R-1 with no microorganisms added also proved to be the most stable as determined by pH measurement. These samples maintained a relatively constant pH reading fluctuating within 0.10 pH unit throughout the 26 day incubation period (Table II). The most unstable sample was cottage cheese alone which had a rise of 0.47 pH unit after 26 days of incubation. Other samples showed a variance of from 0.19 to 0.27 pH unit, demonstrating a rise and subsequent fall in pH measurement. A rise in pH may be attributed to proteolysis by the spoilage organisms or to metabolism of the organic acids present by the yeast and mold. The subsequent drop in pH could be the result of acid production by lactic acid bacteria. These bacteria may have been present in low numbers initially and their growth would then have been aided by the more alkaline environment produced by the other spoilage organisms.

The samples were also rated on the basis of their appearance and odor. As would be expected on the basis of the data presented, the cottage cheese containing 1500 µg/g of R-1 was rated as the most organoleptically acceptable, whereas the cottage cheese alone received the worst overall rating. Organoleptic quality as measured by appearance and odor seemed to be most closely correlated with yeast and mold growth. In general, samples with low yeast and mold counts received the highest overall ratings since yeast and mold produced the most visible changes resulting from surface growth and discoloration. Also, they are probably more lipolytic than the bacteria present and therefore contribute more to the rancid off odors associated with hydrolysis of butterfat (Frazier, 1967).

To test any effects of R-1 on the flavor of the cottage cheese from R-1 itself, two individuals tasted creamed cottage cheese containing either 750 or 1500 µg/g of R-1. Creamed cottage cheese alone was used as a control in the blind test. The sample containing 1500 µg/g of R-1 was decidedly bitter, whereas the sample containing 750 µg/g of R-1 was only slightly bitter. This suggests that lower concentrations of R-1 should be explored to find its minimal inhibitory concentration in creamed cottage cheese as well as its threshold level of detection in this product.

**Sanitizing Activity of R-1.** R-1 is water soluble, has a broad antimicrobial spectrum at low concentrations, is noncorrosive, and should be safe both to food and to humans. Because of these properties a preliminary study was initiated to investigate the potential application of R-1 as

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Table III. Sanitizing Activity of
$N^\alpha$-Palmitoyl-L-lysyl-L-lysine Ethyl Ester Dihydrochloride
(R-1) in Distilled, Deionized Water at 21 °C

| microorganism | µg/mL | 1 min | 2 min | 5 min | 10 min |
|---|---|---|---|---|---|
| *Escherichia* | 0 | 2500 | 2800 | 4600 | 2600 |
| *coli*[a] | 50 | 11 | 340 | 1 | 1 |
| *Pseudomonas* | 0 | 350 | 720 | 480 | 450 |
| *putrefaciens*[a] | 50 | 450 | 1 | 25 | 0 |

[a] No bacterial growth was observed at 100 or 200 µg/mL R-1.
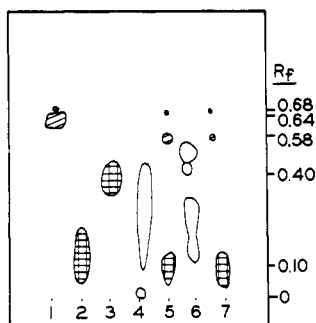


**Figure 4.** Enzymic digestion of $N^\alpha$-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride (R-1) with trypsin and pancreatin. Weight ratio of enzyme to compound is 1:1 in Tris buffer, pH 8, at 31 °C for 24 h. Thin-layer plates developed with butanol–acetic acid–water–pyridine (15:3:12:10, v/v) and spots visualized with ninhydrin: (1) R-1, (2) lysine, (3) lysine ethyl ester, (4) trypsin, (5) trypsin hydrolysate of R-1, (6) pancreatin, (7) pancreatin hydrolysate of R-1.

a sanitizing agent. Use of the test employed is restricted to presumptive evaluations and screening operations. Counts of less than ten organisms in the subculture plates at the longest interval considered potentially significant in the proposed use would be necessary as an index of a significant reduction. The data indicate a significant reduction for both of the test organisms at 50 µg/mL for a 10-min exposure (Table III). Since sanitizing agents are sometimes used at 200 µg/mL, further studies may be warranted to determine the effective level of R-1 as a sanitizing agent.

**Enzymic Proteolysis of R-1.** On the basis of its chemical composition, R-1 would be expected to be completely metabolized to its natural components of lysine and palmitic acid. To verify this, enzymic studies were undertaken using trypsin and pancreatin. Such a study would have a bearing on the safety of R-1 in food related applications.

Proteolysis was followed using TLC, and the results are given in Figure 4. These results indicate that most of R-1 is converted to lysine within 24 h by using either trypsin or pancreatin under the given test conditions. It appeared that both the peptide bond and the ester bond were completely hydrolyzed; no R-1 or lysine ethyl ester was detected in the hydrolysates. The compound having an $R_f$

value of 0.58 is believed to be $N^\alpha$-palmitoyllysine, whereas the remaining compound at an $R_f$ value of 0.68 is a minor contaminant carried over from the R-1 preparation. These results agree with the findings of Vogler et al. (1964), who reported a 90% conversion of R-1 to lysine, using guinea pig intestinal juice at 37 °C for 24 h. This author also reported that the amide bond of R-1 was hydrolyzed much more slowly than was the peptide bond. In our investigation, essentially no change was found in the amount of the compound with an $R_f$ value of 0.58 between 24 and 72 h of treatment.

On the basis of the available data, R-1 has potential application as a safe and effective food preservative. The possibility also exists for its use as a sanitizing agent. Further studies are needed to more completely understand the advantages and disadvantages of using R-1 in food-related applications. Related peptide derivatives should be synthesized to retain the beneficial effects of R-1 without its disadvantage of a bitter taste.

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