Semisynthetic Coumermycins: Structure-Activity Relationships

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The relative antimicrobial activity of a large series of semisynthetic coumermycins has been determined. Most of the derivatives, which were 3-substituted-4-hydroxy-8-methyl-7-[3-O-(5-methyl-2-pyrrolylcarbonyl)noviosyloxy] coumarins, had an in vitro antibacterial spectrum similar to that of the parent compound, coumermycin A₁, but were generally less potent in minimal inhibitory concentration (MIC) tests. Derivatives with an alkylcarboxamido, arylcarboxamido, or arylsulfonamido group in the 3 position had considerably greater in vitro activity than those possessing an amino-, aryl-, or alkureido substituent. Efficacy in Staphylococcus aureus Smith infections of mice was greater for those compounds with branched-chain alkylcarboxamido, unsubstituted, mono- or disubstituted aryl- and heteroaryl-carboxamido groups than for derivatives having an n-alkylcarboxamido, aralkyl-carboxamido, arylsulfonamido, or trisubstituted arylcarboxamido substituent. Significant in vitro activity against Klebsiella pneumoniae and other gram-negative species was restricted to those compounds having a 3-(3-n-alkyl-4-hydroxy-phenyl-carboxamido) group. Only the n-hexyl homologue demonstrated in vivo activity in a K. pneumoniae infection. Many derivatives were two- to threefold more active than coumermycin A₁ in orally treated mouse infections, despite the fact that their MIC values were considerably higher. This result was undoubtedly a reflection of the markedly greater oral absorbability possessed by many of the derivatives. Although peak oral mouse blood levels of some compounds were >25 times higher than those of coumermycin A₁, their toxicity for the host was no greater. In addition, the semisynthetic coumermycins caused much less local irritation than coumermycin A₁ when administered parenterally.

The antibiotic coumermycin A₁ was first isolated from the fermentation broths of Streptomyces rishiriensis by Kawaguchi and his fellow workers (5). The purified compound was found to be an acidic, white crystalline substance with extremely potent antibacterial activity, particularly against staphylococci. The spectrum of microorganisms inhibited by the antibiotic included streptococci, pneumococci, Bacillus, and mycobacteria species, as well as a number of Enterobacteriaceae strains. Coumermycin A₁ was also highly effective in protecting mice from an experimental Staphylococcus aureus infection. In subsequent studies, Grunberg and his co-workers (3, 4) found that the compound successfully controlled infections of mice produced by Streptococcus pyogenes, Diplococcus pneumoniae, Neisseria meningitidis, Klebsiella pneumoniae, and the murine meningoc pneumoniae agent. No therapeutic effect was obtained in infections caused by mycobacterial species or by gram-negative bacteria other than K. pneumoniae.

Although the antibiotic's subcutaneous LD₅₀ of 250 to 380 mg/kg and oral LD₅₀ of >2,000 mg/kg (3, 5) indicate only a moderate level of toxicity, the compound does have several undesirable characteristics. The most important of these are its low degree of oral absorbancy and its irritating effect on tissues at the site of parenteral administration.

Elucidation of coumermycin's structure (1, 6) revealed that it contained several moieties in common with novobiocin (Fig. 1). Structural modification of the latter antibiotic was achieved by Walton, McDaniel, and Woodruff (11) but without any increase in the compound's antibacterial potency or spectrum. Unfortunately, due to the biosynthetic methods employed in these studies, structural changes were restricted to sub-

¹ Deceased, February 1969.
SEMISYNTHETIC COUMERMYCINS

Figure 1. Structural formulae of coumermycin A₁ and novobiocin.

stituting various amide-linked benzoic acids for novobiocin's 3-isopentenyl-4-hydroxybenzamide group. When the decision was made to investigate the effect of structural changes on the antimicrobial and pharmacological properties of coumermycin A₁, it was decided to utilize chemical rather than biosynthetic modifications in order to avoid any such limitations.

Preliminary studies with crude preparations revealed that substitution of other groups for the 5-methylpyrrolyl or derivatization of noviose or coumarin hydroxyl groups resulted in a marked decrease or complete loss in bioactivity. Efforts were therefore concentrated on attempts to degrade the antibiotic into two identical residues composed of a methylpyrrole-noviose-coumarin (PNC) oxazole and a 3-methylpyrrole-2,4-dicarboxylic acid fragment. In the course of fractionating these products, it was discovered that a new amide was present which contained the PNC grouping and the acyl portion of whichever acid chloride or acid anhydride had been added to the reaction mixture (7, 8).

This transacylation procedure permitted many new PNC-amides to be prepared. However, some derivatives were not obtainable in adequate yield by this method. This problem was essentially eliminated when it was found that the PNC-amine could be prepared in quantity. The compound was obtained by catalytic reduction of the PNC-benzyloxycarbamyl derivative which can readily be prepared via the transacylation route (J. G. Keil, I. R. Hooper, R. H. Schreiber, C. L. Swanson, and J. C. Godfrey, presented at the 9th ICAAC, Washington, D.C., 28 October 1969).

The availability of PNC-amine permitted many new semisynthetic coumermycin derivatives to be made in high yield by acylation with acid chlorides and acid anhydrides.

The present report describes structure-activity relationships found for a large series of PNC-amides.

MATERIALS AND METHODS

Compounds. Over 160 derivatives of coumermycin A₁ were synthesized either by direct transacylation of the antibiotic itself or by acylation of the free coumarin amino group possessed by one of its derivatives. The compounds (PNC derivatives) were prepared by the Bristol Laboratories Biochemistry Department and in almost all instances were at least 90% pure. In the case of compounds prepared by transacylation of coumermycin A₁, particular care was taken to insure that they were not contaminated with significant amounts of this potent antibiotic. Since most of the derivatives are not readily soluble when added to water at neutral pH, compounds were usually prepared for testing by adding them to NaHCO₃ solutions in which sodium salts presumably formed with the 4-hydroxy group of the coumarin moiety. In those cases where dimethyl-sulfoxide (DMSO), Tween 40, Tween 80, propylene glycol, or carboxymethyl cellulose (CMC), was used as a solubilizing or suspending agent, a specific notation has been made.

Measurement of in vitro antibacterial activity. The minimal inhibitory concentrations (MIC) of the PNC derivatives were determined by the standard twofold broth dilution technique (2) utilizing Antibiotic Assay Broth (BBL) or the same medium containing the indicated amount of human serum. Compounds were dissolved in DMSO and 1 part added to 9 parts NaHCO₃ solution (5%). After the pH was adjusted to 7.4, all derivatives were tested against one strain of each of three gram-positive species (D. pneumoniae (5% serum), Streplococcus pyogenes, and Staphylococcus aureus Smith) as well as against one strain of each of seven gram-negative species (Proteus morganii, P. mirabilis, Escherichia coli, Salmonella typhosa, S. enteritidis, K. pneumoniae, and Pseudomonas aeruginosa).

It was observed that any structural modification in the test compounds that affected their activity against the highly sensitive Staphylococcus aureus Smith strain appeared to induce a corresponding and proportional change in activity against the other two gram-positive species. Similarly, the degree of inhibitory effect of the compounds against K. pneumoniae, the most sensitive of the gram-negative species, was found to be representative of their activity against the other six members of this group of organisms. Consequently, inhibition values obtained in tests with these two species are considered to indicate the extent of a compound's overall antibacterial potency.

Effect of human serum on in vitro antibacterial activity. The MIC of each of the compounds for S. aureus Smith was determined not only in Antibiotic Assay Broth as indicated above, but also in the same medium supplemented with sufficient human serum to give a final concentration of 25%.

The higher concentrations required for inhibition of the S. aureus strain in the serum-containing medium were attributed to binding of the test compounds by serum proteins. The extent to which the MIC of a given compound increased was employed as a rough measure of its degree of binding to serum.

Measurement of in vivo antibacterial activity. The
median curative dose of each of the PNC derivatives was determined in an experimental S. aureus Smith infection. The mice employed in these studies were male, 18 to 22 g in weight, and of the Swiss-Webster line. They were infected by intraperitoneal (IP) administration of sufficient bacterial cells suspended in 5% mucin (200 LD₅₀) to kill all nontreated mice within 24 hr. The PNC derivatives were dissolved in DMSO and 1 part added to 9 parts NaHCO₃ (5%). Immediately after challenge they were administered per os, by means of hypodermic syringe fitted with a blunt 18-gauge needle, to five animals per dose level. At the termination of the experiments (96 hr), the number of surviving mice was recorded and the CD₅₀, 50% curative dose (the dose in milligrams per kilogram required to cure 50% of the infected mice), was estimated by means of a log-probit plot.

K. pneumoniae infections of mice were induced by IP administration of the bacterial cells in mucin-free saline (minimum of 75 LD₅₀). Oral or intramuscular (IM) treatment was given at both the time of challenge and 4 hr later. After 96 hr, the CD₅₀ was estimated as described above.

Mouse blood level determinations. Male, Swiss-Webster mice ranging in weight from 18 to 22 g were employed in oral blood level studies. Compounds were suspended in a 5% NaHCO₃ solution that contained 0.4% CMC and 0.93% Tween 40. The suspensions were administered by means of a blunt 18-gauge needle to mice that had been fasted for 18 hr. Blood samples were collected from the orbital sinuses by means of heparinized capillary tubes (Clay-Adams) and used to saturate 6.35-mm filter-paper discs (no. 740E; Schleicher & Schuell Co., Keene, N.H.). After drying, discs were assayed against S. aureus Smith ATCC 6538P on thin-layer Seed Agar (BBL) plates at pH 6.0. This is basically the procedure described by Wick and Boniece (12). Diameters of inhibition zones were determined after incubating the plates at 37 C for 18 hr. Antibiotic potency of the samples was estimated by use of a dose response curve that had been prepared by supplementing heparinized mouse blood with known amounts of the PNC derivatives. Differences in average blood levels were analyzed by means of Student’s t test.

Injection pain and irritation studies. Sprague-Dawley, 175 to 200 g, male rats were used to determine whether pain upon injection or local tissue irritation, or both, occurred after injection of coumermycin A₁, or any one of several PNC derivatives into the plantar side of the hind paw.

Preparations (solutions or suspensions) were made up at both 5 and 1% concentrations by adding the compound to a solution comprised of 1 part DMSO and 9 parts 1% NaHCO₃. These were then administered in 0.1-ml volume into one hind paw of each of five rats per dose level. The other hind paw received 0.1 ml of the DMSO-NaHCO₃ vehicle alone. Pain upon injection was estimated subjectively, whereas the degree of irritation or paw edema was determined by the mercury-displacement method. Measurements were made at 1, 2, 4, 24, and 48 hr. A compound causing a mean increase in paw volume of 0.25 ml above the control paw volume, maintained for at least 4 hr, was considered to be irritating.

Acute toxicity determinations. The dosage producing 50% mortality (LD₅₀) after oral (PO) or IP administration was determined for coumermycin A₁ and for several PNC derivatives that had been suspended in propylene glycol containing 0.5% Tween 80. Survivors on the 10th day after administration of a single dose were noted and the LD₅₀ was calculated (9).

RESULTS

Although the bulk of compounds synthesized for this study were PNC-carboxamido derivatives, a few members of two other classes (PNC-sulfonamido and PNC-ureido derivatives) were prepared. Table 1 shows comparative MIC and CD₅₀ values for coumermycin A₁ and representative derivatives from the three classes of PNC compounds.

The marked activity of coumermycin A₁ against S. aureus Smith in in vitro tests was considerably reduced when the medium was supplemented with 25% human serum. Its MIC against K. pneumoniae in serum-free medium, although still low (3.1 μg/ml), was more than 1,000 times higher than that found for the S. aureus Smith culture. The in vitro antibacterial activity of all the PNC derivatives was much lower than that of coumermycin A₁, with the poorest activity being produced by the nonacylated PNC-amine. Although all remaining PNC compounds were more active than the free amine derivative, only four inhibited S. aureus Smith at 1.0 μg/ml or less, whereas none displayed significant activity against the K. pneumoniae strain. The four compounds with the greatest inhibitory effect on the S. aureus strain were representatives of the aryl-, alkyl-, and aralkylcarboxamido and arylsulfonamido classes. The lowest MIC observed among this group of semisynthetic coumermycins was that of the phenylcarboxamido. This compound also proved to be the most efficacious in the S. aureus Smith mouse protection test. The other two carboxamido derivatives (compounds 4 and 5) had poorer in vivo antistaphylococcal activity, whereas the sulfonamido derivative (compound 6) was inactive. The failure of this compound to display in vivo activity and the high CD₅₀ obtained with the aralkylcarboxamido representative can very likely be attributed to their relatively great susceptibility to serum binding. A high degree of binding for these compounds is suggested by the fact that their inhibitory concentrations in 25% serum are at least 50 times higher than in serum-free medium. The poor intrinsic activity of the aryl- and alkylureido derivatives probably is responsible for their lack of in vivo efficacy.

On the basis of results shown in Table 1, it was decided to confine further structure-activity relationship studies to those PNC derivatives possessing carboxamido groups.
Activity of monosubstituted benzamido derivatives. As shown in Table 1, the PNC derivative with the unsubstituted benzamido side chain (compound 3) gave a lower oral *S. aureus* Smith CD₅₀ than coumermycin A₁, despite having an MIC about 80 times higher in serum-free medium and 20 times higher in serum-containing medium. Substitution of a chlorine or methyl group into the ortho, meta, or para position of the benzene ring did not have a favorable effect on in vitro or in vivo activity. Regardless of the substituent's position, MIC and CD₅₀ values were two- to fourfold higher than those obtained with the unsubstituted parent compound. Similar results were obtained with the *p*-ido-, *p*-bromo-, and *p*-fluorobenzamido derivatives.

Several other PNC-benzamido compounds with *para* substituents with varying electron-withdrawing capacities were subjected to in vitro and in vivo evaluation. Results of these studies are summarized in Table 2.

It can be seen that there were no appreciable differences between compounds as regards their in vitro activity versus *S. aureus* Smith and *K. pneumoniae* when tests were conducted in serum-free medium. However, addition of 25% human serum to the test medium resulted in an increase in MIC that paralleled to some extent the increase in electron-withdrawing capacity of the *para* substituent. In vivo efficacy also appeared to be affected since there was a corresponding increase in the *S. aureus* Smith CD₅₀. The most active members of this series had in vivo activity about equal to that of the unsubstituted benzamido derivative (compound 3, Table 1).

Activity of disubstituted benzamido derivatives. In most cases, introduction of two chlorine atoms into the benzene ring had a depressing effect on in

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**TABLE 1.** Antibacterial activity of PNC derivatives having various substituents in the 3-position of the coumarin moiety.

| COMPOUND NUMBER | R GROUP | MINIMUM INHIBITORY CONCENTRATION (μg/ml) | CD₅₀ (mg/kg) |
|-----------------|---------|----------------------------------------|-------------|
|                 |         | **S. AUREUS SMITH** | **K. PNEUMONIAE** | **S. AUREUS SMITH** |
|                 |         | NO SERUM | 25% SERUM | NO SERUM | |
| 1               | COUMERMYCIN A₁ | (SEE FIG. 1) | 0.0008 | 0.016 | 3.1 | 17 |
| 2               | AMINO   | - - - - - | 12.5 | 50 | 100 | >100 |
| 3               | ARYL CARBOXAMIDO | - - - - - | 0.063 | 0.6 | 50 | 5.5 |
| 4               | ALKYL CARBOXAMIDO | - - - - - | 1.0 | 1.8 | 100 | 20 |
| 5               | ARALKYL CARBOXAMIDO | - - - - - | 0.4 | 50 | 100 | 68 |
| 6               | ARYL SULFONAMIDO | - - - - - | 0.8 | 50 | >100 | >100 |
| 7               | ARYLUREIDO | - - - - - | >1 | 12.5 | >100 | >100 |
| 8               | ALKYLUREIDO | - - - - - | 3.1 | 12.5 | 100 | >100 |

*4-HYDROXY-8-METHYL-7-(3-O-(5-METHYL-2-PYRROLYLCARBONYL) NOVIOXYLOXY) COUMARIN.

*Compounds were administered by oral route.*
vivo activity. However, there were profound differences in the compounds' in vivo efficacy, depending on the location of the substituents. Data obtained with several such derivatives are shown in Table 3.

As evidenced by the CD50 obtained with the 2,6-dichlorobenzoyl derivative, dissubstitution in the ortho position had a negligible effect on in vivo efficacy. This was not the case, however, with the 3,4-dichloro derivative which failed to give a measurable protective effect in the experimental mouse infection. Measurable but poor in vivo activity was found for the 2,5- and 3,5-dichlorobenzoyl compounds. The vast range of activity displayed by this group of chlorine-substituted derivatives could not have been predicted from MIC values in serum-free medium, but did correlate well with those obtained in serum-supplemented medium.

The MIC in serum and the S. aureus Smith CD50 of the 3,5-dinitro- and 3,5-diaminobenzoyl derivatives were no better than those of the 3,5-dichlorobenzoyl compound. However, poor efficacy is not a general property of 3,5-disubstituted compounds since the dimethylbenzoyl analogue was only slightly less active in in vitro and in vivo tests than the unsubstituted parent compound.

Among other semisynthetic coumermycins with disubstituted benzoic acid side chains examined were several that contained both hydroxyl and methyl substituents in the benzene ring. Data collected with these compounds are shown in Table 4.

The 4-hydroxy-3-methylbenzoyl derivative was by far the most potent of the three compounds. It had good in vivo activity and its MIC values for S. aureus Smith and K. pneumoniae appeared to be somewhat lower than those of the unsubstituted benzoyl. The excellent activity observed with this dissubstituted compound was not completely unexpected in view of the fact that the closely related antibiotic, novobiocin, also contains a 3-alkyl (2-butetyl-3-methyl)-4-hydroxybenzamido group at the 3 position of the coumarin moiety. Other derivatives with novobiocin-like side chains were then prepared as described by Schmitz et al. (10) and examined to determine whether an even greater increase in antibacterial potency could be obtained with substituents of this type.

**Activity of 3-substituted-4-hydroxybenzamido derivatives.** Derivatives having up to six straight-chain carbons in the 3 position were subjected to in vitro and in vivo studies. In vitro antistaphylococcal activities of compounds with 3, 4, 5, or 6 carbons were up to 20 times better than that of the 3-methyl homologue. The gram-negative activity of the derivatives, based on the K. pneumoniae MIC, was also considerably affected by the size of

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**TABLE 2. Antibacterial activity of PNC-benzamido derivatives having substituents with varying electron-withdrawing capacities in the para-position of the benzene ring.**

| COMPOUND | R GROUP | MINIMUM INHIBITORY CONCENTRATION (μg/ml) | CD50 (mg/kg) |
|-----------|---------|----------------------------------------|-------------|
| NUMBER    | NAME    | S. AUREUS SMITH | K. PNEUMONIAE | S. AUREUS SMITH |
|           |         | NO SERUM | 25% SERUM | NO SERUM |         | |
| 9         | AMINO   | -NH₂     | 0.08     | 0.8       | 50       | 8         |
| 10        | HYDROXY | -OH      | 0.16     | 0.8       | >100     | 8         |
| 11        | METHOXY | -OCH₃    | 0.08     | 3.2       | >100     | 12        |
| 12        | NITRO   | -NO₂     | 0.08     | 50        | 50       | 22        |

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*3-BENZAMIDO-4-HYDROXY-8-METHYL-7-[3-O-(5-METHYL-2-PYRROLYLCARBONYL)-NOVOXYLOXY] COUMARIN.

Compounds were administered by the oral route.
TABLE 3. Antibacterial activity of PNC-NH₂derivatives having side chains prepared from dichlorobenzoic acids.

| COMPOUND NUMBER | R GROUP | MINIMUM INHIBITORY CONCENTRATION (pg/ml) | CD₅₀ (mg/kg) |
|-----------------|---------|---------------------------------------|-------------|
|                 |         | S. AUREUS SMITH | K. PNEUMONIAE | S. AUREUS SMITH |
|                 |         | NO SERUM | 25% SERUM | NO SERUM | NO SERUM |
| 13              | BENZOYL | 0.063   | 0.6       | 100      | 5.5      |
| 14              | 2,6-DICHLOROBENZOYL | 0.031 | 0.31 | 50 | 6 |
| 15              | 2,5-DICHLOROBENZOYL | 0.031 | 2.5 | >100 | 50 |
| 16              | 3,5-DICHLOROBENZOYL | 0.063 | 50 | >100 | 70 |
| 17              | 3,4-DICHLOROBENZOYL | 0.16 | 50 | 50 | >100 |

*3-AMINO-4-HYDROXY-8-METHYL-7-[3-O-(5-METHYL-2-PYRROLYL)NOVIOXY]COUMARIN.

TABLE 4. Antibacterial activity of PNC-NH₂derivatives having side chains prepared from methyl and hydroxyl substituted benzoic acids.

| COMPOUND NUMBER | R GROUP | MINIMUM INHIBITORY CONCENTRATION (pg/ml) | CD₅₀ (mg/kg) |
|-----------------|---------|---------------------------------------|-------------|
|                 |         | S. AUREUS SMITH | K. PNEUMONIAE | S. AUREUS SMITH |
|                 |         | NO SERUM | 25% SERUM | NO SERUM | NO SERUM |
| 18              | BENZOYL | 0.063   | 0.6       | 100      | 9.5      |
| 19              | 4-HYDROXY-3-METHYL-BENZOL | 0.032 | 0.4 | 25 | 7 |
| 20              | 3-HYDROXY-4-METHYL-BENZOL | 0.25 | 3.1 | 50 | 50 |
| 21              | 2-HYDROXY-3-METHYL-BENZOL | 0.06 | 50 | 50 | 4.5 |

*3-AMINO-4-HYDROXY-8-METHYL-7-[3-O-(5-METHYL-2-PYRROLYL)NOVIOXY]COUMARIN.

*COMPOUNDS WERE ADMINISTERED BY THE ORAL ROUTE.
Relative in vitro antibacterial potency against Staphylococcus aureus and Klebsiella pneumoniae of 4-hydroxy-8-methyl-7-[3-O-(5-methyl-2-pyrrollylcarbonyl)noviosyloxy] coumarins substituted in the 3-position of the coumarin moiety with various 3-n-alkyl-4-hydroxybenzamido groups.

Figure 2 shows the antibacterial activity in serum-free medium of this series as a function of the number of carbons in the 3-alkyl group.

The striking increase in activity achieved by lengthening the n-alkyl chain was, with the possible exception of the n-hexyl derivative, almost inapparent in medium containing 25% serum. The six-carbon homologue had an MIC in serum about 10 times lower than that of the other compounds. This derivative gave the lowest S. aureus Smith CD<sub>50</sub>, although all members of the series had a median curative dose that fell within a very narrow range (6 to 14 mg/kg).

Much the same activity pattern was observed with branched chain derivatives. Compounds with 3, 4, or 5 carbons in the 3-position substituent, such as the i-propyl, 1-methylpropyl, tert-butyl, 3-methylbutyl, and the 1,2-dimethylpropyl, displayed good in vitro activity against both S. aureus Smith and K. pneumoniae. The CD<sub>50</sub> values obtained in the S. aureus Smith infection ranged from 4 to 10 mg/kg for these compounds.

Several unsaturated 3-alkyl side chains were also examined. These were the allyl, the 1-methylallyl, and the novobiocin side chain, the 2-butenyl-

**TABLE 5.** Antibacterial activity of PNC-4-hydroxybenzamido<sup>α</sup> derivatives having various substituents in the 3-position of the benzene ring.

| COMPOUND NUMBER | R GROUP | MINIMUM INHIBITORY CONCENTRATION (μg/ml) | CD<sub>50</sub>α (mg/kg) |
|----------------|---------|------------------------------------------|--------------------------|
|                | S. AUREUS SMITH | K. PNEUMONIAE | S. AUREUS SMITH | K. PNEUMONIAE |
|                | NO SERUM | 25% SERUM | NO SERUM |                  |
| 22             | HYDROGEN          | -H           | 0.16 | 0.8 | >100 | 14.3 | - |
| 23             | n-HEXYL           | -(CH<sub>2</sub>)<sub>5</sub>-CH<sub>3</sub> | 0.0016 | 0.02 | 1.6 | 6 | 140 |
| 24             | 3-METHYLBUTYL     | -(CH<sub>2</sub>)<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub> | 0.0032 | 0.2 | 6.2 | 8.6 | >300 |
| 25             | CYCLOHEXYL        | S            | 0.0063 | 0.8 | 3.1 | 12.5 | >200 |
| 26             | COUMERMYCIN A<sub>1</sub> | (SEE FIG. 1) | 0.0008 | 0.016 | 3.1 | 17 | 130 |

<sup>α</sup>Compounds administered by oral route (I)x for S. AUREUS SMITH; by IM (2x) for K. PNEUMONIAE.

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3-methyl. Despite having comparable in vitro activity, both the allyl and 1-methylallyl were much less effective in vivo (CD\textsubscript{50} values of 23.5 and 25 mg/kg, respectively) than their corresponding saturated analogues. However, the 2-butenyl-3-methyl derivative was as active as the saturated compound since their respective median curative doses were 5.0 and 8.6 mg/kg.

A cyclohexyl group was also introduced into the 3 position of the 4-hydroxybenzamido side chain. The resulting derivative displayed antibacterial effects similar to those found with the longer chain n-alkyl analogues. Table 5 shows antibacterial data for this compound as well as for the outstanding representatives among compounds having an n-alkyl or branched-chain alkyl group.

All of the 3-substituted derivatives just discussed had better in vitro antistaphylococcal and anti-\emph{K. pneumoniae} activity in serum-free medium than did the unsubstituted compound. However, in the presence of 25\% serum, only compound 23, the n-hexyl analogue, had an \emph{S. aureus} Smith MIC appreciably lower than that of the compound having no 3-position substituent. All of the derivatives were effective in \emph{S. aureus} Smith infections with oral CD\textsubscript{50} values ranging from 6 to 12.5 mg/kg. This level of in vivo activity compares very favorably with that of coumermycin A\textsubscript{1}. The derivative with the greatest activity in the \emph{S. aureus} Smith infection, the 3-n-hexyl analogue, also proved to be as effective as coumermycin A\textsubscript{1} in an experimental \emph{K. pneumoniae} infection of mice when the compounds were administered by the IM route. Neither was active against this infection when treatment was given orally at doses up to 300 mg/kg \times 2. None of the other compounds shown in Table 5 had measurable in vivo activity against \emph{K. pneumoniae}.

**Activity of trisubstituted benzamido derivatives.** Acylation of the coumarin amine with trisubstituted benzoic acids produced compounds having a higher degree of serum binding and poorer in vivo efficacy than the unsubstituted benzoic acid derivatives (compound 18, Table 4). Among derivatives examined were those having 2,4,6-trimethylbenzamido, 3,5-dimethyl-4-hydroxybenzamido, and 3,5-dipropyl-4-hydroxybenzamido side chains.

**Activity of alkylamido derivatives.** A number of PNC derivatives were prepared by acylation of the coumarin amine with aliphatic acid chlorides. Chain length of this series of homologues varied from 2 to 22 carbons. Figure 3 shows their in vitro potency against \emph{S. aureus} Smith as a function of the number of carbons in the acyl group.

It is apparent that chain length has a profound effect on antistaphylococcal activity since potency of the derivatives varied over a greater than 1,000-fold range (inhibitory concentrations ranged from 0.08 to 100 \(\mu\text{g/ml}\)). An increase in chain length starting from the 2-carbon acetyl group resulted in an increase in potency (about 10- to 20-fold) that peaked with the 10-carbon acyl radical. Activity then fell off rather precipitously as the number of carbons in the chain was increased still further. Compounds containing 21- and 22-carbon acyl radicals had about 1\% of the activity of the 2-carbon derivative. Unfortunately, in serum-containing medium, an \emph{S. aureus} Smith MIC of 50 \(\mu\text{g/ml}\) or greater was obtained with all the alkylamido compounds except the acetamido and propionamido which had inhibitory concentrations of 0.4 and 1.8 \(\mu\text{g/ml}\), respectively. In vivo antistaphylococcal activity was also rather poor for this series since the most active compound (propionamido) had a CD\textsubscript{50} of 30 mg/kg. None of the members of the series displayed significant in vitro activity against \emph{K. pneumoniae}.

In addition to the n-alkylamido compounds discussed above, a number of semisynthetic coumermycins having branched-chain acyl groups were examined. In contrast to the straight-chain analogues, several of the compounds were quite active in vivo. \emph{S. aureus} Smith CD\textsubscript{50} values falling
within an 8 to 13 mg/kg range were obtained with the 2-methylpropionamido, 3-methylbutyramido, and 4-methylpentanamido derivatives. Median curative doses obtained with the 2,2-dimethylpropionamido, 3,3-dimethylbutyramido, 2-ethylbutyramido, and 2-n-propylpentanamido were higher, ranging from 23 to 40 mg/kg.

Activity of aromatic and heteroaromatic derivatives. The relative antibacterial efficacy of some representative aromatic derivatives is shown in Table 6.

Good in vitro and in vivo activity was demonstrated by all compounds except the fused-ring derivative (1-naphthoyl) and the phenyl-substituted isoxazole (compound 33). It seems probable that the bulky nature of these acyl groups increased their degree of binding to serum with the net result that their in vivo effectiveness was decreased.

Activity of aralkylamido derivatives. A series of aralkyl compounds including the 2-phenylacetamido, 3-phenylpropionamido, 3-cyclohexylpropionamido, and a number of related derivatives with various substituents in the ring or on the α-carbon were examined for their antibacterial properties. Although most had a low MIC against *S. aureus* Smith in serum-free medium, addition of 25% serum resulted in a marked and essentially uniform decrease in antistaphylococcal activity. The lowest *S. aureus* Smith CD50 in this series (21 mg/kg) was obtained with the 2-phenylbutyramido derivative.

Oral absorbability of coumermycin A1 and various PNC derivatives. Coumermycin A1 and four semisynthetic derivatives that had demonstrated good in vivo efficacy in the *S. aureus* Smith infection were selected for absorption studies. Blood concentrations of each of the compounds were determined at various time intervals after oral administration of a single 25 mg/kg
dose. Results obtained with coumermycin A₁ and these PNC derivatives are presented in Fig. 4.

Coumermycin A₁ displayed a low blood level peak (0.9 μg/ml at 1 hr posttreatment), but still had a measurable blood concentration (0.5 μg/ml) 5.5 hr after drug administration. In sharp contrast were the blood levels produced by the same dose of the PNC derivatives. Peak concentrations of these compounds, which in all cases occurred at 0.5 hr postadministration, were considerably higher than that of coumermycin A₁. Despite a rather wide range (8.4 to 24.9 μg/ml) of peak levels, all PNC derivatives with aromatic carboxamido side chains had similar blood "disappearance" curves. This was not the case, however, for the derivative with the alkylcarboxamido (2-methylpropionamido) side chain. Its blood concentration appeared to decrease at a significantly slower rate than that of the aromatic carboxamido compounds. The blood level of this derivative at 5.5 hr was only 50% below the peak level as compared to a minimum 75% decrease from the peak for each of the other PNC derivatives. The in vitro and in vivo antimicrobial properties as well as the pharmacological behavior of this alkylcarboxamido derivatives have recently been described in detail (K. E. Price, D. R. Chisholm, F. Leitner, and M. Misiek, presented at the 9th ICAAC, Washington, D.C., 28 Oct., 1969).

Three additional PNC compounds were subjected to oral absorption studies in the mouse. These were benzamido analogues and had peak blood levels ranging from 14.6 to 26.7 μg/ml. In all cases peak blood levels occurred at 0.5 hr, and blood disappearance curves were similar to those found for the other aromatic carboxamido derivatives.

The nature of the relationship between the S. aureus Smith MIC (in 25% serum), peak mouse blood levels, and therapeutic effectiveness in the Smith infection for coumermycin A₁ and the seven PNC derivatives that had been subjected to absorption studies, has been considered in Table 7.

It is apparent that all of the PNC derivatives were quite effective in controlling the S. aureus Smith infection, despite the fact that in most instances their antibacterial potency was inferior to that of coumermycin A₁. It must be presumed, therefore, that their relatively high efficacy in the Smith infection was due to their greater oral absorptivity. When both antibacterial potency (A = 1/MIC) and peak blood antibiotic concentrations (P) are simultaneously considered, a coefficient showing the relative efficacy of the test compound (t) to coumermycin A₁ (c) can be obtained as follows:

$$A_t/A_c \times P_t/P_c = \text{"efficacy" coefficient}$$

Although the CD₉₀ values obtained only vary within a narrow range (6 to 17 mg/kg), there does appear to be some relationship between the efficacy coefficient and therapeutic effectiveness. As a general rule, the higher the coefficient, the lower the CD₉₀. PNC derivatives with a coefficient of 2

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**Fig. 4.** Concentration of coumermycin A₁ and several amide derivatives of 3-amino-4-hydroxy-8-methyl-7-[3-O-(5-methyl-2-pyrrolylcarbonyl)novo-syloxy] coumarin in mouse blood after oral administration of a single 25 mg/kg dose. Each point is the average value for 12 mice.

**Fig. 5.** Relative irritability to the rat foot-pad of coumermycin A₁ and four derivatives of 3-amino-4-hydroxy-8-methyl-7-[3-O-(5-methyl-2-pyrrolylcarbonyl)novo-syloxy] coumarin. One and 3% solutions or suspensions were injected in 0.1-ml volume into the plantar surface (foot-pad) of five rats per dose level. The degree of edema was measured by the volume displacement method.
or higher were at least twice as effective in the *S. aureus* Smith infection as coumermycin A₁ (coefficient of 1).

**Irritability of coumermycin A₁ and various PNC derivatives following parenteral administration.**

Coumermycin A₁ and several semisynthetic derivatives that had displayed a high level of effectiveness in an experimental mouse infection were tested to determine the extent of their irritating properties for the rat foot-pad when injected as 1 or 5% solutions or suspensions. To be considered irritating, a compound must produce a swelling or edema volume of 0.25 ml or greater for at least 4 hr. The degree of edema obtained at representative time periods after administration of the test compounds is shown in Fig. 5. Coumermycin A₁ proved to be extremely irritating at both 1 and 5% concentrations. Although the PNC derivatives had a lesser effect, all were irritating at the 5% dosage level and only two could be considered to be free of irritating properties at the 1% concentration level. The nonirritating derivatives were the benzoyl (com-
TABLE 8. Acute toxicity of coumermycin A₁ and several PNC-NH₂<sup>a</sup> derivatives for mice.

| R GROUP          | STRUCTURE | ROUTE OF ADMINISTRATION | INTRAPERITONEAL | ORAL |
|------------------|-----------|--------------------------|-----------------|------|
| COUMERMYCIN A₁  | (SEE FIG. 1) | 230<sup>b</sup>          | 2400            |      |
| BENZOYL         |           | 370                      | 1980            |      |
| 2,6-DICHLOROBENZONYL |      | 130                      | 940             |      |
| 2-METHYLPROPIONYL |           | 350                      | 1400            |      |

<sup>a</sup>3-AMINO-4-HYDROXY-8-METHYL-7-[3-O-(3-METHYL-2-PYRROLYLCARBONYL) NOVIOSYLOXY] COUMARIN.
<sup>b</sup>LD₅₀ (LETHAL DOSE, 50 %) IN mg/kg.

compound 1) and the p-aminobenzoyl (compound 2). In most cases, the degree of edema reached a maximum at 4 hr postinjection and appeared to be directly related to the dosage administered. The only exception to this was noted with the 3-π-heptyl-4-hydroxybenzoyl analogue (compound 3). This derivative, possibly because of its extremely low degree of solubility, was only slightly more irritating at the 5 than at the 1% level.

It is of interest that the swelling induced by all of the PNC compounds receded much more rapidly than that caused by coumermycin A₁. By 48 hr postinjection (not shown in Fig. 5), no swelling of significance was found for any of the PNC derivatives, whereas the 5% coumermycin suspension still produced an average foot-pad edema volume of >0.5 ml.

No evidence of pain on injection was noted for coumermycin A₁ or any of the PNC derivatives.

Acute toxicity of coumermycin A₁ and several PNC derivatives. IP and PO acute LD₅₀ values in the mouse were determined for coumermycin A₁ and three PNC derivatives. Results are summarized in Table 8.

The acute IP LD₅₀ values of PNC derivatives other than the 2-6-dichlorobenzoyl were about the same or higher than that of coumermycin A₁. The suggestion that the chlorine-containing compound has somewhat greater toxicity for mice is reinforced by the finding that its PO LD₅₀ was also lower than those of the other PNC compounds. On the whole, however, all of the derivatives displayed a surprisingly low level of oral toxicity relative to coumermycin A₁, considering that their degree of absorbability by this route is some 15 to 30 times greater.

DISCUSSION

The objectives of this structural modification program were to maintain or enhance the desirable properties of coumermycin A₁ and to reduce or eliminate some of its undesirable characteristics. There is little question but that a certain degree of success has been achieved. A number of the new semisynthetic antibiotics (acylated methylpyrrole-noviose-coumarin amines) which were prepared by chemical treatment of coumermycin A₁ possess markedly greater oral absorbability in mice. This improvement over the parent compound is of such magnitude (in some cases, >25-fold) that it more than compensates for the lower antibacterial potency possessed by most of the derivatives. The result for some of the more active compounds was a net two- to threefold improvement in vivo antistaphylococcal therapeutic effect. Furthermore, in experimental D. pneumoniae and Streptococcus pyogenes mouse infections, the oral efficacy of such derivatives ex-
ceeded that of coumermycin $A_1$ by more than four- to eightfold (Price, unpublished data). It is noteworthy that the improved oral therapeutic effect of the semisynthetic antibiotics was achieved in most cases without a corresponding increase in toxicity to the host.

Compounds having activity against gram-positive bacteria of the order described above were produced when the 3-amino group of the coumarin moiety was acylated with aromatic or heteroaromatic carboxylic acids, either without or with mono- or dissubstituents on the ring. Whereas several compounds prepared from branched-chain aliphatic acids had comparable in vivo efficacy, those derived from $n$-aliphatic or aryl-substituted aliphatic acids, as well as those prepared from trisubstituted benzoic acids, were much less effective.

The structural requirements for inhibitory activity against gram-negative bacteria would appear to be much more stringent since only those derivatives prepared by acylation with 3-substituted-4-hydroxybenzoic acids had significant activity. However, it is likely that such inhibitory effects are merely a reflection of these compounds' relatively high potency and cannot be attributed to a specifically broadened antibacterial spectrum. The most active representative of this class (the 3-$n$-hexyl-4-hydroxybenzoic acid derivative) and coumermycin $A_1$ had comparable efficacy when administered parenterally in an experimental $K$. pneumoniae infection of mice. However, neither proved to be effective when given by the oral route.

In addition to their superior oral absorbability, most of the semisynthetic derivatives tested proved to be significantly less irritating to local tissues following parenteral administration. Several derivatives, specifically those prepared by acylation with benzoic acid or $p$-aminobenzoic acid, were less irritating when given as a 5% suspension than was coumermycin $A_1$ when given as a 1% suspension. Even the swelling produced by the most irritating derivative receded much more rapidly than that induced by coumermycin $A_1$.

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